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Pathogenesis of rabies virus by ERA and PV strains administered orally in hamsters (*M. auratus*)

Estudo da patogenia do vírus da raiva por meio de amostras ERA e PV administradas por via oral em hamsters (*M. auratus*)

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Summary

Hamsters orally inoculated with ERA and PV strains of rabies virus were sacrificed at 24, 48, 72 hours, 21 and 30 days after inoculation. Brain fragments were examined by Fluorescent Antibody test (FAT) and heminested PCR (hn-PCR). Fragments from stomach, blood, heart, and lung were examined only by hn-PCR. Sera of other hamsters, similarly inoculated, obtained at 30th day after inoculation were submitted to mouse neutralization test. The hamsters were challenged intracerebrally with CVS strain with $10^{2.7}$mouse LD_{50}/0.03mL, 45 days after inoculation. Brains examined by FAT were negative. The hn-PCR detected the presence of rabies virus RNA in the lung of one animal inoculated with ERA, and in the brain, stomach, blood, and lung of PV-infected animals. The orally inoculated virus was capable to infect and replicate in several organs and tissues; however, none of the challenged hamsters did survive after challenge.

Key-words
Rabies.
Oral administration.
ERA and PV strains.
Hn-PCR.
Hamster.

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Introduction

Rabies is a zoonosis caused by viruses of the genus *Lyssavirus*. This genus' genome is constituted by a single-stranded, negative-sense, nonsegmented RNA[1] which codes for five separate proteins, designated N (nucleoprotein), G (glycoprotein), L (polymerase), NS (phosphoprotein), and M (the membrane protein)[2]. The disease has a worldwide distribution with the urban pattern being primarily associated to dogs' bite and still causing thousands of human deaths annually. In many countries where the disease in domestic animals is under strict control, the oral rabies vaccine appeared as an alternative tool for the disease control in wildlife[3].

Although many studies related to different viral strains and types of baits used for specific target species as oral antirabies vaccines are available in literature, papers dealing with the viral spread and its pathogenesis after oral inoculation are very scarce. Also the precise sites or mechanisms of viral penetration have not been established yet for oral infection[4].

The aim of this work was to study the pathogenesis of the ERA and

PV strains of rabies virus administered orally in hamsters, by submitting fragments of brain, stomach, blood, lung and heart, in order to trace the presence of rabies antigens and nucleic acid in these organs and by determining rabies neutralizing antibodies. Additionally, the immune status was assessed through a challenge experiment.

Material and Method

Animals

Forty five male and female golden Syrian hamsters (*Mesocricetus auratus*) aging approximately 90 days old with weights varying through 120 to 160 grams, along with 580 albino Swiss mice (*Mus musculus*) weighing 11 to 14 grams were used for the control of viral pathogenicity, virus titration and for serum neutralization test (SNT).

Virus strains

The rabies virus strains used were ERA, CVS and PV. Vaccine containing attenuated ERA strain had $>10^{3.3}TCID_{50}$/mL and was produced from virus grown in BHK cells (from Laboratório BIO - VET S/A, Vargem Grande Paulista - SP). The PV and CVS strains, provided by the Instituto Butantan de São Paulo, have been maintained through several intracerebral passages in mice until use, having respectively, titers of $10^{6.5}MILD_{50}$/0.03 mL and $10^{5.8}$ $MILD_{50}$/0.03 mL.

Virus titration

Using serial ten-fold dilutions, the virus titration was performed in mice through intracerebral inoculation at a dose of 0.03 mL. Titers were calculated according to the method of Reed and Müench[5] and expressed as log_{10} of the reciprocal dilution killing 50 % of inoculated animals.

Experimental design

After water abstinence of 8 hours, 10 hamsters were administered orally by feeding 0.5 mL suspension of ERA vaccine using 1.0 mL plastic syringes[6] and similarly, other 10 hamsters received the same dose of a PV suspension. Each strain's suspension was inoculated intracerebrally in parallel into 10 mice using 0.03 mL and observed for 21 days, in order to assess the pathogenicity of these strains after this route of inoculation. For each period of 24, 48, 72 hours, and 21 and 30 days after inoculation, two hamsters were randomly chosen and samples of brain, stomach, blood, heart and lung were taken. Brain fragments were examined by Fluorescent Antibody test (FAT). For heminested PCR (hn-PCR) tests, brain fragments and the other specimens were diluted in 20.00% 1.5 M Tris-EDTA buffer solution. After centrifuging at 2,000g, samples were distributed into microtubes and kept frozen at -20°C until use. Brain, stomach, blood, heart and lung collected from a non-inoculated hamster were used as negative control.

Another group of 10 hamsters submitted to water intake restriction treatment of 8 hours has received 0.5 mL dose of ERA vaccine while another 10 animals received PV suspension through oral route[6]. Additionally, strains were similarly inoculated in parallel into mice to test their pathogenicity and then observed for 21 days. Thirty days post inoculation, the hamsters were anesthetized with ether, and submitted to blood collections by intracardiac punction and sera were tested for neutralization test. At the 45th post inoculation day, hamsters surviving

after blood collection were challenged with CVS using a 0.05 mL dose having $10^{2.7}$LD/0.03 mL in mice, adapted from the modified Habel test[7].

Fluorescent Antibody Test

The FAT used to confirm the presence of rabies antigen in brain materials was according to that described by Goldwasser and Kissling[8].

Serum Neutralization Test

Serum samples collected 30 days after inoculation from 24 hamsters (10 inoculated with ERA, 10 inoculated with PV and 4 negative controls) were submitted to SNT[9], and serum dilutions tested were 1:5, 1:10, 1:20 and 1:40. Each serum-virus dilution mixture was inoculated intracerebrally into 5 mice and observed on a daily base for rabies signs during 28 days. Antirabies titers were calculated according to the method described by Reed and Müench[5] and expressed as $\log_{10}$ of the reciprocal dilution that protected 50.00% of inoculated mice.

Heminested-PCR

RNA extraction: Total RNA was extracted directly from tissues by the Trizol LS (Gibco BRL®) method according to the manufacturer's instructions.

Primers: The design of the primer sets was used for amplification of representatives of genotype 1[10].

cDNA synthesis: Reverse transcription was performed with 7 µl of total brain RNA. The RNA was denatured at 95ºC for 5 min, cooled on ice and then added to final volume of 20 µl containing reverse transcription buffer (Gibco BRL®) 1X, 10 mM of each dNTP, 20pmol of primers 505, 20 pmol of primer 937, 10 mM of DTT and 200 U of M-MLV RT (Gibco BRL®). This mixture was incubated for 60 min at 42ºC.

Primary amplification: Amplification of 5 µl of the reverse-transcribed cDNA template was performed in a final volume of 50 µl: 0.2 mM of each dNTP, PCR buffer 1X, 25 pmol of primer 505, 25 pmol of primer 937, 1.5 mM of magnesium chloride, 1.25of Taq DNA polymerase (Gibco BRL®) and ultra-pure water (milli-Q®) qsp. After denaturation at 94ºC for 3 min, the reaction was cycled 35 times at 94ºC for 45 seconds, 55ºC for 60s and 72ºC for 90s, followed by a final elongation step of 72ºC for 10 min.

Heminested amplification: 5 µl aliquot of the external amplified product was diluted in PCR buffer 1 X, 0.2 mM of each dNTP, 25 pmol of primer 505, 25 pmol of primer 779, 1.5 mM of magnesium chloride, 1.25 U of Taq polymerase and ultra-pure water (milli-Q®) with a final volume of 50 µl. Secondary PCR was done as described above for the primary amplification program but this time with 25 rather than 35 cycles. On completion of the amplification program, the samples were analyzed by 2.00% (w/v) agarose gel electrophoresis in a horizontal cube immersed in Tris-borate buffer (0.045 M) and EDTA 1 mM, pH 8.0. The products were analyzed by transilumination in ultra-violet light after ethidium bromide (0.5 µg/mL) staining[11].

Results

The brain materials of 10 hamsters, which received ERA vaccine through oral route, were found negative by FAT examination. By using hn-PCR, the presence of viral nucleic acid was detected in the lung of one hamster,

which had been sacrificed 30 days after virus administration.

Another 10 hamsters inoculated orally with PV virus were found negative through FAT, but the hn-PCR revealed the presence of viral RNA in the lung of one hamster that had been sacrificed 24 hours after inoculation; in the blood of one hamster sacrificed at 72 hours, in the brain of two hamsters, in the stomach and lung of one animal after 21 days, and in the brain of two hamsters, sacrificed after 30 days. The results are shown in Table 1. Among the 10 mice that had been inoculated intracerebrally with ERA vaccine, seven were found ill and died in few days. All the mice inoculated intracerebrally with PV strain died after characteristic signs of rabies and rabies infection was confirmed by FAT.

The neutralization test indicated rabies $\log_{10}$ antibody titers ranged from <0.699 to 1.342 on hamsters inoculated with ERA strain; from <0.699 to 1.602 on the PV inoculated animals; and from 0.699 to 0.778 on the non-inoculated control group.

At challenge experiment, all the hamsters died with signals and symptoms of rabies, which were confirmed by FAT.

Discussion

In this experiment, mice which had been inoculated intracerebrally with ERA strain in parallel to hamsters showed characteristic signs of rabies and died, as described by Abelseth et al.[12]. These authors also reported that ERA vaccine was capable to immunize dogs even when diluted until 1:1000, and the neutralization test indicated the formation of high antibody level and dogs had survived after challenge.

The precise site or mechanism of viral penetration has not been established for oral infection[4], however, Correa-Giron, Allen and Sulkin[13] suggested that after oral administration of a street or fixed virus, the infection occurs by virus penetration through oral and lingual mucosa, spreading to lung and intestine, and apparently the virus would resist the action of gastric juice for many hours. Using the FAT, the authors detected the presence of virus in oral mucosa, lingual mucosa, lung and stomach, just few minutes after administration by oral route. In the stomach, the virus was detected 6 hours after its inoculation as well as at sixth and seventh days. The virus was detected in the lung 10 minutes after its administration, and later at 2, 5, 6 and 7 days.

According to Correa-Giron, Allen and Sulkin[13], the use of FAT could detect the presence of virus in brain materials 5 days after its oral inoculation. In our experiment, none of brains collected from animals that had been administered PV or ERA strains showed any positive results by FAT, however, rabies infection could be detected on placenta of 21 days and 30 days after inoculation of PV strain by means of hn-PCR.

The ERA and PV strains of rabies virus orally administered into hamsters could infect different tissues such as brain, lung, blood and stomach, however, did not produce sufficient rabies antibody to protect against intracerebral challenge using CVS at a dose of $10^{2.7}MILD_{50}/0.03$ mL.

Although there is evidence for a viremic phase following parenteral administration, its role in the pathogenesis of rabies has been considered insignificant[6]. In our experiment, the blood of one hamster inoculated orally with PV strain was found positive after 72 hours. The fact that only one animal showed viremia does not permit us to state that it occurs on a regular base after oral viral

administration. Although Acha and Szyfres[3] reported that viremia could occur with low virus titers in rabies infections, it could be a possible mechanism of how the virus begins spreading to different organs and our opinion is that such statement needs more accurate further studies.

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Resumo

Hamsters inoculados oralmente com as amostras de vírus rábico ERA e PV foram sacrificados após 24, 48 e 72 horas, 21 e 30 dias. Fragmentos do cérebro foram analisados através da imunofluorescência direta (IFD) e heminested-PCR (hn-PCR). Os fragmentos do estômado, sangue, coração e pulmão foram examinados somente com a técnica de hn-PCR. Soros de outros hamsters, inoculados de modo similar e obtidos 30 dias após a inoculação, foram submetidos ao teste de soroneutralização (SNT) em camundongos. No 45º dia pós-inoculação, os hamsters foram desafiados intracerebralmente com a amostra CVS, contendo $10^{2,7}DL_{50}$ em camundongos/ 0,03 mL. Os fragmentos do cérebro foram todos negativos ao teste de imunofluorescência. A hn-PCR detectou a presença de RNA do vírus da raiva no pulmão de um animal inoculado com a amostra ERA e, no cérebro, estômago, sangue e pulmão de hamsters inoculados com a amostra PV. As amostras de vírus inoculadas oralmente foram capazes de se replicar em diferentes órgãos, no entanto, todos od hamsters morreram ao desafio, indicando uma resposta imunológica insuficiente.

Palvras-chave
Raiva.
Administração oral.
Amostras ERA e PV.
hn-PCR.
Hamster.

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