Purified Rabies Vaccine (Suckling Rat Brain Origin)

J. F. LAVENDER

Department of Immunizing Biologicals, Research and Development, Eli Lilly and Company, Greenfield, Indiana 46140

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A 10% suckling rat brain rabies vaccine free from encephalitogenic activity was prepared and inactivated with 1:8,000 beta-propiolactone (BPL), or ultraviolet light, or a combination of ultraviolet light and BPL, or 1% phenol. Potency was excellent in all samples, with the exception of the phenolized product which was marginal. A purified suckling rat brain (SRB) vaccine prepared by zonal centrifugation and inactivated with 1:8,000 BPL contained about 0.01 the amount of protein nitrogen of the unpurified 10% SRB vaccine. This purified product passed the National Institutes of Health potency test for rabies vaccine after administration of a quantity equivalent to a standard 10% brain suspension.

The classical rabies vaccines of neural tissue origin have generally been high-virus-titer antigens, but inoculation of humans with these vaccines has occasionally been complicated by both severe local reactions and various degrees of neuroparalytic involvement. More recently, the duck embryo vaccine has replaced brain tissue vaccines in the United States, practically eliminating postvaccinal reactions (10, 11). However, the relatively high level of extraneous protein and lipid in this product leaves much to be desired.

In this era of cell culture vaccines, extensive efforts have been directed toward producing an ideal vaccine by using chicken, duck, hamster, pig, monkey, rabbit, mouse, dog, and human diploid cells (1, 5, 6, 14–16). Adapting this highly neurotropic virus to a non-neural cell system has been fraught with problems of low virus titers, lack of a suitable serum substitute to obtain acceptable virus titers, and no really specific potency test for a cell culture product.

Recently, attempts have been made to eliminate the encephalitogenic activity associated with neural tissue vaccines (while preserving their high virus titers and excellent antigenicity) by using the brains of immature or suckling animals.

It has been known for some years that the degree of encephalitogenic activity of brain tissue is associated with the extent of myelin development and, therefore, with the ontogeny of the animal used as the vaccine source (7).

In an extensive treatise on experimental allergic encephalitis (EAE), Lumsden et al. concluded that all encephalitogenic activity associated with mature brain tissue extracts may be narrowed to a common denominator, i.e., a dialyzable basic small polypeptide with a molecular weight of 4,400 to 4,800 (9).

Gispen et al., for example, have shown that 4- to 5-day-old rabbit brains are free from encephalitogenic activity when tested with Freund’s adjuvant in guinea pigs (3, 4). However, Svet-Moldavskij et al. were able to produce symptoms of EAE in guinea pigs with 2-day-old rabbit brain, and sheep brain had the encephalitogenic factor on the first day after birth (13). Fuenzalida et al. reported that mouse brain is free from EAE activity for at least 10 days after birth (2). Sikes and Larghi were able to prepare a purified vaccine from suckling mouse brain by a combination of centrifugation and column chromatography (12).

Svet-Moldavskij and his associates showed that the brains of albino rats did not become encephalitogenic until after the 18th day of life (13). Because of their relatively long freedom from the encephalitogenic factor after birth and their size advantage over mice, the suckling rat brain was selected for further investigation.

The purposes of the following experiments have been to repeat and extend the work reported by Svet-Moldavskij et al. on a rabies suckling rat brain (SRB) vaccine, to prepare SRB vaccines inactivated by various techniques, and to prepare a purified vaccine by zonal centrifugation with low protein nitrogen.

MATERIALS AND METHODS

Virus. The CVS strain of fixed rabies virus was passed one to four times in 50 to 100 4- to 8-day-old suckling rats by intracerebral inoculation. The rats were moribund with typical symptoms of fixed virus rabies in 72 to 96 hr. The brains were removed aseptically and ground in a chilled Waring blender
with pyrogen-free water. A 10% suspension of virus emulsion, with titers ranging between $10^{9.4}$ and $10^{10.3}$, was used in the production of both the regular and purified SRB vaccines. All virus seed was stored at −30°C.

**Virus inactivation.** The 10% SRB vaccines were inactivated with 1:8,000 beta-propiolactone (BPL; Betaprone, Fellows-Testagar), or ultraviolet (UV) light (J. J. Dill Machine; four lamps, 180 ml/min), or 1:8,000 BPL and UV light (280 ml/min, four lamps), or 1% phenol. In the combined inactivation procedure, the vaccines were treated with BPL for 2 hr at 4°C and then were processed in the Dill machine. The phenolized vaccines were inactivated for 8 to 10 days at room temperature (25°C).

EAE. The brain and cord were removed from an adult rabbit and ground in a chilled Waring blender with cold, pyrogen-free water for 2 min. A 25% emulsion of this material, mixed with an equal quantity of Freund's complete adjuvant, was used as the EAE control.

A 25% suspension of inactivated suckling rat brain vaccine, mixed with an equal quantity of Freund's complete adjuvant, was used as the EAE test vaccine. Groups of 10 Hartley guinea pigs (400 to 500 g) were inoculated in the pectoral region with 0.1 ml of either control or test EAE vaccine. The animals were observed daily for 43 days (along with uninoculated controls) for symptoms of leg and sphincter paralysis, paresis, and general incoordination. Animals exhibiting any or all of these symptoms were considered clinically positive for EAE. All paralyzed animals were killed when symptoms were obvious. All other survivors and controls were killed at the end of the observation period. The brain and spinal cord were removed from each animal, fixed in 10% Formalin, sectioned, stained, and then microscopically examined for histopathological lesions.

**Testing.** Prior to testing, all vaccines were lyophilized with either lactose or sucrose bulking agents (Stokes Drier; 72 hr). All undiluted vaccines were tested for the presence of live virus by the intracerebral inoculation of at least 20 mice (12 to 14 g). All vaccine lots were tested for sterility in thioglycolate broth (14 days of incubation at both 25 and 36°C). Only samples passing the inocuity and sterility tests were used for potency testing. All vaccines were assayed for potency by the standard National Institutes of Health (NIH) potency test for rabies vaccine. All purified vaccines, although considerably reduced in protein nitrogen, were potency tested as equivalent to a 10% suspension of brain material.

**Purification of SRB vaccine.** Samples (100 ml) of high-titer SRB virus (LD$_{50}$, $10^{-7.6}$) were processed with 1,600 ml of sucrose by density gradient centrifugation in a Beckman LZU centrifuge with a B-IV rotor. Lots of purified vaccine were prepared by isopycnic or rate zonal centrifugation. The isopycnic separation was conducted at 90,871 × g for 3.5 hr in a gradient composed of 17 to 55% sucrose in 0.02 M phosphate saline buffer (pH 7.4).

The rate zonal separation was performed at 35,496 × g for 30 min in a 30% sucrose gradient in 0.02 M phosphate saline buffer (pH 7.4), cushioned with 55% sucrose in the same buffer. After banding of the virus sample, 45 samples (40 ml each) were pumped from the rotor. Each sample was analyzed for protein nitrogen (Lowry method), sucrose concentration (Bausch & Lomb hand refractometer), optical density (at 280 nm with a Vanguard UV light analyzer), and LD$_{50}$.

Selected high-virus-titer fractions from each run were pooled and diluted to a sucrose concentration of 7 to 9% prior to inactivation and potency testing.

**Vaccine pH.** Prior to lyophilizing, if necessary, vaccines were adjusted to a pH of 7.3 to 7.7.

LD$_{50}$. All virus titrations were performed intracerebrally in adult mice (12 to 16 g) by using 10-fold dilutions of vaccine. The LD$_{50}$ was calculated by using the Reed-Muench method and was expressed as the amount of virus contained in 0.03 ml of vaccine.

**Diluent.** All lyophilized vaccines were reconstituted with sterile pyrogen-free water.

**RESULTS**

The initial lots of standard SRB vaccine were prepared essentially the same way as reported by Svet-Moldavskij, with two notable exceptions. (i) The Russian SRB vaccines were all inactivated with 1% phenol and prepared as liquid or lyophilized vaccines with glucose or sucrose bulking agents. The standard vaccines that we were prepared inactivated with phenol, or

<table>
<thead>
<tr>
<th>Lot</th>
<th>Log10 LD$_{50}$/0.03 ml</th>
<th>Inactivated with</th>
<th>NIH potency ratio</th>
<th>Lowry protein nitrogen (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-59174A</td>
<td>6.38</td>
<td>UV only</td>
<td>THTC$^b$</td>
<td>1.32</td>
</tr>
<tr>
<td>T-59174B</td>
<td>6.38</td>
<td>BPL (1:8,000)</td>
<td>THTC</td>
<td>1.53</td>
</tr>
<tr>
<td>T-59174C</td>
<td>6.38</td>
<td>UV and BPL (1:8,000)</td>
<td>2.19</td>
<td>1.51</td>
</tr>
<tr>
<td>T-59174D</td>
<td>6.38</td>
<td>1% Phenol</td>
<td>Failed, TLTC$^c$</td>
<td>0.808</td>
</tr>
<tr>
<td>T-59254A</td>
<td>7.58</td>
<td>UV and BPL</td>
<td>THTC</td>
<td>1.62</td>
</tr>
<tr>
<td>T-59254B</td>
<td>7.58</td>
<td>1% Phenol</td>
<td>0.372</td>
<td>3.04</td>
</tr>
</tbody>
</table>

$^a$ Ratio of 0.30 is needed to pass test.

$^b$ Too high to calculate.

$^c$ Too low to calculate.
BPL, or UV or a combination of BPL and UV. (ii) All vaccines were lyophilized with lactose or sucrose bulking agent. The results of the NIH potency test are included in Table 1. The first lot of vaccine, T-59174, inactivated with either UV or BPL, had such good potency that the ratios were too high to calculate (THTC). The sample inactivated with a combination of BPL and UV had an excellent potency ratio, whereas the vaccine inactivated with phenol failed the NIH potency test.

The second lot of vaccine, T-59254, also had a potency THTC in the UV and BPL combination; however, the phenolized product narrowly passed the NIH test, even though it contained about twice the protein nitrogen of the other samples. Although the Russian workers found phenol to be an acceptable inactivating agent for the SRB vaccines, phenol proved a rather poor substitute for BPL in our studies. The phenolized vaccines either failed the NIH potency test or were marginal, indicating an adverse effect on the antigenicity. Phenol inactivation requires many days to accomplish, and residual phenol in the product is undesirable in human vaccine preparations. BPL at 1:8,000, on the other hand, inactivated all vaccines in about 48 hr, and residual BPL was not detected in the final product after 72 hr.

The combination of BPL and UV, although effective in activating the rabies vaccines, did not prove to have any advantage over BPL alone. Also, the UV-BPL combination required careful control, or antigenicity was adversely affected. Therefore, all purified preparations of rabies SRB were inactivated with 1:8,000 BPL.

**Encephalitogenic activity.** The lots of SRB vaccine tested for encephalitogenic activity were prepared from rat brains harvested at a maximum of 12 days of age. Hartley guinea pigs were selected because of their high sensitivity to experimental allergic encephalitis (8).

Symptoms of EAE were observed in guinea pigs inoculated with the vaccine made of 25', adult rabbit brain suspension in Freund's adjuvant as early as 4 days postinoculation. The most consistent clinical observation in the animals was ascending bilateral paralysis with paralysis of the sphincter muscles. At no time during the 43-day observation period were clinical symptoms of EAE observed in guinea pigs inoculated with the 25' suspension of SRB vaccine in Freund's adjuvant. A few guinea pigs died during the experiment from other causes (usually pneumonia). These animals were negative for EAE when the central nervous system (CNS) was examined for histopathological lesions.

The results listed in Table 2 indicate that a direct correlation existed between clinical symp-

**Table 2. Experimental allergic encephalitis in Hartley guinea pigs**  

<table>
<thead>
<tr>
<th>Tissue inoculated</th>
<th>Clinical signs paralyzed total</th>
<th>Histopathological positive (EAE) total</th>
</tr>
</thead>
<tbody>
<tr>
<td>25', Adult rabbit brain and spinal cord</td>
<td>7/10</td>
<td>7/10</td>
</tr>
<tr>
<td>25', Suckling rat brain</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>Noninoculated controls</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

![Fig. 1. Results of purification of suckling rat brain rabies vaccine, lot T-59402, by isopycnic separation.](http://aem.asm.org/)

Downloaded from http://aem.asm.org/ on October 18, 2017 by guest
FIG. 2. Results of purification of suckling rat brain rabies vaccine, lot T-59468, by rate zonal separation.

toms of EAE and observable histopathological lesions in the CNS.

Purified SRB vaccine. The results of the purification of the SRB vaccines by zonal centrifugation are charted in Fig. 1 and 2. The first lot of vaccine prepared by isopycnic separation left the majority of the virus in about eight 40-ml samples (LD_{50}, >10^{-0.5}) with a sucrose concentration of 32 to 41%. Samples 15, 17, 19, 21, and 23 were pooled and diluted with phosphate-buffered saline with 0.2% gelatin to a sucrose concentration of 7.5%. They were activated with 1:8,000 beta-propiolactone. The pH was adjusted to 7.38, and amorphous, with a 1.1 ml fill dose, were lyophilized. The sucrase served as the bulking agent. The resulting vaccine, T-59402, when reconstituted with diluent, was water-clear, had a protein nitrogen content of 0.0104 mg/ml, and passed the NIH potency test.

The vaccine separated by the rate zonal process (T-59468) had a much broader peak of virus. Samples 15 through 33 contained virus titers ranging between 4.5 and 5.6 logs. All samples between 15 and 33 (sucrose concentration 20 to 34%) were combined and used to produce the second lot of purified SRB vaccine. The combined samples were diluted with sterile pyrogen-free water to a sucrose concentration of 9%, and then were inactivated with 1:8,000 BPL. The pH was adjusted to 7.7. Merthiolate (1:10,000) was added as a preservative. This lot of vaccine also was lyophilized, and again the reconstituted product was water-clear, had a protein nitrogen content of 0.0025 mg/ml, and passed the NIH potency test.

<table>
<thead>
<tr>
<th>Lot</th>
<th>Log 10 LD_{50} (mg/ml final product)</th>
<th>Lowry protein N_{2} (mg/ml)</th>
<th>NIH potency ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-59402</td>
<td>10^{-1.3}</td>
<td>0.0104</td>
<td>0.540</td>
</tr>
<tr>
<td>T-59468</td>
<td>10^{-2.2}</td>
<td>0.0025</td>
<td>0.305</td>
</tr>
</tbody>
</table>

*Ratio of 0.30 is necessary to pass NIH potency test.

DISCUSSION

The results of our experiments with a 10^{-1} lyophilized SRB vaccine confirm the observations of Svet-Moldavskij et al. (13) that a high-titered vaccine with good potency and low encephalitogenic potential can be produced, even after a variety of inactivation procedures.

In the studies of Svet-Moldavskij et al. (13), after over 12,000 human vaccinations, not one case of postvaccinal encephalitis has been seen, and high-titered serum-neutralizing antibody has been observed regularly.

However, the vaccine has the disadvantages of high protein nitrogen and phenol inactivation. Also, some severe local reactions and a significant number of general reactions (headache, rise in body temperature, and nausea) have been seen in human subjects inoculated with this vaccine.

The purified SRB vaccine has the advantages of BPL inactivation and very low protein nitrogen (about 0.01 the amount of the conventional SRB vaccine). Furthermore, both lots of the purified product passed the NIH potency test when tested as equivalent to a standard 10^{-1} suspension of brain tissue (Table 3).
It therefore appears that SRB vaccine can be improved by purification in the zonal centrifuge. A 100-ml sample of high-titered SRB vaccine (10^{-7.0} \text{ ml/cm}^3) diluted with 1,600 ml of buffered sucrose during purification, and then still further diluted about 1:10 after purification, still yielded over 4 logs of virus/0.03 ml.

The isopycnic separation appears to be superior to the rate zonal method in that practically all of the high-titered virus (free from much extraneous protein, hemoglobin, and lipid) was collected in about 10 40-ml fractions.

This purified SRB vaccine, with good potency, has a lower protein nitrogen content than most cell culture rabies vaccines.

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LITERATURE CITED