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PATHOGENICITY OF DIFFERENT RABIES VIRUS ISOLATES AND PROTECTION TEST IN VACCINATED MICE

Elenice M.S. CUNHA(1), Alessandra F.C. NASSAR(1), Maria do Carmo C.S.H. LARA(1), Eliana C.M. VILLALOBOS(1), Go SATO(2), Yuki KOBAYASHI(2), Youko SHOJI(2), Takuya ITOU(2), Takeo SAKAI(2) & Fumio H. ITO(3)

SUMMARY

This study was aimed to evaluate and compare the pathogenicity of rabies virus isolated from bats and dogs, and to verify the efficacy of a commercial rabies vaccine against these isolates. For evaluation of pathogenicity, mice were inoculated by the intramuscular route (IM) with 500MICLD$_{50}$/0.03mL of the viruses. The cross-protection test was performed by vaccinating groups of mice by the subcutaneous route and challenged through the intracerebral (IC) route. Isolates were fully pathogenic when inoculated by the IC route. When inoculated intramuscularly, the pathogenicity observed showed different death rates: 60.0% for the *Desmodus rotundus* isolate; 50.0% for dog and *Nyctinomops laticaudatus* isolates; 40.0% for *Artibeus lituratus* isolate; 9.5% *Molossus molossus* isolate; and 5.2% for the *Eptesicus furinalis* isolate. Mice receiving two doses of the vaccine and challenged by the IC route with the isolates were fully protected. Mice receiving only one dose of vaccine were partially protected against the dog isolate. The isolates from bats were pathogenic by the IC route in mice. However, when inoculated through the intramuscular route, the same isolates were found with different degrees of pathogenicity. The results of this work suggest that a commercial vaccine protects mice from infection with bat rabies virus isolates, in addition to a canine rabies virus isolate.

KEYWORDS: Rabies virus; Pathogenicity; Vaccine**.**

INTRODUCTION

The *Lyssavirus* genus includes 11 recognized species[17]. The rabies virus (RABV) is found in various domestic animals but also in bats. The Lagos bat virus (LBV), Mokola virus (MOKV) and the Duvenhage virus (DUVV) have been isolated in bats, domestic animals and humans in Africa[21]. European bat lyssaviruses 1 and 2 (EBLV1 and EBL2) have been isolated from insectivorous bats in Europe[14]. Australian bat lyssavirus (ABLV) circulates in Australia among insectivorous and pteropid bats and has caused cases of human rabies[35]. Recently, four new lyssavirus species were isolated from bats in Eurasia and were ratified by the International Committee on Virus Taxonomy (ICTV Official Taxonomy: Updates since the 8th Report)[17]: the Irkut (IRKV)[5] Aravan (ARAV)[1], Khujand (KHUV)[22] and the West Caucasian bat virus (WCBV)[5]. KUZMIN *et al*., 2010[21] described the isolation and characterization of a new lyssavirus, which should be considered a new species of the genus. Based on phylogeny, serological cross-reactivity, and peripheral pathogenicity to mice, lyssaviruses can be placed into two phylogroups[2]. Phylogroup I includes RABV, DUVV, EBL1, EBL2, ABLV, ARAV, KHUV and IRKV. Phylogroup II includes LBV and MOKV. The WCBV cannot be included in any of these phylogroups, and was suggested that it be considered as a member of an independent phylogroup III[16,20]. Viruses of phylogroup I have been shown to be pathogenic for mice when inoculated via the intracerebral (IC) and intramuscular (IM) routes. Members of phylogroup II were shown to be pathogenic for mice only when inoculated via the IC route[2].

In Brazil, dog-transmitted rabies has been reduced by an aggressive vaccination program[30]; however, bat-transmitted rabies (particularly the *Desmodus rotundus*) remains endemic, especially in Northern and Northeastern states[26,33]. According to the data of Brazilian Ministry of Health[27], in 2010 (until April) occurred one case of human rabies in the state of Rio Grande do Norte, and the source was associated to bat transmission; in 2009, two cases in the state of Maranhão due to dog transmission; in 2008, there were two cases transmitted by vampire bats respectively in Pernambuco and Goiás State, and one marmoset-transmitted case in Ceará; in 2007 there was one case of dog-transmitted rabies in Maranhão; in 2006 there were nine cases due to dogs and vampire bats, six in the state of Maranhão, and respectively one case in the states of Pernambuco, Alagoas, Minas Gerais and Rio de Janeiro. In the year of 2005, there were reported 42 human rabies cases transmitted by vampire bats, one dog and one due to marmoset transmission. In the majority of these human cases, the possible animal species were drawn based on genetic sequencing analyses of the isolates.

A panel of monoclonal antibodies to rabies virus collections from

(1) Laboratório de Raiva e Encefalites Virais, Instituto Biológico de São Paulo. Av. Conselheiro Rodrigues Alves 1252, 04014-002 São Paulo, SP, Brasil. Tel.: 55 11 5087 1779. E-mail: cunha@biologico.sp.gov.br
(2) College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa, 252-8510 Japan.
(3) Departamento de Medicina Veterinária Preventiva e Saúde Animal, FMVZ-USP, São Paulo, SP, Brasil.
Correspondence to: Elenice M.S. Cunha, Lab. Raiva e Encefalites, Instituto Biológico de S. Paulo, Av. Cons. Rodrigues Alves 1252, 04014-002 Sao Paulo, SP, Brasil. E-mail: cunha@biologico.sp.gov.br

Brazil has identified two major variants, one associated with dogs and other with vampire bats (*Desmodus rotundus*), as well as other variants associated with several insectivorous bats and common marmosets (*Callithrix jacchus jacchus*)[4,12]. Genetic characterization of Brazilian rabies virus isolates, by sequencing of partial or complete N gene[18,34], P gene[4], and G genes sequences[31] also identified two principal variants maintained by vampire bats and dogs. However, a molecular analysis performed with viruses of insectivorous bats identified three variants of rabies virus[19]. Thus, rabies virus isolates from the frugivorous bats (*Artibeus* sp.) were found to be closely related to those viruses isolated from *D. rotundus* vampire bats[19,34]. All these studies have demonstrated that the rabies virus isolates from Brazil belonged to genotype 1 of rabies virus. However, little is known about the biological characteristics of these isolates and about the ability of current rabies vaccines to elicit immune responses which would provide cross-protection. The inactivated commercial rabies virus vaccines for human and animal use, such as the Pitman Moore (PM), Pasteur virus (PV), and Flury Lep (LEP), all belong to genotype 1. These vaccines induce immunity against viruses of the phylogroup I but fail to protect against viruses of the genotype 2 and 3[2,6,23,24] and WCBV[16]. Failures of protection in mice by Brazilian vaccines against wild rabies viruses were reported[36].

The purpose of this study was to evaluate and to compare the pathogenicity of several isolates of rabies viruses isolated from hematophagous, frugivorous, and insectivorous bats with the rabies virus isolated from dog, and the efficacy of a commercially available rabies vaccine against these isolates. The experiments were performed in a mice model.

MATERIALS AND METHODS

Canine rabies virus isolate (BR-C) used in this experiment corresponded to one of the isolates of the 1992's dog rabies epidemic registered in the municipality of Araçatuba, São Paulo. Five bats isolates were chosen based on the nucleocapsid (NC) differences of the several independent lineages of bat rabies viruses in Brazil. One lineage consisted of a vampire bat (*Desmodus rotundus* BR-DR1), and also including the isolate from a frugivorous bat (*Artibeus lituratus* BR-AL3). Other three lineages consisted of insectivorous bat isolates; namely the *Eptesicus* sp. (BR-EF1), *Molossus* sp. (BR-MM1) and *Nyctinomops* sp. (BR-EF1) isolates[19]. The source of each virus isolate is summarized in Table 1. Brain samples were diagnosed as rabies-positive by both the fluorescent antibody test (FAT) and mouse inoculation test (MIT). All isolates were collected from the brain tissue and passed five times in suckling mouse brain using IC inoculation.

The titers of the viral isolates were determined by IC inoculations of tenfold virus dilutions into 4-week-old mice, and 50% mouse IC lethal dose ($MICLD_{50}$) was calculated using the Reed and Müench method[29].

Four-week-old mice were used for pathogenicity and cross protection tests.

Mice were provided by the LANAGRO-SP (Laboratório Nacional Agropecuário de São Paulo), and were housed and handled with ethical principles in animal research adopted by the Bioethics Commission of the Faculty of Veterinary Medicine and Zootechny of University of São Paulo (protocol number 263/2003) .

Table 1
Field isolates of rabies virus used in pathogenicity and vaccine protection tests, according to the original host, year of isolation and GenBank accession numbers

Isolates	Original host	Year of isolation	Accession numbers*
BR-C	Dog	1992	-
BR-EF1	Bat (*Eptesicus furinalis*)	1998	AB201811
BR-NL1	Bat (*Nyctinomops laticaudatus*)	1998	AB201806
BR-AL3	Bat (*Artibeus lituratus*)	1998	AB117971
BR-MM1	Bat (*Molossus molossus*)	1999	AB201815
BR-DR1	Bat (*Desmodus rotundus*)	2000	AB201803

* Sequences retrieved from GenBank.

For evaluation of pathogenicty, one group of Swiss female mice (n = 20), 6 week-old, were injected by IM route in the thigh (0.1 mL) with 500MICLD50/0.03mL of each viral isolates. Control mice were injected using the virus diluent containing 2% inactivated horse serum in distilled water and antibiotics. These mice were observed for signs of rabies for a minimum of 30 days.

For the immunization of mice, a veterinary commercially available Pasteur virus (PV-strain) propagated in BHK-21 cells, chemically inactivated and containing an adjuvant was kindly provided by the Biovet® (RAI-VET - lot no. 466/04). The vaccine titer informed was 3.42 international unit (IU)/dose/mice of 2.0 mL and the vaccine had been approved by the LANAGRO (Brazilian Official Vaccine Testing Laboratory from the Brazilian Ministry of Agriculture, Livestock and Supply).

The mouse intracerebral lethal dose 50% ($MICLD_{50}$/0.03mlL was determined by injecting 0.03 mL of BR-C, BR-EF1, BR-NL1, BR-AL3, BR-MM1, and BR-DR1, in each group consisting of 4 week-old Swiss female mice (n = 8) by the IC route.

For mouse protection studies, 14 groups of six 4-week-old female Swiss mice each were used. We administered 0.2 mL of the commercial inactivated rabies vaccine by the subcutaneous route on day 0 to all groups. A week later, seven groups were inoculated with a second dose (0.2 mL) of the same vaccine. All vaccinated groups were challenged 14 days after the first dose of vaccine, together with an equal number of unvaccinated control mice, with tenfold dilution of BR-C, BR-EF1, BR-NL1, BR-AL3, BR-MM1, and BR-DR1 isolates, by the IC route. Lethal dose (LD_{50}) endpoints in vaccinated and unvaccinated control mice were determined by the modified Habel technique[15] and calculated by the Reed and Müench method[29]**.** The degree of protection (protection index) in mice challenged by the IC route was determined by subtracting the logarithm of the LD_{50} endpoint in vaccinated mice from that of the control mice. A difference of 3 or log 1000 indicated vaccine protection.

RESULTS

Susceptibility: The field isolates BR-C, BR-EF1, BR-NL1, BR-AL3, BR-MM1, and BR-DR1 were fully pathogenic when inoculated

Table 2
Protection of mice vaccinated with a commercial inactivated PV-rabies vaccine and challenged by the IC route with field isolates rabies virus

Challenge virus	$ICLD_{50}$ log 10 Control	$ICLD_{50}$ log 10 Vaccinated 2 doses	$ICLD_{50}$ log 10 Vaccinated 1 dose	Protection index (PI)[a] log 10	
				2 doses	1 dose
BR-C	5.9	1.0	4.3	4.9	1.6
BR-EF1	5.5	<1.0	1.4	5.5	4.1
BR-NL1	5.2	<1.0	1.0	5.2	4.2
BR-AL3	4.8	1.0	1.8	3.8	3.0
BR-MM1	4.7	<1.0	1.0	4.7	3.7
BR-DR1	5.3	<1.0	2.0	5.3	3.3

a) The degree of protection (protection index), in mice challenged by IC route was determined by subtracting the logarithm of the LD_{50} endpoint in vaccinated mice from that of the control mice.

in mice by the IC route, all leading to 100% mortality. However, when injected intramuscularly with a dose 0.1 mL of 500MICLD_{50}/0.03mL, the pathogenicity observed among the viruses showed different mortality rates: 60.0% for the *D. rotundus* isolate; 50.0% for dog and *Nyctinomops laticaudatus* isolates; 40.0% for *A. lituratus* isolate; 9.5% *Molossus molossus* isolate; and 5.2% for the *Eptesicus furinalis* isolate.

Mouse protection studies: The protective indices of vaccinated mice challenged by IC route with the isolates were summarized in Table 2. Mice receiving two shots of vaccine and challenged intracerebrally were protected against all the isolates, and mice receiving only one shot were partially protected against the dog isolate (BR-C).

DISCUSSION

Bats living in rural and urban areas are a complex problem with economic, public health and ecological implications. Rabies virus infected bats found in these environments pose a risk to both humans and domestic animals. In fact, in Brazil, there are reports of vampire bats feeding on the domestic dogs from an urban area of Olinda-Pernambuco[8] and Rio de Janeiro[10,11]. SCHAEFER *et al.*[32] (2002) described, in south of Brazil, a cat rabies isolate found in an area free of urban rabies since 1990 and related that rabies virus could be associated to a bat-related virus. The number of rabid bats detected, mainly in non-hematophagous species, increases each year, due to the increased diagnoses of rabies in bats from the heavy urbanized areas, reflecting the more active surveillance on rabies in non-hematophagous bats. A study describing the detection of bat rabies in the north-northwestern regions of the State of Sao Paulo-Brazil showed that in 7.9% of the cases, bats had contact with humans or domestic animals and in 17.4%, attacked humans and animals[7]. Thus, genetic and biological studies of these isolates would contribute to a better understanding of the bat rabies epidemiology, as well as indicate new ways to prevent the disease in man and animals. With the objective to elucidate some of these questions, the present study analyzed the pathogenicity of the rabies virus isolates obtained from a vampire, frugivorous, insectivorous bats and from a dog, using the mice model. The efficacy of a commercially available inactivated rabies vaccine was assessed using these isolates as a challenge virus.

Previous studies suggested that phylogroup II lyssaviruses were not pathogenic when inoculated peripherally. These assertions suggested that such viruses are generally less pathogenic, and imply that they have limited public health and veterinary significance[2]. In this work we have evaluated the susceptibility of mice to various isolates for hematophagous, frugivorous and insectivorous bats in comparison to a virus isolate from a dog. We showed that all isolates of rabies virus used in this experiment were pathogenic by the IC route in mice. However, when 500MICLD_{50} were inoculated through the IM route, the isolates were found with different degrees of pathogenicity. When inoculated by the IM route the bat rabies isolates BR-DR1, BR-NL1 and BR-AL3 demonstrated almost the same pathogenicity of the dog (BR-C) isolate. On the other hand, rabies virus isolates from the insectivorous bat *E. furinalis* (BR-EF1) and *M. molossus* (BR-MM1) were less pathogenic. MORIMOTO *et al.*[28] (1996) compared the biological properties of rabies virus isolates of the silver-haired bat (SHBRV) to those of the coyote isolates (COSRV) and found that SHBRV is less neurovirulent than COSRV, when administered by the IM route, whereas both viruses were equally neuroinvasive when injected intracranially. BADRANE *et al.*[2] (2001) investigated the biological significance of the phylogrouping in relation to the pathogenicity and immunogenicity of the lyssaviruses and found that when inoculated in adult mice, genotype 1 and 6 viruses (phylogroup I) were pathogenic by both the IC and IM routes and genotype 2 and 3 viruses (phylogrup II) were only pathogenic by IC route. Lagos bat virus (LBV) was reported to have markedly reduced levels of peripheral pathogenicity[2]. MARKOTTER *et al.*[25] (2009) suggest that this affirmative was based on a study of one isolate of LBV and demonstrated that peripheral pathogenicity of representatives of LBV in a murine model is as high as that of the rabies virus. In the same way, in this study regarding the observed decreased virulence, the fact must be analyzed carefully, since only one isolate of BR-EF1 and BR-MM1 was studied. Thus, we suggest that future studies should be accomplished, including rabies virus isolates isolated from different species of bats to provide a better resolution.

In the present study we verified the protection in mice conferred by an inactivated veterinary vaccine and challenged with field rabies virus isolates. Some failures of protection in mice, following experimental immunizations have been reported[13,23,36]. In this work we verified that the vaccine protected mice against the IC challenge, with isolates from bats and a dog. Mice receiving a single dose of the same vaccine were

found to be only partially protected against the challenge with a dog isolate. Other studies showed that animal vaccines protected mice against challenges with Duvenhage and Lagos bat viruses and isolates derived from various species of bats[13]. DIETZSCHOLD & HOOPER[9] (1998) reported that commercial vaccines licensed for use in humans protected mice against silver-haired bat rabies virus. However, immunization with P-G gene of rabies virus did not protect against viruses from genotypes 3 and 2[2]. LAFON *et al.*[23] (1988), however, showed that inactivated vaccine prepared with PV strain protected the mice challenged with a German bat isolate (Duvenhage), but PM or LEP vaccines did not protect mice against the virus infection. The potency of an inactivated animal rabies vaccine for domestic animals by using two types of potency tests; namely the traditional NIH and the CDC test (mice vaccinated once and challenged by the IM route) indicated that protection was highest against raccoon and bat viruses when compared with protection conferred against isolates of skunk, coyote and fox[3]. HANLON *et al.*[16] (2005) showed reduced protection with vaccination with conventional rabies vaccine against four new rabies viruses from bats in Eurasia. Studies performed with field isolates in Brazil showed differences in the degree of protection provided by immunization with PV vaccine and challenged with bovine rabies virus isolates (vampire bat virus lineage)[36]. Although belonging to genotype 1, these isolates turned out to be the most divergent among American and Brazilian rabies viruses studied by genetic characterization[36]. Nevertheless, data related to vaccine cross-protection have not been reported for rabies virus isolated from bats in Brazil. The results of this work suggest that PV vaccine protects mice from infection with vampire, frugivorous and insectivorous bat rabies viruses in addition to a canine rabies virus. Studies on the cross-protection to rabies virus in mice, conferred by a rabies vaccine are controversial and perhaps they do not represent what really takes place in nature with the species involved. The results often depend on the type of vaccine used as well as on the number of isolates tested.

At present, little is known about the epidemiology of the rabies virus in bats in Brazil and the country's surveillance systems need to assess the potential implications and the impact in public health and in veterinary public health.

RESUMO

Patogenicidade de diferentes isolados do vírus da raiva e teste de proteção em camundongos vacinados

O estudo avaliou e comparou as propriedades patogênicas de cinco isolados do vírus da raiva de morcegos e um isolado do vírus da raiva de cão e analisou a eficácia de vacina comercial contra estes isolados, em camundongos. Para o estudo de patogenicidade camundongos foram inoculados pela via IM com 0,1 mL contendo 500MICLD$_{50}$/0,03mL das amostras de vírus. Quando inoculados pela via IC, os isolados do vírus da raiva provocaram a morte de 100% dos camundongos. No entanto, 500MICLD$_{50}$/0,03mL das mesmas amostras, inoculadas pela via IM, ocasionaram mortalidade de: 60,0% quando a amostra era de *Desmodus rotundus*; 50,0% de cão e de *Nyctinomops laticaudatus*; 40,0% de *Artibeus lituratus*; 9,5% de *Molossus molossus*; e 5,2% de *Eptesicus furinalis.* Camundongos que receberam duas doses de vacina foram protegidos quando desafiados pela via IC, com todas as amostras testadas. Quando os camundongos receberam uma dose da mesma vacina, houve proteção parcial daqueles desafiados com a amostra de cão. Todos os isolados do vírus da raiva testados foram patogênicos para camundongos, inoculados pela IC. No entanto, pela via IM, os mesmos isolados mostraram diferentes graus de patogenicidade. Concluiu-se também que a vacina comercial contra raiva protegeu os camundongos desafiados com amostras de vírus isolados de morcegos e de cão.

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