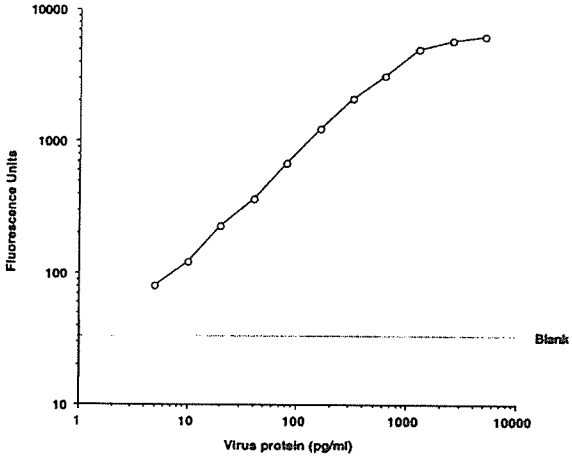

Fig. 5. Antigen-capture ELISA of SARS-CoV with mAbs. Anti-N mAb, SKOT-8, was immobilized on the surface of a 96 well plate. Serially-titrated purified SARS-CoV fractions were reacted for 1 h at room temperature and the bound virus proteins were detected by biotinylated SKOT-9 (anti-N) antibody followed by peroxydase-labeled streptavidin. They were then quantitated by chemiluminescent reaction using 4-methylumberiferril as a substrate. Abscissa: concentration of purified SARS-CoV proteins; ordinate: fluorescent unit.

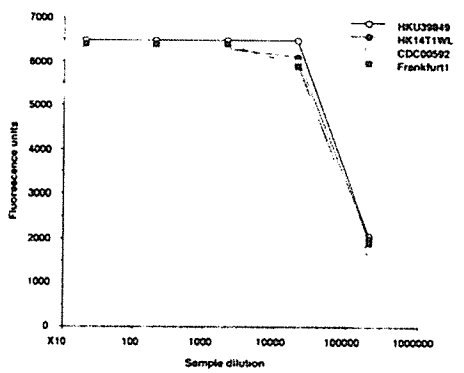

Fig. 6. Comparison of SARS-CoV strains for reactivity to the antigen capture sandwich ELISA. SARS-CoV strains, HKU39849, HK14T1WL, CDC00592 and Frankfurt1 were tested for the reactivity to the antigen-capture ELISA system as described in Fig. 5. Abscissa: sample dilution; ordinate: fluorescent units.

the detection of virus S- and N-proteins by means of immunofluorescence assay, immunohistochemistry, Western blot and antigen-capture ELISA. A summary of selected mAbs is shown in Table 3.

Among the originally selected 42 mAbs that were positive in ELISA for SARS-CoV infected Vero E6 cell lysate, 26 reacted to recombinant-S-protein and only three reacted to N-protein. We could not find hybridoma secreting mAb to M-protein or other protein components of SARS-CoV. These results suggest that S protein is the dominant target in the antibody response. We observed that none of the anti-S mAbs established worked in Western blot, suggesting that these mAbs may recognize 'conformational' epitopes. In contrast, all three anti-N mAbs worked in Western blot and immunohistochemistry, suggesting that these mAbs recognize 'linear' epitopes.

We examined whether our mAbs were applicable for immunofluorescence detection of virus-infected cells. In immunofluorescent staining of Vero E6 cells infected with SARS-CoV, anti-N mAbs stained the Golgi body and anti-S mAbs stained the Golgi body and surface membrane. This difference in localization of N- and S-proteins may reflect the common assembly process of coronaviruses (21). Further analysis is needed to clarify sensitivity and specificity in infected cells for clinical use.

During the course of outbreak of SARS-CoV in Hong Kong, it was reported that more than half the patients were not positively diagnosed by RT-PCR (8). Therefore, the diagnosis was finally confirmed by serum specimens in a convalescent-phase at a late stage of illness (8). To overcome this problem, virus shedding patterns have been extensively analyzed, with results showing that respiratory shedding of the virus increases over the first week and viral shedding in stools begins a few days after respiratory shedding (7,8). From this analysis, it is considered that a combination of stool sampling and pooled throat and nasal swab specimens could be good specimens for safe and highly sensitive SARS-CoV detection.

In general, a single diagnostic test is not conclusively reliable, because of the serious potential for false positives and negatives. Considering the limited sensitivity of RT-PCR, serological screening systems other than antibody detection are currently being examined (22,23). ELISA-based antigen captured assays are known to offer high specificity and reproducibility. Antigen-captured assays have been used in the diagnosis and monitoring of disease in cases of infection with dengue virus (24), human immunodeficient virus p24 (25) and Ebola hemorragic fever (26) and examined in hepatitis B virus and hepatitis C virus (22,23). In this context, extensive analysis in Ebola hemorragic fever suggests that the RT-PCR assay is extremely useful, but should always be utilized in combination with antigen-captured ELISA, which makes the diagnosis more reliable (26).

Table 3. Summary of selected hybridoma clones

Clone	Epitope	Class	IFA	Neutralization[1]	Western-blot	Histology	Avidity (%)[2]
SKOT-7	N	IgG	Golgi	-	50kDa	-	1.2
SKOT-8	N	IgG	Golgi	-	50kDa	Usable	3.2
SKOT-9	N	IgG	Golgi	-	120, 50kDa	Usable	3.8
SKOT-3	S	IgG	Golgi / cell membrane	100	-	-	18.7
SKOT-10	S	IgG	Golgi / cell membrane	10	-	-	29.9
SKOT-19	S	IgG	Golgi / cell membrane	1	ND	ND	ND
SKOT-20	S	IgG	Golgi / cell membrane	1	-	-	35.4
SOAT-5	S	IgG	Golgi / cell membrane	ND	-	-	48.0
SOAT-13	S	IgG	Golgi / cell membrane	ND	-	-	62.0

[1]: Numbers represent minimum concentration (μg/mL) that exerts neutralization. -, no neutralization activity; ND, not determined.
[2]: Avidity index at 6M urea (see Materials and Methods).

In the case of SARS-CoV, the assay has been recently evaluated by using mAbs and polyclonal antibodies directed against recombinant SARS-CoV nucleocapsid protein (22,23). A soluble N-protein was observed to be released from infected cells in culture, which led to the opportunity to evaluate the level in serum specimens from infected patients. N-antigen ELISA employing mAbs reproducibly detected 50% of patients on days 3 and 5 after the onset of illness, with a limitation of the detection of the recombinant protein at 50 pg/ml (22). N-antigen ELISA with use of polyclonal antibodies detected 60-50% of nasopharyngeal aspirate and fecal specimens from patients at day 3 to day 24 after the onset of illness, although the signal was relatively weak in fecal samples (22). These results suggest that antigen-captured assay could be useful for the early diagnosis of SARS-CoV infection.

We developed an antigen-capture ELISA system that detects purified SARS-CoV virion at levels as low as 40 pg/mL. The sensitivity of the system, which comprised two anti-N mAbs, seems high enough to detect virus protein in patient sera when compared to a recently reported antigen-capture ELISA system, which detects 100 pg/mL of purified recombinant N protein, successfully determined the virus protein in patient sera (22). We are now improving the sensitivity of the system and checking its applicability in the diagnosis and monitoring of SARS-CoV infection. Although none of our mAbs cross-reacted to human or other animal coronaviruses (229E, TGEV and MHV) by ELISA, it is also important to define the specificity of these mAbs by other techniques such as Western blot and immunofluorescent staining. This issue is currently under investigation.

Two anti-S mAbs, SKOT-19 and -20 demonstrated significant virus neutralizing activity. It would be interesting to address whether these mAbs interfere with the binding of the virion to its recently reported receptor, ACE2 (27). If this were the case, the humanization of these mAbs by means of either CDR-grafting or mouse-human chimeric antibody would be of interest as a possible application for the therapeutic use of these mAbs.

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The development of vaccines against SARS corona virus in mice and SCID-PBL/hu mice

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Abstract

We have investigated to develop novel vaccines against SARS CoV using cDNA constructs encoding the structural antigen; spike protein (S), membrane protein (M), envelope protein (E), or nucleocapsid (N) protein, derived from SARS CoV. Mice vaccinated with SARS-N or -M DNA using pcDNA 3.1(+) plasmid vector showed T cell immune responses (CTL induction and proliferation) against N or M protein, respectively. CTL responses were also detected to SARS DNA-transfected type II alveolar epithelial cells (T7 cell clone), which are thought to be initial target cells for SARS virus infection in human. To determine whether these DNA vaccines could induce T cell immune responses in humans as well as in mice, SCID-PBL/hu mice was immunized with these DNA vaccines. As expected, virus-specific CTL responses and T cell proliferation were induced from human T cells. SARS-N and SARS-M DNA vaccines and SCID-PBL/hu mouse model will be important in the development of protective vaccines.

Keywords: SARS DNA vaccine; SCID-PBL/hu; Human CTL

1. Introduction

The causative agent of severe acute respiratory syndrome (SARS) has been identified as a new type of corona virus, SARS corona virus (SARS CoV) [1–3]. SARS has infected more than 8400 patients in about 7 months in over 30 countries and caused more than 800 deaths. The deadly epidemic has had significant impacts on many health, social, economic and political aspects. SARS is assumed to resurge in the near future. However, no SARS vaccine is currently available for clinical use. Therefore, we have developed novel vaccine can-

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didates against SARS CoV using cDNA constructs encoding the structural antigens; S, M, E, or N protein. In immunized mice, neutralizing antibodies against the virus and T cell immunity against virus-infected-cells were studied, since these immunities play important roles in protection against many virus infections. In particular, $CD8^+$ CTL plays an important role in T cell immunity dependent protection against virus infections and the eradication of murine and human cancers [4,5]. In the present study, a type II alveolar epithelial cell clone, T7, was used for analyzing precise mechanism of CTL against SARS CoV membrane antigens, as the SARS-CoV infects alveolar epithelial cell in the lungs [6]. Furthermore, the SCID-PBL/hu model, which is capable of analyzing in vivo human immune response, was also used because it is a more relevant translational model for human cases [4].

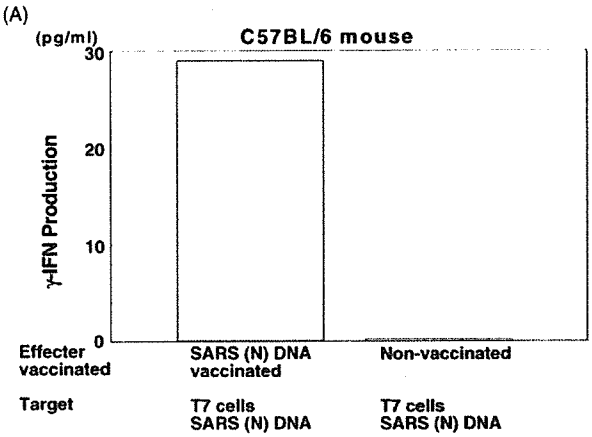
2. Materials and methods

Three kinds of SARS CoV strains: HKU39849(1), TW-1 and FFM-1(2) and their cDNAs were used. S, M, N or E cDNA was transferred into pcDNA 3.1(+) vector and pcDNA 3.1(+)/vs-His Topo (QIAGEN K K, Tokyo, Japan). These genes were expressed in eukaryotic cells and *Escherichia coli*. pcDAN 3.1(+) vector, 50 μg each, containing SARS S, M, N, or E DNA was injected i.m. (M.tibia anterior) into C57BL/6 mice (female, 8 weeks CLEA Japan Inc, Japan) and BALB/c mice (female, 8 weeks) three times, at an interval of 7 days. Neutralizing antibodies against SARS CoV in the serum from the mice immunized with SARS S, M, N or -E DNA vaccines were assayed by use of Vero-E6 cell. CTL activity against SARS CoV was studied using human type II alveolar epithelial cells, T7, expressing SARS antigens [6]. PBL from healthy human volunteers were administered i.p. into IL-2 receptor γ-chain disrupted NOD SCID mice [IL-2R(−/−) NOD-SCID], and SCID-PBL/hu mice were constructed [4]. SARS DNA vaccines at 50 μg were injected i.m. into the SCID-PBL/hu mice. CTL activity of human CD8-positive lymphocytes in the spleen from SCID-PBL/hu was assessed using IFN-γ production and 51Cr-release assay [4,5].

3. Results

3.1. Induction of CTL against SARS CoV by SARS (N) DNA and SARS (M) DNA vaccine

Spleen cells from C57BL/6 mice immunized with SARS-S, -M, -N or -E DNA vaccine were cultured with syngeneic T7 lung cells transfected with S, M, N or E cDNA. pcDNA 3.1(+) SARS (N) DNA vaccine induced significantly CTL activity (IFN-γ production) against N cDNA transfected T7 cells (Fig. 1A). Similarly, SARS M DNA vaccine induced SARS antigen M-specific CTL against T7 cells transfected with SARS M DNA (data not shown).



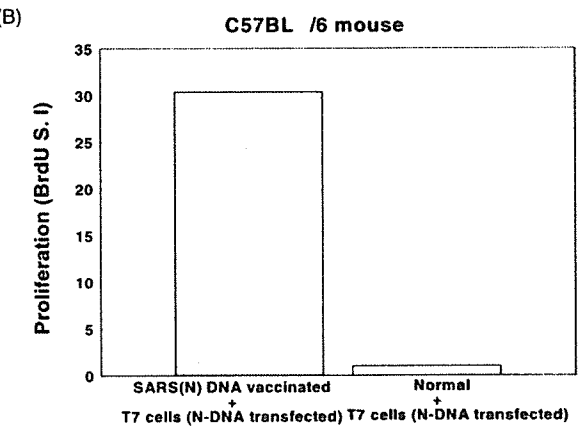


Fig. 1. Induction of CTL and T cell proliferation against SARS (N). (A) Induction of CTL against SARS (N) antigen in the spleen cells from C57BL/6 mice immunized with SARS (N) DNA vaccine. SARS (N) DNA using pcDNA3.1(+) vector was injected i.m. into C57BL/6 mice three times, at an interval of 7 days. CTL activity was assessed by IFN-γ production in the culture of 1×10^6 spleen cells and 1×10^4 T7 lung alveolar type II epithelial cells transfected with SARS (N) DNA at the E/T ratio of 100:1. IFN-γ production was assessed by ELISA assay. (B) Augmentation of lymphocyte proliferation specific for SARS (N) DNA vaccine. 1×10^5 responder cells from vaccinated mice were cultured with Mitomycin C treated 1×10^4 T7 cells transfected with SARS (N) DNA for 48 h and then Bromodeoxy Uridine (BrdU) was added. Proliferative responses were assessed by BrdU assay.

3.2. Augmentation of lymphocyte proliferation specific for SARS CoV antigens by the immunization with SARS (M) DNA and SARS (N) DNA vaccine

The proliferation of splenic T cells stimulated by co-culture either with T7 cells transfected with M DNA or SARS M peptide (TW1 M102-116) was strongly augmented by M DNA vaccine (data not shown). SARS N DNA vaccine also induced proliferation of splenic T cells in the presence of recombinant N protein as well as N DNA-transfected T7 cells (Fig. 1B). Thus, both SARS N DNA vaccine and M DNA vac-

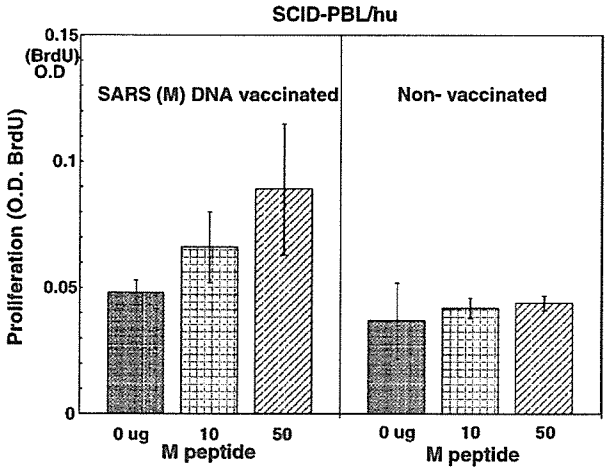


Fig. 2. SARS (M) DNA vaccine induces in vivo human T cell proliferation against SARS CoV in the SCID-PBL/hu human immune systems. 4×10^7 PBL from healthy human volunteers were administered i.p. into IL-2 receptor γ-chain disrupted NOD SCID mice [IL-2R (−/−) NOD-SCID], and SCID-PBL/hu mice were constructed. Fifty micrograms of SARS DNA vaccine was injected i.m. into these SCID-PBL/hu mice. 1×10^5 spleen cells from these vaccinated mice were cultured with 10~50 μg of SARS M peptide for 3 days. Proliferation was assayed by BrdU.

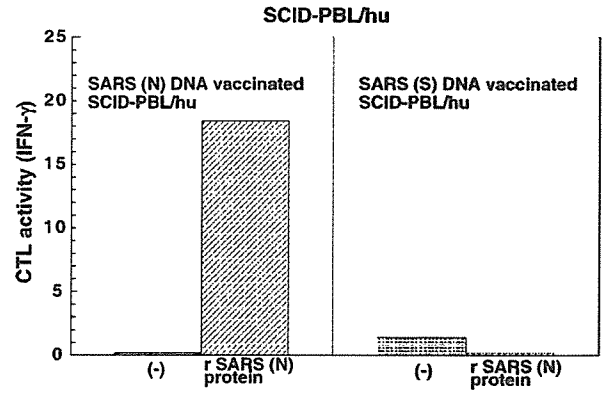


Fig. 3. SARS (N) DNA vaccine induces in vivo human CTL against SARS CoV in the SCID-PBL/hu human immune systems. 4×10^7 PBL from healthy human volunteers were administered i.p. into IL-2 receptor γ-chain disrupted NOD SCID mice [IL-2R (−/−) NOD-SCID], and SCID-PBL/hu mice were constructed 50 μg of SARS (N) DNA vaccine or 50 μg of SARS (S) DNA vaccine. 1×10^5 spleen cells from SCID-PBL/hu were cultured with 10 μg of recombinant SARS (N) protein for 72 h. IFN-γ production in the culture supernatant was assayed using ELISA.

cine were shown to induce T cell immune responses against the relevant SARS CoV antigens.

3.3. SARS M DNA and N DNA vaccines induced human T cell immune responses (CTL and proliferation) in SCID-PBL/hu model

The M DNA vaccine enhanced the CTL activity and proliferation in the presence of M peptide in SCID-PBL/hu mice (Fig. 2). Furthermore, the SARS N DNA vaccine induced CTL activity (IFN-γ production by recombinant N protein or N protein pulsed-autologous B blast cells) and proliferation of spleen cells in SCID-PBL/hu mice (Fig. 3). From these results, it was demonstrated that SARS M DNA vaccine and N DNA vaccine induced human CTL and human T cell proliferative responses.

4. Discussion

We have demonstrated that SARS (M) DNA and (N) DNA vaccines induce virus-specific immune responses (CTL and T cell proliferation) in the mouse systems using type II lung alveolar T cell lines in clone target models [6]. These DNA vaccines induced SARS-CoV-specific CTL and T cell proliferation in vivo human immune systems using SCID-PBL/hu. Gao et al. developed adenovirus based a SARS DNA vaccine encoding S1 polypeptide was capable of inducing neutralizing antibody, while another SARS DNA vaccine encoding N protein generated IFN-γ producing T cells in rhesus monkeys [7]. SARS S DNA vaccine which elicits effective neutralizing antibody responses that generate protective immunity in a mouse model [8]. However its immunogenicity in humans has yet to be established. Therefore, it is very important to evaluate the efficacy of SARS DNA vaccine in a SCID-PBL/hu mice, which is a highly relevant translational model for demonstrating human immune responsiveness. Recently, SARS DNA vaccines capable of inducing human neutralizing antibodies against SARS CoV have been established by our SCID-PBL/hu model. It has been demonstrated that Angiotensin-converting enzyme 2 (ACE2) is a functional receptor for the SARS CoV [9]. A transgenic mouse with human ACE-2 may be useful as an animal model of SARS. Furthermore, ACE-2 transgenic SCID mice should be useful as a human model for pre-clinical trial for SARS vaccines, since ACE-transgenic SCID-PBL/hu model could analyze the human immune responses against SARS infection in vivo. The effect of combination immunization with such SARS vaccines and neutralizing antibody dependent DNA vaccine is now being studied. These DNA vaccines should provide a useful tool for development of protective vaccines.

Acknowledgements

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Receptor-Independent Infection of Murine Coronavirus: Analysis by Spinoculation

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A highly neurovirulent murine coronavirus JHMV (wild-type [wt] JHMV) is known to spread from cells infected via the murine coronavirus mouse hepatitis virus receptor (MHVR) to cells without MHVR (MHVR-independent infection), whereas a mutant virus isolated from wt JHMV, srr7, spread only in an MHVR-dependent fashion. These observations were obtained by the overlay of JHMV-infected cells onto receptor-negative cells that are otherwise resistant to wt JHMV infection. MHVR-independent infection is hypothetically thought to be attributed to a naturally occurring fusion activation of the wt JHMV S protein, which did not occur in the case of srr7. Attachment of S protein on cells without MHVR during the S-protein activation process seems to be a key condition. Thus, in the present study, we tried to see whether wt JHMV virions that are attached on MHVR-negative cells are able to infect those cells. In order to make virions attach to the cell surface without MHVR, we have used spinoculation, namely, the centrifugation of cells together with inoculated virus at 3,000 rpm for 2 h. This procedure forces viruses to attach to the cell surface, as revealed by quantitative estimation of attached virions by real-time PCR and also facilitated wt JHMV infection to MHVR-negative cells, but failed to do so for srr7. Virions of both wt and srr7 attached on MHVR-negative cells by spinoculation were facilitated for infection in the presence of a soluble form of MHVR that induces conformational changes of both wt and srr7. It was further revealed that wt JHMV S1, but not srr7, was released from the cell surface when S protein was expressed on cells. These observations support the hypothesis that attachment of the virion to MHVR-negative cells is a critical step and that a unique feature of wt JHMV S1 to be released from S2 in a naturally occurring event is involved in an MHVR-independent infection.

The binding of virus to its specific receptor on a susceptible cell surface is an initial event in viral infection. A number of molecules classified into the immunoglobulin (Ig) superfamily are known to serve as receptors for various viruses. For example, CD4, signaling lymphocyte-activation molecule (SLAM or CD150), and a cell adhesion molecule in a carcinoembryonic antigen (CEACAM1 or MHVR) are identified as functional receptors for human immunodeficiency virus (HIV) (7, 22), measles virus (43, 46) and murine coronavirus mouse hepatitis virus (MHV), respectively (12, 45). However, additional alternative molecules are thought to be used as a receptor for HIV and measles virus, since they are known to infect cells lacking their specific receptors, CD4 or SLAM (4, 21).

MHV is classified as one of the *Coronaviridae*, consisting of an enveloped virus with a single, positive-stranded genomic RNA of about 31 kb (26). The characteristic spikes on the virion surface are composed of spike (S) protein. S protein is a class I fusion protein of 180 to 200 kDa in molecular mass (5, 26). It is synthesized as a large protein and cleaved by cellular protease into two subunits, N-terminal S1 and C-terminal S2 (37). The N-terminal region of S1 consisting of 330 amino acids (S1N330) is responsible for receptor binding (25, 38), and S2 is not involved in this activity (39). After receptor binding, S1 is dissociated from the membrane-anchored S2 subunit, which triggers the conformational changes of S2 to acquire fusion activity (28). The cell entry mechanism of MHV is very similar to that of HIV proposed by Chen and Kim (6). Likewise, the structural features of MHV S2 subunit are very similar to those of the membrane-anchored subunit of HIV envelope protein gp41. They both have two heptad repeats (HR) upstream of the transmembrane domain (6, 10) that play a crucial role in fusion of viral and cell membranes by forming so-called "six-helix bundles" (5, 6, 10, 14).

The major receptor for MHV, CEACAM1 or MHVR, is composed of four Ig-like ectodomains, a transmembrane domain (TM), and a cytoplasmic tail (Cy) (3, 12). There are four isoforms, two having four immunoglobulin-like domains and the other two having Ig-like domains, one of which has either a long or a short Cy (3). The N-terminal (N) domain is responsible for virus binding (13), induction of S protein conformational changes, and activation for fusion (30), although the N domain linked with TM and Cy and expressed on the cell surface is not functional, which is presumably due to inaccessibility for MHV to short molecules expressed on the cell surface (13, 30).

Although MHVR is a critical receptor for MHV, a highly neurotropic strain of JHMV was revealed by Gallagher et al. (17) to spread from DBT cells initially infected in an MHVR-mediated manner to MHVR-negative BHK cells via DBT-BHK cell fusion, when infected DBT cells were overlaid onto a BHK monolayer (called MHVR-independent fusion/infection by an infected-cell overlay test) (17). We have isolated

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from wild-type (wt) JHMV with this activity, a mutant virus resistant to neutralization by soluble receptor (srr7) that lacks this unique feature of infection (34, 42). Recent studies suggested that the high neurovirulence of wt JHMV was due to the MHVR-independent infection (18, 31).

The mechanism of this MHVR-independent infection and fusion is still speculative, although some unusual feature of wt JHMV S, but not srr7 S, hints that there is a possible mechanism of this unique mode of infection. wt JHMV S1 is reportedly removed from membrane-anchored S2 (23), and the conformational changes in the S2 take place (28). Both of these activities occur naturally, without binding to MHVR. These findings suggest that the spontaneous release of S1 from S2 triggers the conformational changes of the S2 in a similar fashion, since it occurs after binding its receptor. The fusion peptide in S2 could penetrate into the membrane without MHVR, if S protein is attached on the membrane when S1 is being detached from S2. Expectedly, virus inoculation onto BHK cells does not permit virus infection, whereas JHMV-infected DBT cells overlaid onto BHK cells permits the infection, since S proteins on infected DBT cells are attached onto BHK cells, whereas virion S protein fails to attach to the cell membrane because of the absence of the receptor that binds the virus and cell membrane.

If the hypothesis described above is correct, wt JHMV can infect a cell without a receptor under conditions whereby the virion is forced to attach to the cell surface. Thus, the present study is undertaken to attach virions onto the cell surface without MHVR expression. To achieve such conditions, we have used spinoculation, which enabled or facilitated virion attachment onto the cell surface by spinning cells, as well as inoculated viruses for retroviruses (8, 15, 32). We show here that spinoculation enabled wt JHMV and srr7 attachment onto the cell membrane, while it facilitated the infection of wt JHMV, but not that of srr7, into MHV receptor-negative cells. In addition, S1 of wt JHMV but not srr7 S1 was revealed to be free from S2 when S protein was expressed on BHK cells. These results are in good agreement with the hypothesis described above.

MATERIALS AND METHODS

Cells and viruses. The MHVR-positive cell line DBT (mouse delayed brain tumor) and the MHVR-negative BHK (baby hamster kidney), VeroE6 (African green monkey kidney), and HeLa (human cervical cancer) cell lines were maintained in Dulbecco minimum essential medium (DMEM; Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS; Tissue Culture Biologicals, California). A highly fusogenic MHV strain, MHV-JHM cl-2 (defined as wt JHMV) (40), and a soluble receptor-resistant mutant derived from wt JHMV, srr7 (34), were amplified and assayed on DBT cells as described previously (42). Viral infectivity was shown as PFU. srr7 has a single amino acid mutation at position 1114 (Leu to Phe) of the S2 subunit of the S protein relative to wt JHMV (34).

Spinoculation. Spinoculation, with modifications, was done according to the previous reports on retroviruses (8, 15, 32). BHK and DBT cells were seeded at a concentration of 5×10^5 cells in 0.5 ml per well of a 24-well culture plate (Falcon, Franklin Lakes, NJ). After 6 h of incubation, cells were washed once with phosphate-buffered saline (pH 7.2) (PBS) and inoculated with viruses serially diluted by 10-fold in 300 μl of DMEM containing 0.5 μg of concanavalin A (ConA; Wako, Osaka, Japan)/ml. The plates were centrifuged at 3,000 rpm (1,750 × *g*) for 2 h at 4°C. As a control, cells in 24-well plates were inoculated with virus in 50 μl of DMEM and cultured for 1 h at 4°C without centrifugation. After infection, cells were washed three times with ice-cold PBS and incubated with DMEM supplemented with 1% FBS for further 14 h at 37°C. Cells were then fixed and stained with crystal violet, and the syncytia were counted under light microscopy.

In some experiments, a soluble form of MHVR (soMHVR) was added into the culture after spinoculation of viruses as described above. For this experiment, soMHVR1 (1) containing N domain alone of the MHVR (30), which was revealed to have a receptor functionality indistinguishable from soluble MHVR composed of two ectodomains (30), was utilized, after being expressed by recombinant baculovirus and purified by using its tag as described previously (42).

Quantitative estimation of viral RNA by real-time PCR. BHK cells prepared in 24-well plates were inoculated with 10^5 PFU (corresponding to 1.1×10^7 and 1.4×10^7 copies for wt JHMV and srr7, respectively) of viruses/well and then incubated with or without centrifugation as described above. Then, cells were washed three times with PBS, and total cell-associated RNAs were extracted by using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. Five micrograms of yeast tRNA (Sigma, St. Louis, MO) was added as a carrier. The real-time reverse transcription-PCR to estimate the amount of viral RNA was performed as described previously for SARS-CoV mRNA detection (29) with slight modification. A series of reactions were performed by using LightCycler RNA Master mix (Loche Diagnostics, Mannheim, Germany). The target sequence was set at the inside of the N protein. The nucleotide sequences of the forward and reverse primers were 5′-TGTCTTTTGTTCCTGGGCA-3′ and 5′-CAAGAGTAATGGGGAACCA-3′, respectively. To detect the amplified fragments, we used hybridization probes labeled with fluorescence dye, 5′-GCTCCTCTGGAAACCGCGCTGGTAATGG-3′ (3′fluorescein isothiocyanate labeled), and 5′-ATCCTCAAGAAGACCACTTGGGCTGACCAAAC C-3′ (5′LCRed640 labeled). These oligonucleotides were synthesized according to the MHV-JHM N gene sequence (36). PCR analysis was performed under the following conditions (reverse transcription, 61°C for 20 min; PCR, 95°C for 30 s; 40 cycles for 95°C for 5 s, 55°C for 15 s, and 72°C for 13 s). To obtain a calibration line, DNA plasmids encoding target sequences were serially diluted and subjected to real-time PCR analysis. The relationship between the copy number of target sequence (*y* axis) and cycle of real-time PCR to reach a positive level (*x* axis) was obtained. The amounts of virus that attached onto cells were calculated from a calibration line obtained as described above.

Expression of S proteins and evaluation of S1 dissociation. MHV S proteins were expressed in BHK and HeLa cells by either transient expression by the transfection of expression plasmids or transient vaccinia virus expression system as previously reported (42). BHK cells were transfected by using FuGENE6 transfection reagent (Roche) with the plasmids containing wt JHMV or srr7 S gene under the control of the cytomegalovirus and T7 promoters pTarget/cl2-S or pTarget/srr7S (27). These cells were cultured for 40 h after transfection, and the expressed S protein was analyzed by flow cytometry as described below. As for the S-protein expression by vaccinia virus, BHK cells were infected with vTF7.3, a recombinant vaccinia virus harboring the T7 RNA polymerase gene (16) with a multiplication of infection of 5 and incubated at 37°C for 1 h. The cells were then treated with trypsin and transfected with plasmid pTarget/cl2-S or pTarget/srr7S (27) by electroporation using a GenePulser (Bio-Rad, Hercules, CA). Cells were then cultured in DMEM containing 5% FBS, and the culture medium was replaced with fresh medium 3 h after transfection. After an additional 12 h of incubation, the culture supernatants and cells were separately harvested. To detect the released S1 protein, culture supernatants were centrifuged at 9,000 × *g* for 1 min to clarify the cell debris, and a mixture of anti-S monoclonal antibodies (MAbs 2, 3, and 7) (24) and protein G-Sepharose (Amersham Bioscience, Arlington Heights, IL) was added, followed by further incubation at 4°C for 4 h with gentle rotation. After five washes with PBS, an aliquot of 2× sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-Cl [pH 6.8], 4% SDS, 200 mM dithiothreitol, 20% glycerol; volume equal to protein A-Sepharose) was added, and the lysates were subjected to Western blot analysis. To detect S proteins expressed in cells, cells infected with vTF7.3 and transfected as described above were washed twice with PBS and lysed with cell lysis buffer (PBS containing 10% glycerol and 1% Triton X-100). After centrifugation at 9,000 × *g* for 1 min, soluble fractions were mixed with 2× SDS sample buffer as described above.

Western blot analysis. S proteins of wt JHMV and srr7 were prepared as described above and analyzed by Western blotting as described previously (27, 34). Briefly, each sample was separated by SDS–7.5% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). S proteins on the membrane were reacted with anti-S1 (11F) or anti-S2 (10G) MAb (33) and subsequently with anti-mouse immunoglobulin G (IgG) serum labeled with horseradish peroxidase as described elsewhere (27, 34). The bands were then visualized by using Supersignal West Dura (Pierce, Rockford, IL) with LAS-1000PLUS (Fujifilm, Tokyo, Japan).

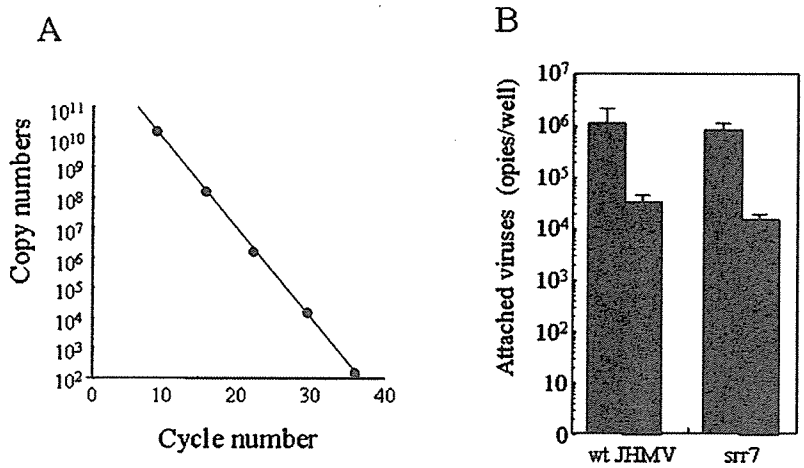

FIG. 1. Quantitative estimation by real-time PCR of virus attached onto cells after spinoculation: (A) Calibration in real-time PCR. Control DNA encoding the target sequence was subjected to real-time PCR. The relationship between the copy numbers of target sequence (*y* axis) and the cycles of real-time PCR to reach a positive level (*x* axis) is shown. (B) Effect of spinoculation on virus binding. BHK cells were spinoculated (black column) or inoculated by ordinary methods (shaded column) with 10^5 PFU of wt and srr7 as described in Materials and Methods. Cell-attached virus copies were estimated by real-time PCR using a total RNA extracted from infected cells. They were calculated from the calibration line shown in panel A. Error bars represent the standard deviations of the results of three independent experiments.

Indirect immunofluorescence assay (IFA). To examine viral antigens in cells infected by spinoculation, cells spinoculated as described above were fixed with acetone-methanol (1:1) for 2 min. After three washes with PBS, cells were incubated with anti-S MAbs (a mixture of MAbs 2, 3, and 7) (24) for 1 h at 37°C. After three washes with PBS, cells were further incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Zymed Laboratories, California).

Flow cytometry. BHK cells transfected with pTarget/cl2-S or pTarget/srr7S as described above were examined for the S protein expression on cell surface by flow cytometric analysis with cytofluorometer FACSCalibur (Becton Dickinson, San Jose, CA). Transfected cells were treated with PBS containing 0.05% EDTA to prepare a single cell suspension and collected into microtubes. The cells were fixed with 4% paraformaldehyde at 4°C for 10 min; reacted with an aliquot of 1:200-diluted anti-S MAbs 2, 3, and 7 for 1 h on ice; and washed with PBS containing 1% FBS. The cells were then incubated with 1:200-diluted anti-mouse IgG (H+L) conjugated with phycoerythrin (Jackson Immunoresearch, West Grove, PA) for 1 h on ice. After three washes with PBS, the fluorescence intensity was analyzed with FACSCalibur.

RESULTS

Enhancement of viral attachment onto MHVR-negative cells by spinoculation. Spinoculation has been reported to force virions attached onto cell membrane for retroviruses (8, 15, 32). We first tested whether the spinoculation applied to those viruses can enhance the attachment of MHV onto MHVR-negative cells. To enhance the adsorption efficiency, Polybrene or DEAE-dextran was included in the medium, which was shown to not enhance JHMV infection (data not shown). Instead, ConA was revealed to enhance the adsorption efficiency. Finally, we spinoculated wt JHMV and srr7 onto BHK cells prepared in 24-well plates in 300 μl of DMEM containing 0.5 μg of ConA/ml/well and spun the sample at 3,000 rpm for 2 h at 4°C. After the cells were washed, the total RNA was extracted from them, and virus genome RNA copies were evaluated by real-time PCR according to the calibration line obtained using cloned DNA of target sequence (Fig. 1A). As shown in Fig. 1B, spinoculation under the above conditions augmented the virus attachment to MHVR-negative BHK by ~100-fold. There was no significant difference between wt JHMV and srr7 in the virus copies attached to MHVR-negative BHK by the spinoculation used in the present study. Unexpectedly, considerable amounts of both wt and srr7 attached onto BHK cells after ordinary infection, although the molecule(s) participating into this binding has not yet been identified.

Enhancement of MHVR-independent infection of wt JHMV by spinoculation. Since spinoculation was revealed to enhance the virus attachment onto cells for both wt JHMV and srr7, we then examined whether it enhances virus infection or not. BHK cells were spinoculated with either wt JHMV or srr7, and syncytium formation was examined at 14 h after incubation. As shown in Fig. 2, a large number of syncytia were produced after infection with wt JHMV but not at all after infection with srr7. To quantitatively examine the effect of spinoculation, we inoculated about 10^4 PFU/well of either wt JHMV or srr7, and the number of syncytia formed was determined. As shown in Fig. 3, spinoculation of wt JHMV augmented the infection on MHVR-negative BHK cells by 200- to 1,000-fold (with a mean value of ca. 800-fold), while it failed to facilitate the infection of srr7. wt JHMV infected BHK cells very inefficiently (2.75 syncytia per well after infection with 10^4 PFU) by standard procedures of infection (nonspinoculated and incubated at 4°C for 1 h). Although the enhancement was not so remarkable, spinoculation of both wt and srr7 enhanced their infection on MHVR-positive DBT cells by ca. 10-fold (Fig. 3).

We then examined whether spinoculation facilitates the wt JHMV infection in other MHVR-negative cells. We have tested HeLa cells and VeroE6 cells, as well as BHK cells, all of which are MHVR negative and resistant to infection to wt JHMV. All of these cell lines were infected by spinoculation with wt JHMV, as described for the infection to BHK cells, and syncytium formation was observed. As shown in Fig. 4, after phase-contrast microscopy no syncytium formation was found in two cell lines, HeLa and VeroE6 cells, whereas syncytia were

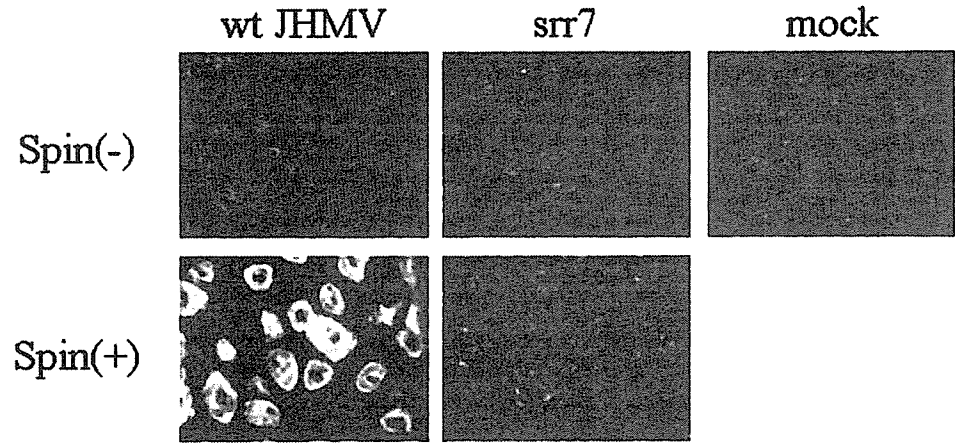

FIG. 2. Syncytium formation of BHK cells with wt JHMV infected by spinoculation: wt and srr7 (10^4 PFU) were spinoculated [spin (+)] or inoculated by an ordinary method [spin (−)] and cultured in DMEM containing 5% FBS for 14 h. Cells were fixed and stained with crystal violet. Mock-infected cells are shown as a control (mock).

clearly visible for infection of BHK cells. We then examined these cells by IFA to determine whether a small number of cells unrecognized by microscopy were infected or not. As shown in Fig. 4, tiny syncytia were detected by IFA on both HeLa and VeroE6 cells, showing that wt JHMV infected various cells without MHVR, when they are forced to attach on these cells by spinoculation. After the spinoculation of 10^5 PFU of wt JHMV, ca. 4×10^3, 3×10^3, and 8×10^2 syncytia were detected on BHK, HeLa, and VeroE6 cells, respectively. Srr7 failed to produce syncytium, and no virus antigen was detected by IFA after srr7 spinoculation in MHVR-negative cell lines (data not shown).

These results collectively suggest that wt JHMV infects MHVR-negative cells if it is forced to attach the cell surface. However, srr7 fails to infect cells in an MHVR-independent fashion, being consistent with our previous results obtained by the infected-cell overlay test.

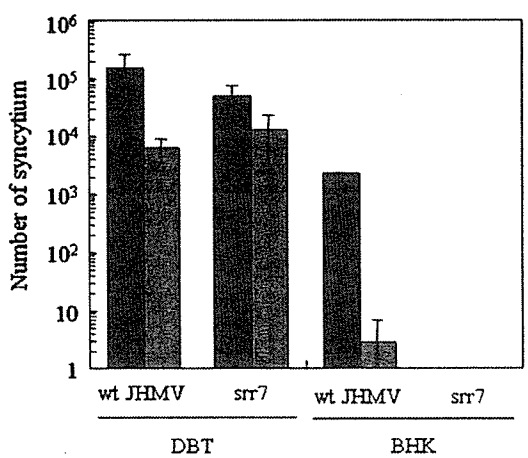

FIG. 3. Quantitative estimation of syncytium formation after spinoculation: DBT or BHK cells were infected by spinoculation (black column) or by ordinary methods (shaded column) with 10^4 PFU of wt JHMV or srr7. Cells were further incubated in DMEM containing 5% FBS for 14 h, and the number of syncytia was determined after staining with crystal violet. Error bars the represent standard deviations of the results of three independent experiments.

Facilitation of infection by spinoculation with soMHVR. Previous studies on the infected-cell overlay test have shown that MHVR-independent infection was facilitated by soluble MHVR (42). DBT cells infected with srr7 did not induce fusion on BHK cell monolayers. However, they induced fusion when cells were cultured in the presence of soMHVR. wt JHMV infection was also augmented by soMHVR in terms of the size of syncytia produced in an MHVR-independent fashion. These findings suggested that S protein adjacent to MHVR-negative target cells can be activated by the soMHVR (42). In the present study, we also addressed whether or not soMHVR enhances MHVR-independent infection. wt JHMV or srr7 infected by spinoculation, as described above, were incubated in the presence of 50 nM soMHVR (1), consisting of the N domain alone of MHVR (30). As shown in Fig. 5, soMHVR enhanced the spinoculated wt JHMV by about 30-fold. Although srr7 attached by spinoculation failed to infect cells without MHVR, soMHVR potentiated the srr7 infection, which shows that srr7 infection is solely dependent upon the presence of MHVR. These results with virions are similar to those found with MHVR-independent infection, when examined by the infected-cell overlay test.

Naturally occurring release of S1 of wt JHMV from the cell surface. S1 of JHMV with MHVR-independent infection activity has been described as being released in a naturally occurring fashion from the cell surface, but this result was not seen in JHMV-derived mutant viruses without this activity (23). Thus, we also examined wt JHMV and srr7, with and without MHVR-independent infection activity, respectively, to assess whether their S1 is released from S2 when expressed on cells. S protein of wt JHMV and srr7 was expressed in BHK cells by transfecting the plasmids harboring wt or srr7 S gene. The S proteins of both viruses were expressed in similar amounts and transported equally onto the cell surface, as revealed by flow cytometric analysis (Fig. 6A). However, S1 proteins were not clearly detected in the culture fluids of cells expressing these S proteins. We then expressed S protein with

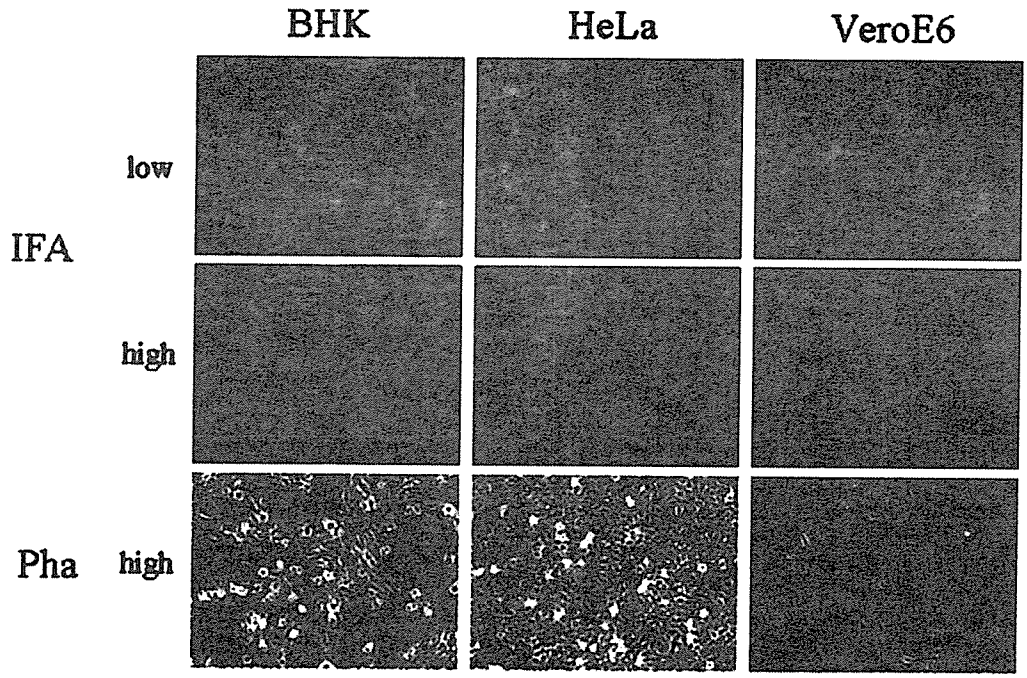

FIG. 4. MHVR-independent infection of wt JHMV in various cell lines by spinoculation: wt JHMV of 10^5 PFU was spinoculated onto cells without MHVR expression, BHK cells, HeLa cells, or VeroE6 cells, and incubated for 14 h. Syncytium formation was microscopically observed by phase-contrast microscopy (Pha) or by immunofluorescence (IFA) with anti-spike MAbs under low and high magnifications (high magnification = 2× low magnification).

the T7 vaccinia virus expression system, which normally permits higher expression of the protein. S1 or S2 subunits released in the culture fluids were detected by immunoprecipitation, followed by Western blotting with subunit specific MAbs, as described in Materials and Methods. MAbs used to precipitate S protein in the supernatant recognize epitopes located in the S1 and were shown to coimmunoprecipitate S2 that interacts with S1 in our previous study (24). It was revealed that large amounts of the S1 subunit of wt JHMV were released into culture supernatants of cells transfected with wt S expression plasmid (Fig. 6B). On the other hand, there was no release of S1 subunit in the supernatants of cells introduced with srr7 S expression plasmid. The S2 subunit of each strain was not released into the supernatant. The amounts of wt and srr7 S proteins expressed were not different, as demonstrated by Western blotting (Fig. 6B). These results agree with the observations by Krueger et al. (23) that S1 of MHV showing MHVR-independent infection is released in a naturally occurring event, whereas this phenomenon was not observed for those without such activity.

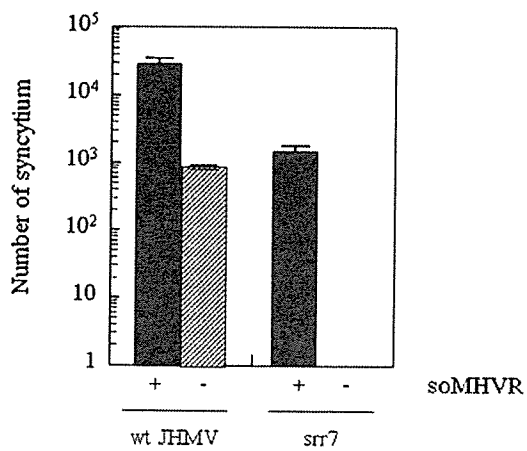

FIG. 5. Effect of soMHVR on the infection after spinoculation: BHK cells were infected by spinoculation with 10^3 PFU of wt and srr7, and infected cells were cultured for 14 h in DMEM containing 5% FBS in the presence (+) or absence (−) of soMHVR (50 nM final concentration). The number of syncytia was counted after staining with crystal violet. Error bars represent the standard deviations of the results of three independent samples.

DISCUSSION

Viral infection is generally restricted to cells that express the receptor molecule, with which the virus binds specifically and invades cells. Some viruses are known to infect cells on which a specific receptor molecule is not expressed, and these infections are generally not as efficient as the infection via an identified receptor. For example, HIV infects cells that do not express its receptor CD4 (4) and measles virus can infect cells lacking both SLAM and CD46, molecules identified as measles virus receptor (21). These infections are supposedly attributed to another cellular receptor (4, 21). MHV mutants derived from cells persistently infected with MHV-A59 strain or cells doubly infected with JHMV and MHV-A59 were reported to infect a variety of MHVR-negative cells derived from different

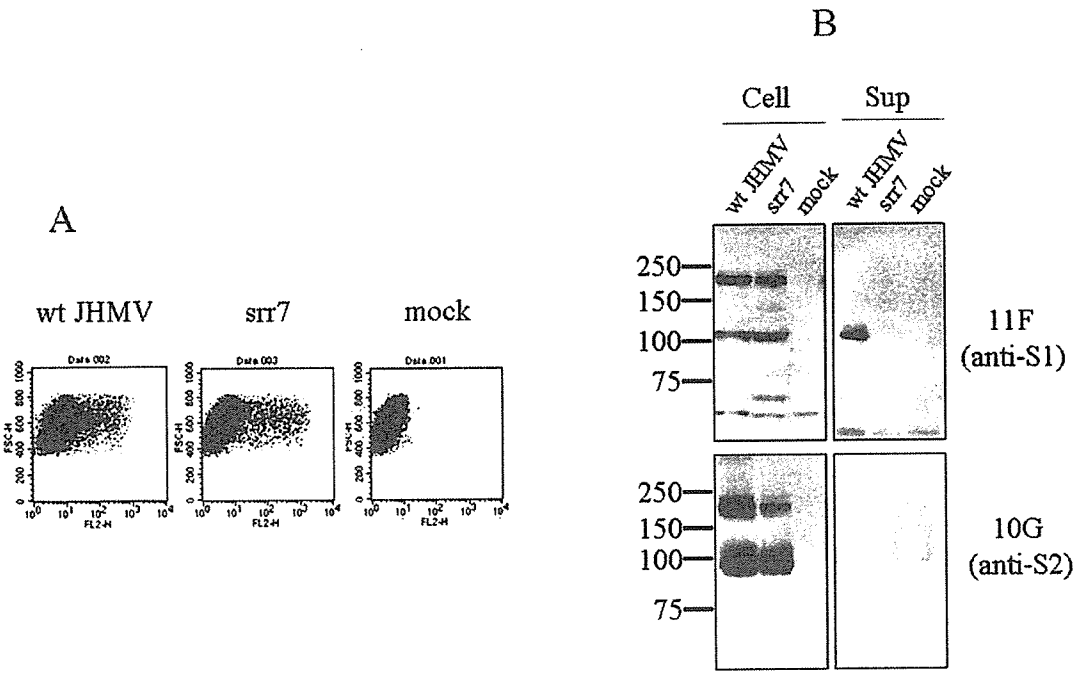

FIG. 6. Analysis of wt and srr7 S proteins expressed in BHK cells: (A) Expression of S protein on cell surface after transfection with expression plasmid containing S gene of wt or srr7. Cells were transfected with plasmid containing wt (wt) or srr7 (srr7) S gene. They were also transfected with plasmid containing no S gene (mock). The cells were harvested at 8 h after transfection and stained with anti-S MAbs and anti-mouse IgG antibody conjugated with phycoerythrin. Flow cytometric analysis was done with FACSCalibur, and the fluorescence intensity was analyzed by dot plot analysis. (B) Western blot analysis of the S protein in culture fluid of BHK cells transfected with S gene. Expression plasmids harboring either wt (wt) or srr7 (srr7) S gene, as well as plasmid alone (mock), were transfected into BHK cells previously infected with vTF7.3 for the S protein expression. S protein in culture supernatants (Sup) was immunoprecipitated by MAbs against S protein and analyzed by Western blotting with MAbs 11F and 10G specific for S1 and S2, respectively. S protein in cells (Cell) was analyzed by using total cell lysates.

species (1, 2, 35). In these cases, infections were induced by using standard protocols and did not require an infected cell overlay or spinoculation, suggesting that their infections are mediated by molecule(s) that function as an alternative receptor. Some of these MHV mutants were revealed to utilize human CEA as a receptor, although some other molecules were supposed to work as a receptor, since these mutants infected cells derived from nonhuman species as well (1). MHV mutant isolated from the above-described persistent infection was recently reported to obtain the ability to use heparan sulfate for entry receptor (11). In contrast, some strains of MHV are reported to spread in an MHVR-independent manner (41). For example, highly neurovirulent MHV-JHMV is able to spread from cells initially infected via its receptor CEACAM1 to CEACAM1-negative cells (17, 31). Although it is clear that MHVR is not involved in this MHVR-independent infection, no other specific molecules are supposedly involved, since this infection differs from those that use alternative, less-functional receptors, such as HIV, measles virus, or MHV mutant isolated from persistent infection (4, 11, 21) in that the infection is not accomplished after standard protocol of infection.

MHVR-independent infection had been restricted from cells initially infected via MHVR to MHVR-negative cells (infected-cell overlay test), whereas viruses failed to directly infect MHVR-negative cells when ordinary infection procedures were used. The proposed mechanism of MHVR-independent infection is that S protein adjacent to the cell membrane is activated for fusion in a naturally occurring manner, which allows it to induce a fusion of the viral envelope and cell membrane without receptor involvement. However, an infected-cell overlay test could not completely rule out the possible contribution of some cellular factors derived from initially infected MHVR-positive cells. Even the possibility is not thoroughly excluded that MHVR expressed on MHV-infected DBT cells plays a part in this infection. To explore the hypothesized mechanism of MHVR-independent infection described above and to determine whether there are possible cellular factors, we have performed spinoculation that was reported by retrovirologists to force virions to attach onto the cell surface (8, 15, 32). For spinoculation in the present study, cells were spun together with inoculated viruses in medium containing 0.5 μg of ConA/ml at 1,750 × *g* for 2 h at 4°C. This procedure enhanced virus attachment by ~100-fold, irrespective of the virus strains used, i.e., wt JHMV and srr7, with and without MHVR-independent infection activity, respectively. It also enhanced the infection of wt JHMV by more than 100-fold but not at all for srr7 infection, which displayed for the first time an MHVR-independent infection by using wt JHMV virions. Since our virus materials used for spinoculation could contain some components of infected cells, we excluded those materials by the purification of MHV with sucrose gradient centrifugation. These viruses also showed the similar enhanced infection by spinoculation (data not shown). These findings

collectively exclude the possible participation of some specific cellular factors into the MHVR-independent infection.

Although the data now available suggest the mechanism of MHVR-independent infection as described above, they fail to exclude the possibility that another receptor molecule is responsible for this infection. Cellular factors such as heparan sulfate or several chemical reagents were reported to function as a receptor (11, 19, 20). However, we think the possibility is quite low for the following reasons. A possible receptor, if there is one, has very weak receptor functionality; it is more than 10^4 and 10^5 less functional compared to CEACAM1 for wt JHMV and srr7, respectively. Actually, it does not function at all as a receptor for srr7. Moreover, such a receptor would permit the binding of wt and srr7 to almost the same extent (Fig. 1B) and be expressed ubiquitously in various species, since not only BHK cells derived from hamster but also HeLa and VeroE6 cells derived from human and the monkey are target cells for MHVR-independent infection. To finally exclude such a molecule to work as a receptor, liposome-binding of wt JHMV but not srr7 in the absence of soMHVR would be a convincing evidence.

The receptor molecule for enveloped viruses has at least two distinct, critical functions, namely, its ability to bind to the virus surface protein and its ability to activate fusion of the protein, which would result in the fusion of envelope and cell membrane, thereby permitting virus entry into the cell. Spinoculation can replace one of these functions, the binding of envelope protein to cell surface. After binding to the cell surface, virus surface protein is activated for fusion if a soluble form of the receptor interacts with the protein. This is evident for the S protein of srr7 that does not show MHVR-independent fusion activity. This feature is usually observed in other viruses, such as the retrovirus avian sarcoma leukosis virus (ASLV), which is able to infect cells lacking its receptor, if ASLV is allowed to attach those cells by spinoculation and soluble ASLV receptors are mixed in the culture (8).

To show that the MHVR-independent infection is not restricted in BHK cells, two other cell lines were used for spinoculation. Although remarkable syncytia were not produced, as seen in BHK cells, infection by wt JHMV of HeLa and VeroE6 cells was detected by IFA with anti-S antibodies. There are two possibilities to explain this difference in cytopathic changes caused by wt JHMV. One is that there are differences in the degree of replication among these cells after spinoculation. wt infection in BHK cells could produce more abundant S protein than in VeroE6 or HeLa cells. The other possibility is that the components of plasma membrane of these cell lines, especially the lipid composition, are varied. Cellular cholesterol was reported to influence replication efficiency and fusion formation ability of MHV (9, 44).

In conclusion, we showed in the present study that the virus with MHVR-independent infection activity is able to infect cells when it is attached to the target cell surface. However, the virus without this activity fails to infect cells, even if it is attached to the cell surface. It is also confirmed that S1 release from S2 in a naturally occurring event is one of the important features for the virus with MHVR-independent infection activity. These observations support the proposed mechanism of MHVR-independent infection; S protein attached to the MHVR-negative cell surface is activated for fusion of the virus-cell membrane, which is mediated by a naturally occurring dissociation of S1 from S2. However, the possibility that the cellular molecule is a receptor with a weak function for wt JHMV, but not for srr7, cannot be thoroughly excluded.

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Reviews in Medical Virology

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.

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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 to 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]

[a]MPL, monophosphoryl lipid A; TDM, trehalose dicorynomycolate.
[b]RBP-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.)	*In vitro* neutralization activity, 10^{-8}–10^{-11} M *In vivo* 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, *in vitro* neutralization	[35]
		In vivo protection in mouse model S1-genotyping co-related with 80R sensitivity	[89]
scFv, phage display screening on irradiated SARS-CoV whole virion	anti-S, -N (CR3014, CR3018, CR3009)	Epitope mapping, immunoelectron microscopy, IFA, *in vitro* 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 *in vitro*	[19]
Transgenic mice with human immunoglobulin gene (Medarex) immunized with recombinant S protein	2 clones (68, 201)	Epitope mapping, *In vivo* protection in mouse model	[21]

[a]scFv; 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.*

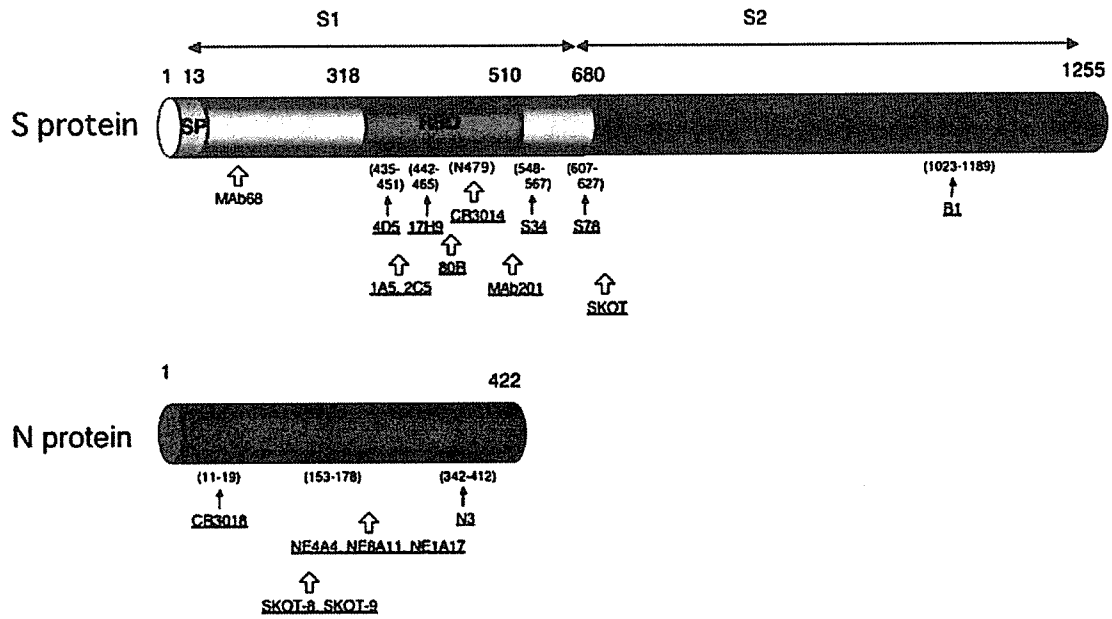

Figure 1. Schematic drawing of S- and N-protein epitopes and the name of the recognizing mAbs. Small arrows indicate that the epitopes are confined to the peptide fragment indicated in the figure. Large arrows indicate that the epitopes are nearly confined to the region indicated. The epitopes identified 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	
			Monkey	i.n versus i.m.	+	
		S	Ferret	i.p. or s.c. hepatitis+	No effect	[71]
	Adenovirus type5	S1, M, N	Monkey	Nab, N-peptide-reactive T	ND	[73]
		N	B6	N-reactive T	ND	[90]
		S	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,	BALB/c	Nab, no contribution of $CD4^+/CD8^+$ T cells	+	[79]
		SCID				
		N-linked to Calreticulin	B6	CTL N peptides	+? (N + Vaccina)	[80]
		N	BALB/c	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.