INFLUENCE OF AGE FACTORS ON IMMUNIZABILITY OF MICE TO RABIES VIRUS

By J. CASALS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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During the course of our studies on rabies infection in mice, it was learned that age factors may affect both susceptibility and immunizability. The experiments on age and susceptibility have already been reported (1); those on immunizability are described here in detail.[1]

Materials and Methods

Both mice and test virus employed in the present experiments have been described in the preceding paper (1).

Vaccination was carried out 3 weeks prior to the test infection with either virulent or avirulent suspensions. The *virulent vaccines* were prepared by removing the brain of a mouse prostrate following intracerebral injection of rabies virus, emulsifying and diluting it in 10 per cent horse serum in distilled water to obtain a 1 per cent suspension. This was centrifuged for 5 minutes at 500 R.P.M. and the supernatant designated as a 1:100 dilution of virus made up in serial tenfold dilutions. Certain dilutions of the brain virus were injected intraperitoneally as a vaccine in 0.5 cc. amounts into mice of different ages. The vaccine was also titred intracerebrally in 20 day old W-Swiss mice to determine the number of intracerebral M.L.D. contained in each dilution employed. The *avirulent vaccines* were commercial preparations containing chloroform or were prepared as described above and rendered non-virulent by exposure to ultraviolet light (2). The number of intracerebral M.L.D. contained in the commercial vaccines before they were rendered non-virulent was unknown; that in the material prepared in the laboratory was determined by intracerebral titrations of each preparation before inactivation in 20 day old mice. All supposedly avirulent vaccines were tested for pathogenicity by injecting them in as concentrated form as possible intracerebrally into ten young mice. Vaccines failing to kill any of these inoculated mice were considered avirulent and were the only ones employed in tests with this type of vaccine.

Mice 7 to 9 days old received the same amount of vaccine as mice 20, 60, or 90 days old, regardless of weight.

3 weeks following vaccination the mice were regarded as having achieved a maximum degree of immunity (3) and hence were tested by an intracerebral or intramuscular inoculation of virulent virus in graded doses. 0.03 cc. was given intracerebrally in ten-

[1] An abstract of some of these experiments was presented at the Forty-First General Meeting of the Society of American Bacteriologists, held at New Haven, Connecticut, December 28 to 30, 1939.

fold dilutions to batches of four mice and 0.01 cc. intramuscularly in two- or fourfold dilutions. Mice were observed 21 days and then discarded.

The presence of neutralizing antibodies was determined in some experiments immediately before test inoculation by bleeding six or eight mice from each group from the heart, under ether anesthesia. The blood from each batch was pooled and the sera were tested by mixing 0.2 cc. with 0.2 cc. of different dilutions of virus, incubating the mixtures for 2 hours at 37°C., and leaving them for 2 hours at room temperature. They were then injected intracerebrally in 0.03 cc. amounts into 4 weeks old W-Swiss mice.

EXPERIMENTAL

Vaccination with Virulent Virus

Immunity against Intracerebral Injection of Virus.—In this group of experiments, comparative tests were limited to 20 and 60 day old mice, since many younger mice succumb to intraperitoneal vaccination with virulent virus even when it is highly diluted. An illustrative test is described below.

Experiment 1.—Strain 79, passed through three mice following its isolation from a "street" dog, was prepared and injected as a vaccine intraperitoneally into batches of 20 and 60 day old mice in dilutions of 10^{-2} to 10^{-5} inclusive. Each mouse received a single injection of 0.5 cc. of a given dilution. The titre of the vaccine, according to a virulence test in 20 day old mice, proved to be 0.03 cc. of a 10^{-6} dilution. Hence 0.5 cc. of the 10^{-2} dilution contained 166,670 intracerebral M.L.D., and 0.5 cc. of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions contained 16,670, 1,670, and 170 M.L.D., respectively. 3 weeks later all mice, plus controls of the same ages, were given an intracerebral test injection of the same strain, No. 79, after its 6th passage. The results are shown in Table I.

The 20 day mice withstood an amount of vaccine containing 1,670 doses but 22 per cent succumbed following vaccination with 16,670 and 79 per cent following 166,670 doses. In contrast, the 60 day old mice withstood quite well vaccine containing 16,670 doses and only 23 per cent died following 166,670 doses.

The titre of the test virus in the 20 day, non-vaccinated mice was 0.03 cc. $\times$ 10^{-5} and was not significantly different in the 20 day mice receiving vaccine containing 170, 1,670, and 16,670 M.L.D. of virus. In those vaccinated with 166,670 doses there may have been an undisclosed one hundredfold difference. The titre of virus in the 60 day old unvaccinated was similar to that in the 20 day old mice—0.03 cc. $\times$ 10^{-5}—and to that in the 60 day old mice vaccinated with only 170 doses. In contrast, however, a one hundredfold difference in titre was obtained in 60 day old mice vaccinated with 1,670 doses of virus, and a ten thousandfold difference with 16,670 and 166,670 doses. These differences may be expressed in terms of units of resistance if one unit is taken as the maximum amount of virus withstood by 51 per cent or more of control mice. Thus the maximum amount of virus

withstood by the 60 day old controls was 0.03 cc. × 10^{-6}. This dilution is regarded, therefore, as one unit of resistance. Since the 60 day mice vaccinated with 166,670 M.L.D. withstood 10,000 times this dilution, they are said to have resisted 10,000 units of resistance.

This test was repeated with several strains of virus. The results, summarized in Text-fig. 1, show that vaccines containing 170 or less M.L.D. of virus, when injected intraperitoneally into 20 or 60 day old mice, fail to

TABLE I

Immunity of 60 and 20 Day Old W-Swiss Mice Following Intraperitoneal Vaccination with Virulent 3rd Passage Rabies Virus, Strain R79. Intracerebral Infection

Age of mice tested	No. of intracerebral M.L.D. in vaccine	Mice dead following vaccination	Fate of vaccinated mice injected intracerebrally with 0.03 cc. of homologous virus in dilutions						Amount of immunity (units of resistance)*
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
days									
60	166,670	4/17	S, S, S	S, S, S, S	S, S, S	S, S, S	—	—	10,000+
	16,670	2/15	S, S, S	Pa?, S, S, S	17, S, S	S, S, S	—	—	10,000
	1,670	0/16	7,†10, 14, S	15, Pa, S, S	21, S, S, S	S, S, S, S	—	—	100
	170	0/15	—	7, 8, 8, S	9, 10, 10, 13	12, 15, S	21, S, S, S	—	1
	None		—	—	9, 9, 10, 10	12, 15, S	S, S, S, S	S, S, S, S	1
20	166,670	19/24	12, S	9, 10, S	—	—	—	—	100 or less
	16,670	4/18	7, 8, 12	10, 12, S, S	10, 12, 13, S	10, 15, S	—	—	1
	1,670	0/15	9, 9, 10	8, 9, 10, 13	10, 10, 11, S	12, 13, S, S	—	—	1
	170	0/15	—	7, 8, 9, 9	9, 10, 12, S	8, S, S	S, S, S, S	—	1
	None		—	—	10, 10, 10, 12	12, 12, 13, 14	21, S, S, S	S, S, S, S	1

S = mouse survived 21 days.

Pa = mouse became paralyzed but survived 21 days.

Pa? = mouse questionably paralyzed and recovered.

— = dilution not tested.

* A unit of resistance is taken as the maximum dose of virus withstood by 51 per cent or more of control mice.

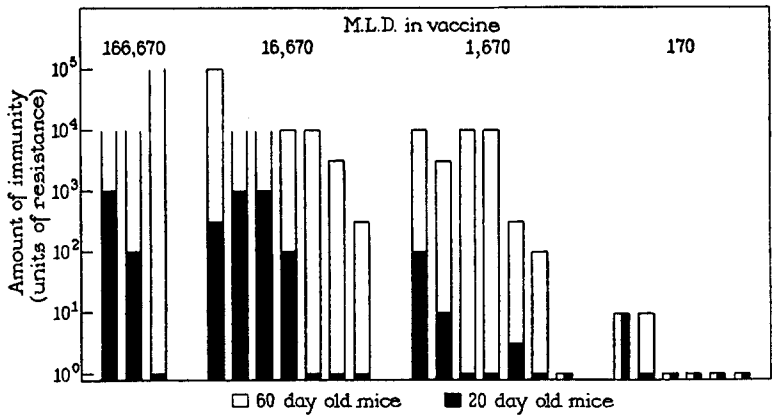
† 7 = mouse died of rabies on the 7th day following infection.

confer a measurable immunity against subsequent intracerebral test injection. Vaccines containing 1,670 M.L.D. confer on 60 day mice 100 to 10,000 units of resistance, whereas the 20 day mice generally show no higher immunity than the controls. Moreover, vaccines containing 16,670 M.L.D. confer on 60 day mice at least 1,000 and usually 10,000 or more units of resistance, whereas in 20 day mice the protection is 10 to 1,000 times less. Finally, 166,670 M.L.D. in a vaccine immunize 60 day old mice solidly, but 20 day mice only partially. At the same time, it should not be overlooked that 16,670 or more doses of virulent virus in a vaccine are fatal to 60 to 90 per cent of 20 day old mice; consequently the individuals surviving represent only a very small and selected part of the original population.

Immunity against Intramuscular Injection of Virus.—In two experiments the test virus was administered to the vaccinated animals intramuscularly rather than intracerebrally. In both instances the results were similar.

Experiment 2.—Batches of 20 and 60 day old mice were vaccinated intraperitoneally with the Pasteur strain, 45th mouse passage, in dilutions of 10^{-4} to 10^{-7} which proved to contain 16,670, 1,670, 170, and 17 M.L.D., respectively. 3 weeks later, all mice, plus unvaccinated controls, were injected intramuscularly with virus No. 15811, passage 53, in fourfold dilutions. The results are shown in Table II.

The titre of the test virus in the 20 day unvaccinated mice was 0.01 cc. of the 1:2,560 dilution. Similar mice, receiving vaccine containing 17 and



TEXT-FIG. 1. Immunity of 60 and 20 day old W-Swiss mice following intraperitoneal vaccination with different amounts of virulent rabies virus.

170 doses of virulent virus were not protected, but those vaccinated with 1,670 or 16,670 doses showed 16 to 64 units of resistance. The 60 day mice, receiving 17 doses in the vaccine, were not protected, but those receiving 170 doses developed 16 units, those receiving 1,670 developed 256, and those receiving 16,670 M.L.D. in the vaccine, 256 or more units of resistance.

The experiments on vaccine containing virulent virus, when taken together, show that 170 to 1,670 doses of virulent virus in the vaccine are required to produce immunity against subsequent intracerebral or intramuscular test injections of rabies virus, and that 60 day mice require fewer M.L.D. in the vaccine for establishment of immunity and show measurably more immunity per M.L.D. of virus in the vaccine than do 20 day old mice.

Vaccination with Non-Virulent Virus

Immunity against Intracerebral Injection of Virus.—The difficulties in immunizing mice with non-virulent vaccines against a subsequent intra-

cerebral test infection have been reported previously (4). In two experiments in this series in which commercial chloroform-treated vaccines were employed, no immunity was produced in 20 day old mice and only 10 to 100 units of resistance in 60 day mice.

Immunity against Intramuscular Injection of Virus.—Commercial vaccines rendered non-virulent with chloroform immunize mice against a subsequent intramuscular infection if given in sufficiently large amounts (3). Three experiments made with 33⅓ per cent brain-virus suspensions con-

TABLE II

Immunity of W-Swiss Mice Following Intraperitoneal Vaccination with Virulent 45th Mouse Passage Rabies Virus, Pasteur Strain. Intramuscular Infection

Age of mice tested	No. of intracerebral M.L.D. in vaccine	Fate of mice injected intramuscularly with 0.01 cc. of virus No. 15811 in dilutions						Amount of immunity (units of resistance)*
		1:10	1:40	1:160	1:640	1:2,560	1:10,240	
days								
60	16,670	10,† S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	256+
	1,670	12, S, S, S	S, S, S, S	14, S, S, S	S, S, S, S	S, S, S, S	—	256
	170	7, 9, 14, S	11, 11, 12, S	S, S, S, S	S, S, S, S	S, S, S, S	—	16
	17	8, 9, 12	10, 15, S, S	9, 9, 10, 21	16, S, S, S	15, S, S, S	—	1
	None	9, 10, 11	8, 9, 9, 10	11, 16, S, S	12, 14, 15, S	S, S, S, S	—	1
20	16,670	9, 10, 16	10, S, S, S	9, S, S, S	10, 14, S, S	S, S, S, S	—	16–64
	1,670	10, 10, 11, S	9, 12, 16, S	9, S, S, S	12, S, S, S	S, S, S, S	—	64
	170	9, 10, 10, S	8, 10, S, S	9, 10, 11, S	16, 21, S, S	10, 10, S, S	—	1
	17	8, 9, 10, 14	9, 9, 9, 10	10, 11, 11, S	9, 11, 12, 16	11, 12, 12, S	—	1
	None	9, 9, 10	9, 10, 11, 11	9, 9, 10, 12	11, 12, 14, 15	10, 11, 14, S	S, S, S, S	1

S = mouse survived 21 days.

— = dilution not tested.

* A unit of resistance is taken as the maximum dose of virus withstood by 51 per cent or more of control mice.

† 10 = mouse died of rabies on the 10th day following infection.

taining 1 per cent chloroform are summarized in Table III. The vaccines were diluted ten times in saline and injected intraperitoneally in one or two doses to mice 20 and 60 days old.

Table III shows that in each test 20 and 60 day mice remained well and were equally protected by the vaccines.

Vaccines made avirulent by ultraviolet light were likewise tested in groups of 60, 20, and 7 day old mice.

The 7 day old mice were kept with their mothers until they reached 3 weeks of age and showed no untoward effects of the vaccine. The vaccine, consisting of a 1 per cent brain-virus suspension exposed to ultraviolet light and proved non-virulent by intracerebral inoculation, was inoculated intraperitoneally in single doses of 0.2 cc. Addi-

tional mice of the same ages were set aside as controls. 3 weeks later, all were given an intramuscular injection of virulent virus in graded dilutions.

Table IV shows that unvaccinated, 60 day old mice succumbed through the 1:160 dilution of virus, whereas the vaccinated, 60 day old mice with-

TABLE III

Immunity of W-Swiss Mice Following Intraperitoneal Vaccination with Chloroformized 33⅓ Per Cent Commercial Canine Antirabies Vaccine Diluted 1:10. Intramuscular Test Infection

Experiment No.	Age of mice tested	Amount of vaccine given	Fate of mice injected intramuscularly with 0.01 cc. of virus No. 15811 in dilutions										Amount of immunity (units of resistance)*
			1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
	days												
1	60	0.5 cc.		0/2	1/4†	1/4	0/4	0/4	0/4	—	—	—	16–32
		0.5 cc. × 2		0/4	0/4	0/4	0/4	0/4	0/4	—	—	—	64+
		None	—	—	—	4/4	2/3	2/4	3/4	0/4	0/4	—	1
	20	0.5 cc.		1/2	1/4	0/3	0/4	1/4	1/4	0/4	0/4	—	16–32
		0.5 cc. × 2	—	0/3	1/4	0/4	0/4	0/4	0/4	0/4	0/4	—	64–128+
		None	—	—	4/4	2/2	3/4	2/4	1/4	3/4	1/4	1/4	1
2	60	0.5 cc.	—	2/6	1/6	2/6	1/6	1/6	—	—	—	—	16
		None	—	—	5/5	3/5	2/6	5/6	3/6	1/6	—	—	1
	20	0.5 cc.		0/4	2/6	1/6	0/6	0/6	—	—	—	—	32
		None	—	—	—	5/6	5/6	3/6	4/6	2/6	—	—	1
3	60	0.5 cc. × 2	0/7	—	1/7	—	0/7	—	0/7	—	0/7	—	64
		None	5/7	—	6/7	—	3/7	—	0/7	—	1/7	—	1
	20	0.5 cc. × 2	1/7	—	1/7	—	0/7	—	0/7	—	0/7	—	64
		None	—	—	6/7	—	3/7	—	2/7	—	1/7	—	1

— = dilution not tested.

*A unit of resistance is taken as the maximum dose of virus withstood by 51 per cent or more of control mice.

† 1/4 = 1 mouse out of 4 died of rabies.

stood the 1:10 dilution, at least a sixty-fourfold difference. Similarly, the vaccinated, 20 day old mice showed 64 units of resistance, whereas the 7 day old mice remained susceptible.

In similar tests in which mostly 20 and 60 day old mice were used, 0.2 cc. and even 0.1 cc. of irradiated vaccine gave results in agreement with the above; namely, definite protection against intramuscular test infection but equal protection in 20 day and 60 day old mice. Finally, 0.01 cc. of vaccine failed to immunize significantly.

TABLE IV

Immunity of W-Swiss Mice Following Intraperitoneal Vaccination with Ultraviolet Light-Inactivated 1 Per Cent Suspension of Rabies Virus, Pasteur Strain. Intramuscular Test

Age of mice tested	Amount of vaccine given	Fate of mice injected intramuscularly with virus No. 15811, 0.01 cc. in dilutions							Amount of immunity (units of resistance)*
		1:10	1:40	1:160	1:640	1:2,560	1:10,240	1:40,960	
days									
60	0.2 cc.	1/6†	0/6	1/6	0/6	0/6	—	—	64
	None	5/5	5/6	5/6	1/6	2/6	—	—	1
20	0.2 cc.	2/4	1/5	0/6	0/6	0/6	—	—	64
	None	—	5/6	6/6	5/6	0/6	1/6	—	1
7	0.2 cc.	3/4	5/5	6/6	3/6	3/6	0/6	—	1–4
	None	—	5/5	5/5	5/5	4/6	3/6	1/6	1

— = dilution not tested.

* A unit of resistance is taken as the maximum dose of virus withstood by 51 per cent or more of control mice.

† 1/6 = 1 mouse out of 6 died of rabies.

TABLE V

Neutralizing Titre of Sera from W-Swiss Mice of Different Ages Following Vaccination with Virulent and Avirulent Rabies Virus Suspensions

Age of mice tested	Treatment of mice furnishing serum	Route of test injection	Amount of immunity (units of resistance)*	Mortality of mice injected intracerebrally with mixtures of serum and virus							Degree of protection afforded by serum of vaccinated mice (units of resistance)*
				Dilution of virus in mixture							
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
days											
60	Virulent suspension: vaccinated	Intracerebral	1,000–10,000	—	1/4†	0/4	0/4	0/4	0/4	—	1,000
	Unvaccinated	"	1	—	—	4/4	4/4	0/4	0/4	—	1
20	Virulent suspension: vaccinated	"	1	—	1/4	0/4	0/4	0/4	0/4	—	10,000
	Unvaccinated	"	1	—	—	4/4	4/4	3/4	0/3	—	1
60	Avirulent suspension: vaccinated	Intramuscular	64	4/4	3/4	1/4	0/4	—	—	—	10,000
	Unvaccinated	"	1	—	—	4/4	4/4	4/4	2/4	—	1
20	Avirulent suspension: vaccinated	"	64	4/4	4/4	1/4	0/4	—	—	—	10,000
	Unvaccinated	"	1	—	—	—	4/4	4/4	3/4	1/4	1
7	Avirulent suspension: vaccinated	"	1–4	4/4	4/4	2/4	0/4	0/4	—	—	1,000–10,000
	Unvaccinated	"	1	—	—	—	4/4	3/4	3/4	0/4	1

— = dilution not tested.

* A unit of resistance is taken as the maximum dose of virus withstood by 51 per cent or more of control mice.

† 1/4 = 1 mouse out of 4 died of rabies.

Presence of Circulating Neutralizing Antibodies in Vaccinated Mice

Webster (5) has shown that following multiple intraperitoneal injections of rabies vaccine, mice develop antibodies and become immune, whereas with a single intraperitoneal injection they develop antibodies to the same extent yet fail to show significant immunity. Recent tests have indicated that mice, regardless of age, respond to antigenic vaccines with the formation of abundant neutralizing antibodies, yet may or may not be immune. Two such tests are summarized in Table V.

In the first test, mice were vaccinated with virulent vaccine and tested intracerebrally. The 60 day old mice showed 1,000 to 10,000 units of resistance to intracerebral infection and had neutralizing antibody titres at the time of the test injection of 1,000 units of resistance. 20 day mice, vaccinated and tested as above, proved non-immune yet showed neutralizing antibody titres of 10,000 units.

In the second test, mice were vaccinated with an avirulent vaccine and tested intramuscularly. The 60 day and 20 day old vaccinated mice showed 64 units of resistance to intramuscular injection and the 7 day old mice were not immune. Yet all age groups showed an equally high titre of neutralizing antibodies,—1,000 to 10,000 units of resistance.

DISCUSSION

That older animals are more readily immunizable than younger ones has recently been shown by Culbertson and Kessler with trypanosome infection of rats (6), by Morgan with Eastern equine encephalomyelitis infection of mice (7), and by Casals and Webster with rabies infection in mice (8). In the case of rabies, however, it has developed that age influences immunizability only under definite sets of conditions. Most conspicuous is the case in which 20 day old mice vaccinated with virulent virus are less immunizable against a test intracerebral infection than 60 day old mice similarly treated. Least conspicuous is the case in which 20 day and 60 day old mice vaccinated with non-virulent virus are equally immunizable against a test intramuscular infection.

The extensive studies on increasing antibody formation with advancing age have been comprehensively reviewed by Baumgartner (9). Whether this increase in titre with age means a corresponding increase in immunity must be determined independently for each infection. Certainly in rabies antibody titre does not parallel immunity titre.

CONCLUSIONS

1. W-Swiss mice 60 or more days old are more readily immunizable against rabies virus infection than 20 day old or younger mice; this difference in immunizability with increasing age is most conspicuous when vaccination with virulent virus is followed by intracerebral test infection and least apparent when vaccination with avirulent virus is followed by intramuscular test infection.

2. The titre of circulating neutralizing antibodies does not parallel the titre of immunity.

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