# *COURSE OF SECONDARY HUMORAL IMMUNE RESPONSE SHORTLY AFTER REVACCINATION WITH BHK-21 CELL CULTURE INACTIVATED RABIES VACCINE ADJUVANTED WITH ALUMINUM HYDROXIDE*

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*SUMMARY*: The phenomenon of "in vivo" blocking effect of antigen, due to the presence of rabies specific neutralizing antibodies in previously vaccinated cattle, and its possible interference on the humoral immunologic response to revaccination was investigated in five zebu-crossbred bovines reared in field condition. The secondary vaccination was performed 180 days from the first immunization, using a commercial inactivated rabies vaccine prepared in BHK-21 cells. Serum samples were taken sequentially after each vaccine administration at intervals of 0 (zero), 24 and 72 hours, and after 7 and 14 days for the determination of neutralizing antibodies. The mean neutralizing antibody titer determined for sera taken immediately before the first vaccination was < 1:5, at 24 and 72 hours post-vaccination, < 1:6.25; the mean value found at 7 days post-vaccination was 1:144 ± 51; and 1:3,460 ± 1,329, at 14 days post-vaccination with mean titer of 1:58 ± 14; 24 hours after revaccination the mean titer was 1:129 ± 80, raising forwardly to 1:277 ± 161, at 72 hours post-revaccination, and increasing levels > 1:6,400 and > 1:25,600 were found at 7 and 14 days post-revaccination, evidenciating a full anamnestic response. The blocking effect of antigen and the consequent fall in antibody levels shortly after the revaccination was not observed.

*UNITERMS*: Rabies of cattle; Antibody formation; Immunization; Immunity of cattle

## *INTRODUCTION*

Epidemic pattern of rabies in cattle was first diagnosed by CARINI [8] (1911) in Santa Catarina State, Southern Brazil, and nearly a century ago the vampire bat-transmitted rabies is still prevalent in most of South and Central American Countries from Northern Argentina to Northern Mexico [3,15].

Referring to the vampire bats, despite the wide variety of control methods currently available, they have been generally ineffective and, by and large the non-selective methods of bat destruction have been criticized by conservationists and ecologists [14]. Thus, vaccination has been used as an important alternative for controlling rabies in cattle, in order to minimize the ecnonomic losses due to this fatal disease.

To achieve this purpose, in the last 30 years research has traditionally been conducted toward the development of more effective and safer vaccines [2] and several live or inactivated vaccines became commercially available in this country [10,17,19].

A number of vaccines have experimentally been demonstrated to be very effective for cattle

[1,4,7,13,16,19] although in field conditions the results were, in some occasions, very disappointing [7,16,17].

Insufficient potency of the vaccines [7,17], antigenic variants of the virus [25,26] host nutrition status and the antigenic stimulus [11], and other factors like the interference of the specific passive maternal antibody in young animals [5,12,21,22,28] have been mentionned as the possible cause of the low protection revealed by such vaccines. In fact, ARNOLD et al. [5] (1973) have recommended carefulness in vaccinating and revaccinating young calves born from dams immunized against rabies. According to these authors, calves possessing maternal antibodies should not be vaccinated until the age of seven months or until the passive immunity has wanned, a booster shot should be given six months later and subsequent annual revaccination should be administered cautiously.

In case of an enduring immunity following vaccination, it has been suggested that injection of a second dose of the same antigen when titer is still detectable from the primary response results in reduction rather than increase in serum antibody titer due to removal of antibody in complex with antigen, before the secondary immune response gets under way [27].

This phenomenon is the so-called "negative phase" and according to TIZZARD [21] (1977) in theory, it is similar to that observed in young calves passively protected by the specific colostral antibodies, the presence of high levels of circulating antibodies may interfere with active immunization against the same antigen.

This "in vivo" neutralization is still a matter of controversy and needs further investigation in a scientifically controlled study, especially in cattle. The subject is important if revaccination has to be considered in herds presenting antibody titers above protective levels and reared in an endemic area where an emergency immunization is needed to rapidly protect the cattle against the disease.

The correlation between neutralizing antibodies and protection against challenge has raised some controversies in rabies, but as quoted by ATANASIU et al. [7] (1968) a relatively high neutralizing antibody level is generally accepted as an evidence of immunity.

The evaluation of the humoral immunity in those experiments involving different types of rabies vaccines has usually been conducted through the mouse serum neutralization (SN) test, a worldwide accepted assay method known to be very sensitive, although CÔRTES; NILSSON [9] (1974) recommended precaution in interpreting the results due to a high degree of variability, since results change gradually over a wide range of virus doses used in the test system.

Based on the facts mentionned above, this work aims to investigate the phenomenon of the "in vivo" blocking effect of antigen by the serum antibodies remaining after its primary immune response. If the "negative phase" does occur as the response of the antigen binding and removing antibodies from the circulation, the SN test would detect the drop in the antibody levels for a few days before the establishment of an enduring secondary immune response.

## *MATERIALS AND METHODS*

### RABIES VACCINE*

A commercially produced PV-strain adapted to BHK-$C_{13}$ cell line and binary ethyleneimine (BEI) - inactivated rabies vaccine, adjuvanted with 2% aluminum hydroxide (GUIDOLIN et al. [10], 1983) was given subcutaneously in a single dose of 5.0 ml per animal.

### ANIMALS

*Cattle* - The bovines used in this study were derived from a ranch located in the State of São Paulo, known to be free of rabies and not practicing vaccination against rabies. Five female Zebu cattle of Nelore breed aged between 12 to 36 months were selected after screening through the mouse SN test at 1:5 dilution.

*Mice* - Swiss albino mice used in the SN test were derived from a laboratory animal breeding station maintained at Santa Isabel - SP by Pfizer, weighing between 10 to 14 grams and divided into groups of five mice each. Groups of 10 mice each were used for the CVS (Challenge Virus Standard) strain virus titration.

*Virus* - The CVS strain 31/2 of fixed rabies virus from Pan American Zoonoses Center (CEPANZO), Argentina, was used for the SN test. The virus had been maintained in mice through two additional intracerebral inoculation and the $LD_{50}$ ($MICLD_{50}$) determined was $10^{-5,35}$/0.03 ml.

*Equine sera - Rabies hyperimmune serum* - This serum was kindly provided by the Butantan Institute - São Paulo, SP, and used as the positive control serum for the SN test.

*Rabies vaccine: RABVAC, Pfizer Co.Ltd., Guarulhos - SP, Brazil.

*Equine normal serum* - Previously tested by SN test, free of antibody against rabies, this foal serum was used for the preparation of the diluent at a concentration of 2% (V/V) in distilled water containing 1.000 IU of Penicillin and 1.25 mg of Streptomycin per ml.

*Mouse serum neutralization (SN) test* - The test carried out was the constant virus - varying serum dilution method and the procedures adopted were described by ATANASIU [6], 1976.

EXPERIMENTAL PROCEDURE

Five cattle were given subcutaneously 5.0 ml of rabies vaccine at the beginning of the experiment (day zero) immediately after the initial bleeding, blood collections were then sequentially performed at the 1st, 3rd, 7th and 14th day post initial vaccination (DPIV). The revaccination was carried out at 180th day after the primary vaccination using the same type of vaccine and dose.

Bleedings were made shortly before the administration of the second vaccine shot and repeated subsequently as described for the first vaccination.

Serum samples taken at each bleeding days were stored at -20 °C until required. After thawing, the sera were inactivated at 56 °C for 30 minutes and then submitted to SN test. The antibody titration was run twice, all samples not having determined the 50% end dilution at the first titration were tentatively assessed for the second time, using the two-fold serial dilutions and the final dilution of the serum-virus mixture ranged from 1:6.25 to 1:25,600. Control serum was also diluted by using two-fold serial dilutions, each dilution was then inoculated intracerebrally into five mice each.

The mice were observed daily for 21 days and only the mice found dead from the 4th day post inoculation were considered for the titer computation, which was made accordingly to REED; MUENCH [18] (1938). The titers were expressed as the reciprocal of dilutions protecting 50% of the inoculated mice.

## *RESULTS*

The time course of the humoral immune response of five cattle to inactivated rabies vaccine, enabled by bleeding at intervals of zero, 1,3,7 and 14 days after primary vaccination and their respective values in titers of neutralizing antibody are summarized in Tab. 1. At day zero, all five animals were found with their SN titers < 1:5 and at 2nd and 3rd DPIV the titers determined were < 1:6.25. The antibody titers corresponding to 7th DPIV the values found ranged from 1:31 to 1:199, with a mean titer of 1:144 ± 51. The mean SN titer found in the serum samples taken at 14th DPIV was 1:3,460 ± 1,329; indicating a mean rise in SN titer of 24 times within a week. By the profiles of the primary humoral immune response, increasing levels of antibodies were noted until 14 DPIV; animal nº 16 presented lower antibody response than did the others, i.e., 1:31 and 1:504, respectively at 7 and 14 DPIV.

The bleeding of five animals carried out on day 180 confirmed the presence of the remaining serum neutralizing antibodies following the primary immune response; the SN titers ranged from 1:26 to 1:70 with a mean titer of 1:58 ± 14, as are summarized in Tab. 2.

Serum samples taken 24 hours following revaccination were slightly increased in their SN titers with a mean titer of 129 ± 80. At this time interval, although statistically not significant, a drop of two units in titer was detected in the serum of animal nº 33, and animal nº 16 maintained its titer of 1:62, whilst the antibody titers of other three animals have increased in terms of their absolute values, titers corresponding to two heifers were found to be increased 3.7 times within 24 hours.

From day 3 after the revaccination the levels of the serum neutralizing antibodies tended to elevate rapidly with the mean titer of 1:277 ± 161 and the mean rise in titer was 2.14 times. Serum samples corresponding to 7th DPR were not determined their final 50% end dilutions but they were found to be greater than 1:6,400, except animal nº 16 with a titer of 1:4,525. Similar results were found for sera corresponding to 14th DPR, their 50% end dilutions were not determined but animal nº 16 with a titer of 1:4,530.

## *DISCUSSION*

In the sera of cattle given a commercially produced rabies inactivated vaccine, detectable amount of specific antibodies could not be found for several days after the primary vaccination. This is known as the "lag period", according to TIZARD [21], 1977. Although the time course of the immune response was not followed at daily intervals, serum neutralizing antibodies were already detectable at 7th DPIV, which are in accordance to many of the previous works reported elsewhere.

The response of cattle given a second dose of the same vaccine six months later was very different from the first in that it occurs much more rapidly and the serum neutralizing titers reach higher levels. The so-

called "lag period" tended to be very transient or undetectable.

The mean titer corresponding to this time point, i.e., 1st DPR, has increased from 58 ± 14 to 129 ± 80 within 24 hours, indicating that the drop in the level of antibodies due to "in vivo" blocking effect did not occur. In fact, the adjuvant contained in the vaccine may have influenced the processing of antigen by the antigen sensitive cells, slowing the rate of antigen release from the vaccine depot and consequent degradation. When the antigen was directly administered through intravenous route, UHR et al. [24] (1962) and SVEHAG; MANDELL [20] (1964) reported the phenomenon of "negative phase" due to the excess of serum antibodies.

The antibody levels of 1:62 and 1:24, respectively derived from sera of animal nº 16 and nº 33 taken at 1st DPR, when compared to the values corresponding to those of 180 DPR are not statistically different, indicating clearly that the "negative phase" did not occur. However, in spite of the high sensitivity of the mouse SN test used in this experiment, this method of antibody quantitation involves assay in living system and the reactions are subejected to a high degree of variability; the results tend to change gradually over a wide range of doses of the virus and, as suggested by CÔRTES; NILSSON [9] (1974), they must be interpreted carefully. Based on the analysis of the overall results, we are inclined in not accepting the phenomenon of "negative phase" to occur with the vaccine used.

Another fact clearly demonstrated in this study was the velocity in the rising of the antibody titers after the presentation of the second dose of the same vaccine; the memory cells as well as the effector cells of the cell-mediated immune response must have effectively been triggered by the vaccine. SVEHAG; MANDEL [20] (1964) reported that the duration of antibody synthesis is related to dose of antigen; high dose gives enduring response and antibody population consisted of IgM and IgG; IgM synthesis proceded IgG by one to two days. UHR; BAUMANN [23] (1961) reported that IgM synthesis has short-lasting immunologic memory but in the presence of high doses, the effective anamnestic response is associated only with IgG.

Analyzing the serum neutralizing antibody profile of animal nº 16 it seems that the rise in the level of antibodies tended to a plateau; this could be due to an individual difference in responding to an antigen stimulus rather than antigen exhaustion. The ideal scheme for the vaccination of young calves, however, is still lacking and needs further investigation.

The anamnestic immune response was fully confirmed, corroborating the findings of other authors that have investigated different types of rabies vaccines. This is of epidemiological importance, especially in situation when Agricultural Authorities, field practitioners and cattle farmers must intervene rapidly and effectively to control the focus of this lethal disease.

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*RESUMO*: O fenômeno do consumo de antígeno, devido à presença de anticorpos neutralizantes específicos para a raiva, em bovinos previamente vacinados e a sua possível interferência na resposta imunológica humoral, decorrente de revacinação, foi investigado em cinco bovinos mestiços azebuados, criados em condições de campo. A revacinação foi realizada 180 dias após a primeira aplicação, utilizando uma vacina comercial inativada, preparada em células BHK-21. As amostras de soros foram obtidas em intervalos sequenciais de 0 (zero), 24, 72 horas, sete e 14 dias, em cada vacinação e foram submetidas à prova de neutralização em camundongos para a pesquisa de anticorpos. O título médio de anticorpos neutralizantes, encontrado para o momento imediatamente antes da primeira vacinação foi < 1:5,24 e 72 horas após a vacinação foi < 1:6,25; no sétimo dia pós-vacinação o valor médio foi de 1:144 ± 51; aos 14 dias pós-vacinação, 1:3.460 ± 1.329. Todos os animais apresentaram títulos detectáveis no 180º dia pós-vacinação, com um valor médio de 1:58 ± 14; no entanto, 24 horas após a dose revacinante, foi detectado um valor médio de 1:129 ± 80, ascendendo para 1:277 ± 161 no 3º dia pós-revacinação, e níveis crescentes no 7º e no 14º dia pós-revacinação, com valores, respectivamente, superiores a 1:6.400 e 1:25.600, indicando o estabelecimento de uma resposta anamnéstica plena. Não foi obervado o efeito do consumo do antígeno e a conseqüente diminuição nos títulos de anticorpos, imediatamente após a revacinação.

*UNITERMOS*: Raiva, bovinos; Anticorpos, formação; Imunização; Imunidade, bovinos

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*TABLE 1* - Antirabies serum neutralizing antibody titers shortly before and after vaccination of cattle with BHK-21 cell culture inactivated rabies vaccine. São Paulo, 1988.

| Animal Number | Neutralizing Antibody Titers* Day 0 | 1st DPV** | 3rd DPV | 7th DPV | 14th DPV |
|---|---|---|---|---|---|
| 10 | < 5 | < 6.25 | < 6.25 | 141 | 4,150 |
| 16 | < 5 | < 6.25 | < 6.25 | 31 | 504 |
| 17 | < 5 | < 6.25 | < 6.25 | 178 | 4,525 |
| 19 | < 5 | < 6.25 | < 6.25 | 170 | 4,592 |
| 33 | < 5 | < 6.25 | < 6.25 | 199 | 4,530 |
| Arithmetic mean | ... | ... | ... | 144 | 3,460 |
| Variance | ... | ... | ... | 3,527 | 2,302,117 |
| Standard deviation | ... | ... | ... | 59 | 1,517 |
| Mean standard error | ... | ... | ... | 26 | 678 |
| Confidence interval | ... | ... | ... | 93 I-I 195 | 2,131 I-I 4,798 |

* Reciprocal of neutralizing antibody dilution, using CVS strain of fixed rabies virus with 30 mouse $ID_{50}$/0.03 ml
** Day Post Vaccination
§ Confidence interval, $\alpha = 0.05$
... Not determined

TABLE 2 - Antirabies serum neutralizing antibody titers shortly before and after revaccination of cattle at 180 days with BHK-21 cell culture inactivated rabies vaccine. São Paulo, 1988.

| Animal Number | Neutralizing Antibody Titers* | | | | |
|---|---|---|---|---|---|
| | 180th day | 1st DPR** | 3rd DPR | 7th DPR | 14th DPR |
| 10 | 67 | 80 | 308 | > 6,400 | > 25,600 |
| 16 | 62 | 62 | 79 | 4,525 | 4,530 |
| 17 | 67 | 224 | 356 | > 6,400 | > 25,600 |
| 19 | 70 | 259 | 565 | > 6,400 | > 25,600 |
| 33 | 26 | 24 | 80 | > 6,400 | > 25,600 |
| Arithmetic mean | 58 | 129 | 277 | ... | ... |
| Variance | 269 | 8,767 | 33,489 | ... | ... |
| Standard deviation | 16 | 93 | 183 | ... | ... |
| Mean standard error | 7 | 41 | 82 | ... | ... |
| Confidence interval | 44 I-I 72 | 44 I-I 209 | 116 I-I 438 | ... | ... |

* Reciprocal of neutralizing antibody dilution, using CVS strain of fixed rabies virus with 30 mouse $ID_{50}$/0.03 ml

** Day Post Revaccination

§ Confidence interval, $\alpha = 0.05$

... Not determined