Original Article

Monoclonal antibodies for characterization of rabies virus isolated from non-hematophagous bats in Brazil

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Abstract

Introduction: In Brazil, various isolates of rabies virus (RABV) show antigenic profiles distinct from those established by the reduced panel of eight monoclonal antibodies (MAbs) determined by the Centers for Disease Control and Prevention (CDC), utilized for the antigenic characterization of RABV in the Americas. The objective of this study was to produce MAbs from RABV isolates from insectivorous bats with an antigenic profile incompatible with the pre-established one.
Methodology: An isolate of RABV from the species *Eptesicus furinalis* that showed an antigenic profile incompatible with the panel utilized was selected. Hybridomas were produced utilizing the popliteal lymph nodes of mice immunized with ribonucleoproteins purified from the isolate.
Results: Two MAbs-producing clones were obtained, BR/IP1-3A7 and BR/IP2-4E10. Fifty-seven isolates of RABV from different species of animals and different regions of Brazil were analyzed utilizing the MAbs obtained. In the analysis of 23 RABV isolates from non-hematophagous bats, the MAbs cross-reacted with ten isolates, of which four were of the species *Nyctinomops laticaudatus*, one of the species *Eptesicus furinalis*, and five of the genus *Artibeus*. Of the nine isolates of non-hematophagous isolates that displayed an incompatible profile analyzed, characteristic of insectivorous bats, BR/IP1-3A7 reacted with five (55.55%) and BR/IP2-4E10 with four (44.44%).
Conclusions: The MAbs obtained were able to recognize epitopes common between the three genera, *Artibeus*, *Eptesicus*, and *Nyctinomops*, thereby allowing the antigenic characterization of RABV isolates in Brazil.

Key words: rabies virus; antigenic characterization; monoclonal antibodies; insectivorous bats; Brazil.

Introduction

Rabies is an acute encephalitis or meningoencephalitis due to a lyssavirus infection, and is responsible for an estimated 61,000 yearly human deaths worldwide despite the existence of effective vaccines for human and veterinary use [1]. The *Rabies virus* (RABV) belongs to the *Mononegavirales* order, the *Rhabdoviridae* family, and the *Lyssavirus* genus [2]. There are 14 lyssavirus species classified by the International Committee on Taxonomy Virus (ICTV) [3]. The bullet-shaped lyssavirus particle is composed of two structural and functional units: an internal helical nucleocapsid, which consists of a ribonucleoprotein complex comprising the genomic nonsegmented RNA of negative polarity and tightly bound nucleoprotein (N) together with the RNA-dependent RNA polymerase (or large protein, L) and phosphoprotein (P); and an external envelope, which is derived from the host cytoplasmic membrane during budding and in which surface trimeric glycoprotein (G) spikes are anchored. The matrix protein (M) forms oligomers that bind to the outside of the nucleocapsid, giving rigidity to the virion structure and providing a binding platform for the viral glycoprotein and the envelope membrane [4,5].

The disease is widespread and is found in all continents except Antarctica. All mammals are susceptible to the rabies virus, mainly the Carnivora and Chiroptera orders. Dogs and, occasionally, cats are mainly responsible for the transmission of urban rabies; in sylvatic rabies, the major host is a different species of wild mammal in different regions of the world [1].

The concept of antigenic variants of RABV and the study of their differences and specific reservoirs were consolidated with the development of the techniques for the production of monoclonal antibodies (MAbs) at the end of the 1970s. These techniques were utilized to produce the first hybridomas-secreting MAbs against proteins G and N of RABV. Since then, MAbs were widely utilized for identifying RABV and other lyssaviruses and classifying them into groups corresponding to antigenic determinants [6-8].

Different panels of MAbs were established, allowing the differentiation of RABV isolates from terrestrial species and bats in the United States and Western Europe and, to a lesser degree, in Africa, Asia, Eastern Europe, and Latin America [8-13].

The disease control centers of various countries and the Pan-American Zoonosis Center (CEPANZO) of the Pan-American Health Organization (PAHO) conducted studies during the period of 1987 to 1992 on different RABV isolates in various countries of the Americas [14]. The data obtained were used to establish a panel of eight MAbs that would allow the detection of the most common variants found in Latin America. This panel is available at the World Health Organization (WHO) Collaborating Center, Centers for Disease Control and Prevention (CDC), to participating laboratories of the PAHO consortium, the reference laboratories for rabies in the Americas [15]. Antigenic characterization of RABV isolates by indirect immunofluorescence (IIF) according to the reactivity of the 8 MAbs defines 12 antigenic profiles, which establishes the 11 antigenic variants found in the isolates of Latin America and differentiates them from the laboratory and vaccine strains Challenge virus standard (CVS), Pasteur virus (PV), Street-Alabama-Dufferin (SAD), and Evelyn-Rokitnicki-Abelseth (ERA) [15,16].

Since 1996, the Rabies Diagnostic Laboratory of Instituto Pasteur of São Paulo/Brazil (IP/SP/Brazil) has been doing the antigenic characterization of viral isolates from different animal species of various regions of Brazil, utilizing the panel of eight MAbs specific for viral nucleoprotein.

An analysis conduct on 330 isolates from humans and different animal species between 1996 and 2000 demonstrated five antigenic variants (AgV) compatible with the profiles observed in the panel: two in dogs (AgV1 and AgV2) and three in bats (AgV3 of *D. rotundus*, AgV4 of *Tadarida braziliensis*, and AgV6 of *Lasiurus* spp.). Six other profiles were identified that were incompatible with the panel

utilized. The greatest variability was observed among the samples isolated from insectivorous bats, and the most common variant found was variant 3 of *D. rotundus* [17].

Various studies have identified RABV isolates from different species and regions in Brazil with profiles incompatible with those pre-established in the antigenic characterization of the CDC panel. There are two distinct variants that circulate in Northeast Brazil and that have as reservoirs *Cerdocyon thous* (crab-eating fox) and *Callithrix jacchus* (common marmoset). These variants are not compatible with those defined by the CDC panel, but they have a consistent antigenic profile [18-22].

The observation of the incompatibility of certain isolates required the complementation of antigenic studies with genetic analyses, which have proven the diversity of RABV isolates in Brazil. All isolates confirmed as antigenic variants belong to the species *Rabies virus* of the genus *Lyssavirus*, like all the other isolates on the American continents and Caribbean [23,24].

In the period of 2000 to 2006, the diagnostic laboratory of IP/SP analyzed 4,057 samples of bats found in urban areas of the city of Ribeirão Preto, in the state of São Paulo, of which 64 were positive for rabies and were frugivorous or insectivorous species. The antigenic study of these isolates identified the majority as the variants AgV3 and AgV6, but two were not compatible with the profiles of the panel utilized [25].

Albas *et al.* [26] antigenically characterized 18 isolates of non-hematophagous bats from the west of the state of São Paulo, and detected the antigenic variants AgV3 of *D. rotundus* and AgV4 of *T. braziliensis*.

Of the 174 species of bats existing in Brazil [27], RABV was isolated from 42 species [28-30]. Of these, approximately 62% showed an insectivorous feeding habit. IP/SP/Brazil receives about 4,000 samples of bats annually for the diagnosis of rabies, where the samples diagnosed positive are subjected to antigenic characterization. In 2008, of the 42 bats positive for rabies, 24 were incompatible with the profiles pre-established in antigenic characterization. In 2009, of the 60 positive bats, 19 showed incompatible profiles. In 2010, there were 60 positive bats, with 28 being incompatible (data from IP/SP/Brazil).

In recent years, a large number of bats of species with insectivorous feeding habits were diagnosed positive for rabies in IP/SP/Brazil, of which about 40% did not show profiles compatible with the panel

utilized in antigenic characterization (data from IP/SP/Brazil).

Therefore, it was necessary to produce MAbs that complement the characterization of the antigenic variants circulating in these species, contributing to a more effective epidemiological monitoring of the disease in Brazil.

In this study, we describe the production of MAbs for RABV isolates from insectivorous bats with profiles incompatible with pre-established antigenic variants in Latin America.

Methodology

Animals

Female BALB/c mice, weighing 20 to 22 g, were used for immunization. The mice were from the central animal facility of Instituto Butantan and were maintained in the animal facility of the Immunopathology Laboratory of Instituto Butantan. The procedures in animals were approved by the Ethics in the Use of Animals Commission of Instituto Butantan (CEUAIB No. 465/08).

Cell lines

Mouse myeloma cells P3X63Ag8.653 (ATCC CRL-1580) and the hybridomas were maintained in RPMI 1640 medium with 25 mM Hepes (Gibco, Grand Island, New York, USA), plus 20 mM sodium pyruvate (Gibco), 0.2 mM L-glutamine (Gibco), 1% gentamicin (Gibco), 1% solution of antibiotics and antifungal (streptomycin, penicillin, and fungizone – Gibco), and 10% fetal bovine serum (FBS – Gibco) at 37°C and 5% CO_2.

Baby hamster kidney cells (BHK-21, ATCC, CCL-10) and murine neuroblastoma cells (N2A, ATCC, CCL-131) were cultivated in Eagle's minimum essential medium (Sigma-Aldrich, Saint Louis, USA), supplemented with 10% FBS (MEM-10% FBS), and 3% gentamicin (Gibco), at 37°C and 5% CO_2. N2A cells were grown in medium with the addition of 3% non-essential amino acids (Sigma-Aldrich).

Preparation of the antigen for immunization

A central nervous system (CNS) sample of an insectivorous bat, *Eptesicus furinalis*, from the city of Ribeirão Preto, state of São Paulo, that was diagnosed positive for rabies by direct immunofluorescence (DIF) and viral isolation (VI) and that showed an antigenic profile incompatible with that established in the antigenic characterization by Diaz *et al*. [15] was selected. From the second passage of the isolate in the

CNS of Swiss albino mice, a 20% (w/v) virus suspension was prepared, which was utilized for the infection of N2A cells for adaptation of the virus to cell culture. Titers of the virus suspensions were determined by DIF in the isolation of virus in N2A cultured cells (IVCC) [31] and calculated at a 50% focus-forming dose (FFD50), as described by Smith *et al*. [32].

The ribonucleoproteins (RNP) of the isolate were concentrated following the protocol described by Caporale *et al*. [33].

Immunization of the animals

Three mice received, on day zero, two subcutaneous doses of 0.05 mL containing 20 µg concentrated RNP, diluted 1:2 in Marcol-Montanide adjuvant [34,35], in the footpad of the hind legs. After 15 days, the animals were given a booster with the same dose, adjuvant, and route used for priming. Three days before cell fusion, a booster of the same dose of antigen in saline was administered. Antibody titers were determined by IIF, from 7 to 10 days after the first booster.

Production of monoclonal antibodies

Three days after the last booster, the mouse that showed the highest antibody titers in IIF was euthanized in accordance with the established guidelines of the ethics of animal handling [36], and the popliteal lymph nodes were aseptically excised. The cells of the lymph nodes and P3X63Ag8.653 myeloma cells in exponential growth phase were mixed in a 2:1 lymph node/myeloma proportion in RPMI without FBS, and cell fusion was carried out by adding 1 mL of a 50% solution of polyethylene glycol 4000 (Merck, Frankfurt, Germany) in PBS, pH 7.4, containing 5% DMSO (Sigma), under agitation, in 1 minute. After 90 seconds of resting at 37°C, the cell suspension was diluted in 1 mL of RPMI, under agitation, in 1 minute, followed by 20 mL of RPMI in 4 minutes. Finally, the cells were allowed to rest for 4 minutes at 37°C, centrifuged for 5 minutes at 500 g and resuspended in RPMI medium with 10% FBS containing 3% hypoxanthine-aminopterin-thymidine medium (HAT medium, Gibco) at a concentration of 1×10^6 cells/mL. After 2 hours of incubation at 37°C and 5% CO_2, the cell suspension was distributed in 96-well plates, 100 µL per well, and kept at 37°C and 5% CO_2.

Ten days after fusion, the supernatants of the hybridomas were analyzed by IIF. The hybridomas

that produced positive MAbs in IIF were subjected to two rounds of the limiting dilution method.

Screening test – indirect immunofluorescence

The hybridomas secreting antibodies against RABV were tested by IIF in BHK-21 cultured cells infected for 24 hours with a fixed virus strain, PV or CVS, maintained at 37ºC in 5% CO_2 atmosphere, or N2A cells infected for 72 hours with RABV isolate of non-hematophagous bat (the same used in antigen preparation). The cells were fixed for ten minutes with 80% acetone (Merck) and kept at 4°C. The microplates containing fixed infected cells were stored at -20°C until use. Fifty microliters of the supernatants of the hybridomas were added as the first antibody and then incubated at 37°C for 30 minutes. MAbs reactions were revealed by incubation for 30 minutes at 37°C with 50 µL of FITC-conjugated anti-mouse polyvalent immunoglobulins (IgG, IgA, and IgM) (Sigma-Aldrich). The results were observed with an inverted fluorescence microscope at 100X magnification.

Characterization of monoclonal antibodies

MAb isotype was determined by capture immunoenzyme assay (ELISA) using a mouse isotyping kit (Pierce, Waltham, USA) according to the manufacturer's protocol. Spectrophotometric readings were at 450 nm (ELISA Multiskan EX reader, Labsystem, Rockfort, USA).

Reactivity of MAbs with RABV isolates

Fifty-seven RABV isolates of different animal species were subjected to antigenic characterization by the IIF technique on a touch impression of CNS on glass slides, utilizing the panel of eight MAbs produced by the CDC and also the new MAbs obtained [15].

Results

Production and characterization of the antibodies monoclonal against RABV

After the fusion of the cells, 40 clones were positive in IIF (of 434 wells tested). After two steps of limiting dilution, two hybridomas that produced specific monoclonal antibodies were selected and designated as BR/IP1-3A7 and BR/IP2-4E10. The two MAbs were characterized as IgG2a, and of the kappa light chain.

The specificity was demonstrated by the positive reaction in IIF with different RABV isolates in the

CNS of infected mice and negative reaction in the CNS of uninfected mice (Figures 1, 2, and 3).

Figure 1. IIF negative in touch impression of CNS of noninfected mouse. A: MAb BR/IP1-3A7; B: MAb BR/IP2-4E10. 200x.
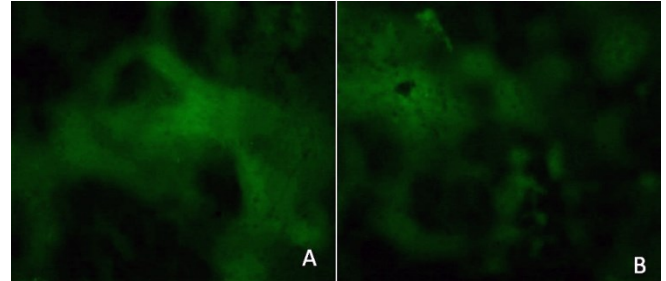
Figure 2. Reactivity of MAb BR/IP1-3A7 with isolate from *Artibeus lituratus* by IIF. Touch impression of CNS of infected mouse with isolate 5861V/09. 200x.
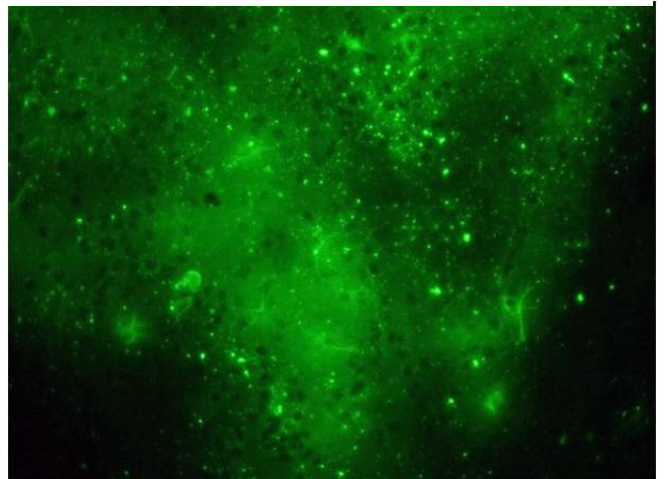
Figure 3. Reactivity of Mab BR/IP2-4E10 with isolate from *Artibeus lituratus* by IIF. Touch impression of CNS of infected mouse with isolate 5861V/09. 200x.
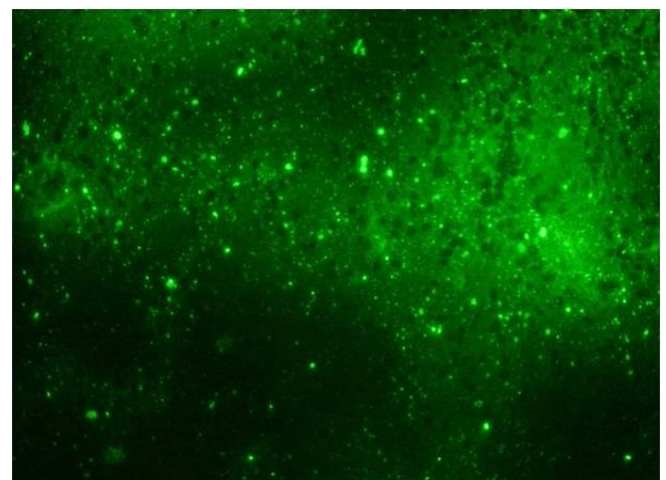
Table 1. Reactivity of BR-IP MAbs and CDC panel with RABV isolates from non-hematophagous bats by IIF

IP/SP No.	Isolate from	Feeding habit	Origin of sample/State	AgV	CDC Panel								BR/IP	
					C1	C4	C9	C10	C12	C15	C18	C19	1-3A7	2-4E10
1	2445/08	*Eptesicus furinalis*	Insectivorous	São Paulo	IC	-	+	-	+	+	-	-	-	-	-
2	3866V/08	*Nyctinomops laticaudatus*	Insectivorous	Minas Gerais	IC	-	±	-	++	++	-	-	-	+	+
3	497V/09	*Myotis nigricans*	Insectivorous	São Paulo	4	-	+	+	+	+	-	-	-	-	-
4	512V/09	*Eptesicus furinalis*	Insectivorous	São Paulo	IC	-	+	-	+	+	-	-	-	+	-
5	542V/09	*Nyctinomops laticaudatus*	Insectivorous	São Paulo	IC	-	+	-	+	+	-	-	-	+	+
6	2271V/09	*Nyctinomops laticaudatus*	Insectivorous	São Paulo	IC	-	++	-	++	++	-	-	-	+	+
7	3176V/09	*Tadarida braziliensis*	Insectivorous	São Paulo	4	-	+	+	+	+	-	-	-	-	-
8	4607V/09	*Artibeus lituratus*	Frugivorous	São Paulo	3	-	+	+	+	+	-	-	+	±	±
9	4616V/09	*Myotis albescens*	Insectivorous	São Paulo	4	-	+	+	+	+	-	-	-	-	-
10	5610V/09	*Artibeus fimbriatus*	Frugivorous	São Paulo	3	-	+	+	+	+	-	-	+	+	+
11	5861V/09	*Artibeus lituratus*	Frugivorous	São Paulo	3	-	+	+	+	+	-	-	+	++	++
12	6207V/09	*Artibeus lituratus*	Frugivorous	São Paulo	3	-	+	+	+	+	-	-	+	±	±
13	6375V/09	Chiroptera	Non-hematophagous	Paraná	4	-	+	+	+	+	-	-	-	-	-
14	6429V/09	*Molossus molossus*	Insectivorous	São Paulo	4	-	+	++	++	+	-	-	-	-	-
15	6746V/09	*Artibeus lituratus*	Frugivorous	Minas Gerais	3	-	+	+	+	+	-	-	+	-	-
16	6945V/09	*Nyctinomops laticaudatus*	Insectivorous	São Paulo	IC	-	+	-	++	++	-	-	-	±	±
17	7279V/09	*Artibeus lituratus*	Frugivorous	Minas Gerais	3	-	+	+	+	+	-	-	+	-	+
18	7952V/09	*Eptesicus braziliensis*	Insectivorous	Paraná	IC	-	+	+	+	-	-	-	-	-	-
19	9404V/09	*Lasiurus ega*	Insectivorous	Paraná	4	-	+	+	+	+	-	-	-	-	-
20	9709V/09	*Molossus molossus*	Insectivorous	Rio Grande do Norte	IC	-	+	-	+	+	-	-	-	-	-
21	9710V/09	*Molossus molossus*	Insectivorous	Rio Grande do Norte	IC	-	+	-	+	+	-	-	-	-	-
22	401V/10	*Nyctinomops laticaudatus*	Insectivorous	Minas Gerais	IC	-	+	-	++	++	-	-	-	-	-
23	402V/10	*Artibeus lituratus*	Frugivorous	Minas Gerais	3	-	+	+	+	+	-	-	+	+	+

IC: incompatible; AgV: antigenic variant; (++) strong positive reaction; (+) positive reaction; (-) negative reaction; (±) weak positive reaction; BR/IP: MAbs obtained from Instituto Pasteur/SP/Brazil; CDC: Centers for Disease Control and Prevention; IIF: indirect immunofluorescence.

Table 2. Reactivity of BR/IP MAbs and CDC panel with RABV isolates from domestic canids, wild canids, domestic felines, and a human by IIF

	IP/SP No.	Isolate from	Origin of sample	AgV	Reactivity of MAbs									
					CDC Panel								BR/IP	
					C1	C4	C9	C10	C12	C15	C18	C19	1-3A7	2-4E10
1	5635V/09	Domestic canid	Rio Grande do Norte	2	+	+	-	+	+	+	-	+	-	-
2	1016V/09	Domestic canid	Paraíba	2	+	+	-	+	+	+	-	+	-	-
3	4194V/09	Domestic canid	Bahia	IC	-	+	-	+	+	+	-	+	-	-
4	7841V/09	Domestic canid	Pernambuco	2	+	+	-	+	+	+	-	+	+	+
5	7847V/09	Domestic canid	Pernambuco	IC	-	++	-	++	++	++	-	++	+	±
6	6634V/09	Domestic canid	Bahia	IC	+	+	±	+	+	-	-	+	±	±
7	5634V/09	Wild canid	Rio Grande do Norte	2	+	+	-	+	+	+	-	+	-	-
8	5636V/09	Wild canid	Paraíba	2	+	+	-	+	+	+	-	+	-	-
9	1043V/09	Wild canid	Sergipe	IC	±	+	+	+	+	+	-	+	-	-
10	7839V/09	Wild canid	Pernambuco	IC	-	+	-	+	+	+	-	+	-	-
11	7840V/09	Wild canid	Pernambuco	2	+	+	-	+	+	+	-	+	-	-
12	7848V/09	Wild canid	Pernambuco	IC	-	+	-	+	+	+	-	+	±	±
13	7853V/09	Wild canid	Pernambuco	IC	-	++	-	++	++	++	-	++	-	±
14	7837V/09	Wild canid	Pernambuco	IC	-	+	-	+	+	+	-	+	-	-
15	7850V/09	Wild canid	Pernambuco	IC	-	+	-	+	+	+	-	+	-	-
16	7851V/09	Wild canid	Minas Gerais	IC	-	+	-	+	+	+	-	+	-	-
17	7852V/09	Wild canid	Pernambuco	IC	-	+	-	+	+	+	-	+	-	-
18	7856V/09	Wild canid	Pernambuco	IC	-	+	-	++	++	++	-	++	-	-
19	8042V/09	Wild canid	Rio Grande do Norte	IC	-	++	-	++	++	++	-	++	+	+
20	7838V/09	Domestic feline	Pernambuco	2	+	+	-	+	+	+	-	+	-	±
21	7849V/09	Domestic feline	Pernambuco	IC	-	+	-	+	+	+	-	+	±	±
22	7488V/09	Human	Maranhão	2	+	+	-	+	+	+	-	+	±	+

IC: incompatible; AgV: antigenic variant; (++) strong positive reaction; (+) positive reaction; (-) negative reaction; (±) weak positive reaction; BR/IP: MAbs obtained from Instituto Pasteur/SP/Brazil; CDC: Centers for Disease Control and Prevention; IIF: indirect immunofluorescence

Table 3. Reactivity of BR/IP MAbs and CDC panel with RABV isolates from herbivores by IIF

	IP/SP No.	Isolate from	Origin of sample	AgV	Reactivity of MAbs									
					CDC Panel								BR/IP	
					C1	C4	C9	C10	C12	C15	C18	C19	1-3A7	2-4E10
1	1023V/09	Cattle	Sergipe	IC	+	+	+	+	+	-	-	+	-	-
2	1024V/09	Cattle	Bahia	IC	+	+	+	+	+	-	-	+	-	-
3	1026V/09	Cattle	Sergipe	IC	+	+	+	+	+	-	-	+	-	-
4	1027V/09	Cattle	Sergipe	IC	+	+	+	+	+	-	-	+	-	-
5	1032V/09	Cattle	Sergipe	IC	+	+	+	+	+	-	-	+	+	+
6	2429V/09	Cattle	São Paulo	3	-	+	+	+	+	-	-	+	-	-
7	6292V/09	Cattle	São Paulo	3	-	++	+	+	+	-	-	+	-	±
8	6462V/09	Cattle	São Paulo	3	-	+	+	+	+	-	-	+	+	±
9	6879V/09	Cattle	São Paulo	3	-	+	+	+	+	-	-	+	+	+
10	7062V/09	Cattle	São Paulo	3	-	++	++	++	++	-	-	+	±	±
11	7063V/09	Cattle	São Paulo	3	-	++	++	++	++	-	-	++	-	+
12	1028V/09	Horse	Sergipe	IC	+	+	+	+	+	-	-	+	-	-

IC: incompatible; AgV: antigenic variant; (++) strong positive reaction; (+) positive reaction; (-) negative reaction; (±) weak positive reaction; BR/IP: MAbs from Instituto Pasteur/SP/Brazil; CDC: Centers for Disease Control and Prevention; IIF: indirect immunofluorescence

Reactivity of MAbs against the RABV isolates by IIF

The results of the antigenic characterization of 23 isolates of non-hematophagous bats are grouped in Table 1. Of the ten isolates considered incompatible (IC), nine exhibited the same profiles, with reactivity to the MAbs C4, C10, and C12 of the CDC panel. Only isolate 7952V/09 showed reactivity with the antibodies C4, C9, and C10.

The MAbs BR/IP1-3A7 and BR/IP2-4E10 recognized 10 of the 23 isolates from the bats analyzed. For nine isolates analyzed that showed an incompatible profile, characteristic of insectivorous bats, MAb BR/IP1-3A7 reacted with five (55.55%) and BR/IP2-4E10 with four (44.45%).

The two MAbs reacted with highest intensity (strong reaction ++) with the isolate 5861V/09 of *Artibeus lituratus*, characterized as AgV3 (Figures 2 and 3). MAb BR/IP2-4E10 reacted with ten isolates of bats, of which five were of the species *Artibeus lituratus*, one of *Artibeus fimbriatus* (characterized as AgV3), and four of *Nyctinomops laticaudatus*, with profiles incompatible with the pre-established panel. Of these ten, MAb BR/IP1-3A7 did not react with the isolate 7279V/09 of *Artibeus lituratus*, but did react with the isolate of *Eptesicus furinalis* (512V/09), with an incompatible profile.

Table 2 groups 22 isolates related to AgV2 of dogs, cats, and wild canids (fox and crab-eating fox), with six being from domestic dogs, thirteen from wild canids, two from domestic cats, and one from a human. Of these isolates, fourteen were incompatible with the characterization profiles, including three from dogs, ten from wild canids, and one from a cat. With the exception of the isolate from a dog, 6634V/09, for the other isolates considered incompatible, the difference in the profile to be considered AgV2 occurred in the lack of reactivity with MAb C1 of the CDC panel. Isolate 6634V/09 also showed an incompatible profile, but was close to an AgV3 profile, differing in a positive reaction to MAb C1.

MAb BR/IP1-3A7 reacted with two isolates from dogs, two from wild canids, one from a cat, and the one from a human. Of these seven isolates, five were incompatible, including isolate 6634V/09, and two were characterized as AgV2. MAb BR/IP2-4E10, besides reacting with these seven isolates, also reacted with isolate 7853V/09 from a wild canid, incompatible, and an isolate from a cat, 7838V/09, characterized as AgV2.

Twelve isolates from herbivores are grouped in Table 3, where eleven are from cattle and one from a horse. Six isolates from cattle were characterized as AgV3, and the rest displayed incompatible profiles, but differing only in the reaction of MAb C1.

MAb BR/IP2-4E10 reacted with five isolates from cattle characterized as AgV3 and one with an incompatible profile. Of these six isolates, MAb BR/IP1-3A7 did not react with two, isolates 6293V/09 and 7063V/09, both AgV3.

In the total 57 isolates analyzed, MAb BR/IP1-3A7 reacted with 22 isolates (38.59%) and MAb BR/IP2-4E10 with 26 (45.61%). Of the 13 isolates characterized as AgV3, MAb BR/IP1-3A7 reacted with eight (61.53%) and BR/IP2-4E10 with eleven (84.61%). Of the nine isolates that showed an incompatible profile related to the non-hematophagous bats (C4+ C10+ C12+), MAb BR/IP1-3A7 reacted with five (55.55%) and BR/IP2-4E10 with four (44.44%).

Discussion

The present study describes the production of two new MAbs for RABV of the isolate from a non-hematophagous bat. The mice in this study were immunized with concentrated RNP from N2A cells infected with the isolate. Two stable MAb secreting-hybridomas were obtained.

The choice of the isolate from the bat of the genus *Eptesicus* for this study was based on the incompatible profile shown in the antigenic characterization done with the reduced panel of eight MAbs of the CDC. Viral antigenic characterization is based on the fact that MAbs bind to conserved antigenic sites in a particular strain or variant and that the antigenic data allow the mapping of particular epitopes to which specific MAbs bind, thereby providing useful information about the amino acid residues critical for the maintenance of their structure. In practice, however, few residues critical for binding of the MAbs have been identified for the variants of RABV [37,38].

The specificity of the MAbs BR/IP1-3A7 and BR/IP2-4E10 for RABV was confirmed by the positive reaction in the antigenic characterization of various isolates, demonstrating a similar reactivity pattern for the two with difference of recognition for a few isolates (Tables 1, 2, and 3). Of the isolates recognized by the MAbs BR/IP1-3A7 and BR/IP2-4E10, there was a predominance of isolates characterized as AgV3. Various studies have demonstrated a predominance of the circulation of the antigenic variant of *Desmodus rotundus* AgV3 in cattle and non-hematophagous bats in Brazil [17,26,39].

Schaefer *et al.* [40], utilizing a panel of 11 MAbs for the antigens of Lyssavirus, antigenically characterized RABV isolates from different animal species of various regions of Brazil and described two main clusters, one for dogs and another for hematophagous bats, and also identified another group for insectivorous bats. In the genetic analyses of the N protein of the isolates analyzed, there was grouping by species because of the occurrence of species specificity.

The method of antigenic characterization more often utilizes protein N as the target, which is produced in large amounts in infected cerebral tissue [41]. Therefore, the RNP concentration of the isolate for production of antigen utilized in this study allowed a satisfactory immune response, which enabled the successful production of MAbs.

Although there are a large number of MAbs against RABV described in the literature, they are not always easily obtained, mainly because the production of MAbs is very limited in Brazil.

In this study, of the 57 isolates of RABV characterized antigenically, 30 showed an incompatible profile. Of these 30 isolates, 9 had a characteristic profile related to isolates from non-hematophagous bats, as has been observed in the laboratory of IP/SP/Brazil, with reactivity with the MAbs C4, C10, and C12 of the CDC panel. This incompatible reaction pattern has been described by Favoretto *et al.* [17].

Of 103 isolates obtained from insectivorous bats in Argentina submitted to antigenic typing with the CDC panel, 22 exhibited 11 distinct atypical reaction pattern (ARP), one of which ARP found in three *Eptesicus* spp. was the same incompatible profile found in non-hematophagous bats in this study [42].

Bernardi *et al.* [43] conducted an antigenic and genetic analysis of 50 isolates from different animal species and regions of Brazil. Genetic analysis carried out by sequencing the *P* gene and antigenic analysis by IIF utilizing 473 MAbs in N2A cells, allowed the selection of 10 MAbs proposed for improving the characterization of different antigenic variants.

The MAbs BR/IP1-3A7 and BR/IP2-4E10 showed a varying reactivity profile for the 57 RABV isolates analyzed (Tables 1, 2, and 3). Similarly, Zanluca *et al.* [44] produced and characterized seven MAbs against RABV from fusions with different immunization protocols and viral antigens, and two of these MAbs also showed a varying reactivity pattern for the RABV isolates from various species, suggesting the possibility of their use for antigenic characterization.

In the process of obtaining MAb-producing hybridomas, certain immunologically dominant antigenic sites often limit the reactivity of the selected hybrid clones. Therefore, a panel of MAbs with appropriate discriminatory power requires the selection of MAbs against different isolates. For the production of a new panel, it is necessary to take into consideration the virus population to be recognized and the regional variations in the circulation of the viral antigenic variant in a particular geographic region [38,40].

Antigenic characterization utilizing the eight monoclonal antibodies developed by the CDC is widely used in Latin America for RABV surveillance [16]. However, antigenic analysis is unable to identify RABV isolates obtained from several reservoirs species because these isolates produced atypical reaction patterns or incompatible profiles. [17-22,42]. The genetic analysis of the viral nucleoprotein sequence allowed further characterization of those isolates.

The major advantage of genetic typing in which sequence determination is employed it is the possibility of using such data for phylogenetic investigation and gaining a totally objective strain identification as well as evolutionary information that is inherent to this type of analysis. However, the costs associated with the performance of this type of variant identification, in terms of technical expertise as well as the acquisition of equipment and reagents, are substantial. Despite costs having decreased with the improvement of new techniques and equipment, cost still remains a difficulty to developing countries to obtain resources to characterize, by sequence analysis, all rabies-positive cases [38,45,46]

The method of antigenic characterization by IIF with MAbs is less expensive and technically simpler than genetic sequencing methods and can thus be routinely employed in large numbers of cases [38]. However, discrimination using antigenic methods depends on the MAb panel used. Obtaining hybridomas that produce MAbs against RNP can also be very useful for the production of anti-RNP conjugates highly specific for use in the laboratory diagnosis of rabies.

Analysis of the *G* and *N* genes of 57 rabies virus isolates from different genera of insectivorous bats in Brazil made it possible to identify specific molecular markers, in the putative N and G proteins, for three strains of the genera *Myotis*, *Eptesicus*, and *Nyctinomops*, which allows their distinction from other strains of RABV [47]. In analyzing the analogy

between the genetic and antigenic classification of these isolates, researchers did not find a relationship between these classifications because isolates with the same antigenic profile grouped with different genetic strains. There can be detectable antigenic differences between these strains, which could be utilized for the standardization of a new panel of MAbs. The sharing of the same epitopes in different RABV strains found in genetic analysis can be one of the reasons for the lack of accuracy in antigenic classification with the panel utilized [47,48].

Conclusions

The MAbs BR/IP-3A7 and BR/IP2-4E10 were capable of recognizing common epitopes between the RABV isolates of the three genera of non-hematophagous bats, *Artibeus*, *Eptesicus*, and *Nyctinomops*, thereby being helpful in the antigenic characterization of the RABV isolates from non-hematophagous bats in Brazil. However, further studies testing the MAbs BR/IP-3A7 and BR/IP2-4E10 with more isolates of distinct bats reservoirs and/or the production of new MAbs are needed to complement the current panel of MAbs utilized for the characterization of the RABV isolates in Brazil that show incompatible profiles.

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