MAJOR ARTICLE

Comparison of an Anti-Rabies Human Monoclonal Antibody Combination with Human Polyclonal Anti-Rabies Immune Globulin

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The World Health Organization estimates human mortality from endemic canine rabies to be 55,000 deaths/year. Limited supply hampers the accessibility of appropriate lifesaving treatment, particularly in areas where rabies is endemic. Anti-rabies antibodies are key to protection against lethal rabies. Currently, only human and equine polyclonal anti-rabies immune globulin (HRIG and ERIG) is available. Replacement of HRIG and ERIG with a safer and more widely available product is recommended. We have recently identified a combination of 2 human monoclonal antibodies (MAbs), CR57 and CR4098, that has high potential. We here describe a head-to-head comparison between an CR57/CR4098 MAb cocktail and HRIG. The MAb cocktail neutralized all viruses from a panel of 26 representative street rabies virus isolates. In combination with vaccine, the MAb cocktail protected Syrian hamsters against lethal rabies when administered 24 h after exposure, comparable with the results obtained with HRIG. Furthermore, the MAb cocktail did not interfere with rabies vaccine differently from HRIG. These results demonstrate that the human MAb cocktail of CR57 and CR4098 is a safe and efficacious alternative to RIG in rabies postexposure prophylaxis.

A recent World Health Organization publication estimated human mortality from endemic canine rabies to be 55,000 deaths/year [1]. When the public health impact is quantified, rabies is ranked above such diseases as dengue [2]. A lethal disease in humans and other animals, rabies can be prevented by postexposure prophylaxis (PEP) through the combined administration of rabies vaccine and anti-rabies immune globulin (RIG). The latter can be derived from pooled serum samples from rabies-vaccinated human donors or horses, to obtain either human RIG (HRIG) or equine RIG (ERIG). However, the existing supply of RIG has been limited for several years, and issues regarding the safety of blood-derived products have been raised. In light of the recent advances in the field of antibody technology, there is now an opportunity to replace RIG with monoclonal antibodies (MAbs) that are capable of neutralizing rabies virus (RV) after exposure to this deadly pathogen.

Mouse MAbs, as well as human MAbs, have been shown to protect rodents from lethal RV challenge [3–9]. In the development of a replacement for HRIG and ERIG, we opted for a combination of 2 MAbs and considered a set of predefined criteria to be of crucial importance for the inclusion of human MAbs in a cocktail aimed at effectively blocking 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 by use of one antibody should be neutralized by the other, nonselecting antibody in the cocktail (and vice versa), thus addressing the issue of natural variation among RV field iso-

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lates. Furthermore, the individual MAbs, in combination with vaccine, must provide protection against lethal RV challenge in a Syrian hamster model.

We recently characterized CR57 [10], a human MAb produced in PER.C6 cells that was based on IgG sequences originally identified by Dietzschold et al. [8]. We then selected highly potent anti-rabies MAbs from rabies immune phage libraries on the basis of complementation with CR57 [11, 12]. A panel of 23 novel human anti-rabies MAbs was identified, one of which, CR4098, proved to be fully compatible with CR57 on the basis of the criteria described above [11].

In the present study, the combination of CR57 and CR4098 was analyzed in a head-to-head comparison with HRIG. We assessed the in vitro breadth of neutralization, using a panel of 26 street RVs; in vivo protection against lethal RV challenge in Syrian hamsters; and vaccine potency in the presence of antibody.

MATERIALS AND METHODS

Cells. Mouse neuroblastoma cells were grown at 37°C/0.5% CO_2 in MEM (Gibco) supplemented with glutamine (Gibco), MEM vitamins (Gibco), and 10% heat-inactivated fetal bovine serum (FBS; Hyclone). PER.C6 cells [13] were grown at 37°C/10% CO_2 in Dulbecco's MEM (Gibco) supplemented with 10% FBS and 10 mmol/L $MgCl_2$.

Viruses. Monolayers of neuroblastoma cells were infected with challenge virus standard–11 or other viruses at an MOI of 0.3 for 15 min at 37°C/0.5% CO_2. The virus inoculum was then removed, fresh medium was added to the cells, and incubation was continued for 40 h at 37°C/0.5% CO_2. The culture supernatants were collected and stored at −80°C until further use.

Antibodies. The heavy and light chain of each phage antibody were cloned indirectly into the pcDNA3002 vector [14] via shuttle vectors containing the constant domains of the IgG1 heavy chain, the κ light chain, or the λ light chain. Antibodies were expressed in PER.C6 cells and purified by protein A chromatography. Antibodies were buffered with PBS (Gibco), filter sterilized, and stored at −20°C. Each antibody and HRIG preparation (BayRab [Bayer] and Imogam Rabies HT [Sanofi-Aventis]) was first titrated by a rapid fluorescent focus inhibition test (RFFIT), to determine the level of IUs per milliliter.

RFFIT. Standard RFFITs for neutralization were performed as described elsewhere [15]. To determine the neutralizing potency of each MAb, their 50% neutralizing titers were compared with the 50% neutralizing titer of standard reference serum (standard RIG, lot R3), which is defined as 2 IU/mL.

In vivo Syrian hamster challenge model. A lethal animal model mimicking rabies exposure was used as described elsewhere [16]. Briefly, Syrian hamsters (Harlan Sprague Dawley) were infected with 0.05 mL of a 1:1000 dilution of a rabid Mexican dog salivary gland homogenate of $10^{6.8}$ MIC LD_{50} (coyote street RV; United States–Mexican border, reference number 323R) on day −1. On days 0, 3, 7, 14, and 28, the hamsters were vaccinated with rabies vaccine (Imovax; Sanofi-Aventis). In addition, HRIG (Imogam Rabies HT) at 20 IU/kg or a MAb cocktail of CR57 and CR4098 (at the doses indicated in the figure legends) was administered on day 0. The hamsters were examined daily for clinical signs of rabies; if present, the hamsters were killed. The hamsters were maintained and evaluated up to day 42 after infection. All animal care and handling was performed in accordance with the guidelines specified by the National Institutes of Health [17]. Postmortem diagnosis of rabies by direct fluorescent antibody testing using a standardized Centers for Disease Control and Prevention protocol was performed for each killed animal [18].

Vaccine potency in the presence of MAbs. Syrian hamsters ($n = 36$ per treatment group) were vaccinated with Imovax on days 0, 3, 7, 14, and 28. HRIG (Imogam Rabies HT) or the CR57/CR4098 MAb cocktail was administered on day 0 (at the doses indicated in the figure legends). On days 1, 3, 7, 14, 28, and 42, 6 hamsters in each treatment group were killed, and blood was collected. Serum samples were analyzed by RFFIT, to quantify the presence of RV-neutralizing antibody (RVNA).

Statistical analyses. To compare the vaccine potency of the CR57/CR4098 MAb cocktail at 5 IU/kg and at 20 IU/kg with the potency of HRIG, the area under the curve (AUC) and 95% confidence intervals (CIs) were calculated, on the basis of the linear trapezoidal rule. Differences in serum RVNA titers on day 42 were analyzed using the Wilcoxon rank-sum test. Finally, survival time after challenge was compared between groups using the log-rank test, and Kaplan-Meier survival curves were obtained. Statistical analyses were performed using SAS (version 9.1; SAS Institute).

RESULTS

Breadth of neutralization against a broad panel of street RVs. To analyze the in vitro breadth of neutralization, we determined the coverage of the CR57/CR4098 MAb cocktail against a broad panel of street RVs and compared it with that of HRIG. The MAb cocktail neutralized the entire RV panel, including virus of canine and bat origin (table 1). Analysis of the HRIG preparations revealed that BayRab also neutralized all of the RVs in the panel. In contrast, Imogam Rabies HT did not neutralize 1 North American bat virus isolate (*Lasiurus cinereus,* NY), in agreement with earlier findings of Hanlon et al. [5]. Overall, the results indicated that the MAb cocktail and HRIG have comparable coverage of genotype 1 lyssaviruses.

Vaccine immunogenicity in nonchallenged Syrian hamsters treated with the MAb cocktail or HRIG. During PEP, there is the possibility that the simultaneous administration of MAbs and vaccine decreases the ability of the vaccine to induce the

Table 1. Breadth of neutralization against street rabies viruses.

Lyssavirus	SRIG	HRIG preparation		
		BayRab	Imogam Rabies HT	CR57/CR4098 MAb cocktail
CVS-11	+	+	+	+
Raccoon, southeast United States	+	+	+	+
Gray fox, TX	+	+	+	+
Gray fox, AZ	+	+	+	+
Arctic fox, AK	+	+	+	+
Coyote, TX	+	+	+	+
Dog/coyote, TX	+	+	+	+
Skunk, north-central United States	+	+	+	+
Skunk, south-central United States	+	+	+	+
Skunk, CA	+	+	+	+
Mongoose, NY/Puerto Rico	+	+	+	+
Dog, Argentina	+	+	+	+
Dog, Sonora	+	+	+	+
Dog, Gabon	+	+	+	+
Dog, Thailand	+	+	+	+
Bat, *Lasiurus borealis,* TN	+	+	+	+
Bat, *Eptesicus fuscus–Myotis* species, CO	+	+	+	+
Bat, *Myotis* species, 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/Mexico	+	+	+	+
Bat, *Desmodus rotundus,* Brazil	+	+	+	+

NOTE. Neutralizing potency was determined by a rapid fluorescent focus inhibition test. +, neutralization; −, no neutralization; CVS, challenge virus standard; HRIG, human rabies immune globulin; MAb, monoclonal antibody; SRIG, standard rabies immune globulin, lot R3.

threshold levels of NAs required for protection [19–23]. Therefore, it is critical to evaluate the degree to which a MAb treatment interferes with vaccination. To determine the effect of the MAb cocktail on vaccine potency, we performed an in vivo animal experiment in the absence of RV (figure 1). For PEP, hamsters were administered 20 or 5 IU/kg MAb cocktail plus vaccine or 20 IU/kg HRIG plus vaccine. On days 1, 3, 7, 14, 28, and 42, 6 hamsters in each group were killed, to determine the serum RVNA titer in each animal. On day 1, serum RVNA titers were equivalent in hamsters that received 20 IU/kg HRIG

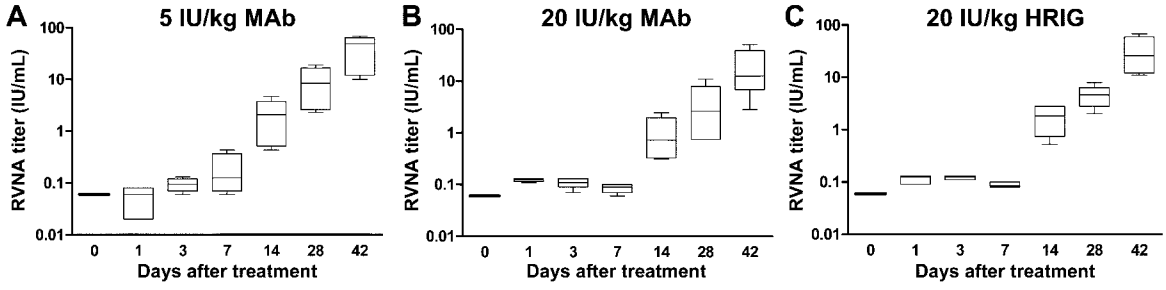

Figure 1. Serum rabies virus–neutralizing antibody (RVNA) titers in nonchallenged Syrian hamsters. The hamsters in each treatment group (n = 36 per group) were vaccinated with rabies vaccine and treated with 5 *(A)* or 20 *(B)* IU/kg monoclonal antibody (MAb) cocktail (CR57 and CR4098) on day 0, and the hamsters in the control group *(C)* received vaccine and 20 IU/kg human rabies immune globulin (HRIG). On days 1, 3, 7, 14, 28, and 42, 6 hamsters in each group were killed, and blood was collected. The RVNA titer in each serum sample was determined by a rapid fluorescent focus inhibition test, and geometric mean titers were calculated and plotted against time. The lines represent means, the edges of the boxes represent interquartile ranges, and the bars represent SEs.

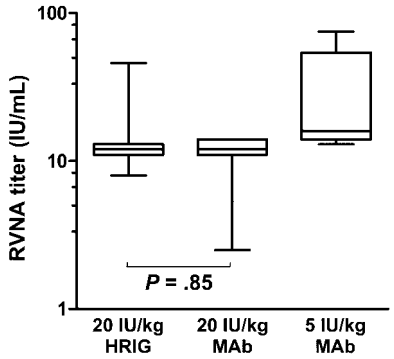

Figure 2. Serum rabies virus (RV)–neutralizing antibody (RVNA) titers in challenged Syrian hamsters. Hamsters ($n = 12$ per group) were challenged with coyote street RV on day −1. One day after challenge (day 0), the hamsters in each treatment group were vaccinated with rabies vaccine and treated with either 5 or 20 IU/kg monoclonal antibody (MAb) cocktail (CR57 and CR4098), and the hamsters in the control group received rabies vaccine and 20 IU/kg human rabies immune globulin (HRIG). On day 42 after treatment, serum was obtained from the surviving hamsters (11 in the control group, 12 in the group that received 20 IU/kg MAb cocktail, and 10 in the group that received 5 IU/kg MAb cocktail) and analyzed for RVNA titer by a rapid fluorescent focus inhibition test. Differences between groups were tested using the Wilcoxon rank-sum test. The lines represent means, the edges of the boxes represent interquartile ranges, and the bars represent SEs.

or 20 IU/kg MAb cocktail and were somewhat lower in hamsters that received 5 IU/kg MAb cocktail, a finding that is consistent with the lower dose of passively transferred neutralizing MAbs they received. The immune response mounted against the vaccine was measurable between days 7 and 14, when hamster RVNA began to appear in animals in all 3 treatment groups, and the serum RVNA titers continued to increase until day 42, when the experiment was terminated and the hamsters were killed. In an AUC analysis, the hamsters treated with HRIG showed a mean of 331 IU/mL*days (95% CI, 197–465 IU/mL*days), the hamsters treated with 20 IU/kg MAb cocktail showed a mean of 215 IU/mL*days (95% CI, 104–327 IU/mL*days), and the hamsters treated with 5 IU/kg MAb cocktail showed a mean of 478 IU/mL*days (95% CI, 312–644 IU/mL*days). There were no statistically significant differences between the 3 treatment groups, as is illustrated by the overlapping 95% CIs.

Vaccine potency in challenged Syrian hamsters treated with the MAb cocktail or HRIG. We analyzed the effect of the MAb cocktail on vaccine potency after lethal RV challenge. Serum RVNA titers in the surviving hamsters in each treatment group (20 IU/kg HRIG, 20 IU/kg MAb cocktail, or 5 IU/kg MAb cocktail) were determined on day 42 (figure 2). The serum RVNA titers observed in the hamsters treated with 20 IU/kg MAb cocktail were similar to those observed in the hamsters treated with HRIG. A higher serum RVNA titer was observed in the hamsters treated with 5 IU/kg MAb cocktail, a finding that is in agreement with those of previous studies [19–23].

Dose-dependent survival of Syrian hamsters treated with the MAb cocktail. We previously demonstrated that both CR57 and CR4098 are highly potent antibodies in vivo, providing efficacy at a dose of 10 IU/kg [11]. In the present study, a dose-titration experiment was performed, to determine the lowest protective dose of the MAb cocktail when administered in combination with vaccine. For this experiment, the doses of the MAb cocktail used were based on the actual neutralizing titer (in IU per milliliter) of a 1:1 (neutralizing potency:neutralizing potency) mixture of CR57 and CR4098 (figure 3). A survival rate of 8% (1/12) was observed in the control hamsters that received vaccine but no HRIG or MAb cocktail, proving that vaccine alone is not sufficient to protect hamsters from rabies, whereas treatment of hamsters with vaccine and HRIG resulted in a survival rate of 92% (11/12). A clear dose effect was observed in the hamsters treated with 20, 5, 2, and 0.1 IU/kg MAb cocktail, which produced survival rates of 100% (12/12), 75% (9/12), 58% (7/12), and 42% (5/12), respectively. This indicated that the MAb cocktail, at both 20 and 5 IU/kg, provided a level of protection against rabies comparable with that provided by 20 IU/kg HRIG ($P = .317$ and $P = .316$, respectively; log-rank test), illustrating the strong neutralizing potency of CR57 and CR4098.

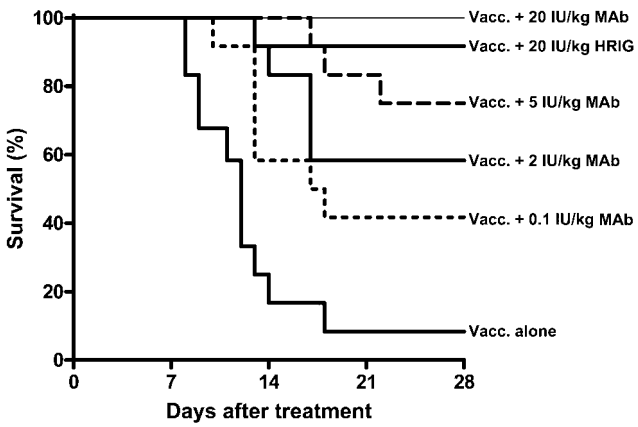

Figure 3. Kaplan-Meier survival curves for Syrian hamsters after rabies virus (RV) challenge. Hamsters ($n = 12$ per group) were challenged with coyote street RV on day −1. One day after challenge (day 0), the hamsters in the treatment groups were vaccinated with rabies vaccine (vacc.) and treated with 20, 5, 2, or 0.1 IU/kg monoclonal antibody (MAb) cocktail, and the hamsters in the control groups received either vaccine alone or vaccine with 20 IU/kg human rabies immune globulin (HRIG). The hamsters were monitored twice daily and were killed when clinical signs of rabies appeared. Kaplan-Meier survival curves are shown for days 0–28. The hamsters were monitored until day 42 after treatment (no additional deaths occurred between days 28 and 42). Nontreated hamsters ($n = 9$) died within 18 days (data not shown).

DISCUSSION

Human MAbs produced in accordance with industrial standards could provide a good solution to the current global shortage of HRIG. Production of MAbs can be done on a large scale (to ensure sufficient availability), and, unlike HRIG, the produced MAbs would be consistent from batch to batch. Furthermore, the health risks associated with blood-derived products such as HRIG would be eliminated by use of MAbs, which are manufactured using a mammalian cell line platform according to a well-defined production process. Our aim has been to develop a combination of 2 MAbs that could replace RIG for use in PEP. The MAb CR57 was previously proven to be highly potent and to have the ability to neutralize a variety of RVs [8, 10]. The second MAb, CR4098, was selected on the basis of stringent criteria from a panel of 23 human anti-rabies MAbs that had been isolated using phage display technology [11, 12]. These 2 MAbs proved to be fully compatible, as was demonstrated by the findings that they recognized nonoverlapping, noncompeting epitopes [10, 11]; displayed high potency; demonstrated broad in vitro neutralization; showed reciprocal in vitro coverage of each other's escape viruses; and were capable of in vivo protection against RV infection in an animal model [11].

In the present study, we characterized the combination of these 2 MAbs for use in PEP. We previously demonstrated that the combination of CR57 and CR4098 has an additive effect on the in vitro neutralization of RV [11]; here, the in vitro breadth of neutralization experiments demonstrated that the combination of CR57 and CR4098 could neutralize all 26 of the street RVs that were tested. The lack of coverage of 1 bat virus isolate (*Lasiurus cinereus,* NY) by Imogam Rabies HT was observed, confirming an earlier finding by Hanlon et al. [5]—despite potential variation among different batches of this bat virus generated over the course of several years, in both cases it was observed that Imogam Rabies HT provides no protection against this isolate. Although no human fatalities have been associated with this bat isolate, recent cases of human rabies have been associated with bat bites, and bat RV variants are considered to be the most common cause of human deaths attributed to rabies in the United States. Thus, the CR57/CR4098 MAb cocktail may provide additional protection when used against bat bites.

We previously reported that both CR57 and CR4098, in combination with vaccine, can protect Syrian hamsters from lethal RV challenge [11]. Here, we tested the combination of these 2 MAbs in the same animal model. When the effect of the MAb cocktail on vaccine potency in the absence of RV infection was analyzed, it was found that the serum RVNA titers in hamsters treated with 5 or 20 IU/kg MAb cocktail were similar to those in hamsters treated with 20 IU/kg HRIG. In rabies-exposed animals, the MAb cocktail, at 5 or 20 IU/kg, provided a level of in vivo protection against lethal RV challenge similar to that provided by 20 IU/kg HRIG ($P = .317$ and $P = .316$, respectively; log-rank test). Analysis (using the Wilcoxon rank-sum test) of serum RVNA titers in treated hamsters that survived lethal RV challenge showed equivalence between HRIG and the highest dose of the MAb cocktail tested ($P = .85$).

In conclusion, in a head-to-head comparison composed of both in vitro and in vivo experiments, the combination of the MAbs CR57 and CR4098 showed equivalence to HRIG with respect to in vitro breadth of neutralization, survival, and level of vaccine interference. Therefore, this human MAb cocktail is a good alternative to HRIG for use in PEP of human rabies.

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