

REVIEW



Severe acute respiratory syndrome (SARS) coronavirus: application of monoclonal antibodies and development of an effective vaccine

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SUMMARY

SARS-CoV is a new type of human coronavirus identified as a causative agent of severe acute respiratory syndrome (SARS). On the occasion of the SARS outbreak, various monoclonal antibodies (mAbs) against SARS-CoV have been developed and applied for diagnosis, clinical management and basic research. In this review, we overview the biochemical and functional properties and applications of these SARS-CoV mAbs. We also focus on a variety of vaccines currently under development and discuss the immune response elicited by these vaccines in animal models, hopefully to better understand what we need to do next to fight against newly emerging pathogens in the future. Copyright © 2006 John Wiley & Sons, Ltd.

Received: 25 November 2005; Revised: 13 December 2005; Accepted: 13 December 2005

INTRODUCTION

The outbreak of fatal severe acute respiratory syndrome (SARS), which originally occurred in 2002 in China and subsequently spread to many countries in early 2003, is now under control due to a concerted world-wide effort led by the World Health Organisation (WHO). A causative pathogen, SARS-CoV, was identified and the entire genome sequence was quickly determined [1,2]. SARS-CoV belongs to a distinct group of known human coronaviruses, such as HCoV-229E (Group 1) and HCoV-OC43 (Group 2), which are only slightly pathogenic in humans. On the contrary, animal coronaviruses have been known to cause clinically serious diseases in livestock and pet animals. Based on the nucleotide sequence homology of SARS-CoV-like virus in wild animals traded in Chinese markets, it is considered that SARS-CoV emerged through the interspecies transmission of such animal coronaviruses (review in [3]). A recent report strongly suggested that the Chinese horseshoe bat was a natural host of SARS-CoV

(bat-SARS-CoV) and that caged animals such as Himalayan palm civets and raccoon dogs are the amplification hosts [4].

SARS-CoV is readily transmissible through close contact between family members, and health-care and laboratory workers. Since SARS-CoV infects and replicates in the respiratory and intestinal tissues, a vaccine eliciting a mucosal neutralising antibody, particularly in the broncho-alveolar lumen would be most desirable. It has been reported that high titres of neutralising IgG antibodies against SARS-CoV are present in SARS patient [5] and that passive administration of serum antibodies from previously infected patients improved the conditions in newly infected recipients [6]. Furthermore, in the mouse model, the passive transfer of mouse immune serum against SARS-CoV has been demonstrated to reduce pulmonary viral titres in mice infected with SARS-CoV [7]. These results support the notion that the neutralising antibodies are crucial for protection against SARS-CoV infection.

On the occasion of the SARS outbreak, several laboratories endeavoured to identify the monoclonal antibodies (mAbs) that act against SARS-CoV in order to provide tools for diagnosis, in

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combination with viral detection by RT-PCR analysis, and for application in the clinical treatment of SARS patients. In this context, we overview the biochemical and functional properties of SARS-CoV mAbs, as established by different technical approaches, together with the applications which utilise these mAbs in both clinical and basic research. We also discuss the immune response elicited by a variety of vaccines in animal models to better understand what is required for developing an effective vaccine against SARS-CoV infection.

MONOCLONAL ANTIBODIES AGAINST SARS-CoV

The SARS-CoV encodes four major structural proteins; the spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins. Up to now, a variety of mouse and human monoclonal antibodies (mAbs) recognising these proteins have been established and are listed in Table 1, for mouse, and Table 2 for human mAbs. In addition, the fine epitope-mapping studies of these mAbs are summarised in Figure 1. We now know that the S protein binds to the cellular receptor ACE2 [8] and that ACE2 recognises the S-protein at the amino acid residues aa318–510 [9]. This region is designated as a receptor binding domain (RBD). Thus, the S protein, especially RBD, is a major

target for eliciting neutralising antibody. We will focus on this issue in the next section.

Mouse mAbs (Table 1)

When whole virions were used as an antigen, established mAbs frequently recognised the S protein. We used a highly purified whole virions of SARS-CoV as an immunising antigen, which had been inactivated by UV-irradiation to avoid unwanted denaturation of virus protein [10]. The majority of mAbs established (26 out of 29) recognised 'conformational' epitopes on the S protein with virus-neutralising ability *in vitro*, whereas 3 mAbs recognised 'linear' epitopes on the N protein. On the other hand, Chou *et al.* established several mAbs which recognise the linear epitopes of S protein by using detergent-disrupted virions as an antigen [11]. Such mAbs, designated 1A5 and 2C5, had high neutralisation ability, suggesting that neutralising activity is mediated by a variety of antibody recognition sites on the S protein. Gubbins *et al.* used whole virions inactivated with β -propiolactone and obtained 9 mAbs with virus neutralising ability [12]. Surprisingly, the majority of mAbs are encoded by the V_H-gene family, V_HJ558, coupled with variety of light chains, suggesting a correlation between neutralisation ability and specific V_H-gene allele.

Table 1. Establishment of mouse monoclonal antibodies

Host	Antigen (adjuvant)	Clones	Application	Reference
1	Whole virion UV-inactivated (FCA)	26 clones of anti-S and 3 clones of anti-N (SKOT-8, -9 etc.)	Sandwich-ELISA, Western-blot Histology, IFA, neutralization	[10]
2	Whole virion detergent-inactivated (FCA)	7 clones of anti-S (1A5, 2C5 etc.)	Epitope mapping on S, Neutralization Western-blot	[86]
3	Whole virion beta-propiolactone- inactivated (FCA)	5 clones to S (F26G18, F26G19 etc.)	MAbs belong to single V _H -gene family	[87]
4	Recombinant N (MPL + TDM) ^a	9 clones to anti-rN (NE4A4, NE8A11, etc.)	Sandwich-ELISA, Western-blot	[88]
5	Recombinant S fragment (FCA)	4 clones to S (S26, S34, S84, S78)	Neutralization, Western-blot, ELISA, cyto-staining	[13]
6	RBP-Fc ^b	27 RBP-specific mAbs (4D5, 17H9 etc.)	Epitope mapping Neutralization	[14]

^aMPL, monophosphoryl lipid A; TDM, trehalose dicorynomycolate.

^bRBP-Fc, fusion protein containing the receptor binding domain (RBP) linked to a human IgG1 Fc fragment.

Table 2. Human monoclonal antibodies for SARS-CoV

Method	Clones	Notes	References
Improved EBV-transformation of memory B cells from patients	anti-S, -N, -E (many clones, S3.1, S102.1 etc.)	<i>In vitro</i> neutralization activity, 10^{-8} – 10^{-11} M <i>In vivo</i> protection in mouse model	[48]
scFv ^a , phage display screening on recombinant S1 protein	8 scFv clones (80R etc.)	Memory B cell repertoire representation Epitope mapping, <i>in vitro</i> neutralization <i>In vivo</i> protection in mouse model	[35] [89]
scFv, phage display screening on irradiated SARS-CoV whole virion	anti-S, -N (CR3014, CR3018, CR3009)	Epitope mapping, immunoelectron microscopy, IFA, <i>in vitro</i> neutralization	[36]
scFv, the library constructed from SARS convalescent patients, selection against inactivated whole virion	anti-S2 (B1 etc.)	Mapped to 1023–1189 of S2 protein, potent neutralization activity <i>in vitro</i>	[19]
Transgenic mice with human immunoglobulin gene (Medarex) immunized with recombinant S protein	2 clones (68, 201)	Epitope mapping, <i>In vivo</i> protection in mouse model	[21]

^ascFv; single-chain variable antibody fragments.

Another approach is to use the recombinant SARS-CoV proteins as an immunising antigen. Zhou *et al.* prepared a recombinant protein fragment of S protein, S-II (aa 485–625), which was predicted to include the RBD [13]. This recombinant protein fragment binds to the surface of Vero cells, and the mAbs against this fragment had potent *in vitro* neutralisation ability. He *et al.* used a small fragment of recombinant RBD, which was fused with human IgG1 Fc fragment (RBD-Fc) as an immunising antigen [14]. Twenty-seven hybridomas were established and their antigen specificities were mapped into 6 different conformation-dependent and 2 adjacent linear epitopes (see below) [14]. Using recombinant N protein, Che *et al.* isolated anti-N mAbs, useful for the sensitive antigen-capture ELISA system and Western blot [15].

Human mAbs (Table 2)

Considering the clinical approach to block SARS-CoV infection by utilising neutralising antibodies,

human mAbs have been established by different techniques. Traggiai *et al.* developed a unique method of EBV transformation of B cells, which enabled rapid generation of human neutralising antibodies against SARS-CoV [16]. The memory B cell pools of recovered SARS patients were efficiently immortalised, and high affinity (10^{-8} M to 10^{-11} M) human mAbs were successfully obtained. In this case, about 80% of EBV-transformed B cell clones secreted IgG antibody against S protein and about 15% against N protein, most likely reflecting the actual memory B cell repertoire in those individuals who recovered from SARS-CoV infection.

Sui *et al.* obtained eight clones of two single-chain variable antibody fragments (scFvs) by screening the scFv library constructed from non-immune human antibodies on the S1 fragment of SARS-CoV S protein [17]. Among these clones, 80R bound to the S protein RBD region and showed an efficient neutralisation ability [17]. Using a similar approach, van den Brink *et al.*

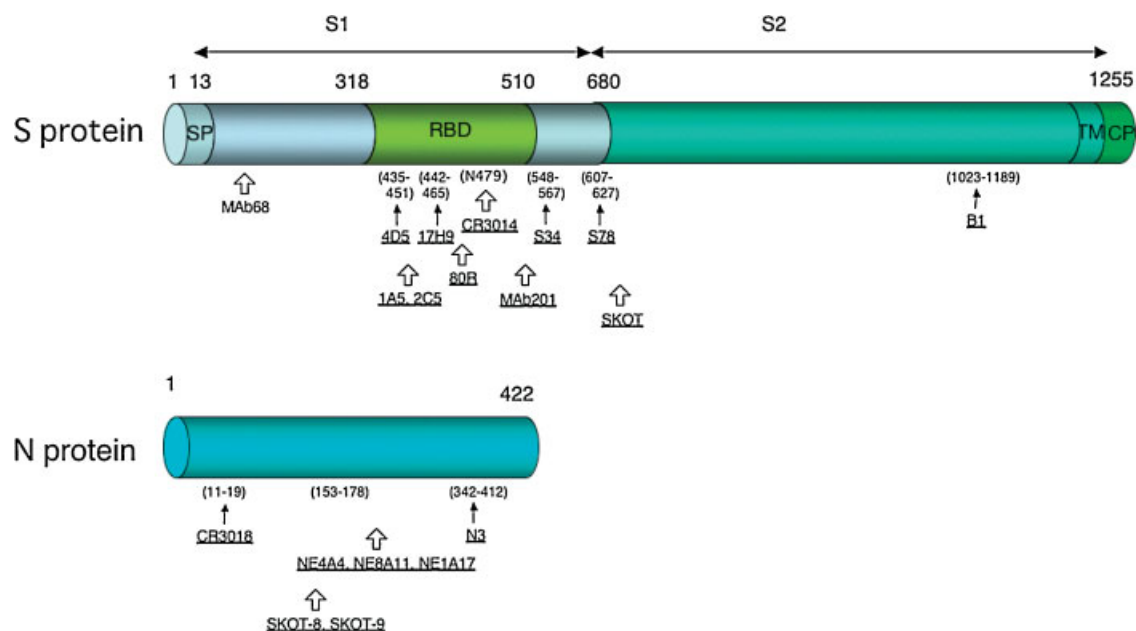


Figure 1. Schematic drawing of S- and N-protein epitopes and the name of the recognizing mAbs. Small arrows indicate that the epitopes are confined to the peptide fragment indicated in the figure. Large arrows indicate that the epitopes are nearly confined to the region indicated. The epitopes identified only in polyclonal antibodies are not listed

obtained eight scFv clones, of which two clones could be mapped to the N protein and four to the S protein [18]. One of the two anti-N scFv clones recognises a linear epitope and all of the others recognise conformational epitopes. Three of the four anti S scFv clones recognised the RBD and retained an *in vitro* neutralising activity. Duan *et al.* also utilised a phage-display library technique to construct a library from convalescent SARS patients. One of the scFv clones, B1, recognises the aa1023–1189 S2 protein region and has potent neutralising activity [19].

Greenough *et al.* obtained human mAbs against SARS-CoV structural proteins by immunising transgenic mice with human immunoglobulin genes [20,21]. One of these mAbs, 201, recognises the RBD (aa490–510) and another mAb, 68, recognises the aa130–150 region, outside the RBD. Thus, a variety of approaches makes it possible to establish mAbs of human origin against SARS-CoV. Of interest, the technique for establishing mAbs from recovered patients, in which high-affinity antibodies with potent biological activity can be selected during virus elimination, is useful for other infections to establish antibodies with potent activity

for diagnosis and, quite probably, for clinical approaches as well.

Epitopes on SARS-CoV proteins

Spike (S) protein: SARS patient's sera recognise the major conformational and linear S protein epitopes [22–24]. The S protein of SARS-CoV (GenBank accession no. 29836496) is 1225aa residues long and contains a leader sequence at its N-terminal [1,2] (Figure 1). The S protein is divided into two regions, S1 (aa1–690) and S2 (aa691–1255), although it is not cleaved by a protease as other corona-viruses are. The S2 region contains a transmembrane and cytoplasmic region and the S1 contains the RBD. The S1 domain of Group-I coronaviruses, including human CoV-229E and transmissible gastroenteritis virus (TGEV), recognises aminopeptidase N (CD13) as a cellular target molecule [25,26]. The binding site was mapped to aa407–547 in the case of Hu-CoV 229E [27] and aa506–655 in TGEV [28]. Group-II coronaviruses, such as mouse hepatitis virus (MHV), recognise the carcinoembryonic antigen-related cell adhesion molecule (CEACAM1) as a cellular receptor [29], and the N-terminal 330aa residues contain

Table 3. SARS Vaccine Studies in Animal Models

Type of vaccines	Vaccine preparation	Target or antigen	Animal model	Route and immune control etc	Protection	References	
Inactivated SARS-CoV	UV-irradiation	Whole virion	BALB/c	s.c., twice, Nab and T-cell activation	ND	[56]	
	Formaline- β -propyolactone	Whole virion	BALB/c	Nab	ND	[65,67]	
		Whole virion	BALB/c	Nab	ND	[66]	
Recombinant virus vectors	Attenuated vaccinia (MPA)	S	BALB/c	i.n. = i.m., twice	+	[69]	
		S	BALB/c	i.m.	+	[70]	
		(S-DNA)	Rabbit	i.m.	ND		
		S	Monkey	i.n versus i.m.	+		
		S	Ferret	i.p. or s.c.	No effect	[71]	
	Adenovirus type5	S1, M, N	Monkey	Monkey	hepatitis+ Nab, N-peptide-reactive T	ND	[73]
			B6	B6	N-reactive T	ND	[90]
			B/6 and BALB/c	B/6 and BALB/c	S-peptide-reactive T epitopes in S1 CD4 and CD8 epitopes	ND	[64]
		Parainfluenza virus	S	Monkey	Nab	+	[77]
		S, E, M, N	Hamster	Nab, S only	+	[76]	
Rabiesvirus	S and N	BALB/c	i.m., Nab, S only	ND	[74]		
VSV	S	BALB/c	i.n., Nab, passive imm.	+	[75]		
DNA vaccines	S, SdTM, SCID	BALB/c	BALB/c	Nab, no contribution of CD4 ⁺ /CD8 ⁺ T cells	+	[79]	
		N-linked to Calreticulin	B6	CTL	++ (N++)	[80]	
	N	BALB/c	N peptides CTL: N-expressing cells	ND	[81,82]		
	M, N	B/6, SCID	CTL response	ND	[91]		
Recombinant protein	Soluble polypeptide	S 14-762	BALB/c	Nab, with adjuvant (MPL + TDM)	+	[85]	

Nab, neutralizing antibody; i.m, intramuscular; i.n, intra nasal; i.p., intraperitoneal; s.c., subcutaneous; ND, not done.

the receptor binding site [30]. Upon binding to the target molecule, the S protein undergoes a conformational change leading to exposure of the fusogenic region in the S2 part resulting in membrane fusion between the viral and cellular membranes [31,32].

Mouse mAbs against recombinant protein fragments of S-II (aa485–625), S34 and S78, obtained by Zhou *et al.* [13], had potent neutralisation ability. The epitopes of S34 and S78 were localised to aa548–567 and aa607–627, respectively. These epitopes, however, are slightly downstream of the RBD shown by Wong *et al.* [9] and Xiao *et al.* [33]. On the other hand, among the mAbs against RBD fused with human IgG1-Fc fragment (RBD-Fc), two mAbs, 4D5 and 17H9 recognised linear epitopes such as aa435–451 and aa442–465, respectively [34], whereas the remaining mAbs recognised conformational epitopes, classified into six groups as Conf-I to Conf-VI. This study showed that the ability to inhibit ACE2 binding of SARS pseudovirus was retained in all conformational epitopes, especially in Conf-III, -IV and V, but not in the linear epitopes.

Likewise, human mAbs against S protein, such as 80R [35], CR3014 [36] and 201 recognise the RBD and have potent neutralising activity against SARS-CoV, as discussed below. The clone, CR3014, recognises the S1 fragment in the context with aa479, suggesting the importance of this amino acid in the high binding affinity of CR3014.

Spiga *et al.* tried to model the tertiary structure of the S1 and S2 S protein domains by the homology modelling and molecular dynamics methods [37]. Their model predicted two hydrophobic pockets, Phe850-Phe870 and Phe1077–1079, for the putative receptor binding site. This model would be useful in evaluating the antigen drift caused by the mutations in the S1 region, which has already been deposited in the NCBI database. Interestingly, when Yi *et al.* studied the immunogenicity of S protein in mice by DNA immunization, a single amino acid substitution, the R441A mutation, failed to induce neutralizing antibodies and abolished viral entry. The R453A mutation, however, retained the capacity to induce neutralizing antibodies, although it also abolished viral entry [38]. Thus, a single amino acid mutation here easily affects the virus-to-cell interaction, and therefore, this region is an ideal target structure for neutralization.

Nucleocapsid (N) protein: The nucleocapsid (N) protein of SARS-CoV is a highly basic structural protein of 422 amino acids. The N protein is thought to be involved in the packaging of the viral RNA-genome and, thus, crucial for viral replication and pathogenesis as in other CoVs [39,40] (Figure 1). Anti-N-protein antibodies are generally found in the sera of SARS patients, suggesting that the N-protein is one of the immunodominant structural proteins of SARS-CoV [41,42]. In addition, SARS patient's sera recognise the N-protein at a large number of linear epitopes [23,24] and, at least, two major conformational epitopes [43].

In patient's sera, Wang *et al.* identified the peptides N66 (aa66–87) and N371–401 (aa371–422) as the most immunogenic epitopes [24]. On the other hand, He *et al.* identified two major epitopes in the C-terminal region (aa362–412) and middle region (aa153–178), in addition to several minor immunodominant epitopes [44]. They also showed that the most potent antibody response was elicited against the C-terminal region, which contains a short lysine-rich sequence (aa362–381; KTFPPTEPKKDKKKKTDEAQ); however, the functional role of this stretch remains unknown. Van den Brink *et al.* established two human anti-N scFvs, CR3018 and CR3009, which recognised a linear epitope, RSAPRITFG (aa11–19) and a nonlinear epitope, respectively [18].

In mice immunised with inactivated SARS-CoV, we identified one major epitope in the middle part (aa110–210), which was recognised both by the SKOT-8 and SKOT-9 mAb [10], whereas He *et al.* identified one major epitope adjacent to the N-terminal region (aa76–101) in addition to epitopes in the C-terminal and middle regions [44].

Together, these mapping studies of the SARS CoV N protein suggest that there are three major epitopes localised to the N-terminal, middle and C-terminal regions, respectively, which are responsible for the potent immune response in both humans and mice.

Envelop (E) protein: The small E protein is a 76aa residue long protein involved in the envelop morphogenesis. As far as we know, there is only one mAb against this protein in humans [16], and this E protein antibody is detected in SARS convalescent patient sera [23].

Matrix (M) protein: The M membrane glycoprotein is a 221aa residue long integral membrane protein which is functionally involved in the budding of virions from cells. Pang *et al.* reported that rabbit antiserum raised against recombinant M protein has a potent neutralising ability *in vitro*, suggesting that the M protein could be one of the target proteins chosen for the vaccine development [45]. Wang *et al.* reported that the synthetic peptide M137 (aa137–158) is one of the most immunogenic regions of the SARS-CoV structural proteins [24]. Recently, Zhong *et al.* reported a systematic search for the viral epitopes by a bio-panning of the M13 phage display dodecapeptide library using antibodies found in plasma samples of convalescent SARS patients [46]. They determined

the continuous viral epitopes including an 'epitope-rich region' on the S2, M and E proteins. This contiguous epitope map of SARS-CoV would greatly help to develop an effective vaccine for SARS-CoV.

Antibody cross-reactivity to OC43 and 229E

We confirmed the absence of cross-reactivity in the anti-N mAbs, SKOT-8 and SKOT-9, to human coronaviruses, HCoV-OC43 and HCoV-229E, by ELISA [10] and IFA (Figure 2). However, at the polyclonal level, there are reports of cross-reactivity between SARS and other human coronaviruses. Wo *et al.* reported false-positive results in their recombinant SARS-CoV N-protein-based ELISA assay, mainly due to the presence of cross-reactive

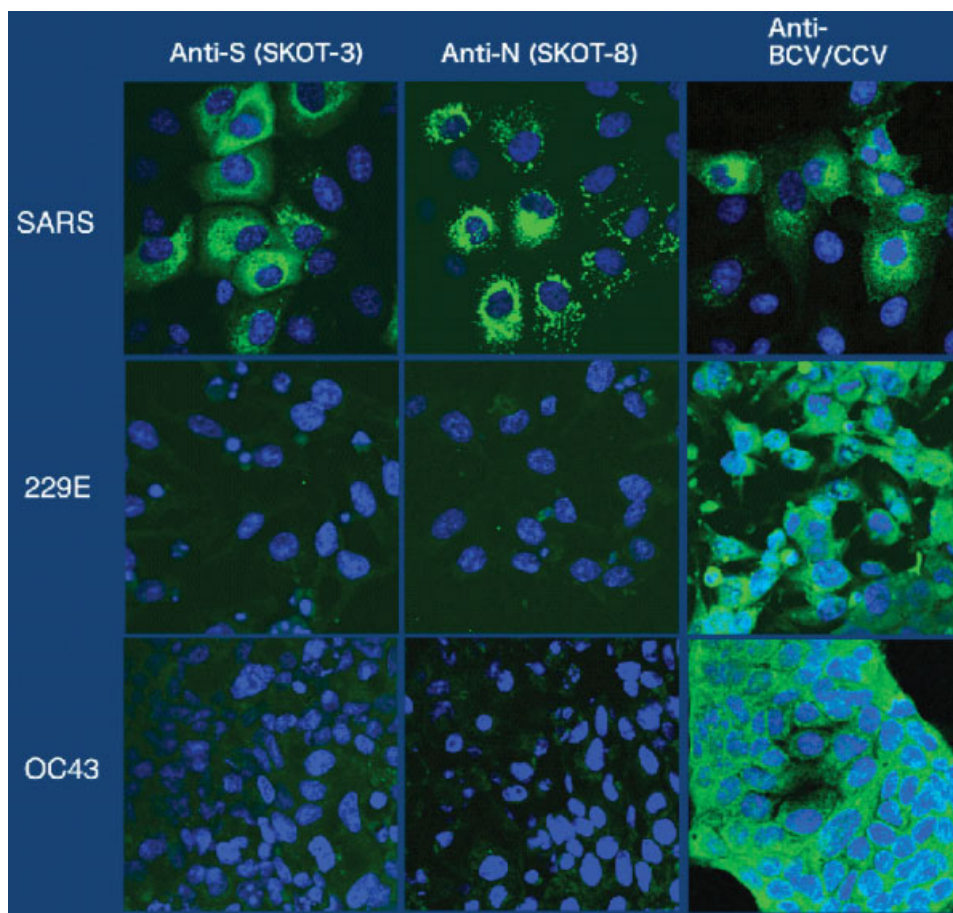


Figure 2. Immuno-fluorescent assay showing the absence of cross-reactivity with anti-SARS-CoV S-protein mAb, SKOT-3 and anti-N-protein mAb SKOT-8, with Hu-CoV 229E and OC43. SARS-CoV-infected VeroE6 cells (upper lane), Hu-CoV 229E-infected L132 cells (middle lane) and Hu-CoV OC43-infected HRT-18 cells are stained with anti-SARS-CoV S- and N-protein mAbs. SKOT-3 (left columns) and SKOT-8 (middle column) and polyclonal anti-BCV/CCV (right columns)

antibodies to SARS-CoV N-protein in the sera [47]. They found that 3 out of 21 and 1 out of 7 sera from convalescent patients from HCoV-OC43 and HCoV-229E, respectively, contained antibodies cross-reactive with the SARS-CoV N-protein, suggesting the presence of a common epitope(s) in these human CoV N-proteins. The sera from convalescent patients, however, did not react with SARS-CoV S-proteins in the Western blot analysis. Thus, highly specific SARS-CoV mAbs are required for sensitive and accurate laboratory diagnosis.

NEUTRALISING ABILITY OF ANTI-SARS-CoV ANTIBODIES

Neutralising antibodies are crucial for establishing protection from SARS-CoV infection. Notably, the analysis of the memory B cell repertoire at a clonal level suggested that the memory B cell repertoire in recovered SARS patients is biased towards neutralising antibodies recognising the S protein [48]. As described above, the main neutralising S protein epitope was at the aa318–510 position, which binds to the cellular receptor ACE2 (RBD; see Figure 1). Therefore, under the expectation that a passive immunotherapy that utilises neutralising antibodies against SARS-CoV could be a promising therapeutical method, many efforts have been focused on the development of mAbs as discussed above.

Analysis of rabbit anti-sera against recombinant S, N and M proteins suggested significant neutralising abilities in anti-S and anti-M antibodies, but not in anti-N antibodies [45]. The main neutralising epitope on S protein was in the RBD, whereas some parts irrelevant to the ACE2 binding site were also shown to be involved in neutralisation, presumably via steric hindrance. Furthermore, a competitive neutralisation assay suggested the presence of an additional target site other than ACE2 binding site [45]. In this regard, by continuous epitope mapping using the M13 phage display dodecapeptide library, Zhong *et al.* reported that the 'epitope-rich region' on the S2 protein (aa787–809) represents one of the major neutralising targets for SARS patients and that is blocked by the peptide of the corresponding region [46].

Human mAbs against SARS-CoV with neutralising activity are attractive reagents for the treatment of SARS patients. For example, all EBV-transformed memory B cells recovered from

SARS patients have specificity for the S protein with neutralising activity [48]. Curiously, one of them, S3.1, conferred efficient protection in a mouse model with SARS-CoV infection [16]. In addition, one of mAbs established from the human scFv library by Sui *et al.*, designated 80R, was shown in a mouse model to display effective protection from infection with wild-type or mutant viruses isolated from patients [49]. Furthermore, when 80R IgG1 was administered into the mice prophylactically, SARS-CoV replication was reduced by more than four orders of magnitude as compared with untreated mice. Human mAbs, 201, established from transgenic mice that harboured human immunoglobulin genes [20] also had neutralising activity and significant protective effect in the mouse infection model upon administration with a 1.6–40 mg/kg dose [21]. Together, all these human mAbs could be candidates for chemoprophylaxis and therapy for SARS, and, in fact, clinical trials are being planned for some of these human mAbs [16,18,21,49].

APPLICATION OF ANTI-SARS-CoV mAbs FOR LABORATORY DIAGNOSIS

Laboratory diagnosis of SARS-CoV infection was based on a combination of serologic tests, reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation [50–53]. Hence, the mAbs against SARS-CoV structural proteins have been established by several groups and their utility in antigen-capture ELISA, immuno-fluorescence assay, Western blot analysis has been tested [10,51,54](see Table 1).

Immuno-fluorescence assay (IFA) and immuno-histochemistry (IHC)

IFA is the simplest serological test for detecting the pathogen. The IFA for SARS diagnosis is carried out by using several polyclonal and monoclonal antibodies against N-protein or those against S-protein, irrespective of the specificity for conformational or linear epitopes [10,15,18,53]. We showed that SKOT-8 and SKOT-9 mAbs against N protein were quite useful for diagnosis, in terms of both specificity and sensitivity [10].

He *et al.* developed a system that uses insect sf9 cells expressing the N195-Sc fusion protein as a target cell, by which 23 serum samples from SARS patients were scored at a rate comparable

to that of conventional IFA and a commercial SARS-CoV IFA kit [55].

Western blot: The majority of anti-N mAbs are directed against linear epitope(s) and thus used in Western blot to detect the 47 kDa band of the N-protein [10,15]. In contrast, the epitopes on native S-protein are mostly conformational and the anti-S mAbs we obtained were not useful for Western blot [10]. However, by using SDS-denatured S protein or recombinant S protein fragments as an immunogen, several mAbs for Western blot were obtained [11,13]. Although the sera from patients and from immunised mice detected both the E and M protein [23,24,56], no useful mAb for detecting either of these proteins by Western blot have been established.

ELISA: Because the immunological test for the presence of SARS-CoV virions in a patient's specimen is an important complement to the RT-PCR test, three groups have so far reported the development of such an antigen capture ELISA system for SARS-CoV. We intended to establish an antigen-capture sandwich ELISA system by using a total of 29 mouse mAbs with specificity for S- and N-proteins, and found that a virus protein load as low as 40 pg/mL was successfully detected by use of the anti-N mAb, SKOT-8, as a capturing antibody and the biotinylated mAb, SKOT-9, as a probing antibody [10]. The sensitivity of the system did not differ between at least four strains of SARS-CoV tested. In contrast, any combination of anti-S mAbs failed to yield high titres.

Che *et al.* [15] utilised three mouse anti-N mAbs as a capturing antibody and a rabbit polyclonal anti-N antibody as a detecting antibody, and were able to detect the recombinant N protein at 50 pg/mL. This assay system scored positive for approximately 84% of SARS patients serologically confirmed and 1.5% in 1272 healthy individuals [15].

Di *et al.* tested 829 serum samples from 643 patients and compared them to 197 control sera from healthy donors [57]. After the onset of symptoms, the N protein was detected by day 10 with a sensitivity of 100% in the case of 27 patients who had been positive with the neutralisation test. After 10 days, the N-protein level in the sera decreased and was undetectable beyond days 19. The specificity of the assay calculated from the

results of 66 serological test-negative patients and 197 healthy donors was reported to be 100%. The positive rate, 96–100%, was obtained from the sera at day 3–5 after the onset of symptoms for 27 neutralisation-test positive SARS patients and 298 serologically confirmed patients.

Lau *et al.* reported the development of polyclonal antibody-based ELISA for the N protein by utilising guinea pig and rabbit antibodies against recombinant His₆-tagged SARS-CoV N-protein [58]. They tested nasopharyngeal aspirates, urine and faecal samples, and detected N protein in the nasopharyngeal aspirate samples (day 6–24 after the onset of the disease), in urine samples (day 11–31) and in faecal samples (day 8–32), though the sensitivity was not very high except for the day 11–15 nasopharyngeal aspirates (83% sensitivity).

Apart from an antigen-capture detection system, the high-throughput assay for anti-SARS-CoV IgG antibody detection system, DETECT-SARS™, was reported to be highly sensitive (95.9%) for convalescent serum samples [59]. These immunological detection systems would be necessary compliments of RT-PCR-based diagnosis, and any future improvement in these detection systems will certainly contribute to control the SARS pandemic.

IMMUNITY TO SARS-CoV AND ANIMAL MODELS (Table 3)

Immune responses have been studied using animal models including macaques, ferrets, cats, Golden Syrian hamsters and mice (review in [3]). However, these animal models showed only transient viral replication in the respiratory tracts without manifestation of the disease, except in ferrets and hamsters. Ferrets and hamsters develop pathological disorders in the lung and infected ferrets and cats transmit SARS-CoV to naïve counterparts housed together [60]. Thus, it seems that some aspects of the SARS-CoV infection in humans were reproduced in these animal models, but the disease entities themselves are quite different. For example, in humans, a rapid progression of pulmonary and intestinal complications is observed and almost 20% of SARS patients develop watery diarrhoea [61], which is not seen in animal models. Despite the different outcomes, animal models are still useful for vaccine development and evaluation.

While antibody responses in SARS patients have been studied extensively, we know little about T-cell responses in patients. Two A-2-restricted epitopes of the S2 domain were recognised by memory CD8⁺ T cells of SARS-CoV-infected patients [62]. However, a study using HLA-A0201 transgenic mice or *in vitro* primed PBMCs of healthy donors identified a slightly different dominant epitope in the S2 domain [63]. In mice, epitopes of CD8⁺ T cell were within the S1 domain [64]. Current strategies to elicit an effective immunity against SARS-CoV are listed in Table 3.

VACCINE STUDIES

Attenuated or inactivated SARS-CoV

Because we know little about the pathogenesis of severe acute respiratory syndrome associated with SARS-CoV infection, a live attenuated SARS-CoV vaccine will not be feasible as a candidate for a SARS vaccine. Instead, a whole inactivated virion will be the first choice. Mice subcutaneously or nasally injected with a whole virion, that had been inactivated either by UV-irradiation [56] or formalin [65–67], with or without a variety of adjuvant, induced a high level of antibodies against SARS-CoV. Surprisingly, we observed that the UV-inactivated virion *per se* elicited a considerably high level of serum IgG-type neutralising antibody without an alum adjuvant [56]. Furthermore, the level of serum IgG antibody was retained at the peak for more than 6 months after a single injection, probably reflecting the generation and maintenance of long-term AFCs (antibody-forming cells, i.e. effector memory cells) [68]. No IgA response was elicited in mucosal tissues by subcutaneous injection, whereas a response was elicited in mice immunised nasally with inactivated virions by the aid of potent mucosal adjuvants, such as cholera toxin (CT) or CpG [66]. The Chinese government recently announced that they completed a phase I study of an inactivated virion vaccine, and that no serious complications were observed so far (<http://my.tdctrade.com/airnewse/index.asp?id=8856>).

Component vaccines

Virus vector-based vaccines: As described in the previous section, S and N proteins are two major viral proteins which induce a high level of antibody response. Current strategies to develop an effective vaccine generally rely on the induction

of potent neutralising antibodies on the mucosal surfaces. The study of attenuated parainfluenza virus suggest that immunisation with recombinant S proteins, but not E, M or N proteins, results in protection by eliciting neutralising antibodies, indicating the advantage of S antigen-expressing vaccines currently in development.

Likewise, the modified vaccinia virus Ankara (MVA) expressing the S protein (MVA-S) has been used successfully in mice [69], rabbits and monkeys [70]. MVA-S vaccination induced a high level of neutralising antibodies, which in turn results in protecting the monkeys from virus infection. In contrast, inoculation of attenuated vaccinia expressing the S protein into ferrets had no effect on the viral load and caused hepatitis upon challenge with SARS-CoV in MVA-S-immunised ferrets [71]. However, it remains unknown whether this has been caused by the mechanism of antibody-dependent enhancement of virus infectivity or some other immunopathological effect on the liver. In feline coronavirus infection, an antibody-dependent enhancement of the disease was observed in vaccinated animals with vaccinia vector [72]. Thus, such an unfavourable side effect of MVA-S vaccination has to be carefully considered for application.

Adenovirus type 5 vector expressing codon-optimised S, M or N was also developed as one of the candidate vaccines [73]. When six monkeys were immunised with these three recombinant adenoviruses, all developed antibodies against S1 fragments with neutralising activity. They also developed various levels of IFN- γ -producing T cells reactive to pooled 15-mer N peptides. However, no challenge experiment was carried out. Whether or not pre-existing immunity against adenovirus reduces the efficacy of recombinant adenovirus vaccine in humans needs to be further investigated.

Rhabdovirus-based vaccines were also developed, using a recombinant technology for rabies virus (RV) [74] and vesicular stomatitis virus (VSV) [75]. Both viruses, which encode the S protein in their genome, induced a high level of neutralising antibodies after a single injection into mice. In the case of VSV-based vector, pre-existing immunity is not a concern, and mice were protected from SARS-CoV infection by intranasal immunisation. RV can replicate in mucosal membrane cells and this is one of the advantages of

an RV-based vaccine. Intramuscular injection of RV-based vector expressing N protein did not elicit a neutralising antibody. Of note, the size of the S protein expressed by recombinant RV is smaller (~140 kDa) than that observed in other reports (~200 kDa) [10,69,75–77], suggesting that the S protein may not be modified by glycosylation during the replication of RV. In any case, the protective ability of the RV-based vaccine needs to be tested further.

DNA vaccine: DNA immunisation has been developed as a safe and stable vaccine technology for protection against a variety of infectious diseases. Since then, many efforts have been made to improve the efficiency of DNA vaccine, for example, by adding cytokines and costimulatory DNA adjuvants (review in [78]). Using modified codons to optimise expression, Yang *et al.* prepared plasmids encoding S protein and analysed their ability to elicit antiviral immunity in mice [79]. Both CD4⁺ and CD8⁺ T cells reactive to overlapping S peptides were detected and these mice generated neutralising antibodies, which accounts for their protection from respiratory infection. Depletion of both CD4⁺ and CD8⁺ T cells or adoptive T-cell transfer of immunised T cells did not affect protective immunity, whereas passive transfer of IgG from immunised mice provided immune protection. This study, along with that of attenuated parainfluenza virus [76], indicated that humoral immunity against S protein alone can confer protection. However, since SARS-CoV replicates in mice only transiently, it remains to be elucidated whether T-cell immunity is also important especially when neutralising antibodies fail to clear the virus.

The other studies used the plasmid-expressing N protein, expecting that a high level of intracellular N protein expression may induce strong CTL responses [80–82]. In order to increase the efficiency, Kim *et al.* [80] linked the *calreticulin* (*CRT*) gene to the N gene, which is a Ca²⁺-binding, heat shock family protein abundantly present in the endoplasmic reticulum. When mice were injected three times with CRT/N DNA by a gene gun, humoral and T-cell immune response were enhanced and these immunised mice were protected from infection by recombinant vaccinia expressing N protein. This is the only approach so far which demonstrated the protective role of

N-reactive T cells by vaccination. However, it remains unknown whether or not N-reactive T cells can contribute to suppressing the progression of SARS during natural infection.

Lastly, a combination of DNA vaccine with recombinant S protein [83] or whole-killed virus [84] were designed, with the expectation that this regimen may enhance not only the titre of neutralising antibody but also T-cell response, though the importance of the latter for protection is not yet clear.

Recombinant proteins for vaccination: Because the S protein in SARS-CoV is as heavily glycosylated as is the gp120 of HIV, the large scale production of recombinant protein is economically difficult. Nevertheless, a component vaccine has a great advantage with respect to safety, if it is effective enough. A baculovirus system was developed by Bisht *et al.* and they produced soluble recombinant polypeptide containing an N-terminal segment of the spike glycoprotein (nS) [85]. When mice were injected subcutaneously three times with nS with a Ribi (MPL + TDM) adjuvant, they were protected from SARS-CoV infection 4 weeks later. It would be interesting to study whether the neutralising antibody elicited by this protein subunit vaccine is sustained and is able to protect susceptible animals from disease.

CONCLUSION

The mAbs against the structural proteins of SARS-CoV have been rapidly established and are being applied not only for basic research but also in clinical practice, including SARS patient therapy. Studies utilising these antibodies provide us enormous information with respect to the immune responses elicited by the SARS-CoV and for the development of vaccines against the SARS-CoV. What we learned from the emergence of SARS will be potently useful for preparing against any future outbreaks of new pathogens likely coming from wild animals.

ACKNOWLEDGMENT

We thank a member of the Department of Immunology, who shared this work regarding mAb establishment and basic SARS-Co vaccine development. We are also grateful to Mr. Hirotaka Takagi for preparing cell lines infected with human coronaviruses. This work was supported

in part by grants from the Ministry of Health, Labour and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science and Technology of Japan..

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