Ethylidenimine-Inactivated Rabies Vaccine of Tissue Culture Origin

O. P. LARGHI, V. L. SAVY, A. E. NEBEL, AND A. RODRIGUEZ*
Pan American Zoonoses Center, Pan American Health Organization/World Health Organization,*
and Instituto Pasteur, Buenos Aires, Argentina

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The replication of seven rabies virus strains (CVS, HEP, PV, ERA, WIRAB, CPZ, and BOLIVAR) in BHK cells and the inactivation dynamics of these strains by beta-propiolactone, acetyladenimine, and ethylidenimine were studied to find the most immunogenic strain and the most economic and stable inactivating agent for the production of an inactivated tissue culture rabies vaccine for animal use. The seven strains reached the peak of virus production 3 to 5 days after inoculation of the cell culture; PV yielded the highest virus titer (10^6 plaque-forming units/ml). The infectivity of virus suspensions containing 10^7 to 10^8 plaque-forming units/0.1 ml was inactivated by beta-propiolactone in 0.5 h, acetyladenimine in 3.0 h, and ethylidenimine in 1.0 h. Most of the vaccine lots prepared with the different strains and inactivating agents passed a modified National Institutes of Health potency test. The vaccines prepared with the PV strain had consistently higher antigenic values (equal or better than four) than the other six strains. This difference was highly significant (F_{6,11} = 59.8), whereas there were no statistically significant differences among the antigenic values of the vaccine lots prepared with the three inactivating agents. Batches of lyophilized and liquid vaccine stored at 4°C maintained potency for over 1 year. Ten dogs vaccinated with a vaccine prepared with the PV strain and inactivated with ethylidenimine developed a good antibody response and resisted challenge 60 days after vaccination, while seven of eight nonvaccinated controls died of rabies. This information indicates that an inactivated, stable, economic, and easy-to-prepare rabies vaccine can be produced in BHK cells by using the PV strain and ethylidenimine as an inactivating agent.

Immediately after the report of rabies virus replication in non-neural tissue culture (12, 19) investigations were made on the possible use of this system to develop vaccines (13, 20) which would be safer and more potent than those currently in use. A few tissue culture vaccines have been used experimentally in man (2, 5, 14, 42) and many which have been prepared with different cell systems and virus strains (38) are already used for animals. Some of these vaccines protected 100% of the vaccinated dogs when challenged 1 year after vaccination, but none protected all dogs 3 years after vaccination (34). Although the suckling mouse brain (SMB) (17) and chicken embryo (21, 22) vaccines have been very effective for the protection of animals against rabies (16, 34, 37), most of the countries in this hemisphere have not been able to control the disease for which lack of sufficient vaccine has sometimes been blamed (15).

Since inactivated vaccines are more stable and easier to handle under field conditions than attenuated virus vaccines, and because BHK-21 C_{13} (36) are the cells yielding the highest rabies virus titers (39), studies were conducted to develop an inactivated tissue culture vaccine produced in that cell line. This report describes the investigations done to find the most immunogenic strain of rabies virus and the most economic and stable inactivating agent for the production of an inactivated tissue culture rabies vaccine for use in animals. Preliminary information on the stability of the vaccine and the duration of immunity it afforded to dogs is also presented.

MATERIALS AND METHODS

Cells. The BHK-21 C_{13} cells were grown either in milk dilution or Blake bottles, using growth and maintenance media, and the inoculation procedure (including diethylaminoethyl-dextran) has been described in detail elsewhere (25). The inoculated cells were incubated at 33°C.

Virus strain. Seven rabies virus strains were used in the study; these had been passaged four to
five times in BHK in our laboratories. The strains CPZ and Bolivar were isolated from cattle dying of rabies and were adapted to BHK cells in this Center, as was the ERA strain isolated from a commercial vaccine (1). The following strains were received already adapted to tissue culture: the PV (4) strain was kindly provided by the Pasteur Institute, Paris, France; and the CVS (19), HEP (41), and WIRAB (mentioned for identification purposes and does not constitute priority for publication) strains were obtained from the Wistar Institute, Philadelphia, Pa. The WIRAB strain had been isolated from the commercial ERA vaccine and cloned on BHK-21 138 cells (31) at the Wistar Institute.

Dynamics of virus replication. To determine the time required for the different strains to reach the peak of viral production, BHK monolayers grown in milk dilution bottles were inoculated, as described elsewhere (25), with the seven rabies virus strains at different multiplicities of infection (MOI). Samples of the maintenance medium were taken daily for virus titration.

Virus inactivation. Each of the seven virus strains harvested at the peak of virus infectivity was centrifuged at 25 × g for 5 min and inactivated at 37°C for 4 h with each of the following agents: beta-propiolactone (BPL) (Fellows Testagar, Anaheim, Calif.), acetyleneimine (AEI) (Burroughs Welcome Co., Dartford, Kent, England), and ethyleneimine (EI) (Schuchardt, Munich, West Germany). BPL was used at a concentration of 0.025%, whereas AEI and EI were used at 0.05%. Samples of the virus-inactivating agent mixtures were taken at different intervals during the inactivation procedure for titration.

Virus titrations. Each sample of the different strains obtained during replication and inactivation was titrated by a plaqueing technique (31). A few samples were titrated by mouse inoculation, mostly at the beginning of this project.

Experimental vaccines. The rabies virus strains were inoculated into Blake bottles containing 10⁵ to 1.5 × 10⁶ cells. Seventy milliliters of maintenance medium was added and cultures were harvested at the peak of viral infectivity. After inactivation, sodium thioglycollate at a final concentration of 2% was added to the virus-inactivating agent mixture, the pH was adjusted to 7.4 to 7.6 with sodium bicarbonate, and a stabilizer described elsewhere (33) was also added. Sterility and safety (intracerebral inoculation into 20 suckling and 20 adult mice) were determined on the final vaccine, which was dispensed in 5-ml volumes and stored at −20°C until potency tested by the National Institutes of Health (NIH) method (32), following the modification described earlier (33). Three lots of vaccine prepared with the WIRAB strain (one with each inactivating agent) were lyophilized and stored at 4°C. A lot of vaccine prepared with the PV strain was inactivated with EI and kept at 4°C in two portions, one liquid and the other lyophilized.

Immunofluorescence. When necessary, the brains of the animals dying of rabies were studied by the rabies immunofluorescence technique. The method, reagents, and equipment used are described elsewhere (24).

Virus neutralization. The virus neutralization (VN) technique described by Atanasiu et al. (3) was used to titrate serum antibodies.

RESULTS

Dynamics of virus replication. Figure 1 presents the kinetic curves for the PV strain at different MOI. The shapes of the curves for all the strains were similar to these at the multiplicities studied. In general, the seven strains reached the peak of virus production between 3 and 5 days after infection of the cells. Each strain required a different MOI to obtain the best titer at the time of harvest. Table 1 shows the averages of the results for each strain; PV was the strain producing the highest titers: equal to or greater than 10⁸ plaque-forming units per ml (PFU/ml).

Virus inactivation. At least two sets of experiments were run for each virus strain and each inactivating agent. The infectivity of virus suspensions containing 10⁴ to 10⁶ PFU/0.1 ml was inactivated in 0.5 h by BPL, in 1.0 h by EI, and in 3.0 h by AEI. Figure 2 presents the inactivation kinetics for the PV strain by these inactivating agents and shows that this is a first-order reaction. Similar results were obtained with the other strains.

Experimental vaccines. The averages of the antigenic values (AV) for the NIH potency tests run for two lots of vaccine per strain and inactivating agent are presented in Table 2. The titers for each strain before inactivation were similar to those presented in Table 1. All the vaccine lots except those prepared with the CPZ strain passed the potency test. The PV strain gave consistently high AVs; these values were statistically different from those obtained with the other six strains (F<sub>6,12</sub> = 59.8). On the other hand, differences among the AVs for the three inactivating agents were not statistically significant.

Stability of the vaccine. The lyophilized and liquid vaccines stored at 4°C maintained their potency for a period of 12 to 14 months. The results of the potency tests performed on each vaccine during this period are presented in Table 3.

Canine vaccination and challenge. Sikes et al. (34) found that an experimental inactivated vaccine with an AV of 3 protected 100% of vaccinated dogs 3 years after vaccination. In light of the results obtained with the PV strain, a new lot of vaccine was prepared with this strain using EI as inactivating agent (PV-BHK-EI). This vaccine was lyophilized and an
**FIG. 1.** Dynamics of PV strain replication in BHK-21 cells.

**TABLE 1.** MOI and titer at time of harvest, for seven strains of rabies virus in BHK-21 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>MOI</th>
<th>Titer at harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV</td>
<td>5.50</td>
<td>$1.0 \times 10^9$</td>
</tr>
<tr>
<td>WIRAB</td>
<td>0.56</td>
<td>$2.9 \times 10^8$</td>
</tr>
<tr>
<td>ERA</td>
<td>0.02</td>
<td>$1.6 \times 10^8$</td>
</tr>
<tr>
<td>CVS</td>
<td>0.11</td>
<td>$1.1 \times 10^8$</td>
</tr>
<tr>
<td>HEP</td>
<td>0.60</td>
<td>$1.0 \times 10^8$</td>
</tr>
<tr>
<td>CPZ</td>
<td>0.34</td>
<td>$5.5 \times 10^6$</td>
</tr>
<tr>
<td>Bolivar</td>
<td>0.59</td>
<td>$2.0 \times 10^6$</td>
</tr>
</tbody>
</table>

a MOI (PFU per cell).
b PFU per milliliter.

AV equal to or greater than 4.3 was obtained for the NIH potency test. Ten dogs under 1 year of age were each inoculated intramuscularly with 2 ml of the vaccine. These dogs were bled periodically for VN testing. Sixty days after vaccination these dogs, together with eight nonvaccinated dogs of the same age, were challenged in the masseter muscle with a 20% salivary gland suspension from rabid dogs, titering $10^{5.2}$ mouse mean lethal doses/0.03 ml. The 10 vaccinated dogs and four controls received 0.6 ml of the 20% suspension, whereas the other four controls received a 1:5 dilution of the 20% suspension.

The antibody response of individual dogs is presented in Table 4. Figure 3 presents the geometric mean titers for the 10 dogs, expressed in international units per milliliter. These results were obtained using the NIH Rabies Standard Serum no. 2/1970. All dogs converted to VN positive when first checked (7
Fig. 2. Inactivation dynamics of PV strain from BHK-21 cells, by three chemical agents at 37 C. (1) BPL, Beta-propiolactone; AEI, acetylenimine; EI, ethylenimine.

Table 2. NIH potency test results for inactivated vaccines prepared with seven rabies virus strains

<table>
<thead>
<tr>
<th>Inactivating agent (4 h, 37 C)</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PV</td>
</tr>
<tr>
<td>BPL 0.025%</td>
<td>5.48</td>
</tr>
<tr>
<td>AEI 0.050%</td>
<td>5.98</td>
</tr>
<tr>
<td>EI 0.050%</td>
<td>4.66</td>
</tr>
</tbody>
</table>

* Averages of two determinations.

days) after vaccination and reached maximum VN antibody titers 3 to 4 weeks after vaccination; challenge produced a very good booster effect.

The results of the challenge experiment are presented in Table 5. The brain of every dog dying during the observation period was positive for rabies by immunofluorescence.
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Table 3. Stability at 4 C of rabies vaccines produced in BHK-21 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inactivating agent</th>
<th>State</th>
<th>Months of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>WIRAB</td>
<td>BPL</td>
<td>Lyophilized</td>
<td>0.96a</td>
</tr>
<tr>
<td></td>
<td>AEI</td>
<td>Lyophilized</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>EI</td>
<td>Lyophilized</td>
<td>1.05</td>
</tr>
<tr>
<td>PV</td>
<td>EI</td>
<td>Lyophilized</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>EI</td>
<td>Liquid</td>
<td>3.90</td>
</tr>
</tbody>
</table>

a Antigenic value, NIH potency test.
b ND, Not done.

Table 4. Antibody responsea of 10 dogs vaccinated with the PV-BHK-EI vaccine

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>60</th>
<th>132</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>&lt;2</td>
<td>8</td>
<td>47</td>
<td>38</td>
<td>56</td>
<td>65</td>
<td>1,284</td>
</tr>
<tr>
<td>41</td>
<td>&lt;2</td>
<td>56</td>
<td>65</td>
<td>84</td>
<td>104</td>
<td>47</td>
<td>773</td>
</tr>
<tr>
<td>42</td>
<td>&lt;2</td>
<td>125</td>
<td>956</td>
<td>424</td>
<td>277</td>
<td>125</td>
<td>1,284</td>
</tr>
<tr>
<td>44</td>
<td>&lt;2</td>
<td>1,219</td>
<td>2,062</td>
<td>1,637</td>
<td>1,187</td>
<td>925</td>
<td>6,968</td>
</tr>
<tr>
<td>46</td>
<td>&lt;2</td>
<td>11</td>
<td>47</td>
<td>56</td>
<td>49</td>
<td>49</td>
<td>393</td>
</tr>
<tr>
<td>49</td>
<td>&lt;2</td>
<td>1,219</td>
<td>1,400</td>
<td>1,650</td>
<td>1,387</td>
<td>625</td>
<td>4,500</td>
</tr>
<tr>
<td>53</td>
<td>&lt;2</td>
<td>20</td>
<td>190</td>
<td>202</td>
<td>184</td>
<td>56</td>
<td>75</td>
</tr>
<tr>
<td>54</td>
<td>&lt;2</td>
<td>66</td>
<td>56</td>
<td>65</td>
<td>125</td>
<td>47</td>
<td>526</td>
</tr>
<tr>
<td>55</td>
<td>&lt;2</td>
<td>5</td>
<td>32</td>
<td>65</td>
<td>25</td>
<td>56</td>
<td>1,268</td>
</tr>
<tr>
<td>57</td>
<td>&lt;2</td>
<td>284</td>
<td>1,400</td>
<td>280</td>
<td>277</td>
<td>56</td>
<td>451</td>
</tr>
<tr>
<td>Median</td>
<td>&lt;2</td>
<td>61</td>
<td>127</td>
<td>143</td>
<td>154</td>
<td>56</td>
<td>1,021</td>
</tr>
</tbody>
</table>

a Reciprocal of dilution protecting 50% of the mice.
b Bled before challenge.

DISCUSSION

A variety of animal rabies vaccines of tissue culture origin are presently in use. Of these, the modified live virus vaccines have generally protected dogs better than the inactivated ones. However, the work reported by Sikes et al. (34) showed that the opposite could also be true, provided the inactivated vaccine contained a good immunogenic mass. In the present report it was shown that such a mass can be obtained using the BHK cells to propagate rabies virus. As demonstrated by Wiktor et al. (40), viral production reaches a peak in this cell line 3 to 5 days after infection with each of the seven rabies virus strains included in our study. The PV strain at an MOI of 5.5 PFU/cell produced the highest titer (Table 1).

Rabies virus has been inactivated by various chemical and physical agents for vaccine production. BPL has been used extensively for the production of rabies vaccines (18, 27, 28) after LoGrigippo and Hartman (26) reported its action on rabies virus. In our study, the inactivation dynamics of BPL for the seven strains propagated in tissue culture were faster than those obtained by Wiktor et al. (40) for purified and concentrated rabies virus; while the infectivity of virus preparations containing 107 to 108 PFU/0.1 ml was inactivated by 0.025% BPL in 30 to 60 min, it required 6 h to inactivate 5 logs of virus in the experiments of Wiktor et al. (40). This difference could be due to the fact that these workers used an inactivation temperature of 4 C, whereas we used 37 C.

The inactivation of rabies virus by AEI has already been reported (8, 9, 40). We have used the same concentration of this reagent and the same inactivation temperature indicated in these papers and obtained inactivation dynamics similar to those reported by Wiktor et al. (40); complete inactivation was achieved in approximately 3 h.

Although several EI derivatives have been used as antimicrobial agents (11), EI has only been used for inactivation of foot-and-mouth disease virus (6, 10). This compound is approximately 20 times less costly than BPL and is very stable. EI can be kept at room tempera-
tured (10), whereas BPL and AEI require storage at -20°C. In the experiments reported in this paper, the infectivity of virus suspensions containing 10^2 to 10^8 PFU/0.1 ml was inactivated by EI in 1 h with no reduction in the immunogenic properties of the inactivated virus, as demonstrated by the fact that there were no statistically significant differences among AVs of the vaccine lots prepared with the three inactivating agents.

The results obtained by Sikes et al. (34) would indicate that inactivated vaccines with an AV equal to or greater than three could protect 100% of vaccinated dogs for 3 years. In the same experiments, an SMB vaccine with an AV of 0.3 protected 100% of the dogs 1 year after vaccination and 80% 3 years after vaccination. Most of the 42 lots of vaccine prepared by us with the seven strains of rabies virus gave AVs consistently better (Table 2) than the SMB vaccine used by Sikes et al. The vaccine lots prepared with the CPZ strain were the exception; this is not surprising if one considers that this last strain yielded the lowest virus titer (10^6 PFU/ml, Table 1) at harvest. On the other hand, the vaccine lots prepared with the PV strains gave AVs consistently higher than the purified vaccine used by Sikes et al. which protected 100% of the animals 3 years after vaccination. The single tissue culture-derived, inactivated vaccine (23) used by Sikes et al. protected only 70% of the dogs 1 year after vaccination (34). A vaccine prepared by Atanasius et al. (4) in a system similar to ours protected 70% of the dogs 50 days after vaccination. The results for the potency test of these two vaccines could not be compared with ours; in these two studies the guinea pig and Habel tests were used, respectively, whereas we used a modified NIH test. The latter appears to be the best method for testing inactivated vaccines (34). Petermann et al. (29) have produced a BPL-inactivated vaccine in Nil-2 cells with AVs of 5 to 10; this vaccine has protected 100% of the dogs 20 months after vaccination (30).

Batches of vaccine stored in the liquid state or lyophilized at 4°C maintained potency for 12 or more months (Table 3). Variations of individual antigenic values may be inherent in the NIH test.

In a preliminary duration of immunity study, 100% of the dogs were protected against challenge 60 days after vaccination (Table 5) by a vaccine prepared with the PV strain in BHK cells using EI as an inactivating agent. All the vaccinated dogs converted to VN positive 1 week after vaccination. The median antibody titers observed during the period of study (Table 4) were similar to those obtained by Sikes et al. (34) for dogs vaccinated with SMB vaccine and a good booster response was obtained in the dogs after challenge with street rabies virus. The VN antibody titers were somewhat lower than the titers of the dogs vaccinated with the purified vaccine in the latter study. This lower VN titer might not indicate lower immunity. Cabasso et al. (7) found that the rabies VN titers of the same sera could differ consistently in tests conducted in two different laboratories. For future comparison, and following the recommendations of the World Health Organization Expert Committee on Rabies (38),

<table>
<thead>
<tr>
<th>Dog vaccination</th>
<th>Challenge virus dilution</th>
<th>Rabies mortality</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Undiluted</td>
<td>0/10</td>
<td>11,11,16,31</td>
</tr>
<tr>
<td>No</td>
<td>Undiluted</td>
<td>4/4</td>
<td>11,14,17</td>
</tr>
<tr>
<td>No</td>
<td>1:5</td>
<td>3/4</td>
<td>11,14,17</td>
</tr>
</tbody>
</table>

* 10^5.3 mouse mean lethal doses/0.03 ml.
* Dead/challenged.

**Fig. 3. Geometric mean titers** of rabies antibodies in 10 dogs vaccinated with PV-BHK-EI vaccine.
we have also presented the antibody profile for the vaccinated dogs expressed as international units per milliliter (Fig. 3). The findings presented here indicate that an inactivated, stable, economic, and easy-to-prepare rabies vaccine of high potency can be produced in BHK cell cultures using the PV strain and EI as the inactivating agent.

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LITERATURE CITED

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O. P. LARGHI, V. L. SAVY, A. E. NEBEL, AND A. RODRIGUEZ*

Pan American Zoonoses Center, Pan American Health Organization/World Health Organization*
Ramos Mejia, Argentina, and Instituto Pasteur, Buenos Aires, Argentina

Volume 3, no. 1, p. 26. A. Rodriguez is not affiliated with the Pan American Zoonoses Center, as indicated by the asterisks, but with the Instituto Pasteur in Buenos Aires. Please address reprint requests to: Dr. O. P. Larghi, Casilla 3092 Correo Central, 1000 Buenos Aires, Argentina.

Significance of Urinary Isolates of Coagulase-Negative Micrococcaceae

D. N. WILLIAMS,* MARLYS E. LUND, AND DONNA J. BLAZEVIC

Departments of Medicine* and Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455

Volume 3, no. 6, p. 559, reference 13. The chapter title and pages should read: "Culture media, p. 881–929." On p. 913, oxidation-fermentation test medium, formulation B, is the formulation of King, as used in the investigation reported.