

# Effect of heterogeneity of rabies virus strain and challenge route on efficacy of inactivated rabies vaccines in mice

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**Objective**—To determine effect of route of challenge and strain of rabies virus on efficacy of inactivated rabies vaccines in mice.

**Animals**—3,056 mice.

**Procedure**—Challenge was performed with fixed and street rabies virus strains by use of footpad and intracerebral routes as well as IM injection into the hip, shoulder, neck, and masseter muscles. Intraperitoneal and IM vaccination was performed with 1 or 2 doses of 1 of 3 vaccine-strain inactivated rabies vaccines. For 2 of the vaccine strains, the vaccines were adjuvanted and nonadjuvanted.

**Results**—Incubation periods were dependent on route, dose, and virus strain used for challenge. Use of an intramuscular challenge route with challenge virus-strain rabies virus, which more accurately models natural exposure to rabies virus, resulted in reproducible mortality rates in mice. Use of this route revealed that differences among vaccines and challenge virus strains affected mortality rate less than that observed in the National Institutes of Health potency test, even when street isolates of widely variant origin were used for challenge.

**Conclusions and Clinical Relevance**—These results, combined with earlier data, support a proposal for a new rabies potency test that more closely models current vaccine administration practices and natural infection routes. (*Am J Vet Res* 2003;64:499–505)

The National Institutes of Health (NIH) potency test<sup>1</sup> is presently recommended by the World Health Organization (WHO)<sup>2</sup> to measure protection from rabies virus infection elicited by commercial vaccines for human and veterinary use. In this test, mice are administered 2 doses of test vaccine by IP injection and are challenged 2 weeks after initial vaccination by intracerebral (IC) injection with a lethal dose of fixed-strain rabies virus. Vaccine potency is determined relative to the protection observed in control mice that re-

ceive a standard reference vaccine preparation.<sup>1</sup> However, the accuracy of this test in measuring a vaccine's ability to induce protection against natural infection (peripheral exposure) has been questioned, because the severe IC challenge route does not occur naturally<sup>3-6</sup> and places this neurotropic virus in direct contact with the tissue in which it best replicates.<sup>7-11</sup>

A second concern is variation resulting from use of different virus strains in vaccine preparation. Pasteur and his coworkers developed the first rabies vaccination methodology with rabbit spinal cords infected with a rabies virus isolated from a rabid dog.<sup>12</sup> This was the strain from which all rabies vaccines were initially developed.<sup>13</sup> Other strains isolated from more recent rabid dog or human exposures are also used for vaccine production.<sup>13</sup> Examples of the latter are the Flury strain, which has derivatives (low egg passage [LEP], high egg passage [HEP]) of varying pathogenicity that are based on the level of attenuation,<sup>14</sup> the street Alabama Dufferin (SAD) rabies strain and its derivatives (ERA and Vnukovo 32-37),<sup>15</sup> and the Kelev<sup>15</sup> strain. These viruses have been passaged many times in animals and tissue culture. Some of the more attenuated derivatives were used as live virus vaccines for veterinary use,<sup>16</sup> and all are presently used in production of inactivated tissue culture-derived vaccines.<sup>17</sup>

Despite the multiplicity of viral isolates used in vaccine production, the rabies virus strains used for development of vaccine reference preparations (eg, the Pitman-Moore [PM] strain) and the virus strain used for challenge (challenge virus standard [CVS]) in vaccine potency testing are derivatives of the original Pasteur rabies virus (PRV) isolate.<sup>17</sup> A number of investigators have identified problems that may result from this reliance on a single strain origin for all rabies vaccine testing.<sup>18-23</sup> The WHO organized collaborative studies that determined that virus strain heterogeneity affected standardized in-vivo potency test results,<sup>2</sup> influencing serum neutralizing antibody production and protection of laboratory animals, but heterogeneity made virtually no difference in the protection elicited against street rabies virus (SRV) isolates.<sup>13</sup> The investigators concluded only that vaccine manufacturers should be aware of heterogeneous virus strain effects on potency-testing assays.<sup>13</sup>

A more important consideration to vaccine manufacturers is that antigenic differences exist between rabies virus isolates from diverse regions of the world and from various wildlife species.<sup>24-27</sup> Challenge with divergent rabies strains affects protection after vaccination with commercially licensed vaccines.<sup>28-30</sup>

In earlier investigations,<sup>31,32</sup> the nature of the immune response resulting in protection in the NIH test was examined to assess the factors present in vaccines and the test that affect protection results. The results provided evi-

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dence that the correlation between IV administered humoral antibody and protection from IC challenge may occur as a result of virus-neutralizing activity, although there was also evidence that immune cell populations contribute to the clearance of infection and mortality rate after rabies virus infection in the CNS. Many factors inherent in rabies vaccine strain differences and the characteristics of the NIH test influence protection in mice, including administration route and number of doses,<sup>6</sup> the duration between vaccination and challenge, and the age of mice at the time of initial vaccination.<sup>2,32,33</sup>

The studies reported here were performed to investigate the effect of rabies vaccine and CVSs on protection in mice induced by commercial vaccines. The effects of various vaccination and challenge routes were also examined to determine how protection against different virus strains might be influenced.

## Materials and Methods

**Mouse strains**—Five<sup>2,33</sup> to 9-week-old female ICR mice were used for this study; ICR mice are an outbred strain routinely used for rabies vaccine testing and were purchased commercially.<sup>a</sup>

**Veterinary care**—The experiments were approved by the Institutional Animal Care and Use Committee at the Centers for Disease Control and Prevention. The NIH test is presently the only method approved for evaluating the potency of rabies vaccines prepared for use in humans and animals, although it requires that mice are challenged IC with a fixed-strain rabies virus that causes illness and death. Although it is difficult to reduce the severity of the disease in test animals given the nature of the infection, mice were observed daily after injection for signs of rabies infection and euthanatized when signs of rabies were detected.

**Vaccines and virus**—Vaccines used were commercial products prepared from inactivated preparations of rabies virus grown in tissue culture. The exception was a PRV strain-derived vaccine<sup>b</sup> of PM origin and grown in BHK tissue culture, provided in adjuvanted and nonadjuvanted form that was not licensed for commercial distribution at the time these studies were conducted. The potency of this vaccine was determined by use of NIH methodology to be 4.4 U/mL. A second adjuvanted vaccine was of SAD<sup>b</sup> tissue culture origin, grown on porcine kidney cell tissue culture, and had an NIH potency of 0.8 U/mL. Flury LEP vaccines,<sup>c</sup> adjuvanted (relative potency, 2.1 U/mL) and nonadjuvanted (relative potency, 3.2 U/mL), were grown on chicken fibroblast tissue culture. Another adjuvanted PRV strain-derived vaccine<sup>d</sup> (PRV-2) of PM origin and grown in BHK tissue culture had relative potency of 8.5 U/mL. Veterinary reference vaccine<sup>e</sup> was prepared from PM-strain rabies virus grown in BHK tissue culture.

**Challenge virus**—The identity of rabies challenge virus strains was confirmed by use of **monoclonal antibody (MAB)** typing, sequence analysis of **polymerase chain reaction (PCR)** products derived from a highly conserved region of the rabies N protein,<sup>34</sup> or both, and included CVS-11,<sup>1</sup> CVS-23,<sup>6</sup> Flury-LEP rabies virus,<sup>8</sup> LEP-C26,<sup>c</sup> dog (Thailand) rabies virus isolate 2960,<sup>1</sup> dog (Mexico) virus isolate 5951,<sup>1</sup> bat (Poland) SRV isolate,<sup>1</sup> and a vampire bat origin SRV isolated from a human (Peru) with rabies.<sup>1</sup>

Titers of these 8 rabies strains, which were prepared from mouse brain suspension and cell culture, were determined by inoculation of mice via multiple routes and sequential 10-fold dilutions. Where specified for vaccine potency testing, virus preparations were diluted to contain 10- to 100-

mouse IC LD<sub>50</sub>s in 0.03 mL for challenge by that route and equivalent mouse LD<sub>50</sub> in 0.03 mL for footpad or intramuscular challenge. Vaccines were diluted in NIH buffer,<sup>1</sup> and virus was diluted in 2% horse serum.

**Serum collection and viral neutralizing antibody titration**—Blood samples were collected from the ocular sinus of individual mice. Where specified, mice were uniquely marked with dye immediately after bleeding for identification. Serum separated from blood was inactivated for 30 minutes at 56°C. The **rapid fluorescent focus inhibition test (RFFIT)** was performed as described<sup>35</sup> to measure the **viral neutralizing antibody (VNA)** in the individual serum samples. The number of fluorescent foci was counted to estimate the infectious CVS dose administered to mice by the IC route. Fluorescein isothiocyanate-labeled antirabies antibody was purchased from a commercial source.<sup>h</sup>

**Vaccination and challenge**—Mice were vaccinated with various dilutions of the vaccines by use of equivalent doses administered either IP (0.5 mL/dose) or IM in the muscle of the hip region (0.1 mL/dose) as specified for individual experiments. Challenge was conducted by IC, intramuscular, or footpad injection with a volume of 0.03 mL. Deaths from rabies were confirmed by examining brain impression smears with fluorescein isothiocyanate-labeled antirabies antibodies<sup>36</sup> or by use of clinical signs, and mice were monitored for the time periods specified in each experiment. In every experiment, unvaccinated control mice were also challenged to ensure that the challenge was lethal. Virus titers and relative potency were determined by the method of Reed and Muench.<sup>37</sup>

**Challenge route titration**—In the first experiment, various routes of inoculation were used with CVS-11 grown in suckling mouse brains as the challenge virus. Five groups of 10 mice were challenged with 10-fold dilutions (starting with 10<sup>-1</sup>) of CVS-11 to determine the LD<sub>50</sub> for each route. Titration via the IC route was performed with 10<sup>-6</sup> through 10<sup>-8</sup> dilutions of stock virus. Multiple experimental titrations (5 IC, 3 intramuscular, 3 footpad) and single IM challenges in the hip, shoulder, or neck regions were used to determine the incubation period, defined as the time at which 50% of the rabies-induced deaths had occurred.

Seven replicate intramuscular challenges of 9-week-old unvaccinated mice in groups of 10 were performed with 3 sequential 10-fold dilutions of CVS-11 rabies virus to determine the reproducibility of mortality rate resulting from this route of administration. In addition, 9-week-old mice (10/group) were challenged by intramuscular inoculation with five 10-fold dilutions of CVS-11 and each of the 4 SRV strains. Mortality rate was recorded for 3 weeks after challenge. This experiment was repeated with similar or identical dilutions of SRV and CVS-11.

**Challenge route and vaccine protection**—For the second experiment, 5 groups of 60 mice were vaccinated IM with 1 dose of 1 or 2 dilutions (1:2 and 1:20 for PRV and SAD; 1:2 for LEP) of 1 of the 3 adjuvanted vaccine preparations. Intramuscular, footpad, and IC challenges were performed 4 weeks after vaccination. Two 10-fold dilutions of CVS-11 challenge inoculum were chosen for estimated LD<sub>50</sub>s between 5 and 50 and 100 and 500 for each route to examine protection with various challenge doses. Challenge was administered by each route to 10 mice from each vaccine and challenge dosage group. Ten mice from each vaccine and dilution group were chosen at random, bled, and marked for identification for titration of serum VNAs. Unvaccinated mice were challenged (10/group) with the equivalent doses and a dilution 10-fold less than that given to vaccinated mice for the purpose of determining the LD<sub>50</sub> administered by all 3 routes.

**Vaccine and challenge virus strain heterogeneity**—In a third experiment, the NIH test (2-dose IP vaccination, IC challenge at 2 weeks) was compared with 1-dose IM vaccination

followed by intramasseter challenge at 4 weeks with adjuvanted and nonadjuvanted PRV and LEP vaccines and challenge with  $\geq 50$  LD<sub>50</sub>s of CVS 11, CVS 23, LEP, or LEP-C26. There were 10 to 20 mice in each treatment group, as specified. In later vaccine potency tests, 10 mice were used for each group because older mice had reduced variation, compared with that observed with 4-week-old mice.<sup>33</sup> Five to 10 mice were bled and marked for individual titration of serum VNAs.

In the final experiment, 6 sets of 150 mice each were administered 1 dose of vaccine (diluted 1:10) IM with either adjuvanted or nonadjuvanted PRV, adjuvanted PRV-2, adjuvanted or nonadjuvanted LEP, or a 1:5 dilution of SAD and challenged intramasseter 4 weeks later with three 10-fold dilutions of CVS 11 and the 4 SRV isolates (10 mice challenged for each strain and dilution of virus). An additional titration in unvaccinated control mice was performed concurrently to determine the virus dose used for challenge.

**Statistical analyses**—Analysis of variance was performed on all geometric mean serum VNA titers. The Fisher exact test or the  $\chi^2$  test was used to analyze the mortality rate data. For all comparisons, values of  $P < 0.05$  were considered significant.

## Results

Intracerebral and peripheral challenge routes were compared to establish the relative doses needed to achieve an LD<sub>50</sub> with CVS-11 (Table 1). Significant ( $P < 0.001$ ) differences were observed in the incubation period for rabies after challenge with CVS (3 sequential dilutions) for 3 of these routes (IC, intramasseter, and footpad), as indicated by the geometric mean time in days to achieve LD<sub>50</sub> and, to a lesser extent, the challenge dose (significant [ $P = 0.003$ ] only for footpad doses). Intramasseter challenge resulted in the shortest incubation periods across all doses, with mean time to death of 8.8, 7.9, and 7.4 days for 1, 10, and 100 LD<sub>50</sub>s, respectively. Although 1 LD<sub>50</sub> yielded similar mean times to death for footpad and IC challenges (10.4 and 10 days, respectively), 10 and 100 LD<sub>50</sub>s resulted in 8.9 and 8.1 days to deaths, respectively, for footpad injection, whereas 10 LD<sub>50</sub>s by the IC route yielded similar time to death (10.1 days) as 1 LD<sub>50</sub>, and 100 LD<sub>50</sub>s resulted in mean time to death of 9.1 days.

Intramasseter challenge with dilutions of CVS-11 stock virus resulted in reproducible death of unvaccinated mice (Table 2). Significant ( $P < 0.001$ ) variation was observed between incubation periods when different SRV strains were inoculated by the intramasseter route, and the Thai dog isolate (2960) had the longest incubation period (13.8 and 12.1 mean days to 50%

mortality rate at 4 and 40 LD<sub>50</sub>s, respectively). The Polish bat isolate injected by the intramasseter route resulted in means of 12 and 11.9 days to 50% mortality rate at 1 and 10 LD<sub>50</sub>s, respectively. Significant differences were observed in the incubation periods after intramasseter challenge with 1 or 10 LD<sub>50</sub> doses between that of CVS-11 (8.6 and 8.4 mean days for 5 and 50 LD<sub>50</sub>s, respectively) and either dog (Mexico) isolate 5951 (11.4 and 10.3 days for 8 and 80 LD<sub>50</sub>s, respectively [ $P = 0.008$ ]) or human (Peru) rabies virus isolates (11.3 and 10.7 days for 2 and 20 LD<sub>50</sub>s, respectively [ $P < 0.001$ ]). The highest challenge doses for CVS, Mexican dog, and Peruvian human isolates resulted in mean incubation periods that were not significantly different (7.9, 9.1, and 8.6 days, respectively) by use of 500, 800, and 200 LD<sub>50</sub>s, respectively.

Single-dose IM vaccination (1:2 dilution) of mice with PRV, SAD, or LEP resulted in significantly ( $P = 0.02$ ) different mean serum VNA titers between vaccines (Table 3). Although there was no significant difference between VNA titers elicited by the 1:20 doses of PRV and SAD, mice vaccinated with PRV vaccine had a mean titer more than 5 times greater than those administered SAD vaccine.

Challenge of single-dose IM vaccine recipients was performed with 20 to 200 LD<sub>50</sub>s of CVS virus by all 3 challenge routes. Regardless of serum VNA titers, no mice that received a 1:2 dilution of any of the 3 vaccines succumbed to even 200 LD<sub>50</sub>s of CVS-11 administered in the masseter muscle or footpad (Table 3). There were no significant differences in mortality rates between the PRV and SAD low-dose (1:20 vaccine dilution) recipients when 200 LD<sub>50</sub>s were inoculated by footpad ( $P = 0.16$ ); although 3 of 20 mice that received the 1:20 dilution of SAD died, no mice that received the low-dose PRV vaccine died.

There was high mortality rate in the SAD vaccine

Table 2—Mortality rates (%) in unvaccinated mice (groups of 10/dilution) administered 0.03 mL of CVS-11 stock rabies virus solution of various dilutions into the masseter muscle during 7 experiments

CVS dilution	Experiment No.							Mean $\pm$ SD
	1	2	3	4	5	6	7	
10 <sup>-2</sup>	100	100	100	100	100	100	100	100 $\pm$ 0.0
10 <sup>-3</sup>	100	90	100	90	80	100	100	94 $\pm$ 8
10 <sup>-4</sup>	70	70	70	70	30	80	60	64 $\pm$ 16

Table 3—Geometric mean  $\pm$  SD (U/mL) serum virus neutralizing antibody (VNA) titers obtained from 1-dose IM vaccination with Pasteur rabies virus (PRV), street Alabama Dufferin (SAD), and low egg-passage (LEP) vaccines and mortality rates (proportion) after challenge by footpad, intramasseter, or intracerebral routes 4 weeks after vaccination

Strain	Vaccine dilution	Mortality rate <sup>a</sup>			
		VNA	Footpad	Masseter	Cerebral
PRV	1:2	13.8 $\pm$ 7.1	0/20	0/20	1/10
	1:20	6.3 $\pm$ 8.3	0/20	0/20	3/10
SAD	1:2	4.4 $\pm$ 7.3	0/20	4/20	6/10
	1:20	1.1 $\pm$ 1.7	3/20	8/20	18/20
LEP	1:2	30.6 $\pm$ 19.9	0/20	0/20	2/10

<sup>a</sup>Cumulative totals (footpad and IM) from challenge with 20 to 200 LD<sub>50</sub>s of CVS as titrated in unvaccinated control mice.

Table 1—Mean time to death (days) and LD<sub>50</sub> estimates for challenge virus strain (CVS)-11 dose titration in mice challenged by intracerebral and peripheral routes

Challenge route	No. of replicate experiments	Mean time to death	Mean LD <sub>50</sub> <sup>*</sup>
Intracerebral	5	10	10 <sup>-7.5</sup>
Masseter muscle	3	8.8	10 <sup>-4.3</sup>
Neck region (IM)	1	NC	10 <sup>-3.7</sup>
Shoulder region (IM)	1	NC	10 <sup>-3.6</sup>
Hip region (IM)	1	NC	10 <sup>-3.4</sup>
Footpad	3	10.5	10 <sup>-3.8</sup>

<sup>\*</sup>Dilution of CVS-11 stock virus grown in brains of suckling mice. NC = Not calculated because only 1 experiment was performed.

recipients after intramuscular challenge (Table 3), with significant ( $P = 0.002$ ) differences between the mortality rates in this group and the PRV group at the low vaccine dose (1:20 dilution) but not between the high dose recipients of any of the 3 vaccines. There were no deaths in either group of PRV vaccine recipients or in the 1:2 dilution LEP recipients, regardless of the challenge virus dose, by either the footpad or intramuscular challenge routes.

Intracerebral challenge resulted in death in all vaccine treatment groups (Table 3). There were significant ( $P = 0.035$ ) differences in the mortality rates with this inoculation route between the SAD dose recipients and mice administered the same doses of PRV vaccine.

Mean serum VNA titers and mortality rates of mice were determined from NIH testing methods with adjuvanted and nonadjuvanted LEP vaccines and challenged with approximately 100 LD<sub>50</sub>s of CVS-11 and LEP virus stocks (Table 4). There was no significant difference between the mean serum VNA titers of mice that received adjuvanted or nonadjuvanted LEP preparations. Relative potencies determined for these 2 vaccines were slightly higher against the CVS-11 challenge than against the LEP virus strain (2.06 and 3.56 with adjuvanted vs 1.96 and 3.28 for nonadjuvanted vaccines, respectively). Differences observed in mean serum VNA titers or mortality rates with the 2 formulations and the 2 challenge strains were not significant ( $P > 0.1$ ).

This experiment was repeated with nonadjuvanted vaccines of PRV and LEP strain origin and a second set of rabies CVSs (CVS-23 and LEP-C26 [Table 5]). There was no significant difference ( $P = 0.32$ ) between mean serum VNA titers elicited by vaccination with the 2 vaccines of unique rabies strain origin. The relative potencies of the PRV vaccine with CVS-23 and LEP-C26 were almost identical (9.9 and 10.0 units, respectively), whereas the potency for the LEP vaccine with the homologous challenge virus strain (LEP-C26) had 2.5-fold greater potency than with the heterologous CVS-23 challenge (12.78 and 4.98 units, respectively). However, there were no significant differences in the mortality rates between vaccine groups or from use of different challenge strains.

Vaccination with a single dose and 1 dilution of these vaccines was done with adjuvanted formulations administered by IP and IM routes and IC challenge with CVS-11 and LEP challenge strains (Table 6). There was no significant difference between the mean serum VNA titers with either vaccine administered by IP or IM route at 4 weeks after vaccination or between mortality rates resulting from either of the challenge strains.

Table 4—Geometric mean  $\pm$  SD (U/mL) serum VNA titers after 2-dose IP vaccination with sequential dilutions of LEP vaccine in adjuvanted (+) or nonadjuvanted (–) formulae and mortality rates (proportion) from day-14 intracerebral challenge with CVS-11 or Flury-LEP rabies virus strains

Challenge adjuvant	strain	Vaccine dilution (reciprocal)			
		10	50	250	1250
+	VNA	22.8 $\pm$ 7.8	1.9 $\pm$ 2.1	0.3 $\pm$ 0.4	< 0.1
	CVS	1/16	5/16	12/16	15/16
	LEP	1/16	3/16	14/16	16/16
–	VNA	11.3 $\pm$ 10.3	0.6 $\pm$ 0.6	0.6 $\pm$ 0.5	< 0.1
	CVS	1/16	2/16	9/16	15/16
	LEP	0/16	3/16	9/16	16/16

Single-dose vaccination was repeated with only IM administration, by use of 3 dilutions of the nonadjuvanted preparations of the PRV and LEP vaccines and the CVS and LEP challenge strains injected into the masseter muscle (Table 7). There were no significant differences between the 2 vaccine preparations in the ability to elicit a serum VNA response or in the ability to protect against heterologous challenge strains.

In the final experiment, the efficacy of 4 commercially available vaccines derived from the 3 rabies strains most commonly used for preparing inactivated rabies vaccine was compared to test for differential protection between CVS challenge and a variety of SRV strains. The vaccines were administered IM with a single dose (groups of 10 mice for each SRV and for each virus challenge titer) to determine ability to protect the mice from intramuscular challenge at 4 weeks. Adjuvanted and nonadjuvanted formulations of PRV

Table 5—Geometric mean  $\pm$  SD (U/mL) serum VNA titers after 2-dose IP vaccination with sequential dilutions of nonadjuvanted PRV or LEP vaccine and mortality rates (proportion) from day-14 intracerebral challenge with CVS-23 or LEP-C26 rabies virus strains

Vaccine	Virus	Vaccine dilution (reciprocal)			
		10	50	250	1250
PRV	VNA	18.8 $\pm$ 20.1	1.1 $\pm$ 0.9	0.9 $\pm$ 0.9	< 0.1
	CVS	0/10	1/10	4/10	6/10
	LEP	0/10	0/10	2/10	9/10
LEP	VNA	10.3 $\pm$ 6.2	3.0 $\pm$ 4.9	0.2 $\pm$ 0.3	< 0.1
	CVS	0/10	0/10	6/10	8/10
	LEP	0/10	0/10	2/10	7/10

Table 6—Geometric mean  $\pm$  SD (U/mL) serum VNA titers after 1-dose IP or IM vaccination with adjuvanted PRV or LEP vaccine and mortality rates (proportion) from day-28 intracerebral challenge with CVS-11 or LEP rabies virus strains

Strain	Vaccination route	Challenge strain	Serum VNA	Mortality rate
PRV	IP	NA	11.5 $\pm$ 6.7	NA
		CVS		1/20
		LEP		1/20
	IM	NA	10.6 $\pm$ 10.2	NA
		CVS		3/20
		LEP		0/20
LEP	IP	NA	9.9 $\pm$ 6.4	NA
		CVS		2/20
		LEP		0/20
	IM	NA	6.4 $\pm$ 1.8	NA
		CVS		1/20
		LEP		1/20

NA=Not applicable.

Table 7—Geometric mean  $\pm$  SD (U/mL) serum VNA titers after 1-dose IM vaccination with nonadjuvanted PRV or LEP and mortality rates (proportion) from day-28 intramuscular challenge with CVS-23 or LEP-26C rabies virus strains

Vaccine	Challenge strain	Vaccine dilution (reciprocal)		
		2	20	200
PRV	VNA	15.8 $\pm$ 16.9	3.9 $\pm$ 3.8	0.6 $\pm$ 1.3
	CVS	0/20 (0)	2/20 (10)	7/20 (35)
	LEP	1/20 (5)	0/20 (0)	15/20 (75)
LEP	VNA	5.2 $\pm$ 3.8	1.7 $\pm$ 0.7	0.2 $\pm$ 0.3
	CVS	0/20 (0)	1/20 (5)	9/20 (45)
	LEP	0/20 (0)	3/20 (15)	8/20 (40)

Table 8—Percentage mortality rates in mice (groups of 10/viral dilution) vaccinated IM with adjuvanted (+) or nonadjuvanted (–) rabies vaccines of PRV, LEP, or SAD strain origins and challenged by intramasseter inoculation with serial dilutions of CVS and 4 street rabies virus isolates of dog, bat, or human

Challenge strain	Virus titer†	Mortality rate					
		PRV*		PRV2	LEP		SAD
		+	–	+	+	–	+
CVS	200	0	40	0	0	40	80
	20	0	10	0	0	0	20
	2	0	10	0	0	0	0
Dog (Mexico)	2,000	10	30	0	0	10	20
	200	0	0	0	0	10	0
	20	0	0	0	0	0	0
Dog (Thailand)	150	0	0	0	0	0	10
	15	0	0	0	0	0	0
	1.5	0	0	0	0	0	0
Bat (Poland)	90	0	0	10	0	30	10
	9	0	0	0	0	30	0
	0.9	0	0	10	0	0	0
Human (Peru)	300	10	0	10	0	0	20
	30	0	0	0	0	0	0
	3	0	0	0	0	0	0

\*Mice were administered 1 dose of a 1:10 dilution of all vaccines except SAD (1:5 dilution) by IM injection and challenged 4 weeks later. †Titer is given as the number of mouse intramasseter 50%-lethal doses determined by titration in unvaccinated mice.

and LEP vaccines were used. To control for the elicited protection against the parental strain, a second Pasteur-derived vaccine, PRV2, of equal or higher efficacy than the PRV preparation used in previous experiments was included in the study.

Both adjuvanted PRV vaccines, diluted 1:10, protected  $\geq 90\%$  of recipient mice against all dilutions of all viruses (Table 8). There were few differences between these 2 vaccines in protection against the various CVSs. The same dose of nonadjuvanted PRV protected recipients less effectively ( $P = 0.039$ ). Adjuvanted and nonadjuvanted LEP vaccines yielded similar results, with significantly ( $P = 0.002$ ) better protection in the adjuvanted vaccine recipients.

The SAD vaccine, diluted 1:5, did not elicit complete protection against any SRV isolate, although  $\geq 80\%$  of vaccinated mice were protected from high-dose challenge ( $> 50$  LD<sub>50</sub>s) of all but the CVS. This vaccine is of lower relative potency than the other vaccines used by NIH testing.

## Discussion

Results of other investigators<sup>38</sup> are similar to ours, which indicated that peripheral virus challenge required at least 1,000 times more virus to reach LD<sub>50</sub>, compared with IC challenge. Also in agreement with earlier studies, viral LD<sub>50</sub> increased as distance of the challenge site from the brain increased, whereas the incubation period was dependent on the distance of the inoculation site from the brain<sup>39</sup> and, to a lesser extent, the virus dose.<sup>4</sup>

Longer incubation periods were observed with the IC challenge, compared with intramasseter inoculation, most likely because the virus dose was less by a factor of  $> 1,000$ . By the IC route, 80 to 90% of the inoculum is deposited into the circulation,<sup>40</sup> and few infectious virions may remain at the site of inoculation. Although adequate to cause 100% mortality rate in mice, the virus dose that infects brain cells may be less than the dose that reaches the brain via the intramasseter route. As would be expected, increasing the IC virus dose shortened the incubation period.

Results of the experiments reported here support

earlier data that suggest that the incubation period for rabies virus infection is dependent on both inoculation site<sup>26</sup> and dose.<sup>38</sup> The results also provide evidence that the rabies virus strain can affect the incubation period when the inoculation site and the dose of virus administered remain relatively constant.

The intramasseter (peripheral) challenge route, proposed as an alternative to IC inoculation for rabies vaccine testing,<sup>4,38</sup> is relatively easy to perform. It also results in reproducible mortality rates in unvaccinated mice with a standardized CVS-11 stock virus and allows the use of more dilute inoculum than other peripheral routes. Because intramasseter challenge is also used for target species,<sup>41,42</sup> this route appears optimal for replacing IC challenge in rabies vaccine testing. In support of results described previously,<sup>4,38</sup> intramasseter challenge of mice provided a more accurate model of target species efficacy testing and has potential for use in a sensitive, rapid, and reproducible method for measuring rabies vaccine efficacy.

Intramuscular vaccination is a common and efficacious route of vaccination for target species<sup>42</sup> and accurately models practical use. Results of previous experiments indicate that single-dose IM vaccination of mice results in equal or better protection than the IP route when IC challenge is performed 4 weeks after vaccination or later.<sup>32</sup> To determine whether the challenge route affected protection outcome, 3 commercially available rabies vaccines (2 with well-documented efficacy in target species) were used to compare vaccine efficacy against intramasseter, footpad, and IC challenge with fixed strain rabies virus. Adjuvanted PRV, SAD, and LEP vaccines had been evaluated by use of the NIH test with relative potencies of 4.4, 0.8, and 2.1 U/mL, respectively. After IM vaccination with a single dose of each vaccine, significant differences in antibody titer were observed with each of these vaccines at 4 weeks. No significant differences in mortality rates among the 3 vaccines were observed after footpad challenge at any of the doses administered, but there were significant differences in mortality rates after intramasseter challenge between the PRV and SAD vaccines when low vaccine doses were

administered. Overall, IC challenge resulted in significantly higher mortality rates in the SAD vaccine groups than in the other vaccine groups.

The rabies virus strain used for challenge has been repeatedly shown to affect NIH potency-testing results.<sup>1,4,30</sup> In that test, the reference vaccine to which the test vaccines are compared and the challenge virus are both of Pasteur strain origin. As a result of this, it is not surprising that vaccines prepared from rabies strains of Pasteur derivation have a potency advantage over non-Pasteur rabies strain-origin vaccines.<sup>1,4,30,43</sup> This potency advantage is not observed when these comparisons are made with vaccines and non-Pasteur origin rabies CVSs of direct homology to the test vaccine preparation.<sup>2,4,43</sup>

In an attempt to repeat those earlier results,<sup>1,4,30,43</sup> nonadjuvanted PRV and LEP vaccines were evaluated to attempt to maximize the effect of virus strain heterogeneity. Our results failed to duplicate the results associated with differences among NIH test strains reported by other investigators using nonadjuvanted LEP vaccine against challenge with the available stock CVS (Pasteur) and LEP (Flury) virus strains. However, a 2.5-fold difference in potency was detected in the NIH test when CVS-23 was compared with the stock virus strain (LEP-C26) from which the LEP vaccine was produced. Remarkably, similar potency differences were observed when comparing the NIH potency results with the ATCC strain LEP rabies virus challenge and the LEP-C26 strain, although the 2 virus strains are indistinguishable by MAb typing (antigenic analysis) and PCR sequence typing. The replicate experiment using nonadjuvanted PRV vaccine (Pasteur origin) also revealed an equal potency difference between the 2 CVSs, despite the fact that these 2 virus strains are also indistinguishable by use of antigenic analysis. In fact, this potency difference occurs despite the fact that the differences in mortality rates were not significant.

More important for this study, the CVS-11 and LEP stocks used to challenge both forms of the LEP vaccines resulted in nearly equivalent potencies when used for challenge in the NIH test, and the CVS-23 and LEP-C26 strains used to challenge PRV-vaccinated mice resulted in similar NIH potency values. These results appear to indicate that the effect of vaccine strain on potency may be less important than previously reported<sup>1,4,30,43</sup> and may be an artifact of the potency model and mortality rate analysis that were used.

This conclusion is supported by results obtained with single-dose vaccination (IP or IM) with these vaccines and a 4-week interval between vaccination and IC challenge, which indicated similar protection results irrespective of the virus strain used for challenge. There were only non-significant differences in the mortality rates for the 2 vaccines when the LEP and CVS challenges were compared (2.5 and 5%, respectively), across the vaccine administration routes. When a single-dose vaccination was used and followed by intramuscular challenge at 4 weeks, there were again no significant differences in results obtained with various adjuvanted vaccines or CVSs.

Another concern with NIH testing is the observation that fixed-strain CVS rabies virus has infectivity and pathogenesis characteristics quite different from SRV isolates,<sup>4</sup> causing doubt that protection can be directly correlated with protection against SRV strains. However,

in 1 report<sup>2</sup> of vaccine testing that included vaccines of the same 3 strain derivations used in our studies and a SRV strain (fox isolate) challenge, good protection was noted after mice were vaccinated with all 3 vaccines.

Protection failures in potency testing are reported to be more pronounced when the CVS is an SRV strain that differs markedly (by antigen reactivity to antibodies) from the typical canine SRV strains, and the vaccine strains associated with poor protection appear to vary in their ability to protect against the more divergent SRV isolates used for challenge.<sup>17-19</sup> For these reasons, our experiments were performed with 2 dog SRV isolates of widely differing geographic origin (Mexico and Thailand) with distinctive nucleocapsid gene bases.<sup>33</sup> In addition, 2 bat rabies isolates of European and American origin were selected to study the effect of these divergent SRV isolates on protection. These SRVs were compared for ability to overcome protection with several vaccines of high relative potency (as determined by NIH and 1-dose intramuscular testing) and of 3 different strain origins.

The rabies viruses were inoculated into the masseter muscle of vaccinated mice because this is the most severe challenge site for peripheral (natural) rabies virus infection.<sup>38</sup> Of the virus strains tested, fixed-strain CVS rabies virus appeared to be the most pathogenic for mice. All 3 vaccines resulted in better protection in recipient mice against even 150 to 200 LD<sub>50</sub>s of the 2 canine SRVs than against CVS challenge. Even when 2,000 LD<sub>50</sub>s of dog (Mexico) virus were used to challenge recipients, good protection was induced by all the vaccines. All vaccines also protected mice against infection with the bat (Poland) and human (Peru) SRV isolates.

Although small sample sizes prevented conclusive determination of the effect of vaccine strain on protection, it did appear that adjuvanted vaccines had an advantage over nonadjuvanted vaccine preparations in single-dose vaccination (using a 4-week vaccination-challenge interval and an intramuscular route with the SRV strains used), as observed for the PRV and LEP vaccines.

Combined with previous results,<sup>32</sup> the observations reported here allow the definition of a new method of rabies vaccine testing by single-dose, IM vaccination of mice followed at 4 weeks by intramuscular challenge with a lethal dose of fixed-strain virus. Such a test would more closely model the practiced routes of vaccination and natural exposure in target species and will provide accurate information regarding the primary immunogenicity of a given vaccine. In addition, this test would be easier to perform, because only a single dose of vaccine is administered, and because it eliminates the unnatural, difficult, and traumatic IC challenge presently recommended. Such a procedure would offer improvements that could increase the efficiency of vaccine potency testing conducted both by vaccine producers and agencies responsible for approving the use and distribution of vaccines. This could result in faster approval of vaccine production changes and a reduction in the number of animals required to complete the testing and approval process. Support for this methodology has been revealed by use of an extensive and direct evaluation of protection elicited by a rabies vaccine used to compare protection across a range of SRV strains.<sup>44</sup>

<sup>a</sup>Harlan Sprague Dawley Inc, Indianapolis, Ind.

<sup>b</sup>Provided by Keith Haffer, Pfizer Laboratories, Lincoln, Neb.

<sup>c</sup>Provided by Volker Franke, Chiron Inc, Marburg, Germany.

<sup>d</sup>Provided by Jeannine Gilbert, Merial Inc, Athens, Ga.

<sup>e</sup>National Veterinary Service Laboratory, USDA, Ames, Iowa.

<sup>f</sup>Centers for Disease Control and Prevention, Atlanta, Ga.

<sup>g</sup>American Type Culture Collection, Rockville, Md.

<sup>h</sup>BBL Microbiological Systems, Cockeysville, Md.

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