

Biological and immunological studies of five Brazilian rabies virus isolates

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Abstract

Aspects of biological behavior of Brazilian rabies virus isolated from canine, bovine, equine, vampire and insectivorous bats were studied in mice. The oral infection occurred in mice fed with infected brain of insectivorous bat (8.82%), canine (8.57%) and equine (3.03%). The mean period of incubation to all the isolates was 6 days after mice intracerebral inoculation, however, symptoms were variable, since hyperexcitability (canine sample), progressive paralysis of lower limbs and prolonged clinical course until death (equine sample), and mice without clinical signs before death (insectivorous bat). By immunohistochemistry IFN was detected in brains of mice inoculated with bovine and insectivorous bat samples, TNF and iNOS were detected in brains of those inoculated with insectivorous bat, bovine and canine samples, and positive GFAP astrocytes were found in all five samples. Two commercial inactivated rabies vaccines, one imported (vaccine 1) and another manufactured in Brazil (vaccine 2) were compared to evaluate their efficacy to protect against experimental rabies infection in mice, through the NIH and the CDC potency tests, using these street isolates as challenge virus. There was no statistical significant difference between the efficacy of both vaccines, when comparing the same potency test and challenge virus strain suggesting no need to produce specific vaccines with street isolates.

Key-words:

Rabies virus.
 Biological and immunological studies.
 Brazilian strains.

Introduction

Some aspects of rabies virus infection such as incubation period, viscerotropism, early death phenomenon, paralysis and encephalitis may be modulated by the immunological response. Also, they may be related to the presence of specific antibodies and to the site of viral replication within the central nervous system^{1,2}.

During infection it might be observed high levels of interferon in the CNS, according to viral reproduction. The increase

of IL-1, TNF and IFN- γ in the brain of infected animals is related to the development of symptoms, however it does not affect the mortality, because rabies virus is capable to induce apoptosis in the brain cells before any inflammatory response^{3,4,5}.

The etiopathogeny of rabies may also be modulated by the excessive production of nitric oxide, due to the activation of the enzyme iNOS in macrophages and microglia, and by the decrease of the enzyme cNOS activity⁶.

In Brazil and other countries where

rabies in domestic animals is not under control, the recommendation is to annually vaccinate at least 70% of dogs and cats population and to vaccinate herbivorous in endemic areas⁷.

The potency tests for rabies vaccines were developed with the objective of determinate the ability of a vaccine to induce protection. The NIH (National Institute of Health) is considered the gold standard for inactivated virus vaccines, and it is adopted to control commercial vaccines. The test developed by the CDC of Atlanta-USA is closer to natural conditions, performed with an intramuscular challenge with street virus isolates^{8,9,10}.

The objective of this article is to study in mice, some aspects of the biological behavior of rabies virus isolated from different species, observing the oral transmission, incubation period, symptoms, mortality, brain inflammatory response and the efficacy of two commercial inactivated rabies vaccines used in Brazil in order to provide additional information for the rabies prevention and control authorities.

Materials and Methods

Mice

Swiss albino mice (*Mus musculus*), CH-3 Rockfeller lineage, weighting 14-16g were used.

Virus

The street isolates selected were identified as M288/99 (horse, Guararema-SP, 1999); M44/89 (dog, São João da Boa Vista – SP, 1989); M45/97 (insectivorous bat *Histiotus velatus*, Ibiúna – SP, 1997); T11/95 (vampire bat *Desmodus rotundus*, Taubaté – SP, 1995); M186/00 (bovine, Poços de Caldas – MG, 2000). The CVS 31/2 strain was used for the potency test and virus neutralization.

Titration

Samples were diluted from 10-2 to 10-6 and volumes of 0,03ml/animal were intracerebrally (i.c.) inoculated in groups of

10 mice for each dilution. Titers were expressed as LD₅₀/0,03 ml/mice/ i.c¹¹. It was calculated the standard error for the LD₅₀ and $\alpha = 0,05$ ¹².

Vaccine potency test

It was used a commercial imported vaccine* (vaccine 1), and a national one** (vaccine 2), both prepared with the PV strain propagated in BHK cell culture, inactivated by betapropiolactone and having aluminum hydroxide as adjuvant.

Both the CDC/ Atlanta – USA² and NIH²⁰ potency tests were also performed. The last one was adapted using the street isolates of this study as challenge virus. Results were expressed as LD₅₀/0,03 ml/mice/ i.c¹³. It was calculated the standard error for the DP₅₀ and $\alpha = 0,05$ ¹².

Neutralization

Sera of surviving animals of oral inoculation were submitted to the neutralization test¹³.

Oral infection

For each sample, 35 mice were offered 1g of infected brain/ mouse. Those ones which refused to feed were discarded of the experiment. The other were maintained with water and food “*ad libitum*” and observed for 60 days.

Biological behaviour

Each viral sample was diluted at 20% and volumes of 0.03 ml/animal were intracerebrally inoculated in 20 mice. Daily observation for symptoms and death was recorded.

Histopathology

For each sample, infected mice brain were fixed in 10% neutral formalin, and stained by routine hematoxilin-eosine.

Immunohistochemistry

The expression of IFN γ (interferon gamma), TNF α (tumor necrosis factor alpha), iNOS (inducible nitric oxide synthase) enzyme

* Laboratories Smith Kline – Beecham. (Defensor®), indicated for dogs, cats, bovines and ovines.

** Laboratory BioVet. (RaiVet®), indicated for dogs, cats, bovines and equines.

and GFAP (glial fibrillary acidic protein) in the brain of mice infected with the selected samples was verified¹. Primary antibodies were used as follow:

IFN: policlonal antibody – goat anti human IFN - Santa Cruz Biotechnology, Inc; TNF: monoclonal antibody - mouse anti human TNF - Santa Cruz Biotechnology, Inc; iNOS: policlonal antibody – rabbit anti human NOS2 - Santa Cruz Biotechnology, Inc; GFAP: monoclonal antibody - rabbit anti bovine GFAP - DAKO CORPORATION, Denmark.

As secondary antibody it was used the Kit Duet for primary antibodies of rabbit and mouse - STREPTABC HRP Duet, Dako Corporation, USA.

Results

Oral transmission occurred for the insectivorous bat sample (8,82%; LD₅₀ = 3,76); dog (8,57%; LD₅₀ = 3,55) and equine (3,03%; LD₅₀ = 4,28). The LD₅₀ of the bovine and vampire bat isolates were 2,60 e 3,92, respectively, without rabies symptoms during the observation period. No animal

presented titer equal or superior to 5 at the neutralization test. CVS titer was 10^{-6,28} LD₅₀/0,03ml/mice/i.c.

The histological examination of the brain of infected mice showed areas of focal and perivascular gliosis, and focal areas of haemorrhage in the parenchyma and pia mater. Degenerated neuronons and Negri bodies inclusions were noticed. Through immunohistochemistry a positive staining for IFN g was verified only in the brain of mice inoculated with the insectivorous and vampire bats isolates.

There was no statistical difference between the ED₅₀ of both vaccines, for the same potency test and virus challenge strain. It was not possible to calculate the variation of the ED₅₀ for both potency tests performed with the insectivorous bat isolate; for the bovine isolate at the CDC test for vaccine 1 and for the isolates of dog, vampire bat and equine for vaccine 2, due to the fact that the effective doses were higher than 250.

The expression of TNF and iNOS was observed in the brains of mice infected with the samples originated from dog, bovine

Table 1 – Results of the mice intracerebral inoculation of five Brazilian rabies virus isolates. São Paulo – Brazil, 2002

Sample	First day of symptom	Last day of death	Mean period of incubation	symptoms
Vampire bat (<i>Desmodus rotundus</i>)	5	10	7.5	Paralysis, frizzy hair, emaciation
Insectivorous bat (<i>Histiotus velatus</i>)	7	12	9.5	Death without symptoms
Bovine	8	13	9.0	Paralysis, frizzy hair, emaciation
Equine	6	12	7.0	Paralysis
Canine	6	12	8.0	Hyperexcitability, convulsion, sensitivity to noise

Table 2 – Effective dose 50% (ED₅₀) of two antirabies vaccines according to the potency test, vaccine and viral isolate. Sao Paulo - Brazil, 2002

Virus isolate	Vaccine 1		Vaccine 2	
	CDC	NIH	CDC	NIH
Vampire bat (<i>Desmodus rotundus</i>)	4,57	14,80	120,22	26,91
Insectivorous bat (<i>Histiotus velatus</i>)	> 250,00	144,54	131,82	123,27
Bovine	77,62	17,38	38,90	50,00
Equine	14,79	8,91	> 250,00	28,18
Canine	5,00	43,65	> 250,00	54,95

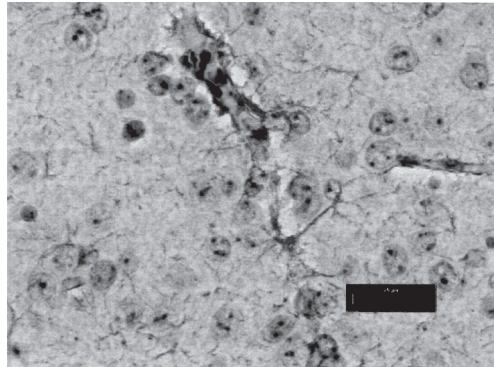


Figure 1 – Encephalon mice inoculated with a bovine rabies virus isolate showing increased GFAP expression surrounding blood vessel (anti – GFAP scale bar 25mm)

and insectivorous bat, with cytoplasmatic staining of macrophages at glial nodes and blood vessels areas. There was an increase in GFAP expression in the brain of mice infected with all five isolates, with astrocytic hypertrophy, mostly surrounding blood vessels (Figure 1).

The results of the potency tests are presented at table 2.

Discussion and Conclusions

Results obtained at this study reveal higher oral transmission in mice fed with the insectivorous bat isolate (8.82%). The bovine isolate was not pathogenic by this route of infection, despite the fact that infective doses were similar. The difference in the infectivity for rabies virus samples isolated from the same animal specie and the difference of susceptibility of rodent species were previously reported¹⁴.

All the animals intracerebrally inoculated with any of the five isolates died until the thirteenth day of observation and the clinical stage was 5 to 6 days. The mean incubation period for all the isolates was 7 to 9.5 days. For routine diagnostic, the observation period is 21 days, consequently in all cases it would be possible to isolate and identify positive materials.

Rabies viruses isolated from bovine and vampire bat are related to the same epidemiological cycle¹⁵. Mice inoculated with the vampire bat strain showed symptoms

and death at the fifth and seventh days post inoculation respectively. These animals had symptoms similar to those mice inoculated with the bovine strain, presenting frizzy hair, emaciation and paralysis. However, the incubation period for the bovine isolate was eight days and death occurred between the ninth and thirteenth days.

Histological observation was compatible with literature, blood vessels surrounded by linfocytes and macrophages, degenerated neurons, focal gliosis and haemorrhages observed in this study were also reported in goats, equines and bovines naturally infected^{16,17}. “Status spongiformis” were observed in all the isolates from this study and also occur in naturally infected animals. Thalamus and inner layer of cerebral cortex are the most affected regions¹⁵. The presence of Negri bodies was verified in all five isolates, however in a natural infection they may not be seen¹⁶. In this work it was observed high expression of GFAP and activated and hypertrophic astrocytes were localized mostly surrounding blood vessels. Although literature about the role of the astrocytes in rabies virus infection is rare, this finding is compatible with previous articles which described morphology and distribution of these cells during infection^{4,15,17}. In naturally infected bovine, the astrocyte activation may follow viral dissemination⁴.

Mortality and rabies symptoms are associated to viral replication and IFN, TNF and nitric oxide production, modulating the immunologic response and inflammatory reaction^{3,18}. During natural infection there are high concentrations of IFN in the brain, proportional to viral replication, while experimentally intracerebral infected animals produce lower levels of IFN¹⁹.

In our work, a consistent positive staining for IFN was verified only in the brains of mice infected with rabies virus isolated from insectivorous bat and bovine. TNF was detected in the brains of mice infected with dog, bovine and insectivorous bat isolates. Although differences in the incubation period and symptoms were

observed, the mortality rate for all five samples was 100%. This fact is in accordance to the study of Lodmell et al. (1989)²⁰, in which lineages of mice naturally resistant to rabies infection and submitted to an anti-IFN treatment did not have alteration in the survival rate, suggesting that this immune modulator is not essential in the protection against infection.

Mice deficient to TNF receptors and experimentally infected with CVS virus have a longer clinical illness, although mortality is not altered³. So, the prolonged clinical phase observed in mice inoculated with the equine isolate may be related to the absence of TNF detection. However, for the vampire bat isolate there was no positive staining for IFN, TNF or iNOS and the duration of the disease was similar to the other isolates of this study. In this case, symptoms and death may have developed before the production of these modulators, since rabies virus induce apoptosis in brain cells⁵.

The production of nitric oxide by the iNOS positive cells in rabies infected animals, has been studied and related to the severity of the symptoms and also to the presence of inflammatory cells⁶. In this work it was observed macrophages positively stained for iNOS in the brains of mice inoculated with the isolates from dog, bovine and insectivorous bat, those presenting aggressive behavior, paralysis and no clinical signs, respectively.

At the NIH test both vaccines were

more effective against the insectivorous bat isolate, followed by the dog and then the bovine strains. Difference was recorded for vaccine 2 showing its worst result for the challenge with vampire bat isolate and for vaccine 1. The worst result was for the challenge performed with the equine isolate. A reason for this result might be that vaccine 1 is recommended only for dog, bovine and ovine. However its worst result at the CDC test occurred when challenge was performed with vampire bat ($ED_{50}=4.57$) and dog ($ED_{50}=5.00$) isolates.

Major failure for both vaccines was observed when challenge was performed with the vampire bat and bovine strains. In Brazil paralytic rabies in herbivorous transmitted by vampire bat is frequent and the annual vaccination of endemic areas is recommended⁷. Vaccines are submitted to control tests before they can be commercially used and they are considered effective. In our study no statistical difference was observed when comparison was made for both vaccines and the same potency test and challenge viral isolate. Also it was not verified statistical difference between ED_{50} for each vaccine within each test. Immunological studies have been performed with the purpose to aim the development of vaccines which protect against different rabies virus strains. The results presented in this study and in previous articles suggested no need to produce specific vaccines with street isolates. Annual vaccination and animal population control still are important measures to prevent rabies transmission.

Estudos biológicos e imunológicos de cinco amostras brasileiras do vírus da raiva

Resumo

Estudou-se em camundongos aspectos do comportamento biológico de amostras brasileiras de vírus rábico isoladas de cão, bovino, equino, morcegos hematófago e insetívoro. Observou-se transmissão oral em camundongos alimentados com cérebros infectados de morcego insetívoro (8,82%), cão (8,57%) e equino (3,03%). O período de incubação médio para todas as amostras foi de 6 dias após a inoculação intracerebral, com sintomas variando, desde hiperexcitabilidade (amostra canina), paralisia progressiva principalmente de membros posteriores e maior duração do curso clínico até a morte (equino) e

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morte repentina, sem sintomas aparentes (morcego insetívoro). Pela imunohistoquímica detectou-se produção de IFN nos cérebros dos camundongos inoculados com amostra de bovino e morcego insetívoro, TNF e iNOS nos animais infectados com amostra de cão, bovino e morcego insetívoro e reação astrocitária com aumento da expressão de GFAP em todas as cinco amostras. A eficácia de 2 vacinas comerciais inativadas, uma nacional e outra importada, para a proteção contra a infecção experimental em camundongos foi avaliada através dos testes NIH e CDC, usando as amostras de campo para o desafio. Não houve diferença significativa entre o desempenho das vacinas, quando comparadas para um mesmo teste de potência e amostra de desafio sugerindo que não há necessidade de se produzir vacinas com amostras isoladas de campo.

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