INFECTION AND IMMUNITY, July 1975, p. 98-103

Vol. 12, No. 1
Printed in U.S.A.

Prolonging Morbidity in Rabid Dogs by Intrathecal Injection of Attenuated Rabies Vaccine

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Received for publication 28 January 1975

Dogs vaccinated intrathecally with attenuated rabies vaccine developed antibodies that were detected in the cerebrospinal fluid, blood, and brain; dogs similarly vaccinated but with an inactivated vaccine developed no antibodies in the brain. When the attenuated vaccine was administered to rabid dogs, a prolongation of the morbidity period was noted and, in some dogs, recovery from the disease. Rhesus monkeys died when administered any of the available attenuated vaccines intrathecally, and further studies with that species could not be undertaken.

Once rabies virus invades the central nervous system (CNS), it produces an almost invariably fatal disease (19). Strenuous treatment procedures including intensive care (7, 20) have been instituted in people with symptoms of the disease. In one case these efforts actually resulted in the patient's recovery (10), and in that case a high level of neutralizing antibody was found in the cerebrospinal fluid (CSF), similar to the levels seen in the other human cases in which intensive care resulted in prolonged survival periods (7). Moreover, in animal experiments, only those dogs recovering from CNS disease have had antibody in the CSF (3, 5), suggesting that there may be a relationship between those antibodies and recovery. It occurred to us that the production of CSF antibody without accompanying disease might be achieved by injecting high-titered attenuated virus into the CSF, and that the resultant antibody might affect recovery from rabies. The experiments described here were designed to answer those questions.

MATERIALS AND METHODS

Animals. The animals used were purebred 2-year-old beagles, juvenile rhesus monkeys (*Macaca mulatta*), and yearling black Angus steers. None had been vaccinated against rabies, and no animal had neutralizing antibody (17) to the virus. The white Swiss mice used for virus titrations were of the ICR strain, either suckling (1- to 3-day-old) or weanling (3-week-old); those used for vaccine potency testing were 4 weeks old.

Vaccines. The attenuated vaccine administered to dogs was ERA (1; M. K. Abelseth, Ph.D. thesis, Univ. of Minnesota, Minneapolis, 1966) grown on BHK cells (13) with a mouse intracerebral mean lethal dose ($MICLD_{50}$) titer of $10^{6.5}$/0.03 ml. This vaccine was also administered to dogs after being inactivated with a 1:4,000 dilution of beta-propiolactone. Three vaccines were administered to rhesus monkeys: a highly potent (and inactivated) ERA-BHK vaccine concentrated by the method of Schneider (16) with an antigenic value of 125 when tested by the NIH test against reference lot no. 178; live ERA grown on BHK cells ($MICLD_{50}$ titer of $10^{6.5}$ to $10^{7.5}$) or on primary porcine kidney cells (titer of $10^{4.5}$); and a high egg passage (HEP) Flury vaccine grown on primary canine kidney cells (6) with a suckling $MICLD_{50}$ titer of $10^{4.7}$/0.015 ml.

Challenge virus. The challenge virus used for the dogs was a salivary gland suspension from a rabid fox (kindly supplied by T. Hosty, Alabama State Health Department) with an $MICLD_{50}$ titer of $10^{6.7}$; each dog was inoculated bilaterally in the masseter muscles with 0.6 ml of various dilutions of the material, as described below.

Intrathecal vaccination. Dogs and monkeys were vaccinated by injecting 2 and 1 ml, respectively, of vaccine into the cisterna magna with a 1.5-inch (about 3.7 cm), 22-gauge needle; a similar CSF liquid volume had been withdrawn before administration to avoid increasing the intracerebral pressure. The dogs were anesthetized with ether in the first experiment, but Brevane (Eli Lilly and Co., Indianapolis, Ind.) was used in the second since it gave better analgesia and immobilization. The monkeys were immobilized by intramuscular administration of ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, N.Y.). The steers were immobilized with Rompun (Chemagro Corp., Kansas City, Mo.), and the vaccine was administered with a 4-inch (about 10 cm), 18-gauge needle, with needle insertion approximately 2 in (5 cm) caudal to the occipital ridge and 1 inch (about 2.5 cm) from the median line; 2 ml of ERA-BHK was injected.

Serum neutralization tests. All serum, CSF, and brain neutralization titers for rabies were determined by the rapid fluorescent-focus inhibition test developed by Smith et al. (17).

Interferon determinations. Serum and CSF interferon titers were tested through the kindness of S. Baron and H. Levy, National Institutes of Health, Bethesda, Md.; the determinations were made in human foreskin fibroblasts by the method of Armstrong (4). No interferon determinations were made with canine or bovine CSF or serum.

Fluorescent antibody technique. The brain of each animal that died was examined for the presence of viral antigen by classic fluorescent antibody procedures (8).

Mouse inoculation. Weanling mice were inoculated intracerebrally with a 20% brain suspension of all animals that died to test for infective virus (12).

Animal observation. Dogs, monkeys, mice, and steers were observed daily for signs of rabies.

RESULTS

The first observations were made on four dogs to determine whether intracisternal vaccination resulted in antibody development. The beagles inoculated intrathecally with either live (two dogs) or inactivated (two dogs) ERA-BHK ($10^{6.5}$ $MICLD_{50}$/0.03 ml before inactivation) remained healthy, yet CSF antibody developed by the 4th day after inoculation (Table 1). The brain suspensions of those that received the live vaccine, however, had neutralizing antibody, whereas those that received the inactivated one did not.

Since intrathecal administration of vaccine had resulted in the production of CSF antibody without causing illness, we decided that we should then try to save animals sick with rabies by administering attenuated vaccine. The first such attempt was made in 12 challenged dogs, and we were primarily interested in determining the appropriate anesthetic agent and precise inoculation site. We vaccinated these dogs (experiment 1) when the first signs of rabies appeared. Three dogs (no. 87, 92, and 84) died of trauma while being inoculated, and six others died soon after being treated. The remaining three (no. 80, 44, and 8), however, had markedly prolonged morbidity periods and recovered from the disease (Fig. 1). These three, while still

TABLE 1. *Neutralizing antibody levels in cerebrospinal fluid (serum) and brain of dogs inoculated intrathecally with attenuated or inactivated rabies vaccines*

Dog no.	Neutralizing antibody levels on day:					Terminal titers	
	1	2	3	4	12	83	83 (Brain)
Attenuated							
35	<2	<2	<2 (25)[a]	280 (625)	3,125 (7,000)	NT[b] (125)	11
70	<2	<2	<2 (125)	240 (125)	125 (7,000)	NT (360)	11
Inactivated							
185	<2	<2	<2 (70)	56 (230)	25 (3,125)	11 (390)	<5
242	<2	<2	<2 (NT)	<5 (125)	625 (5,900)	125 (125)	<5

[a] Numbers in parentheses refer to serum titers.
[b] NT, not tested.

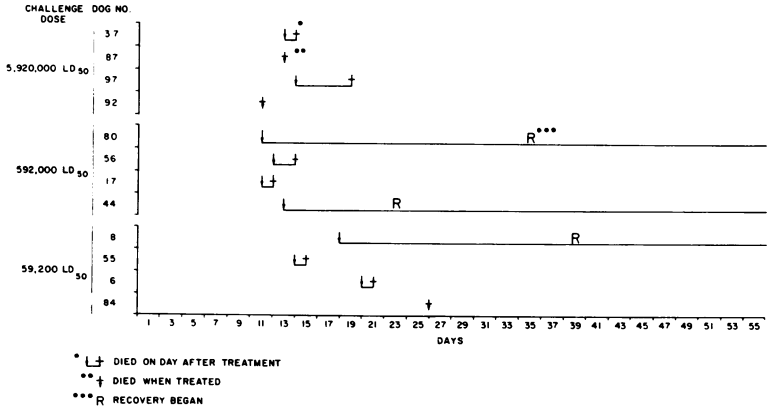

FIG. 1. *Group mortality, incubation periods, and morbidity periods in dogs vaccinated intrathecally with rabies vaccine after challenge (experiment no. 1).*

paralyzed, had other clinical signs than those usually seen in rabid animals; they were "lucid," and their ocular movements followed the movements of the people around them, with none of the accompanying excited trembling and convulsions that are usually noted.

In the next experiment 19 dogs were challenged by inoculation of the same salivary gland suspension used in experiment 1 (59,200 LD_{50}); the animals were then divided into two groups, one of 5 dogs and one of 14. The first group was inoculated intrathecally with the vaccine virus 1 week after challenge. Eight of the 14 in group 2 sickened, and every other one (412, 333, 188, and 243) was vaccinated; thus four (411, 356, 293, and 138) served as untreated controls. Moreover, tests were then done to show that those animals that might sicken and recover actually had been rabid. CSF was tested for rabies when it was drawn at the time of intrathecal vaccination, salivary swabs were taken (12), and skin biopsies (18) were excised at three sites for fluorescent antibody examination.

None of the dogs treated 1 week after challenge died (Fig. 2). Four control animals died with incubation periods that varied from 12 to 16 days and with morbidity periods of 1 to 3 days. Two of the four symptomatic dogs that were vaccinated intrathecally had markedly longer morbidity periods than the control animals (8 and 12 versus 1 to 4). The signs in these paralyzed animals were again notably different from the control animals. Virus was isolated from the saliva of the dog that survived for 12 days on two occasions, the 1st and 7th days of illness. The isolation attempts on the other dogs were negative.

The next trials were with rhesus monkeys. Because of the known susceptibility of that species to intracerebral administration of HEP (11), the most attenuated live rabies vaccine available to us, we began working with the concentrated inactivated vaccine we had prepared. Four monkeys were inoculated with 1 ml each of this material, two intrathecally and two intravenously via the femoral vein. Serum samples and CSF were drawn at vaccination, at 3, 6, 24, and 48 h, and then at 3, 4, and 7 days; the first five samples were tested for interferon levels, the last three for neutralizing antibody determination. Interferon appeared in both serum and CSF of all four monkeys, with higher CSF levels in the two monkeys given the vaccine intrathecally (Table 2). Neutralizing antibody appeared earlier in the sera of the three surviving monkeys than in CSF, and reached higher levels. When the last two monkeys were killed and examined 1 year after vaccination, both had neutralizing antibody in their sera, but only the one originally given vaccine intrathecally had antibody in the CSF and (a low level) in brain suspension.

With the high CSF interferon and neutralizing antibody levels in mind, we planned an experiment evaluating the efficacy of intrathecal administration of the potent inactivated vaccine in rabid monkeys. Ten rhesus monkeys were inoculated in the neck muscles with 1.2 ml of a fox salivary gland suspension containing

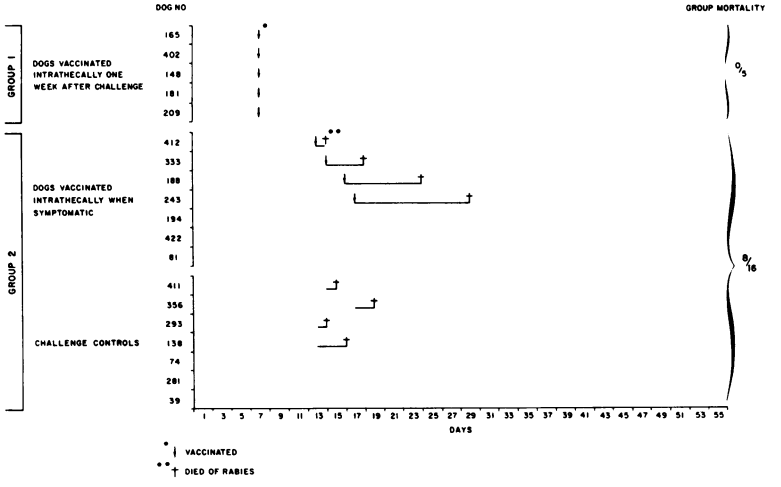

FIG. 2. *Group mortality, incubation periods, and morbidity periods in dogs vaccinated intrathecally with rabies vaccine after challenge (experiment no. 2).*

TABLE 2. *Interferon and neutralizing titers in serum and CSF of four monkeys administered concentrated inactivated rabies vaccine (two intrathecally, two intravenously)*

Inoculation route	Monkey no.	Material	Interferon levels at					Neutralizing antibody levels				
			0 h	3 h	6 h	24 h	48 h	2 days	3 days	4 days	7 days	1 year post-vaccination
Intrathecal	1	Serum	<5	60	50	NT[a]	NT	NT	NT	NT	NT	NT
		CSF	<1	16	50	NT	NT	NT	NT	NT	NT	NT
Intrathecal	2	Serum	<5	3,000	3,000	25	<1	<5	180	1,100	4,600	1,400
		CSF	<1	300	110	300	25	<5	<5	33	70	280
		Brain										14
Intravenous	3	Serum	16	110	600	300	5	5	56	2,200	4,200	NT
		CSF	NT	<1	16	25	1	<5	<5	70	625	NT
		Brain										NT
Intravenous	4	Serum	25	110	80	5	<1	<5	15	800	4,600	360
		CSF	NT	<1	<1	5	<1	<5	<5	15	125	<5
		Brain										<5

[a] NT, Not tested (monkey no. 1 died of trauma the evening of the 1st day).

approximately 8 monkey peripheral LD_{50} of virus, and 1 ml of the vaccine was administered intrathecally when they became ill; as with dog experiment no. 2, the animals were divided alternately into two groups, with the first sick monkey being vaccinated, the second serving as a control, the third vaccinated, etc. The intrathecal vaccination not only failed to prolong the morbidity periods but actually hastened death (Table 3).

We next turned to the intrathecal administration of attenuated ERA vaccine in monkeys. Every dilution of vaccine inoculated from the very concentrated to the very dilute produced rabies in the monkeys, in spite of the appreciable interferon and neutralizing antibody levels in the CSF long before the animals sickened or died (Table 4). Another attenuated vaccine, HEP, was also injected and again proved lethal (11) with, however, surprisingly long incubation periods in the animals given the higher dilutions of vaccine. Most of the animals that died after receiving either ERA or HEP had neutralizing antibody in the brain.

The last species inoculated was the bovine (Table 5); their susceptibility appeared to be similar to that of dogs, with no animals dying after receiving ERA-BHK ($MICLD_{50}$ of $10^{6.5}$/0.03 ml) and subsequent development of antibodies in both CSF and serum. Both the cisternal and lumbar routes were used in the experiment since it was not known which would be the easier route of administration.

TABLE 3. *Incubation and morbidity periods of rhesus monkeys challenged and then vaccinated intrathecally with inactivated rabies vaccine on appearance of signs*

Monkey no.	Incubation period (days)	Morbidity period (days)
Vaccinated		
8A	16	2
6A	17	2
18A	20	1
4A	25	1
15A	30	1
Controls		
16A	15	5
2A	17	4
1A	18	3
21A	36	7
9A	38	5

DISCUSSION

Recovery from rabies in the CNS has only rarely been noted, but on those few occasions high levels of neutralizing antibodies were present in the CSF (3, 5, 10). This suggests that reticuloendothelial cells not usually reached were stimulated by the virus. Arko et al. (3) showed that neutralizing antibody does not "cross over" to the CSF, even when present at very high levels in the face of a fatal encephalitis, adding further weight to this initial evidence. The relationship between cerebral resistance to virus infection and the presence of neutralizing antibody in the CSF has long been known; Morgan et al. (14) reported that rabbits vaccinated subcutaneously with Eastern equine encephalomyelitis virus resisted intracerebral challenge only when spinal fluid antibody was induced, the resistance being "a true immunity, dependent on strain-specific neutralizing antibodies." They also stated that "another type of resistance, not strain specific, and not de-

TABLE 4. *Incubation periods, interferon titers, and neutralization titers in cerebrospinal fluid and serum of rhesus monkeys vaccinated intrathecally with attenuated rabies vaccines*

Vaccine and titer ($MICLD_{50}$/0.03 ml)	Monkey no.	Incubation period and days of death	Interferon titers in CSF (24 h)	Neutralizing antibody titer in CSF (and serum)		Terminal neutralizing titers	
				3 days	4 days	CSF (serum titer)	Brain
ERA-BHK[a]							
$10^{8.5}$[a]	68	4-5[b]	NT[c]	NT	NT	NT	17
	69	4-5	NT	NT	NT	NT	NT
$10^{7.5}$	31	—[d]	$10^{1.4}$	<5 (70)	11 (250)	70 (1,300)	45
	32	12-14	$10^{2.1}$	<5 (70)	10 (625)	NT	4
$10^{6.5}$	33	13-[e]	$10^{2.1}$	<5 (11)	<5 (10)	360 (1,800)	250
	34	16-	$10^{2.3}$	<5 (5)	<5 (33)	56 (1,000)	56
$10^{5.5}$	35	13-21	$<10^{1.7}$	<5 (<5)	11 (16)	NT	350
	36	12-14	$10^{2.1}$	9 (42)	29 (270)	NT	>625
$10^{4.5}$	37	16-21	$<10^{1.7}$	<5 (13)	10 (42)	NT	<8
	38	15-21	$<10^{1.7}$	<5 (<5)	<5 (<5)	NT	18
Commercial ERA[f]							
$10^{4.5}$	39	12-	$10^{2.1}$	<5 (<5)	<5 (<5)	210 (1,300)	625
	40	14-	$10^{2.1}$	<5 (<5)	<5 (13)	85 (1,300)	50
$10^{3.5}$	41	12-	$10^{1.1}$	<5 (<5)	<5 (6)	1100 (>3,125)	1,000
	42	5-7	$10^{1.0}$	<5 (<5)	<5 (6)	NT	<2
$10^{2.5}$	43	17-	$<10^{1.7}$	<5 (<5)	<5 (<5)	<5 (11)	<5
	44	20-	$<10^{1.7}$	<5 (<5)	<5 (<5)	<5 (50)	<5
Commercial HEP[g]							
$10^{4.7}$	45	32-36	$<10^{1.7}$	NT	<5 (<5)	NT	NT
	46	33-36	$10^{2.1}$	NT	<5 (<5)	NT	NT
$10^{3.7}$	47	53	$<10^{1.7}$	NT	<5 (<5)	<5 (8)	<2
	48	57[h]	$<10^{1.7}$	NT	<5 (<5)	<5 (<5)	<2
$10^{2.7}$	49	40-45	$<10^{1.7}$	NT	<5 (<5)	NT	<2
	50	54[h]	$<10^{1.7}$	NT	<5 (<5)	>5 (>125)	<2
$10^{1.7}$	51	—[i]	$<10^{1.7}$	NT		NT	NT
	52	63[h]	$<10^{1.7}$	NT	<5 (<5)	<5 (<5)	<5

[a] First group given concentrated material.
[b] Sick on day 4, died on day 5.
[c] Not tested.
[d] Remained healthy.
[e] Survivor with sequelae, sickened on day indicated.
[f] Grown on primary porcine kidney cells.
[g] Grown on primary canine kidney cells.
[h] Sickened on day indicated, then recovered completely.
[i] Died from aftereffects of spinal tap on day 1.

TABLE 5. *Neutralizing antibody titers in cerebrospinal fluid (and serum) of three calves administered ERA-BHK rabies vaccine*

Route	Neutralizing antibody titers on day:			
	0	4	6	15
Intrathecal (cervical)	<5 (<5)	11[a], <5[b] (350)[c]	50, 50 (1,500)	230, 170 (250)
Intrathecal (lumbar)	<5 (<5)	<5, <5 (56)	625, 50 (1, 300)	<5, 7 (1,400)
Intravenous	<5 (<5)	<5, 5 (480)	50, 11 (1,600)	<5, <5 (1,900)

[a] CSF from cervical region.
[b] CSF from lumbar region.
[c] Serum titer.

pendent on neutralizing antibody, was encountered on repeated intracerebral inoculations of active virus."

Our results indicate that although CSF antibodies (and interferon) may be elicited by both attenuated and inactivated vaccines, recovery from CNS infection and prolonged morbidity periods appear to be related to additional factors, since highly concentrated inactivated vaccine (when given at the first signs of the disease) not only failed to prolong the morbidity period but markedly shortened it.

In contradistinction to this, attenuated vaccine tended to prolong the morbidity period and to radically affect the symptomatology of some sick animals. We found that the intrathecal inoculation of the attenuated vaccine in dogs and rhesus monkeys resulted in the development of cerebral antibodies (i.e., in suspensions of brain material), whereas the inoculation of the inactivated vaccine did not. We thus conclude that the stimulation of brain immunocompetent cells, interference, or other factors dependent on replication of attenuated virus were the critical factors in the prolonged illness periods noted and occasional canine recovery. Some dogs recovered even without the intensive care essential for "treatment" of rabies in man (monitoring of blood gases, administration of anticonvulsant drugs, reduction of increased CSF pressure and tracheostomy), and if those procedures had been instituted in addition to the intrathecal vaccination, even more animals might have recovered.

Perhaps the immune response to the live rabies vaccine is similar to the local antibody response found in the CNS of rhesus monkeys given intrathalamic inoculations of the Mahoney and Sabin strains of poliovirus (15); no local (CNS) antibody was produced after similar injection of inactivated vaccine, and it was suggested that the CNS, when stimulated locally by a potent, replicating vaccine, elicits a local antibody response. We were unfortunately not able to test the possible saving effect of such a local antibody response in monkeys given attenuated rabies vaccines intrathecally, since animals inoculated with those vaccines died of vaccine-induced rabies. Further studies in subhuman primates must thus await the development of a more highly attenuated virus. The ultimate aim, of course, would be to develop procedures permitting the same general conditions of disease alleviation in man.

The results in cattle suggest that intrathecal vaccination experiments with ERA-BHK should be carried out in an attempt to alter the disease course in cattle, especially in those Latin American areas where bovine rabies is a definite problem (2).

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