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Failure of protection induced by a Brazilian vaccine against Brazilian wild rabies viruses

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Summary. This report shows that the SMB vaccine currently used in Brazil for human immunisation provides different degrees of protection in mice, depending on the rabies virus strain used as challenge. Using the NIH and Habel potency tests to evaluate the protective activity of rabies vaccine, we observed that vaccinated mice showed a higher resistance to a challenge with a fixed rabies virus (CVS – Challenge Virus Strain). The vaccine potency using the Habel or NIH tests was respectively > 6.4 (log 10) and 1.0 (Relative Potency-RP) when the fixed rabies virus was used for challenge, and from 2.9 to 4.3 (log 10) or 0.13 to 0.8 (RP) when different wild rabies viruses were used for challenge. The presence of virus neutralising antibodies (VNA) could not explain the differences of susceptibility after vaccination, since sera of vaccinated animals had similar VNA levels against both fixed and wild strains before virus challenge (respectively, 5.6 ± 0.24 and 5.0 ± 0.25 IU/ml of VNA against the fixed rabies virus and the 566-M strain of wild rabies virus in sera of mice vaccinated with 0.2 units of vaccine). Only cell-mediated immunity parameters correlated with the protection induced by vaccination. The IFNγ titers found in sera and brain tissues of animals challenged with CVS strain were higher (from 36.7 ± 5.7 to 293.3 ± 46.2 IU/ml) than those found in mice challenged with 566-M virus strain (from 16.7 ± 5.8 to 36.7 ± 5.8). The proliferation index of spleen cells obtained with CVS stimulation reached a maximal value of 15.1 ± 0.7 while spleen cells from vaccinated mice stimulated with 566-M virus failed to proliferate. The implications of these data in human protection by vaccination are discussed.

Introduction

Rabies encephalitis is caused by viruses belonging to the *Lyssavirus* genus of the *Rhabdoviridae* family. Based on serological relationships, they were classified in four serotypes: serotype 1 comprises the "classical" rabies viruses, including

the wild and fixed strains; serotypes 2, 3 and 4 are rabies-related viruses whose prototypes are Lagos bat, Mokola and Duvenhage viruses, respectively. Recently, molecular phylogenies constructed from either nucleotide or aminoacid sequence data have described the *Lyssavirus* genus with six distinct genetic lineages [1]. In addition to the four serotypes previously characterised, which are equivalent to genotypes 1–4, the European bat lyssaviruses (EBL_1 and EBL_2) were identified and classified in genotypes 5 and 6.

In view of antigenic differences among isolates of rabies viruses, several studies were carried out with the purpose of detecting cross-reactions among the isolates, mainly in relation to rabies virus strains used in the preparation of vaccines [2–5]. Blancou et al. [6] identified a bovine rabies strain whose experimental infection was only partially avoided by previous immunisation with commercially available rabies vaccine for veterinary use. Lafon et al. [7], studying mouse protection against rabies virus infection elicited by different human rabies vaccine strains, showed that the immunisation with PV (Pasteur Virus) rabies strain was capable of inducing protection against an experimental infection with EBL strain but not against a Mokola virus strain. It was also shown that the immunisation with PM (Pitman-Moore) rabies strain failed to induce protection against either EBL or Mokola virus infections [8].

Antigenic variations were also shown among rabies virus isolates from Brazil, and contradictory data are found in the literature concerning the efficiency of protection conferred by veterinarian rabies virus vaccine against different isolates of rabies virus. As far as we know, there are no studies regarding the ability of different human rabies virus vaccines to induce protection against natural or experimental infection. Wiktor [9], working with Brazilian isolates, suggested that at least two antigenic groups of rabies virus could be found in Brazilian animals, one among dogs and another among bats, which in turn were transmitted to bovines.

Hayashi et al. [10] tested a SMB vaccine employed in canine mass vaccination in Brazil. Doses of this vaccine, which protected 100% of the mice against 10 000 LD_{50} of the vaccinal virus strain (CVS), conferred variable protection levels against 24 other wild rabies strains isolated from different animals. The lower range of protection was observed when virus strains isolated from bovines were used to challenge the mice (15 to 20%).

Cordeiro et al. [11] evaluated the protection levels in mice of an attenuated rabies vaccine of ERA origin, prepared in kidney tissue culture. Animals of different experimental groups were challenged by i.c. route with six Brazilian antigenic variants of rabies virus, isolated from dogs, vampire bats and foxes and compared to the protection conferred to CVS. The authors concluded that the ERA vaccine studied was effective against all antigenic variants of street and sylvatic rabies virus tested.

Erbolato et al. [12] showed that a similar ERA rabies vaccine, also prepared in kidney tissue culture, protected 100% of mice challenged by i.m. route with four different antigenic strains of rabies virus isolated in Brazil from dogs and bats.

Although veterinary rabies vaccines were efficient when tested against some Brazilian variant strains, rabies vaccines for human treatment had never been tested.

The human rabies vaccine currently used in Brazil is produced with SMB infected with either the CVS or PV rabies virus strain [13]. Only vaccine batches with a relative potency > 1.0 IU/dose in the NIH test are released.

In the present study we examined, in a mouse model, the protective efficacy of a Brazilian human rabies vaccine produced with the PV strain, against several wild rabies viruses isolated in Brazil, mainly in the State of São Paulo. The levels of protection were compared with virus neutralising antibody (VNA) titers, production of interferon in sera and supernatants of brain homogenates of vaccinated mice, and proliferation assays of lymphocytes from these mice.

Materials and methods

Mice

Swiss mice weighing 11 to 14 g from Instituto Pasteur, São Paulo, were used for determination of vaccine potency in both NIH [14] and HABEL [15] tests. The same mice were used for study of virus neutralising antibody (VNA) and interferon gamma (IFN_γ) synthesis. 30 day-old inbred BALB/c mice from Instituto Butantan were used for the lymphocyte proliferation studies.

Viruses

Seven samples of wild strains of rabies virus were isolated from different animals and regions of the State of São Paulo. They were isolated from naturally infected animals after 3 i.c. passages in Swiss mice. The brains of mice showing paralysis were collected and homogenised with 20% phosphate buffer solution (PBS), and the supernatant was aliquoted and kept at $-80\,^{\circ}$C after virus titration by intra-cerebral (i.c.) mouse inoculation [16]. We performed the modified Habel and the NIH tests using as a challenge the wild virus isolates and the Challenge Virus Standard (CVS/31-2 obtained from CEPANZO), that is a "fixed" strain included as reference. The DR-19 strain [17], which was firstly isolated from a vampire bat (*Desmodus rotundus*), was also included in the tests, since it is an autoctone strain routinely used in Brazil as a virus challenge to evaluate veterinary rabies vaccine potency. The viral strains are presented in Table 1, as well as the animals they were isolated from. The wild rabies virus 566-M, isolated from a cow infected by a vampire bat, was previously adapted to BHK-21 cells [18] and used for spleen cell in vitro stimulation and virus-neutralising antibody titration.

Rabies vaccine

The modified-Fuenzalida and Palacios [13] vaccine, prepared by Instituto Butantan, São Paulo, for human treatment, was used. Basically, each 1-ml dose of vaccine contains a 2% suspension of suckling mouse brain (SMB) infected with the fixed PV rabies virus and inactivated by UV radiation, phenol in a concentration of 1: 1 000, and thiomersal in a concentration of 1:10 000. The vaccine is prepared using mice no older than 24 h at the time of inoculation, and the brain suspension is centrifuged at 17 000 **g** for 10 min. Batches are released for human use when NIH potency values ≥ 1.0 IU/dose.

Table 1. Vaccine potency obtained in the Habel and NIH tests when different rabies virus strains were used in the challenge

Virus challenge		Vaccine potency test	
Strain	Origin	Habel (log 10)[a]	NIH (R.P.)[b]
26-V	Man	4.24	n.r.
40-M	Cat	3.90	n.r.
65-M	Cow	3.01	0.13
221-M	Cow	3.24	0.20
251-M	Cow	3.90	0.13
330-M	Cat	3.96	n.r.
566-M	Cow	2.90	0.38
DR-19	Bat	4.30	0.80
CVS	Fixed	>6.40	1.00

Mice were vaccinated with PV vaccine as described in the Habel and NIH protocols and challenged with rabies virus samples of different origins

[a]Minimal index accepted for the veterinary vaccine release = 4

[b]Minimal Relative Potency (R. P.) accepted for human vaccine release = 1

Protective activity of rabies vaccine

The protective activity of rabies vaccine was determined by the NIH potency test [14] in mice (20 mice per vaccine dilution), where two vaccine injections (0.5 ml i.p.) at days 0 and 7 were followed by an i.c. virus challenge (30 to $50LD_{50}$/0.03 ml i.c.) at day 14 with the different rabies virus strains, or by the Habel test [15] where six doses of vaccine administered at days 0, 2, 4, 7, 9, 11 were followed by i.c. virus challenge (virus dilutions ranging from 10^{-1} to 10^{-7}–10 mice per virus dilution) at day 14. Infected mice were observed for the following 4 weeks for symptoms indicative of rabies infection, and the 50% mortality end-point of vaccine dilution was calculated by the method of Reed and Muench [16]. The results shown are representative of three experiments.

Determination of virus neutralising antibody titers (VNA)

Blood samples from vaccinated mice were obtained by retro-orbital puncture on day 14, before the virus challenge. Sera were prepared, heat-inactivated, batched and stored at $-20\,^{\circ}$C. VNA were determined on BHK-21 (Baby Hamster Kidney) cells by focus inhibition immunofluorescence, using supernatants of BHK-21 cells infected with PV or 566-M virus according to FAVORETTO et al. [19], employing as reference an equine hiperimmune antirabies serum diluted to contain 20 IU/ml. Samples were assayed in duplicate in serial two-fold dilutions starting with a dilution of 1:5. Data are expressed as International units/ml (IU/ml), and standard deviations are within 10% for any given experiment.

Interferon assay

A cytopathic effect reduction test technique, described in detail elsewhere [20], was used for the IFN titer determination. Briefly, monolayers of L929 cells in microplates were incubated for 18h with different dilutions of sera or supernatants of brain homogenates collected six

days after the virus challenge. Sera and brain supernatants were removed and the monolayers were infected with 200 tissue culture infective doses 50% ($TCID_{50}$) of encephalomyocardits virus. Unadsorbed virus were removed 2 h later by washing the monolayers, and fresh minimum essential medium (MEM) (Gibco) supplemented with 10% foetal calf serum (FCS) was added. Microplates were incubated for 24 h and the IFN titers, in Units/ml (U/ml), were expressed as the reciprocal dilution of the supernatant able to inhibit 50% of virus replication. For characterisation of IFNγ in the samples, polyclonal antibodies to mouse IFNα, β and monoclonal antibodies to mouse recombinant IFN$_\gamma$ (Holland Biotechnology, Leiden, Holland) with activity of 2×10^3 and 2×10^5 neutralising units per mg, respectively, were used throughout. The antibodies showed no cross-reactivity, and controls for IFN characterisation included internal and well-known preparations of IFNα, β, IFNγ and a mixture of both to assure the assay specificity.

Lymphocyte proliferation assay [21]

A pool of three immunised BALB/c mouse spleen cells were collected on day 14 and cultured (2×10^5 cells in 200 μl/well) in RPMI-1 640 (GIBCO) medium supplemented with 2 mM L-glutamine, 10% FCS, 1% Hepes-buffer, 1mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and gentamicin (40 mg/1). The spleen cells were stimulated with 50 μl of an inactivated 5% suspension of mouse brain infected with CVS, PV or 50μl of inactivated 1:30 supernatants of BHK-21 cells infected with 566-M or with Concanavalin A (5μg/ml) and 5% suspension of non-infected mouse brain or supernatants of BHK-21 non-infected cells used as controls. Microplates were then incubated for 96 h at 37 °C, 5% CO_2, and the cultures were pulsed during the last 16 h of incubation with 1μCi/well of [^{3}H] Thymidine, being harvested and washed through a glass fibre filter. Results of cell proliferation assay performed in triplicates were calculated by the mean counts per minute of [^{3}H] Thymidine incorporated into DNA and expressed as the proliferation index calculated as the ratio of mean of incorporation obtained in the presence of infected brain suspension to a mean of incorporation without antigen (non-infected brain suspension).

Results

Vaccine protective activity against a challenge with wild and "fixed" virus

Wild and fixed rabies virus strains were used as the challenge virus in Habel and NIH vaccine potency tests and the results are presented in Table 1. Results are extremely variable, ranging from 2.9 to $>$ 6.4 log 10 in the Habel test and relative potency from 0.13 to 1.0 in the NIH test.

94.4% of mice were protected by the vaccine diluted to 1:5 against a CVS challenge, and under the same conditions only 36.2% were protected when challenged with the 566-M virus. Even when a vaccine dilution of 1:125 was used, 100% of the animals succumbed after a challenge with the 566-M virus, and more than 60% of the animals survived a CVS challenge (data not shown).

Characterisation of the protective immune response

Figure 1 shows the level of VNA against CVS and 566-M rabies virus at day 14 after vaccination following the NIH protocol. It can be observed that the levels of VNA against CVS were similar to those elicited against 566-M virus, and the titers obtained correlated with the concentration of vaccine used in immunisation.

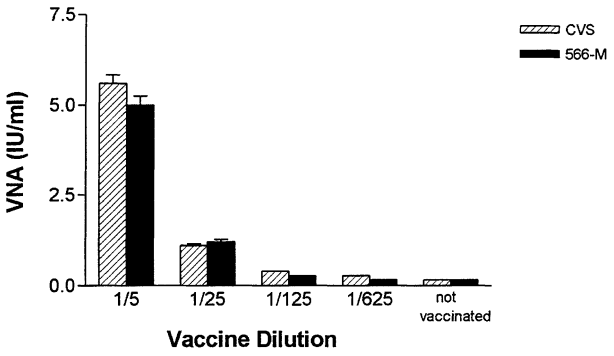

Fig. 1. Virus neutralising antibodies (VNA) induced by PV vaccine and tested against CVS and 566-M rabies virus. Mice were vaccinated at days 0 and 7 with a PV vaccine as described in the NIH test protocols. Blood samples were collected at day 14 before the challenge with virus and the sera were tested for the presence of VNA against CVS or 566-M virus by focus inhibition immunofluorescence. Data are expressed as units per ml (U/ml)

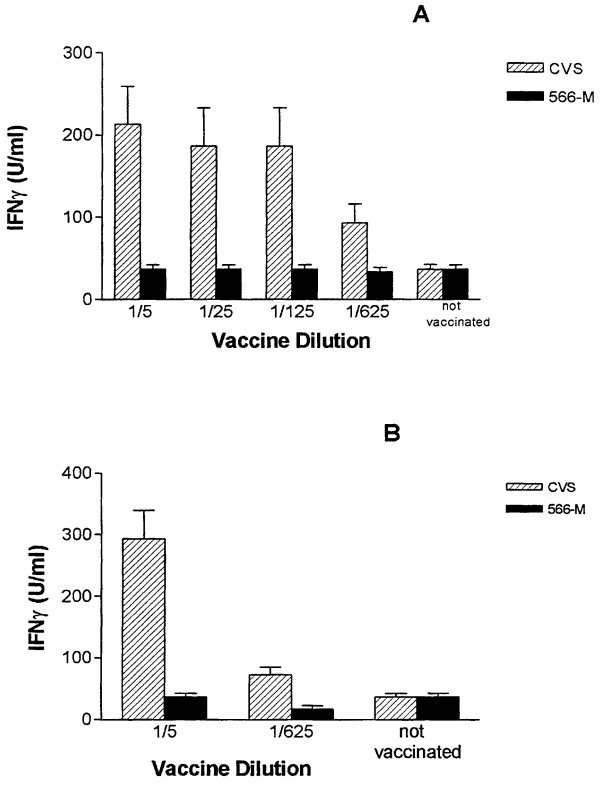

Fig. 2. IFNγ titers in mouse sera (**A**) or in brain tissue (**B**) of mice vaccinated with PV and challenged with CVS or 566-M rabies virus. Mice were vaccinated at days 0 and 7 with a PV vaccine as described in the NIH protocols and challenged at day 14 with CVS or 566-M virus. Blood samples and brain tissues were collected six days later (day 20) and analysed for IFNγ production

Figure 2A shows the titers of IFNγ found in the serum samples of the animals, 6 days after the challenge (20 days after the onset of vaccination) with both virus strains studied. The IFNγ titers found on day 14, before the virus challenge, were always < 20 U/ml (data not shown) showing that the vaccine alone did not elicit IFNγ production. Non-vaccinated animals were unable to synthesise IFNγ after infection with either of the virus strains studied. Animals vaccinated and

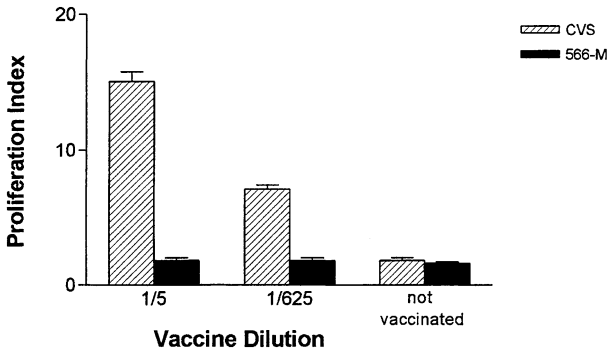

Fig. 3. Proliferation indexes of spleen cells obtained from PV vaccinated mice, stimulated in vitro with inactivated antigens of CVS or 566-M virus. Mice were vaccinated at days 0 and 7 with a PV vaccine, as described in the NIH test protocols. At day 14 the spleen cells were collected and stimulated in vitro with inactivated antigens of CVS or 566-M virus. Microplates were incubated for 96 hours and pulsed during the last 16 h with ^{3}H-Thymidine. Results were performed in triplicates and expressed as proliferation index calculated as ratio of mean of thymidine incorporation obtained in the presence of antigen or mitogen, to mean of thymidine incorporation obtained without them

challenged with CVS virus were capable of producing IFNγ, showing in their sera, 6 days after the challenge, titers that reached values as high as 213.3 ± 46.2.

The IFNγ titers obtained 6 days after virus challenge (20 days after the onset of vaccination) in the brain of mice are shown in Fig. 2B. Non-vaccinated mice, infected either with CVS or 566-M virus, were unable to produce IFNγ in the brain, and among the vaccinated animals, those challenged with CVS virus were capable of synthesising high levels of IFNγ (293.3 ± 46.2 U/ml) when compared to those challenged with 566-M virus, which produced very low levels of IFNγ (36.7 ± 5.7 U/ml). As indicated for the IFN$_\gamma$ detection in the sera, we observed no synthesis of IFNγ in the brain of vaccinated mice before the virus challenge (data not shown).

The proliferation indexes of spleen cells from vaccinated mice, stimulated in vitro with inactivated CVS or 566-M rabies virus, are shown in Fig. 3. The indexes obtained with spleen cells from non-immunised animals were always below 2.0, as also were those with spleen cells from immunised animals stimulated in vitro with a suspension of non-infected mouse brain (data not shown). The proliferation indexes of spleen cells obtained with CVS virus stimulation correlated with the concentration of vaccine used to immunise the animals (proliferation index of 7.1 ± 0.3 and 15.1 ± 0.7 for respectively 1/625 and 1/5 vaccine dilutions). Mouse spleen cells from vaccinated mice stimulated with 566-M virus failed to proliferate and the indexes were always below 2.0.

Discussion

We have shown in this paper that, after rabies vaccination with a SMB vaccine prepared with PV rabies strain, protection is clearly dependent upon the rabies

virus strain used for the challenge. In Brazil, a batch of antirabies vaccine is officially released for human use when the relative potency, evaluated by the NIH test, is equal to or higher than 1.0 IU/dose. The PV vaccine used in the present work only showed a relative potency, enough to be released, when the CVS was used for the challenge. When wild strains were used, instead of CVS, the relative potency of the NIH test was always lower than 1.0, clearly indicating a failure of protection induction by vaccination (Table 1).

Since the potency of inactivated veterinary rabies vaccines in Brazil is still evaluated by the Habel test we also included this assay using CVS and wild rabies virus strains as challenge viruses. Only when the Habel test was performed using CVS, DR-19 or 26-V strains did the PV vaccine show potency indexes > 4 ($\log_{10}$) (Table 1), which confirmed the lack of large spectrum protection elicited by the vaccination.

Hayashi et al. [10] had already reported reported different protection indexes when mice were immunised with a canine modifed SMB vaccine and challenged with 24 different rabies viruses isolated in Brazil from different animal species (surviving rates ranging from 15% to 100%). Curiously, the lowest levels of protection were observed against bovine isolates. These results contrasted with previous studies reporting good protection levels induced by an attenuated ERA (Evelyn-Rokitnicki-Albelset) vaccine against antigenic variants of Brazilian rabies wild virus strains, as well as against fixed rabies virus [11, 12]. However, in these previous studies the authors selected the variant strains by antigenic features with monoclonal antibody staining, while in the present study we selected them performing NIH and Habel tests with different challenge viruses. SMB vaccines are known to contain low quantities of rabies glycoprotein inducing lower levels of neutralising antibodies. It was observed that neural tissue vaccines induced immune responses directed mainly against rabies virus ribonucleoproteins with a high synthesis of antinucleocapsid non-neutralising antibodies [22, 23]. Since processing and presentation of an inactivated virus vaccine (PV strain) and an attenuated virus vaccine (ERA) follow different inter and intracellular pathways in the antigen presenting cells [24–27], this could determine differences in protection on the vaccine potency assays. In addition, ERA virus infection induces glycoprotein synthesis, which stresses the importance of this antigen as a key element for producing the neutralising antibodies.

In our study, however, the quantitative presence of VNA is unable to explain the differences of mouse protection against fixed and wild rabies virus strains induced by the PV vaccination, since sera of animals receiving different amounts of the rabies vaccine had similar levels of antibodies against CVS and 566-M virus at day 14 after vaccination. There was a correlation between VNA levels and mouse protection against CVS challenge, but this was not the case when the 566-M rabies virus strain was used in the challenge (Fig. 1). With a PV vaccine dilution of 1: 25, for instance, the VNA titers against CVS and 566-M virus were very similar (1.10 ± 0.05 for CVS and 1.20 ± 0.06 for 566-M), but the mortality levels were completely different (14.3% for CVS and 91.2% for 566-M virus – not shown).

Although the 566-M rabies virus had been adapted in BHK-21 cells during the isolation procedure [18], the adaptation process did not change the antigenic characteristics of the virus, since it continued displaying the same monoclonal profile of the original wild sample when tested by a monoclonal panel from the Pan-American Health Organisation (PAHO) (data not shown). The 566-M rabies virus, characterised as a strain from a hematophagous bat (Desmouds rotundus), the most common agent of bovine rabies in Brazil, was sent to the Lyssavirus Laboratory of the Institut Pasteur of Paris, where it was submitted to molecular biology studies. Although belonging to genotype 1, this strain turned out to be the most divergent among all the American strains studied by the laboratory (Dr. Noël Tordo, pers. comm.).

To investigate the contribution of cellular immunity in protecting mice against i.c. challenge against a fixed (CVS) and a wild rabies virus strain (566-M), we evaluated, in the present study, the titers of IFNγ in the serum and in the brain tissues as well as the in vitro lymphocyte proliferation of spleen cells of mice vaccinated with a PV strain and challenged with CVS or 566-M rabies virus.

Concerning the IFN assays, we observed that 6 days after the i.c. challenge with CVS, the IFNγ titers present in the serum and in the brain of PV vaccinated mice correlated with the protection rates (Fig. 2 and Table 1). The 566-M virus challenge did not elicit a detectable IFNγ production in the tissues of PV vaccinated mice, correlating also with a lack of protection rates (Fig. 2 and Table 1). As expected, in view of the IFNγ dependence on the immune system stimulation, non-vaccinated mice showed much lower IFNγ titers than the PV vaccinated and CVS challenged mice. Perrin et al. [18], assaying the biological activity by the polymerase chain reaction, had shown that splenocytes from BALB/c mice infected with 566-M (WRCVBB) were able to produce IFNγ and other cytokines up to the moment when the animals showed the first signs of illness (8 days after infection). Afterward, the production of the cytokines was hugely abolished. In our study we were not able to detect the IFNγ production on day 6 after the infection of non-immunised animals with 566-M virus. Only vaccinated animals showed IFNγ production. These differences may be due to several factors, such as a lower sensibility of the techniques used for cytokine detection, differences in the kinetics of infection in our conditions (we used a higher dose of virus – about 50 LD_{50} injected in the brain – than those authors who used 6 LD_{50} injected by i.m. route), and the significant fact that we analysed the presence of IFNγ directly in the serum and in the brain while other authors measured this cytokine or its mRNA after in vitro stimulation of splenocytes. Taken together, the correlation between IFNγ production (and lack of production) by PV vaccination followed by challenge, and the rates of protection (or lack of protection) against the challenge, suggest a participation of IFNγ in the resistance mechanisms of vaccinated animals against rabies infection.

Since the IFNγ synthesis correlated with the protection induced by PV vaccination, and as it mirrors a cell-mediated immune response [28], we performed studies of lymphocyte proliferation with in vitro stimulation with inactivated virus antigens to further investigate a possible correlation of cell-mediated immune re-

sponse and protection conferred by rabies vaccination against a challenge. While the proliferation of spleen cells from PV vacinated mice was elevated when stimulated with CVS, there was no cell proliferation at all when the spleen cells from PV vaccinated mice were stimulated with 566-M virus. Nevertheless, in animals previously immunised with inactivated 566-M virus, the proliferation index of spleen cells observed with homologous in vitro stimulation was similar to that obtained with spleen cells from mice vaccinated with CVS and stimulated in vitro with this virus (data not shown), indicating that the 566-M virus is not only capable of in vivo priming of the spleen cells during an immunisation process but is also capable of efficiently in vitro stimulating these cells, in order to proliferate like those primed and stimulated by the CVS. Giving further support to the data concerning the IFNγ synthesis and its correlation with the protection against rabies infection, which implicates the cellular compartment of the immune response in the virus resistance, the cell-mediated immunity study, as measured by the proliferation of the spleen cells from PV vaccinated mice, showed that these primed cells failed to respond to wild rabies virus strain (566-M virus) stimulation but efficiently responded to a fixed rabies virus strain (CVS).

To summarise, we have shown in this paper that important differences in degree of protection provided by immunisation with a PV-SMB vaccine depend on the rabies virus strain used for challenge. The failure in protection seems to be due to the inability of this vaccine to induce an effective cell-mediated immune response against wild rabies strains. Five out of seven wild rabies virus strains tested in this study probably originated from bats, which are the most important agents of human rabies transmission in Brazil, second only to dogs. In addition, they are responsible for hundreds of cases of bovine rabies deaths. Considering the great number of bat species found in Brazil and the number of other wild animals whose roles in rabies transmission have not yet been investigated, we must be aware that, at least theoretically, the current virus strains elected for vaccine preparation and the methodology of vaccine production should be re-evaluated. The vaccine used for pre or post-exposure profilaxis of human and animal rabies might not induce protection against all the wild rabies virus strains. Based on information provided by the Brazilian Health Ministry, there are in Brazil no cases of rabies diagnosed in persons vaccinated with SMB vaccines. It would not be difficult to speculate, however, that such cases might not have been notified, since in rural areas the medical care is inefficient. Actually, in a study under field conditions, an SMB vaccine produced with the CVS strain failed to induce satisfactory VNA titers in 20% of the vaccinees receiving the currently used pre-exposure anti-rabies immunisation schedule in Brazil [29].

Since cross-protection studies and the understanding of the immune mechanisms involved in protection are essential to the choice of both suitable virus strains and vaccine preparation for different regions, investigations on rabies virus antigenic variants must be encouraged. In addition, more modern rabies vaccines with higher potency values should be made available to avoid the potential possibility of failure in human treatment.

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