THE PRODUCTION OF RABIES IMMUNE SERUM

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THE PRODUCTION OF RABIES IMMUNE SERUM

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I. INTRODUCTION

The observations by Pasteur that the infective agent in rabies could be made non-infective for dogs by serial passage in rabbits, and that bacterial cultures become non-infective by desiccation, led him to apply these principles to the preparation of a vaccine for human prophylaxis. The first known attempt to produce an immunity to rabies in humans was in 1885 when a child, bitten by a rabid dog, was treated with a suspension of desiccated spinal cord from an infected rabbit. The treatment was successful, and the method came into general use. The vaccines in use today, however, are not generally of this type, but of the Semple (1) type containing phenol inactivated brain virus or the Habel (2), Levinson (3), Hodes (4) type containing ultra-violet inactivated virus. The disadvantage in the use of a brain tissue vaccine is that a series of injections (seven to twenty-one) must be administered and sensitivity to the brain tissue may develop. Neuro-paralytic accidents occur in one of every 600 patients (5,6) and thus it would seem advisable to decrease the number of treatments and to supplement them with an agent less likely to cause an unfavorable reaction. Antirabies serum might be used as such a supplementary agent. It would also serve to neutralize virus immediately, whereas it takes ten to fourteen days for antibodies to appear in individuals being treated with vaccine. During this period virus may reach the central nervous system and no longer be accessible to the antibody.

II. REVIEW OF THE LITERATURE

As early as 1889 Babes and Lepp (7) conjectured as to the feasibility of using the fluids and cells of rabies refractive animals for the prophylactic treatment of dogs exposed to rabies virus. Remlinger (8) reported differences in the antirabies serum titers from one rabies-immunized sheep to another and therefore advised careful titration of the serum from each bleeding.

The first clinical trials noted were those of Marie (9), who reported satisfactory results with serum-vaccine therapy in 300 humans bitten on the face and the extremities. Koprowski (10) reported on clinical trials in Georgia with twenty individuals who received serum and vaccine. None of them showed any signs of rabies at the time the article was published. He also reported on an individual bitten by a guinea pig with active street virus in its saliva. Another individual was bitten on the hands by her own dog which proved to be rabid by the presence of Negri bodies in its brain. Both persons were treated with serum followed by vaccine. These patients developed neither rabies nor any ill effects from the treatment.

Koprowski and Cox (11) reported on another clinical survey conducted in 1948 in the United States in which practitioners returned questionnaires to the authors on individuals treated with serum concentrates. None of the forty-eight patients included in the survey had shown any signs of rabies at the time of publication. Some of these treated cases

received a sheep serum and others a rabbit serum. There were some unfavorable side effects in patients who received the sheep serum but none in those receiving the rabbit serum.

The experiments by Hoyt et al. (12) and Yen (13) using mice as test animals indicated a higher percentage of survival among the serum-treated animals than in the untreated controls. Hoyt et al. (14) were of the opinion that the blood-brain barrier must be broken in order for treatment to be successful. Habel (15) also reported on extensive studies using mice, guinea pigs and monkeys as test animals. His findings indicated that serum administered at the site of infection within seventy-two hours was the most effective. Also a much higher percentage of animals survived, if the serum prophylaxis was followed with a series of daily injections of phenolized vaccine.

Most workers agree that in order to be effective the serum concentrates must be given not later than three to five days after exposure, followed by a series of vaccine treatment, with the number of treatments dependent upon the severity and location of the exposure. The illustrated publication of Blatt et al. (16) serves a good purpose in showing the anguish and suffering of patients infected with rabies. The number of such cases could probably be lessened with wider use of antirabies immune serum.

III. EXPERIMENTAL METHODS

A. PREPARATION OF IMMUNIZING ANTIGEN

Rabbits were infected with the Michigan Department of Health (MDH) strain of rabbit adapted fixed rabies virus. This strain is one reportedly fixed by Pasteur and has had continuous rabbit passage in these laboratories since 1932. The animals were inoculated intracerebrally with 0.2 ml of a 1:100 dilution of the centrifuged supernatant fluid of rabbit brain suspension. Symptoms of fixed virus rabies appeared four days after infection. The rabbits were sacrificed, using air embolism, within forty-eight hours after exhibiting symptoms and the brains removed aseptically. The brains were weighed and stored at -55°C until the time of emulsion. Emulsification to a twenty percent tissue concentration in distilled water was done in a Waring Blendor. The emulsions were dispensed into glass ampoules, flame sealed, the contents shell frozen and stored at -55°C.

The mouse brain virus (NIH-CVS) was obtained from the National Institutes of Health. White Swiss mice of eighteen to twenty grams were inoculated intracerebrally with 0.03 ml of a 1:100 dilution of the mouse brain virus. Four days after inoculation they exhibited symptoms of fixed virus rabies and twenty-four to thirty-six hours later were sacrificed under ether. The brains were removed, pooled in a common container, weighed and stored at -55°C until the time of emulsion. Emulsification was performed in the same manner as for the rabbit brain.

B. INOCULATION OF RABBITS

Healthy young white rabbits weighing approximately six pounds were selected for this study. Age was considered important, since it was necessary to carry the study over a period of eight months.

The backs of the rabbits were closely clipped. The intracutaneous route was selected because the work of Habel (15) indicated that it yielded the highest antibody titers. In general, inoculations were started with 0.25 ml of the centrifuged supernatant liquid of a ten percent brain emulsion. Inoculations were made twice weekly for a period of three weeks, and the dosage increased by 0.25 ml every three weeks until a maximum of 1.5 ml was reached. Inoculations were then made only weekly. No more than 0.5 to 0.75 ml was inoculated into any one site. Twelve rabbits were hyperimmunized with the MDH virus and eighteen with the NIH-CVS virus. Eight rabbits inoculated with the NIH virus developed fixed virus rabies, data which are in agreement with the findings of Habel (15). None of the rabbits that received the MDH virus exhibited symptoms.

C. BLEEDING OF RABBITS

The rabbits were bled from the heart four times at monthly intervals, beginning two months after the start of the experiment. They were then bled twice monthly for the last two months of the experiment, sacrificing the animals at the final bleeding. The animals received no immunizing injection one week prior to bleeding. Serum was separated from the clotted blood the day after it was drawn. Approximately 1.5 ml from each rabbit was set aside for complement fixation tests. For

virus neutralization testing, pooled serums were used. The rabbits were divided into groups of five or six each, two groups for MDH virus immunization (groups 1 and 2) and two groups for NIH-CVS virus immunization (groups 3 and 4). At each bleeding a serum pool was prepared for each group, using 0.5 ml of serum from each rabbit. The remainder of the serum was pooled in two bottles according to the strain of virus used in the immunizing injections. All serums were shell frozen and stored at -55°C.

D. IN VITRO NEUTRALIZATION

All tests were performed with sixteen to eighteen gram white Swiss mice of random sex and six weeks of age. The first tests were carried out with the raw serum mixed with mouse brain fixed virus, but later tests were done with both the mouse and the rabbit brain fixed viruses. The following dilutions of fixed virus were prepared: 1:5, 1:50, etc., through 1:500,000,000. Serum dilutions of 1:2, 1:4, 1:8 were also prepared. Serum and fixed virus mixtures were prepared by adding equal volumes of the diluted serum to diluted virus. Thus, the final dilution of virus would be 1:10, 1:100, 1:1,000 and 1:10,000, and the final serum dilutions 1:2, 1:4, 1:8 and 1:16. The higher dilutions of virus were combined with equal volumes of normal rabbit serum for inoculation into the control mice. The mixtures with fixed virus were incubated in a 37°C water bath for one hour, immediately chilled, and inoculated intracerebrally into mice in 0.03 ml amounts. Mice were observed for fourteen days.

In the experiments with street virus, cow or dog brain suspensions were used. They were first treated with 1,000 units of penicillin and 2,000 units of streptomycin per ml to destroy or inhibit any bacteria that may have been present. Saline was used as the diluent for making

the virus suspensions. The virus-serum mixtures were made as described in the preceding paragraph except that the final serum dilutions were made as high as 1:16,000. Only purified serum was used in these tests. The mixtures were incubated at 37°C for one hour prior to inoculation of the mice. The animals were observed for symptoms of street virus rabies for twenty-eight days.

The final dilutions of both the fixed and street viruses for inoculation of the control mice were made with undiluted normal rabbit serum.

E. COMPLEMENT FIXATION

The method of Kolmer (17) was used in the performance of these tests. The reagents were prepared in the laboratory except for the hemolysin, which was purchased from Difco Laboratories.* The normal mouse brain and infected mouse brain antigens were prepared according to the procedure outlined by Espana and Hammon (18).

F. PREPARATION OF REFINED SERUM

The serums were removed from storage at -55°C and kept overnight at +5°C for thawing. Serum obtained from rabbits immunized with MDH antigen and that obtained from rabbits immunized with NIH antigen were purified separately. The method used in purification was that of Heidelberger et al. (19). The protocols are as follows:

Concentration and Refinement of Rabies Antiserum

1. The volume of raw serum was MDH 1280 ml and NIH 1100 ml.
2. A temperature of 25°C was used for the concentration of the serum.

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* Difco Laboratories, Detroit, Michigan

3. The pH was adjusted to 7.3 with 1:5 glacial acetic acid.
4. Anhydrous sodium sulfate was added to a 16% concentration and the solution was stored overnight at 25°C to permit the precipitate to reach equilibrium with the supernatant solution.
5. The precipitate was separated by centrifugation and then washed four times with 16% sodium sulfate by resuspending and recentrifuging.
6. The precipitate was dialyzed through two layers of #300 cellophane* (.001 inch thick) for eighteen hours at 15°C in running tap water and for forty-eight hours at 5°C in running distilled water.
7. The volume of concentrated serum was MDH 100 ml and NIH 108 ml.
8. Phenol was added to 0.5% concentration.
9. NaCl crystals were added to 0.85% concentration.
10. Super-cel** was added to 1.0% concentration to assist in the removal of suspended particles.
11. The solution was then centrifuged for one hour at 3000 R.P.M. and the precipitate discarded.
12. Analysis of the final solution for total solids indicated the following: MDH 14.4% and NIH 13.7%.
13. The solution was sterilized by filtration through one ST-3 pad,*** 6 cm diameter.

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* E. I. Dupont De Nemours Company, Cellophane Division, Wilmington, Delaware.
** Johns Manville Company, 832 Fisher Building, Detroit, Michigan.
*** Hercules Filter Company, 204 Twenty-First Avenue, Patterson, New Jersey.

14. In both lots the sterility test on the bulk products was performed by planting two tubes consisting of 15 ml of thioglycollate medium with 0.5 ml each and observed for seven days. No evidence of growth was observed.

15. The final solutions of purified antiserums were dispensed into bottles at a volume of 10 ml each and immediately frozen. The frozen serum was then dried from the frozen state by sublimation.

16. The sterility test on the reconstituted dried serum was performed in the same manner as for the bulk product. No evidence of growth was observed.

17. The pyrogen test was performed in rabbits. They were injected intravenously with 3.0 ml of serum for each kilogram of body weight. Two animals were used for each serum. No significant changes in body temperature were noted during the required three-hour observation period.

18. A safety test was performed on the reconstituted dried serum and consisted of the intraperitoneal inoculation of 0.5 ml into each of two 20 gram mice and 5 ml into each of two 300 to 350 gram guinea pigs. They showed no reaction or loss in weight over the seven-day observation period.

IV. EXPERIMENTAL RESULTS

A. IN VITRO NEUTRALIZATION TESTS

The method of Reed and Muench (20) for calculating fifty percent endpoints was followed for determination of the neutralization index. To obtain an index, the fifty percent endpoint dilution of the protected mice was subtracted from the fifty percent endpoint dilution of the unprotected control mice, with the difference being the neutralization index.

Animals can be a source of considerable variation when they are used for biological titration purposes. It is generally accepted that it is necessary to obtain nearly a ten-fold difference in the results from one test to another in order for them to be significant.

1. Fixed virus. The indices obtained for the unpurified serum pools from each bleeding are given in Table I. The serums of all the pools showed a satisfactory index at the first bleeding, but only in pool 1 when tested with NIH-CVS virus was there a significant rise in titer from the first to the last bleeding. The titer of the serum pools showed considerable variation from one bleeding to another.

   The raw serums from the rabbits immunized with MDH and NIH antigens contained less antibody against MDH challenge virus at the conclusion of the experiment than was present in the serum at the time of the initial bleeding. Three of the serum pools showed an increase in neutralization power against the NIH challenge

TABLE I

NEUTRALIZATION INDICES OF RAW SERUMS WHEN TESTED AGAINST FIXED VIRUS

Chronological order of bleeding	MDH immunizing antigen				NIH immunizing antigen			
	Pool 1		Pool 2		Pool 3		Pool 4	
	Serums tested against virus							
	MDH	NIH - CVS	MDH	NIH - CVS	MDH	NIH - CVS	MDH	NIH - CVS
1	100,000 +	10,000	100,000 +	7,500	100,000 +	17,100	67,600 +	7,500
2	100,000 +	1,330	100,000 +	1,330	100,000 +	661	100,000 +	631
3	3,160	31,600	3,160	21,400	3,160	10,500	3,160	5,630
4	3,160 +	5,620	3,160 +	7,940	3,160 +	10,000	3,160	5,630
5	4,270 +	1,000	4,270 +	759	4,270 +	1,000	4,270 +	759
6	4,270 +	7,590	4,270 +	7,590	4,270 +	4,680	2,140	3,160
7	4,270 +	112,000	4,270 +	42,700	4,270 +	24,000	4,270 +	34,700
8	4,270	345,000	4,270 +	46,800	4,270 +	11,200	4,270 +	31,600

Final serum dilutions were 1:2
Serum was not inactivated

virus during immunization, while in the remaining pool the neutralization index was essentially unchanged.

The neutralization indices of the purified serums are given in Table IIa and IIb. They were tested only against NIH virus. Tests 1 and 2 in Table IIa illustrate the fact that animals can exhibit wide variation when used for biological titration. These tests were not done simultaneously, but with an interval of three weeks between them. The differences in titer of NIH serum between test 1 and test 2 were not as large as those for MDH serum.

The titers of the serums after drying (Table IIb) were nearly identical when tested against both viruses. The uninactivated serums were tested simultaneously with the inactivated serums. It was suspected that some loss of antibody would occur in the purification process, and there were only slight differences in the neutralization index of the MDH raw serum from the last bleeding as compared to the inactivated purified serum.

2. Street virus. The data on the neutralization capacity of the purified and dried serums tested against street virus are shown in Table III. The results of tests 1, 2 and 3 were inconclusive because the serum was not diluted sufficiently to obtain an endpoint. The outcome of tests 4 and 5 did not show appreciable differences between the two serums. Even at a dilution as high as 1:16,000 the serum did have a neutralization index of from three to fourteen. Since street virus usually has a low titer for mice, high neutralization index values could not be expected.

TABLE II

NEUTRALIZATION INDICES OF PURIFIED ANTISERUM AGAINST NIH - CVS VIRUS

a. Before drying

Serum produced by	Test No.	Final serum dilution			
		1:2	1:4	1:8	1:16
MDH antigen	1	1,410 +	1,410 +	1,410 +	
	2		436,000 +	436,000 +	245,000 +
NIH antigen	1	100,000 +	39,800 +	31,600 +	
	2		436,000 +	251,000	100,000

Serums not inactivated

b. After drying

Serum produced by	Treatment of serum	Final serum dilution			
		1:2	1:4	1:8	1:16
MDH antigen	Uninactivated	25,100 +	25,100 +	25,100	25,100 +
	Inactivated 56°C 20 min.		4,270 +	4,270 +	1,780
NIH antigen	Uninactivated	25,100 +	25,100 +	25,100 +	7,940
	Inactivated 56°C 20 min.		4,270 +	4,270 +	1,780

TABLE III

NEUTRALIZATION TESTS WITH PURIFIED ANTISERUM AND STREET VIRUS

Experiment number	Final serum dilution	Neutralizing index	
		MDH serum	NIH serum
1	1:2	320 +	
	1:4	320 +	
	1:8	320 +	
	1:16	320 +	
2	1:32	240 +	
	1:64	240 +	
	1:128	240 +	
	1:256	240 +	
3	1:500	32 +	
	1:1000	32 +	
	1:2000	21	
	1:4000	30 +	
4	1:500	10 +	10 +
	1:1000	10 +	10 +
	1:2000	10 +	10 +
	1:4000	10 +	10 +
	1:8000	7	1:3
	1:16000	3	1:7
5	1:500	43 +	43 +
	1:1000	43 +	43 +
	1:2000	14	14
	1:4000	29	14
	1:8000	14	14
	1:16000	14	14

B. COMPLEMENT FIXATION

The data in Table IV are a summary of the results of complement fixation tests. The endpoint of the reaction was taken as the tube with the highest dilution of serum showing 2+ fixation. A correlation between these titers and those for virus neutralizing antibodies could not be made, since neutralization tests were not done on individual rabbit serums. There was no definite pattern in the rise or fall of the titers for an individual rabbit. In general, if the titer of serum from the first bleeding was 1:10 or 1:20, the serum maintained that titer for the duration of the experiment. Exceptions were rabbit 960 and 683 of those immunized with MDH antigen, and 965, 662, 695 and 627 of those immunized with NIH antigen.

TABLE IV

COMPLEMENT FIXING ANTIBODY TITER OF RABBITS HYPERIMMUNIZED WITH RABIES ANTIGEN

Serum produced with	Rabbit number	Months of immunization					
		3	4	5	6	7	8
MDH Antigen	819	1:10	1:20	1:20	1:20	1:40	1:20
	960	1:20	1:10	-	1:10	1:5	1:5
	904	1:2.5	-	-	1:5	Dead	
	635	-	-	1:10	-	1:5	-
	683	1:20	1:20	1:20	1:20	-	Dead
	971	1:20	1:20	1:5	1:20	1:10	Dead
	000	1:20	1:20	1:10	1:20	1:40	1:40
	968	1:10	1:20	1:20	1:10	1:10	1:5
	986	1:10	1:20	1:20	1:10	1:20	*
	615	1:10	1:20	1:5	1:20	1:5	1:10
	690	1:20	1:20	1:20	1:20	1:20	1:10
NIH Antigen	907	1:20	1:40	1:40	1:40	1:40	1:10
	965	*	1:5	1:5	-	*	1:20
	662	1:2.5	*	1:5	1:10	1:10	1:10
	941	1:5	1:5	1:10	1:5	-	1:20
	939	*	1:20	1:20	1:40	1:20	1:20
	627	1:2.5	-	1:10	1:20	1:20	1:20
	695	1:10	1:2.5	1:2.5	1:10	1:5	-
	660	1:20	1:40	1:20	1:40	1:20	1:20
	667	1:10	*	1:10	1:20	1:10	1:10

* Unsuccessful bleeding

V. CONCLUSIONS AND SUMMARY

A small supply of antirabies serum (rabbit) was produced in order to resolve any technical problems dealing with its routine production.

If complement fixation is an indication of immune antibody production in rabbits, the complement-fixing antibodies were produced in a short period of time. However, as is true with most hyperimmunization procedures, a few animals appeared quite refractory to immunization. These animals could be excluded in routine production of serum, and, if this had been done after the first or second bleeding, the neutralization index of the serum pool in which their serums were placed might have been slightly higher.

Since the value of immune serum in the prophylaxis of rabies is related to an exposure to street virus, neutralization tests against fixed virus could be indicative, but of limited value to forecast its ability to neutralize street virus. The high dilution of serum needed in order to obtain an endpoint in the animal titration with street virus could be attributed to the low titer of the inoculum in the controls. This necessity for high dilution does indicate that considerable antibody against street virus was present.

It was concluded that an immune serum against rabies could be produced with little difficulty and would likely be of value in the treatment of human beings bitten by rabid animals.

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