A NON-VIRULENT, SINGLE-DOSE RABIES VACCINE FOR PROPHYLACTIC IMMUNIZATION OF DOGS

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Experiments have been described indicating that a vaccine containing approximately 50,000 mouse intracerebral lethal doses of rabies virus properly irradiated will immunize mice, and that 25,000,000 doses will immunize dogs against a subsequent test inoculation of virulent virus. Additional experiments showed that a volume of 0.1 cc. of an irradiated vaccine prepared from the supernatant of centrifuged virus-containing dog brain contained the required 50,000 doses and immunized mice adequately (1). It remained to determine whether the required number of mouse doses to immunize dogs could be concentrated into a small volume and whether a single injection of such a vaccine would in fact protect them adequately.

Technique

To prepare vaccine, 2-months-old beagle dogs are injected intracerebrally with 0.5 cc. of a 1:10 dilution of dog brain virus, Pasteur strain. When the animals become prostrate, they are sacrificed, their brains are removed, triturated in an electric mixer, and diluted in distilled water containing 2 per cent citric acid-sodium diphosphate buffer (McIlvaine), pH 7.2—to make a 1 or 5 per cent emulsion. This emulsion is then centrifuged at 500 R.P.M. for 5 minutes and the supernatant removed. The supernatant must be relatively free from large particles to insure that ultraviolet light will render it non-virulent and yet not destroy its immunizing potency. It must also titre at least 330,000 mouse doses per ml., that is, prove fatal to W-Swiss mice when injected intracerebrally in 0.03 cc. volumes and in a dilution of 10^{-4}, to insure that small volumes of the final vaccine will immunize animals effectively. The material is rendered non-virulent by exposure to ultraviolet light for 35 minutes (2). Tests for sterility are made, merthiolate in a dilution of 1:10,000 is added as preservative, and the material, ready for use, is stored in the ice box at 40°F.

The technique which we have employed for testing rabies vaccines in dogs has been described in a previous paper (3). Beagle dogs, aged 4 to 6 months, are used for the most part in all tests. In some, however, it has been necessary to include animals of mixed small breeds of the same age. They are wormed as soon as received on the premises and then given 10 to 15 cc. of distemper immune serum, followed by one or two injections of non-virulent distemper vaccine.

About 7 days after arrival, groups of six to twelve animals receive rabies vaccine and a remaining group is set aside as controls.

Three weeks later all are given a test injection of rabies virus, 0.25 cc. properly diluted, deep into the neck muscles of each side. They are then watched for signs of

rabies for 2 to 5 months. The animals which become prostrate are sacrificed, their brains removed, tested for the presence of Negri bodies in stained impressions, and for the presence of rabies virus by inoculation of diluted brain suspension intracerebrally into mice. The survivors are also sacrificed when the experiment is terminated and their brains tested for the presence of rabies virus.

Results of Tests on Unvaccinated Dogs

In attempting to evaluate experiments on the immunizing effects of vaccines on experimental animals, a factor of prime importance is the regularity of response of unvaccinated controls to the test virus. When small animals, such as mice, are employed, it is possible to exert such a degree of control that not only unvaccinated animals respond in a uniform and predictable manner within narrow limits, but the actual amount of virus administered can be determined in terms of minimum lethal doses. When large and expensive animals are employed, such as the monkey or dog, the problem becomes more difficult, although the aims remain the same, namely, to set up conditions which will insure uniform and predictable mortalities among unvaccinated animals.

These aims have been realized in part only. English investigators in India (4, 5), studying the potency of rabies vaccines in monkeys, found that mortalities in unvaccinated groups following the test injection of virus varied from 20 to 100 per cent in different experiments. More recently, Johnson and Leach (6) carried out repeated tests of vaccines in thirteen groups of seven to ten dogs and found that the mortalities among unvaccinated dogs varied unpredictably from 20 to 91 per cent. They attribute such "fallacious results" to chance and carried out repeated tests until the total number of animals "satisfied statistical requirements" (6). They also performed a single experiment with fifty-five control dogs which they regarded as sufficient to eliminate the effects of chance variables encountered in their type of test. We have been more fortunate in controlling chance variables in our experiments with smaller groups of animals and have obtained results in unvaccinated dogs which show little variation from experiment to experiment. In six tests shown in Table I, the mortalities among the unvaccinated dogs averaged 83 per cent and were never less than 71 nor more than 88 per cent except in one instance in which the mortality was 100 per cent. These results have been similar in all eighteen tests in that the controls showed mortalities as great as 100 per cent but never less than 71 per cent except on two occasions when mortalities were 60 and 50 per cent respectively. The duration of life of these animals varied widely: 39.6 per cent in 12 to 19 days; 27 per cent in 20 to 29 days; 16.6 per cent in 30 to 60 days, and 16.6 per cent remained well more than 90 days. This means that 66.6 per cent of the animals died within the first month. Under natural conditions of street infection, probably not more than 20 per cent die during a similar period.

Although we have been successful in achieving relatively uniform and pre-

dictable results in mortalities following injection of groups of five to eleven control dogs with rabies virus, we have not been successful in eliminating completely complicating factors of intercurrent infection—in our case, distemper. The British workers referred to above lost as many as 20 per cent of their animals from intercurrent infections—chiefly among the vaccinated groups—and hence compared vaccinated and unvaccinated groups at 2 rather than 3 months following infection. Leach and Johnson, in their thirteen tests with small batches of dogs, lost not only 49 per cent of the original 149 controls from the experimental inoculation with rabies virus, but also 19 per cent from other

TABLE I

Mortality of Unvaccinated Control Dogs, Tests 10–18, Following Intramuscular Inoculation of Virus

Test	Virus	Dilution	No. of dogs dying from rabies on given days following inoculation																	Dead/Total	Dead
			12	13	15	16	17	18	19	20	21	23	24	25	27	28	30	34	37	41	Survivors		
																							per cent
10	Fixed No. 15811	1:4,000			1	1		1									1				1	4/5	80.0
12	Fixed No. 15811	1:400				2	1		1		1	1			1		1		1		2	9/11	81.8
13	Fixed No. 15811	1:400			1		1	2		1						1		1			2	7/9	77.7
15	Fixed No. 15811	1:200										3	1			1					2	5/7	71.4
16	Street (1 passage)	1:20					1			1				1	1		2		1	1	0	8/8	100.0
18	Street (1 passage)	1:20	1	4					2												1	7/8	87.5
			19/48—39.6 per cent 12 to 19 days							13/48—27 per cent 20 to 29 days							8/48—16.6 per cent 30 to 60 days				8/48—16.6 per cent Survived	40/48	83.3

causes; they also lost 18.6 per cent of the original 140 vaccinated dogs from the experimental inoculation and an additional 25 per cent from other causes. The effect of uncontrolled variables was therefore equal to 40 per cent of, or even exceeded, the total effect of the test inoculation and was, therefore, highly selective. From the biological viewpoint, it is questionable whether results with such selected animals are completely reliable. Bearing this in mind, we have chosen to present for main consideration only those tests on vaccines in which mortalities from other causes have been not greater than 5 per cent of the original population.

Results of Tests on Vaccinated Dogs

The first experiment was planned to determine whether a calculated amount of irradiated vaccine given in a single dose intraperitoneally would immunize

dogs and, at the same time, whether the immunity obtained would compare favorably with that achieved by commercial canine vaccines.

Experiment 1, Test 10, October 16, 1939.—A 1 per cent mouse brain irradiated vaccine was prepared as described above. Prior to irradiation the 1 per cent vaccine titred 330,000 mouse doses per cc. 8 days later, ten dogs, groups B and D, were each given 40 cc. of this vaccine in a single dose intraperitoneally and 5 dogs, group E, were each given 10 cc. An additional five dogs, group F, were each given a single 5 cc. dose of a commercial 33 per cent chloroformized canine vaccine subcutaneously, and still

TABLE II

Immunizing Effects of Antirabies Vaccines on Beagle Dogs

Experiment 1, Test 10

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus No. 15811		
		Day of death following inoculation	No. dead/ No. injected	Dead
				per cent
A. No vaccine	1:400	13,20,S	2/3	66
B. Vaccine: irradiated, 40 cc., intraperitoneally	"	S,S,S,S	0/4	0
C. No vaccine	1:4,000	15,16,18,30,S	4/5	80
D. Vaccine: irradiated, 40 cc., intraperitoneally	"	53,S,S,S,S	1/5	20
E. Vaccine: irradiated, 10 cc., intraperitoneally	"	S,S,S,S,S	0/5	0
F. Vaccine: chloroform, 5 cc., subcutaneously	"	15,19,S,S,S	2/5	40
G. Vaccine: phenol, 5 cc., subcutaneously	"	14,16,17,43,S	4/5	80
H. No vaccine	1:40,000	12,39,S,S	2/4	50

S = animal remained well following injection. Survivors discarded after 70 days.

another five dogs, group G, a single 5 cc. dose of a commercial phenolized canine vaccine subcutaneously. Twelve remaining dogs were set aside as controls. 3 weeks later, all save one dog in group B, which died of distemper, were ready for the test inoculation of virulent virus. A strain of dog passage virus, No. 15811, which had been employed in previous tests (2), was prepared as described above and given into the neck muscles of the dogs in the following manner. Group B, plus a group of three controls (group A), received an injection of 0.25 cc. of a 1:400 dilution into the neck muscles of each side; groups D, E, F, and G, plus five controls (group C), the same inoculation of a 1:4,000 dilution, and four controls (group H), a 1:40,000 dilution. Animals were observed 70 days.

The results of this test are shown in Table II. Of greatest interest are the animals receiving the 1:4,000 dilution of virus which, in previous tests, has

been shown to produce rabies in 50 per cent and less than 100 per cent of controls and to contain roughly about ten lethal doses for dogs (3). In the present test, this amount of virus was fatal to four of five unvaccinated dogs (80 per cent), (group C), and one-tenth of this amount was fatal to two of four unvaccinated dogs (group H). The 1:4,000 dilution of virus was likewise fatal to four of five dogs receiving phenolized vaccine (80 per cent), (group G), to two of five receiving chloroformized vaccine (40 per cent), (group F), but to only one of five receiving 40 cc. of irradiated vaccine (20 per cent), (group D), and to none of five receiving 10 cc. of the irradiated vaccine (group E). Finally, ten times this dose of test virus was fatal to two of three controls (66 per cent), (group A), but to none of four dogs receiving 40 cc. of irradiated vaccine (group B). Clearly the irradiated vaccine in 40 or 10 cc. amounts conferred a strong

TABLE III

Immunizing Effects of an Irradiated Antirabies Vaccine on Beagle Dogs

Experiment 2, Test 13

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus No. 15811		
		Day of death following inoculation	No. dead/ No. injected	Dead
				per cent
A. No vaccine	1:400	15,17,18,18,20,28,34,S,S	7/9	77.7
B. Vaccine: irradiated, 10 cc., intraperitoneally	"	17,19,24,29,31,38,S,S,S,S	6/10	60.0
C. Vaccine: irradiated, 2 doses, 10 cc. each, intraperitoneally	"	17,17,S,S,S,S,S,S	2/8	25.0

immunity to thirteen of fourteen dogs, whereas phenolized vaccine failed and chloroformized vaccine succeeded only partially in immunizing the animals.

Inasmuch as 10 cc. or more of irradiated vaccine immunized dogs successfully in Experiment 1, the test was repeated using 10 cc. or 20 cc. of vaccine per dog.

Experiment 2, Test 13, April 8, 1940.—A 1 per cent dog brain irradiated vaccine was prepared in the manner described above. Prior to irradiation, 1 per cent vaccine titred 3,300,000 mouse doses per cc. 15 days later, ten dogs, group B, were given a single dose of 10 cc. of the vaccine intraperitoneally, and eight dogs, group C, two doses of 10 cc. 1 week apart. Ten dogs, group A, were set aside as controls. 3 weeks later, all dogs were given the stated inoculation of virulent dog passage virus, strain 15811, diluted 1:400, and observed for 70 days.

The results of this test are shown in Table III. One of the control dogs (group A) died of distemper on the 22nd day. Of the remaining nine, seven died of rabies (77.7 per cent). Six of ten dogs receiving 10 cc. of vaccine (group B) also died of rabies (60 per cent), whereas only two of eight receiving

two 10 cc. doses of vaccine (group C) succumbed (25 per cent). Evidently in this test 10 cc. of vaccine produced little immunity and 20 cc. a good immunity.

In the following experiment a larger amount of irradiated vaccine was employed—30 cc. in a single dose and in three doses of 10 cc. each.

Experiment 3, Test 16, October 10, 1940.—A 1 per cent dog brain virus was prepared and tested as in the previous experiment. Its titre prior to irradiation was 3,300,000 mouse doses per cc. 9 days later eight dogs were given a single injection of 30 cc. of vaccine intraperitoneally (group B), and eight dogs three injections of 10 cc. each (group C). Eight dogs (group A) were reserved as controls. 3 weeks later, all but one dog in group B and one in C, which died of distemper, were inoculated into the

TABLE IV

Immunizing Effects of Irradiated Antirabies Vaccine on Beagle Dogs

Experiment 3, Test 16

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of one-passage virus No. 17456		
		Day of death following inoculation	No. dead/ No. injected	Dead
				per cent
A. No vaccine	1:20	17,20,25,27,30,30,37,41	8/8	100
B. Vaccine: irradiated, 30 cc., intraperitoneally	"	S,S,S,S,S,S,S	0/7	0
C. Vaccine: irradiated, 3 doses, 10 cc. each, intraperitoneally	"	S,S,S,S,S,S,S	0/7	0

neck muscles with one-passage street virus, No. 17456, diluted 1: 20. The animals were observed for rabies for 70 days.

The results of this test, shown in Table IV, were clear-cut. All of the controls died of rabies but none of the fourteen vaccinated dogs. A total of 30 cc. of irradiated vaccine either in single or multiple doses conferred a strong immunity, as already indicated in Experiment 1.

It was apparent from these tests that 25 to 30 cc. of 1 per cent irradiated vaccine would immunize young beagle dogs against an inoculation of fixed or street virus fatal to nearly all unvaccinated dogs. It remained to discover whether the same amount of vaccine concentrated to a smaller volume would be equally effective as an immunizing agent.

Experiment 4, Test 18, May 27, 1941.—A 5 per cent rather than a 1 per cent supernatant dog brain virus was prepared as a vaccine, thus effecting at once a 5 times concentration of virus per cubic centimeter. This material titred 33,000,000 mouse doses per cc. as did preparations in other tests prior to irradiation. Following irradiation,

the 5 per cent vaccine was concentrated 6 times at low temperature *in vacuo*. 27 days later eight dogs (group B) were given a single dose of 5 cc. intraperitoneally, eight dogs (group C) 5 cc. subcutaneously, eight dogs (group D) two injections of 5 cc. each intraperitoneally, and eight dogs (group E) 5 cc. of a commercial chloroformized vaccine subcutaneously. Eight dogs were reserved as controls. 3 weeks later, all received the standard inoculation into the neck muscles of one-passage street virus, No. 17825, diluted 1:20. The animals were observed 70 days for signs of rabies.

The results of this test were again striking (Table V). Seven of the eight control dogs (group A) died of rabies in 12 to 19 days (87.5 per cent), indicating an unusually virulent street virus. Three of the eight dogs given chloro-

TABLE V

Immunizing Effects of Irradiated Antirabies Vaccine on Beagle Dogs

Experiment 4, Test 18

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of one-passage street virus		
		Day of death following inoculation	No. dead/ No. injected	Dead
				per cent
A. No vaccine	1:20	12,13,13,13,13,19,19,S	7/8	87.5
B. Vaccine: irradiated, concentrated, 5 cc., intraperitoneally	"	S,S,S,S,S,S,S,S	0/8	0.0
C. Vaccine: irradiated, concentrated, 5 cc., subcutaneously	"	S,S,S,S,S,S,S,S	0/8	0.0
D. Vaccine: irradiated, concentrated, 2 doses, 5 cc. each intraperitoneally	"	S,S,S,S,S,S,S,S	0/8	0.0
E. Vaccine: chloroform, 5 cc. subcutaneously	"	12,13,19,S,S,S,S,S	3/8	37.5

formized vaccine (37.5 per cent) likewise died of rabies (group E), whereas all of the twenty-four dogs given irradiated vaccine remained well. It appears, therefore, that 5 cc. or more of this concentrated vaccine protects highly susceptible dogs against rabies.

Having demonstrated that a single 5 cc. dose of our non-virulent rabies vaccine immunized dogs effectively, we sought information from all of our tests as to (1) the comparative effectiveness in dogs of irradiated, phenolized, and chloroformized vaccines, and (2) the comparative effectiveness of the intraperitoneal as opposed to the subcutaneous route of injecting the vaccine.

With respect to the relative immunizing potencies of irradiated as compared to phenolized or chloroformized vaccines, the following information is at hand. Summarizing all data in nineteen dog tests (Table VI), we noted that the mortality of 141 controls given test virus in dilutions corresponding to those given

to vaccinated animals was 92.2 per cent. Similarly, the mortality of fifty-three dogs given commercial phenolized vaccine according to directions was 77.3 per cent and that of fifty dogs given commercial chloroformized vaccine, 46.0 per cent. In contrast, of thirty-eight dogs given 20 to 40 cc. of irradiated vaccine intraperitoneally, 13.1 per cent succumbed, and of thirty-one given 5 to 10 cc. of irradiated vaccine, concentrated, only one succumbed to the test inoculation of virulent virus. Clearly the course of vaccination with irradiated vaccine appears far superior to that of vaccination with commercial vaccines.

From the figures of Table VI alone, however, one would rightly question whether the superior results with irradiated vaccine were due only to the quality of the material, or to the greater amounts employed and to the routes of inoculation. It is our belief that all three factors contribute to the potency of the

TABLE VI

Comparison of Commercial (Phenolized, Chloroformized) and Irradiated Canine Antirabies Vaccines

Groups	Total dead/ Total tested	Dead
		per cent
A. Controls. Fixed virus, dilution 1:50 to 1:10,000; street virus, dilution 1:20	130/141	92.2
B. Vaccinated. Phenolized vaccine, 5 cc., subcutaneously	41/53	77.3
C. Vaccinated. Chloroformized vaccine, 5 cc., subcutaneously	23/50	46.0
D. Vaccinated. Irradiated brain tissue supernatant:		
1. 20–40 cc., intraperitoneally	5/38	13.1
2. 5–10 cc. concentrated, intraperitoneally	1/31	3.2

irradiated vaccine and the following reasons are emphasized. That irradiated vaccine given intraperitoneally is superior to equal quantities of chloroformized vaccine given by the same route is indicated by the quantitative results of three experiments (1) in which one part of a single pool of virus material was inactivated by chloroform and another part by irradiation. Each inactivated preparation was then given to mice as vaccine and in each instance the irradiated sample proved equal in immunizing potency or superior to the chloroform-treated sample. In further tests, the irradiated vaccine was compared with commercial canine vaccines for ability to immunize dogs. The results shown in Table VII, though not critical, confirm the findings in mice. The vaccines contained widely different amounts of brain-virus—33 per cent to 1 per cent—and were inoculated into dogs in different amounts. Hence, for purposes of comparison, it seemed best to reduce the dose of each vaccine to an equivalent amount of 1 per cent emulsion. Thus, dogs of group A, given 5 cc. of 20 per cent vaccine, are said to have received 100 cc. of 1 per cent vaccine. On this basis, among dogs of groups A, B, and C receiving vaccine subcutaneously,

group C, given irradiated vaccine in a dose equivalent to 150 cc., suffered only 9.1 per cent mortality, in contrast to 46 per cent by group B, given a similar equivalent dose of chloroformized vaccine, and 77.3 per cent by group A, given phenolized vaccine. Again, among groups vaccinated intraperitoneally, group D, given phenolized vaccine in a dose equivalent to 500 cc., suffered a mortality of 22.2 per cent and group E, with a similar dose of chloroformized vaccine, 18.5 per cent, as contrasted with 10 per cent by group F, given only 30 to 40 cc. of 1 per cent irradiated vaccine, or less than one-tenth of that given to groups D and E, and 3.2 per cent by group G, given 150 to 300 cc. of irradiated vaccine.

TABLE VII

Relative Amounts of Irradiated, Phenolized, and Chloroformized Vaccines Required to Immunize Dogs

Group	Equivalent amount of 1 per cent vaccine	Total dead/Total for test	Dead
	cc.		*per cent*
A. Vaccinated subcutaneously—phenolized 20 per cent, 5 cc.	100	41/53	77.3
B. Vaccinated subcutaneously—chloroformized 33 per cent, 5 cc.	150	23/50	46.0
C. Vaccinated subcutaneously—irradiated 5 per cent, concentrated 6 times, 5 cc.	150	1/11	9.1
D. Vaccinated intraperitoneally—phenolized 20 per cent, 25 cc.	500	2/9	22.2
E. Vaccinated intraperitoneally—chloroformized 33 per cent, 10–25 cc.	330–825	5/27	18.5
F. Vaccinated intraperitoneally—irradiated 1 per cent, 30–40 cc.	30–40	3/30	10.0
G. Vaccinated intraperitoneally—irradiated 5 per cent, concentrated 6 times, 5–10 cc.	150–300	1/31	3.2

Actually these comparisons should be made not in terms of equivalent dose of 1 per cent vaccine but number of mouse lethal doses contained in the original vaccine material (1). This information is not obtainable for the commercial canine vaccines but for the irradiated preparations we find from the present tests that 10 cc. of the 1 per cent vaccine contains about 3×10^7 mouse doses and that this does not give the best immunity according to our rigid test; 5×10^7 or 10^8 doses, however, immunize dogs well.

The question of whether vaccine is more effective if given intraperitoneally rather than subcutaneously has not yet been definitely answered. Certainly in mice, the intraperitoneal is the route of choice (1). In dogs, however, the evidence is less convincing. Johnson and Leach (6) interpret their tests as showing the subcutaneous route to be more effective than the intraperitoneal,

whereas our data, though not based on tests especially planned to decide this question, suggest that there is little difference between the two routes but that the intraperitoneal is slightly preferable.

COMMENT AND SUMMARY

Our studies on rabies vaccines thus far have led us to the view that in order to develop and test vaccines, quantitative methods are necessary, and that such quantitative methods may be exploited to greatest advantage by using mice, preferably W-Swiss, as the test animal. Dogs, due to their variability and susceptibility to intercurrent infections when kept under experimental conditions, are useful chiefly to check whether or not a vaccine produces a high grade of immunity; they remain of limited value in testing the comparative potencies of weak vaccines. A second point is that the Pasteur strain of virus has proved as potent as any tested for the preparation of vaccines.[1] Another point is that virus material for preparing vaccines must titre at least 330,000 mouse doses per cc. to be effective. This requirement has eliminated all culture vaccines thus far reported, with the possible exception of Plotz's (7) and leaves virus-containing brain tissue as the sole potent source of vaccine.

In summary, we believe that a single injection of non-virulent irradiated vaccine, prepared as herein described, immunizes mice and dogs effectively against a subsequent test inoculation of virulent rabies virus and does so to a greater degree than do other vaccines now obtainable. It is easily and quickly prepared, keeps well, and has a low nitrogen content.

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[1] Casals, in unpublished studies, has found the Pasteur strain to be somewhat superior as an antigen to several other recently isolated strains.