

Evaluation of Human Monoclonal Antibody 80R for Immunoprophylaxis of Severe Acute Respiratory Syndrome by an Animal Study, Epitope Mapping, and Analysis of Spike Variants

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In this report, the antiviral activity of 80R immunoglobulin G1 (IgG1), a human monoclonal antibody against severe acute respiratory syndrome coronavirus (SARS-CoV) spike (S) protein that acts as a viral entry inhibitor in vitro, was investigated in vivo in a mouse model. When 80R IgG1 was given prophylactically to mice at doses therapeutically achievable in humans, viral replication was reduced by more than 4 orders of magnitude to below assay limits. The essential core region of S protein required for 80R binding was identified as a conformationally sensitive fragment (residues 324 to 503) that overlaps the receptor ACE2-binding domain. Amino acids critical for 80R binding were identified. In addition, the effects of various 80R-binding domain amino acid substitutions which occur in SARS-like-CoV from civet cats, and which evolved during the 2002/2003 outbreak and in a 2003/2004 Guangdong index patient, were analyzed. The results demonstrated that the vast majority of SARS-CoVs are sensitive to 80R. We propose that by establishing the susceptibility and resistance profiles of newly emerging SARS-CoVs through early S1 genotyping of the core 180-amino-acid neutralizing epitope of 80R, an effective immunoprophylaxis strategy with 80R should be possible in an outbreak setting. Our study also cautions that for any prophylaxis strategy based on neutralizing antibody responses, whether by passive or active immunization, a genotyping monitor will be necessary for effective use.

Severe acute respiratory syndrome (SARS), is a highly communicable illness consisting of fever and respiratory symptoms that can progress to pneumonia, respiratory failure, and death. Infrequently, a subclinical or nonpneumonic form of the syndrome may also exist (6, 28). The disease emerged in southern China's Guangdong province in late 2002 and quickly spread in early 2003 to several countries in Asia, Europe, and North America. International public health measures led to the rapid identification of the etiologic agent, a novel coronavirus (SARS-CoV) and successful containment of the outbreak (8, 14, 21). During the winter of 2003/2004, four cases of SARS were reported in Guangdong and all patients recovered. With the exception of one case from this group, the epidemiologic link to SARS-CoV exposure has not been established. Laboratory-acquired SARS was responsible for two isolated cases in Taiwan (http://www.who.int/csr/don/2003_12_17/en/) and Singapore (12) and for the very recent outbreak in Beijing and Anhui province, China. The latter outbreak resulted in secondary and tertiary human-to-human transmissions, including one fatal infection (http://www.who.int/csr/don/2004_04_30/en/).

SARS-CoV is readily transmissible by close contact within households and the health care environment. Evidence of air-

borne transmission is now well documented (17, 34). However, in numerous human cases the exact mode of transmission cannot be established (29) and the reasons for heterogeneity of transmission, particularly superspreader events, remain unknown (13, 22). The role of wild animals as reservoirs for SARS-CoV has been suggested by the detection of SARS-like coronaviruses (SARS-like-CoVs) in the Himalayan palm civet (civet cat) and raccoon dog, which were tested from markets selling wild animals for human consumption. In addition, antibody studies in people working in these markets have shown that some have had prior infection with SARS-like-CoV (5). Moreover, a recent study confirmed that all new human cases in the resurgence of SARS in 2003/2004 in Guangdong were caused by independent and multiple interspecies transmissions from animals to humans (4). Although the mass culling of civet cats in Guangdong likely provided a temporary break in this chain, the virus reservoir has almost certainly not been eliminated.

Currently, prevention of SARS has largely relied on improved awareness, surveillance, and institution of local, regional and international public health care measures (23). Significant efforts in the area of SARS vaccine research have been initiated, and several recent reports have documented that transfer of immune serum from mice with prior SARS-CoV infection, or from mice vaccinated with a DNA plasmid encoding SARS S protein or a vaccinia virus expressing the S

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protein, can prevent virus replication in the lungs and upper respiratory tract (1, 24, 31). In addition, in SARS-CoV infection of humans, decreasing virus titers from nasopharyngeal aspirates, serum, urine, and stool have been observed to be coincident with the development of neutralizing antibodies (9, 19). Treatment of SARS with convalescent plasma has been reported (2, 27). These studies support the importance of humoral immunity in protection against SARS-CoV and suggest that a specific and effective human monoclonal antibody (MAb) should be developed to provide a prophylaxis and early treatment against SARS in the event that episodic or even widespread reemergence into the human population occurs.

We have recently isolated a high-affinity recombinant human MAb (80R) against the S1 domain of the SARS-CoV S protein, which acts as a viral entry inhibitor (25). Through blocking the association of S protein to its receptor ACE2 (11), 80R potentially neutralizes SARS-CoV infection in vitro. In the present study, we further investigated the prophylactic effectiveness of 80R immunoglobulin G1 (IgG1) in vivo in a mouse model of SARS. We also defined the unique features of the 80R neutralizing epitope, and an extended panel of 80R sensitivity and resistance markers has been established that will serve as a useful tool to guide the potential prophylactic use of this antibody in an outbreak setting.

MATERIALS AND METHODS

Mouse studies. All mouse studies were approved by the National Institutes of Health Animal Care and Use Committee and were carried out in an approved animal biosafety level 3 facility, and personnel entering the facility were powered air-purifying respirators (3M HEPA Air-Mate; 3M, Saint Paul, MN). Sixteen-week-old female BALB/c mice were housed four per cage. Mice were lightly anesthetized with isoflurane before receiving injections of antibodies. On day 0, three groups of mice ($n = 4$ for each) were intraperitoneally injected with three different doses of 80R IgG1 (500 μ l of 500 μ g/ml, 100 μ g/ml, and 20 μ g/ml of 80R IgG1 in phosphate-buffered saline [PBS]). The control group ($n = 4$) was injected with 500 μ l of 500 μ g/ml of a human IgG1 isotype control antibody in the same buffer as 80R IgG1. One day later, mice were challenged with 10^4 50% tissue culture infective doses (TCID₅₀) of SARS-CoV (Urbani strain) intranasally, and they were sacrificed 2 days later. The lungs were removed and homogenized in a 10% (wt/vol) suspension in Leibovitz 15 medium (Invitrogen, Carlsbad, CA), and virus titers were determined in Vero cell monolayers in 96-well plates.

Production of 80R scFv and whole human IgG1. 80R single-chain antibody (scFv) and IgG1 were expressed and purified as previously described (25). Briefly, six-His-tagged 80R scFv was expressed in *Escherichia coli* XL1-Blue (Stratagene) in the prokaryotic expression vector pSyn1 and purified from the periplasmic fraction by immobilized metal affinity chromatography. Whole human IgG1 of 80R was expressed in 293T cells by transient transfection and purified by protein A-Sepharose affinity chromatography. The S1-binding activities of purified soluble 80R scFv and IgG1 of 80R were confirmed by enzyme-linked immunosorbent assay.

Construction of full-length spike, S1-Ig, truncation variants, and mutants. The plasmid encoding a codon-optimized form of the S1 gene (residues 12 to 672 were defined as the S1 domain of the SARS-CoV S protein), fused with the Fc portion of human IgG1 (S1-Ig), was previously described (10, 11, 26). Plasmids encoding residues 318 to 510, 327 to 510, 318 to 490, and other truncation variants of S1 were generated by PCR using S1-Ig as a template. Mutations within S1-Ig or within S1(318-510)-Ig were generated by site-directed mutagenesis using the QuikChange method (Stratagene). S1 or full-length spike genes of SARS-CoV Tor2 (accession number AY274119), GD03T (AY525636) and SARS-like-CoV SZ3 (AY304486) were generated de novo by recursive PCR. Full-length spike proteins for immunoprecipitation were fused with a carboxyl-terminal nine-amino-acid (C9) tag (11, 15). All variants and mutations were confirmed by DNA sequencing.

Radioimmunoprecipitation of S1, truncation variants, and mutations. S1-Ig, S1(318-510)-Ig, and other truncations and mutations were expressed in 293T cells, secreted in culture supernatants, and metabolically labeled for 24 h with

TABLE 1. Protection from virus replication in the lower respiratory tracts of mice following passive transfer of anti-SARS MAb 80R

Passive-transfer MAb ^a (500 μ g/ml)	Virus replication in lungs of challenged mice ^b		P value ^c
	No. infected/ no. tested	Mean (\pm SE) virus titer ^d	
Undiluted control MAb	4/4	5.7 \pm 0.1	
80R undiluted	0/4	$\leq 1.5 \pm 0^e$	0.00000001
80R diluted 1:5	3/4	2.0 \pm 0.2	0.0000035
80R diluted 1:25	4/4	4.6 \pm 0.26	0.007

^a The indicated dilutions of MAb in 500 μ l were administered to recipient mice by intraperitoneal injection.

^b Mice were challenged with 10^4 TCID₅₀ of SARS-CoV intranasally.

^c P values comparing titers with those seen in mice that received the control antibody in a two-tailed Student *t* test.

^d Virus titers are expressed as log₁₀ TCID₅₀/g of tissue.

^e Virus not detected; the lower limit of detection of infectious virus in a 10% (wt/vol) suspension of lung homogenate was 1.5 log₁₀ TCID₅₀/g.

[³⁵S]cysteine and [³⁵S]methionine (NEN Life Science). One microgram of 80R scFv was conjugated to 20 μ l of anti-His6 agarose beads in PBS buffer by incubating 2 h at 4°C and followed by washing two times with 1 ml of PBS. The 80R scFv beads were used to precipitate Fc-tagged S1 or its derivatives. Five hundred microliters of culture supernatants which contains metabolically labeled S1 or its derivatives was incubated with beads for 4 h at 4°C, and then beads were washed three times with PBS containing 0.25% Nonidet P-40. Full-length S protein was also expressed in 293T cells and metabolically labeled, and cells were lysed with 1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane-sulfonate (CHAPSO)/PBS. One microgram of 80R IgG1 or anti-C9 antibody 1D4 was used to precipitate S protein from cellular lysate. Bound proteins were eluted in reducing Laemmli sample buffer at 100°C for 5 min. Proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, visualized by phosphorimaging, and quantified using ImageQuant software.

80R IgG1 inhibition of infection by S protein-pseudotyped viruses. S protein-pseudotyped lentiviruses expressing a luciferase reporter gene were produced as described previously (16, 32). Briefly, 293T cells were cotransfected with a plasmid encoding S protein variants with a modified carboxyl-terminal ht2 (15), a plasmid pCMVΔ8.2 encoding human immunodeficiency virus type 1 (HIV-1) Gag-Pol, and a plasmid (pHIV-Luc) encoding the firefly luciferase reporter gene under control of the HIV-1 long terminal repeat. Forty-eight hours posttransfection, viral supernatants were harvested and 5 μ l of S protein-pseudotyped virus was used for infection of 6,000 ACE2-expressing 293T cells in a 96-well plate (human ACE2-expression cells for Tor2, GD03T; civet ACE2-expression cells for SZ3). Infection efficiency was quantitated by measuring the luciferase activity in the target cells with an EG&G Berthold Microplate Luminometer (LB 96V).

RESULTS

Passive administration of 80R efficiently protects mice from infection by SARS-CoV. We previously showed that 80R IgG1 potentially neutralized SARS-CoV in an in vitro microneutralization assay. To further evaluate the prophylactic effect of 80R in vivo, we used a recently developed SARS-CoV small-animal model employing BALB/c mice (24). Intranasal inoculation with SARS-CoV results in rapid virus replication in the respiratory tract. Although the mice showed no evidence of clinical illness or disease, the high level of replication is sufficient for evaluating the efficacy of vaccines and antiviral agents. In this study, 80R IgG1 was given intraperitoneally to BALB/c mice 1 day before SARS-CoV (10^4 TCID₅₀) intranasal challenge and 2 days later the virus titer of lung tissue was determined. As shown in Table 1, at the highest 80R dose tested (undiluted, 250 μ g/mouse, \approx 12.5 mg/kg of body weight), 4/4 mice had a more-than-4-log reduction in viral load (to below the assay limit), whereas the equivalent amount of human

IgG1 had no effect. At a dose of 50 μ g/mouse, 1 of 4 mice again showed a viral load reduction to below the limit of detection and 3 of 4 mice showed a nearly 4-log reduction in viral titer. At the lowest dose (1:25 dilution, 10 μ g/mouse), 4/4 mice became infected and the virus load was reduced about 10-fold. This excellent level of protection is comparable to that seen when the animals were injected with convalescent-phase sera from previously infected mice (24). These results indicate that the prophylactic administration of 80R can efficiently protect mice from SARS-CoV infection.

80R neutralizing determinants are located within the ACE2 receptor-binding domain on S protein. In our previous report, primary characterization of the 80R epitope showed that it was not sensitive to denaturing conditions (25). This prompted us to try to identify the epitope by enzyme-linked immunosorbent assay screening of plates coated with a series of 18-mer peptides comprising the entire SARS S1 protein with 10-amino-acid overlaps between sequential peptides (National Institutes of Health AIDS Research and Reference Reagent Program). However, no binding of 80R IgG1 was detected (data not shown). This indicated that the epitope could be conformation dependent. Preliminary epitope mapping of 80R also showed that it was located within amino acids (aa) 261 to 672 of the S protein and that the neutralizing activity of 80R was achieved by blocking the association of S protein to its cellular receptor, ACE2 (25). A 193-aa region consisting of residues 318 to 510 of the S1 domain was recently shown to contain the ACE2 receptor-binding domain of the SARS-CoV S protein. Two truncation variants expressing fragments smaller than the 193-aa fragment, residues 318 to 490 and 327 to 510, did not bind to ACE2 by radioimmunoprecipitation (26). In this study, to more precisely map the binding domain of 80R and to better understand how 80R blocks the binding of S protein to ACE2, we tested the ability of these three fragments of the S1 domain (residues 318 to 510, 318 to 490, and 327 to 510) to bind to 80R. 80R scFv-conjugated agarose and protein A-Sepharose were individually used to precipitate metabolically labeled Fc-tagged soluble forms of these three S1 truncations. The amount of S1-Ig protein precipitated by 80R scFv was normalized to the amount precipitated by protein A. As shown in Fig. 1a, protein in the supernatants was assayed by protein A precipitation. 80R scFv precipitated the 193-aa fragment S1(318-510)-Ig as efficiently as protein A. Under similar conditions, 80R scFv did not precipitate the smaller deletion of S1(318-490), and it precipitated only 5% of the amount of S1(327-510) that protein A precipitated. This is the same precipitation pattern that was seen in the ACE2 precipitation studies with these variants. These data indicate that the 80R neutralizing determinant and the ACE2 receptor-binding domain are located within the same domain of the S protein, residues 318 to 510. Both smaller N-terminal and C-terminal deletion variants of this domain, 318 to 490 and 327 to 510, lost 80R-binding activity. This implied that some residues in the N-terminal and C-terminal parts of S1(318-510) contribute either directly to the binding of 80R with this domain or to the folding of the correct antibody-binding domain. Hence, we made a further series of slightly smaller Fc-tagged N-terminal and C-terminal deletions of S1(318-510) to define the smallest binding domain of 80R. As shown in Fig. 1a, the 321-503 variant was expressed as well as S1(318-510) and displayed the same 80R-binding

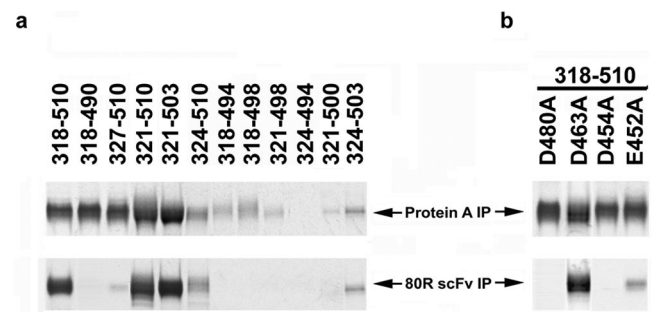


FIG. 1. Truncations and point mutations of S1(318-510) were analyzed to define the 80R antibody epitope. S1 residues 318 to 510 fused to the Fc region of human IgG1 and truncation or mutation variants of S1(318-510) containing the indicated residues were metabolically labeled and precipitated by protein A or 80R scFv. (a) S1(324-503) was the smallest fragment bound to 80R. Either N-terminal or C-terminal truncation variants slightly smaller than that either had decreased expression or lost binding activity to 80R. (b) Critical residues for the 80R epitope were observed. Individual alanine substitution of glutamic acid 452 or aspartic acids 454 and 480 in the S1(318-510) fragment impaired or abolished binding to 80R. IP, immunoprecipitation.

activity as S1(318-510). Only a small amount of secreted protein of the 321-500 variant was detected due to inefficient expression or secretion, and 80R binding was not detectable; however, the binding of variant 324-503 remained the same as for S1(318-510) even though its expression was greatly reduced. Therefore, the smallest 80R-binding domain is located within amino acids 324 to 503.

Identification of some important residues for 80R binding to the ACE2 receptor-binding domain of S1 protein. Some acidic residues between 318 and 510 (glutamic acid 452 and aspartic acids 454, 463, and 480) were previously individually changed to alanine to test their effect on association with ACE2. It was shown that E452 and D454 individually made important contributions to the S1 interaction with ACE2 and D463A alteration also resulted in a decrease in ACE2 binding, but no effect was found with a D480A alteration (26). We therefore further tested these point substitution variants for 80R-antibody binding. They were mutated to alanine individually in both S1(318-510)-Ig and full-length S1-Ig. Protein A and 80R-scFv-conjugated beads were used to precipitate metabolically labeled S1(318-510)-Ig, S1-Ig, or their variants. A ratio of 1 was set for the amount of 80R-scFv-precipitated S1(318-510)-Ig or S1-Ig to that of protein A-precipitated S1(318-510)-Ig or S1-Ig, and the amount of 80R-scFv-precipitated variants was normalized accordingly. As shown in Fig. 1b, the E452A and D463A variants were precipitated ~10% and 100% of wild-type S1(318-510)-Ig by 80R scFv, respectively. D454A and D480A variants were not detectable on precipitation with 80R scFv. The same result was obtained for variants in S1-Ig (data not shown). These data demonstrated that E452 and D454 contribute to the association of S protein with 80R antibody similarly to that of S1 with ACE2. D463 does not affect 80R binding to S1 but affects the association of ACE2 to S1. D480 plays no significant role in S protein association with ACE2 but is critically important for 80R-antibody binding.

80R-binding characteristics of variant S proteins with amino acid substitutions in the 80R-binding domain that occur in SARS-like-CoV from civet cats and that evolved during

TABLE 2. Amino acid changes in SARS-like-CoVs and SARS-CoVs from human cases

Viral isolate	Example		Amino acid at position:						
	Name of genomic sequence	GenBank accession no.	344	360	472	479	480	487	
Middle/late phase 2002/2003	Tor2	AY274119	K	F	L	N	D	T	
Early phase 2002/2003	GD01	AY278489	R/K	F	L	N	D	T	
Palm civet	SZ3	AY304486	R	S	L	K	D	S	
Guangdong index patient 2003/2004	GD03T0013	AY525636	R	S	P	N	G	S	

the 2002/2003 outbreak and in a 2003/2004 Guangdong index patient. The S1 domains of spike proteins contain neutralizing epitopes for most known coronaviruses and therefore are likely to encode determinants for host tropism, neutralizing antibody, and viral virulence. Recent molecular epidemiological studies of SARS-CoVs have shown that amino acid changes are mainly located in the S1 region, which contains its receptor (ACE2)-binding domain (3). We compared 78 reported human SARS-CoV sequences and SARS-like-CoV sequences from the Himalayan palm civets for amino acid sequence variations in the ACE2- and 80R-binding domains (residues 318 to 510) and summarized all variations in Table 2 (3, 5, 30, 33). A total of six differences are observed at positions 344, 360, 472, 479, 480, and 487 in this region. Among the human SARS-CoVs isolated from the 2002/2003 epidemic, the amino acid arginine or lysine was present at position 344 in the early isolates (K344K/R) but lysine was present in the middle and late isolates (3). The amino acids at positions 472 and 480 were lysine and aspartic acid in all reported SARS-like-CoV S sequences and human SARS-CoV S sequences but were proline and glycine in the S sequence of the 2003/2004 Guangdong index patient (GD03T0013, referred as GD03T) (L472P and D480G). Major amino acid variations in the spike between human SARS-CoVs of 2002/2003 and SARS-like-CoVs are located at positions 360, 479, and 487 (F360S, N479K, T487S, SARS-CoV/SARS-like-CoV). The above sequence variations in the 80R antibody-binding domain could be associated with significant changes in the binding activity of 80R. The 80R antibody was originally screened against the S1 domain of S protein of a late-phase human SARS-CoV isolate (Tor2, NC_004718). For substitution analysis, each amino acid in Tor2 was individually replaced with a corresponding changed amino acid in order to examine the effect of these residues on 80R antibody binding. For example, K (Tor2) at position 344 was replaced with R (Civet SARS-like-CoV, isolate SZ3). We also replaced each amino acid with alanine to investigate whether these residues normally contribute to form the 80R epitope. As shown in Fig. 2a, no effect on the binding of 80R was found for the variants of either F360S and L472P or F360A and L472A in S1(318-510) and no significant changes in binding were observed with the variants K344R and T487S. However, mutation to alanine with the variants R344A and T487A resulted in ~20% and ~50% reductions in binding, respectively. At position 479, N479K substitution resulted in an ~50% decrease in 80R binding, whereas N479A substitution resulted in only ~20% reduction. These results implied that lysine 344, asparagine 479, and threonine 487 normally contribute to some degree to the binding of 80R to the S1 domain, either by forming part of the 80R-binding site or by facilitating correct folding of the protein. As shown in Fig. 1b and 2a,

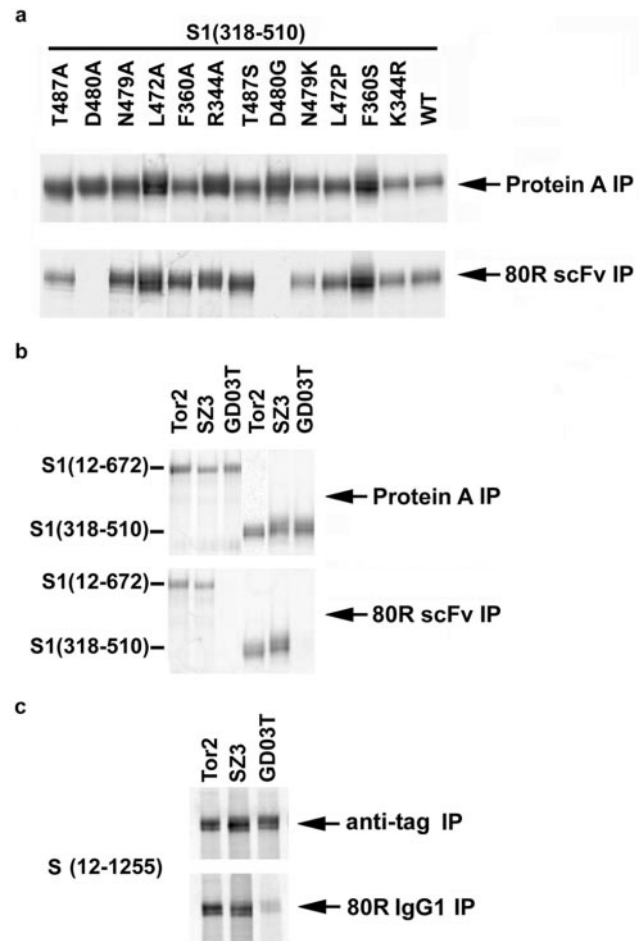


FIG. 2. Effects on 80R binding of variant amino acid substitutions of S protein that occur in animal SARS-like-CoVs and human SARS-CoVs. (a) Indicated amino acid residues in S1(318-510) of Tor2 were individually replaced with corresponding variant amino acids found in SARS-like-CoVs or other human SARS-CoVs. These residues also were replaced with alanine. Alterations of K344A, N479A, and T487A affected the binding to 80R to some degree. N479K substitution resulted in an ~50% decrease in 80R binding, and D480G substitution totally abolished binding to 80R. (b) Multiple substitutions with the amino acids of civet SZ3 virus (344R/360S/479K/487S) in the S1(318-510)-Ig construct of Tor2 had no effect on 80R binding, as well as full-length S1(12-672) of SZ3, which was de novo synthesized. Multiple substitutions with the amino acids of human GD03T virus (344R/360S/472P/480G/487S) in the S1(318-510)-Ig construct of Tor2 and full-length S1(12-672) of GD03T completely lost binding to 80R scFv. (c) Full-length S protein of Tor2 and variants containing amino acid substitutions of isolates SZ3 or GD03T were precipitated by 1D4, which recognizes a C9 tag present at the carboxyl terminus of each S protein, or by 80R IgG1 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The binding activities of these full-length S proteins to 80R IgG1 were consistent with those of their receptor-binding domains (318 to 510) or S1 domains (12 to 672) to 80R scFv. WT, wild type; IP, immunoprecipitation.

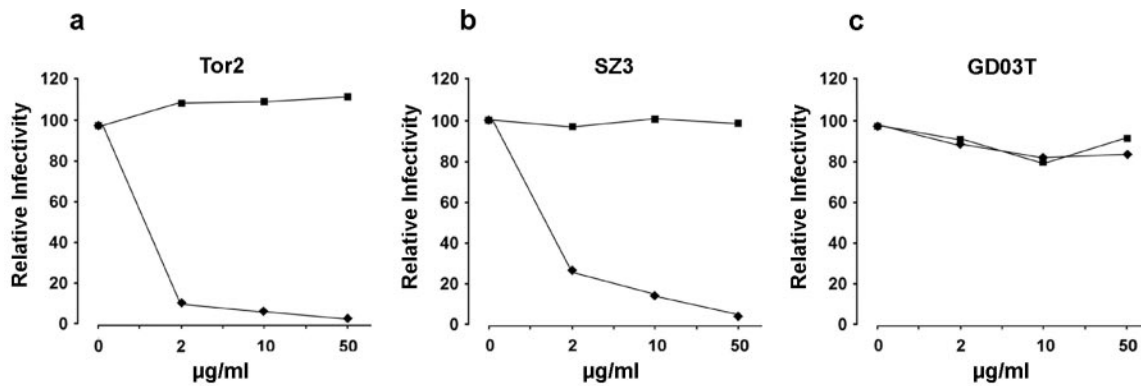


FIG. 3. 80R IgG1 neutralization of pseudoviral infection mediated by full-length SARS-CoV spike variants. HIVs pseudotyped with the S protein from Tor2, SZ3, or GD03T isolates were incubated with the indicated concentration of 80R IgG1 (solid line, diamonds) or nonrelevant human IgG1 (solid line, squares) for 1 h prior to infection. At 48 h after infection, luciferase activities in target cells were measured and relative viral inhibition was calculated as the ratio of luciferase activity in the presence to absence of 80R IgG1 or nonrelevant human IgG1. (a) 80R IgG1 efficiently blocked Tor2 S protein-pseudotyped HIV infection, with a 90% inhibitory concentration around 2 µg/ml. (b) 80R IgG1 also efficiently neutralized SZ3 S protein pseudoviral infection. (c) In contrast to Tor2 and SZ3, GD03T S protein-pseudotyped virus was essentially resistant to the neutralization of 80R IgG1 with a concentration up to 50 µg/ml. These results are representative of two experiments with similar results.

D480A substitution completely abolished binding to 80R. The same result was observed when aspartic acid was changed to glycine, which is found in the sequence of the 2003/2004 Guangdong index patient GD03T.

In addition, we made S1(318-510)-Ig corresponding to the civet SZ3 and human GD03T viral isolates by introducing multiple amino acid substitutions 344R/360S/479K/487S for civet SZ3 and 344R/360S/472P/480G/487S for human GD03T in the Tor2 S1(318-510)-Ig construct. Also, the S1 genes (12 to 672) encoding the entire S1 protein of civet SZ3 and human GD03T were synthesized *de novo* as previously described for the entire human SARS-CoV S1 protein of the Tor2 isolate (11). These two full-length S1 variants include amino acid changes in residues 318 to 510, as well as changes outside this region. We characterized these S1 protein variants' binding activities to 80R scFv by immunoprecipitation. Both S1(318-510) and S1(12-672) of civet SZ3 bound to 80R similarly as did Tor2 (Fig. 2b). Surprisingly, the variant containing the single amino acid substitution N479K, which reduced binding to 80R, did not appear to affect binding in the context of the multiply substituted variant. Neither S1(318-510) nor S1(12-672) of GD03T bound to 80R scFv. This is consistent with the critical role of D480 in the binding of 80R to S1 protein. A D480G change in the S protein of GD03T conferred complete resistance to 80R scFv. Finally, we also evaluated the effect of these alterations for binding to 80R IgG1 in the context of the full-length S protein. As shown in Fig. 2c, consistent with the binding of the S fragment of the receptor-binding domain and S1 to 80R scFv, 80R IgG1 efficiently precipitated the full-length S proteins of Tor2 and SZ3, but not GD03T, although very weak binding of GD03T to 80R IgG1 was detectable due to much higher affinity of 80R IgG1 over 80R scFv.

80R IgG1 neutralization of pseudoviral infection mediated by full-length S protein variants. The neutralization activity of 80R IgG1 against variant SARS-CoV was evaluated by using a pseudovirus system. Single-round HIV luciferase reporter viruses pseudotyped by S protein variants of Tor2, SZ3, or GD03T were tested for neutralization sensitivity to increasing concentrations of 80R IgG1 or nonrelevant human IgG1. As

expected, Tor2 is very sensitive to neutralization of 80R IgG1, with a 90% inhibitory concentration around 2 µg/ml (Fig. 3a); 80R IgG1 could also efficiently block SZ3 pseudoviral infection (Fig. 3b); in contrast, GD03T is essentially resistant to neutralization by 80R IgG1 in the concentration range assayed (Fig. 3c).

DISCUSSION

A current challenge facing the global scientific and medical community is how to provide specific immunoprophylaxis against SARS in an outbreak setting. In this study, the prophylactic use of the anti-SARS-CoV human MAb 80R was investigated in a mouse model for SARS-CoV infection, in which SARS-CoV can replicate to high titers in the lower respiratory tract. The results demonstrated that passive transfer of 80R IgG1 can completely protect mice from SARS-CoV replication in lung tissue, at doses that are therapeutically achievable in humans. Critical amino acids and unique structural features of the 80R neutralizing epitope were also defined. In addition, the 80R-binding characteristics of variant S1 proteins with amino acid substitutions in the 80R epitope were analyzed, including substitutions found in SARS-like-CoVs from civet cats, and which evolved during the 2002/2003 outbreak and in an index patient with SARS in Guangdong in 2003/2004. These variants were further evaluated by 80R immunoprecipitation in the context of full-length S protein, and the neutralization activity of 80R against these variants was also confirmed by a pseudotyped reporter virus system. The results of these studies demonstrate that the vast majority of viruses remain sensitive to 80R. The potent virus-neutralizing activity of 80R likely reflects the overlap between its epitope and the ACE2-binding domain of S protein.

One surprise of this study was the absolute conformational dependence of the neutralizing epitope of 80R, since we showed previously that 80R can recognize the denatured S1 protein (25). However, binding of 80R was not detected when a complete series of 18-mer S1 peptides with 10-amino-acid overlap, or some larger linear and conformationally constrained peptides that centered around essential residues for

80R binding, were examined (data not shown). The 80R neutralizing epitope was shown to be located in the same region as the ACE2 receptor-binding domain (residues 318 to 510). In addition, the 80R epitope could be further reduced but minimally required a 180-amino-acid fragment lying between 324 and 503 of the S1 domain. Interestingly, Zeng et al. recently showed that a plasmid DNA vaccine encoding residues 18 to 495 elicited high antibody titers in mice but no SARS-CoV neutralizing activity (35). Our data are consistent with this observation, since the carboxy-terminal deletion prior to amino acid 503 in S1 likely failed to maintain the conformationally sensitive neutralizing epitope. Thus, this 180-amino-acid region encompassing the 80R epitope can now be considered an essential core region of the S protein for subunit vaccines aimed at eliciting potent neutralizing antibody responses. Whether other neutralizing epitopes exist on the S1 protein remains to be determined.

Some acidic residues previously analyzed for ACE2 receptor binding were tested by alanine substitution for their effect on the binding of 80R in this study. Glutamic acid 452 and aspartic acid 454 contribute to both the association of S protein with 80R and the association of S1 with ACE2. Aspartic acid 463 reduces the association of ACE2 with S1 but has no effect in 80R binding to S1. Substitution of aspartic acid 480 abolishes the binding to 80R but has no effect on the binding to ACE2. These data strongly suggest that the S determinants involved in the binding of receptor and of neutralizing antibodies are in part overlapping and are likely to result from both common and unique contact residues. Further studies of 80R antibody binding through an analysis of amino acid variants between civet SARS-like CoVs and human SARS-CoVs found that lysine 344, asparagine 479, and threonine 487 contribute to the binding of 80R with the S1 domain. Asparagine 479 of human SARS-CoV S1 changed to lysine of civet SARS-like-CoV S1 resulted in partial resistance to 80R antibody. However, this resistance was not seen in the context of the complete civet SZ3 S protein. Glycine at position 480 of S1 of SARS-CoV isolated from the 2003/2004 Guangdong index patient conferred complete resistance to 80R. Critical amino acids for the 80R epitope were not found to vary among all the human SARS-CoVs from the 2002/2003 epidemic. Therefore, the vast majority of SARS-CoVs, including the well-adapted and more pathogenic human SARS-CoVs from the 2002/2003 epidemic and SARS-like CoVs isolated to date, are likely to be sensitive to 80R.

For passive immunotherapy to be effective in a SARS outbreak, 80R IgG1 would have to be active against a plethora of viral isolates that may emerge. We propose that testing for susceptibility to 80R neutralization can be managed in an outbreak by rapid and early genotyping of the DNA fragment encoding the 180 amino acids of the 80R epitope from the outbreak strains. For example, in some outbreaks, such as the one which recently occurred as a result of a laboratory exposure at the National Institute of Virology in Beijing, it should be possible to predict the susceptibility of the SARS-CoV to 80R by comparing the amino acid sequences of the S1 protein of the laboratory strain with the 80R neutralizing epitope elucidated by this study. However, in a natural reemergence such as that which occurred in 2003/2004 in Guangdong, it would not be possible to know a priori whether the new viruses would

be susceptible to 80R. The resistance to 80R neutralization of the SARS-CoV from the 2003/2004 Guangdong index patient because of the D480G mutation provides one such sobering example. Although the GD03T virus appeared to be only weakly pathogenic and no secondary infections with SARS-CoVs of this genotype have been reported, other mutations that have different properties on viral tropism and replication kinetics may emerge in future outbreaks. Variations in the genomes of SARS-like CoVs in the animal reservoirs will likely continue to occur because of the high mutation rate of RNA viruses. Indeed, recent surveillance of the known animal reservoirs of SARS-like-CoVs have confirmed that the 2003/2004 viruses are different from those that circulated in animals and humans in the outbreak of 2002/2003 (4). Thus, our study cautions that any prophylaxis strategy based on neutralizing antibody responses, whether by passive immunotherapy or active immunization, should be monitored closely by rapid and early genotyping of the target neutralizing epitope.

At present, management of potentially SARS-CoV-exposed individuals is still based on observation and quarantine, even on a large scale (http://www.who.int/csr/don/2004_04_28/en/). We propose that 80R IgG1 may be useful for emergency prophylaxis of potentially SARS-CoV-exposed individuals by treatment with a single intravenous or intramuscular dose (half-life of IgG1, circa 21 days) sufficient to achieve a serum level that would result in $\geq 99\%$ reduction in the expected virus titers in the serum or tissue of humans or animals based on previously published studies (18, 20).

The results of these 80R studies also provide further validation of the results from several recent vaccine studies that have demonstrated the importance of the spike protein in eliciting neutralizing antibody responses (1, 31). However, the pros and cons of passive immunoprophylaxis versus vaccination requires additional consideration. Since heightened awareness and public health care measures that are now in place, it is uncertain if a worldwide epidemic or even a regional epidemic of the magnitude seen in 2002/2003 will reoccur (13). Therefore, the cost effectiveness of vaccinating a susceptible population with a low incidence of infection versus employing passive immunotherapy in an outbreak setting must be considered.

In summary, we propose that 80R IgG1 may be useful for the emergency immunoprophylaxis of SARS. The vast majority of SARS-CoVs isolated so far remain sensitive to 80R. In an outbreak setting, early and rapid genotyping of the 540-bp S1 gene fragment encoding the 80R epitope from index cases may provide an accurate guide to susceptibility or resistance to 80R prophylaxis. Our study also cautions that for any prophylaxis strategy based on neutralizing antibody responses, whether by passive or active immunization, a genotyping monitor will be necessary for effective use. Therefore, there should be ongoing surveillance in place to monitor closely, on a regular basis, the SARS-like-CoV genotypes in the known animal reservoirs, as is now being routinely performed with influenza virus (7).

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