Effects of vaccine route and dosage on protection from rabies after intracerebral challenge in mice

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Objective—To evaluate the effect of various routes of administration and number of doses of 3 commercially produced rabies vaccines on serum antibody responses and protection in mice challenged by intracerebral injection with fixed-strain rabies virus.

Animals—2,213 mice.

Procedure—Inactivated, adjuvanted rabies vaccines were administered to mice in either 2, 1, or 0 (control) doses via IP, IM, and SC routes, and mice were challenged intracerebrally with fixed-strain rabies virus.

Results—Vaccination route and dose number significantly influenced serum antibody responses and protection from rabies virus challenge, independent of vaccine strain origin and mouse strain, although mouse age significantly affected results. Extended challenge studies revealed that IM vaccination of mice resulted in the highest serum neutralizing antibody responses and protection levels equivalent to IP vaccination. Even multiple doses administered SC (a vaccination route used in dogs) resulted in poor serum anti-rabies neutralizing antibody responses in mice and were far less protective than other routes.

Conclusions and Clinical Relevance—Findings suggest possible improvements for the current rabies vaccine potency test in mice by using 1 dose, the IM route, and a delayed time of challenge. These modifications would more closely model vaccination practices in target species and yield more accurate information regarding primary immunogenicity of a vaccine. (*Am J Vet Med* 2003;64:491–498)

The **National Institutes of Health (NIH)** potency test for rabies vaccines[1] has been criticized, because the testing methodology differs markedly from accepted immunization and natural infection routes in humans or domestic animals,[2-5] but it remains the officially recommended means of evaluating the protective potential of commercial vaccines developed for use in all target species.[3,6] The most notable differences are the following: 1) the **challenge virus strain (CVS)** has infectivity and pathogenesis characteristics that differ from street rabies virus strains[5]; 2) vaccination in the NIH test incorporates a booster dose that may allow a vaccine with poor primary immunogenicity to test well[3,6]; 3) the NIH test has virus-strain bias[6-11]; 4) the weight, age, and strain of a mouse cause variation in test results; 5) the **intracerebral (IC)** challenge route is quite different from the natural route of infection that typically results from bite wounds; and 6) the interval between vaccination and challenge varies greatly in nature[12] but is quite brief in this test. However, no other test has been developed that meets the requirements of a vaccine protection model along with the simplicity, speed, and reproducibility needed for inexpensive and timely potency testing.[6] Rabies vaccine development would be facilitated by an efficacy test in mice that corrects the deficiencies of the NIH test.[12,13]

Protection in the NIH potency test correlates well with serum **virus neutralizing antibody (VNA)** responses to rabies virus **glycoprotein (G protein)**.[2,14-20] Nevertheless, the immunologic parameters of the response to the IP vaccination and IC challenge routes are not well understood. Protection from lethal challenge may result from humoral VNAs that simply neutralize free rabies virus in the CNS before it can infect nerve cells.[20] Moreover, following IC injection, most of the inoculum enters the bloodstream,[21] perhaps allowing for neutralization of challenge virus to occur in circulation.

The purpose of the study reported here was to evaluate the effect of various routes of administration and number of doses of 3 commercially produced rabies vaccines on serum antibody responses and protection in mice challenged by IC injection with fixed-strain rabies virus. These and other factors were studied with the aim of developing a method for testing inactivated rabies vaccines that more closely resembles natural infection and current immunization practices. In providing a more accurate and predictive method, there is also the potential that fewer animals and less severe manipulations might be necessary to evaluate preparations of rabies vaccines for potency.

Materials and Methods

Mouse strains—Four- to 7-week-old female ICR (an outbred strain routinely used for testing rabies vaccines) and BALB/c mice were used for this study. Both mouse strains were purchased from commercial sources.[a,b]

Veterinary care—The experiments were approved by the Institutional Animal Care and Use Committee at the Centers for Disease Control. The NIH test is presently the only method approved for evaluating the potency of rabies vaccines prepared for use in humans and other animals. The study reported here required the use of such methods for the purpose of finding a better potency model and a more natural and less adverse route of challenge. Although the ability to reduce the severity of the disease in test animals is difficult because of the nature of the infection, mice were observed daily after infection for signs of rabies infection, and whenever the signs indicated onset of rabies, mice were euthanatized.

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Vaccines and virus—The **Pasteur rabies virus (PRV)** strain-derived vaccine[c] virus used in these experiments was grown in BHK tissue culture. **Street Alabama Dufferin (SAD)** strain-derived vaccine[c] virus was grown on a porcine kidney cell line. Flury **low egg-passage (LEP)** strain-derived vaccine[d] virus was grown on cultured chick fibroblast cells. Each of these commercially prepared vaccines is adjuvanted and intended for veterinary use. The National Veterinary Service Laboratory[e] provided veterinary reference vaccine lots 84-3-1 and 89-3-1. Vaccines were diluted in NIH buffer.[1]

Titer of CVS-11 rabies virus in mouse brain suspension was determined before use, and the virus was diluted in 2% horse serum to contain 10 to 50 mouse 50% IC challenge lethal doses in a 0.03-mL volume for a single challenge.[1]

Vaccination and challenge—Mice (4 and 6 weeks old) were vaccinated with 1 (day 0) or 2 (days 0 and 7) doses via various dilutions of PRV, SAD, or LEP vaccines and various administration routes, as specified. Mice were administered 0.5 mL of vaccine by the IP route, and the equivalent vaccine dose (diluted one-fifth the dilution of the IP vaccine) was administered IM in the thigh or hip region and SC in the neck region by use of 0.1 mL, because a 0.5-mL volume was too great for these routes. The challenge in all experiments was IC[1] at the times specified after vaccination.

Serum collection and assay—Blood samples were collected from the orbital sinus of 3 to 8 mice 2 days before and, in some experiments, at various times after challenge, as specified. To prevent multiple bleeding of a single mouse, mice were individually marked with dye. Serum samples were inactivated for 30 minutes at 56°C. Rabies VNA titers were determined by the **rapid fluorescent focus inhibition test (RFFIT)**.[22] The detection limit of this assay is approximately 0.1 U/mL, and samples with values less than the limit of detection were assigned a value of zero in the data analysis. Where specified, **2-mercaptoethanol (2-ME)** treatment[23] was used to estimate IgM neutralizing antibody in serum samples by RFFIT titer comparison performed on treated and untreated aliquots. Fluorescein isothiocyanate-labeled anti-rabies antibody was purchased from a commercial source.[f]

Effect of mouse age—To test the effect of age on potency-test results, groups of 40 to 60 ICR and BALB/c mice were vaccinated with 1:100 and 1:200 dilutions of PRV vaccine at 4 and 6 weeks of age by use of NIH methods.[1] Serum VNA titers and mortality rate from IC challenge were determined for individuals in each group.

Vaccine route effects on pre- and postchallenge serum VNAs—Two groups of twenty-five 6-week-old BALB/c mice were administered 2 doses (days 0 and 7) of either 1:10 or 1:100 vaccine dilutions by the IP route. Three groups of twenty 6-week-old BALB/c mice were administered 1 equivalent dose (day 0) of PRV vaccine by the IP, IM (in the right hip region), or SC route. Two additional groups of 20 control mice each were not vaccinated.

All treatment groups and the untreated controls were challenged 14 days after vaccination. Four mice from each of the treatment and control groups were chosen at random for bleeding before challenge. Three mice from each double-dose group were bled and marked for identification on days 1, 4, 6, 8, 11, 14, and 21 after challenge, and 3 mice from each of the single dose groups were bled on days 4, 7, 10, 14, and 21 after challenge or until all mice in the treatment groups had died from rabies infection. Three nonvaccinated controls were bled at the same time after challenge until there were no survivors. Rabies VNA was determined for individuals in the single-dose groups with serum samples that both had or had not been treated with 2-ME to determine the proportion of the titer derived from IgM anti-rabies antibody.

Vaccine booster dose and route response—Six-week-old ICR and BALB/c mice were vaccinated with 1 or 2 doses of PRV vaccine dilutions with a 7-day interval between doses. Treatment groups consisted of 20 ICR and 15 BALB/c mice each, and mice were vaccinated either IP, IM, or SC. For vaccination by the IP route, 4 groups of ICR and 1 group of BALB/c mice were used. The ICR mice were administered 1 or 2 doses of vaccine diluted either 1:10 or 1:100, whereas BALB/c mice were administered 2 doses of the 1:100 vaccine. Mice admininstered vaccine by the IM route were similarly grouped and received 1:2 or 1:20 dilutions of vaccine, which was equivalent to the 1:10 and 1:100 doses by the IP route, respectively, because of the reduced injection volume used. For this route, the BALB/c mice received only 1 dose of the 1:2 vaccine dilution and 2 doses of the 1:20 vaccine dilution. For the SC route, 2 groups of ICR mice received 1 or 2 doses of either 1:2 or 1:20 vaccine dilutions. Four mice from each group were bled 2 days before challenge to determine individual serum rabies VNA titers. Challenge was performed 14 days after initial vaccination, and mortality rate was monitored for an additional 2 weeks.

Varying time of challenge in the NIH test—The PRV and LEP vaccines were each administered to 3 sets of ICR mice by use of the NIH method (days 0 and 7). Each set contained 4 groups of 16 mice that received vaccine diluted 1:10, 1:50, 1:250, or 1:1,250. Four to 8 mice were bled from each vaccine dilution group of each set and for each vaccine for rabies VNA determination. One of the 3 sets of mice (and nonvaccinated controls) was challenged at either 2, 4, or 8 weeks after initial vaccination, and mortality rate was monitored.

Varying time of challenge with 1-dose vaccination—Six-week-old ICR mice, in 2 sets of 3 groups of 20, were vaccinated by the IP (set 1) or IM (set 2) route with 1 dose of PRV diluted 1:10 or 1:2, respectively. Eight mice from each treatment group were bled 2 days before challenge; 1 group from each vaccine route and nonvaccinated controls were challenged 2, 4, or 8 weeks after vaccination. In addition, 1 group of 20 mice was vaccinated SC with a single 1:10 dose equivalent of PRV. Five mice were bled from this group 12, 26, and 54 days after vaccination to determine individual serum rabies VNA titer. These mice were challenged 8 weeks after vaccination. The experiment was repeated with the SAD vaccine, except only 5 mice were bled from each group, IM vaccination was in the hip region, and mice were bled at 2, 4, and 8 weeks, although challenge was only performed on 1 group of mice at 4 weeks.

Vaccine and route comparison—Four groups of 20 mice were vaccinated with a single dose (1:10 or 1:2) of either PRV or LEP vaccine by the IP or IM route. Five mice from 1 group of each vaccine treatment set were bled 5 or 26 days after vaccination and challenged 2 days later (1 and 4 weeks after vaccination).

Extended 1-dose vaccine and route comparison—Two sets of 240 mice were vaccinated with 1 dose of either PRV or LEP vaccines; for each vaccine, 4 groups of 60 mice were injected IP with either 0.5 mL of a 1:10 or 1:100 dilution or IM with 0.1 mL of a 1:2 or 1:20 dilution. Those 60 mouse groups were divided into 3 sets of 20 mice, and 1 set was challenged IC at either 4, 12, or 20 weeks. An additional 60 control mice were challenged with each group. Two days before each challenge, 5 mice from each vaccine and dilution group were bled to determine serum VNA titers.

Statistical analyses—Geometric mean serum VNA titers were determined for each treatment group, and ANOVA was used to measure the significance of observed differences. Differences in protection results among treatment groups were determined by use of the Fisher exact test or χ^2 analyses. Values of $P \leq 0.05$ were considered significant.

Results

Effect of mouse age—For both mouse strains, analysis (χ^2) of mortality rates revealed significant differences between 4- and 6-week-old mice at both vaccine dilutions (1:100 dilution, $P = 0.003$; 1:200 dilution, $P < 0.001$; **Table 1**). There were also 5-fold and ≥ 2-fold increases in mean serum VNA titers of the 6-week-old 1:100- and 1:200-dilution vaccine recipients, respectively, compared with those same vaccine preparations administered to 4-week-old mice.

Vaccine route effects on pre- and postchallenge serum VNAs—The BALB/c mice that were administered two 1:10 (protected and immune, 0% mortality rate) or 1:100 (marginally immune, 70% mortality rate) vaccine dilutions by IP route had serum rabies VNA titers that were remarkably different both before and for the first week after IC challenge, although these titers had become equivalent in survivors at 3 weeks after challenge (**Fig 1**). Mice that succumbed to rabies in the 1:100 dilution treatment group died 7 to 12 days after challenge.

Table 1—Geometric mean ± SD serum virus neutralizing antibody (VNA) titers and mortality rate in ICR and BALB/c mice vaccinated IP with 2 doses (weeks 0 and 2) of Pasteur rabies virus (PRV) strain-derived vaccine and challenged intracerebrally (IC) with challenge virus standard virus 4 weeks after intial vaccination

Mouse strain	Age*	Serum dilution	VNA (U/mL)	Mortality rate (dead/total [%])
ICR	4	1:100	0.34 ± 0.46	29/48 (60.5)
		1:200	0.22 ± 0.32	43/46 (93.5)
	6	1:100	1.87 ± 1.41	15/53 (28.3)
		1:200	0.64 ± 0.49	28/51 (54.9)
BALB/c	4	1:100	0.32 ± 0.39	31/48 (64.5)
		1:200	0.24 ± 0.17	51/56 (91.1)
	6	1:100	1.79 ± 1.06	11/45 (24.6)
		1:200	0.42 ± 0.53	22/41 (54.1)

*Mouse age in weeks at time of initial vaccination.

Mean serum rabies VNA titers (VNA titer after reduction with 2-ME and the fraction that was lost to reduction) and mortality rate before and after IC challenge of BALB/c mice that had been administered a single dose of PRV vaccine by IP, IM, or SC route were determined (**Fig 2**). Mice vaccinated IP had the highest mean serum rabies VNA titers before challenge, and the antibody was mostly non-IgM. After challenge, serum VNA titers increased rapidly and converted to non-IgM exclusively, and 4 of 10 mice died from challenge. Mice vaccinated IM had low VNA titers prior to challenge (mean, 0.08 U/mL), although titers increased quickly after challenge, and composition of the VNAs changed from mostly IgM antibodies to primarily non-IgM antibodies. Mortality rate in that group was 60%. Mice administered PRV by SC injection had no detectable VNA titers before challenge. Serum VNA concentrations increased slowly beginning 4 to 10 days after challenge, but all mice died or were euthanatized by day 14. Nonvaccinated mice died before any serum VNA could be detected.

Vaccine and route comparison—The effect of vaccine booster dose and route of administration on protection from rabies was determined by measuring the mean serum rabies VNA titers, as well as the mortality rate for each mouse strain, dose, and route (**Table 2**).

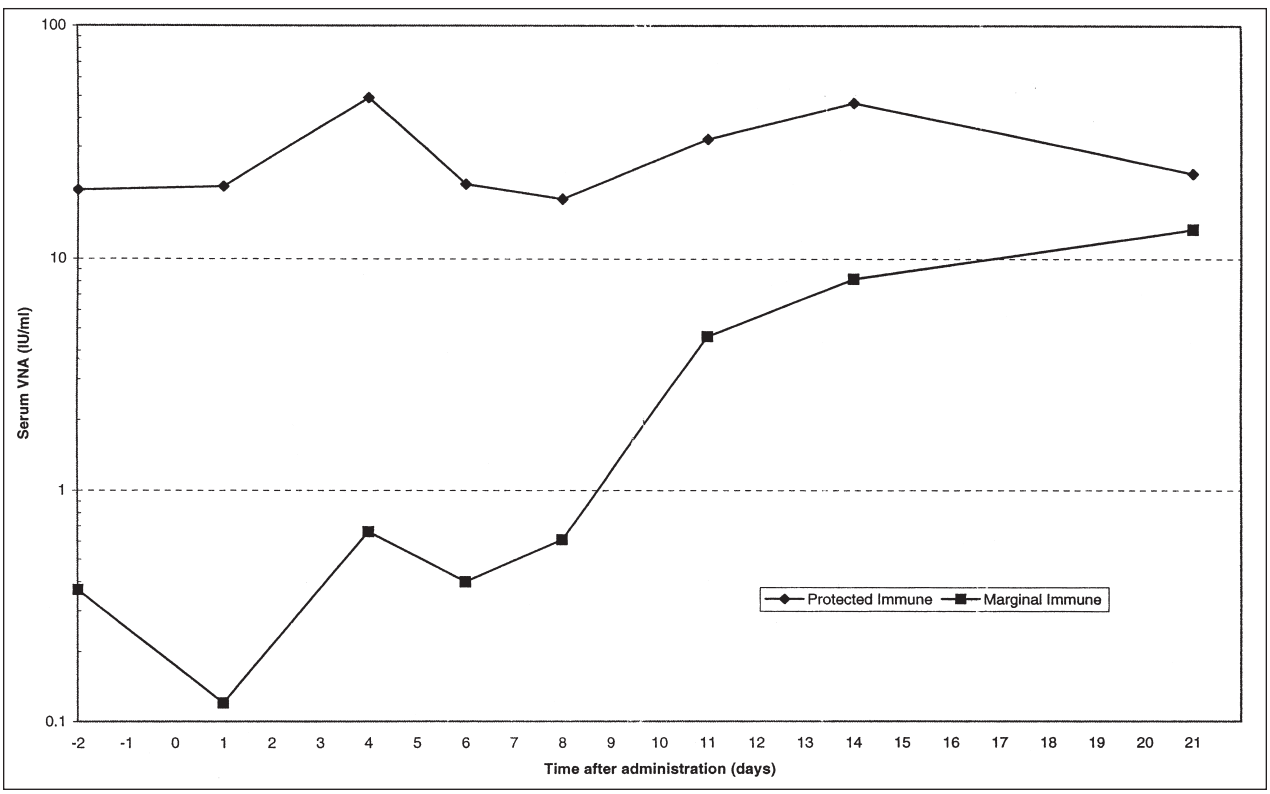

Figure 1—Geometric mean serum virus neutralizing antibody (VNA) titers before and after lethal intracerebral challenge (day 0) in BALB/c mice administered 2 doses of either a 1:10 (protected immune) or 1:100 (marginal immune) dilution of Pastuer rabies virus (PRV) vaccine by IP injection (National Institutes of Health protocol).

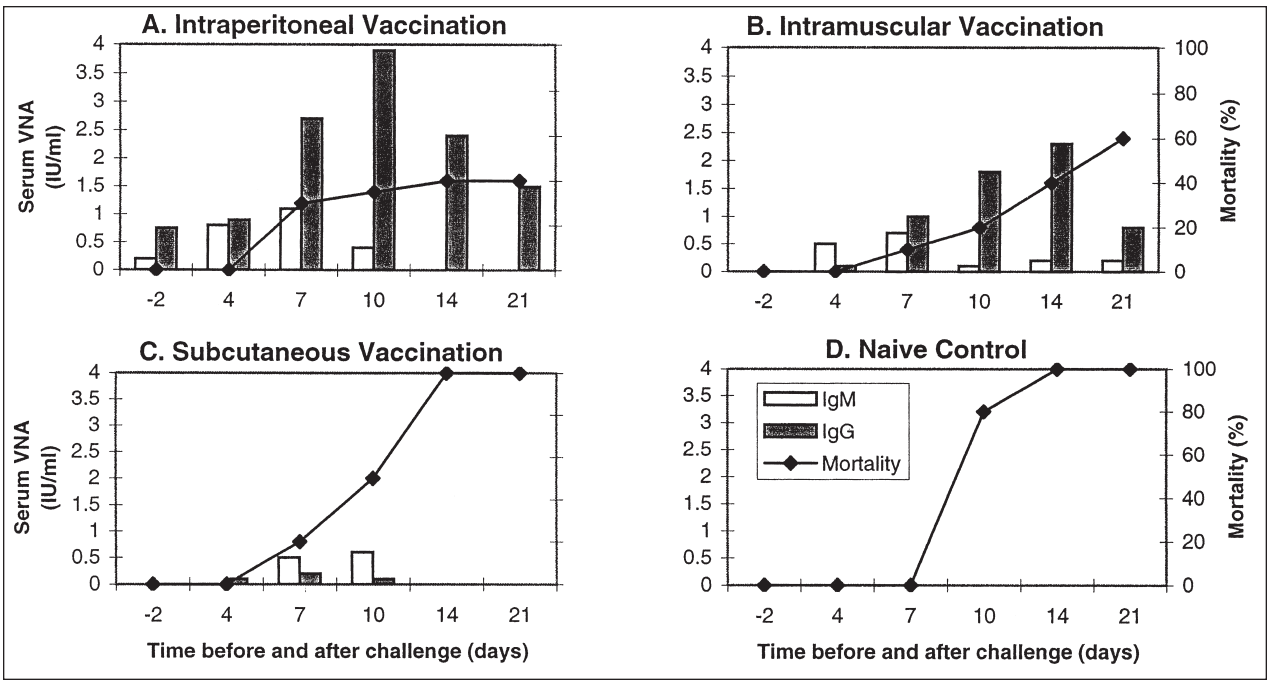

Figure 2—Geometric mean serum VNA titer: IgM (solid bars), IgG (open bars), and mortality rates (line) before and after intracerebral challenge in BALB/c mice administered a single equivalent dose of PRV vaccine by various routes 14 days before virus inoculation. IgM-derived VNA titer determined from 2-mercaptoethanol-treated serum; IgG VNA is total VNA less that determined for treated serum.

The results of serum rabies VNA testing indicated that 2 doses of vaccine administered IP or IM to ICR mice induced detectable antibodies in all recipients by day 12. All 4 tested mice that had been administered 2 doses of the 1:10 vaccine dilution IP had serum VNA titers > 16 U/mL, as did 3 of 4 mice that received 2 doses of the 1:2 dilution IM (the fourth mouse had 0.3 U/mL). The ICR treatment groups that received 2 doses of the 1:2 IP and 1:20 IM vaccines responded with significantly less VNAs, although all mice responded. Only 1 mouse of 4 had detectable serum VNAs (0.3 U/mL) after administration of 2 doses of the vaccine diluted 1:2.

All mice vaccinated IP with a single dose and tested for VNAs had positive results (0.1 to 3.1 U/mL). Six of 8 ICR mice vaccinated IM with a single 1:2 dose had rabies VNAs, whereas all 5 of the BALB/c mice vaccinated IM with a single 1:2 dose had VNAs (0.1 to 0.42 U/mL). No mice that received 1 dose of the 1:2 vaccine dilution by the SC route had detectable rabies VNAs by day 12 after vaccination; neither did any of the mice that received 1 dose of the 1:100 IP or 1:20 IM vaccine. Five of 8 ICR and 6 of 8 BALB/c mice administered a single dose of the 1:100 dilution IP had detectable VNAs.

The ICR mice that received the single-dose PRV vaccine had significantly (P = 0.002) lower geometric mean VNA titers before challenge (day 12) and higher mortality rate after challenge (P = 0.006) than those that received a second dose. There was no significant difference between the 1- versus 2-dose SC vaccinations. There was no significant difference between the BALB/c mouse strain data and the ICR strain data. A significant difference was detected in serum VNAs between the 2-dose 1:20 IM and 1:100 IP route data (P = 0.023) and also between the single-dose 1:2 IM and 1:10 IM route data (P = 0.038), but there was no significant difference in mortality rates between the 2 routes. Both IM and IP routes induced significantly (P < 0.001) higher serum VNA titers and protection results than the SC route.

Table 2—Effect of booster dose and route of administration on VNA titer (geometric mean ± SD) and mortality rate of mice vaccinated with PRV vaccine and challenged 14 days after initial vaccination

Mouse strain	Vaccine* route	Dilution	No. of doses	VNA (U/mL)	Mortality rate (dead/total [%])
ICR	IP	1:10	1	1.13 ± 1.12	8/20 (40)
			2	21.23 ± 3.10	0/20 (0)
		1:100	1	< 0.1	20/20 (100)
			2	0.28 ± 0.05	7/20 (35)
	IM	1:2	1	0.20 ± 0.23	13/20 (65)
			2	15.92 ± 10.45	0/20 (0)
		1:20	1	< 0.1	20/20 (100)
			2	1.85 ± 1.11	3/20 (15)
	SC†	1:2	1	< 0.1	20/20 (100)
			2	0.08 ± 0.04	15/20 (75)
BALB/c	IP	1:100	2	0.57 ± 0.51	9/15 (60)
	IM	1:2	1	0.18 ± 0.14	10/15 (60)
		1:20	2	0.85 ± 0.33	10/15 (67)

*1:10 and 1:100 dilutions for IP administration are equivalent to doses of 1:2 and 1:20, respectively, for IM and SC administration.

†Values for the SC route differ significantly from values for the IM route for VNA (P = 0.001) and mortality rate (P < 0.001) and from values for the IP route for VNA (P < 0.001) and mortality rate (P = 0.006).

Varying times of challenge in the NIH test—The ICR mice administered 1:10 dilutions of PRV and LEP vaccines by the NIH method had high (> 19 U/mL) serum rabies VNA titers through 8 weeks after initial vaccination (**Table 3**). Despite these titers, mice that received either the PRV or LEP vaccine were not completely protected at 2 weeks. There was no significant

difference between the serum VNA and mortality data derived with these 2 vaccines by this testing method, irrespective of time of challenge, but there was a significant ($P = 0.003$) vaccine dosage effect.

Various times of challenge and route with 1-dose vaccination—Mice vaccinated IP with a single 1:10 dose of PRV or SAD had higher serum antibody responses 12 days after vaccination than were detected with either of the other 2 routes (**Table 4**). All of the mice sampled after IP vaccination with PRV had titers ranging from 0.1 to 3.4 U/mL (geometric mean ± SD titer, 1.1 ± 1.1 U/mL). Intraperitoneal vaccination also appeared to give slightly better protection than the IM route with PRV vaccine at 2 weeks, and all mice vaccinated IP with PRV were protected from IC challenge at 4 and 8 weeks after vaccination. Six of 8 mice (thigh region) and 8 of 8 mice (hip region) administered a single IM dose of PRV and SAD vaccines, respectively, had detectable VNA titers at 2 weeks (0.2 ± 0.2 and 1.6 ± 1.4 U/mL). Thirteen of 20 mice in the IM PRV treatment group died from challenge at 2 weeks (Table 4), whereas 3 of 20 died when challenged either 4 or 8 weeks after vaccination. Intraperitoneal vaccination with SAD resulted in slightly better protection than IM administration at 4 weeks, although there was no difference in the results at 8 weeks. Serum VNA titers at 2 weeks in individual mice, whether vaccinated IP or IM, were significantly ($P = 0.012$) higher in mice that survived challenge (0.77 ± 0.43 U/mL) than in mice that died (0.24 ± 0.24 U/mL). Mortality rate was reduced in the IM SAD vaccine treatment group between 4 and 8 weeks, although serum VNA titers were not changed substantially (range, 0.3 to 13.0 U/mL; geometric mean titer, 6.8 ± 4.1 and 7.5 ± 4.1 U/mL, respectively).

None of the mice vaccinated SC with PRV or SAD vaccine had detectable serum VNAs at 2 weeks. At 4 weeks, no recipients of PRV vaccine and only 1 of 5 recipients of the SAD vaccine (0.9 U/mL) had serum VNAs; all of the SAD-vaccinated mice died after IC challenge. By 8 weeks, 1 of 4 (0.1 U/mL) and 0 of 5 mice vaccinated SC with PRV and SAD vaccines, respectively, had serum VNAs, and no mice were protected from rabies challenge by either vaccine (Table 4).

There were significant ($P < 0.01$) increases in serum VNA titers and protection (for PRV-vaccinated mice) in each 1-dose vaccine treatment group by all but the SC administration routes between 2 and 4 weeks. No significant difference was detected between serum VNA titers elicited by the 2 vaccines at any time point, although PRV vaccine induced significantly ($P <$

Table 3—Geometric mean ± SD serum VNA titers and mortality rates of ICR mice challenged at various times after IP vaccination (National Institutes of Health protocol) with PRV and Flury low egg-passage (LEP) vaccines

		Time of challenge after vaccination					
		2 wk		4 wk		8 wk	
Strain	Dose	VNA (U/mL)	Mortality rate (dead/total [%])	VNA (U/mL)	Mortality rate (dead/total [%])	VNA (U/mL)	Mortality rate (dead/total [%])
PRV	1:10	27.0 ± 17.1	0/16 (0)	17.5 ± 13.5	3/16 (19)	19.5 ± 15.1	1/16 (6)
	1:50	6.5 ± 11.5	3/16 (19)	14.5 ± 9.2	6/16 (38)	5.0 ± 0.5	3/16 (19)
	1:250	1.1 ± 1.1	9/16 (56)	1.1 ± 0.5	11/16 (72)	5.5 ± 5.5	10/16 (63)
	1:1,250	< 0.1	16/16 (100)	0.1 ± 0.1	13/16 (81)	0.4 ± 0.4	13/16 (81)
LEP	1:10	23.2 ± 8.1	1/16 (6)	22.9 ± 2.5	0/16 (0)	19.5 ± 11.0	0/16 (0)
	1:50	1.9 ± 1.9	4/16 (25)	7.1 ± 7.0	3/16 (19)	23.5 ± 6.5	3/16 (19)
	1:250	0.1 ± 0.1	10/16 (63)	1.1 ± 1.0	8/16 (50)	5.8 ± 2.2	4/16 (25)
	1:1,250	< 0.1	15/16 (94)	0.5 ± 0.5	12/16 (75)	< 0.1	8/16 (50)
Control		ND	16/16 (100)	ND	16/16 (100)	ND	16/16 (100)

ND = Not determined.

Table 4—Geometric mean ± SD serum VNA titers and mortality rates of ICR mice challenged at various times after single-dose vaccination via different administration routes

			Time of challenge after vaccination					
			2 wk		4 wk		8 wk	
Vaccine strain	Route*	Dose	VNA (U/mL)	Mortality rate (dead/total [%])	VNA (U/mL)	Mortality rate (dead/total [%])	VNA (U/mL)	Mortality rate (dead/total [%])
PRV	IP	1:10	1.1 ± 1.1	8/20 (40)	10.3 ± 6.4	0/20 (0)	11.8 ± 7.2	0/20 (0)
	IM	1:2	0.2 ± 0.2	13/20 (65)	12.5 ± 7.7	3/20 (15)	11.5 ± 8.2	3/20 (15)
	SC	1:2	< 0.1	NC	< 0.1	NC	< 0.1	20/20 (100)
SAD	IP	1:10	1.6 ± 1.4	NC	6.8 ± 4.1	10/20 (50)	7.5 ± 4.1	8/20 (40)
	IM	1:2	0.7 ± 0.6	NC	4.2 ± 3.9	16/20 (80)	8.5 ± 5.2	6/20 (30)
	SC	1:2	< 0.1	NC	0.2 ± 0.2	20/20 (100)	< 0.1	NC
Control	NA	NA	ND	15/16 (94)	ND	16/16 (100)	ND	16/16 (100)

*Equivalent doses were delivered by diluting 1:10 for IP and 1:2 for IM or SC administration.
SAD = Street Alabama Dufferin strain virus. NC = Not challenged until 4 or 8 weeks. NA= Not applicable.
See Table 3 for remainder of key.

0.02) greater protection than did the SAD vaccine at 4 and 8 weeks after IP and IM vaccination. Serum VNA titers and protection were maintained through 8 weeks after vaccination for both vaccines (Table 4). All the mice tested at 4 and 8 weeks had serum VNAs from 0.3 to 20 U/mL (IP and IM vaccine recipients), with no significant differences between these routes.

One-dose vaccine and route comparison at 1 and 4 weeks—To clarify the differences between single-dose vaccination by the IP and IM routes, serum rabies VNA responses and protection were monitored in mice administered undiluted PRV and LEP vaccines and challenged 1 and 4 weeks later. Mean VNA titers 2 days before challenge (days 5 and 26) and mortality rate from IC challenge were determined **(Table 5)**. All mice vaccinated IP with either vaccine had responded by 5 days after vaccination (0.1 to 1.1 U/mL) and had the same mortality rate after challenge at 7 days, and both vaccines elicited a significant ($P = 0.037$) increase in VNA titer and protection by 4 weeks.

Four of 8 mice tested for serum VNAs that received LEP vaccine IM responded after 5 days, whereas only 1 of 8 mice that received PRV vaccine IM had VNAs. The differences in VNAs and mortality rates were significant ($P = 0.006$ and $P = 0.002$, respectively) in these 2 vaccine groups (Table 5). All mice vaccinated IM with PRV vaccine died as a result of challenge on day 7, whereas mortality rate was 60% in the matched LEP group. There was a slight protective advantage at 1 week with administration of LEP vaccine by the IP route and a significant ($P = 0.002$) advantage with PRV vaccine by the IM route. When challenged 4 weeks after vaccination, there was no significant difference in either VNA titer or mortality rate in these treatment groups. Antibody and protection increased with time after vaccination with a highly significant ($P < 0.001$) P value.

Table 5—Geometric mean ± SD serum VNA titers and mortality rates of ICR mice administered a single dose of diluted PRV or LEP vaccine by IP or IM routes and challenged 1 or 4 weeks after vaccination

		Time of challenge after vaccination			
		1 wk		4 wk	
Vaccine strain	Route	VNA (U/mL)	Mortality rate (dead/total [%])	VNA (U /mL)	Mortality rate (dead/total [%])
LEP	IP	0.40 ± 0.3	9/20 (45)	9.9 ± 6.4	2/20 (10)
PRV		0.45 ± 0.4	9/20 (45)	11.5 ± 6.7	1/20 (5)
LEP	IM	0.13 ± 0.2	12/20 (60)	6.4 ± 1.8	1/20 (5)
PRV		0.02 ± 0.06	20/20 (100)	10.6 ± 10.2	3/20 (15)
Control		ND	15/15 (100)	ND	15/15 (100)

Extended 1-dose vaccine and route comparison—Serum VNA and protection responses were monitored from 4 to 20 weeks after mice were vaccinated with a single dose of either PRV or LEP vaccine by the IM and IP routes **(Table 6)**. Mice vaccinated IP with PRV vaccine had the highest titers at 4 weeks with the 1:10 and 1:100 dilutions, after which time the titers and mortality rate after IC challenge remained fairly constant through 20 weeks.

Mice administered a 1:2 dilution of PRV vaccine by the IM route had highest VNA titers at 12 weeks, and as titers decreased at 20 weeks, mortality rate increased, although not significantly, from 0 to 20%. The mice that received the same vaccine IM at a lower (1:20) dose had increased titers at 20 weeks and were never completely protected, although protection was maintained at 95% through 20 weeks.

Mice administered a 1:10 dose of LEP vaccine by the IP or IM route had increasing titers through the course of testing and were completely protected at 12 and 20 weeks. In the lower-dose IP administration group, mean serum VNA titers and mortality rate were almost constant through 20 weeks. The 1:20 vaccine dilution administered IM gave increased protection with time, and mortality rate decreased significantly ($P < 0.001$) from 45% at 4 weeks to 0% at 12 weeks, whereas the mean serum VNA titer increased from 2.88 to 11.82 U/mL ($P = 0.002$). Variation in the sera tested decreased 2-fold (4 week SD, 4.07; 12 week SD, 1.84). By 20 weeks, although the mean titer for the treatment group doubled (22.18 U/mL), titers of the mice tested had a 10-fold increase in SD (23.47). Mortality rate after IC rabies challenge at 20 weeks was increased, although not significantly, compared with the 12-week group.

Table 6—Mean serum VNA titers mortality of ICR mice challenged at various times after 1-dose IP and IM vaccination with 2 dilutions of PRV and LEP vaccines

			Time of challenge after vaccination					
			4 wk		12 wk		20 wk	
Strain	Vaccine route[a]	Dose	VNA[b] (U/mL)	Mortality rate (dead/total [%])	VNA (U/mL)	Mortality rate (dead/total [%])	VNA (U/mL)	Mortality rate (dead/total [%])
PRV	IP	1:10	13.5 ± 10.5	4/20 (20)	11.5 ± 2.0	0/20 (0)	11.5 ± 0.5	0/20 (0)
		1:100	3.2 ± 1.9	8/20 (40)	1.1 ± 0.5	12/20 (60)	2.4 ± 2.3	11/20 (55)
IM		1:2	8.9 ± 9.0	0/20 (0)	16.1 ± 2.5	0/20 (0)	9.2 ± 6.8	4/20 (20)
		1:20	2.9 ± 1.6	3/20 (15)	2.1 ± 1.9	2/20 (10)	12.6 ± 9.4	2/20 (10)
LEP	IP	1:10	4.5 ± 2.5	2/20 (10)	12.5 ± 0.5	0/20 (0)	28.0 ± 22.5	0/20 (0)
		1:100	1.4 ± 1.9	10/20 (50)	1.9 ± 0.6	8/20 (40)	1.1 ± 0.5	10/20 (50)
IM		1:2	6.9 ± 7.0	0/20 (0)	12.1 ± 0.9	0/20 (0)	38.8 ± 25.2	0/20 (0)
		1:20	2.9 ± 4.1	9/20 (45)	11.8 ± 1.8	0/20 (0)	22.1 ± 23.4	2/20 (0)
Control			ND	20/20 (100)	ND	20/20 (100)	ND	20/20 (100)

[a]Equivalent doses were delivered by diluting 1:10 for IP and 1:2 for IM or SC administration. [b]Expressed as geometric mean titer ± SD.
See Table 3 for remainder of key.

Discussion

The age of mice has been observed to affect potency in the NIH potency test.[12] Mice are chosen for vaccination on the basis of weight (11 to 15 g), but recommendations for selection on the basis of age (4 weeks) have also been made.[1] Our results suggest that delaying vaccination by as little as 2 weeks can substantially affect results of potency testing, perhaps by allowing for additional maturation of immune response. Increased immunoresponsiveness has been correlated with age for a variety of vaccines and species; because the G protein is the rabies antigen most directly implicated in eliciting a protective response, this may result directly from slower maturation of responses to carbohydrate epitopes. Our results indicate that delaying the time of initial vaccination of mice from 4 to 6 weeks of age will produce more reliable data for potency testing.

Earlier experiments also revealed a correlation between protection and serum VNA responses[2,14-18,20] and indicated that anti-rabies neutralizing antibody, particularly that directed against the G protein, is of primary importance in protection against rabies in this test.[2,14,15] Studies[24-27] of cellular immune responses indicate that they are elicited by rabies virus infection or vaccination. Observations that rabies nucleocapsid protein is capable of protecting animals[28-29] and observations of protection failures in animals despite high serum VNA concentrations[4] have raised further questions regarding immunity to rabies in natural infection and vaccine potency tests.[4,28,29] The results of experiments described here support the correlation between serum rabies VNA response and protection from IC challenge, regardless of vaccination route and the number of doses administered.

Concerns regarding NIH potency testing include vaccine administration route and number of doses.[2-4] Commercial rabies vaccine preparations are never administered IP for any species, nor are booster doses of vaccine typically administered < 6 to 12 months after the initial dose. Our studies were performed to compare the serum VNA responses of mice administered vaccine IP (as in the NIH potency test) with the single dosage and routes (IM and SC) used in rabies vaccination. Results indicated that the booster effect of a second vaccine dose in the NIH test was dramatic, particularly at the higher (1:10 dose equivalent) vaccine dilutions. It not only resulted in decreased mortality rate, compared with the single-dose vaccination at 2 weeks, but also greatly enhanced the serum rabies VNA response. This effect occured with IM vaccination as well, but not when vaccines were administered to mice SC.

Serum rabies titers and protection results at 2 weeks in mice given 1 vaccine dose IP appear to be consistently higher than mice given 1 vaccine dose IM. However, in both groups, VNA titers of mice given 1 dose increased quickly after the 2-week challenge, and the composition of the VNAs converted from ≤ 30% to mostly (≥ 70%) or completely non-IgM antibody during the following 3 weeks. Serum VNA titers in both groups peaked 10 to 14 days after challenge and then declined. This is different than what is observed with the 2-dose vaccination method used in the NIH test, in which serum VNA titers begin at much higher concentrations, are relatively insensitive to 2-ME reduction (< 15%),[20] and have no clearly discernible peak after challenge. Mice administered higher doses (1:10 dilution) maintain titers > 20 U/mL after challenge, and titers apparently decline only slightly after 14 days. The titers of the 1:100 group (survivors) were still increasing 14 days after IC challenge. These latter observations are typical of a multiple booster dose response with the challenge acting as a third antigen dose.

At low vaccine dosage (1:20 dilution), 2 doses administered IM resulted in significantly greater titers and marginally better protection, compared with the IP route. By 4 weeks, there was no difference in titers of mice vaccinated with the 1:10 dose by IM and IP routes, as determined for 2 vaccines derived from different virus strains.

Extended protection in the treatment groups administered the more dilute dose (1:100 equivalent) of PRV and LEP vaccines revealed the advantage of the IM route, compared with the IP route. Serum VNA production increases during a longer period after administration, mean titers at ≥ 4 weeks are higher, and better protection is observed and maintained through 20 weeks. Mice vaccinated once or even twice by the SC route had not responded with detectable concentrations of serum VNA by 2 weeks after vaccination. Even when challenge was delayed until 8 weeks after mice were administered 2 doses of PRV vaccine SC, only 1 of 13 mice had rabies VNAs. Single-dose recipients had little detectable VNA even 4 days after a 2-week IC challenge. Mice vaccinated SC were not well protected from IC rabies challenge when 2 doses were administered, although 1 SC dose (1 mL) of the PRV vaccine protects dogs from challenge with street rabies virus 1 year after vaccination.[c] Although the IP vaccination route resulted in slightly better protection results than the IM route even 8 weeks after vaccination with PRV, such differences may result from difficulty of vaccine administration into the thigh region. In early experiments, this route of injection resulted in visible SC vaccine deposition in 10 to 20% of mice, which suggests that such deposition would result in greater incidence of treatment failure in mice, compared with the IP route. In our study, PRV vaccine injected into muscle of the thigh region stimulated VNA production in only 75% of mice (with low titers and poor protection), whereas the less protective and immunogenic (at 4 and 8 weeks) SAD vaccine administered into the hip region resulted in a VNA-positive rate of 100%. Further evidence of this effect is that the mice that died after challenge had significantly lower titers than the treatment group mean. Switching the IM administration from the thigh region to the hip region decreased the variation among individual serum VNA titers and increased 4- and 8-week challenge protection after IM vaccination. Despite the SC deposition of LEP vaccine in some mice vaccinated IM in the thigh region, the mice responded and were protected from IC challenge at 12 weeks.

Results of these experiments indicate that serum rabies VNAs and protection from IC challenge elicited by inactivated rabies vaccines in mice vary with the number of doses, route of administration, and time of challenge.

Such findings have important implications for the development of an improved potency test in laboratory mice. An improved method should be one that retains the speed and relative simplicity of the NIH test, while using practical vaccine administration routes to model the situation in which commercial vaccines are used, whether for veterinary or human application, and thus enable vaccines to be tested for efficacy more accurately than presently possible with the NIH vaccine potency test.

Because of the proven efficacy of SC administration of veterinary rabies vaccines in several target species, that route was tested in our studies. Results suggest that this route may not be effective for rapid single-dose evaluations of vaccine protection that use a mouse model. Use of an IM vaccination route reflects common practices; our results suggest that better efficacy predictions may be made by the IM route than that presently obtained by the NIH test, and it also has practical application for those vaccines not intended to be administered by the SC route.

Mouse age is an important factor in protection of mice in vaccine efficacy trials, and vaccination at 6 weeks of age appears to give more reproducible results and more reliable potency testing data than that obtained with younger mice. Serum rabies VNA response and protection from IC challenge by inactivated rabies vaccines in a mouse model vary significantly as a result of vaccination dose number, route of administration, and the time between vaccination and challenge. Single-dose IM administration of inactivated rabies vaccines has proven effective in target species and protected mice in our study, even against direct IC challenge. A rapid vaccination and challenge combination appears possible by the IM route with no greater time interval needed than that presently used for the NIH potency test.

Our results suggest that changes could possibly be made in mouse models for rabies vaccine potency, including retaining the speed of the NIH test and more accurately modeling practical vaccine administration, primary immunogenicity, and efficacy of protection. Such changes could offer improvements that would increase the efficiency of vaccine potency testing conducted by vaccine producers and agencies responsible for approving the use and distribution of vaccines. This could result in faster approval of changes in vaccine production and a reduction in the number of animals required to complete the testing and approval process.

[a]Charles River Laboratories, Wilmington, Mass.
[b]Harlan Sprague Dawley Inc , Indianapolis, Ind.
[c]Provided byKeith, Haffner, Pfizer Inc, Lincoln, Neb.
[d]Provided by Volker Franke, Chiron Vaccines, Marburg, Germany.
[e]National Veterinary Service Laboratory, Ames, Iowa.
[f]BBL Microbiological Systems, Cockneysville, Md.

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