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IMPROVEMENT OF THE ANTIGENICITY OF ANTIRABIES VACCINE BY POOLING CHECKED BY POST-CHALLENGE VACCINATION OF GUINEA-PIGS

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SYNOPSIS

The author describes some studies carried out at the Pasteur Institute of Southern India, Coonoor, with the object of developing an antirabies vaccine of uniform potency.

It was found that by pooling batches of vaccine from several infected sheep brains a vaccine was produced which was superior in antigenicity (as determined by potency tests in mice) to the NIH (United States National Institutes of Health) Reference Vaccine 155-D as well as to most of the individual batches of vaccine tested. Furthermore, the pooled vaccine conferred a significant degree of protection on guinea-pigs challenged with virulent strains of street virus, even when not administered until an hour after infection.

A brief outline is given of the method used for pooling the vaccine.

During studies on the standardization of the potency tests for antirabies vaccine, considerable variation was found in the antigenicity of batches of vaccine prepared under identical conditions from different sheep brains. Some of the batches were very good compared with the standard reference vaccine while others were poor. Experiments have been in progress at the Pasteur Institute of Southern India to evolve a method by which a vaccine of uniform potency could be obtained.

It was found that a vaccine of reasonably uniform potency was obtained when 12 batches of vaccine were pooled before being put into ampoules. In the pools so far tested, the antigenicity was always greater than that of the reference vaccine. The good results obtained in potency tests in mice as well as in the post-infection treatment of guinea-pigs challenged with virulent strains of street virus has led to the adoption of the method for the routine manufacture of antirabies vaccine at the Institute since March 1958.

A brief summary of the work done in this connexion is presented here.

Materials and Methods

Preparation of antirabies vaccine

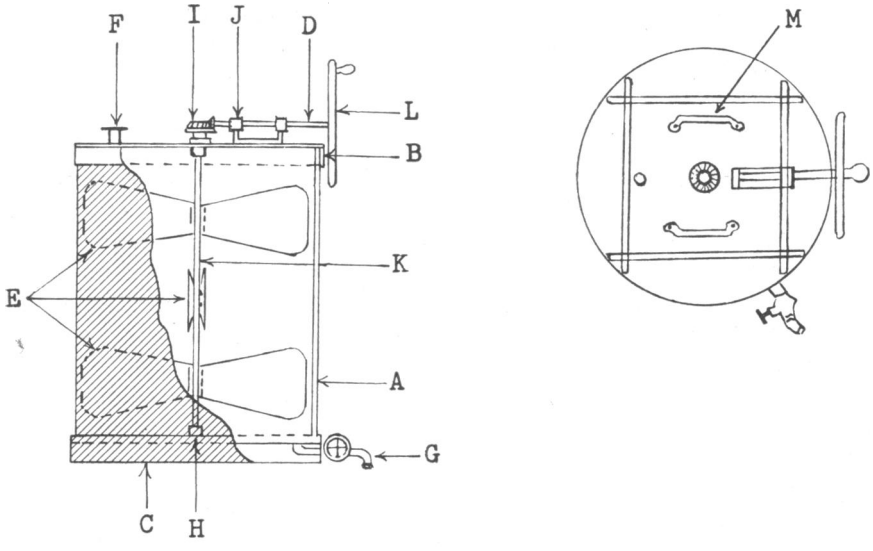
The brains of sheep completely paralysed and moribund after inoculation with the Paris strain of rabies fixed virus were removed aseptically. Each brain was washed free of blood and an 8% suspension in 1% carbol-saline was prepared in a Waring blender. This suspension was incubated at 37°C for 24 hours and stored at 4°C for 4 weeks. It was then diluted to a 5% suspension with normal saline, filtered through gauze, tested for sterility and avirulence, and put into ampoules.

Pooling of vaccine

In the early stages of the work equal quantities (not less than 50 ml) of vaccine from each batch (usually, 12 batches were taken) were pipetted out and mixed.

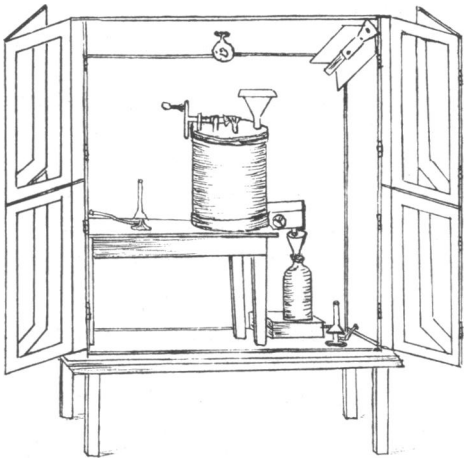
When it was found that the results with the pooled vaccine were good, a mixer was devised in which 12 batches of vaccine prepared from 12 different sheep brains could be pooled. The details of this mixer are shown in Fig. 1.

FIG. 1. SECTION AND PLAN OF MIXER FOR POOLING VACCINE



A. Container
B. Lid
C. Band, 3.8 cm wide
D. Shaft
E. Agitator blades
F. Inlet
G. Stop-cock outlet
H. Bottom bearing
I. Bevel gear, ratio 1 : 2
J. Bearing bracket
K. Shaft
L. Hand wheel
M. Lid handle

FIG. 2. ASSEMBLY FOR POOLING VACCINE



It consists essentially of a stainless-steel drum, 30 cm in diameter and 37.5 cm high, covered with a lid containing an inlet, 2.5 cm in diameter, for pouring the vaccine. There is a 1.9-cm stop-cock outlet at the bottom through which the vaccine can be withdrawn. The mixer is fitted with a stainless-steel shaft containing three pairs of agitator blades. These can be rotated by a hand wheel fitted to the top of the lid or by an electric motor.

The procedure adopted for pooling was as follows: The special chamber in which the pooling was done was irradiated by an ultra-violet lamp for an hour. After sterilization the mixer funnel, etc., were arranged in the chamber as shown in Fig. 2 and the chamber was then irradiated for another half an hour. With strict aseptic precautions each batch of vaccine from a Winchester quart bottle was poured into the mixer, the contents being stirred continuously by rotation of the agitator blades. When the contents of 12 bottles had been emptied into the mixer, the vaccine was stirred for another 15 minutes. The pooled vaccine was then withdrawn in measured quantities into dishes for ampouling or into bottles if the ampouling was to be done at a later date. The vaccine was stirred continuously during this process.

Tests for antigenicity

(*a*) The antigenicity of the vaccine was determined in mice by the method described by Kaplan (1954), the NIH (United States National Institutes of Health) Rabies Reference Vaccine, 155-D, being used as the

standard of comparison. In these experiments the criteria of a valid potency test were satisfied in that the dilutions of the vaccines used generally encompassed the 50% end-point—i.e., the majority of mice receiving the highest dose of vaccine survived and the majority of those receiving the lowest dose of vaccine died.

With the mouse strain employed at the Pasteur Institute of Southern India, the ED_{50} of the reference vaccine fell below 10 mg only when the challenge dose was about 5 LD_{50}. With challenges of 10 LD_{50} or more, there was a disproportionate rise in the ED_{50} of the vaccine. This would explain some of the high figures obtained. The high values may also be due partly to a slight deterioration in the antigenicity of the reference vaccine (M. M. Kaplan, personal communication).

(*b*) The immunizing potency of these vaccines was also studied by determining their value in the post-infection treatment of guinea-pigs challenged with the NYC and Marimuthu strains of street virus as well as with a lyophilized suspension of the submaxillary gland of a naturally infected jackal (J. 154). The method of preparing the lyophilized challenge material and the technique used for challenge are described elsewhere (Veeraraghavan, 1957; Veeraraghavan, Balasubramanian & Subrahmanyan, 1957).

Results

Antigenicity of individual batches and of pools prepared from 50 ml of each batch

Twelve serial batches of vaccine prepared from 12 rabid sheep brains were taken. From each batch 50 ml of vaccine were pipetted out and thoroughly mixed. In the first experiment, the antigenicity of three individual batches (1, 6 and 12) as well as that of the pool of all 12 batches was determined, NIH Reference Vaccine 155-D being used for comparison. In the second experiment, the antigenicity of two individual batches of vaccine (1 and 6) as well as that of two other pools prepared from the same 12 batches was tested, to determine whether reproducible results could be obtained with the testing methods employed. The results are presented in Table 1.

It will be seen that in the first experiment the ED_{50} of batch 1 was greater than 25 mg, while those of batches 6 and 12 were 3.9 mg and 5.0 mg, respectively, as compared with a value of 5.0 mg for the reference vaccine. The ED_{50} of the pooled vaccine was only 2.6 mg.

In the second experiment, the ED_{50}'s of batches 1 and 6 were 3.4 mg and 2.5 mg, respectively, as compared with 3.8 mg for the reference vaccine. It is interesting to note that the values for the two pools prepared from the same batches of vaccine were 1.8 and 1.0 mg.

TABLE 1. ANTIGENICITY OF INDIVIDUAL BATCHES OF VACCINE AND OF POOLS PREPARED FROM 50 ml OF EACH BATCH

Experiment	Test vaccine	ED_{50} of test vaccine (mg)	ED_{50} of reference vaccine 155-D (mg)	CVS * dose (LD_{50})
1	Batch 1	> 25.0	5.0	5
	,, 6	3.9	,,	,,
	,, 12	5.0	,,	,,
	Pool of batches 1 to 12	2.6	,,	,,
2	Batch 1	3.4	3.8	3
	,, 6	2.5	,,	,,
	1st pool of batches 1 to 12	1.8	,,	,,
	2nd pool of batches 1 to 12	1.0	,,	,,

* Standard challenge virus

These results indicate that while individual batches of vaccine were better than, as good as or poorer than the reference vaccine in their antigenic value, the pooled vaccine was superior both to the individual batches tested and to the reference vaccine. A reasonable degree of agreement was observed between the antigenicity of two pools made from the same batches of vaccine.

Antigenicity of individual batches and of pools prepared in a mixer

In these experiments the entire contents of each of 12 serial batches were pooled in the mixer. The immunizing potency of three individual

TABLE 2. ANTIGENICITY OF INDIVIDUAL BATCHES OF VACCINE AND OF POOLS PREPARED IN A MIXER

Experiment	Test vaccine	ED_{50} of test vaccine (mg)	ED_{50} of reference vaccine 155-D (mg)	CVS * dose (LD_{50})
1	Batch 1	> 25.0	> 25.0	21
	,, 6	> 25.0	,,	,,
	,, 12	> 25.0	,,	,,
	Pool of batches 1 to 12	6.4	,,	,,
2	Batch 1	11.2	> 50.0	10
	,, 6	5.0	,,	,,
	,, 12	11.2	,,	,,
	Pool of batches 1 to 12	5.0	,,	,,

* Standard challenge virus

batches (1, 6 and 12) and of the pool was determined. The results of two experiments are presented in Table 2.

In the first experiment, the ED_{50} values of the three individual batches as well as that of the reference vaccine proved to be over 25 mg (the highest level obtained with any of our individual batches of vaccine), while that of the pool was only 6.4 mg.

In the second experiment, the ED_{50} values of batches 1, 6 and 12 were 11.2 mg, 5.0 mg and 11.2 mg, respectively, while that of the pool was 5.0 mg. The ED_{50} of the reference vaccine was over 50 mg.

It will thus be seen that while there was considerable variation in the antigenicity of the individual batches of vaccine, the antigenicity of the pooled vaccine did not vary greatly from one pool to another and was on the whole appreciably greater than that of either the individual batches or the reference vaccine.

Antigenicity of pools prepared in a mixer on different days

In order to determine whether there was a marked variation in the antigenicity of vaccine pools prepared on different days, an experiment was carried out in which the immunizing potency of four pools of vaccine prepared on four consecutive days was tested. The results are presented in Table 3.

TABLE 3. ANTIGENICITY OF POOLS OF VACCINE PREPARED IN A MIXER ON FOUR CONSECUTIVE DAYS

Test vaccine	ED_{50} of test vaccine (mg)	ED_{50} of reference vaccine 155-D (mg)	CVS * dose (LD_{50})
Pool 1	20.2	100.0	26
,, 2	12.6	,,	,,
,, 3	7.5	,,	,,
,, 4	7.7	,,	,,

* Standard challenge virus

It was found that the ED_{50}'s of the different pools were 20.2 mg, 12.6 mg, 7.5 mg and 7.7 mg, as compared with 100 mg for the reference vaccine. Thus the antigenicity of the pools was not only fairly uniform but also superior to that of the reference vaccine.

Post-infection treatment of guinea-pigs with pooled vaccine against different strains of street virus

The value of pooled vaccine in the post-infection treatment of guinea-pigs challenged with different strains of street virus was investigated. The

TABLE 4. POST-INFECTION TREATMENT WITH POOLED VACCINE IN GUINEA-PIGS CHALLENGED WITH DIFFERENT STRAINS OF STREET VIRUS

Experiment	Antigenicity of vaccine			Vaccine dosage **	Challenge LD_{50} and strain	Mortality	Control mortality
	ED_{50} of test vaccine (mg)	ED_{50} of reference vaccine 155-D (mg)	CVS * dose (LD_{50})				
1	1.8	3.8	3	14 × 0.075 ml	8 NYC	1/10	16/20
	"	"	"	14 × 0.15 ml	"	"	"
2	1.0	4.0	3	14 × 0.075 ml	9 Marimuthu	2/10	10/10
	"	"	"	14 × 0.15 ml	"	3/10	"
	"	"	"	14 × 0.075 ml	38	8/10	"
	"	"	"	14 × 0.15 ml	"	6/10	"
	"	"	"	14 × 0.075 ml	75	10/10	"
	"	"	"	14 × 0.15 ml	"	8/10	"
3	5.0	> 50	10	14 × 0.075 ml	42 J. 154	10/20	20/20
4	2.6	> 5.0	5	14 × 0.075 ml	46 J. 154	14/20	19/20
	"	"	"	14 × 0.15 ml	"	13/20	"
	"	"	"	14 × 0.075 ml	92	19/20	"
	"	"	"	14 × 0.15 ml	"	17/20	"
	"	"	"	14 × 0.075 ml	184	19/20	"
	"	"	"	14 × 0.15 ml	"	18/20	"
5	18.9	> 50	32	14 × 0.15 ml	101 J. 154	18/25	25/25
6	6.4	> 50	10	14 × 0.15 ml	105 J. 154	11/25	25/25

* Standard challenge virus
** Vaccine administered daily, subcutaneously; started one hour after challenge

challenge viruses used were the lyophilized canine submaxillary gland suspensions of the NYC and Marimuthu strains and the lyophilized submaxillary gland material from a jackal (J. 154) which had died of natural rabies infection. The vaccine treatment in all cases was started one hour after administration of the challenge dose. The results with various challenge doses of the same strain of virus as well as with different strains are presented in Table 4. The particulars regarding the antigenicity of each pool of vaccine as compared with that of the reference vaccine are also given.

In the first two experiments it was found that excellent protection against 8 LD_{50} of the NYC strain and 9 LD_{50} of the Marimuthu strain

was afforded by treatment with 14 doses of 0.075 ml or 0.15 ml of pooled vaccine. There was fairly good survival (4 animals out of 10) with 14 doses of 0.15 ml of vaccine against 38 LD_{50} of the Marimuthu strain, but against 75 LD_{50} of the same strain there was no significant protection.

In the third experiment, 10 of the 20 animals which received 14 doses of 0.075 ml were protected against 42 LD_{50} of the J.154 strain. In the next experiment it was found that the protection afforded by the same dosage of vaccine against 46 LD_{50} of the same strain was not statistically significant, but that at a higher dosage (14 doses of 0.15 ml) the survival was significantly better in the vaccinated group than in the untreated control group. There was, however, no evidence of protection in animals challenged with 92 and 184 LD_{50} of the J.154 strain and treated with 14 doses of either 0.075 ml or 0.15 ml of vaccine.

In the fifth and sixth experiments, two groups of 25 animals were challenged, respectively, with 101 and 105 LD_{50} of the J.154 strain of virus and treated with 14 doses of 0.15 ml of vaccine; only 18 and 11, respectively, of these vaccinated animals died, as compared with all 25 animals in each of the untreated control groups. These results, which show a statistically significant survival, indicate the degree of protection that can be obtained with pooled vaccines in experimental animals, even when the treatment is not started until after infection.

Discussion

Habel (1940) reported that the immunizing power of strains of rabies fixed virus was related to the ability of the strains to resist phenol, to the rapidity of their passage transfer, and possibly to the length of the incubation period in rabbits. He found that with any one strain of virus the amount of virus injected in mice, whether in the form of live or phenolized vaccine, determined, in part, the degree of immunity produced (Habel, 1941). The virus content of the brains used in the preparation of vaccine seemed, in turn, to depend on the species of animal used, the amount of virus injected, the stage of the disease at which the animal was killed, and the time elapsing between removal of the brain and emulsification. Habel (1948) also found that the important factors which influenced the mouse potency test for rabies vaccine were the strain of mouse and the strain of test virus used.

Earlier work at the Pasteur Institute of Southern India indicated that there was considerable variation in the antigenicity of batches of vaccine even when the different variables mentioned above were controlled. For example, batches of vaccine prepared from sheep, inoculated with an equal quantity of the same virus suspension and having the same incubation period and duration of illness, were found to vary not only in virus content

but also in immunizing potency. It was also found that the antigenic value was not always related to the virus content of the brain from which it was prepared. The antigenicity of some of the batches was very good in comparison with the reference vaccine, while that of others was poor. In countries like India, where rabies is widely prevalent and where it is not uncommon for an institute to use 50-75 sheep a week to meet the demands for antirabies vaccine, it would obviously be impracticable to test the antigenic potency of each and every batch of vaccine prepared from a sheep brain, even if facilities for doing so should exist. It is therefore obvious that a method should be evolved by which a vaccine of uniform potency could be readily prepared.

The results of pooling a dozen batches of vaccine prepared from a dozen sheep brains are interesting. While the individual batches of vaccine were better than, equal to or poorer than the reference vaccine in their antigenic value, the pooled vaccine was consistently superior to the reference vaccine as well as to most of the individual batches tested. It was also found that the immunizing potency of different pools was fairly uniform and that it was invariably better than that of the reference vaccine. These findings indicate that in practice it is possible to obtain a vaccine which is fairly uniform in its antigenic value and is at least 3 to 4 times as potent as the reference vaccine.

The results of post-infection treatment with pooled vaccine in guinea-pigs challenged with strains of street virus are particularly interesting. Webster (1939), in a critical review of the immunizing potency of antirabies vaccine, has pointed out that (1) all workers, save Fermi, have failed to demonstrate a significant protective effect of vaccination following experimental exposure to rabies virus by any route; and (2) vaccine, virulent or non-virulent, given before exposure has generally been found effective only when (*a*) the test virus is given peripherally rather than centrally into the nervous tissue and in amounts fatal to less than 100% of the controls, and (*b*) the vaccine employed is given in multiple doses and in large amounts, at least 1% of the body-weight. Veeraraghavan, Balasubramanian & Subrahmanyan (1957) showed that with doses of phenolized antirabies vaccine comparable to those administered to human beings it was possible to confer solid protection on experimental animals against virulent strains of street virus, provided the treatment was started seven days before challenge. Moreover, even when the treatment was started after a challenge with 8 LD_{50} of the NYC strain of virus, there was evidence of some survival (3/10) among animals given the vaccine corresponding to the human dose in 14 instead of 7 doses. These results, however, were not statistically significant. On the other hand, in the present study excellent protection was obtained with half the dose (14×0.075 ml) of pooled vaccine started one hour after a challenge with 8 LD_{50} of the NYC strain or 9 LD_{50} of the Marimuthu strain, the mortalities being 1/10 and 2/10, respectively. In

other experiments statistically significant protection could be obtained with 14×0.15 ml against 38 LD_{50} of the Marimuthu strain, with 14×0.075 ml against 42 LD_{50} of the J.154 strain and with 14×0.15 ml against 46, 101 and 105 LD_{50} of the latter strain. These results clearly indicate that it is possible to demonstrate a significant protection with antirabies vaccine administered after infection in doses corresponding to those used in the treatment of humans. Further, the results with pooled vaccine are superior to those reported earlier with individual batches of vaccine.

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RÉSUMÉ

Les vaccins antirabiques préparés à l'Institut Pasteur de l'Inde méridionale, à partir de cerveaux de moutons, présentaient entre eux de notables variations de pouvoir antigénique, par rapport à un vaccin de référence. Dans un institut où la demande de vaccin antirabique exige l'emploi de 50-70 moutons par semaine, le titrage de l'activité du vaccin obtenu à partir de chaque cerveau est impossible. On a donc cherché à mettre au point une technique assurant aux vaccins une activité uniforme. Cette nouvelle méthode, appliquée depuis mars 1958, décrite en détail dans l'article, consiste à mélanger 12 lots de vaccin, à raison de 50 ml chacun, avant la mise en ampoules. L'antigénicité de ces mélanges était toujours supérieure à celle du vaccin 155-D des National Institutes of Health des Etats-Unis d'Amérique. L'activité des vaccins a été évaluée sur des cobayes, infectés expérimentalement par du virus des rues (souches NYC, Marimuthu et J. 154).

Les résultats de la vaccination pratiquée *après* infection sont particulièrement intéressants. Il a été possible de protéger 9 sur 10 cobayes infectés par 8 DL_{50} de la souche NYC et 8 sur 10 cobayes ayant reçu 9 DL_{50} de la souche Marimuthu, en leur administrant 14 doses de 0,075 ml du vaccin décrit dans cet article, en commençant le traitement une heure après l'injection du virus d'épreuve. Dans une autre série d'essais 14 doses de 0,15 ml ont assuré une protection statistiquement significative contre 38 DL_{50} de la souche Marimuthu et 105 DL_{50} de la souche J. 154.

Ces expériences ont montré que le mélange de vaccins antirabiques administré après infection à des doses correspondant à celles qui sont utilisées en thérapeutique humaine, assurait une protection significative chez les cobayes.

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