

## IMMUNOPATHOLOGY OF RABIES INFECTION IN MICE SELECTED FOR HIGH OR LOW ACUTE INFLAMMATORY REACTION

ACHKAR S. M. (1), SINHORINI I. L. (2), RIBEIRO O. G. (3), CARRIERI M. L. (1), CERETTA R. S. (1), CONSALES C. A. (1)

(1) Pasteur Institute, São Paulo, Brazil; (2) Laboratory of Pathology, University of São Paulo, USP, São Paulo, Brazil; (3) Laboratory of Immunogenetics, Butantan Institute, São Paulo, Brazil.

**ABSTRACT:** Rabies is a severe and lethal disease that produces a slight inflammatory response during the infection process. We analyzed the immunopathological mechanisms that occur in the central nervous system (CNS) using mice genetically selected for maximal or minimal acute inflammatory reaction (AIRmax or AIRmin). As viral samples, we adopted the antigenic variant 3 (AgV3) of rabies virus from hematophagous bats and a fixed virus strain (PV1 43/3). Titration of specific antibodies was performed using enzyme-linked immunosorbent assay (ELISA). We observed a slight increase in IgG and IgG<sub>1</sub> isotypes in infected AIRmax mice. Incubation period, determined by intracerebral inoculation with 100 LD<sub>50</sub>, was 6-7 days for PV1 43/4 strain and 9-10 days for AgV3. No difference in viral replication was noticed between AIRmax and AIRmin mice. Mortality was 100% with both viral strains. Histopathological analysis of brains and spinal cords showed inflammatory foci in all regions of the CNS. No differences were noticed in the number of neutrophils. Negri bodies were observed in practically all sites analyzed. Results suggested that inflammatory reaction is not a determining factor in the susceptibility to rabies infection.

**KEY WORDS:** rabies, virus replication, pathology, inflammation, immunoglobulin isotypes.

**CONFLICTS OF INTEREST:** There is no conflict.

### CORRESPONDENCE TO:

SAMIRA M. ACHKAR, Instituto Pasteur de São Paulo, Avenida Paulista 393, Cerqueira César, São Paulo, SP, Brasil. Phone: + 55 11 3288 0088 ext. 116.  
Email: [samira\\_achkar@yahoo.com.br](mailto:samira_achkar@yahoo.com.br).

## INTRODUCTION

Rabies is considered the most important zoonosis because it is often fatal, its reservoirs are particularly efficient at maintaining and disseminating the virus, there is a high cost involved in assisting individuals exposed to it and, even with all the existing subsidies and the advent of vaccine, it still represents a serious public health problem. It is caused by a rhabdovirus, genus *Lyssavirus*, which is extremely neurotropic and lethal.

In Brazil, dog was the most important species in the transmission of human rabies until 2004. Since then, hematophagous bats have been the main transmitters in Brazil and Latin America (4, 5). All bat species can transmit the rabies virus but the species *Desmodus rotundus* is the most important in epidemiological terms as it feeds on the blood of mammals, thus adversely affecting the health of economically important animals. Circulation of the virus in colonies of non-hematophagous bats has been demonstrated by the isolation of rabies virus AgV3, characteristic of *Desmodus rotundus*, from non-hematophagous bats as well as from domestic animals (7).

In the CNS, immune response is unusual, as migration of T cells is restricted and antigen-presenting cells are absent. In addition, specific immune response is intimately related to inflammation. Therefore, a weak inflammatory reaction is observed in the CNS during infection (2).

The present study aimed at analyzing the possible mechanisms involved in the CNS inflammatory response during infection by the rabies virus. To this end, we used mice presenting high (AIRmax) or low (AIRmin) acute inflammatory reaction, selected by bidirectional selective breeding. The main phenotypical characteristic selected in these animals was their capability to attract polymorphonuclear leukocytes to the inflammatory focus (11, 16, 17).

## MATERIALS AND METHODS

### *Virus*

Three virus strains were used:

1. Inactivated fixed virus, PV (Pasteur virus), produced in VERO cells;
2. Lyophilized fixed virus, PV1 43/4, in a 20% mouse brain suspension;

3. A virus strain isolated from a hematophagous bat (*Desmodus rotundus*) from the rural area of the town of Tapiratiba, São Paulo State, Brazil. The strain was identified as AgV3 at the Pasteur Institute, São Paulo. Characterization was performed through indirect immunofluorescence using a panel of antinucleocapsid monoclonal antibodies for antigenic typing, produced by the Centers for Disease Control and Prevention (CDC), Atlanta, USA (8).

### **Animals**

The following male and female mice were used:

1. AIRmax and AIRmin mice, 38<sup>th</sup> and 39<sup>th</sup> generation, 6 to 8 week old;
2. heterogeneous N:NIH mice, 11 to 14 g;
3. heterogeneous Swiss mice and sucklings, 11 to 14 g.

### **Infection**

To calculate the median lethal dose (LD<sub>50</sub>), AIRmax and AIRmin mice received intracerebral injections of 30 µl of PV1 43/4 and AgV3 suspensions containing 100 LD<sub>50</sub>. Animals were daily observed for 30 days. Average survival time was calculated as a function of the average mortality after infection.

For the immunological study, groups of 30 AIRmax and AIRmin mice were infected with 30 µl of the virus strains containing 100 LD<sub>50</sub>. Animals were anesthetized, bled by the retroorbital plexus and euthanized for subsequent collection of the brains.

As the present study is focused on acute inflammation, brain and spinal cord samples were collected from some animals before symptoms manifested: on days 3, 4 and 5 from animals inoculated with PV1 43/4, and on days 6, 7 and 8 from animals injected with AgV3. Brains and spinal cords were stored in liquid nitrogen for subsequent determination of the viral replication kinetics and for histopathological study. Sera were stored at -20°C for titration of anti-rabies IgG, and IgG<sub>1</sub> and IgG<sub>2a</sub> isotypes.

Each brain and spinal cord sample collected before symptoms manifested was used to prepare a pool of three samples (20% suspensions [weight/volume] in 0.01 M of 0.075% PBS/BSA buffer solution). Suspensions were diluted from 1:10 to 1:100,000

and groups of 10 heterogeneous Swiss albino mice were inoculated with 30  $\mu$ l of each dilution. Animals were daily observed and virus titer was determined using the Reed & Muench method (13).

### ***Antibodies titration***

To study viral replication kinetics, titrations of anti-rabies IgG, and IgG<sub>1</sub> and IgG<sub>2a</sub> isotypes were carried out using classic and capture enzyme immunosorbent assay (ELISA), respectively (9). Sera from AIRmax and AIRmin mice were used to prepare a pool of three samples on each collection day after infection. Antibody titration was expressed as log<sub>2</sub> of the inverse of the highest serum dilution.

### ***Histopathological analysis***

Brains and spinal cords were fixed separately in 10 ml of Karnovsky solution, dehydrated and embedded in paraffin. Each sample was subjected to random histological sections, 5 $\mu$  thick, to prepare slides, which were stained using the hematoxylin-eosin technique (HE) for detection of morphological details and Negri bodies. The brains of AIRmax and AIRmin animals that had been kept as negative controls were also used to prepare slides as described above. Slides were read on a NIKON E800 eclipse microscope at 400x magnification with a 10x10 reticule in the ocular lens. Images were captured using a CoolSNAP-ProCF color camera. For the reading and subsequent count of neutrophils, each slide was divided into 10 random fields identified according to the region of the CNS (hippocampus, cortex, cerebellum and spinal cord).

### ***Statistical analysis***

The following statistical tests were used: Levene's test, to compare the samples variances; Kolmogorov-Smirnov test, to verify whether the frequency distribution of a given variable could be considered normal; and Student's t-test, to compare the means of two independent samples. When the frequency distribution of a given variable was not considered normal, Mann-Whitney test was employed (10, 15). All tests were carried out using SPSS 10.0 software for Windows at 5% significance level.

## RESULTS

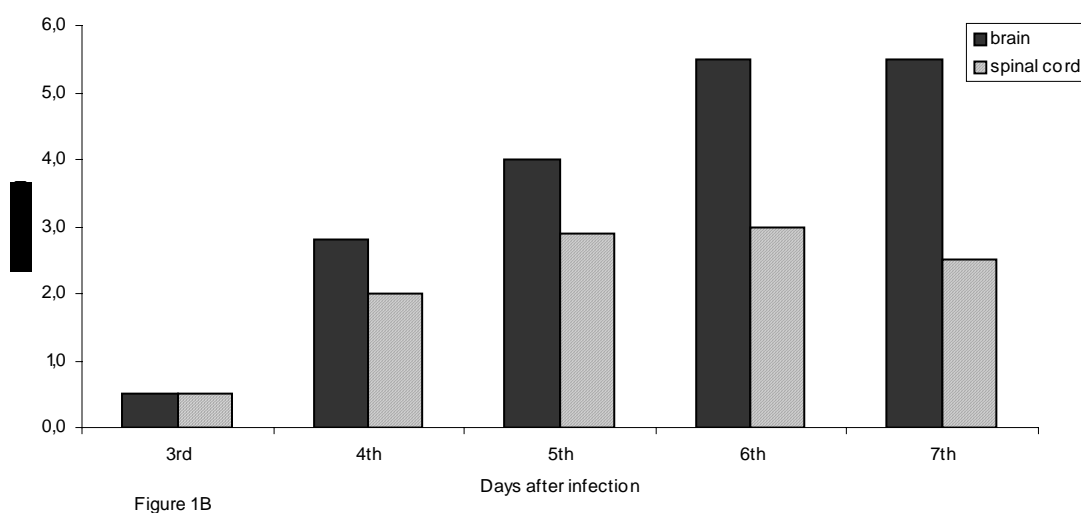
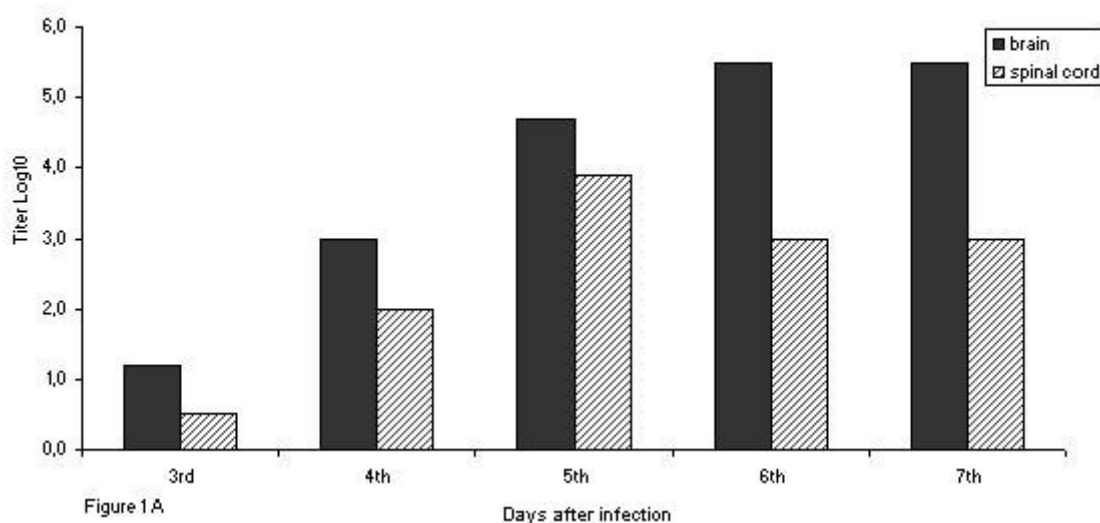
The ideal dose of cerebral injections into heterogeneous N:NIH mice was previously defined as 100 LD<sub>50</sub>. Incubation period was 6-7 days in animals inoculated with PV1 43/4 strain and 9-10 days in animals injected with AgV3. From the incubation period onwards, all animals had clinical symptoms and lethality rate was 100%. The PV1 43/4 strain replicated more rapidly after infection in both AIRmax and AIRmin mice and presented higher titers in brains than in spinal cords (Figures 1A and 1B). AgV3 virus, however, showed irregular replication and titers were the same in the brains and spinal cords (Figures 1C and 1D).

All IgG titers for PV1 43/4 strain remained high during the infection period, and practically no differences were observed between lines. IgG<sub>1</sub> isotype titers were three and five times higher in AIRmax mice on days 6 and 7 after infection, respectively. IgG<sub>2a</sub> isotype titers remained constant during the whole period of infection, and no difference was observed between lines (Figures 2E and 2F).

For AgV3 infection in AIRmax animals, IgG titers were three times higher on days 8 and 10, and IgG<sub>1</sub> isotype titers were five times higher on day 10 after infection, compared with those in AIRmin mice. As observed for PV1 43/4 strain, IgG<sub>2a</sub> isotope titers remained constant and were the same for both lines (Figures 2G and 2H).

For histopathological analysis, all slides including negative controls were photographed while reading was being carried out (Figures 3 and 4). The presence of inflammatory foci did not prevent neutrophils count (Figures 5 and 6). The percentages of neutrophils found in animals inoculated with PV1 43/4 samples were slightly higher in AIRmin mice. There was practically no difference between mouse lines inoculated with AgV3 samples (Table 1). There were more neutrophils in the brain than in the spinal cord with both viral samples. In addition to neutrophils, a greater number of inclusion bodies were observed in neurons infected with the fixed virus sample (Figure 7), and evidence of inflammatory foci such as lymphocyte populations (Figure 8) was noticed.

Variances during viral replication were equal according to the statistical tests, as the results for both lines were  $p > 0.05$ , i.e. not significant. The distribution frequency for all variables was considered normal ( $p > 0.05$  for all parameters analyzed). Student's t-test showed there was no significant difference between the results obtained. Analysis of anti-rabies antibodies using the Mann-Whitney test revealed no significant difference in the distribution of IgG, and IgG<sub>1</sub> and IgG<sub>2a</sub> isotypes, as U was greater than 0.05.



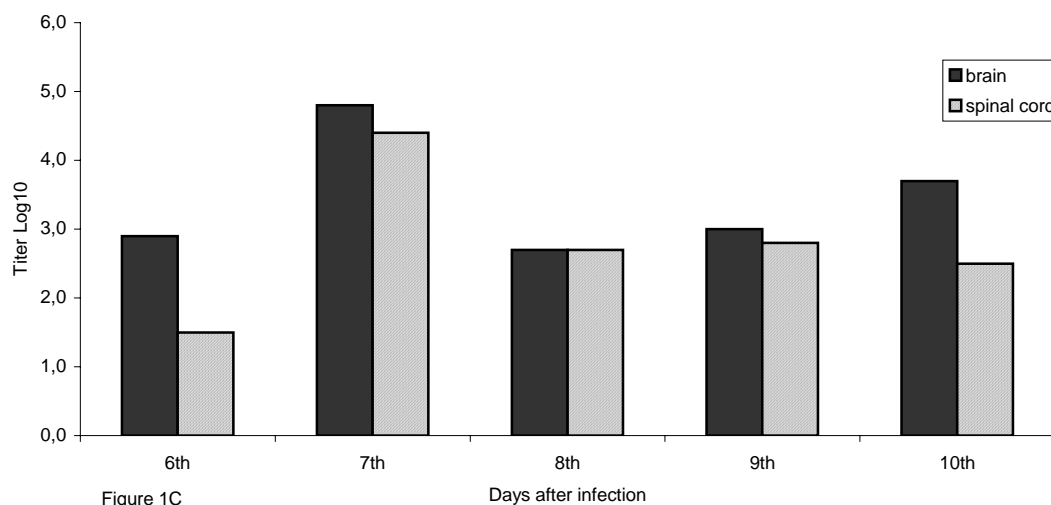


Figure 1C

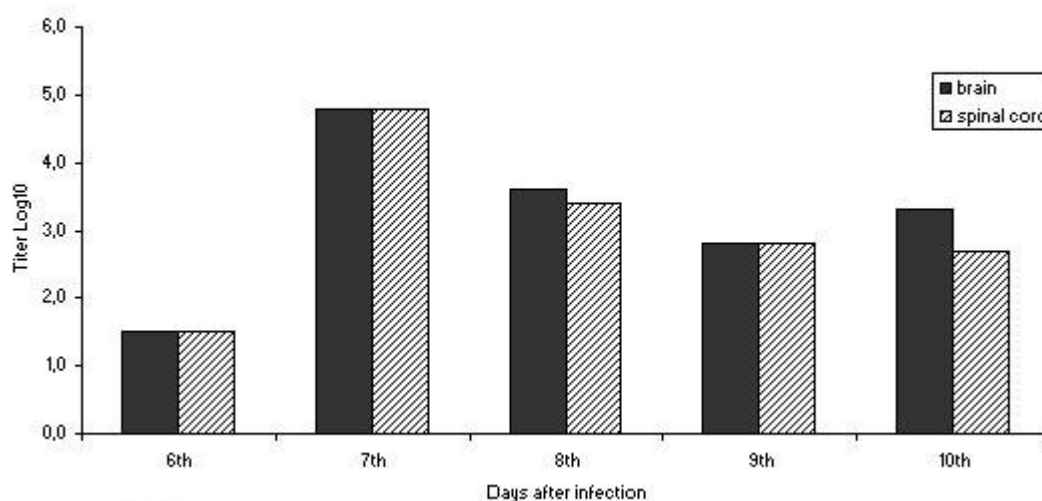


Figure 1D

Figure 1: Kinetics of virus replication in the central nervous system (CNS) of mice selected for maximal and minimal acute inflammatory reaction (AIRmax and AIRmin) during infection by the rabies virus. Animals received intracerebral injections with 100 LD<sub>50</sub> of PV1 43/4 and AgV3 strains. Brains and spinal cords were collected at different times from animals showing rabies symptoms and the virus titer was detected using mouse inoculation assay.

- A. Kinetics of response to PV1 43/4 strain in the CNS of AIRmax mice.
- B. Kinetics of response to PV1 43/4 strain in the CNS of AIRmin mice.
- C. Kinetics of response to AgV3 in the CNS of AIRmax mice.
- D. Kinetics of response to AgV3 in the CNS of AIRmin mice.

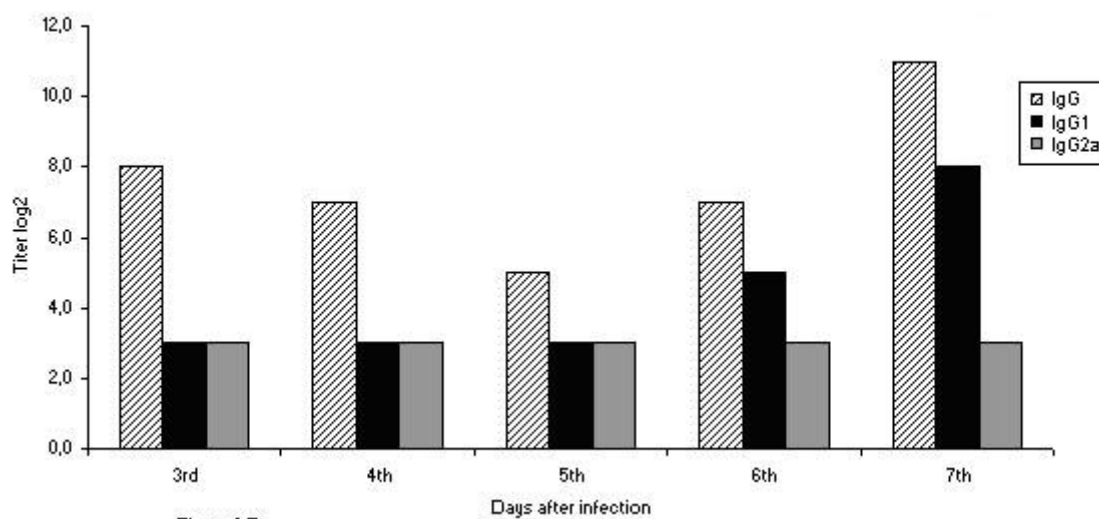


Figure 2 E

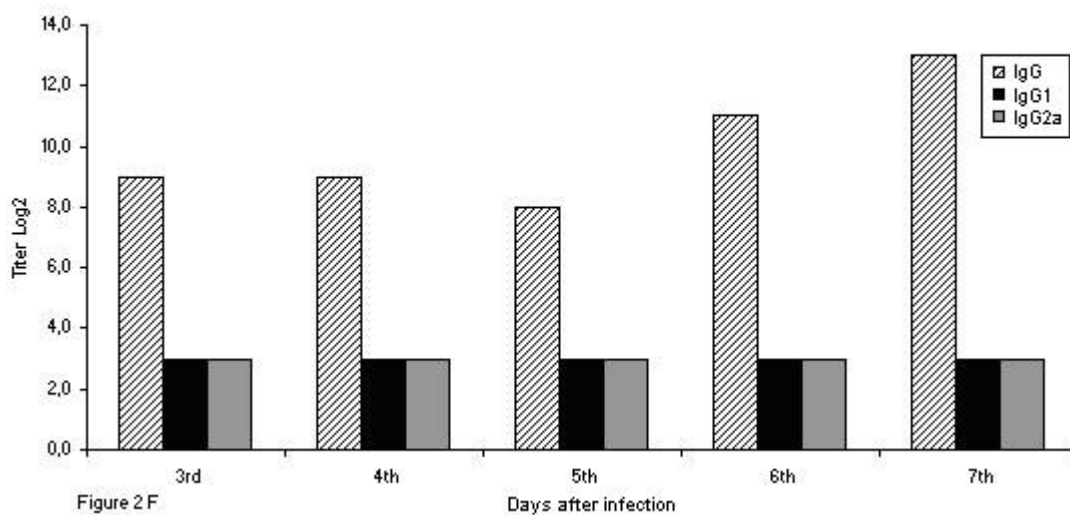


Figure 2 F



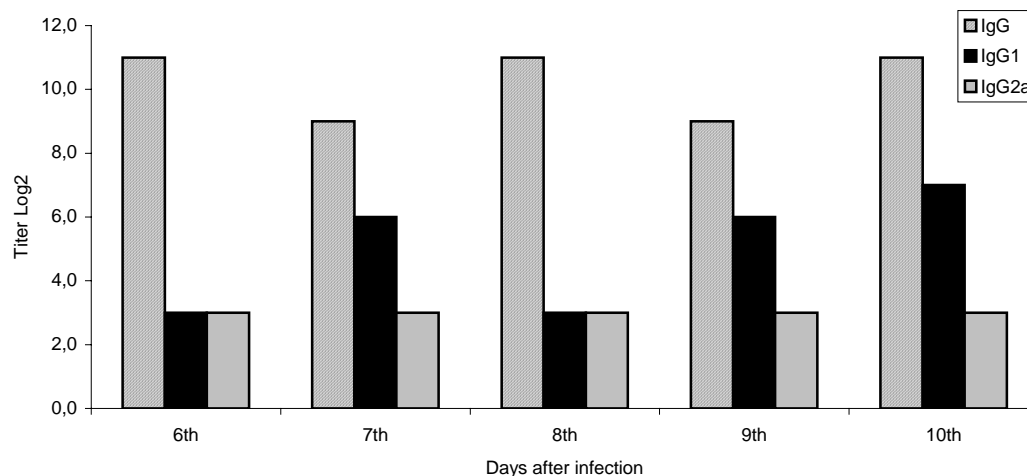


Figure 2 G

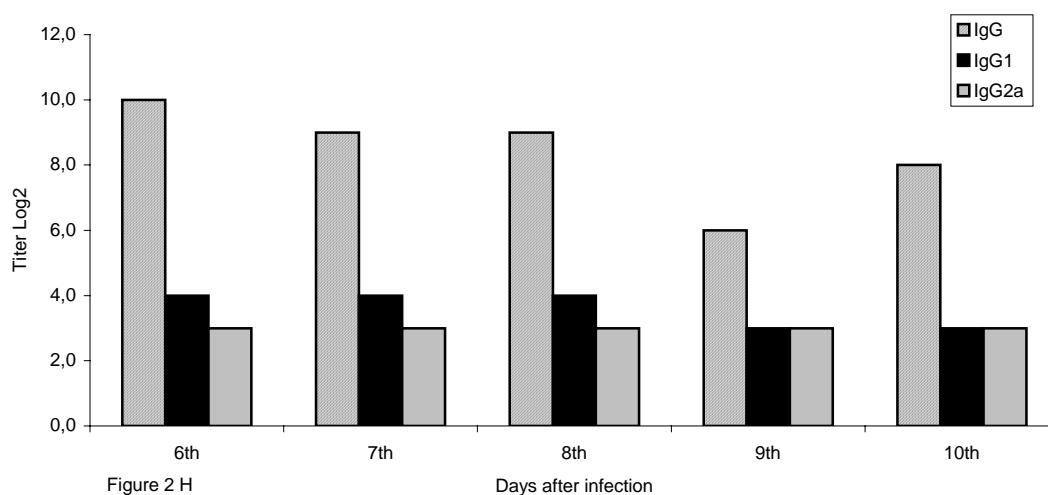


Figure 2 H

Figure 2: Kinetics of IgG, and IgG<sub>1</sub> and IgG<sub>2a</sub> isotypes synthesis in mice selected for maximal and minimal acute inflammatory reaction (AIRmax and AIRmin) during infection by the rabies virus. Animals received intracerebral injections with 100 LD<sub>50</sub> of PV1 43/4 and AgV3 strains. Sera were collected at different times from animals showing rabies symptoms and IgG, IgG<sub>1</sub> and IgG<sub>2a</sub> antibodies were determined using the ELISA method.

- E. Anti-rabies IgG, and IgG<sub>1</sub> and IgG<sub>2a</sub> isotypes titers in AIRmax mice after infection with PV1 43/4 strain.
- F. Anti-rabies IgG, and IgG<sub>1</sub> and IgG<sub>2a</sub> isotypes titers in AIRmin mice after infection with PV1 43/4 strain.
- G. Anti-rabies IgG, and IgG<sub>1</sub> and IgG<sub>2a</sub> isotypes titers in AIRmax mice after infection with AgV3.
- H. Anti-rabies IgG, and IgG<sub>1</sub> and IgG<sub>2a</sub> isotypes titers in AIRmin mice after infection with AgV3.

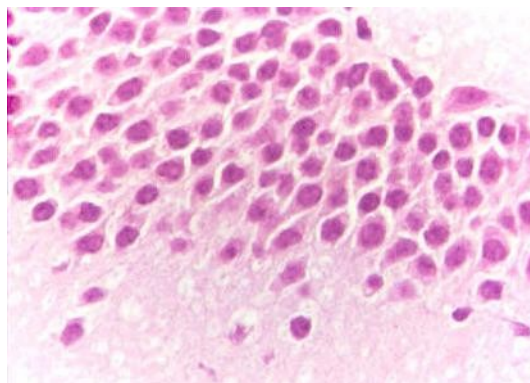


Figure 3: Histological section from the hippocampus of AIRmax mice used as negative controls.

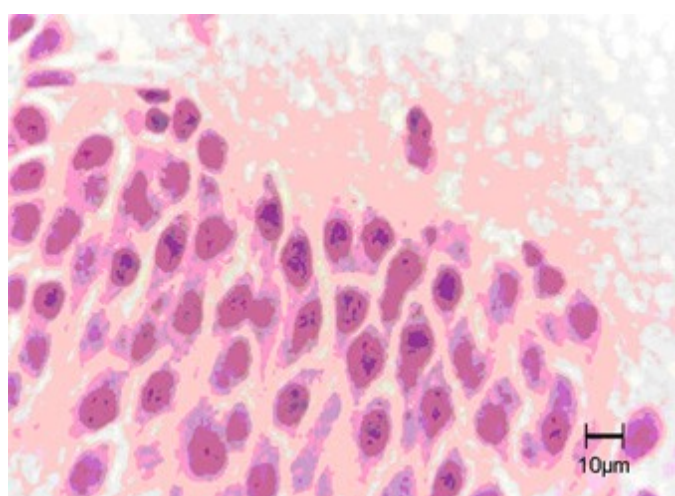


Figure 4: Histological section from the hippocampus of AIRmin mice used as negative controls.

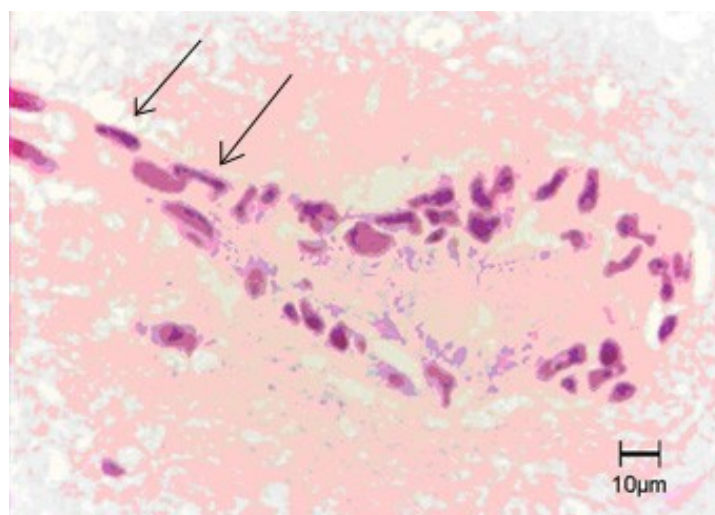


Figure 5: A large number of neutrophils (arrows) in AIRmin mice infected with PV1 43/4 strain.

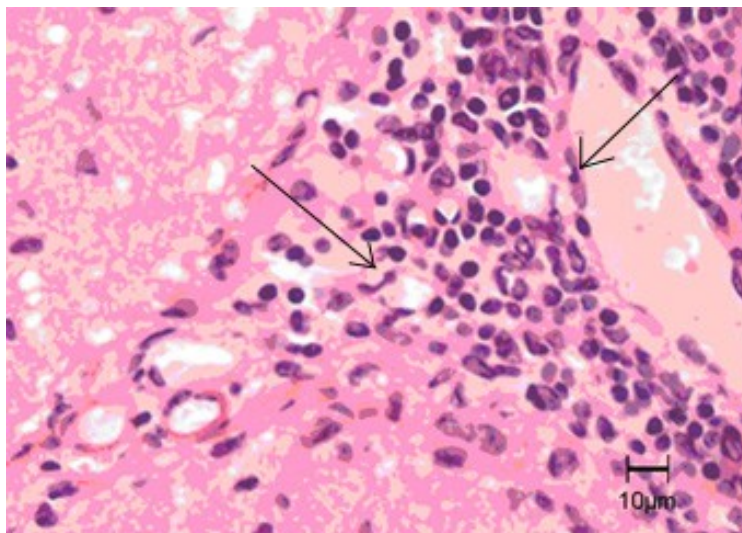


Figure 6: An inflammatory focus with neutrophils (arrows) in AIRmax mice infected with AgV3.

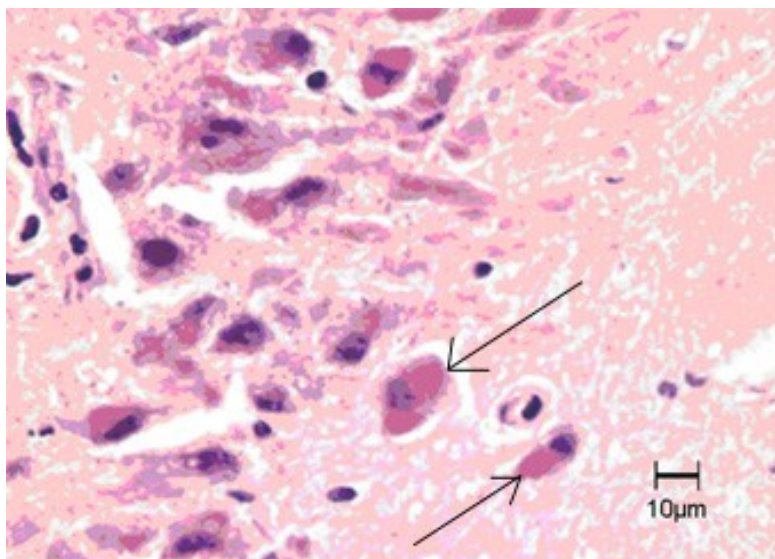


Figure 7: Histological section from the hippocampus of AIRmax mice. Arrows indicate the Negri bodies.

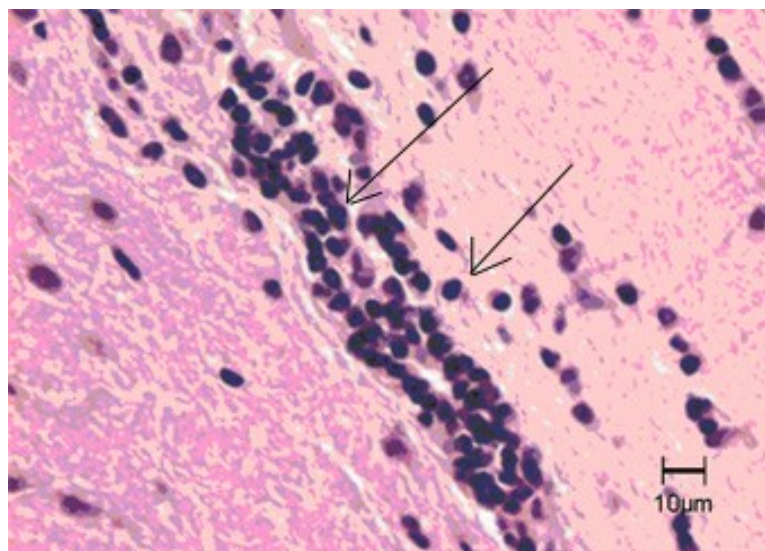


Figure 8: Histological section from the cortex of AIRmin mice. Arrows indicate an inflammation focus with lymphocytes.

Table 1: Percentages of neutrophils in the brains and spinal cords of mice selected for maximal and minimal acute inflammatory reaction (AIRmax and AIRmin) infected with PV1 43/4 and AgV3 strains on different days after infection.

LINE	TISSUE	PV1 43/4		AgV3	
		DAY	% Neutrophils	DAY	% Neutrophils
AIRmax	Brain	5	70	8	70
		6	42	9	87
		7	56	10	75
	Spinal Cord	5	30	8	30
		6	58	9	13
		7	44	10	25
AIRmin	Brain	5	63	8	70
		6	59	9	78
		7	92	10	66
	Spinal Cord	5	37	8	30
		6	41	9	22
		7	8	10	34

## DISCUSSION

The majority of infections that occur in the CNS are lethal, as it is an immunoprivileged site, in which immune functions are limited. Circulation in the cerebral tissue is characterized by the blood-brain barrier, which restricts diapedesis and therefore helps restrain lymphocytic infiltration and development of inflammatory

responses in the brain, which would surely prejudice the integrity of functional connections between neurons. Furthermore, some viruses have developed efficient mechanisms that allow them to adapt and escape the immune response. The rabies virus becomes invisible to the immune system after invading the CNS, probably as a result of apoptosis mechanisms, i.e. the programmed death of infected neurons (18). Infection by the rabies virus causes few neuropathologic lesions or inflammatory foci; clinical symptoms, however, are severe and lead to death. This can be explained by the fact that the neurons cease to function correctly rather than die. The use of animal models to study the mechanisms involved during rabies infection can help to better understand this neuronal dysfunction (12).

Inflammatory reaction is an important defense mechanism that interacts and modulates immune reactions; therefore, in the present study we used mouse lines genetically selected for high or low acute inflammatory reaction, which was measured by cellular infiltrate and protein concentration in the exudates produced as a result of inflammation induced by Biogel inoculation (11).

Researches using AIRmax and AIRmin mice inoculated with heterologous proteins, bacterial antigens and *Bothrops jararaca* venoms showed that the selective process affected neither the specific immune response nor the cell-mediated response. However, the lines did differ in terms of local inflammatory response, and AIRmax animals presented greater inflammatory reaction and a significant increase in cellular infiltrate at the inoculation site. This indicates that the difference in the local inflammatory response between these selected animals does not depend on the agent used to induce inflammation (1, 6, 19).

Infection by the fixed virus sample CVS (Challenge Virus Standard) limits inflammation in the CNS since it stimulates the production of neuroprotective agents such as IL-6 and destroys migrating T cells. Infection in the CNS by the PV virus strain leads to more severe inflammation; consequently, infected neurons are destroyed and propagation of the infection through the brain is interrupted (3).

Infected bats may not only affect livestock industry, but also reduce productivity through repeated attacks, which causes secondary infections following an initial wound, anemia due to bleeding as well as a decrease in hide value and represents a threat to human health.

The fact that rabies can manifest in bats from both rural and urban areas represents an emerging public health problem, as does the extreme versatility of these animals. In a search for food after having lost their natural habitat, they can adapt to urban areas thus spreading the disease over a greater area. Results of antigenic studies of samples isolated from dogs and cats confirm that the hematophagous bat can be found in urban areas and that the virus circulates in the aerial cycle (7).

The number of human rabies transmitted by dogs has decreased and that transmitted by bats has increased. This change requires an effective epidemiological investigation, as a number of factors such as nocturnal habits, greater geographic distribution due to their capability to fly and adapt to new habitats facilitate the proliferation of bats and consequently the spread of rabies when the virus circulates in the colony.

As infection in the CNS progresses, cytokines and different mediators such as nitric oxide and other endogenous neurotoxins are produced, resulting in the migration of T cells across the hematoencephalic barrier. However, this process is unable to control the disease progress. Nitric oxide production in infected animals has been the subject of many researches and is related to the severity of clinical symptoms and the presence of inflammatory cells (2).

In the present study, both the incubation period and the disease progress in animals inoculated with the fixed virus sample were expected, which was not the case of AgV3 virus because it is a street virus. As a result of the highly neurotropic nature of the rabies virus and the poor immune response of the CNS, all the infected animals had severe clinical manifestations, and mortality rate was 100%.

There was little difference in anti-rabies antibody production between mouse lines; production in AIRmax animals was higher. With regard to the isotypes, IgG1 titers

were higher in AIRmax animals, suggesting that stimulation of T-helper 2 population induces IL-4 secretion, which activates antibody production by B lymphocytes (14).

Histological data were compatible with those available in literature. The use of mouse lines reactive to acute inflammation allowed the count of neutrophils, which are the predominant cells during acute inflammatory response and the first line of defense against different infections. The fact that AIRmin animals inoculated with the fixed virus sample had a greater number of neutrophils can be explained by their much faster acute inflammatory response to the antigenic stimulus, which meant that the inflammation had already progressed from the acute to the chronic phase.

Results of the present study point to the following conclusions:

- ✓ There was no difference between mouse lines in the viral replication kinetics of both samples. With regard to the incubation period, PV1 43/4 sample replicated within the expected time, with higher titers in the brain than in the medulla; replication by AgV3 sample varied, and titers in the brain and medulla were practically equal;
- ✓ There were few differences in specific humoral immune response between mouse lines during replication. IgG1 production was higher in AIRmax mice, which may be related to a tendency for preferential stimulation of Th2 subpopulations in this mouse line;
- ✓ During viral replication, Negri bodies were observed in neurons infected with both viral samples; however, a greater number of Negri bodies were noticed in those infected with PV1 43/4 sample. Both samples produced inflammatory foci, but a higher number of foci were observed in AIRmin mice.

Results suggested that the inflammatory reaction is not a determining factor in the susceptibility to rabies virus infection and pointed to future studies related to the immunopathology of this disease.

## ACKNOWLEDGMENTS

This study was supported by Pasteur Institute, São Paulo, Brazil; Laboratory of Immunogenetics, Butantan Institute, São Paulo, Brazil; and Department of Pathology, Faculty of Veterinary Medicine, University of São Paulo, Brazil.

The authors would like to thank Maria das Graças Silva for her help with the references review and the figures configuration.

## REFERENCES

- 1 ARAUJO LMM., RIBEIRO OG., SIQUEIRA M., DE FRANCO M., STAROBINAS N., MASSA S., CABRERA WHK., MOUNTON D., SEMAN M., IBANEZ OM. Innate resistance to infection by intracellular bacterial pathogens differs in mice selected for maximal or minimal acute inflammatory response. *Eur. J. Immunol.*, 1998, 28, 2913-20.
- 2 BAER GM., YANGER PA. A mouse model for post exposure rabies prophylaxis: The comparative efficacy of two vaccines and of antiserum administration. *J. Gen. Virol.*, 1977, 36, 51-8.
- 3 BALOUL L., LAFON M. Apoptosis and rabies virus neuroinvasion. *Sci. Direct.*, 2003, 85, 777-88.
- 4 BELOTTO A., LEANES LF., SCHNEIDER MC., TAMAYO H., CORREA E. Overview of rabies in the Americas. *Virus Res.*, 2005, 111, 5-12.
- 5 BRASIL. Ministério da Saúde. Programa Nacional de Profilaxia da Raiva. *Casos de raiva humana notificados e percentual de casos transmitidos segundo a espécie animal*. Brasília: Ministério da Saúde, 2004.
- 6 CARNEIRO AS., RIBEIRO OC., DE FRANCO M., CABRERA WH., VORRARO F., SIQUEIRA M., IBANEZ OM., STAROBINAS N. Local inflammatory reaction induced by *Bothrops jararaca* venom differs in mice selected for acute inflammatory response. *Toxicon*, 2002, 40, 1571-9.
- 7 CARRIERI ML., MATTOS CA., CARNIELI P. JR., MATTOS C., FAVORETTO SR., KOTAIT I. Canine and feline rabies transmitted by variant 3 *Desmodus rotundus* in the state of São Paulo, Brazil. In: *SEMINÁRIO INTERNACIONAL MORCEGOS COM TRANSMISSORES DA RAIVA*, São Paulo, 2001. Programas e resumos. São Paulo, 2002. p. 51-2.
- 8 CENTER FOR DISEASE CONTROL. Rabies in a laboratory worker. *Morb. Mortal. Wkly. Rep.*, 1977, 26, 183-84.
- 9 CONSALES CA. *Avaliação de vacinas anti-rábicas produzidas em cultivo celular, utilizando modelo de camundongos imunogeneticamente selecionados*. São Paulo: Universidade de São Paulo, Instituto de Ciências Biomédicas, 1999. [PhD thesis]
- 10 DAWSON B., TRAPP RG. *Bioestatística básica e clínica*. 3.ed. Rio de Janeiro: MacGraw-Hill, 2003.



- 11 IBAÑEZ OM., STIFFEL C., RIBEIRO OG., CABRERA WHK., MASSA S., FRANCO M., SANT'ANNA OA., DECREUSEFOND C., MOUTON D., SIQUEIRA M., BIOZZI G. Genetic of nonspecific immunity: I: Bidirectional selective breeding of lines of mice endowed with maximal or minimal inflammatory responsiveness. *Eur. J. Immunol.*, 1992, 22, 2555-63.
- 12 JACKSON AC. Pathogenesis. In: JACKSON AC., WUNNER WH. *Rabies*. San Diego: Academic Press, 2002: 246-74.
- 13 REED LJ., MUENCH HA. A simple method of estimating fifty percent end points. *Am. J. Hyg.*, 1938, 27, 493-7.
- 14 ROMAGNANI S. Induction of Th1 and Th2 responses: a key role for the natural immune response? *Immunol. Today*, 1992, 13, 379-81.
- 15 SIEGEL S. *Estatística não-paramétrica para Ciências do comportamento*. São Paulo: MacGraw-Hill, 1975.
- 16 STIFFEL C., IBAÑEZ OM., RIBEIRO OG., DECREUSEFOND C., SIQUEIRA M., MOUTON D., BIOZZI G. Genetic regulation of the specific and non-specific component of immunity. *Immunol. Lett.*, 1987, 16, 205-17.
- 17 STIFFEL C., IBAÑEZ OM., RIBEIRO OG., DECREUSEFOND C., MOUTON D., SIQUEIRA M., BIOZZI G. Genetic of acute inflammation: inflammatory reactions in inbred lines of mice and in their interline crosses. *Exp. Clin. Immunogen.*, 1990, 7, 221-33.
- 18 THEERASURAKARN AND UBOL. Apoptosis induction in brain during the fixed strain of rabies virus infection correlates with onset and severity of illness. *J. Neurol.*, 1998, 4, 407-14.
- 19 VIGAR ND., CABRERA WH., ARAUJO LMM., RIBEIRO OG., OGATA TRP., SIQUEIRA M., IBAÑEZ OM., FRANCO M. Pristane-induced arthritis in mice selected for maximal and minimal acute inflammatory reaction. *Eur. J. Immunol.*, 2000, 30, 431-7.