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Studies on Chick Embryo Adapted Rabies Virus. III. Duration of Immunity in Vaccinated Dogs. (19640)

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Although attempts have been made to immunize dogs against rabies as far back as 1826(1) and Pasteur succeeded in doing so in 1885(2), only few extensive studies have been conducted on duration of immunity following vaccination of dogs against this disease. A summary of these studies is shown in Table I. Pasteur reported(2) that resistance to intracerebral infection still appeared to be manifest 2 years after the "immunizing" injection. Data obtained by other investigators and summarized by Schnürer(3) seemed to indicate that immunity can be observed even 363 days after vaccination with living virus, although lack of adequate controls makes interpretation of their data more difficult. In the experiments of Miessner and Baars(4), dogs were found to be solidly immune to challenge inoculation with street virus 8 to 14 months after vaccination with fixed virus vaccine. More recently, a series of experimental studies designed to determine the duration of immunity following vaccination of dogs has been published by Johnson(5), and these are also summarized in Table I. It may be observed that preparations of phenolized rabies vaccine conferred an effective immunity upon dogs challenged with street virus one year after vaccination. Of those animals immunized with a single injection of vaccine, 11.5% died after challenge inoculation, as compared with 79% of the controls. Conversely, if the vaccination process consisted of 3 injections given at weekly intervals, none of the vaccinated dogs came down with rabies after challenge one month later, in contrast with 68% which died in the control group.

In view of the fact that an effective single injection method of canine vaccination against rabies was found in the recently developed chick embryo vaccine containing the living Flury strain of rabies(6), it seemed to be of interest to determine the duration of immunity conferred upon dogs by inoculation with this vaccine.

Materials and methods. Virus strains. The Flury egg-adapted strain of rabies virus used in this study has been described(7). An infected chick embryo suspension desiccated from the frozen state was used for vaccination of dogs. It represented the 40th egg passage of the virus, and was prepared 8 months prior to vaccination. The PM (Pitman-Moore 980) rabbit-fixed strain of virus(8) was used for the preparation of phenolized vaccine of horse brain origin. This particular lot of vaccine, bearing Lab. No. 6273-1580, was prepared on April 13, 1948, and the Habel mouse potency test was performed on May 4, 1948, with results indicating that the vaccine protected against 8166 LD_{50} of fixed virus. For challenge purposes the NYC strain of rabies street virus representing either the 6th or 7th passage canine salivary glands in this laboratory was used on the vaccinated animals. The preparation of the challenge inoculum followed that described in detail elsewhere(6).

Animals. Mongrel dogs over 6 months old

TABLE I. Some Observations on Duration of Immunity Following Vaccination of Dogs Against Rabies.

Immunization		Time interval between immunization and challenge	Challenge inoculation		Mortality ratio		Author
Virus	Route		Virus	Route	Vaccinated	Controls	
Fixed or Street	Subcut.	1 yr	Street	Intracer.	3/14	?	Pasteur(2)
		2 "	"	"	2/6	?	
Fixed	Subcut. or intraper.	18–120 days	Fixed	"	3/18	?	Summarized by Schnürer (3)
			Street	"	8/22	?	
		4–363 "	Same	Intraocular	2/24	?	
				"	1/10	?	
		23– 56 "	Same	Intramusc.	0/2	?	
				"	0/14	?	
		15– 62 "	Street	Bite	0/3	?	
Fixed	Intraper.	8– 14 mo	"	Intramusc.	0/9	3/3	Miessner & Baars(4)
Phenolized vaccine	Subcut.*	1 yr	"	Intramasseter	6/52	41/52	Johnson(5)
	" †	1 mo	"	"	0/25	17/25	

* One inj. of phenolized vaccine. † Three inj. of phenolized vaccines.

were used in the experiments. The animals were kept under observation for a period of 3 months prior to vaccination. Following challenge inoculation, the animals were kept in individual cages throughout an observation period of 1½ years. An autopsy was performed on every animal that died and portions of the brain tissues and of the submaxillary salivary glands were removed. Impression smears of Ammon's horn of each dog were examined for presence of Negri bodies. In addition, 10% suspensions of brain and of salivary gland tissues were each inoculated intracerebrally into six 28- to 35-day-old Swiss albino mice, which were observed for a period of 3 weeks. In case of doubt as to the cause of death, brain tissues from such mice were subinoculated into 6 other mice. If the latter died as a result of infection, neutralization tests were performed using immune rabbit serum to determine whether the agent was rabies virus. All material containing living virus used in the experiments was titrated intracerebrally in 28- to 35-day-old mice; each of 6 mice received .03 ml of a given dilution. *Sera.* All nonvaccinated control animals were bled prior to challenge inoculation, and their sera immediately tested in order to exclude those animals whose blood showed neutralizing antibodies. In several instances, serum was also obtained from dogs prior to vaccination with either Flury or phenolized vaccines, and then at certain regular intervals prior to challenge inoculation. *Neutralization tests.* Undiluted sera were mixed in equal volume with a dilution of guinea pig brain suspension of the CVS rabbit-fixed strain which contained approximately 25-50 LD_{50} of the virus. The serum virus mixtures were incubated for 90 minutes at 37°C before being injected intracerebrally in Swiss albino mice.

Experimental. On Feb. 8, 1949, 120 dogs which had been under observation for 3 months were divided into 2 equal groups. One group of animals was immunized by a single injection in the upper quadrant of the posterior surface of the thigh muscle with 5 ml of a 20% suspension of chick embryos infected with the 40th egg passage of the Flury strain. This material was rehydrated at the time of vaccination and kept in iced water during the inoculation period. Simultaneously titrated in mice, it had an LD_{50} of $10^{-3.99}$. The other group of 60 animals received a divided dose of 5 ml of a 20% suspension of horse brain containing phenol-inactivated rabies virus, 2½ ml being administered in each of 2 sites. During the ensuing 2 years all animals were closely observed, and while some losses occurred in both groups as a result of fights or intercurrent infections, in not a single instance could death of an animal be attributed to ill

TABLE II. Results of Challenge Inoculation with Street Virus of Dogs Vaccinated with Rabies Vaccines.

Interval between vaccination and challenge	Vaccine	Results of challenge: No. of dogs Inoculated	Dead	% dead	Day of death after challenge	Rabies virus isolated from: Brain tissue	Salivary gland
1 yr	Flury strain living virus	25	0	0		—	—
	Phenolized horse brain origin	22	3	14	18, 21, 260	3	1
	Nonvaccinated controls	25	18	72	15, 18, 18, 18, 18, 18, 18, 21, 23, 25, 25, 25, 26, 27, 29, 29, 33, 33	18	16
2 yr	Flury strain living virus	25	3	12	21, 22, 24	3	0
	Phenolized horse brain origin	19	8	42	14, 16, 17, 18, 18, 19, 20, 33	6	0
	Nonvaccinated controls	23	21	91	13, 15, 16, 16, 16, 17, 17, 17, 17, 18, 18, 18, 19, 20, 21, 22, 22, 22, 22, 22, 23	21	11

effects of the chick embryo vaccine. On the other hand, out of the 60 animals injected with the phenolized rabies vaccine, one died 16 and another 24 days following vaccination. Both deaths were preceded by paralysis and were found to have been caused by CNS demyelination characteristic of that seen following inoculation with material containing nervous tissue(9).

One year after vaccination, one-half of the surviving dogs in the groups immunized with the Flury strain or the phenolized vaccine were challenged with a 1:90 dilution of canine salivary gland infected with the 6th passage NYC strain of street virus. This preparation, titrated in mice, had a $10^{-4.85}$ LD_{50} titer. The challenge dose consisted of a total of 0.2 ml injected bilaterally into the masseter muscle, 0.1 ml into each site. Inoculation was performed with a 1 cc tuberculin syringe equipped with a 1-inch, 20-gauge needle. The inoculum was deposited in 3 different sites next to the periosteum in order to assure better tissue penetration of the virus. As mentioned previously, the challenged dogs were observed for 18 months following inoculation. The remaining groups of vaccinated animals were challenged 2 years after vaccination with 1:75 dilution of canine salivary gland representing the 7th passage of the NYC strain of street virus. This time, the LD_{50} titer of the challenge virus in mice was found to be $10^{-6.50}$.

The results of the 2 experiments are summarized in Table II. It may be observed that in the one-year challenge experiment, 18 out of 25 non-vaccinated controls died after exhibiting signs of clinical rabies, and the challenge virus was isolated from the brain tissue of all of the 18 animals and from the salivary gland tissue of 16 out of the 18. In contrast, none of the dogs with the Flury strain vaccine succumbed to rabies following challenge inoculation. Out of 22 dogs which were vaccinated with phenolized vaccine, 3 died following challenge, and challenge virus was isolated from the brain tissue of 3 animals and from the salivary gland tissue of one. The latter isolation occurred in a dog which died 260 days after challenge inoculation. Since this animal had been confined during the entire observation period in a separate cage with no contact with any other infected animals, his death must be attributed to the original challenge inoculation even though the incubation period was prolonged.

The results of challenge inoculation 2 years after vaccination show that 21 out of 23 nonvaccinated control animals died following rabies injection. Virus was isolated from the brain tissue of 21 animals but from the salivary gland tissue of only 11 dogs. Out of

TABLE III. Results of Neutralization Test on Sera of Vaccinated Dogs Challenged 1 Year after Vaccination.

Vaccine	Dog	Survival ratio of mice inoculated with virus and serum bleedings A	B	C	D	E	Results of challenge with street virus*
Flury	A	0/5	5/5	—	5/5	4/5	S
	B	0/5	0/5	0/5	0/5	0/5	S
	C	0/5	4/5	4/5	5/5	3/4	S
	D	0/5	4/5	2/5	3/5	5/5	S
	E	0/5	5/5	4/5	4/5	3/5	S
	F	0/5	5/5	4/5	1/5	5/5	S
Phenolized	G	0/5	4/5	5/5	0/5	0/5	D
	H	0/5	0/5	1/5	0/5	0/5	S
	I	0/5	5/5	5/5	0/5	0/5	S
	J	0/5	0/5	0/5	0/5	0/5	D
	K	0/5	5/5	5/5	5/5	2/5	S
	L	0/5	4/5	2/5	—	0/5	S

* One year after vaccination.

A—Prevaccination bleeding; B—1 mo after vaccination; C—3 mo after vaccination; D—6 mo after vaccination; E—9 mo after vaccination. S—survived; D—died.

25 animals vaccinated with the Flury strain, 3 died following challenge, and virus was isolated from the brain tissue of these 3 animals but not from the salivary gland tissue of any of the dogs. Out of 19 animals vaccinated with phenolized vaccine, 8 died following challenge inoculation, and rabies virus was isolated from the brain tissue of 6 of the dead animals. However, the 8 dead animals in this group were all included as having died of rabies since clinical signs of either paralytic or furious rabies were observed in all 8 dogs. Perhaps the absence of the virus in the central nervous system tissue of the 2 animals may be ascribed to the phenomenon of "self-sterilizing neuro-infection"(10). Although evidence obtained in a previous communication(8) indicated that it is difficult to correlate serological evidence of immunity with the resistance of the vaccinated animal to experimental infection with rabies virus, another attempt was made in the present study to determine the presence of homologous antibodies in serum in dogs vaccinated with the 2 types of experimental vaccines.

In Table III are summarized the results of neutralization tests on sera of 6 dogs in each vaccinated group challenged one year after immunization. It may be observed that out of 6 animals vaccinated with the Flury strain, none had neutralizing antibodies prior to vaccination, and in all but one animal, immunization with the Flury strain elicited homologous antibodies. Interestingly enough, even this animal (dog B), which showed no neutralizing antibodies at any time after vaccination, survived challenge with street virus. Out of 6 animals inoculated with the phenolized vaccine, 2 did not develop neutralizing antibodies following vaccination; whereas in the remaining 4, a positive serological response was noted. This response, however, seemed to fade out after 9 months following vaccination. The results of challenge inoculation seemed again to corroborate previous evidence that serological response following immunization against rabies cannot be regularly correlated with the actual resistance of the animal to experimental infection with street virus.

In view of the above results, no further attempts were made to bleed vaccinated animals to determine the presence or absence of neutralizing antibodies in their sera. However, the control dogs which accompanied those challenged 2 years after vaccination were bled and their serum was submitted to neutralization tests. Every animal whose serum had neutralizing antibodies was deleted from the control group.

Comment. The difference between the challenge results obtained in the one-year and two-year immunity tests suggests a decline in specific resistance to rabies during the second year after vaccination. However, this decline

may be more apparent than real. It will be noted that the challenge inoculum employed in the one-year test had a mouse LD_{50} titer of $10^{-4.85}$, while that used in the 2-year test was $10^{-6.5}$. Calculated on the basis of these mouse titers, the dogs in the one-year test were challenged with 5,194 LD_{50}'s and those in the 2-year test received 278,276 LD_{50}'s. This relatively heavier challenge is reflected in the 91% mortality and the 18.4 day average incubation period in the non-vaccinated controls on the 2-year tests, as compared with 72% mortality and 23.2 day average incubation period for the one-year test. Had challenge virus of the same potency as that used in the one-year test been employed in the 2-year challenge, the degree of protection shown in the 2-year test might have been enhanced.

In any attempt to translate the results of these laboratory experiments in terms of the protection afforded dogs against field exposure, it should be borne in mind that the amount of virus introduced by natural exposure is usually greatly exceeded by that employed in the laboratory challenge tests. Thus, if one may assume that the death of 3 dogs out of 25 challenged with street virus 2 years after vaccination with Flury strain was conditioned by the excessive dosage of the challenge virus, then it may perhaps be permissible to state that for all practical purposes vaccination with Flury strain confers immunity for at least 2 years.

Conversely, study of the trend of results of challenge inoculation of animals vaccinated with phenolized vaccine does not permit the drawing of as favorable a conclusion as in the case of vaccination with Flury virus. Although the phenolized vaccine certainly proved effective when dogs were challenged one year after vaccination, the death of 8 animals out of 19 challenged 2 years after vaccination seems to indicate that inoculation with phenolized vaccine constitutes a less effective method of protection than with the Flury strain. This statement may be supported by a statistical analysis of the results. By calculation of the exact probabilities, and by combination of these probabilities for the one- and 2-year results,* using the method of R. A. Fisher(11), one obtains a value of X^2 of 10.166 with 4DF corresponding to a probability of 0.038. The latter figure is lower than 0.05 and therefore indicates a statistically significant difference in favor of the immunizing power of the Flury strain vaccine as compared with the phenolized vaccine.†

Mass field vaccination of the canine population with living fixed strain of rabies virus (rabbit brain origin) has been attempted only once in the past, so far as we know, and with excellent results. Hindersson(12) immunized 2,908 dogs in the Nykyrka, Rautu, Salmis, and Suistamo regions of Finland beginning in 1929 through 1932, and by this means was actually able to eradicate rabies in the entire region of Finland which borders on Soviet Russia. He later observed the first, and as far as can be determined, the only case of rabies in the immunized group 2 years and 3 months after vaccination.

Conclusions. Hindersson's observations, coupled with the results of the present experiments, seem to indicate that: 1. Vaccination with living rabies virus is perhaps a more effective method of immunization of the canine population than any of the others presently available. 2. Immunity conferred by vaccination with living rabies virus, as exemplified by Flury strain in these experiments, lasts at least 2 years. 3. These observations on the duration of immunity following live virus vaccination indicate the need for reconsidering local ordinance and other regulations concerning rabies vaccination.

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* This seems to be particularly permissible since animals in both experiments were vaccinated on the same day with the same material.

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Effects of Aureomycin on Renal Lesions, Liver Lipid, and Tissue Choline in Choline Deficiency. (19641)

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The impression was obtained in a previous study(1) that it might be possible, by causing certain alterations in the conditions in the gut, to reduce considerably the damage produced in rats by diets deficient in choline. Succinylsulfathiazole was studied at that time, but it was not observed to be effective in modifying the damage, although it did produce enlargement of the caeca. More recently, with the same idea in mind, the newer antibiotics have been examined. The present report presents evidence that the severe renal injury which occurred regularly as a result of choline deficiency was prevented in most cases by aureomycin. Fatty changes in the liver also appeared to be diminished somewhat and alterations in tissue and fecal choline were observed. However, it was not demonstrated conclusively that these effects of aureomycin were actually results of changing the conditions in the gut and not results of a direct metabolic effect.

Methods and materials. The animals employed were male rats of a Sherman strain, which were 20 to 23 days old and weighed 30 to 40 g when started on the experimental diets. The basal diet was the same as that described previously(1) with the carbohydrate in the form of sucrose, and with Wesson's modification of the Osborne-Mendel salt mixture with Zn and Co added in most experiments. The unsupplemented diet contained negligible amounts of choline, and presumably contained relatively small amounts of vit. B_{12}. The animals were maintained in individual cages with large-mesh screen bottoms, in a room with controlled temperature. The diets were fed *ad libitum* in some experiments. In others, each animal received 3.6 or 5.0 g of food each afternoon. In the latter experiments, the food was consumed completely and the food intake of all animals was the same. The supplements under study were incorporated in the diet in most cases in the form of pure, crystalline substances. After 10 days on the diets the surviving animals were autopsied after decapitation, and the results evaluated. It had been observed previously that few additional animals developed renal lesions or died after this period. In 4 experiments with 8 animals in each group, employing controlled feeding, total liver lipid, after 4 days on the diets, was determined gravimetrically as the petroleum ether-soluble portion of the material obtained by wet alcohol-ether extraction of homogenized tissue. Choline was determined by the method of Glick(2), either directly on the material extracted by alcohol-