

Effect of the Contents and Form of Rabies Glycoprotein on the Potency of Rabies Vaccination in Cattle

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One of the methods used for controlling cattle rabies in Brazil consists of vaccination. Sometimes, however, rabies occurs in cattle supposedly protected. Since rabies vaccine batches are officially controlled by tests performed on laboratory animals, it is questionable whether the minimal mandatory requirements really correspond to immunogenicity in the target species. We have analyzed the association among potencies of rabies vaccines tested by the NIH test, the contents and form (free-soluble or virus-attached) of rabies glycoprotein (G) in the vaccine batches, and the virus-neutralizing antibodies (VNA) titers elicited in cattle. No correlation was found between G contents in the vaccine batches and the NIH values, whatever the presentation of G. There was no correlation either between NIH values and VNA titers elicited in cattle. There was, however, a positive correlation ($r = 0.8681$; $p = 0.0001$) between the amounts of virion-attached G present in the vaccine batches and VNA elicited in cattle. This was not observed when the same analysis was performed with total-glycoprotein or free-soluble glycoprotein. The study demonstrated that NIH values can not predict the effect of the immunogen in cattle. On the other hand, the quantification of virus-attached rabies glycoprotein has a strong correlation with VNA elicited in cattle.

Key words: rabies - cattle vaccination - NIH test - rabies glycoprotein

Cattle are infected with rabies virus transmitted by different animals in different regions of the world. In Latin America, the vampire bat (*Desmodus rotundus*) is mainly responsible for transmitting the disease to livestock (Larghi & Nebel 1985). According to unofficial information from the Brazilian Ministry of Agriculture, it is presumed that nearly 30,000 cattle are lost each year in Brazil. In order to reduce the important economic losses, one of the methods for controlling the disease consists of reducing vampire bat colonies with anticoagulants (Linhart et al. 1972). Since this is not selective and also affects non-hematophagous bats, this approach has been heavily condemned by conservationist groups. For this reason, at the moment, vaccination seems to be the most suitable measure for the protection of cattle. In Brazil, however, the occurrence of rabies among cattle supposedly protected by rabies immunization is not uncommon, even though all vaccine batches are officially controlled by the LARA (Reference Animal Laboratory, Campinas, SP, Brazil) following the instructions of the Brazilian Ministry of Agriculture and the World Health Organization (WHO) (Habel 1996, Koprowski 1996). Several factors could be responsible for this shortcoming. Among them (discussed in detail by Zanetti et al. 1998, Rodrigues da Silva et al. 2000) a point that must be raised is the fact that, because rabies vaccines are officially controlled in Brazil by tests performed on laboratory animals (mice or guinea-pigs), it is always questionable whether the minimal mandatory

requirements for a vaccine being approved really correspond to high immunogenicity in the target species. We have recently reported that an attenuated ERA vaccine officially approved by the LARA evoked very low and transient titers of neutralizing antibodies in cattle (Rodrigues da Silva et al. 2000). Another point to be stressed is the importance of the content of rabies glycoprotein (G) in the vaccine. G induces virus-neutralizing antibodies (VNA) (Wiktor et al. 1973) and protection against intracerebral challenge (Perrin et al. 1985). G also induces the production of cell-mediated immunity (Celis et al. 1988). Under certain conditions, however, rabies glycoprotein is shed into the culture medium in a free soluble form (FSG), which lacks some amino acids from the C-terminal of the G protein and has a low immunogenicity when employed in a purified form. In vitro assays for quantification of protective antigen in rabies vaccine are recommended by WHO as an alternative potency test for rabies vaccine (Perrin et al. 1996). Commercial veterinary vaccines, however also contain undetermined amounts of ribonucleoproteins (RNP) (Dietzschold et al. 1987, Drings et al. 1999) which are also known to be immunogenic. In the present study we have analyzed the association among the potencies of rabies vaccines tested by the NIH test, performed in mice, the contents of FSG and virion-attached G (VAG) in the vaccine batches, appraised by ELISA, and the VNA titers induced in cattle, in an attempt to add new information on this matter.

The experimental rabies vaccines were produced with clarified BHK-21 cell suspensions and infected with PV strain (obtained from CEPANZO, Buenos Aires). Cell cultures were maintained and infected under standard conditions, with culture medium containing 10% of new born calf serum (NCS). After the cell-infection procedure, the culture medium was replaced by NCS-free medium. Ra-

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bies virus was inactivated by adding 0.05 M-BEI (binary ethyleneimine), in a volume corresponding to 2% of the virulent suspension volume (37°C, 24 h). Neutralization of BEI was achieved by sodium tiosulphate. The inactivated bulks were tested for residual virulent virus by intracerebral inoculation of mice. The vaccine batches were adjuvanted with a 30%-aluminum hydroxide suspension. Six different batches were selected to contain different amounts of FSG and VAG, as shown in the Table. The protective activity of rabies vaccine batches was determined by the NIH potency test (Wilbur & Aubert 1996) in Swiss 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 50LD₅₀/0.03 ml i.c) at day 14 with the Challenge Virus Standard. Infected mice were observed for the following four weeks for symptoms indicative of rabies infection, and the 50% protection endpoint of vaccine dilution was calculated by the method of Reed & Muench (Aubert 1996). Due to the number of animals used in each test, only undiluted vaccine batches were evaluated by NIH. Mice were bred and maintained under conventional laboratory conditions in accordance with International rules. The amount of total rabies glycoprotein (TG), FSG and VAG present in each one of the six vaccine batches produced was measured by ELISA, as previously described by Perrin et al. (1996). Vaccine batches were ultracentrifuged for 2 h at 80,000 g and the pellets were resuspended with the initial volume of PBS. FSG was evaluated in the supernatant whereas VAG was measured in the pellet. The assays of FSG and VAG were performed before the addition of the adjuvant. TG was

assayed before centrifugation. Briefly, microtitration plates were sensitized by adding 200 µl of a 1:300 dilution of polyclonal anti-G antibodies in carbonate buffer to each well. The antibodies were obtained from New Zealand rabbits immunized with purified G protein (150 µg per injection). The sera were tested by the RFFIT, and sera exhibiting a VNA titer higher than 100 UI/ml were selected for IgG purification. After blocking and washing steps, sensitized plates were incubated with 200 µl of serial, twofold dilutions of a reference vaccine and the experimental vaccine batches. A 1:200 dilution of peroxidase-labelled anti-G conjugate (produced with the same anti-G IgG used for sensitization) was then added to the wells. Following the addition of chromogen/substrate solution, the optical density (OD) was determined at 492 nm. The glycoprotein content of the experimental samples was then determined by comparing the OD values of the samples with those of the reference vaccine. Based upon the G contents we selected four batches (1 to 4) for bovine immunization. One hundred fifty-eight healthy cattle, belonging to Vallée farm in the State of Minas Gerais, Brazil, were divided into four groups (Group I to IV). All animals were immunized with one s.c. 5 ml-dose, following the producer's instructions. Each group was divided into four subgroups containing 10-12 animals each (excepting Group 1), which received serial two fold dilutions of the vaccine (1/1, 1/2, 1/4 and 1/8). The number of animals and the vaccine batch used in each group are shown in the Table. Blood samples from vaccinated cattle were obtained on days 0, 30, 60, 90 and 120 after vaccination. Serum samples were prepared, heat-inactivated, batched and stored at -20°C. VNA were de-

TABLE
Characteristics of animal groups studied, glycoprotein amounts, NIH values of vaccine batches, and virus-neutralizing antibody (VNA) titers elicited

| Group (dilution) | No. of cattle | TG | VAG | FSG | NIH value | VNA (IU/ml) ^a |
|------------------|---------------|------|------|------|-----------------|--------------------------|
| 1 (1/1) | 30 | 0.60 | 0.31 | 0.16 | ND ^f | 10.31 (±4.39) |
| 2 (1/1) | 12 | 1.0 | 0.55 | 0.25 | 4.87 | 12.16 (±8.48) |
| (1/2) | 12 | 0.5 | 0.28 | 0.13 | ND | 10.59 (±5.54) |
| (1/4) | 12 | 0.25 | 0.14 | 0.06 | ND | 10.06 (±5.56) |
| (1/8) | 12 | 0.13 | 0.07 | 0.03 | ND | 6.24 (±4.28) |
| 3 (1/1) | 10 | 0.70 | 0.30 | 0.25 | 12.1 | 8.07 (±5.87) |
| (1/2) | 10 | 0.35 | 0.15 | 0.13 | ND | 8.78 (±3.72) |
| (1/4) | 10 | 0.18 | 0.08 | 0.06 | ND | 8.45 (±5.27) |
| (1/8) | 10 | 0.09 | 0.04 | 0.03 | ND | 6.25 (±3.31) |
| 4 (1/1) | 10 | 1.70 | 0.08 | 1.60 | 0.59 | 7.45 (±8.77) |
| (1/2) | 10 | 0.85 | 0.04 | 0.8 | ND | 2.3 (±0.79) |
| (1/4) | 10 | 0.43 | 0.04 | 0.4 | ND | 3.82 (±2.79) |
| (1/8) | 10 | 0.22 | 0.04 | 0.2 | ND | 4.74 (±3.79) |
| 5 | ND | 0.43 | 0.08 | 0.20 | 1.12 | ND |
| 6 | ND | 0.09 | 0.08 | 0.0 | 0.48 | ND |

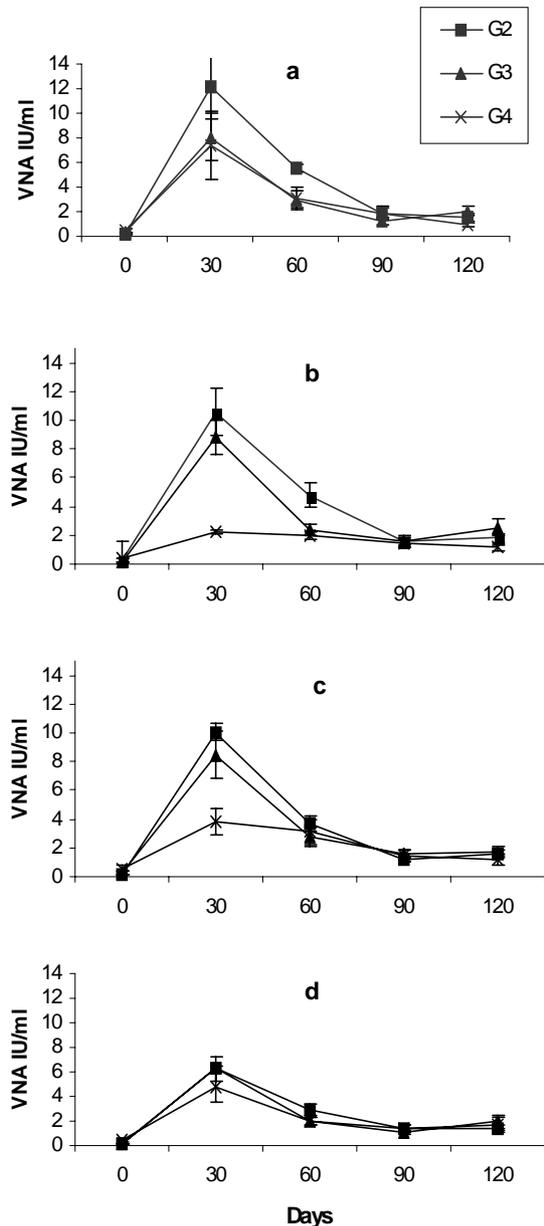
a: VNA on the day 30; TG: total glycoprotein (µg/ml); VAG: virus-attached glycoprotein (µg/ml); FSG: free-soluble glycoprotein (µg/ml); ND: not done

terminated on BHK-21 cells by focus inhibition immunofluorescence, using PV virus according to Favoretto et al. (1993). An equine hyperimmune antirabies serum diluted to contain 20 IU/ml was used as reference. 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 bars represent the SEM. The NIH values and VNA titers elicited were analyzed for statistical correlation with glycoprotein contents (TG, VAG and FSG) by the nonparametric Spearman Rank Correlation test. The comparisons between VNA titers in different groups and on different days were analyzed for statistical significance by analysis of variance (ANOVA) for repeated measures.

The six vaccine batches used in this study contained different concentrations of G, ranging from 0.09 to 1.7 µg/ml. They may clearly be split in two main groups: batches 1 to 3 with high VAG content and batches 4 to 6 with lower VAG concentrations. This distribution was not observed when FSG concentration was focused. All batches had similar FSG concentrations (0.16 to 0.25 mg/ml), except for batch 4, which showed a higher FSG content (1.6 µg/ml) and batch 6, which had no detectable FSG. The latter batch had only traces of G and it was included as a negative control for the NIH test. No correlation was found between the G contents in the vaccine batches and the NIH values, regardless of the presentation of G ($r = 0.300$ and $p = 0.6833$ for NIH X TG; $r = 0.7826$ and $p = 0.133$ for NIH X VAG; $r = 0.3591$ and $p = 0.5167$ for NIH X FSG). Although the number of data points was not great enough for an accurate analysis, there is no correlation either between NIH values and VNA titers elicited in cattle ($r = 0.5$ and $p = 1.0$). There was a positive correlation ($r = 0.8681$; $p < 0.001$) between the amounts of VAG present in the vaccine batches and VNA elicited in cattle. This was not observed however, when the same analysis was performed with TG ($r = 0.2198$, $p = 0.4706$) or FSG ($r = 0.2320$, $p = 0.4455$). In order to evaluate the duration of the VNA titers, different groups of cattle were immunized with serially diluted vaccine batches and the VNAs were followed for up to four months. As can be seen in Figure, the dilution of vaccine batches did not strongly affect the titers and duration of VNA. In general, there were no significant differences in the VNA titers from day 30 to 120 and the VNA titers were maintained above 0.5 IU/ml for up to 4 months in all groups.

In contrast to other authors who found a correlation between NIH values and VNA (Crick & Brown 1978, Wunderli et al. 1991) we could not find any correlation in cattle. Despite the huge differences among NIH values (ranging from 0.59 to 12.1) the VNA titers evaluated on day 30 ranged from 7.45 to 12.16 IU/ml. The results were similar on the consecutive days studied, as it can be seen in the Figure. Although a lower NIH value coincided with a lower elicited VNA titer (Group 4), this was not true for other groups. The lack of a correlation between an antibody's neutralizing activity in vitro and its protective activity in vivo has also been revealed elsewhere (Schumacher et al. 1989). Those differences could result from the different mechanisms by which VNA acts. Dietzschold (1993) extensively reviewed the role of virus-

neutralizing antibodies in viral clearance from the CNS. In addition to the classical functions of VNA (neutralization, ADCC and complement-dependent lysis) the author emphasizes that some (but not all) antibodies can mediate viral clearance by restricting viral gene expression, or by mechanisms not completely elucidated. It is important to stress however, that prior studies in rabies have evaluated the VNA in mice while our results were obtained from cattle, the principal target species for the vaccines studied. The vaccine batches used in Groups 3 and 4, for instance, showed NIH values of 12.1 and 0.59, respec-



Virus-neutralizing antibody (VNA) titers elicited in cattle immunized with inactivated experimental rabies vaccines, assayed by the cell-culture neutralization test. Results are expressed as international units per ml (IU/ml). Values are reported as mean \pm SEM. Bovine groups ($n = 12$) were immunized with different vaccine batches, which were serially two-fold diluted; a (1/1); b (1/2); c (1/4) and d (1/8)

tively, but induced very similar VNA titers on day 30. As shown by Côrtes et al. (1993) VNA profile was indicative of protective levels in cattle challenged with a wild rabies virus strain. Regarding the presence and form of presentation of G in the vaccine batches, we showed that the level of VAG (but not TG and FSG) correlated with the VNA elicited in cattle. This data corroborate those from other studies (Dietzschold et al. 1983, Adamovicz et al. 1983) even though they were performed in mice. To the best of our knowledge, this is the first study performed in cattle that correlates the G presentation form and VNA. In relation to the duration of the VNA titers, it can be seen in Figure that the immunization of cattle with serial dilutions of vaccine batches did not affect the titers and duration of VNA, insofar as these parameters were followed. There were no significant differences in the VNA titers from day 30 to 120 and the VNA titers were maintained above 0.5 IU/ml for up to four months in all groups. The reason for that could be that the vaccines used were not free of other rabies proteins. The immunological properties of rabies ribonucleoprotein (RNP) has been reported in several studies. RNP may act as an adjuvant (Dietzschold et al. 1987, Hooper et al. 1994, Astoul et al. 1996), primes animals for production of VNA induced by vaccines, induces protection against a peripheral i.m. challenge and seems to be the most effective rabies antigen for inducing cross-reactions between different rabies strains (Dietzschold et al. 1987, Drings et al. 1999). Consequently, we can not exclude that the lower but significant VNA titers obtained in Group 4 were due to the presence of RNP, which could be adjuvanted to the FSG present in that formulation.

In the light of the results here presented, we conclude that NIH values, obtained in mice, can not predict the effect of the immunogen on cattle. In addition this test has no correlation with the glycoprotein content in the vaccine. On the other hand, the quantification of virus-attached rabies glycoprotein, but not total glycoprotein nor free soluble glycoprotein, has a strong correlation with VNA elicited in the target species.

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