REPLACEMENT OF ERIG & HRIG BY HUMAN MONOCLONAL ANTIBODIES (MAbs) FOR RABIES TREATMENT

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Rabies is a zoonotic (transmitted from animals to human) viral infectious disease. This infection is transmitted to human by the animals already suffering from it. The animals which are mainly reported as causes of rabies are; dogs, raccoons, skunks, bats, and foxes. Rabies or "Hydrophobia" is a disease which makes the dogs sick. In many eastern and western countries dogs are vaccinated against it, but it is not controlled yet. Rabies is caused by a virus that, attacks on the nerves system and later excreted in saliva.[1] A person or animal can become a victim of rabies in many ways including.[2],

a. Bites

b. Non-bites exposure

c. Human to Human Transmission

Bites from rabid animal to human are very common but the other two factors are rare.[2] Rabies affects the brain and spinal cord (central nervous system) with initial symptoms like; flu, fever, headache, but the infection can progress quickly to hallucinations, paralysis, and eventually death.[3]

Genome and virology

Rabies virus is the "type species" of the *Lyssavirus* genus of *Rhabdoviridae* family. The virus is enveloped and has a single stranded, negative sense RNA genome.[4] The RNA genome of the virus encodes five genes whose order is highly conserved. These genes codes for: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and a viral RNA polymerase (L). All rahbdoviruses have two major structural components; helical ribonucleoprotein core (RNP) and surrounding envelops. The two proteins, P and L are associated with RNP. The glycoprotein forms approximately 400 trimeric spikes, which are tightly arranged on the surface of the virus.[5] The virus nucleoprotein (N) plays critical role

in replication and transcription. Both viral transcription and replication are reduced, if the nucleoprotein is not phosphorylated.[6] Rhabdoviruses cell surface receptors are not identified but some researches point outs the phospholipids, especially phophatidyl serine as the cell surface receptor molecule. After endocytosis, pH-dependent fusion with the membrane of the endocytic vesicle occurs. The polymerase which is carried out by the virus make five individual mRNA for each protein. These mRNAs are capped, methylated and polyadenylated. The polymerase then transcribes the negative-sense genomic RNA into positive sense strand. The switch between transcription and replication of genomic RNAs are controlled by the level of N protein.[7]

Pathogenesis

The common mode of transmission of rabies in man is by bite of a rabid animal or the contamination of scratch wounds by virus infected saliva. Rabies is an acute infection of the central nerves system (CNS) which is almost invariably fatal. Following inoculation, the virus replicates in the striated or connective tissue at the site of inoculation and enters the peripheral nerves through the neuromuscular junction. It then spreads to the CNS in the endoneurium of the Schwann cells. Terminally, there is widespread CNS involvement but few neurons infected with the virus show structural abnormalities. The nature of the profound disorder is still not understood.[8]

Clinical diagnosis in human

Clinical diagnosis of rabies divided upon three stages; prodromal, excitement (furious) and paralytic (dumb). But all these stages cannot be observed in an individual.[10] The very first clinical symptom is neuropathic pain at the site of infection or wound due to viral replication. Following by the prodromal phase either or both the excitement or paralytic forms of the disease may be observed in the particular species. It is also documented that cats are more likely to develop furious rabies than dogs.[11] In some cases, no signs are observed and rabies virus has been identified as the case of sudden death.[12] Diagnosis can only be confirmed by laboratory tests preferably conducted post mortem on central nervous system tissue removed from cranium.[13] Tests are also performed on the samples of saliva, serum, and skin biopsies of hair follicles at the nape of the neck.[9]

Pre-exposure prophylaxis

The people who are considered as high risk group need pre-exposure prophylaxis. These groups includes; a-veterinarian, animal handlers and laboratory workers; b-the people whose

activities bring them in contact with rabies virus or rabid animals; c-international travelers likely to come in contact of the animals in the rabies threaten areas. All these groups should be treated with rabies vaccines to avoid the chances of sudden infection.

Post-exposure prophylaxis

If a person is bitten by an animal, the wound and scratches should be washed thoroughly with soap and water to decrease the chances of infection. Post-exposure prophylaxis involved one dose of rabies immune globulin and five doses of rabies vaccine within the 28 days period. Rabies immune globulin contains antibodies from blood donors who were given rabies vaccine. The rabies vaccine works by stimulating a person's immune system to produce antibodies that neutralize the virus.

Treatment

The WHO-recommended minimum potency is 2.5 IU per IM dose and the WHO recommended volume of a single dose of rabies vaccine administered per ID site is 0.1 ml. It is a multisite (upper arms, lateral thighs, suprascapular or upper trunk region, and lower quadrant of abdomen) vaccination technique which elicits a prompt and highly protective immune response with a small dose. Initially eight-site and four-site ID inoculation were conducted but clinical trials and immunological studies clearly demonstrated that two-site ID inoculation is sufficient. RIG is a biological product which is used to provide immediate readymade antibodies until the patient's own immune system responds to immunization. RIG may be of human or animal origin. ● Equine rabies immunoglobulin (ERIG) ● Human rabies immunoglobulin (HRIG). The dose calculation is done as follows: ● ERIG – 40 IU/kg body weight with a maximum of 3000 units ● HRIG – 20 IU/kg body weight with a maximum of 1500 units. For all category III bites, RIG should be given immediately after the incident. RIG should be infiltrated as much as possible in and around all wounds. After infiltration of the wounds, if there is any remaining RIG, it should be given intramuscularly on the anterolateral region or deltoid region. Anti-rabies vaccines should then be administered, preferably on the same day, but at a different site (right arm for vaccine and left arm for serum, or vice versa). ● RIGs remain in short supply throughout the world.[12] Further in the 21st century invention of Human Monoclonal Antibodies (MAbs) took place by serum institute of Pune.

Mouse MAbs, as well as human MAbs, have been shown to protect rodents from lethal RV challenge. 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 isolates. Furthermore, the individual MAbs, in combination with vaccine, must provide protection against lethal RV challenge in a Syrian hamster model.

We recently characterized CR57[17], a human MAb produced in PER.C6 cells that was based on IgG sequences originally identified by Dietzschold et al.[15] We then selected highly potent anti-rabies MAbs from rabies immune phage libraries on the basis of complementation with CR57.[18,19] 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.[19]

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[20] 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[21] 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.[22] 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 modelA lethal animal model mimicking rabies exposure was used as described elsewhere.[23] 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.[24] 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.[25]

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 & amp;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 Bay Rab 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. Overall, the results indicated that the MAb cocktail and HRIG have comparable coverage of genotype 1 lyssaviruses.

Lyssavirus	SRIG	HRIG preparation		CR57/CR4098 MAb cocktail
		BayRab	Imogam Rabies HT	
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.

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 threshold levels of NAs required for protection.[26,30] 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 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.
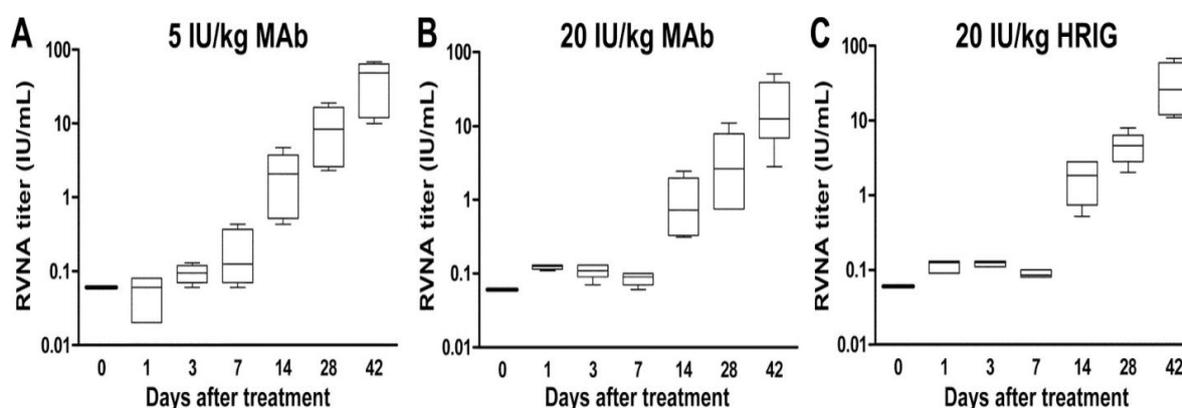
Serum rabies virus–neutralizing antibody (RVNA) titers in nonchallenged Syrian hamsters. The hamsters in each treatment group (n=36 per group) were vaccinated with rabies vaccine and treated with 5 *(A)* or 20 *(B)* IU/kg monoclonal antibody (MAb) cocktail (CR57 and CR4098) on day 0, and the hamsters 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.

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.[26,30]
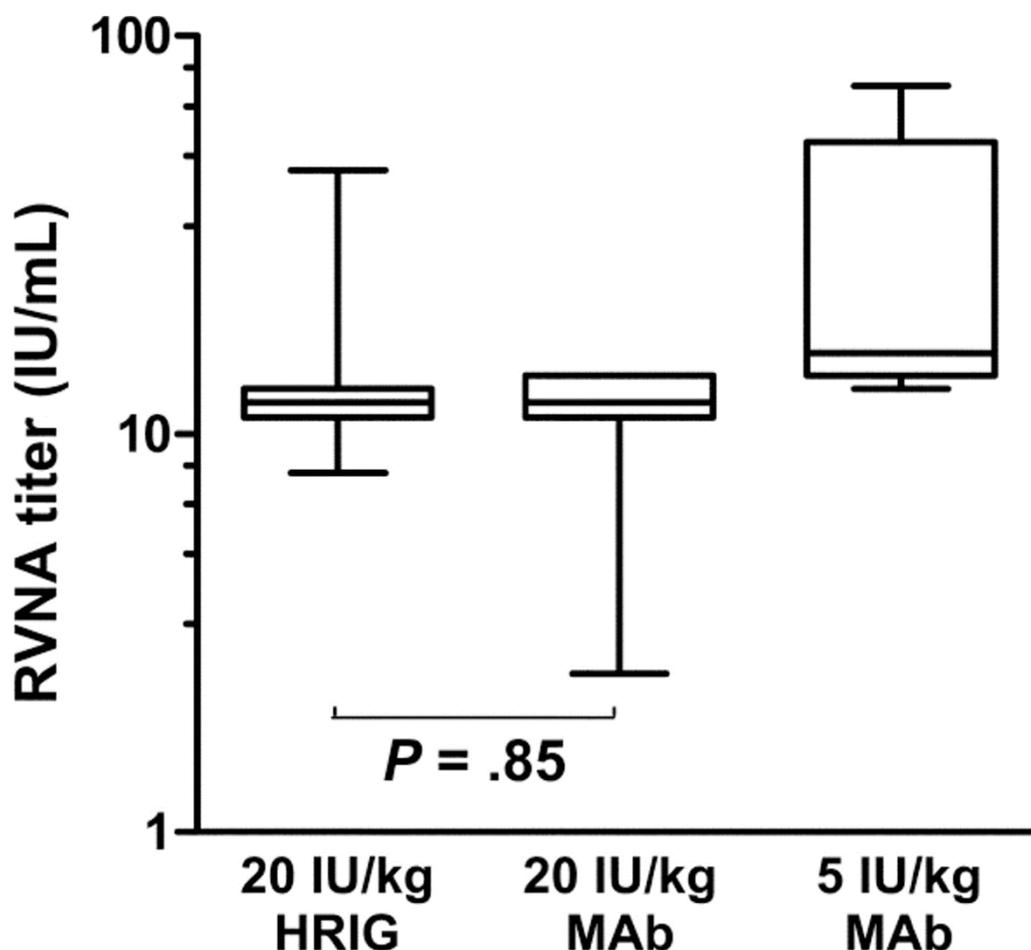
Serum rabies virus (RV)–neutralizing antibody (RVNA) titers in challenged Syrian hamsters. Hamsters (n=12 per group) were challenged with coyote street RV on day −1. One day after challenge (day 0), the hamsters in each treatment group were vaccinated with rabies vaccine and treated with either 5 or 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 & amp;rank-sum test. The lines represent means, the edges of the boxes represent interquartile ranges, and the bars represent SEs.

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.[18] 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.
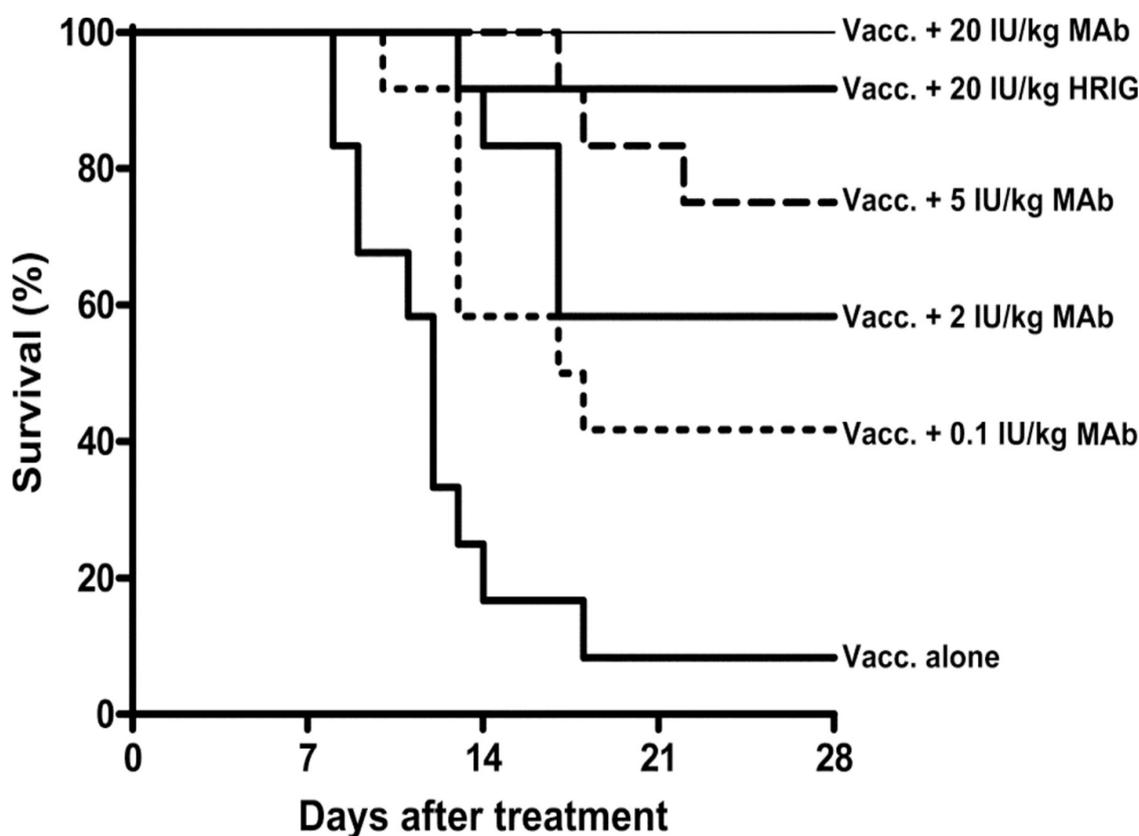
Figure 3

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.

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