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Novel Human Monoclonal Antibody Combination Effectively Neutralizing Natural Rabies Virus Variants and Individual In Vitro Escape Mutants

Alexander B. H. Bakker,[1]† Wilfred E. Marissen,[1]† R. Arjen Kramer,[1] Amy B. Rice,[2] William C. Weldon,[3] Michael Niezgoda,[3] Cathleen A. Hanlon,[3] Sandra Thijsse,[1] Harold H. J. Backus,[1] John de Kruif,[1] Bernhard Dietzschold,[2] Charles E. Rupprecht,[3] and Jaap Goudsmit[1]*

Crucell Holland BV, Leiden, The Netherlands[1]; Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, Pennsylvania[2]; and Centers for Disease Control and Prevention, Division of Viral and Rickettsial Diseases, Rabies Section, Atlanta, Georgia[3]

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The need to replace rabies immune globulin (RIG) as an essential component of rabies postexposure prophylaxis is widely acknowledged. We set out to discover a unique combination of human monoclonal antibodies (MAbs) able to replace RIG. Stringent criteria concerning neutralizing potency, affinity, breadth of neutralization, and coverage of natural rabies virus (RV) isolates and in vitro escape mutants were set for each individual antibody, and the complementarities of the two MAbs were defined at the onset. First, we identified and characterized one human MAb (CR57) with high in vitro and in vivo neutralizing potency and a broad neutralization spectrum. The linear antibody binding site was mapped on the RV glycoprotein as antigenic site I by characterizing CR57 escape mutants. Secondly, we selected using phage display a complementing antibody (CR4098) that recognized a distinct, nonoverlapping epitope (antigenic site III), showed similar neutralizing potency and breadth as CR57, and neutralized CR57 escape mutants. Reciprocally, CR57 neutralized RV variants escaping CR4098. Analysis of glycoprotein sequences of natural RV isolates revealed that the majority of strains contain both intact epitopes, and the few remaining strains contain at least one of the two. In vitro exposure of RV to the combination of CR57 and CR4098 yielded no escape mutants. In conclusion, a novel combination of human MAbs was discovered suitable to replace RIG.

Lethal rabies is prevented by postexposure prophylaxis (PEP) through the combined administration of a rabies vaccine and rabies immune globulin (RIG). Two types of RIG are used: human RIG (HRIG) and equine RIG, both derived from pooled sera of human donors or horses vaccinated against rabies, respectively. The need to replace these hyperimmune serum preparations is widely recognized (29), and monoclonal antibodies (MAbs) that neutralize rabies virus (RV) offer the opportunity to do so.

Mouse MAbs, as well as human MAbs, have been shown to protect rodents from a lethal RV challenge (6, 9, 12, 14, 20, 22, 24). One of the most potent human MAbs, SO57, neutralizing a variety of RV strains, was described by Dietzschold et al. (6). A cocktail of three human MAbs including SO57 and SOJA and SOJB showed effective protection of mice from a lethal dose of RV (22). We reformatted these three MAbs (renamed CR57, CRJA, and CRJB) into our own expression system for production in PER.C6 cells (19). However, we showed that the CRJA and CRJB MAbs were not suitable in combination with CR57 for use in PEP (19) because of overlapping epitope recognition, lack of neutralizing potency, and shared escape mutants. Novel anti-RV MAbs were generated using phage display technology and were characterized with special emphasis on CR57 complementarity.

We considered several criteria to be of crucial importance for the inclusion of human MAbs into a cocktail aimed at effectively blocking an RV infection in humans. First, the MAbs should target distinct, nonoverlapping epitopes and should not compete for binding to RV glycoprotein. Second, in vitro generated antibody-resistant RV variants selected using one antibody should be neutralized by the nonselecting other antibody in the cocktail (and vice versa), thus addressing the issue of natural variation among RV street isolates. Both MAbs should have an in vitro neutralizing potency higher than 500 IU/mg. Furthermore, the MAb, in combination with vaccine, must provide protection against a lethal RV challenge in an appropriate animal model system, such as a system using Syrian hamsters.

In the current study, we analyzed a large panel of neutralizing MAbs selected from RV phage display antibody libraries obtained from the B-cell repertoire of rabies-vaccinated individuals. The selection procedure yielded complementing MAbs that fulfilled the criteria described above, of which CR4098 was the best candidate. CR57 and CR4098 form the optimal combination for use in PEP of rabies.

* Corresponding author. Mailing address: Crucell Holland B.V., Archimedesweg 4, P.O. Box 2048, 2301 CA Leiden, The Netherlands. Phone: 31 71 5248701. Fax: 31 71 5248702. E-mail: j.goudsmit@crucell.com.

† A.B.H.B. and W.E.M. contributed equally to this work.

MATERIALS AND METHODS

Cells. Mouse neuroblastoma (NA) cells were grown at 37°C in 5% CO_2 or at 37°C in 0.5% CO_2 in RPMI 1640 medium (Gibco) or minimal essential medium (Gibco), respectively, supplemented with 10% heat-inactivated fetal bovine se-

rum (FBS). BSR cells (a subclone of baby hamster kidney cells) were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS. PER.C6 cells (10) were grown at 37°C in 10% CO_2 in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS and 10 mM $MgCl_2$.

Virus. Monolayers of NA cells were infected with CVS-11 (challenge virus standard) or MAb-resistant escape viruses at a multiplicity of infectivity of 0.1 for 1 h at 37°C in 0.5% CO_2. The virus inoculum was then removed, fresh medium was added to the cells, and cells were subsequently incubated for 40 h at 37°C in 0.5% CO_2. The culture supernatants were collected and stored at −80°C until further use.

RFFIT. Standard rapid fluorescent focus inhibition test (RFFIT) neutralization assays were performed as described previously (26). Modified RFFIT neutralization assays were performed as described previously (6). To determine the neutralizing potency of each MAb, its 50% neutralizing titer was compared to the 50% neutralizing titer of a standard reference serum (standard RIG, lot R3), which is defined at 2 IU/ml.

Generation of escape viruses. Escape viruses of CR4098 (E98 escape viruses) were generated as previously described (19). Each escape virus was amplified on NA cells in the presence of CR4098 before further characterization. Escape viruses of the combination CR57/CR4098 or HRIG (Imogam rabies HT; Sanofi-Aventis) were selected in the presence of 4 IU/ml of either MAb or HRIG, respectively, and further processed as described above.

Determination of the neutralization index. The neutralization index (NI) for each E98 escape virus was determined as described previously (19) using the following formula: NI = log(focus-forming units [FFU]/ml − immunoglobulin G [IgG]) − log(FFU/ml + IgG). An index lower than 2.5 was considered as evidence of escape from neutralization by the antibody.

cDNA sequencing. To identify possible mutations in the open reading frame (ORF) of the glycoprotein, cDNA was prepared from each escape virus and sequenced as described previously (19).

Affinity and ranking. Surface plasmon resonance (SPR) analysis was performed on a BIAcore 3000TM analytical system. Purified RV glycoprotein (Evelyn-Rokitnicky-Abelseth strain) was immobilized as a ligand on a research grade CM5 four-flow channel sensor chip (Biacore AB, Uppsala, Sweden) using amine coupling. Ranking was performed at 25°C with HBS-EP (10 mM HEPES buffer, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20) (Biacore, Uppsala, Sweden) as running buffer. Fifty microliters of each antibody (500 nM) was injected at a constant flow rate of 20 μl/min. Then, running buffer was applied for 750 s, followed by regeneration of the CM5 chip with 5 μl of 2 M NaOH, 5 μl of 45 mM HCl, and 5 μl of 2 mM NaOH. The resonance signals expressed as resonance units (RU) were plotted as a function of time. The increase and decrease in RU as a measure of association and dissociation, respectively, were determined and used for ranking of the antibodies. To further determine the association rate, dissociation rate, and affinity (K_D), a concentration series from 0.09 to 200 nM of each antibody (50 μl) was injected at a constant flow rate of 20 μl/min.

Biacore epitope mapping. SPR analysis was also used for epitope mapping. Fifty microliters of each antibody (1 μM) was injected at a constant flow rate of 20 μl/min to reach saturation on the RV glycoprotein. After a 150-s period of buffer flow, 50 μl of the second antibody (1 μM) was injected, followed by regeneration as described for ranking analysis. At the end of each antibody injection period, the RU level was determined as a measure for binding.

In vivo hamster challenge model. A lethal animal model mimicking rabies exposure was employed as described previously (13). Briefly, Syrian hamsters (Harlan Sprague-Dawley) were infected with 0.05 ml of $10^{6.8}$ mouse intracerebral median lethal dose of a salivary gland homogenate of a rabid Mexican dog (coyote street RV [COSRV]; U.S.-Mexican border; reference number 323R) at day −1. On days 0, 3, 7, 14, and 28, animals received rabies vaccine (Imovax; Sanofi-Pasteur). In addition, CR57, CR4098, or CR4144 at a dosage of 10, 20, or 40 IU/kg or HRIG (Imogam rabies HT; Sanofi-Pasteur) at 20 IU/kg was administered on day 0. Hamsters were checked daily for clinical signs of rabies, upon which they were euthanized. Animals were followed up to day 90 after infection. Postmortem diagnosis of rabies by direct fluorescent antibody testing using a standardized protocol of the Centers for Disease Control and Prevention was performed for each euthanized animal [http://www.cdc.gov/ncidod/dvrd/rabies/professional/publications/DFA_diagnosis/DFA_protocol-b.htm].

RESULTS

Characterization of anti-RV glycoprotein MAbs. Previously, we reported that CR57 was directed against the highly conserved antigenic site I (19). To find a novel anti-RV MAb that was complementary with CR57, phage display selections using two RV-immune phage antibody libraries were performed. This yielded a panel of 21 IgG1 antibodies specific for RV glycoprotein (R. A. Kramer et al., unpublished). To further characterize these novel MAbs (designated CR4xxx) we determined their neutralizing potency, affinity, and compatibility with CR57. The neutralizing potency of the novel antibodies was determined in a standard RFFIT on CVS-11 as described in Materials and Methods. A neutralizing potency of 500 IU/mg was used as selection criterion for the following reasons. First, the cutoff was set high to compensate for the variation in this bioassay. Second, the neutralizing potency is determined for the fixed laboratory strain CVS-11, which typically results in higher values than for street RV. Third, it has been reported that in vitro neutralizing potency does not necessarily correlate with the protective activity in vivo (24). Overall, this should ensure that the novel MAb efficiently neutralize street RV both in vitro and in vivo. Table 1 shows that 14 of 21 MAbs had a neutralizing potency of >500 IU/mg, thereby fulfilling our neutralizing potency criterion as mentioned above. None of these 14 MAbs competed significantly with CR57 for binding to RV glycoprotein, with the exception of CR4126 (Kramer et al., unpublished). This suggested that 13 of 14 MAbs were compatible with, and recognized, a different epitope than CR57. To further analyze the binding properties of the antibodies, SPR analysis was performed. The antibodies were ranked based on association levels and dissociation rates, showing a wide range of affinities for RV glycoprotein (Fig. 1). This ranking revealed that a subset of the MAbs, i.e., CR4010, CR4098, CR4103, and CR4144, had an affinity in the same order of magnitude as CR57. There was no apparent correlation between the in vitro neutralizing potency of the antibodies and their respective affinities for the RV glycoprotein (Fig. 1). This is exemplified by two of the most potent antibodies,

TABLE 1. Neutralizing potencies of anti-RV antibodies

IgG	Neutralizing potency (IU/mg)[a]
CR4008	139
CR4010	2133
CR4018	24453
CR4021	367
CR4026	1
CR4031	36
CR4038	2411
CR4040	550
CR4060	6
CR4073	5817
CR4097	0
CR4098	1071
CR4103	1502
CR4104	17213
CR4108	2142
CR4120	2624
CR4125	3476
CR4126	1382
CR4140	431
CR4144	7140
CR4164	3481
CR57	1689

[a] Determined with standard RFFIT.

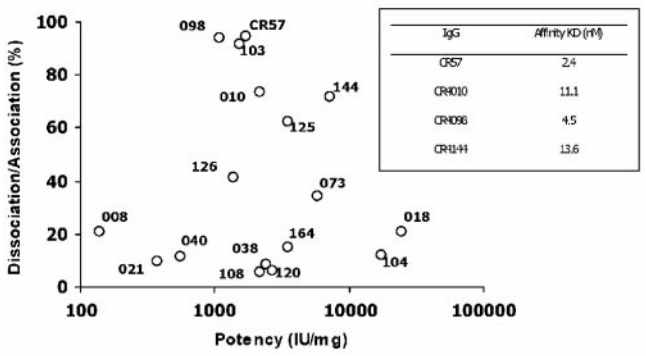

FIG. 1. Lack of correlation between affinity and neutralizing potency of anti-RV antibodies. SPR binding analysis of anti-RV antibodies on glycoprotein from the Evelyn-Rokitnicky-Abelseth strain. Neutralizing potency as measured in a standard RFFIT is shown on the *x* axis in IU/mg. The reduced response after 12.5 min of subsequent dissociation is given on the *y* axis, calculated as a percentage of the association response. Antibodies with a relative high affinity are located in the upper part of the graph. Data points are labeled with the last three digits of the novel MAbs for reasons of clarity. The inset table shows the actual affinity K_D values for a subset of antibodies.

CR4018 and CR4104, which displayed a relatively low affinity. In contrast, CR57, with a neutralizing potency of 1,689 IU/mg, had the highest affinity at 2.4 nM. Actual K_D values were determined for three of the novel MAbs, which revealed that CR4098 had an affinity of 4.5 nM, whereas CR4010 and CR4144 had affinities of 11.1 and 13.6 nM, respectively (Fig. 1, inset).

Coverage of E57 escape viruses by the novel IgG panel. To determine whether the novel IgGs were compatible in a cocktail with CR57, we analyzed the neutralizing activity of the 14 IgGs against the previously generated CR57 MAb-resistant escape viruses (E57 escape viruses) (19). Several IgGs displayed very weak neutralizing activity (between 0% to 20%; designated "+/−") or no longer neutralized (designated "−") the E57 escape viruses compared to neutralization of wild-type CVS-11 (Table 2). The 20% cutoff was based on the variation in the bioassay. By taking a cutoff of 20%, only IgGs with very weak neutralizing activity against E57 escape viruses will be omitted. In contrast, 8 of 14 IgGs, CR4010, CR4038, CR4040, CR4073, CR4098, CR4103, CR4104, and CR4144, showed potent neutralizing activity against all six E57 escape viruses. These eight antibodies were eligible for use in combination with CR57 in a cocktail for PEP and were further characterized.

TABLE 2. Coverage of E57 escape viruses by the novel anti-RV antibodies[a]

IgG	E57A2	E57A3	E57B1	E57B2	E57B3	E57C3
CR4010	+	+	+	+	+	+
CR4018	+	−	+	−	+/−	+
CR4038	+	+	+	+	+	+
CR4040	+	+	+	+	+	+
CR4073	+	+	+	+	+	+
CR4098	+	+	+	+	+	+
CR4103	+	+	+	+	+	+
CR4104	+	+	+	+	+	+
CR4108	+	+/−	+	+/−	+	+/−
CR4120	+	+/−	+/−	+/−	+/−	+/−
CR4125	+/−	+/−	+/−	+/−	+/−	+/−
CR4126	+/−	−	+/−	−	+/−	−
CR4144	+	+	+	+	+	+
CR4164	+	+/−	+	+/−	+/−	+/−

[a] Neutralizing potency was determined in a modified RFFIT. Neutralizing potency towards wild-type CVS-11 is indicated as follows: +, 20 to 100%; +/−, 0 to 20%; −, no neutralization.

TABLE 3. Breadth of neutralization against street rabies viruses[a]

Lyssavirus (source and/or strain)	SRIG	CR57	CR4038	CR4098	CR4144
CVS-11	+	+	+	+	+
Raccoon, SE US	+	+	+	+	+
Gray fox, TX	+	+	+	+	+
Gray fox, AZ	+	+	+	+	+
Arctic fox, AK	+	+	+	+	+
Coyote, TX	+	+	+	+	+
Dog/Coyote, TX	+	+	+	+	+
Skunk, north central	+	+	+	+	+
Skunk, south central	+	−	+	+	+
Skunk, CA	+	+	+	+	+
Mongoose, NY/Puerto Rico	+	+	+	+	+
Dog, Argentina	+	+	+	+	+
Dog, Sonora	+	+	+	+	+
Dog, Gabon	+	+	+	+	+
Dog, Thailand	+	+	+	+	+
Bat, *Lasiurus borealis*, TN	+	+	−	+	−
Bat, *Eptesicus fuscus-Myotis* spp., CO	+	−	+	+	+
Bat, *Myotis* spp., WA	+	+	−	+	+
Bat, *Lasiurus cinereus*, AZ	+	+	−	+	−
Bat, *Lasiurus cinereus*, NY	+	+	−	+	−
Bat, *Pipistrellus subflavus*, AL	+	+	+	+	+
Bat, *Tadarida brasiliensis*, AL	+	+	+	+	+
Bat, *Lasionycteris noctivagans*, WA	+	+	+	+	+
Bat, *Eptesicus fuscus*, PA	+	+	−	−	−
Bat, *Pipistrellus hesperus*, CA	+	+	−	−	−
Bat, *Desmodus rotundus*, TN/MX	+	+	+	+	+
Bat, *Desmodus rotundus*, Brazil	+	+	+	+	+

[a] Neutralizing activity was determined in a standard RFFIT. SRIG, standard rabies immunoglobulin, lot R3; +, neutralization; −, no neutralization; SE US, southeastern United States; MX, Mexico.

Breadth of neutralizing activity toward a representative panel of street RV and lyssaviruses of genotypes 2 to 7. To further explore the applicability of both CR57 and the eight novel anti-rabies IgGs, we analyzed the breadth of neutralization against a broad panel of representative street RV. Initially, we analyzed the breadth of neutralizing activity of CR57, which revealed that CR57 neutralized all but two RV isolates, i.e., south central skunk, and bat, *Eptesicus fuscus-Myotis* spp. (Table 3). We tested our panel of eight IgGs against these two RV isolates (data not shown). The experiments showed that the antibodies CR4038, CR4098, and CR4144 displayed neutralizing activity against these two RVs, and they were subsequently tested against the representative RV panel (Table 3). The results show that CR4098 demonstrated the broadest coverage of the full RV panel (24/26), whereas CR4038 and CR4144 did not cover six and five RV isolates, respectively.

In addition, analysis of representative lyssaviruses of genotypes 2 to 7 revealed that all four MAbs had neutralizing activity against genotype 7, while CR57 showed additional neutralizing activity towards genotypes 4, 5, and 6 (data not shown). Overall, CR4098 displayed the broadest coverage of

TABLE 4. Characterization of E98 escape viruses[a]

Virus	NI		Codon change	Amino acid change
	CR4098	CR57		
E98-2	0	4.2	AAT to GAT	N336D
E98-4	0	3.9	AAT to GAT	N336D
E98-5	0.7	2.8	AAT to GAT	N336D
			CAT to CAG	H354Q
E98-6	0	2.8	AAT to GAT	N336D
E98-7	0	2.5	AAT to GAT	N336D

[a] Neutralization index (NI) is shown as the log10 value for each antibody. NI of <2.5 was considered as escape. Amino acid numbering is from the mature glycoprotein minus the signal peptide. Wild-type CVS-11 glycoprotein sequence was determined in a similar fashion from the original virus stock used to generate the escape viruses.

the RV panel and was thus the best candidate MAb to complement CR57.

Generation and characterization of escape viruses of CR4098. To further investigate whether CR4098 and CR57 recognized distinct epitopes, we generated five CR4098-resistant escape viruses (E98 escape viruses). The escape viruses had altered growth characteristics and were difficult to amplify, suggesting that amino acid changes in the virus had occurred. Neutralization of the E98 escape viruses by CR57 was examined by determination of NI for each escape virus. Each escape virus was amplified in the absence or presence of either selecting antibody, i.e., CR4098 or the nonselecting antibody CR57. Virus titers were determined and used to calculate the NI as described in Materials and Methods (Table 4). Each E98 escape virus was no longer neutralized by CR4098 but was still neutralized efficiently by CR57, which was in agreement with the earlier finding that CR4098 neutralized the E57 escape viruses (Table 2). This suggested that both antibodies recognized different nonoverlapping epitopes on the RV glycoprotein and that binding of the antibodies is not affected by mutations in either epitope. To further confirm this, the glycoprotein ORF of each E98 escape virus was analyzed. DNA sequencing revealed one common point mutation in the glycoprotein ORF of all five E98 escape viruses, N336D (Table 4), and one additional mutation (H354Q) in the glycoprotein ORF of the E98-5 escape virus. The N336D mutation is located in antigenic site III (Fig. 2) and has been observed previously in escape viruses that were generated using mouse MAbs recognizing antigenic site III (25). This finding may also

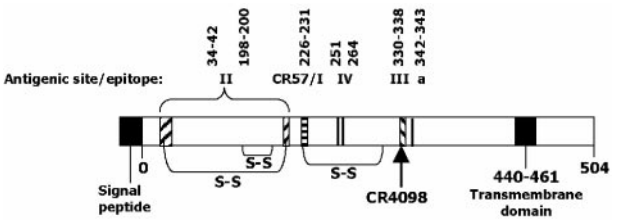

FIG. 2. Neutralizing epitopes on RV glycoprotein. A schematic drawing of the RV glycoprotein is shown depicting the antigenic sites including the recently described CR57 epitope (19). The arrow indicates the location of the CR4098 epitope (antigenic site III). The signal peptide (19 aa) and transmembrane domain are indicated by black boxes. Disulfide bridges are indicated. Amino acid numbering is from the mature protein minus the signal peptide.

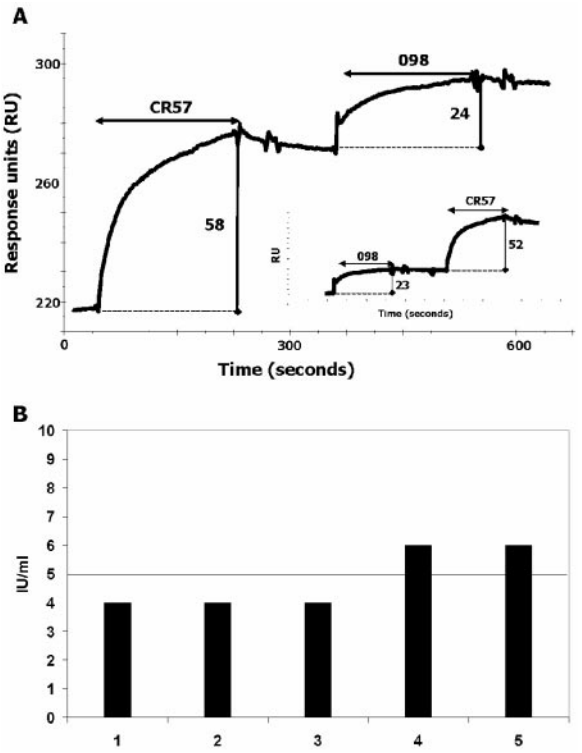

FIG. 3. CR57 and CR4098 bind to different epitopes on RV glycoprotein. (A) SPR experiments were performed by injecting CR57 and CR4098 (1 μM) without regeneration between these injections. Time in seconds (*x* axis) is plotted against RU (*y* axis). RU levels are indicated for each injected antibody. Inset graph shows the reverse experiment. (B) CR57 and CR4098 were mixed in different ratios to obtain a 5-IU/ml antibody solution, which was then tested in a modified RFFIT for neutralizing activity. The activity (in IU/ml) of each combination is shown. Horizontal line indicates 5 IU/ml. The bars on the graph represent the following antibodies: 1, 100% CR4098; 2, 25% CR57 plus 75% CR4098; 3, 50% CR57 plus 50% CR4098; 4, 75% CR57 plus 25% CR4098; 5, 100% CR57.

explain the altered growth characteristics observed for each escape virus, since the antigenic site III has been implicated in viral fitness (4, 8, 25). Epitope mapping studies using Biacore on RV glycoprotein-coated chips confirmed that CR57 and CR4098 bind to different epitopes on RV glycoprotein (Fig. 3A). Injection of CR57 resulted in a response of 58 RU. After injection of CR4098, an additional increase in response levels (24 RU) was obtained, suggesting that binding sites for CR4098 were not occupied by CR57. Similar results were observed when the reverse order was applied, showing that each antibody reached similar RU levels regardless of the order of injection. This formally demonstrated that CR57 and CR4098 bind to different epitopes on RV glycoprotein. Functionally, this was illustrated by the additive effects of different combinations of CR57 and CR4098 on in vitro neutralization of CVS-11 (Fig. 3B). Each mixture of CR57 and CR4098 had a precalculated neutralizing activity of 5 IU/ml. The results showed that the actual activity varied between 4 to 6 IU/ml, suggesting that both MAbs contribute to neutralization in an additive rather than a synergistic manner. Overall, the results are in agreement with data indicating that CR57 and CR4098 do not compete for binding to RV glycoprotein and cross-

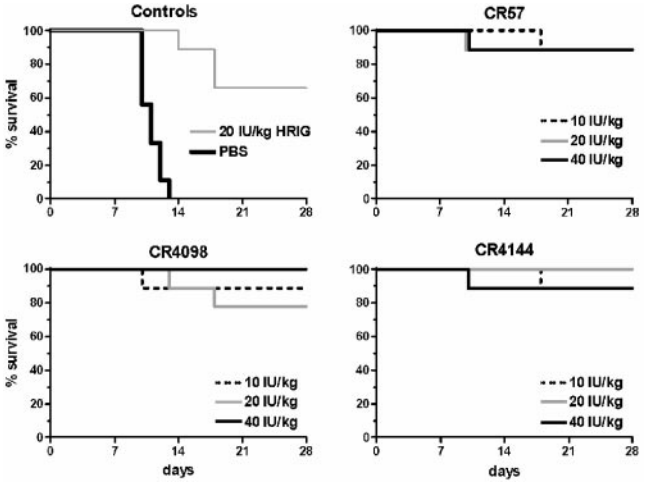

FIG. 4. Anti-RV MAbs protect Syrian hamsters from a lethal RV infection. Syrian hamsters were challenged with COSRV on day −1. Animals were vaccinated with rabies vaccine and treated with 10, 20, or 40 IU/kg of body weight of CR57, CR4098, or CR4144 on day 0. Control groups received either phosphate-buffered saline or 20 IU/kg HRIG. Animals were monitored twice daily and euthanized when clinical signs of rabies appeared. Kaplan-Meier survival curves are shown by plotting percent survival against time (in days).

neutralize each other's escape viruses (Tables 2 and 4). Historically, these data agree with the observation that the antigenic site I antibody 509-6 neutralized antigenic site III mutant viruses (8) and antigenic site III antibodies neutralized SO57 (antigenic site I) escape viruses (6).

Sequence analysis of antigenic site III among rabies street viruses. Previously, we analyzed the glycoprotein sequences of a large set of natural RV isolates showing that 3/229 isolates (~1%) contained mutations that would most likely abrogate binding of CR57 (19). This indicated that the CR57 binding region (KLCGVL) is highly conserved, which may be explained by the involvement of the central cysteine residue in the folding of the glycoprotein. In a similar analysis for CR4098, 123 glycoprotein sequences could be analyzed from the database. From the 123 naturally occurring RV samples, 5/123 (4%) harbored a nonconserved amino acid mutation in antigenic site III (N336D), all five RV being isolated in central Africa. None of the 123 RV samples displayed mutations in both the CR57 epitope and CR4098 epitope simultaneously.

In vivo protection by anti-rabies IgGs from a lethal RV infection. To investigate whether the antibodies display neutralizing activity against a lethal RV infection in vivo, we performed a Syrian hamster study. At 24 h after administration of a lethal dose of COSRV, prophylaxis was initiated with vaccine alone, vaccine plus HRIG (20 IU/kg), or vaccine plus 10, 20, and 40 IU/kg of a single MAb (CR57, CR4098, or CR4144). Animals were monitored during the course of the experiment and were euthanized when signs of clinical rabies occurred. Rabies was confirmed by a direct fluorescent antibody test for each euthanized animal (data not shown). Figure 4 shows the overall results for three antibodies, i.e., CR57, CR4098, and CR4144. All three antibodies provided equal protection against the lethal RV infection at each of the three antibody dosages tested. No clear dose-response effect was observed, illustrating the strong neutralizing potency of each of the MAbs in vivo. The MAbs provided protection against rabies, with a survival range of 78% (7/9) to 100% (9/9), whereas treatment with HRIG resulted in a survival rate of 67% (6/9) in this study. In conclusion, each of the MAbs tested protected hamsters equally as well as HRIG after exposure to a lethal RV infection.

DISCUSSION

Current PEP for severe bites by rabid animals involves the use of HRIG or equine RIG. Blood-derived products, like HRIG, have potential health risks inherently associated with these products. In addition, HRIG can display batch-to-batch variation and may be of limited availability in case of sudden mass exposures (2, 3). HRIG could be replaced by human MAbs produced according to pharmaceutical industry standards. The concern arising from the use of blood-derived products could thereby be circumvented, and consistent batches of antibodies could be produced in large quantities. Therefore, we initiated a study to identify and characterize human MAbs that are compatible with each other for potential use in rabies PEP. Recently, we characterized the human MAbs SO57, SOJA, and SOJB that displayed potent neutralization of several RV strains as described by Prosniak et al. (22). After reformatting these MAbs into the PER.C6 expression system (renamed MAbs to CR57, CRJA, and CRJB) and further characterization, it became evident that the neutralizing potency of CRJA was too low and that antibodies CR57 and CRJB were not compatible in an anti-RV MAb cocktail (19). The latter study also revealed that CR57 recognized antigenic site I, which is highly conserved among a large panel of street RVs, most likely due to the structural constraints of this region of the glycoprotein. We considered CR57 an attractive candidate based on its neutralizing characteristics for use in combination with other MAbs. To complement CR57, we opted to find a MAb that recognizes a different antigenic site. Our preference for antigenic site III over antigenic site II was based on reports describing its major role in viral propagation in the nervous system and pathogenicity (4, 5, 8, 25, 28) as well as its role in receptor binding (27). Nonoverlap between antigenic sites I and III was predicted from historical data. An escape virus of SO57 (antigenic site I) was neutralized by several mouse MAbs directed against antigenic site III (6), and an escape virus harboring an antigenic site III mutation was still neutralized by the mouse MAb 509-6, which is like CR57 directed against antigenic site I (8). Since antibody-induced mutations in antigenic site III can have an adverse effect on the pathogenicity of RV, apparently more so than antigenic site II mutations, such mutant viruses would be rendered harmless in vivo and ineffective in causing disease in their host. In fact, the attenuated RV vaccine strains HEP-Flury, Kelev, SAD-B19, and CTN have a mutated antigenic site III (23, 26) (GenBank accession no. AY009100).

We aimed to develop a combination of antibodies against antigenic sites I and III. Addition of a third MAb specific for antigenic site II would not improve the product since it has been shown that some MAbs against antigenic sites II and III compete with each other for binding to glycoprotein (17, 18). This is most likely caused by the close proximity of antigenic

sites II and III in the tertiary structure of the glycoprotein (16). Interference between antigenic site II and III MAbs could result in antagonistic effects on binding and reduced neutralizing potency.

The antigenic structure of the RV glycoprotein was initially defined by Lafon et al. (18). The antigenic sites were identified using a panel of mouse MAbs and their respective MAb-resistant virus variants. Since then, the antigenic sites have been mapped by identification of the amino acid mutations in the glycoprotein of MAb-resistant variants (1, 21, 25). Antigenic site III is a continuous conformational epitope at amino acids (aa) 330 to 338 and harbors two charged residues, K330 and R333, that play a role in viral pathogenicity (4, 8, 25), likely due to interaction with the receptor (27). The conformational antigenic site I was initially defined by only one MAb, 509-6, and located at aa 231 (1, 18). Recently, we have shown that CR57 recognizes a linear epitope at position 226 to 231, and we have redefined antigenic site I as a neutralization epitope complex harboring both conformational (MAb 509-6 and CRJB) and linear (MAb CR57) epitopes (19). Our current results demonstrate that the antibodies CR57 and CR4098 recognize two distinct epitopes localized in antigenic sites I and III, respectively.

Several mechanisms of virus neutralization exist, of which blocking of the virus-receptor interaction is the most common. Other mechanisms include interference with postattachment interactions, virus internalization, fusion of the viral and cellular or endosomal membrane, or uncoating of virus. Several of these mechanisms have been described for RV as well (7, 11). The exact mechanism of action for CR57 and CR4098 is unknown but is most likely a result of coating of the virion by the MAb, thereby blocking the interaction with the receptor. PEPSCAN analysis of binding of CR57 to peptides harboring a mutated CR57 epitope (as observed in E57 escape viruses) showed that interaction of CR57 was abolished (data not shown). Interestingly, CR4098 still bound to the mutated glycoprotein (N336D) expressed on PER.C6 cells, as measured by flow cytometry (data not shown), even though viruses containing this mutation were no longer neutralized (Table 4). To our knowledge only two other MAbs have been described that still bound but no longer neutralized their MAb-resistant viruses (11, 15). The observation that some MAbs with an apparent low affinity have a very high neutralizing potency may indicate that such MAbs employ a different neutralization mechanism than MAbs with a higher affinity, like CR57. This suggests that our phage display selection method is capable of isolating MAbs that use different modes of virus neutralization.

Global coverage of natural RV isolates by the combination of CR57 and CR4098 MAbs is predicted by both the in vitro neutralization spectrum and the analysis of available RV glycoprotein sequences; not a single natural isolate was identified harboring mutations in both epitopes recognized by CR57 and CR4098. Phylogenetic analysis of these RV glycoprotein sequences revealed that the rare isolates harboring critical mutations in the CR57 epitope were only distantly related to those isolates harboring critical mutations in the CR4098 epitope (data not shown). In addition, these isolates were segregated with respect to their geographic location. Since rabies PEP employing the human antibody combination will not occur in the natural hosts, i.e., rabid carnivores or bats, the potential occurrence of a virus in nature with both the CR57 and the CR4098 epitopes disrupted is virtually nonexistent. This was underlined by our observation that escape viruses under selective pressure of CR57 and CR4098 could not be obtained in vitro (data not shown). Similarly, attempts using HRIG also did not yield escape viruses (data not shown). This suggests that the selective pressure by two antibodies is too high, most likely due to the structural and functional constraints of the antigenic sites that are recognized.

TABLE 5. Summary of selected optimal MAb combination

Parameter	Result for MAb:	
	CR57	CR4098
Isotype	IgG1(λ)	IgG1(κ)
Lyssavirus coverage	Genotypes 1 and 4–7	Genotypes 1 and 7
Epitope	Antigenic site I, linear	Antigenic site III, conformational
Neutralizing potency (IU/mg)	1,689	1,071
Affinity (K_D)	2.4 nM	4.5 nM

In conclusion, we have identified a combination of two potent antibodies, CR57 and CR4098, that fulfilled our criteria for use of human MAbs in a cocktail (Table 5). The MAbs recognize nonoverlapping, noncompeting epitopes, cross-protect against each other's escape viruses, and neutralize a broad range of street RVs. In addition, the combined coverage of genotypes 2 to 7 by the two MAbs was equal to that of HRIG. Furthermore, in vivo protection studies using the single MAb indicated that treatment of Syrian hamsters with each antibody resulted in protection equivalent to that offered by HRIG when hamsters were challenged with a lethal RV dose. Taken together, these results indicate that the selected antibody combination is optimally suited to replace RIG.

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