Immune response in cattle induced by inactivated rabies vaccine adjuvanted with aluminium hydroxide either alone or in combination with avridine


Summary: In a comparative study of two commercial baby hamster kidney rabies vaccines produced in Brazil, the authors were able to demonstrate the following:

a) both vaccines provoked a high level of antibody response and protection against challenge in cattle

b) in primary vaccination, at least, the addition of avridine (a synthetic lipoidal amine) enhances the immune response in terms of the level and persistence of antibody

c) over 90% of cattle vaccinated with either vaccine were protected against experimental challenge one year after revaccination, and the antibody response profile indicated that these vaccines were capable of maintaining antibody titres above protective levels for more than two years after revaccination.

On the basis of these results, the authors recommend optional revaccination of young animals (i.e. "primo-vaccinates") at six months of age. Thereafter, annual revaccination should be sufficient to ensure high levels of antibody between vaccination cycles.


INTRODUCTION

In Brazil, as in most Central and South American countries, two distinct epidemiological patterns exist for urban and rural rabies (3).

In urban areas, rabies is predominantly associated with dogs, which constitute a major public health problem, as canine rabies is often of the "furious" form. In contrast, rural rabies occurs mainly in herbivores, as paralytic rabies, and the reservoir hosts are the various species of vampire bats. Cattle are the most common victims of rural rabies.
count and immunofluorescence. The virus harvest was inactivated by 1 mM BEI at 36°C for 14 h. The safety of the inactivated virus culture was tested in 24 suckling mice and 20 three-week-old mice (weight 11-14 g) by intracerebral (i.c.) inoculation of each mouse with 0.03 ml of a 1:10 dilution of the antigen. The mice were observed over a period of 21 days for the possible presence of any nervous signs and/or rabies-related death.

**Formulation of vaccines**

The inactivated virus culture was adsorbed onto 2% aluminium hydroxide and completed as vaccine by the addition of preservatives and 0.65% antifoaming agent. This constituted the standard aluminium hydroxide-adjuvanted commercial vaccine. For the avridine-adjuvanted vaccine, avridine was added to the adsorbed antigen to a concentration of 4 mg/ml of vaccine. This was achieved in the form of an oil-in-water emulsion in the proportion 1:25 (oil:aqueous phases) as previously described (25).

The potency of both vaccines evaluated by the Habel test (15) showed a log index of 5.97/ml and 5.99/ml, respectively, for aluminium hydroxide- and avridine-adjuvanted vaccines.

**Virus and serum assays**

For both assays, 21 one-day-old Swiss albino mice weighing 11-14 g were used. The virus strain used for neutralisation tests was the challenge virus standard (CEPANZO, strain 31/2).

Virus titrations were performed by ten-fold serial dilutions of virus in distilled water containing 2% inactivated normal horse serum. At each dilution step, groups of 10 mice were each inoculated i.c. with 0.03 ml. The inoculated mice were observed for 21 days. Mice which died after day 4 post-inoculation and showing nervous signs were recorded as positive for rabies. Periodically, smears of brain tissue were examined by immunofluorescence (13) for confirmatory diagnosis. Titres were calculated by the method described by Reed and Muench (23) and expressed as log_{10} LD_{50}/0.03 ml (50% lethal dose per 0.03 ml).

Virus neutralisation tests were performed by the “fixed virus/varying serum” method (4). Serum samples were serially diluted in distilled water containing 2% inactivated normal horse serum by two-fold steps starting from 1:5. Each serum dilution was mixed with an equal volume of virus pre-diluted to contain approximately 200 LD_{50}/0.03 ml, and the mixtures were incubated at 37°C for 90 min. For each serum dilution step, 5 mice were each inoculated i.c. with 0.03 ml of serum/virus mixture. During the inoculation procedures, the serum/virus mixtures were maintained on an ice-bath. The mice were observed over a period of 21 days for signs of rabies. Numbers of protected and dead mice were recorded and serum titres were calculated following the method described by Reed and Muench (23) and expressed as log_{10} SN_{50} (50% serum neutralisation titre).

**Cattle challenge virus**

The DR (Desmodus rotundus) 19.3 strain of a street virus from CEPANZO was used. The sample was passaged twice in mice. A pool of brain tissue was then collected from moribund mice and macerated to obtain a 20% suspension which constituted the challenge virus seed. Titration of this seed revealed a log_{10} LD_{50} value of 6.41/0.03 ml. The LD_{50} final challenge virus consisted of a 1:25 dilution of this suspension.
Experimental procedure

Cattle used in this experiment were derived from a region of the State of São Paulo known to be free of rabies and from a farm which had not been practising rabies vaccination of cattle. In addition, the animals were confirmed as susceptible to rabies by a screening serum neutralisation test of sera at 1:5 dilution. The cattle used were all female zebu of the Nelore breed, aged between 12 and 36 months. They were distributed homogeneously into seven groups of 10 animals each, in order to even out the effect of age. Treatment for each group was administered according to the following scheme:

- Group I was vaccinated with the aluminium hydroxide vaccine on day 0 and revaccinated with the same vaccine on day 180.
- Group II was vaccinated with the aluminium hydroxide vaccine on day 0 and revaccinated with the same vaccine on day 360.
- Group III was vaccinated with the aluminium hydroxide vaccine on day 0 and not revaccinated.
- Group IV was vaccinated with the avridine vaccine on day 0 and revaccinated on day 180 with the same vaccine.
- Group V was vaccinated with the avridine vaccine on day 0 and revaccinated on day 360 with the same vaccine.
- Group VI was vaccinated with the avridine vaccine on day 0 and not revaccinated.
- Group VII consisted of non-vaccinated control animals.

The vaccination procedure consisted of a subcutaneous injection of 5 ml of vaccine per animal.

Blood samples were collected from all animals on days 0, 30, 60, 90, 120, 150, 210, 360, 390 and 480 after initial vaccination. Sera were separated, aliquoted and stored at -20°C until tested. For assay of virus neutralising antibody, the samples were put in random order.

On day 480 after initial vaccination, all the cattle were challenged with virulent rabies virus by injecting 2.5 ml into the muscle on either side of the neck, approximately 10 cm behind the mandible and 10 cm below the dorsum of the vertebral column. Thereby, each animal was challenged with 8.63 mouse log_{10}LD_{50}.

The challenged cattle were kept in two closed isolation compounds, which had been rendered secure and vermin-proof. Animal handlers and all laboratory workers were required to wear overalls, rubber boots and rubber gloves. On leaving the compound, each person had to take shower before changing into normal street clothing. The animals were kept under clinical observation for 40 days. During this period, signs of rabies were noted and the brain of any animal which died was removed and examined for confirmatory rabies diagnosis (13, 17). After 40 days, all surviving animals were transferred to the University experimental farm where they have remained for 36 months without developing any signs of rabies.
RESULTS

Primary vaccination antibody response

Table I shows the mean values (log$_{10}$SN$_{50}$) of neutralising antibody response up to 480 days after single vaccination.

Both vaccines induced a high antibody response, attaining titres in excess of 2 log$_{10}$SN$_{50}$ in all vaccinated cattle at 30 days post-vaccination. Thereafter, there was a curvilinear decrease in antibody level with time as shown in Figure 1.

Analysis of variance demonstrated that the response following vaccination with avridine-adjuvanted vaccine was significantly greater than with vaccine adjuvanted with aluminium hydroxide alone (P<0.001). Avridine induced a higher initial response which persisted throughout the 480-day study period, as confirmed by linear regression (Fig. 2) (P<0.01).

Until 180 days post-vaccination, each vaccine group consisted of 30 animals. Figure 3 shows the frequency distribution of antibody titre 6 months after primary vaccination, again demonstrating the superiority of the avridine adjuvant over aluminium hydroxide alone. For example, 25 (83%) of the 30 animals in the avridine-adjuvanted vaccine

<table>
<thead>
<tr>
<th>No. of days post-vaccination</th>
<th>No. of animals</th>
<th>Avridine Mean log$<em>{10}$SN$</em>{50}$</th>
<th>SD</th>
<th>No. of animals</th>
<th>Al (OH)$<em>3$ Mean log$</em>{10}$SN$_{50}$</th>
<th>SD</th>
</tr>
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<tr>
<td>28</td>
<td>30</td>
<td>2.92</td>
<td>0.49</td>
<td>30</td>
<td>2.76</td>
<td>0.42</td>
</tr>
<tr>
<td>60</td>
<td>29</td>
<td>2.12</td>
<td>0.53</td>
<td>30</td>
<td>1.70</td>
<td>0.60</td>
</tr>
<tr>
<td>90</td>
<td>30</td>
<td>1.62</td>
<td>0.27</td>
<td>30</td>
<td>1.58</td>
<td>0.30</td>
</tr>
<tr>
<td>120</td>
<td>27</td>
<td>1.73</td>
<td>0.34</td>
<td>28</td>
<td>1.64</td>
<td>0.37</td>
</tr>
<tr>
<td>150</td>
<td>29</td>
<td>1.56</td>
<td>0.25</td>
<td>30</td>
<td>1.46</td>
<td>0.23</td>
</tr>
<tr>
<td>180</td>
<td>30</td>
<td>1.75</td>
<td>0.23</td>
<td>30</td>
<td>1.33</td>
<td>0.46</td>
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<td>210</td>
<td>19</td>
<td>1.49</td>
<td>0.48</td>
<td>20</td>
<td>1.53</td>
<td>0.42</td>
</tr>
<tr>
<td>360</td>
<td>19</td>
<td>1.76</td>
<td>0.46</td>
<td>20</td>
<td>1.56</td>
<td>0.44</td>
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<td>390</td>
<td>10</td>
<td>1.59</td>
<td>0.58</td>
<td>10</td>
<td>1.20</td>
<td>0.25</td>
</tr>
<tr>
<td>480</td>
<td>10</td>
<td>1.69</td>
<td>0.48</td>
<td>9</td>
<td>1.53</td>
<td>0.18</td>
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</tbody>
</table>

SN$_{50}$: 50% serum neutralisation titre
FIG. 1
Primary antibody response of cattle to inactivated baby hamster kidney cell-culture rabies vaccine

FIG. 2
Primary antibody response of cattle to inactivated baby hamster kidney cell-culture rabies vaccine
group maintained titres above $1.65 \log_{10} SN_{50}$, while only 11 (37%) of 30 animals in the aluminium hydroxide group had titres above this level.

**Secondary vaccination antibody response**

Revaccination at either 180 days (Groups I and III) or 360 days (Groups II and IV) after initial vaccination resulted in booster secondary responses to high antibody levels (i.e. $> 3.0 \log_{10} SN_{50}$) for both vaccines (Figs 4 and 5).

There was no significant difference in the response induced using the two adjuvants. Following revaccination at 180 days (Tables IIa and IIb; Fig. 4) antibody titres above $2.38 \log_{10} SN_{50}$ were maintained for at least 300 days after revaccination, with no appreciable further decline during the last 4 months of the experiment (i.e. between days 360 and 480).

**Protection against challenge**

The protection rate obtained against challenge 480 days after initial vaccination is summarised in Table III.

For primo-vaccinates, the score for the avridine group was on borderline of significance against the control group ($P=0.06$) whereas, at this stage, the score for the aluminium hydroxide group was not significantly different from the score for the controls.

The protection rate for all revaccinated groups was significantly different from the control group ($P<0.01$).

**FIG. 3**

Rabies antibody distribution 180 days after initial vaccination

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$SN_{50}$: 50% serum neutralisation titre

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**FIG. 4**
Rabies antibody response of cattle revaccinated 180 days after initial vaccination

**FIG. 5**
Rabies antibody response of cattle revaccinated 360 days after initial vaccination
**TABLE IIa**

Rabies antibody response (log_{10}SN_{50}) of cattle revaccinated with avridine vaccine 180 days after initial vaccination

<table>
<thead>
<tr>
<th>Animal identification no.</th>
<th>No. of days from initial vaccination</th>
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<tbody>
<tr>
<td></td>
<td>210</td>
</tr>
<tr>
<td>10</td>
<td>3.71</td>
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<tr>
<td>16</td>
<td>2.16</td>
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<td>17</td>
<td>3.76</td>
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<td>19</td>
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<td>33</td>
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<td>48</td>
<td>2.97</td>
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<td>49</td>
<td>3.66</td>
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<td>50</td>
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<td>66</td>
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<tr>
<td>70</td>
<td>3.07</td>
</tr>
<tr>
<td>Mean</td>
<td>3.48</td>
</tr>
<tr>
<td>SD</td>
<td>0.60</td>
</tr>
</tbody>
</table>

SN_{50}: 50% serum neutralisation titre
SD: standard deviation

**TABLE IIb**

Rabies antibody response (log_{10}SN_{50}) of cattle revaccinated with aluminium hydroxide vaccine 180 days after initial vaccination

<table>
<thead>
<tr>
<th>Animal identification no.</th>
<th>No. of days from initial vaccination</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>210</td>
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<tr>
<td>6</td>
<td>3.77</td>
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<tr>
<td>13</td>
<td>3.94</td>
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<tr>
<td>22</td>
<td>3.66</td>
</tr>
<tr>
<td>23</td>
<td>3.63</td>
</tr>
<tr>
<td>29</td>
<td>2.97</td>
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<tr>
<td>38</td>
<td>3.96</td>
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<tr>
<td>47</td>
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</tr>
<tr>
<td>Mean</td>
<td>3.71</td>
</tr>
<tr>
<td>SD</td>
<td>0.32</td>
</tr>
</tbody>
</table>

SN_{50}: 50% serum neutralisation titre
SD: standard deviation
Table III

Rabies challenge protection score of cattle 480 days after initial vaccination

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>No. of days post-revaccination</th>
<th>No. of animals protected</th>
<th>No. of animals dead</th>
<th>Total no. of animals</th>
<th>Chi-square vs control</th>
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<tbody>
<tr>
<td>Avridine</td>
<td>None</td>
<td>6</td>
<td>4</td>
<td>10</td>
<td>P = 0.06</td>
</tr>
<tr>
<td>Al (OH)₃</td>
<td>None</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>P = 0.25</td>
</tr>
<tr>
<td>Avridine</td>
<td>300</td>
<td>9</td>
<td>0</td>
<td>9</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Al (OH)₃</td>
<td>300</td>
<td>9</td>
<td>1</td>
<td>10</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Avridine</td>
<td>120</td>
<td>9</td>
<td>1</td>
<td>10</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Al (OH)₃</td>
<td>120</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>2</td>
<td>8</td>
<td>10</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 6 demonstrates a relationship between the level of neutralising antibody and protection of cattle against challenge with virulent virus. By probit transformation (Fig. 7), a linear relationship could be established from which a titre of $1.65 \log_{10} SN_{50}$ was calculated as equivalent to 1 PD$_{50}$ (median protective dose).

Figure 8 shows the proportion (%) of vaccinated cattle which survived challenge at each antibody titre step. From these data, the probability of survival for animals with a given antibody titre can be deduced. For example, at a titre of $1.3 \log_{10} SN_{50}$, 84% of the

\[ \text{SN}_{50} : 50\% \text{ serum neutralisation titre} \]

**FIG. 6**

Correlation of rabies antibody titre with protection in cattle
Antibody titre (log_{10} SN_{50})

SN_{50}: 50% serum neutralisation titre

**FIG. 7**

Correlation between antibody titre and protection

SN_{50}: 50% serum neutralisation titre

**FIG. 8**

Relationship between rabies antibody titre and probability of challenge survival
vaccinated cattle were protected, compared to a calculated probability of 87%. Applying this calculation to the antibody data at 180 days post-vaccination reveals that 29 of 30 and 13 of 30, respectively, of the avridine- and aluminium hydroxide-vaccinated cattle had titres above $1.3 \log_{10}\text{SN}_{50}$ (Fig. 3). Using the predicted values from the regression line analysis, it can be calculated that the probability of challenge survival would have been 84% and 38%, for the avridine and aluminium hydroxide groups, respectively. Applying the same calculation to the revaccinated groups enables the prediction that, for both vaccines, these animals would have had a high probability of challenge survival for more than two years after revaccination.

**DISCUSSION**

The present study shows that a BHK cell-culture inactivated rabies vaccine commercially produced in Brazil provoked a high antibody response and protection against challenge in cattle. The authors have further demonstrated that – in primo-vaccinates, at least – the addition of avridine, a synthetic lipoidal amine (16, 25), enhances the immune response in terms of the level and persistence of antibody. The authors have also shown that 12 months after revaccination with either adjuvant (avridine or aluminium hydroxide), over 90% of vaccinated cattle were protected against experimental challenge. Larghi and Nebel (20), obtained similar results in Argentina using experimentally-produced BHK cell-culture rabies vaccine.

From the antibody response profile, it seems that the BHK cell-culture inactivated rabies vaccine studied was capable of maintaining antibody titres above protective levels for more than two years after revaccination. Thus, during application of this vaccine in the field over the past ten years in Brazil, and five years in Venezuela, the authors have not been informed of a vaccination breakdown in animals properly vaccinated with the BHK cell-culture PV strain rabies vaccine used in this study. Specific follow-up investigations need to be conducted to confirm this.

The direct association between neutralising antibody and protection against challenge by rabies virus seems to have been the cause of some controversy (7, 9, 27, 28). However, in this study, the authors were able to clearly establish a satisfactory correlation between virus neutralising antibody titre and protection against challenge. Similar results were reported by Surreau and colleagues (26), Ribeiro-Netto and colleagues (24), Blancou and colleagues (5) and Larghi and Nebel (20). The discrepancy observed by some authors could be related to variations in the serum assay techniques (7), the role of cell-mediated immunity (28), antigenic differences between the vaccine, serum assay and challenge viruses (9), or the more recently recognised phenomenon of antigenic variation among field rabies virus isolates (27).

Nevertheless, the results of this study lead the authors to recommend revaccination of young animals (i.e. primo-vaccinates) at six months of age. Thereafter, annual revaccination should be sufficient to ensure high levels of antibody (i.e. $>2.38 \log_{10}\text{SN}_{50}$) between vaccination cycles.

Résumé : A l’issue d’une étude comparative sur deux vaccins antirabiques préparés sur cellules rénales de hamstiers nouveau-nés, disponibles dans le commerce et produits au Brésil, les auteurs ont abouti aux conclusions suivantes :

a) les deux vaccins suscitent une forte réponse immunitaire et confèrent une protection solide contre l’infection chez les bovins ;

b) l’addition d’avridine (une amine lipoïde synthétique) renforce, tout au moins lors de la primo-vaccination, le titre neutralisant et la persistance des anticorps ;

c) plus de 90 % des bovins ont été protégés contre une infection expérimentale pratiquée un an après le rappel ; d’après l’évolution de la réponse immunitaire, les titres d’anticorps obtenus avec ces vaccins sont supérieurs aux niveaux de protection plus de deux ans après le rappel.

Compte tenu de ces résultats, les auteurs recommandent qu’une vaccination facultative de rappel soit pratiquée sur les jeunes animaux « primo-vaccinés » à six mois. Par la suite, une revaccination annuelle suffit pour qu’ils aient des taux d’anticorps élevés entre chaque rappel vaccinal.


Resumen: Tras un estudio comparativo de dos vacunas antirrábicas preparadas a partir de células renales de hámsteres recién nacidos, disponibles en el comercio y producidas en Brasil, los autores llegaron a las siguientes conclusiones:

a) las dos vacunas provocan una fuerte respuesta inmunitaria y ofrecen una protección importante contra la infección experimental en los bovinos;

b) la adición de avridina (una amina lipoide sintética) refuerza, al menos en la primovacunación, la respuesta inmunitaria en términos de presencia y persistencia de los anticuerpos;

c) más del 90% de los bovinos vacunados con una y otra de estas vacunas se mostraron protegidos contra una infección experimental provocada un año después de la revacunación, y la evolución de la respuesta inmunitaria muestra que los títulos de anticuerpos obtenidos con estas vacunas se mantienen...
superiores a los niveles que garantizan la protección, durante más de dos años después de la revacunación.

A partir de estos resultados, los autores recomiendan una revacunación opcional en los animales jóvenes (<primovacunados>) a los seis meses de edad. Una revacunación anual debería luego bastar para conferir a los animales un nivel elevado de inmunidad entre las revacunaciones.


REFERENCES


