Conservation of Binding Epitopes for Monoclonal Antibodies on the Rabies Virus Glycoprotein

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Abstract

The global need for rabies immune globulin (RIG) for post-exposure prophylaxis (PEP) is significant. The cost of RIG, either of equine or human origin, is prohibitive for most patients in developing countries. Limitations of supply may occur worldwide. Several virus-neutralizing monoclonal antibodies (MAbs), binding to the rabies virus glycoprotein have been proposed as a replacement of conventional RIG in human PEP due to the ability of large-scale production at a reduced cost. In the present study we analyzed 1,042 rabies virus glycoprotein sequences, generated de novo and retrieved from GenBank, to determine the conservation of binding epitopes for several well characterized rabies virus-neutralizing MAbs. Our analysis demonstrated that the use of a single MAb for rabies PEP is inappropriate, because certain viral sequences had critical amino acid substitutions in binding epitopes for each MAb. Rather, a cocktail of MAbs, targeting non-overlapping epitopes, offers a reliable alternative, as no sequences from our study harbored critical substitutions in binding sites for two or more MAbs simultaneously.

Introduction

Rabies is an acute progressive viral encephalomyelitis with the highest case fatality rate (nearly 100%) among conventional infectious diseases. The disease is caused by single-stranded negative-sense RNA viruses from the genus *Lyssavirus* that circulate in mammals (predominantly from the *Carnivora* and *Chiroptera* orders) worldwide, except Antarctica and several isolated islands [1]. Rabies causes more than 5 tens of thousands of human deaths every year. Most cases occur in developing countries of Asia and Africa, affected by dog rabies [2]. Although after onset of symptoms rabies is almost invariably fatal, the disease may be prevented by prompt wound care and post-exposure prophylaxis (PEP). Modern PEP includes combined administration of rabies vaccine and anti-rabies immune globulin (RIG). An active immune response from rabies vaccination develops after the first week of PEP, whereas RIG provides immediate passive protection against rabies virus. Antibodies directed against the rabies virus (RV) glycoprotein (G) neutralize virions and mediate the complement lysis and/or antibody-dependent cellular cytotoxicity of RV-infected cells [3].

Two types of RIG are currently available in the market, either heterologous equine (ERIG) or homologous human (HRIG) origin. RIGs are derived from pooled sera of horses or human donors immunized against rabies. Historically, allergic reactions are a risk connected with ERIG use, and therefore HRIG is recommended for PEP [3]. However, HRIG is prohibitively expensive, especially in developing countries. In addition, the global supply of RIGs is restricted. Even in developed countries, such as the United States, there are periodic constraints on the availability of HRIG, particularly associated with mass human exposures [4,5]. In addition, supply alterations may occur from changes in market directions, regulatory procedures, screening methods of donor serum for infectious agents, and other changes in production techniques [6]. Although donor serum is screened for known pathogens, inherent potential health risks (associated with emerging pathogens, coagulants, immunogens, or other factors) continue to be associated with human blood-derived products.

Therefore, the need for RIG alternatives, such as production of monoclonal antibodies (MAbs), is widely recognized [7]. Both mouse and human MAbs binding to the RV G can neutralize virus *in vitro* and protect animals against experimental rabies challenge [8-14].

The RV G forms a homo-trimer structures projecting from the surface of the virion. As the only outer protein of the virus, the RV G has a pivotal role in virion attachment to host cell receptors, and is a crucial component in development of immune responses against RV [15]. In previous studies, the antigenic structure of RV G was defined using panels of MAbs and their respective neutralization-resistant virus variants. Antigenic site I harbors both conformational and linear epitopes and is located at position 226-231 [16]. Antigenic site II is a discontinuous conformational epitope at residues 34-42 (IIb) and 198-200 (IIa). Antigenic site III is a continuous conformational epitope at residues 330-338. Antigenic site IV has one amino acid at position 251. Minor site "a", also referred to as G1, is located at position 242-243. The G5 antigenic site is a linear epitope at residues 261-264, and includes antigenic site VI, described earlier as a site with only one amino acid at position 264 [17-20]. The placement of the recognized antigenic sites on the RV G is demonstrated in figure 1.
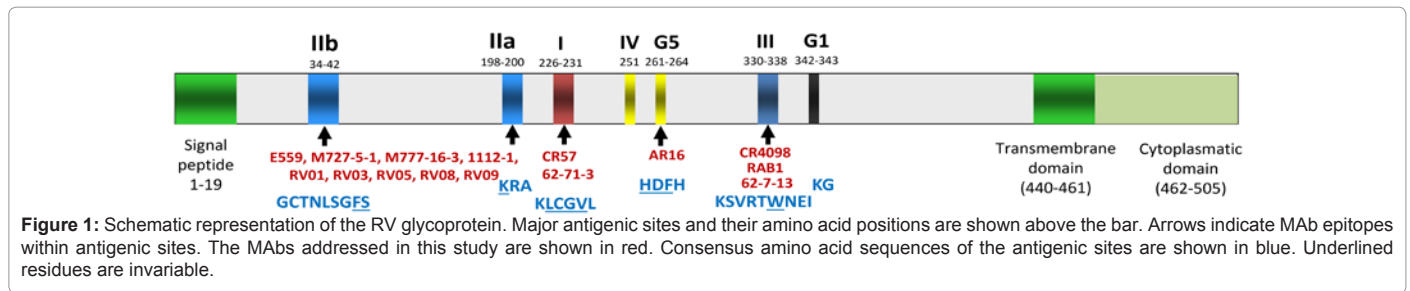
Several MAbs were offered for replacement of the conventional RIG in the human rabies PEP. Not all MAbs have been sufficiently studied and characterized to date. Three human MAbs, SOJA, SOJB, and SO57 [8], which initially were shown to be as efficient as HRIG in protection of mice against RV [12], were reformatted for production in PER.C6 cells and renamed CRJA, CRJB and CR57, respectively.

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Figure 1: Schematic representation of the RV glycoprotein. Major antigenic sites and their amino acid positions are shown above the bar. Arrows indicate MAb epitopes within antigenic sites. The MAbs addressed in this study are shown in red. Consensus amino acid sequences of the antigenic sites are shown in blue. Underlined residues are invariable.

CR57 was demonstrated to be the most potent MAb [16]. Because of natural variability of RV, and particularly the observed variability of the MAb binding epitopes, a cocktail of at least two MAbs, covering non-overlapping epitopes, was recommended to assure adequate replacement of polyclonal products. To address this, a panel of novel human anti-rabies MAbs was identified, one of which, CR4098, proved to be fully complementary CR57 [21]. As demonstrated, CR57 recognizes a linear epitope within antigenic site I of the G ectodomain, amino acids 226 to 331, with critical for binding residues at positions 226, 228, 229 and 230 [16]. The conformational epitope for CR4098 comprises antigenic site III. Screening of 478 glycoprotein sequences available in GenBank demonstrated significant conservation of both epitopes [16,21]. Based on RV G sequence analysis and *in vitro* neutralization studies, the CR57/CR4098 MAb combination, CL184, was shown to neutralize an extensive panel of RV [22].

Another research group developed a human MAb RAB1 [23], also referred to as 17C7 [24] and SII RMAb [25]. This MAb binds to the antigenic site III with critical amino acids in positions 336 and 346 [23,24]. As the authors declared, this single MAb should be capable to neutralize all street RV variants, because the only critical combination of amino acid substitutions that precluded MAb binding in the laboratory escape mutants (N336D/R346K) was not detected in any RV G sequence available in public domain [23].

Chimeric MAb 62-71-3, directed against antigenic site I, was adapted to plant-based production [26]. Critical substitutions that diminished binding of this MAb to the RV G in the escape mutant studies were K226R and G229E. Other substitutions within antigenic site I and their potential significance for MAb binding, as well as breadth of neutralization were not described.

A panel of mouse MAbs (MoMAbs) was offered by a group of WHO collaborating centers. Five MoMAbs, E559, 1112-1, 62-7-13, M727-5-1 and M777-16-3, were selected from available panels based on stringent criteria (i.e. non-overlapping binding epitopes and absence of interference). Four of these MoMAbs recognize epitopes in antigenic site II and one recognizes an epitope in antigenic site III of the RV G. Unfortunately, specific information on binding sites for these MoMAbs is very limited. Muller et al. (2009) described critical substitutions for MoMab E559 at positions L38R and K198E, and G34E for MoMab 1112-1. The substitutions K198E and A286T were listed for MoMab M777-16-3. Three MoMAb combinations had an equal or superior effectiveness to HRIG *in vitro* and *in vivo* for a limited RV panel studied [14].

Another human MAb, AR16, binds to the epitope HDFR (aa 261-264) within antigenic site G5, with critical residues HDF (aa261-263). The substitutions H261A, D262A and F263A abolished binding of this MAb [27]. However, whether other residues at these positions would abolish binding is unknown. Additionally, R264H and R264N

substitutions had no effect on binding activity of AR16. Although conservation of the critical residues is apparent within Phylogroup I lyssa viruses, neutralization data has only been published using the RV laboratory strain CVS-11.

Other RV-neutralizing MAbs, such as No. 254 and 4D4 [28], a panel of MAbs developed by the National Institute of Mental Health and Neurosciences of India [29], and a panel of MAbs developed by the National Institute for Viral Disease Control and Prevention, China CDC [30], have not been sufficiently characterized. In the present study, we generated G sequences from RV variants of worldwide origin, supplemented the dataset with those available in GenBank, and analyzed sequence conservation in described MAb binding sites.

Material and Methods

The RV-positive brain tissue samples were obtained via routine surveillance activity of CDC (Atlanta, GA, USA), as well as via national and international requests in the framework of activity of the WHO Collaborating Center for Reference and Research on Rabies. On several occasions where quality of the original field sample was poor or limited, and the amount of tissue did not allow reliable amplification of RV G in RT-PCR, a single suckling mouse brain passage was performed.

Total RNA was extracted from infected brain tissues using TRIZol reagent (Invitrogen). Primers were designed according to the sequences of rabies viruses available from GenBank. The RT-PCR was performed as described elsewhere [31]. When multiple product bands were obtained in the RT-PCR with certain primer sets, separation in low-melting agarose gel was performed [32]. The RT-PCR products were purified and subjected to direct sequencing. The sequencing products were processed on an ABI 3730 DNA Sequencer (Applied Biosystems). The complete nucleotide G sequences were assembled and converted into amino acid sequences using the Bio Edit program [33]. The dataset was supplemented with complete and partial G sequences available from GenBank. Amino acid sequences of the aligned MAb binding epitopes were compared across the dataset. To evaluate the phylogenetic origin of the RV variants incorporated in the study, Bayesian analysis was implemented in the BEAST, v. 1.6.1 under the GTR+G+I model, and visualized in Fig Tree, v. 1.3.1 [34].

Results

In total, 658 RV G sequences were generated during the study (GenBank accession numbers KC791791 - KC792277). These were supplemented with 384 complete and partial G sequences available from GenBank (total n=1042). As shown in the phylogenetic tree (Figure 2), we encompassed all known major RV lineages circulating worldwide.

Binding epitope for MAb CR57

The binding epitope for MAb CR57 within antigenic site I was relatively conserved (Table 1). The substitution which abolished
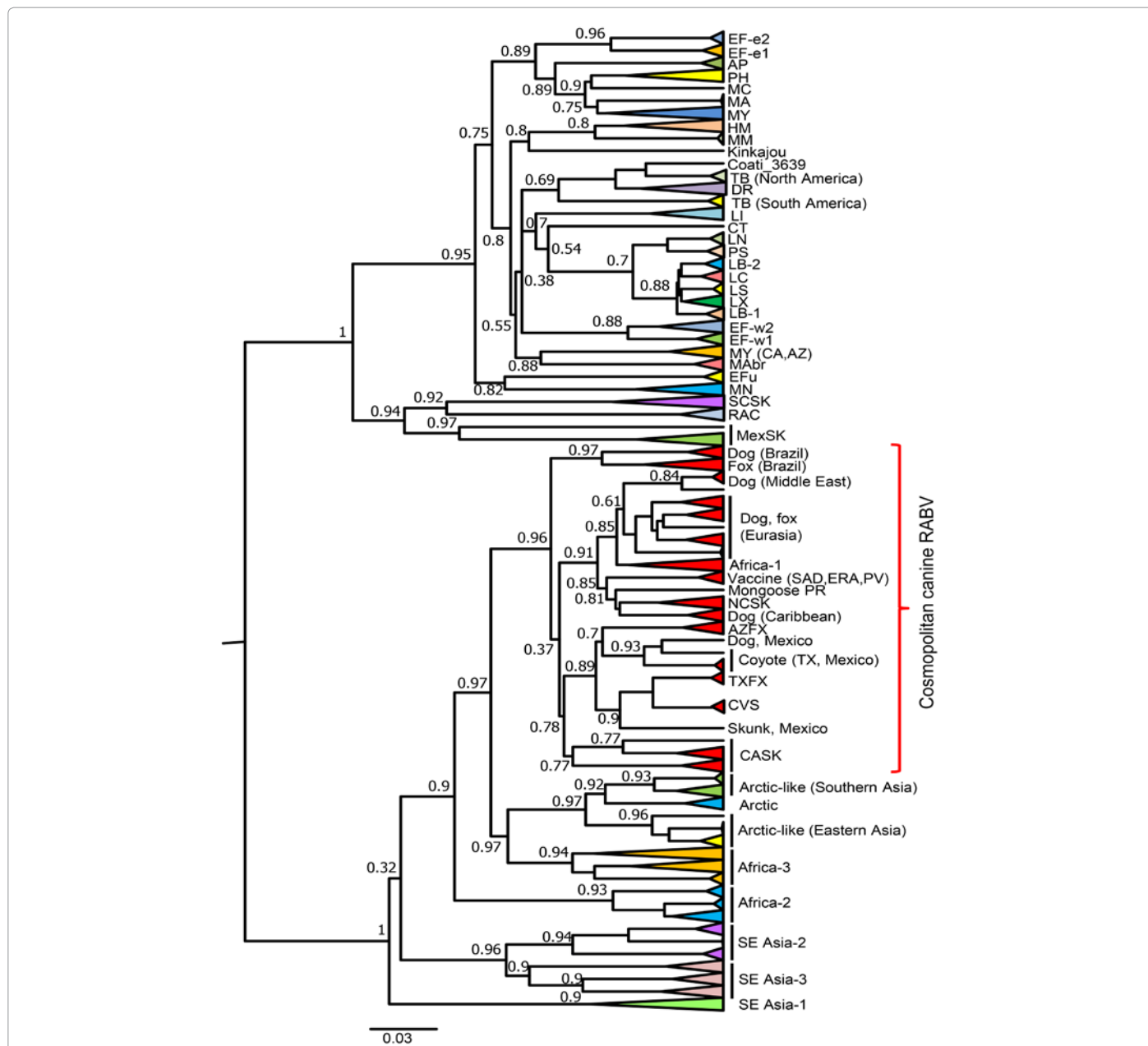
Figure 2: Bayesian tree of RV G sequences analyzed in this study. Phylogenetic lineages that contain more than one sequence are collapsed. Posterior probabilities are shown for key nodes. Lineage abbreviations: EF-e1, *Eptesicus fuscus* bats, eastern lineage 1; EF-e2, *E. fuscus* bats, eastern lineage 2; AP, *Antrozous pallidus* bats; PH, *Parastrellus hesperus* bats; MC, *Myotis californicus* bats; MA, *Myotis austroriparius* bats; MY, *Myotis* spp. bats; HM, *Histiotus montanus* bat; MM, *Molossus molossus* bats; Kinkajou, *Potos flavus*; Coati_3639, a virus from an outbreak among *Nosua nosua* (Oaxaca, Mexico); TB (North America), *Tadarida brasiliensis* bats from North America; DR, *Desmodus rotundus* bats; TB (South America), *T. brasiliensis* bats from South America; LI - *Lasiurus intermedius* bats; CT, *Corynorhinus townsendii* bat; LN, *Lasionycteris noctivagans* bats; PS, *Perimyotis subflavus* bats; LB-1, *Lasiurus borealis* bats, lineage 1; LB-2 *L. borealis* bat, lineage 2; LC - *Lasiurus cinereus* bats; LS - *Lasiurus seminolus* bat; LX - *Lasiurus xanthinus* bat; EF-w1, *E. fuscus* bats, western lineage 1; EF-w2, *E. fuscus* bats, western lineage 2; MY (CA, AZ): *Myotis* spp. bats from Arizona and California; MAbr, *Molossus abrasus* bats; EFu, *Eptesicus furinalis* bats; MN, *Myotis nigricans* bats; SCSK, south-central skunk; RAC, raccoon; MexSK, skunk RV from Mexico; NCSK, north-central skunk; AZFX, Arizona gray fox; TXFX, Texas gray fox; CVS, challenge virus standard (laboratory strain); CASK - California skunk.

binding of this MAb to laboratory-generated escape mutants [16,21], such as K226M, was detected in only one RV sequence, from a Chinese dog (GenBank accession no. FJ418881, phylogenetic lineage SE Asia-2; Figure 2). Other substitutions detected (such as K226R in a raccoon isolate and in African dog isolates from phylogenetic lineage Africa-1; L231P/S in several skunk, raccoon, and various bat RV lineages) did not preclude virus neutralization from previously published studies

[16,21]. We did not encounter substitutions K226E and G229E that abolished binding of MAb CR57 to the escape mutants generated from the laboratory RV strain CVS-11 [30]. However, we encountered another mutation, K226W in all three available sequences of RV from a black myotis bat (*Myotis nigricans*; GenBank accession no. GU123648-GU123650) from Brazil (0.29% in the complete sequence dataset), and substitution K226Q in a vaccine RV strain used in Nepal (GenBank

accession no. KC792230). The significance of these latter two substitutions for MAb binding had not been addressed in published *in vitro* and *in vivo* experiments.

Binding epitope for MAb CR4098

In contrast, the binding epitope for MAb CR4098 within antigenic site III was more variable (Table 2). The critical substitution that precluded binding of MAb CR4098 in the escape mutant studies, N336D, was detected in 63 (6%) sequences. These viruses originated from North American big brown bats (*Eptesicus fuscus*), a South American small big-eared brown bat (*Histiotus montanus*), dogs from phylogenetic lineage Africa-2 and mongooses from lineage Africa-3. Other substitutions, such as K330N (bat isolates from South America), S331L in RV from North American red bats (*Lasiurus borealis*), R333K/N/Q in several bat RV lineages from North and South Americas, V332I/F in several isolates from *E. fuscus*, I338V in the RV from bats of *Lasiurus* genus, N336S in the raccoon RV variant and in the RV variant found in *Myotis austroriparius* bat (USA), did not preclude virus neutralization by MAb CR4098 in the escape mutant studies and previous neutralization reports for field RV isolates [16,21]. In addition, we found a new substitution N336G in several raccoon isolates (GenBank accession no. KC792019, KC792027, KC792031), not observed in previous studies. It is unknown whether viruses with this substitution can be neutralized by MAb CR4098.

Nevertheless, it is important to note that no RV isolates had substitutions in CR57 and CR4098 MAb binding sites simultaneously.

Binding epitope for MAb RAB1

Among all sequences analyzed, only one had the critical amino acid combination 336D/346K in the antigenic site III that abolished virus neutralization by MAb RAB1 in the escape mutant studies [23]. This virus originated from a *H. montanus* bat from Peru (GenBank accession no. KC792200; Table 2). Another substitution, N336G, detected in several raccoon RV isolates (GenBank accession no. KC792019, KC792027, KC792031), was not addressed in the previous studies on MAb RAB1 [23,24], and therefore it is unknown whether it would abolish binding of this MAb to the RV G.

Binding epitope for MAb AR16

The AR16 MAb binds to the linear epitope within antigenic site G5 with critical amino acids HDF at positions 261-263. As the only information published addresses substitutions H261A, D262A and F263A that abolished binding of MAb AR16 [27], we assessed overall conservation of these three amino acids across our dataset (Table 3). Substitution H261P was found in RV from a vampire bat (*Desmodus rotundus*), although in only one sequence (GenBank accession no. AB110669) of 37 examined (0.1% of the complete dataset). Substitution H261Y was detected in RV from a coyote, in one sequence of seven examined (GenBank accession no. AF325477). Substitution D262N was detected in South African mongoose RV (phylogenetic lineage Africa-3; two sequences out of 26 examined (GenBank accession no. FJ465390, FJ465403), and in a single available RV isolated from a kinkajou (*Potos flavus*) from Peru (GenBank accession no. KC792208). Substitution F263L was detected in all 150 sequences from the south-central skunk RV lineage (SCSK), which constituted 14.4% of our complete dataset, whereas substitution F263I was identified in one RV isolate from a *Myotis* sp. bat from USA (GenBank accession no. JQ685921).

Binding epitope for MAb 62-71-3

This MAb binds within antigenic site I, and only two critical

Variant	Number of sequences	Fraction of sequences
K**L**C**G**VL	396	40.53%
KLCGV**S**	326	33.37%
KLCGV**P**	248	25.38%
WLCGVP	3	0.31%
MLCGVL	1	0.10%
QLCGVL	1	0.10%
RLCGVL	1	0.10%
RLCGV**S**	1	0.10%
Total	1033	

Table 1: Amino acids in the antigenic site I (226-231) of the rabies virus glycoprotein in the dataset studied (amino acid substitutions compared to the consensus sequence are shown in **bold italics** and invariable residues are underlined).

Variants	Number of sequences	Fraction of sequences
KSVRT**W**NEI	486	49.59%
KSVRTW**S**EI	143	14.59%
KS**I**RTWNEI	126	12.86%
KSVRTWNE**V**	66	6.73%
KSV**K**TWNE**V**	46	4.69%
KSVRTW**D**EI	37	3.78%
KS**I**RTW**D**EI	24	2.45%
NSVRTWNEI	9	0.92%
KSV**N**TWNEI	6	0.61%
KSVRTWN**DV**	5	0.51%
KS**IQ**TWNE**V**	4	0.41%
KS**IQ**TWNEI	3	0.31%
KSVRTW**G**EI	3	0.31%
KSVRTWN**D**I	3	0.31%
KSVRTW**SE**V	3	0.31%
KSV**K**TWNEI	2	0.20%
KSV**Q**TWNEI	2	0.20%
ESVRTWNEI	1	0.10%
K**L**V**K**TWNE**F**	1	0.10%
KS**FR**TW**D**EI	1	0.10%
KS**IK**TWNE**V**	1	0.10%
KS**I**RTWN**K**I	1	0.10%
KSV**H**TWNE**V**	1	0.10%
KSV**KN**WNE**V**	1	0.10%
KSV**Q**TWNE**V**	1	0.10%
KSVR**N**WNEI	1	0.10%
KSVRTWNE**T**	1	0.10%
NSVRTW**D**EI.....K346*	1	0.10%
TSVRTWNEI	1	0.10%
Total	1031	

*Sequence of RV G from *Histiotus montanus* bat with the residue combination 336D/346K which abolished binding of MAb RAB1 in the escape mutant studies [23].

Table 2: Amino acids in the antigenic site III (330-338) of the rabies virus glycoprotein in the dataset (amino acid substitutions compared to the consensus sequence are shown in **bold italics** and invariable residues are underlined).

substitutions K226R and G229E that diminished neutralization were described for laboratory-generated pseudotypes [26]. Both these substitutions, as well as other substitutions in position 226 (such as M, W and Q) not addressed by Both et al. [26] but encountered in our dataset, are described above in the section dedicated for MAb CR57 and in table 1.

Binding epitopes for MoMAbs E559, 1112-1, 62-7-13, M727-5-1 and M777-16-3

Although limited information is available for binding sites of the WHO MoMAbs, some specific residues and the indicated antigenic sites for each MoMAb identified by Muller et al. (2009) were evaluated. Viral escape mutant analyses were described for MoMAb E559 and 1112-1, but not for MoMAbs 62-7-13, M727-5-1 and M777-16-3. The binding sites for the latter three MoMAbs were assessed on the basis of cross-neutralization [14]. The critical substitution L38R (antigenic site II) that precluded binding of MoMAb E559 in the escape mutant studies, was detected in one of the three available California skunk isolates (phylogenetic lineage CASK; GenBank accession no. KC792197). The critical substitution G34E (antigenic site II) that precluded binding of MoMAb 1112-1, was detected in one of 13 available raccoon dog isolates from Korea (GenBank accession no. DQ076099), belonging to the Arctic-like RV lineage from Eastern Asia (Tables 2 and 4).

Binding epitopes for MAbs RV01, RV03, RV05, RV08 and RV09

Assessment of the significance of sequence diversity in the antigenic site II for binding of MAbs RV01, RV03, RV05, RV08 and RV09, developed at the National Institute for Viral Disease Control and Prevention, China CDC, was challenging due to a lack of published information on their binding epitopes [30]. According to the available data, the same substitution G34E that we found in a raccoon dog RV isolate from Korea abolished binding of all these MAbs. Another critical substitution described in the escape mutants of the laboratory RV strain CVS, K198E (antigenic site II), was not detected in our dataset of field RV isolates (Tables 4 and 5).

Discussion

The significance of passive immunization via administration of immune globulins at early stages of human rabies PEP has been well recognized [3,35,36]. The absence of passive immunization increased mortality in animal challenge experiments [10,22,37] and led to PEP failures in human patients with severe bites [38-40]. Conventional RIG preparations are prohibitively expensive in developing countries, where the burden of rabies is greatest [14,40]. Replacement of RIG with

Variants	Number of sequences	Fraction of sequences
HDFH	462	44.55%
HDF**R**	412	39.73%
HD**L**H	150	14.46%
HDL**R**	7	0.68%
HD**IR**	1	0.10%
H**N**FH	1	0.10%
H**N**F**R**	2	0.19%
PDF**P**	1	0.10%
YDF**R**	1	0.10%
Total	1037	

Table 3: Amino acids in the antigenic site G5 (261-264) of the rabies virus glycoprotein in the dataset (amino acid substitutions compared to the consensus sequence are shown in ***bold italics***).

Variants	Number of sequences	Fraction of sequences
KRA	911	88.28%
K**K**A	118	11.43%
KR**V**	2	0.21%
K**G**A	1	0.10%
Total	1032	

Table 4: Amino acids in the antigenic site IIa (198-200) of the rabies virus glycoprotein in the dataset (amino acid substitutions compared to the consensus sequence are shown in ***bold italics*** and in variable residues is underlined).

Variant	Number of sequences	Fraction of sequences
GCTNLSG<u>F</u>S	439	42.75%
GC**NS**LSGFS	329	32.04%
GCT**S**LSGFS	188	18.31%
GC**N**NLSGFS	21	2.04%
GCT**T**LSGFS	12	1.17%
GC**DS**LSGFS	21	2.04%
GC**S**NLSGFS	8	0.78%
GC**NS**LSG**I**S	2	0.19%
GC**SS**LSGFS	2	0.19%
GCTNLS**E**FS	2	0.19%
ECTNLSGFS	1	0.10%
GCTNL**P**GFS	1	0.10%
GCTNLS**D**FS	1	0.10%
GCTN**R**SGFS	1	0.10%
G**G**TSLSGFS	1	0.10%
Total	1026	

Table 5: Amino acids in the antigenic site IIb (34-42) of the rabies virus glycoprotein in the dataset studied (amino acid substitutions compared to the consensus sequence are shown in ***bold italics*** and invariable residues are underlined).

more affordable synthetically-produced MAbs offers an alternative strategy to save human lives [12-14,16,21]. Large-scale production of MAbs is sufficient to provide global needs. Unlike RIG, MAbs can be produced consistently from batch to batch. Moreover, health risks associated with human blood-derived products, such as HRIG, would be eliminated by use of MAbs.

The challenge of using MAbs to replace RIG is to ensure that these biologics provide adequate protection against a wide natural variety of lyssaviruses. In the present study, we performed analysis of binding sites of several well-characterized MAbs within the greatest panel of RV G sequences available to date, representing viruses from all known phylogenetic lineages circulating worldwide.

The most comprehensive amount of published data has been available for MAbs CR57 and CR4098 [16,21,22]. Therefore we could perform the most rigorous analysis of binding sites for these MAbs. Our analysis demonstrated the presence of critical amino acid substitution in the binding site of MAb CR57 in only one RV G sequence (originating from a dog from China) out of 1044 analyzed (0.1%). Critical substitutions in the binding site of MAb CR4098 were detected in 63 RV G sequences (6%), originating from several lineages of American bat RVs, African dogs and mongooses. The significance of a new substitution N336G that we encountered in several raccoon

isolates, never addressed in previous studies, should be additionally evaluated in biological tests (*in vitro* virus neutralization as a minimum). Nevertheless, no RV G sequences had substitutions in binding sites for MAbs CR57 and CR4098 simultaneously. Therefore, the proposed use of these MAbs as a cocktail (referred to as CL184 [21,40]) is expected to neutralize all RV isolates analyzed in our study.

In contrast, the use of MAb RAB1 [23], also referred to as 17C7 [24] and SII RMAb [25] as a single MAb for human PEP may be insufficient, as we encountered RV that had the critical amino acid combination 336D/346K, which abolished binding of MAb RAB1 in the escape mutant studies [23]. This RV originated from the South American small big-eared brown bat (*H. montanus*), broadly distributed from Venezuela to Argentina [41]. Mutations elsewhere in antigenic site III also may have an impact, such as N336G that we identified in several raccoon RV isolates.

The MAb AR16, binding to a linear epitope within the antigenic site G5, has not been studied sufficiently. Only substitutions H261A, D262A and F263A, used for mapping, were reported to abolish antibody binding [27]. We found a number of other amino acid substitutions in these positions, such as H261P/Y, D262N, F263L/I, but their significance for MAb binding is unclear. As MAb AR16 was proposed for use in a cocktail with MAb CR57 [27] it may be of a great utility, as we did not detect sequences that had critical substitutions in the binding sites of both these MAbs simultaneously.

A panel of mouse MAbs (MoMAbs) developed by the WHO Collaborating Centers, represents a promising solution for development of a MAb cocktail for human PEP, based on the cross-neutralization studies [14]. Although, these candidate MAbs need more rigorous characterization in terms of binding sites, to ensure that the breadth of their neutralization will cover all variety of RV variants. For now, we were able to identify critical substitutions in binding sites for MAbs E559 and 1112-1. The first substitution, L38R, encountered in a California skunk isolate was detected by Muller et al. (2009) [14] both in the escape mutant studies and in the neutralization assay against a similar California skunk RV isolate. Of note, the authors mentioned that the California skunk RV was not efficiently neutralized by all MAbs except 62-7-13. However, based on the sequence data we did not find suggestions for lack of neutralization of several available California skunk RV isolates by MAb 1112-1, which suggests that binding epitope for this MAb should be further characterized. The critical substitution that abolished binding of MAb 1112-1 in the escape mutant studies, G34E, was detected in our study in an RV isolate from a raccoon dog from Korea. Binding epitopes for MAbs 62-7-13, M727-5-1 and M777-16-3 should be characterized before any sequence-based assumptions on a potential breath of their neutralization can be performed conclusively. Nevertheless, the preliminary empirical results suggest that at least several of these MAbs after additional characterization can be considered as candidates for a cocktail in replacement of the conventional RIG [14]. The same is true for the panel of MAbs RV01, RV03, RV05, RV08 and RV09 developed in the National Institute for Viral Disease Control and Prevention, China CDC [30]. Available data suggest that at least one of the field isolates from our dataset (a RV from a raccoon dog from Korea) may not be neutralized by any of these MAbs because of the critical substitution G34E.

In conclusion, in keeping with current WHO recommendations, a single MAb product should not be considered a suitable alternative for current RIG products, as was demonstrated in the present study. Conversely, MAb cocktails may supersede RIG in safety, affordability, and availability. The MAbs CR57 and CR4098 have been characterized most rigorously [16,21,22,42]. The cocktail of these MAbs, CL184, offers a suitable replacement for conventional RIG. Indeed, the use of CL184 should be evaluated in animal models in comparison to conventional RIG, and in clinical trials to evaluate potential interference with vaccine and adverse effects. Other MAbs proposed for replacement of RIG should be additionally characterized [14,26,28-30]. It is possible that several successful candidates will be selected for development of MAb cocktails in response to national and international demands of biologics for rabies PEP in the near future.

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