A MOUSE TEST FOR MEASURING THE IMMUNIZING POTENCY OF ANTIRABIES VACCINES

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Recent knowledge of immunity in central nervous system virus infections indicates a need for a restudy of rabies. Questions are now raised as to whether non-virulent vaccines are capable of inducing an active immunity against subsequent test exposure and whether vaccines administered after exposure are capable of controlling the infection. Moreover, with respect to rabies, statistics fail to show superiority of "live" over "killed" vaccines, nor indeed any advantage gained for the most part in commencing vaccine treatment within 14 days after bite (1). Finally, published experiments on the immunizing potency of antirabies vaccines contain meagre evidence that vaccines will immunize against rabies and this evidence is for the most part inconsistent, non-quantitative, and fails to indicate a superiority of one vaccine over another (2).

The need for a quantitative practical test for determining the immunizing potency of rabies vaccines seemed to be met by our finding that inbred W-Swiss mice were highly susceptible and uniform in their response to rabies virus (3). With these mice methods have been developed for the testing of vaccines and reports made of the results of these tests (4). In the present paper these procedures and results are described in more detail.

W-Swiss Mice as Animals of Choice for Testing Antirabies Vaccines

The W-Swiss mice, selectively bred in our laboratory for susceptibility to central nervous system (C.N.S.) virus infections, have proved especially satisfactory for testing antirabies vaccines for the following reasons.

These Mice Are Highly Susceptible and Relatively Uniform in Their Response to Rabies Virus.—Virus-containing brain tissue diluted 1 to 100 with 10 per cent horse serum plus distilled water and injected intracerebrally in 0.03 cc. amounts into 3 weeks old Swiss mice is passed on serially from the brains of the injected mice when they become prostrate. Such virus from prostrate mice gives reliable titration results in repeated tests, as illustrated in Table I.

It will be noted that with increasing dilutions of virus, 50 per cent or more of tested mice succumb regularly until a critical point is reached beyond which less than 50 per cent succumb. This dilution is regarded as the titration end point and as containing 1 minimum lethal dose (M.L.D.) of virus. Duration of life varies little from an average which depends on the amount of virus administered. Other strains of mice and species of animals have given less uniform results. The susceptibility of the 3 weeks old Swiss mouse is high, as shown in Table I, 0.03 cc. of the 10^{-6} or 10^{-7} dilution proving fatal to the majority of individuals tested. This sensitivity is at least 10 times that of guinea pigs or rabbits injected similarly per gram of body weight. Certain other strains of mice have proved equally susceptible (5) but not equally uniform in their response to the virus.

TABLE I

Intracerebral Titrations of Early Passage Dog 15811 Virus in 3 Weeks Old W-Swiss Mice

Test	Passage	Fate of mice given 0.03 cc. mouse-brain virus intracerebrally in dilutions					Titre
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
1	8	*7, 8, 8	8, 8, 11	9, 14, 15	—	—	0.03 cc. 10^{-6}+
2	17	8, 8	9, 11	12, 14	S, S	—	0.03 " 10^{-6}
3	22	8, 8, 9	10, 10, 11	9, 12, 14	15, 15, S	—	0.03 " 10^{-7}
4	24	—	—	8, 10, 10	10, S, S,	13, S, S	0.03 " 10^{-7}
5	24	7, 9	9, 10, 10, 11	9, 9, 11, 12	11, 11, 12, 12	14, S, S, S	0.03 " 10^{-7}

* Day of death from rabies following injection.

S = mouse remained well. — = dilution not tested.

A similarly uniform response and high susceptibility are obtained if mouse brain passage virus is titred intramuscularly.

Mouse passage virus is prepared in twofold dilutions and 0.01 cc. injected through a 0.3 cm., No. 26 needle pointed distally into the lower third of the gastrocnemius muscle. At least four mice are injected with each dilution of virus. A series of such titrations is illustrated in Table II. The twofold dilutions used in testing this muscle route of infection give less uniform results than the tenfold dilutions employed in testing by the intracerebral route. Nevertheless, 50 per cent or more mice succumb with fair regularity to increasing dilutions of virus, until a point is reached beyond which less than 50 per cent succumb. Duration of life increases regularly with increase in dilution of virus given. The susceptibility of 3 weeks old Swiss mice to this route of infection is high, 0.01 cc. of virus diluted 1 to 320 to 1 to 2,560 generally proving fatal. Other animal species have thus far

proved so irregular in their response to peripheral injection of rabies virus that no quantitative peripheral test has been developed (2).

Certain variables which modify uniformity, regularity, and end points of titrations require careful control. (*a*) Strains of virus differ in virulence. (*b*) Strains of virus increase in virulence with mouse brain passage. (*c*) The age of the mouse modifies the virulence of the virus markedly. These variables will be discussed in detail in a later report.

These mice contract the classical type of disease after injection of a very small amount of virus, 0.01 cc. of a 1 to 320 dilution, into the gastrocnemius muscle. If passage virus is injected, it first appears in the lumbar cord 3 to 5 days later and passes quickly to the brain where it multiplies rapidly. The first signs of disease usually appear on the 9th to 14th days in the form of flaccid paralysis of the injected limb. Convulsions and prostration

TABLE II

Intramuscular Titrations of Early Passage Dog 15811 Virus in 6 Weeks Old W-Swiss Mice

Test	Passage	Fate of mice given 0.01 cc. of mouse-brain virus intramuscularly in dilutions							Titre
		1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
1	7	*11, 16, 21	13, 14, S, S	11, 14, S, S	—	—	—	—	0.01 cc. 1:320+
2	11	13, 13, 19, S	—	12, 12, 16, S	—	15, 17, S, S	—	—	0.01 " 1:1,280+
3	14	—	—	13, 16, 22	26, S, S, S	13, S, S, S	S, S, S, S	—	0.01 " 1:640
4	17	—	—	11, 13, S	15, 16, S, S	S, S, S, S	S, S, S, S	—	0.01 " 1:640
5	18	—	11, 12, 14, S	14, 17, 18, S	12, 17, S, S	11, 16, 17, S	14, S, S, S	15, S, S, S	0.01 " 1:2,560
6	19	—	15, 15, 21, S	12, 15, 17, S	14, 14, 19, S	12, 14, 14, S	12, 15, S, S	S, S, S, S	0.01 " 1:2,560
7	23	—	13, 13, 14, 16	10, 13, 16, 21	18, 20, 25, S	11, 13, 20, S	13, S, S, S	—	0.01 " 1:1,280
8	23	10, 10, 16, 18	10, 13, 13, 18	10, 13, 13, 15	13, 18, S, S	13, 13, 16, S	13, 13, S, S	—	0.01 " 1:2,560+

* Day of death from rabies following injection.

S = mouse remained well. — = dilution not tested.

follow after 24 hours, terminated usually by death in a varying period of time. An occasional mouse survives. At autopsy lesions are generally limited to the posterior root ganglia, spinal cord, and brain and consist of the characteristic perivascular cuffs of round cells plus an occasional focus of similar cells near a necrotic nerve cell. A few nerve cells in Ammon's horn, posterior ganglia, and cord are usually affected. If street virus is injected, the incubation period and duration of disease are longer. Some of the animals become vicious before paralysis sets in. At autopsy Negri bodies are found in the cells of the lumbar cord but most frequently in the region of Ammon's horn.

The response of 5 weeks old mice to 12th passage virus from dog 15811 is shown in Table III. Depending upon the amount of virus injected, paralysis appeared on the 9th to 17th day and terminated with convulsions

and prostration 2 to 40 days later. One paralyzed mouse recovered and remained well 3 months.

The W-Swiss mice are readily immunizable against measurable doses of virus given intracerebrally or intramuscularly. 10,000 intracerebral lethal

TABLE III

Response of 5 Weeks Old Mice to 0.01 Cc. Rabies Virus, Dog 15811, Twelfth Passage, Injected into the Gastrocnemius Muscle

Dilution injected	Mouse No.	Response of mice on days following injection																						
		8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	30	35	40	45	50	55	60
1:40	1	Pa	D																							
	2	Pa	Pr	D																						
	3		Pa	D																						
	4		Pa	D																						
	5		Pa	Pa	Pa	Pa	D																			
	6			Pa	Pa	Pa	Pr	D																		
	7							Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	D	
	8																									
1:80	1		Pa	D																						
	2		Pa	D																						
	3					Pa	Pr	D																		
	4						Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pr	Pr	D										
	5						Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	Imp	Imp	Imp	Imp	Imp	Imp	Imp
	6																									
	7																									
	8																									
1:160	1		Pa	Pr	Pr	D																				
	2					Pa	D																			
	3						Pa	Pr	D																	
	4						Pa	Pr	Pr	Pr	D															
	5							Pa	Pr	Pr	Pr	D														
	6							Pa	Pa	Pa	Pa	Pr	Pr	Pr	D											
	7									Pa	Pa	Pa	Pa	Pa	Pa	Pa	Pa	D								
	8																									
	9																									
	10																									
1:320	1		Pa	D																						
	2				Pr	Pr	D																			
	3				Pa	Pa	Pr	Pr	Pr	D																
	4				Pa	Pa	Pa	Pr	Pr	Pr	D															
	5						Pa	Pa	Pa	Pa	Pr	Pr	D													
	6							Pa	Pa	Pa	Pa	Pa	Pa	D												
	7																									
	8																									
	9																									
	10																									

Pa = paralyzed. Pr = prostrate. D = dead. Imp = improved.

doses or more of virus injected intraperitoneally in a single dose will immunize these mice against a subsequent intracerebral injection of 10 to 1,000 lethal doses.

Table IV shows the results of such a test. Batches of 30 day old mice received an intraperitoneal injection of 0.5 cc. of 175th passage mouse brain virus from dog R-1 in dilutions of 1:100, 1:1,000, 1:10,000, and 1:100,000 respectively. A virulence titration run at the same time indicated that the vaccinated mice had received 160,000, 16,000, 1,600, and 160 lethal doses respectively. 3 weeks later their immunity was tested by injecting them intracerebrally with the same strain of virus in dilutions of 1:1,000 through 1:10,000,000. The controls succumbed through the 1:1,000,000 dilution, while the mice vaccinated with 160,000 doses survived 1,000 intracerebral lethal doses; the mice vaccinated with 16,000 doses survived 10 to 100 lethal doses, those vaccinated with 1,600 doses possibly would have survived one lethal dose, and finally those vaccinated with 160 doses were not immunized.

TABLE IV

Immunity of Mice Following Vaccination with Virulent 175th Passage Dog Virus R-1

Vaccine given	No. of intracerebral lethal doses in vaccine	Fate of mice given 0.03 cc. of test virus intracerebrally in dilutions						Amount of immunity in intracerebral lethal doses
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
None	None	—	—	—	*7, 9, S	9, 11, 12, 12, 13	S, S, S, S, S	
0.5 cc. 1:100	160,000	S, S	S, S, S	13, S, S	S, S, S, S	—	—	1,000
0.5 " 1:1,000	16,000	7, S, S	13, S, S, S	13, S, S, S	20, S, S, S	—	—	10-100
0.5 " 1:10,000	1,600	—	7, 7, S, S	7, S, S, S	8, 9, S, S	—	—	1+
0.5 " 1:100,000	160	7, 13, 21	12, 12, 13	11, 12, 13, 21	11, 13, 17, 21	—	—	0

* Day of death from rabies following injection.

S = mouse remained well. — = dilution not tested.

It will be noted besides that the vaccinated mice, unlike the non-vaccinated controls, occasionally showed individual irregularities in response.

This active immunity is influenced by a number of factors which must be carefully controlled. (*a*) Different strains of virus differ in their immunizing capacity. (*b*) Different strains of virus differ in their tendency to induce rabies following intraperitoneal injection. (*c*) The susceptibility of the mice to intraperitoneal injection and their subsequent capacity to be immunized are markedly affected by age. These factors will be considered in detail in a forthcoming paper.

For the purpose of this paper it is sufficient to note that under the conditions described above, immunity can be demonstrated in 7 days and for at least 9 months. Virus given subcutaneously as a vaccine is more active in inducing rabies and less effective in immunizing the mice. Neutralizing antibodies in the serum appear in the intraperitoneally vaccinated mice at

the same time as does immunity. In subcutaneously vaccinated mice, they usually are not demonstrable.

W-Swiss mice fail to react to vaccination commenced subsequent to a test exposure to the virus. Despite the use of a peripheral route of inoculation with a minimum infecting dose of virus, a method of inoculation that is followed by long incubation periods and a mortality rate of less than 100 per cent (Table III), vaccines of all sorts have proved ineffective in our hands in altering the mortality rates or the course of infection from those in unvaccinated mice.

Development of Swiss Mouse Immunity Test

Having learned that W-Swiss mice are highly susceptible and uniform in response to rabies virus and that they can be readily immunized against subsequent, though not prior test exposure to the virus, we set about developing a simple quantitative test for measuring the immunizing potency of a given vaccine against subsequent measured doses of virulent virus.

The first problem had to do with standardizing the size and number of doses of vaccine for injection into the mice: Since most of the commercial vaccines in this country, whether designed for the treatment of man or prophylaxis of animals, have been rendered non-virulent through treatment with phenol or chloroform, they received first consideration. Undiluted vaccine was poorly tolerated by the mice, whether given intraperitoneally as a vaccine or intracerebrally as a test for virulence. Apparently this poor tolerance was due to the inactivating agent. Consequently, each preparation was diluted 1 to 10, regardless of the concentration of brain emulsion or of inactivating agent. This dilution of vaccine proved entirely harmless. The dose of vaccine prescribed for man or dogs obviously could not be employed for mice. ⅛th of the prescribed dose, however, was readily tolerated by the mice. Hence a standard practice was adopted of diluting each vaccine 1 to 10 and injecting ⅛th of the stated volume, making a total dilution of 1:80. In the case of the vaccines for treatment of man put up in 2 cc. doses, ¼ cc. of a 1 to 10 dilution was given; when put up in ½ cc. doses, 0.06 cc. In the case of the vaccines for animal prophylaxis put up in a single 5 cc. dose, 0.6 cc. was given. This diluted dose for a 15 gm. mouse is still approximately 5 times that prescribed for a 10 kilo child or animal per gram of body weight and is therefore regarded as an ample amount for testing.

The intraperitoneal route of injecting the vaccine was chosen on the basis of tests with virulent virus mentioned above. The difference in effectiveness of inactive vaccines given by intraperitoneal and subcutaneous routes is shown later in Experiment 1. A single injection was given if a canine vaccine was being tested. In the case of vaccines for man, six daily doses proved somewhat less effective in mice than fourteen, but three usually sufficed to demonstrate whether the vaccine was antigenic. The difference in effectiveness of a different number of doses is also illustrated in Experiment 1 described below. The time of testing immunity was chosen at 3 weeks after the first dose of vaccine. At this point experience showed immunity to be at a maximum. The test dose was given intracerebrally in the early tests in dilutions containing 1, 10, 100, and

1,000 M.L.D., as shown in Table I. Later, when a study of canine vaccines was made to demonstrate the presence of even a minute amount of immunizing potency, the more sensitive gastrocnemius muscle route was employed in 2, 4, 8, 16, and 32 M.L.D. doses, as shown in Table II. The tested animals, including unvaccinated controls which were of the same age as the vaccinated animals and which were set apart at the outset of the experiment, were observed for 60 days for signs of rabies and then discarded.

To determine whether or not a vaccine as marketed contained virulent virus, it was diluted 1 to 10 and injected intracerebrally in 0.03 cc. amounts into at least five 2 weeks old Swiss mice.

Briefly, the mouse test for measuring the immunizing potency of any antirabies vaccine is carried out as follows: (*a*) Dilute the vaccine tenfold. (*b*) Segregate sixteen 3 weeks old Swiss mice for vaccination and sixteen of the same age as controls. Provide at least five additional 2 weeks old mice for the virulence test. (*c*) Inject the 2 weeks old mice intracerebrally with 0.03 cc. of the diluted vaccine to determine the presence of virulent virus. (*d*) If the vaccine is designed for the treatment of man, inject sixteen mice with $\frac{1}{8}$th the stated dose of diluted vaccine intraperitoneally daily for 3 or 6 days. If the vaccine is for canine prophylaxis, inject a single dose of $\frac{1}{8}$th the stated amount. (*e*) 3 weeks after the first injection, test vaccinated mice plus controls with 2, 4, 8, and 16 intramuscular lethal doses of virulent virus respectively (or 1, 10, and 100 intracerebral lethal doses).

Details for maintaining titre of the test virus plus additional precautions necessary for consistent results are given above.

Results of Mouse Tests on Commercial Vaccines

Virulent Vaccines for Treatment of Man.—A few vaccines prepared according to the dilution methods of Högyes or Harris were tested with positive results. Upon intracerebral injection they invariably proved virulent but no attempt was made to titrate the amount of virulent virus in the various samples. Injected intraperitoneally as a vaccine, they proved harmless yet immunized the mice within 10 days to 100 intracerebral lethal doses of test virus. The immunity persisted at least 9 months.

Non-Virulent Vaccines for Treatment of Man.—Thirty-two phenolized (Semple) and one chloroformized (Kelser) vaccine for treatment of man obtained from nine different manufacturers have been tested with results shown in Table V. All proved non-virulent. The phenolized products of seven manufacturers have not immunized mice consistently to any significant degree, according to tests on two to four different lots of each. The phenolized vaccine from another manufacturer (No. II), however, has induced a high grade of immunity in mice, as evidenced by the results of tests

of twelve separate preparations. Finally, a chloroformized vaccine from firm No. I also immunized mice readily.

The active phenolized product from firm No. II has been studied in detail with results illustrated in the following protocol.

Experiment 1.—No. II vaccine, a 4 per cent phenolized preparation put up in 14 doses of 2 cc. each, was tested for virulence by diluting it 1 to 10 and injecting 0.03 cc. intracerebrally into 10 mice. It was then given as a vaccine to batches of 75 mice in the following manner. Group A received 1 dose of 0.25 cc. of vaccine diluted 1 to 10 intraperitoneally. Group B received the same dose intraperitoneally for 6 days; group C the same dose intraperitoneally every other day for 6 days; group D 1 dose daily intra-

TABLE V

Results of Mouse Potency Tests of Commercial Non-Virulent Vaccines Designed for Human Treatment

Manufacturer's number	Type of vaccine	Number of preparations tested	Amount of immunity in lethal doses
I	Chloroformized	1	10
II	Phenolized	12	0, 1, 10, 10, 10, 10, 10, 10, 100, 10, 100, 10
III	"	4	1, 0, 0, 0
IV	"	2	0, 0
V	"	3	0, 0, 0
VI	"	3	0, 1, 0
VII	"	3	1, 1, 0
VIII	"	3	1, 1, 1
IX	"	2	0, 0
Total: 9		33	Samples positive from 2 of 9 companies

peritoneally for 14 days. Group E received the fourteen daily doses subcutaneously, and group F received no vaccine. The vaccinated mice were then tested for immunity and for the presence of serum-neutralizing antibodies, 8, 16, 28, 78, 136, and 387 days after the first injection of vaccine.

The results of this experiment are summarized in Tables VI and VII and Text-figs. 1 and 2. The vaccine proved, first of all, to be non-virulent according to the intracerebral test. It immunized in 8 days mice of groups B, C, and D, which had received multiple doses of vaccine intraperitoneally, against 10 to 100 test intracerebral lethal doses of virus. At 16 and 28 days the results were approximately the same and at 78 and 136 days these intraperitoneally vaccinated groups still showed immunity; but at 387 days, when next tested, they were no longer immune. A single dose given to

TABLE VI

Immunity of Mice Following Vaccination with No. II Phenolized Vaccine

Mouse group	Type of vaccination	Time of test after vaccination	Fate of vaccinated mice given 0.03 cc. of test virus intracerebrally in dilutions				Amount of immunity in intracerebral lethal doses
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	
		days					
A	i.p., 1 dose	8	—	*10, 10, 12	12, 13, S	S, S, S	1
B	" 6 doses (1 per day)	"	—	13, S, S	S, S, S	S, S, S	100
C	" 6 doses (1 every other day)†	"	—	S, S, S	11, 15, S	S, S, S	1–10
D	" 14 doses (1 per day)	"	—	13, S, S	S, S, S	S, S, S	100
E	s.c., 14 doses (1 per day)	"	—	8, 11, 12	12, 12, 13	13, S, S	1
F	No vaccination		—	8, 8, 8	9, 12, 12	11, 11, S	—
A	i.p., 1 dose	16	—	12, 12, 12	12, 12, 12	11, 15, S	0
B	" 6 doses (1 per day)	"	—	14, 14, S	S, S, S	15, S, S	10
C	" 6 doses (1 every other day)	"	—	28, S, S	20, S, S	S, S, S	10–100
D	" 14 doses (1 per day)	"	—	S, S, S	S, S, S	S, S, S	100
E	s.c., 14 doses (1 per day)	"	—	9, 9, 11	11, 11, S	12, S, S	1
F	No vaccination		—	8, 10, 10	10, 12, 12	12, 20, S	—
A	i.p., 1 dose	28	—	9, 9, 9	12, 12, S	14, 16, S	0
B	" 6 doses (1 per day)	"	11, 21, S	16, S, S	S, S, S	—	10+
C	" 6 doses (1 every other day)	"	14, 15, S	20, S, S	S, S, S	—	10+
D	" 14 doses (1 per day)	"	S, S, S	S, S, S	S, S, S	—	100+
E	s.c., 14 doses (1 per day)	"	—	8, 9, 10	10, 11, 11	11, S, S	0
F	No vaccination		—	8, 9, 9	12, 14, 14	14, S, S	—
A	i.p., 1 dose	78	—	8, 8, 11, 17	10, 11, 17, S	11, S, S, S	0
B	" 6 doses (1 per day)	"	—	10, S, S, S	13, S, S, S	S, S, S, S	1
C	" 6 doses (1 every other day)	"	—	13, S, S, S	S, S, S, S	S, S, S, S	10
D	" 14 doses (1 per day)	"	—	13, 13, S, S	S, S, S, S	13, S, S, S	1
E	s.c., 14 doses (1 per day)	"	—	10, 10, 10, 10	10, 11, 12, S	S, S, S, S	0
F	No vaccination		—	10, 10, 10, 10	9, 10, 10, 16	S, S, S, S	—
A	i.p., 1 dose	136	—	8, 9, 9, 12	12, 13, 13, 13	—	<10
B	" 6 doses (1 per day)	"	—	10, 10, 14, S	13, S, S, S	—	10
C	" 6 doses (1 every other day)	"	—	9, S, S, S	18, 21, S, S	—	10
D	" 14 doses (1 per day)	"	—	S, S, S, S	15, S, S, S	—	100+
E	s.c., 14 doses (1 per day)	"	—	10, 10, 11, 13	12, 13, S, S	—	<10
F	No vaccination		—	8, 9, 12, 12	12, 13, 13, 14	12, 12, S, S	—
A	i.p., 1 dose	387	—	8, 8, 8, 8	8, 9, 10, 10	10, 11, 16, S	0
B	" 6 doses (1 per day)	"	—	9, 10, 11, 11	12, 13, 17, S	—	0
C	" 6 doses (1 every other day)	"	—	9, 10	9, 12	—	0
D	" 14 doses (1 per day)	"	—	8, 8, 9, 10	9, 9, 9, 11	—	0
E	s.c., 14 doses (1 per day)	"	—	8, 9, 10, 11	9, 9, 9, 10	12, 13	0
F	No vaccination		—	6, 7, 8	9, 9, 10	—	0

* Day of death of mouse following injection.

† Only four of total six injections received at time of 8 day test.

S = mouse remained well 30 days. — = dilution not tested.

group A intraperitoneally and 14 doses to group E subcutaneously failed to immunize.

The results of tests for resistance were paralleled by those for neutralizing antibodies except that group A mice, which had received the single intraperitoneal dose and showed no immunity on inoculation, did show circulating antibodies in every case.

Many preparations of this particular phenolized vaccine (No. II) have been tested. At no time has it been found to contain virulent virus and

TABLE VII

Neutralizing Antibodies in Sera of Mice Vaccinated with No. II Phenolized Vaccine

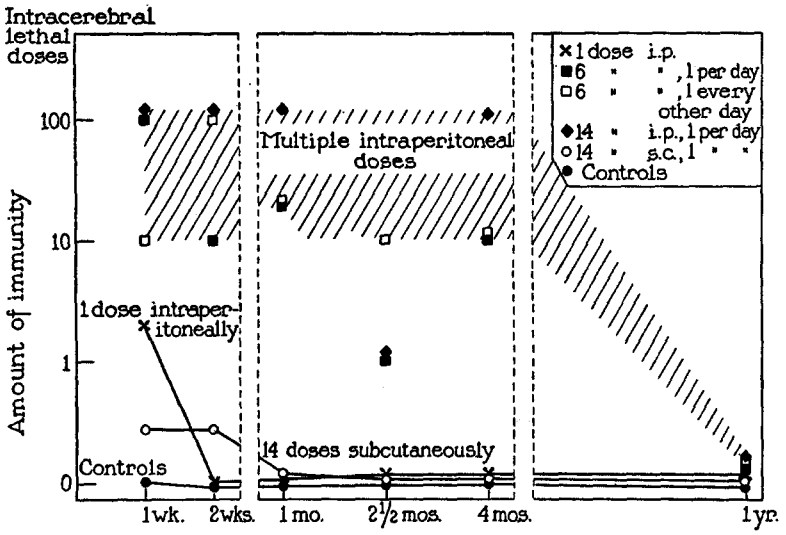
Source of sera		Time of test after vaccination	Fate of mice given 0.03 cc. of serum plus virus in dilutions				Amount of protection in intracerebral lethal doses
Mouse group	Type of vaccination		10^{-3}	10^{-4}	10^{-5}	10^{-6}	
		days					
A	i.p., 1 dose	8	—	—	S, S, S, S	S, S, S, S	100
B	" 6 doses (1 per day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
C	" 6 doses (1 every other day)†	"	—	—	S, S, S, S	S, S, S, S	"
D	" 14 doses (1 per day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
E	s.c., 14 doses (1 per day)	"	—	*8, 9, 10, 10	10, 10, 12, 12	13, S, S, S	1
F	No vaccination		—	8, 8, 8, 10	10, 11, 11, 14	14, 14, 15, S	—
A	i.p., 1 dose	15	—	S, S, S, S	S, S, S, S	S, S, S, S	100+
B	" 6 doses (1 per day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
C	" 6 doses (1 every other day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
D	" 14 doses (1 per day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
E	s.c., 14 doses (1 per day)	"	—	9, 10, 10, 11	10, 11, 12, 12	12, 13, S, S	0
F	No vaccination		—	9, 10, 10, 10	11, 12, 13, 13	13, 13, S, S	—
A	i.p., 1 dose	28	11, 12, 12, S	S, S, S, S	S, S, S, S	—	10
B	" 6 doses (1 per day)	"	S, S, S, S	S, S, S, S	S, S, S, S	-	100+
C	" 6 doses (1 every other day)	"	12, S, S, S	S, S, S, S	S, S, S, S	—	"
D	" 14 doses (1 per day)	"	S, S, S, S	S, S, S, S	S, S, S, S	—	"
E	s.c., 14 doses (1 per day)	"	—	9, 10, 10, 10	10, 12, 13, 13	13, S, S, S	0
F	No vaccination		—	9, 9, 11, 11	10, 11, 13, 13	13, S, S S	—
A	i.p., 1 dose	136	9, 9, 9, 9	9, 10, 11, 12	11, 11, 11, 13	—	0
B	" 6 doses (1 per day)	"	S, S, S, S	S, S, S, S	S, S, S, S	—	1,000
C	" 6 doses (1 every other day)	"	S, S, S, S	S, S, S, S	S, S, S, S	—	"
D	" 14 doses (1 per day)	"	12, S, S, S	13, 14, S, S	S, S, S, S	—	100
E	s.c., 14 doses (1 per day)	"	—	8, 8, 8, 9	10, 10, 11, 11	12, 13, 13, 13	0
F	No vaccination		—	9, 9, 9, 10	11, 11, 13, 13	11, 15, S, S	—

* Day of death of mouse following injection.

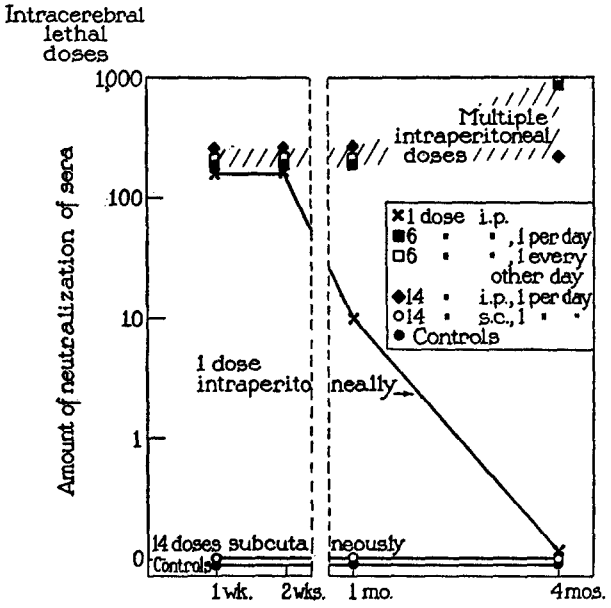
† Only four of total six injections received at time of 8 day test.

S = mouse remained well 30 days. — = dilution not tested.

yet it has never failed on test to induce neutralizing antibodies in human beings or in experimental animals and has rarely failed to render experimental animals immune. The immunity corresponds to that produced with virulent virus. It appears on or about the 8th day and endures approximately a year. Six doses give somewhat less immunity than the full



TEXT-FIG. 1. Immunity of mice to rabies following injection with avirulent phenolized vaccine.



TEXT-FIG. 2. Neutralizing antibodies in blood serum of mice following injection with avirulent phenolized antirabies vaccine.

fourteen doses. The material must be given intraperitoneally, not subcutaneously, for an appreciable or consistent effect. Antibodies appear following a single intraperitoneal injection, although this amount is insufficient to render the animal immune to an intracerebral test dose. The minimum quantity required for immunization of mice is at least 5 times that prescribed for human beings per gram of body weight.

The vaccines described above which failed to immunize mice against a subsequent intracerebral test inoculation likewise failed or showed very little tendency to immunize against the peripheral test inoculation. The two vaccines which successfully immunized against the intracerebral were likewise effective against the peripheral inoculation. The following

TABLE VIII

Immunizing Potency of No. I Chloroform Vaccine in Mice against a Subsequent Intramuscular Test Infection

Mouse group	Fate of mice inoculated intramuscularly with virus in dilutions					Amount of immunity in intramuscular lethal doses
	1:20	1:40	1:80	1:160	1:320	
Non-vaccinated controls	*7, 8, 8, 13, 16	9, 9, 10, 10, 13	8, 9, 9, 9, 13	9, 10, 10, S, S	9, 11, S, S, S	
Vaccinated	S, S, S, S, S	S, S, S, S, S	S, S, S, S, S	S, S, S, S, S	S, S, S, S, S	8+

* Day of death of mouse following test injection.

S = remained well 30 days.

protocol illustrates the immunity conferred by non-virulent No. I chloroformized vaccine against a subsequent intramuscular injection of virus.

Experiment 2.—Twenty mice received 0.06 cc. of No. I chloroformized vaccine, diluted 1:10, daily for 14 days. Twenty-five similar mice were set aside as controls. 3 weeks later both groups were tested intramuscularly. 0.01 cc. of virus in dilutions of 1:20 through 1:160 was given to each of five vaccinated mice respectively. The same dilutions plus 1:320 were likewise given to the non-vaccinated controls.

The results in Table VIII show that 100 per cent of non-vaccinated mice succumbed to the test virus through the 1:80 dilution, 60 per cent in the 1:160, and 20 per cent in the 1:320 dilution. The end point, therefore, is taken as 1:160. All vaccinated mice were immune to the 1:160, 1:80, 1:40, and 1:20 dilutions and hence to at least eight intramuscular lethal doses.

The next experiment illustrates the effect of decreasing dosage of vaccine on the amount of immunity obtained.

Experiment 3.—Batches of sixteen 3 weeks old mice were vaccinated in the following manner. Batch A received six daily intraperitoneal doses of 0.25 cc. and batch B 0.05 cc. of No. II phenolized vaccine, diluted 1 to 10. Batch C received six daily intraperitoneal doses of 0.06 cc. and batch D 0.012 cc. of No. I chloroformized vaccine diluted 1 to 10. Batch E was reserved as controls. To detect the presence of virulent virus, each diluted vaccine was injected intracerebrally in 2 weeks old mice. 3 weeks later the vaccinated and control mice were tested for immunity against the intramuscular injection of virus.

The virulence test showed no evidence of virulent virus in the vaccines and the immunity tests gave results shown in Table IX. No. II vaccine given in six 0.25 cc. doses, which is a total amount equivalent to about 5

TABLE IX

Immunizing Effects on Mice of Antirabies Vaccines Phenolized and Chloroformized Vaccines for Treatment of Man

Mouse group	Fate of mice inoculated intramuscularly with 0.01 cc. of virus in dilutions					Amount of immunity in intramuscular lethal doses
	1:80	1:160	1:320	1:640	1:1,280	
A. Phenolized No. II 0.25 cc.	*10, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	4
B. Phenolized No. II 0.05 cc.	16, 21, S, S	11, 25, S, S	S, S, S, S	9, S, S, S	—	1
C. Chloroformized No. I 0.06 cc.	S, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	4
D. Chloroformized No. I 0.012 cc.	10, 15, S, S	24, S, S, S	11, S, S, S	S, S, S, S	—	1
E. Controls	9, 10, 11, 16	—	9, 12, 19, 22	—	S, S, S, S	—

* Day of death from rabies following injection.

S = mouse remained well. — = dilution not tested.

times that for a 10 kilo child per gram of body weight, protected the mice against at least four lethal intramuscular doses, whereas when given in ⅕th that amount, which more nearly approximates the relative amount given to a child, the result was negative. The same proved true for the chloroformized No. 1 vaccine. Apparently the minimum total dosage sufficient to induce immunity in mice is in the neighborhood of 5 times that regularly prescribed for human beings per gm. of body weight.

Non-Virulent Vaccines for Canine Prophylaxis.—According to United States Government regulations, all vaccines for animal antirabies prophylaxis must be non-virulent. The questions at issue in this study were therefore twofold: First, are the products now on the market non-virulent

according to the mouse test; and second, do they immunize mice against at least two lethal doses of test virus given under controlled and yet as nearly natural conditions as possible?

The following results have been obtained on tests of five or more lots of vaccine from each of ten commercial firms, 50 preparations in all:—

None has contained virulent virus. None has immunized mice against a subsequent test intracerebral injection of one lethal dose. When tested by the intramuscular method (Table X), twenty-seven phenolized prepara-

TABLE X

Results of Potency Tests (Intramuscular) of Canine Vaccines

Manufacturer's number	Type of preparation	Number of preparations tested	Amount of immunity in lethal doses
1	Chloroformized	7	8, 8, 2, 4, 4, 8, 8
3	"	3	4, 16, 8
2	"	4	4, 0, 2, 2
3	Phenolized	4	0, 4, 2, 2
4	"	4	0, 2, 0, 0
5	"	4	0, 0, 0, 0
6	"	4	0, 1, 0, 0
7	"	4	0, 0, 2, 2
8	"	3	0, 0, 0
9	"	4	2, 4, 0, 0
Total: 9 manufacturers		41 preparations	10 of 10 chloroformized vaccines from 2 manufacturers, positive; 3 of 4 chloroformized vaccines from 1, and 27 phenolized vaccines from 7 manufacturers, negative

tions from seven manufacturers for the most part proved negative. Chloroformized vaccines, on the other hand, especially from manufacturers Nos. 1 and 3, have given results which merit further study.

The following protocols illustrate the intramuscular potency test for canine vaccines and the type of result obtained.

Experiment 4.—Batches of sixteen 3 weeks old Swiss mice were given a single injection of commercial vaccine diluted 1 to 10 in the following manner. Batch A received 0.6 cc. of No. 1, 20 per cent chloroformized vaccine intraperitoneally, and batch B 0.1 cc. of the same dilution. Batch C received 0.6 cc. of No. 1, 33⅓ per cent chloroformized vaccine intraperitoneally, and batch D 0.6 cc. of the 20 per cent preparation subcutaneously.

Batch E was given 0.6 cc. of No. 2 vaccine intraperitoneally, batch F 0.1 cc. of the same preparation, while batch G received 0.6 cc. subcutaneously. Batch H was reserved without vaccination as controls. At the same time 0.03 cc. of each vaccine diluted 1 to 10 was injected intracerebrally into five mice.

3 weeks later the vaccinated and control mice were tested against an intramuscular injection of a mouse brain passage strain, 0.01 cc. of virus in dilutions of 1:20 to 1:320 being injected into the gastrocnemius muscle.

The chloroformized vaccines proved non-virulent. In the immunity test (Table XI) all dilutions of virus through 1:320 proved fatal to the non-vaccinated mice. Since no further dilutions were tested, 1:320 is taken as the end point, although the titre may have been still higher. The No.

TABLE XI

Immunizing Effect of Canine Antirabies Vaccine on Mice
Comparison of Effects of Subcutaneous and Intraperitoneal Routes of Injecting Vaccine

Mouse group	Route of vaccination	Fate of mice inoculated intramuscularly with virus in dilutions					Amount of immunity in intramuscular lethal doses
		1:20	1:40	1:80	1:160	1:320	
A. No. 1 chloroformized 20% 0.6 cc.	i.p.	*8, 9, 21	9, 10, 25, S	10, S, S, S	S, S, S, S	—	4
B. " " " 0.1 "	"	7, 8, 15, 29	8, 8, 11, S	8, 8, 10, S	8, S, S, S	—	2
C. " " 33⅓% 0.6 "	"	8, S	9, 9, S	8, 14, 16	S, S, S	—	2
D. " " 20% 0.6 "	s.c.	8, 8, 12	7, 7, 10, S	8, 12, 13, S	9, 9, 10, S	—	0
E. No. 2 " " 0.6 "	i.p.	8, 8, 18	8, 8, 9	8, 9, 9, 13	11, 11, 13, S	—	0
F. " " " 0.1 "	"	7, 8, 8, 8	8, 9, 9, 9	11, 11, 18, S	9, 10, 13, 15	—	0
G. " " " 0.6 "	s.c.	7, 10, 15	8, 9, 9	8, 8, 13	8, 9, 11, 14	—	0
H. No vaccine		—	8, 8, 8	9, 15, 22	10, 10, 11, 15	8, 10, 22, 23	

* Day of death from rabies following test injection.
S = mouse survived 40 days. — = dilution not tested.

2 vaccine failed to immunize, whether given subcutaneously or intraperitoneally. The No. 1 vaccine likewise failed to immunize when given subcutaneously, whereas intraperitoneally in 0.6 cc. or 0.1 cc. doses it immunized against four and two lethal doses respectively. The dose of 0.6 cc. represents 5 times the dose for 10 kilo dogs per gram of body weight, while 0.1 cc. corresponds to the canine dose.

0.6 cc. of chloroformized vaccine given intraperitoneally appeared to irritate the peritoneum. The mice seemed to be in pain for about an hour, were hyperirritable, and occasionally developed transitory convulsions. The discomfort, although causing loss of appetite for a day or so, seemed relatively harmless.

Experiment 5.—Batches of thirty Swiss mice 3 to 4 weeks of age were given a single intraperitoneal injection of vaccine diluted 1 to 10 in the following manner. Batch A

received 0.6 cc. of No. 3 chloroformized vaccine and batch B 0.1 cc. of the same preparation; batch C 0.6 cc. of No. 3 phenolized vaccine, and batch D 0.1 cc. of the same sample; batch E received 0.6 cc. of No. 2 chloroformized vaccine, batch F 0.1 cc., and batch G 0.6 cc. of No. 7 phenolized vaccine. Batch H remained unvaccinated as con-

TABLE XII

Immunizing Effects of Canine Antirabies Vaccine on Mice
Comparison of Chloroformized and Phenolized Vaccines

Mouse group	Test virus	Fate of mice inoculated intramuscularly with 0.01 cc. virus in dilutions							Amount of immunity in intramuscular lethal doses
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
A. No. 3 chloroformized 0.6 cc.	15811	*20, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	—	—	16
B. No. 3 chloroformized 0.1 cc.		9, 15, 20, S	20, S, S, S	14, S, S, S	18, S, S, S	—	—	—	2
C. No. 3 phenolized 0.6 cc.		8, 10, 11, 11	12, 15, S, S	15, 18, S, S	14, S, S, S	—	—	—	2
D. No. 3 phenolized 0.1 cc.		9, 18, 20, 20	17, 18, 18, S	12, 26, S, S	12, 15, S, S	—	—	—	2
E. No. 2 chloroformized 0.6 cc.		10, 11, 12, 20	15, 17, 18, S	15, S, S, S	20, S, S, S	—	—	—	2
F. No. 2 chloroformized 0.1 cc.		12, 15, 19, S	10, 12, 15, S	10, 17, 18, 27	16, 24, S, S	—	—	—	<2
G. No. 7 phenolized 0.6 cc.		10, 16, 20, 20	13, 18, 18, S	14, 23, S, S	S, S, S, S	—	—	—	2
H. No vaccine		10, 10, 12	9, 11, 13, 18	11, 17, 28	13, 14, S, S	10, 15, S, S	—	—	
A. No. 3 chloroformized 0.6 cc.	Sk.	—	9, 17, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	—	8
B. No. 3 chloroformized 0.1 cc.		—	12, 16, S	S, S, S, S	S, S, S, S	15, S, S, S	—	—	8
C. No. 3 phenolized 0.6 cc.		—	9, 17, S	9, 9, 9, S	11, 11, 15, S	10, S, S, S	—	—	2
D. No. 3 phenolized 0.1 cc.		—	7, 9	9, 9, S	9, 10, S, S	9, 10, 14, S	—	—	0
E. No. 2 chloroformized 0.6 cc.		—	7, 9, 15	9, S, S	29, S, S, S	28, S, S, S	—	—	2
F. No. 2 chloroformized 0.1 cc.		—	7, 9, 10	9, 10, 13	9, 10, 12, S	10, 12, 15, S	—	—	0
G. No. 7 phenolized 0.6 cc.		—	9, 10, 15	10, 10, S	12, 15, S, S	10, S, S, S	—	—	2
H. No vaccine		—	—	9, 9, 10, 11	9, 11, 12, 12	9, 9, 10, 10	9, 15, S, S	S, S, S, S	

* Day of death from rabies following test injection.
S = mouse survived 40 days. — = dilution not tested.

trols. At the same time, 0.03 cc. of each vaccine diluted 1 to 10 was injected intracerebrally into five mice.

3 weeks later the vaccinated and control mice were divided into two equal lots for testing against two strains of rabies virus, one recently isolated from a rabid dog and passed through seven mice, the other isolated from a skunk and passed through 156 mice.

0.01 cc. of virus in dilutions from 1:20 to 1:1,280 was injected into the gastrocnemius muscle.

The results of this experiment are shown in Table XII. All vaccines proved non-virulent. The early passage 15811 test virus was somewhat irregular in its effects but was fatal to at least two of four mice through the 1:320 dilution. 0.01 cc. of 1:320 is taken, therefore, as 1 lethal dose, although it is possible that the titre may have been somewhat higher. None of the vaccines gave more than 2 M.L.D. protection except the No. 3 chloroformized preparation. This vaccine, in the 0.6 cc. dose, which is approximately 5 times that for dogs per gram of body weight, protected the vaccinated mice against sixteen lethal muscle doses. 0.1 cc., the dose corresponding to that for dogs, actually seemed to give some immunity, although if one considers the fatalities in high dilutions as retroactive, the irregularities bring the immunity below the level of significance. The results following injection of similar batches with the 156 mouse passage Sk. strain were more regular and similar to those with the 15811 strain. The virus in non-vaccinated mice was fatal regularly through the 1:320 dilution and to two of four in the 1:640 dilution. This is taken, therefore, as the end point. No vaccine except the No. 2 chloroformized preparation immunized the mice against more than 2 M.L.D. of test virus. This latter vaccine, however, again immunized not only against eight muscle doses, when given in a 0.6 cc. dose, but equally as well in a 0.1 cc. dose.

The chloroformized vaccines given intraperitoneally again proved irritative in contrast to the phenolized preparations which caused no reaction.

Experiment 6.—Batches of fifteen mice, 3 to 4 weeks old, were given a single intraperitoneal injection of vaccine diluted 1 to 10 in the following manner. Batch A received 0.6 cc. of No. 1, 20 per cent chloroformized vaccine and batch B 0.1 cc. of the same preparation. Batch C received 0.6 cc. of No. 1, 33⅓ per cent chloroformized vaccine, and batch D 0.1 cc. of the same. Batch E was given 0.6 cc. of No. 5 phenolized vaccine, batch F 0.6 cc. of No. 6 vaccine, and batch G 0.6 cc. of No. 8. Batch H was left unvaccinated as controls. At the same time, 0.03 cc. of each vaccine diluted 1 to 10 was injected intracerebrally into five mice.

3 weeks later the unvaccinated and vaccinated mice were given the early passage 15811 strain of rabies virus, precisely as described in Experiment 5.

The results of this test are given in Table XIII. The vaccines proved non-virulent. Again, the early passage strain proved somewhat irregular in its effects but killed 50 per cent or more mice through the 1:1,280 dilution which was regarded as the end point. The phenolized vaccines again proved generally non-effective but the No. 1 chloroformized preparation, whether in 20 per cent or 33⅓ per cent concentration, immunized the mice

against at least eight intramuscular lethal doses. 0.1 cc. of the 20 per cent vaccine likewise immunized against four lethal doses.

Taken together, the experiments show that no vaccines given subcutaneously, and no phenolized vaccines given either subcutaneously or intraperitoneally immunized mice against more than two intramuscular lethal doses of test virus. Chloroformized vaccines from two manufacturers, however, immunized against four to sixteen doses when given intraperitoneally in amounts 5 to 10 times that advocated for dogs per gram of body

TABLE XIII

Immunizing Effects of Canine Antirabies Vaccines
Further Comparison of Chloroformized and Phenolized Vaccines

Mouse group	Fate of mice inoculated intramuscularly with virus in dilutions						Amount of immunity in intramuscular lethal doses
	1:40	1:80	1:160	1:320	1:640	1:1,280	
A. No. 1 chloroformized 20% 0.6 cc.	*13, 14, 23	17, S, S, S	15, S, S, S	18, S, S, S	—	—	8
B. No. 1 chloroformized 20% 0.1 cc.	12, 12, 13	12, 12, 13, 15	12, 13, 15, 16	17, S, S, S	—	—	4
C. No. 1 chloroformized 33⅓% 0.6 cc.	21, S	16, 20, S, S	14, S, S, S	12, S, S, S	—	—	8
D. No. 1 chloroformized 33⅓% 0.1 cc.	11, 15, 17	12, 13, 14, S	17, 19, S, S	16, 17, 22, S	—	—	<4
E. No. 5 phenolized 33⅓% 0.6 cc.	—	12, 13, 17	13, 14, 15, 17	14, 29, S, S	S, S, S, S	—	2
F. No. 6 phenolized 33⅓% 0.6 cc.	—	13, 16, S	12, 22, S, S	12, 12, 20, S	12, 19, S, S	—	0
G. No. 8 phenolized 33⅓% 0.6 cc.	—	13, 17, S	12, 16, S, S	12, 30, S, S	13, 17, 31, 2	—	0
H. No vaccine	13, 17, 25	12, 12, 30, S	—	12, 12, 17	—	15, 16, S, S	—

* Day of death from rabies following test injection.
S = mouse survived 40 days. — = dilution not tested.

weight, and occasionally when given in amounts corresponding to the canine dose. The chloroformized vaccines given intraperitoneally in 0.6 cc. doses caused transitory irritative phenomena.

DISCUSSION

The mouse test described in this paper is believed to be reliable and at the same time to reproduce field conditions of exposure adequately. According to the results presented, vaccination with 10,000 intracerebral lethal doses of virus, although potentially dangerous, nevertheless immunizes 3 weeks old mice against four or more intramuscular lethal doses

of test virus. Most commercial phenolized vaccines fail to immunize but Kelser's chloroformized preparations generally give positive results. The original virus-containing brain tissue probably contains about 3.3×10^6 mouse intracerebral lethal doses per cc. before inactivation and 1.1×10^6 doses in the $33\frac{1}{3}$ per cent suspension. Following inactivation with chloroform, about 0.5 cc. of a 1 to 10 dilution, or 55,000 inactivated intracerebral mouse doses, is required to immunize a 3 weeks old mouse against four or more intramuscular test doses. These comparative figures must be regarded, of course, as crude comparative approximations not as definite values.

The commercial vaccine of choice for further study is, according to the mouse test, the Kelser chloroformized vaccine; the dose for mice, 2 to 5 times that now advocated for man or animals; the route, intraperitoneal instead of subcutaneous. This preparation requires 60 to 90 days for complete inactivation and under the above conditions is irritating to the peritoneal cavity.

These findings in mice require rigid checking in other animal species. Such experiments are now in progress in dogs.

CONCLUSIONS

1. A quantitative practical mouse test is described for measuring the immunizing potency of antirabies vaccines.

2. Virulent virus, injected intraperitoneally as a vaccine, immunized mice within 10 days and for a period of at least 9 months. Demonstrable neutralizing antibodies accompanied this immunity. Virus given subcutaneously failed to immunize as effectively. The margin between immunizing and infecting dose of vaccine was small.

3. Commercial vaccines containing virulent virus prepared for the treatment of man gave results similar to those obtained with laboratory virus.

4. Commercial vaccines inactivated with phenol and prepared for the treatment of man in general failed to immunize mice. None contained virulent virus. The phenolized preparation from one commercial firm, however, as also the chloroformized preparation from another, immunized mice consistently when given intraperitoneally in quantities approximating 5 times that advocated per gm. of body weight in man.

5. Commercial canine vaccines inactivated with phenol proved non-virulent and failed to immunize mice.

6. Commercial canine vaccines inactivated with chloroform (Kelser) proved non-virulent but capable of immunizing mice provided a single

intraperitoneal injection of 2 to 5 times that prescribed for dogs per gm. of body weight was given.

7. Chloroformized vaccines proved irritative to the peritoneum of mice.

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