ON THE RABICIDAL PROPERTY OF ANTI-RABIES SERUM.*

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Introduction.

Antirabic vaccination is now commonly practised on men and animals, but the mechanism of it is not yet clear, in spite of numerous investigations which have hitherto been made on this subject by many authors.

Högyes described in his preliminary report that antirabic immunization produces a specific refractory condition of the central nervous system, but this opinion is not yet generally believed. In 1889, Babes and Lepp proved for the first time that a specific protective substance is produced in the blood serum of animals treated with rabic virus. In the same year, Babes and Tizzoni, working with serum therapy against rabies, have made great progress in the study of the mechanism of antirabic vaccination.

Centanni reported a few years later that in the case of immunizing an animal against rabies the specific substance in the serum does not increase proportionally to the degree of immunity. He noticed that for several days after vaccination an animal is still susceptible to rabies infection in spite of the presence of the specific substance in its blood serum.

In 1900 Marx stated that an immune body is produced in the blood serum of a vaccinated animal, owing to the stimulation of the inoculated virus which is supposed to be destroyed in the body before it reaches the central nervous system. This fact was confirmed two years later by Kraus, Keller, and Clairmont. They reported that the serum taken from an animal immunized against rabies possesses a rabicidal property while the serum from a normal animal has no specific rabicidal power, and that the immunized animal resisted an intracerebral or intravenous injection of rabic virus, proving that the blood serum of the animal has a rabicidal property.

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According to Babes, the inoculation of virus acts on the animal body in such a way as to produce in the blood a substance which kills the virus and the leucocytosis which generally occurs in the blood of a rabid animal takes part in the production of this substance.

In 1902, Kraus and Kreissl published their report proving that a protective substance is present in the blood serum taken from a human being inoculated with rabic virus, and that the protective substance is always produced in the blood serum of men treated by Pasteur's method. They thought that the unsatisfactory results of the Pasteur treatment might be attributable to the insufficient formation of the specific immune body.

Marie stated that, since the virus acts only as an antigen, an antitoxic and an antiparasitic substance must be produced in the blood serum of an immunized animal by the stimulation of the inoculated virus.

It seems to me that it might be of interest to ascertain the results obtained by the authors above mentioned.

In April, 1922, I already submitted my preliminary report on the subject to the First Annual Meeting of the Japanese Society of Veterinary Science, so I should like here to describe briefly the essential points of my investigations on the nature of what they call the rabicidal property of serum.

1. Properties of Serum from Artificially Immunized Animals.

In investigating the mechanism of antirabic immunization, it is necessary, as a first step, to study the properties of the serum taken from animals artificially immunized against rabies, especially antibodies contained in the serum.

Rabbits have been used for this purpose and they were treated to intravenous injections with a small amount of living fixed virus every day or every other day during a period of 42 days or more. Twenty-one days after the final injection, the rabbits were bled for serum which was sealed in a series of capillary tubes by means of a blow pipe and then kept in an ice-box.

Table I shows the protocol of one of the immunized rabbits.

Table I. Preparation of immune serum.
Rabbit No. 615 B.

Injection	Date	Material *	Injection	Date	Material
1	10- 5-20	1 c.c.	23	10-30-20	1 c.c.
2	10- 6-20	do.	24	11- 2-20	do.
3	10- 7-20	do.	25	11- 3-20	do.
4	10- 8-20	do.	26	11- 4-20	do.
5	10- 9-20	do.	27	11- 5-20	do.
6	10-11-20	do.	28	11- 6-20	do.
7	10-12-20	do.	29	11- 8-20	do.
8	10-13-20	do.	30	11- 9-20	do.
9	10-14-20	do.	31	11-10-20	do.
10	10-15-20	do.	32	11-11-20	do.
11	10-16-20	do.	33	11-12-20	do.
12	10-18-20	do.	34	11-13-20	do.
13	10-19-20	do.	35	11-15-20	do.
14	10-20-20	do.	36	11-16-20	do.
15	10-21-20	do.	37	11-17-20	do.
16	10-22-20	do.	38	11-18-20	do.
17	10-23-20	do.	39	11-19-20	do.
18	10-25-20	do.	40	11-20-20	do.
19	10-26-20	do.	41	11-22-20	do.
20	10-27-20	do.	42	11-23-20	do.
21	10-28-20	do.		12- 1-20	Bled for serum
22	10-29-20	do.			

* Suspension of living rabic virus in proportion of 1:10 (broth), intravenously.

The serum thus obtained was, for the purpose of determining the presence of a specific antibody, tested in the following manner.

I have found that the serum possessed a marked rabicidal property neutralizing in a dilution of from 1:10,000 to 1:100,000 the rabic virus in a hundredfold minimum lethal dose for a guinea-pig, i. e. the titer of the serum being higher than any of those mentioned in the literature on the subject. No agglutinins, precipitins, or complement-fixing substances could be demonstrated in the serum.

Technique for the demonstration of antibodies in serum:
Unnecessary details regarding agglutination, precipitation, and

and complement-fixation test, all of which gave negative results, are omitted.

In order to demonstrate the rabicidal property of the serum a constant quantity of virus was added to an increasing quantity of serum, as described in the following procedure. Several small glass tubes were placed in a row and each of them was filled with 0.9 c.c. of the serum diluted with 0.85 % saline solution in a proportion of from 1 : 5 to 1 : 10,000,000 and mixed with 0.1 c.c. of emulsion prepared from a brain of a rabid animal which was diluted with broth in a proportion of 1 : 1,000. The tubes were then gently shaken and placed in an incubator at 37°C. After the lapse of two hours the glass tubes were taken out and guinea-pigs were inoculated with 0.1 c.c. of the contents of each tube.

The rabicidal titer of the serum was determined by the death or the survival of the inoculated animals; the survival of the animal indicating that the virus was already neutralized in the test tube by the serum while the death denoted the presence of the virus in the mixture.

The results of these experiments are recorded in Table II.

Table II. Results of titration of the immune serum.

Dilution	Serum No. 615 B.				Serum No. 16 m.		
	1	2	3	4	5	6	7
1:100	—	—	—	—	—	—	—
1:1,000	—	—	—	—	—	—	—
1:10,000	—	—	—	—	—	—	—
1:100,000	—	—	—	+(19-20)	—	—	—
1:1,000,000	+(6)	—	+(4)	+(15-20)	+(4)	+(5)	—
1:10,000,000	+(4)	+(7)	+(5)				+(5)
1:100,000,000	+(4)						
Control	+(4)	+(5)	+(4-6)	+(5)	+(4)	+(5)	+(5)

Remark: In this and in subsequent tables, + indicates the death, and — the survival of the inoculated guinea-pigs; the figures in brackets designate the period of incubation; Controls indicate the results of observation on guinea-pigs inoculated with virus alone.

2. *Rabicial Property of Serum from Naturally Immune (Refractory) Animals.*

It will be of interest to determine whether the blood serum of the naturally immune animal has a property similar to that found in the serum of the artificially immunized animal.

It seems to me that one can have very few opportunities to make such a study, as animals naturally immune against rabies are rarely found.

In 1918, Remlinger reported one case in which a rabbit was naturally immune.

During the last several years I have employed various kinds of animals, especially guinea-pigs, rabbits, and dogs for my investigation in rabies, and have occasionally found two dogs which were highly immune against experimental infection.

A small amount of blood was taken from each of the dogs and the serums were diluted in from 1:5 to 1:100. A suspension of rabic virus in a dilution of 1:1,000 was added to each of the serums which, after two hours' incubation, was injected into guinea-pigs.

As will be seen from Table III, the serum from these dogs was rabicidal.

Table III. Results of titration of the serum from naturally immune (refractory) dogs.

Dilution	Serum from	
	Dog No. 108	Dog No. 338
1:5	—	—
1:10	—	—
1:20		+
1:50	—	
1:100	—	
Control	+	+

3. *Specificity of the Rabicial Property of Anti-rabies Serum.*

Further experiments should be made for the determination

of the specificity of the rabicidal property of the immune serum against rabies.

To determine whether the rabicidal property is specific to an immunized animal or not, I tested the serum taken from normal animals (cattle, horses, sheep, dogs, rabbits, guinea-pigs and domestic fowls). Table IV shows that the serum of the normal animals, with a few exceptions, possesses very weak rabicidal property.

Table IV. Results of titration of normal serum.

Dilution of Serum	Kind of Serum						
	Cattle serum	Horse serum	Sheep serum	Dog serum	Rabbit serum	Guinea-pig serum	Domestic fowl serum
Undiluted	±	±	±	±	±	+	+
1:5	±	±	±	±	±	+	+
1:10	±	+	+	+	+	+	+
1:20	+	+	+	+	+	+	+
1:50	+	+	+	+	+	+	+

Remark: ± indicate that some of the guinea-pigs inoculated with the mixture of virus and serum died, and some survived, according to the property of serum.

This result is in agreement with that of Kraus, Keller, and Clairmont, who claim the non-existence of rabicidal property in normal serum.

Furthermore, it would be necessary to determine whether the receptor of anti-rabies serum has an affinity with the rabic virus only, in other words, whether an immune serum prepared by injecting normal nerve materials is capable of neutralizing the rabic virus.

Rabbits were used for this purpose and they were inoculated several times intravenously with a saline suspension prepared from the brain of a normal rabbit.

The serum from these animals was tested for the possession of rabicidal property against rabic virus, but the presence of any

specific antibodies against rabic virus was not discovered in the serum. See Table V.

Table V. Results of titration of the serum from rabbits immunized by injecting normal nerve materials.

Dilution of Serum						Control
1:10	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	
+(4-5)	+(4)	+(4-5)	+(5)	+(5)	+(5)	+(4)

4. *Protective Value of the Anti-rabies Serum.*

The important problem in the practical application of the anti-rabies serum for protective or curative purposes in rabies infection still remains unsolved. This is surely due to failure to make a strong immune serum. I have made some experiments in connection with this problem, since the immune serum which I have obtained was very strong. Although my work on this subject is not yet in an advanced stage, the results obtained may be worthy of note.

The immune serum was administrated intravenously, intraperitoneally, or intracranially into guinea-pigs, after which they were inoculated intramuscularly with a hundredfold minimum lethal dose of rabic virus, suspended in 0.1 c.c. of broth. The results are as follows:

Table VI. Results of protective injection of the immune serum into guinea-pigs.

Guinea-pig	Dose (c.c.)	Method of injection	Results
B. 57 a.	2	Intraperitoneal	—
B. 57 b.	2	do.	—
B. 57 c.	3	do.	—
B. 57 d.	0.75	do.	+(4)
S. 1	0.5	Intravenous	—
S. 2	0.5	do.	+(11)
S. 3	0.2	do.	+(30)
S. 4	0.2	do.	—

S. 5	0.2	Intravenous	—
S. 6	0.2	do.	—
S. 7	0.1	do.	+(24)
S. 8	0.1	do.	—
S. 9	0.1	do.	—
S. 10	0.1	Subcutaneous	+(27)
S. 11	0.1	Intravenous	+(18)
S. 12	0.1	Intracranial	+(5)
S. 13	0.1	do.	+(11)
S. 14	0.1	do.	+(4)
S. 15	0.1	do.	+(5)
S. 16	0.1	do.	+(4)

As shown in the above table, the immune serum has to a certain extent a protective power against rabies infection in animals.

Further experiments will, however, have to be carried out in order to find the practical value of the immune serum in protective or curative treatment against rabies infection in men and animals.

*5. Optimum Time and Temperature for the Union of
Virus with Rabicidal Substance contained
in the Immune Serum.*

The following comparisons have been made in order to determine optimum time and temperature for the union of virus with receptor of the serum; the mixture of serum and virus was placed in an incubator at 37°C. for 5 and 30 minutes, 1, 2 and 3 hours, and then stored in a dark room for 2 and 20 hours at 18°–20°C.

The results of these comparisons are given in Table VII.

Table VII. Results of experiments in the optimum time and temperature for the union of virus with receptor.

Dilution of Serum	37°C.				18–20°C.	
	5 min.	30 min.	1 hr.	2 hrs.	3 hrs.	20 hrs.
1:100	—	—	—	—	—	—

1:1,000	+(4)	+(6)	—	—	—	—
1:10,000	+(4)	+(7)	+(4)	—	—	—
1:100,000	+(4)	+(4)	+(5)	+(5)	+(5)	+(5)
1:1,000,000	+(4)	+(4)	+(5)	+(5)	+(5)	+(4)

Incubation for 2 hours at 37°C. gave the most satisfactory result. Storing for 20 hours at from 18°–20°C. in a dark room gave a result similar to that of 2 hours at 37°C. Incubation within 30 minutes gave an unsatisfactory result. Storing for 2 hours at from 18°–20°C. in a dark room gave a worse result than the incubation for 30 minutes.

6. *Influence of Heating upon the Rabicidal Property of Anti-rabies Serum.*

The following observations have been made to determine whether the rabicidal property of anti-rabies serum is thermostabile or thermolabile. A number of capillary glass tubes containing immune serum were heated in a water bath at various temperatures and were then titrized in the manner mentioned above. It will be seen from Table VIII that the rabicidal property of serum vanishes by heating at 80°C. for 3 minutes, while it remains quite unchanged by heating at from 65°–70°C. for the same length of time.

Table VIII. Influence of heating on the rabicidal property of anti-rabies serum.

Dilution of Serum	Unheated	30 min. at 65°C.	30 min. at 70°C.	30 min. at 80°C.
1:10	—	—	—	—
1:100	—	—	—	—
1:1,000	—	—	—	+(4)
1:10,000	—	—	—	+(5)
1:100,000	+(4)	+(4)	+(4)	+(4)

7. *Quantitative Relation in the Union of Rabic Virus with Anti-rabies Serum.*

The union of toxin with antitoxin generally takes place in multiple proportion while no union of bactericidal serum with bacteria takes place in such proportion. It is important to determine whether the union of rabic virus with rabicidal substance takes place in multiple proportion or not. The results of the experiments which I have made are shown in Table IX.

Table IX. Quantitative relation in the union of serum with virus.

Dilution of serum	Dilution of virus				
	1:5	1:10	1:100	1:1000	1:10000
1:10	—				
1:50	—				
1:100		—			
1:500	+(16)				
1:1,000		+(5)	—		
1:5,000	+(4)				
1:10,000		+(5)	+(5)	—	
1:50,000	+(4)				
1:100,000		+(5)	+(5)	+(4)	—
1:1,000,000			+(5)	+(4)	—
1:10,000,000				+(4)	+(5)

As will be seen from the above table, the quantity of serum required for neutralizing virus is in direct proportion to the quantity of virus. It is very interesting to know that the relation between anti-rabies serum and rabic virus closely resembles that of toxin to antitoxin.

Summary.

The investigations just outlined indicate that

1. The anti-rabies serum from animals artificially immunized possesses a specific rabicidal property, while no other antibodies can be found in it.

2. The serum from animals immunized by repeated injections with normal nerve material has a non-specific property against rabic virus.

3. The rabicidal property of serum from naturally immune (refractory) animals can be a direct index of resistance against rabies infection.

4. The union of rabic virus with anti-rabies serum takes place in multiple proportion as in the case of toxin and antitoxin. This fact indicates that even a slight degree of resistance against rabies in an individual can be detected by titrating the serum of the individual with a small dose of virus. From a practical point of view, it would be very interesting to indirectly standardize the value of a rabies vaccine by titrating the rabicidal property of serum taken from animals inoculated with the vaccine.

5. The rabicidal property of the anti-rabies serum vanishes by heating at 80°C. for one-half hour, while no change takes place in rabicidal power of the serum by heating below 70°C. for one-half hour.

6. The optimum temperature and time for the union of rabic virus with anti-rabies serum are 37°C. and two hours respectively. A somewhat similar result is obtained by storing them at a temperature of from 18°–20°C. for twenty hours in a dark room.

7. The protective injection of the anti-rabies serum into guinea-pigs inoculated with rabic virus has given satisfactory results. This may throw a light on the problem of utilizing anti-rabies serum for protective or curative purposes.

In conclusion, I wish to express my best thanks to Prof. N. NITTA for his valuable suggestions and interest in this work.

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狂犬病血清ノ抗狂犬病毒作用ニ就テ

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狂犬病豫防接種ハ現今人獸共ニ應用セラレ且ツ其ノ成績極メテ良好ナリト雖モ其ノ免疫ノ機制ニツキテハ未ダ充分ナル説明ナシコレ其ノ病原體ノ純粹培養ノ不可能タルト及ビ免疫造抗原ノ摘出困難ナルヲ以テ免疫材料ハ何レモ病毒ヲ含有スル複雑ナル神經物質ヲ供用セザル可ラザルヲ以テ他ノ比較の性狀ノ判明セル細菌性免疫ト其ノ趣ヲ異ニスレバナリ古來本題ニ就キテハ幾多ノ説明アリ或ハ血清ノ殺菌性ニ歸シ或ハ白血球ニ歸スルガ如シ余モ亦本題ノ試験ニ著シ未ダ充分ノ成績ヲ得ザルモ免疫セル動物ノ血清ニ就テノ成績ハ比較的見ルベキモノアルヲ以テ茲ニ報告セル所以ナリ今其ノ結果ヲ概述スレバ

1. 狂犬病ニ免疫セル動物ノ血清ハ抗狂犬病毒作用ヲ有スル物質ヲ含有ス然レドモ他ノ抗體(沈澱素凝集素補體結合素)ハ殆ンド之ヲ證明セズ

2. 天然ニ免疫セル犬ノ血清中ニモ同様ノ物質ヲ有スルモ健康動物ノ血清ハ殆ンド之ヲ有セズ

3. 抗狂犬病毒作用物質ハ70度30分以下ノ加熱ニ堪ユルモ80度以上ノ溫度ニテハ破壊セラル

4. 狂犬病免疫血清ノ豫防的注射ハもるもつとニ於ケル試験ノ結果或ル程度ノ奏效ヲ示セル極メテ興味アル事實ヲ與ヘタリ

本試験ニ就キテハ目下尙試験中ナリト雖モコノ少數ノ結果ヨリ見ルモ本血清ノ實地應用上ニ光明ヲ與ヘタルモノト信ズ

5. 免疫血清ト病毒トノ結合關係ハ恰モ毒素ト抗毒素ノ間ニ存スル倍加律ヲ以テ行ハル、ハ極メテ興味アル事實ニシテ本免疫體ハ比較的僅少ト雖モ檢定シ得ルヲ以テ實際上直接ニ各動物個體ノ抵抗力ノ差異ヲ見ルコトヲ得ルト共ニ間接ニ接種液ノ效果ヲモ想定シ得ルモノナリ

(自抄)