

Acta Medica Okayama

Volume 8, Issue 1

1952

Article 2

JANUARY 1952

Studies on Complement Fixation Test in a Mouse Immuni-zed with Japanese B Encephalitis Virus.

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Abstract

1) I designed a new micro-method for complement fixation test by means of a capillary pipette. 2) By this method, the complement-fixing antibodies in an individual mouse could be tested without taking its life. 3} The complement fixation titers in mice immunized with Japanese B encephalitis had a considerable individuality. 4) An adjuvant containing anhydrous lanoline and paraffin-oil, when mixed with Japanese B encephalitis vaccine, was effective to potent complement-fixing antibody productions in mice to this antigen.

From the Department of Hygiene
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**Studies on Complement Fixation Test in a Mouse
Immunized with Japanese B Encephalitis Virus.**

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Received for publication on Aug. 31, 1951.

Japanese encephalitis virus was first inoculated into the monkey's cerebrum by *M. Hayashi*¹⁾, then was made able to be transmitted to the mouse similar to St. Louis encephalitis. Since then, mice have become universally used for serological tests in Japanese encephalitis.

Complement fixation test in Japanese encephalitis virus is performed with antigens prepared from a mouse's infected brain, and is applied to diagnose such patients or differentiate this virus from another encephalitis viruses^{2, 3)}. In the latter, mice are used as a source of antisera. Because, by immunizing mice with the mouse's infected brain, formation of antibodies affined to brain tissues from normal mouse could be avoided.

And also mice are used in order to examine the relationship between the complement fixing and neutralizing antibody production and the resistance following vaccination in experimental encephalitis infections⁴⁾.

In such cases mice were bled from their heart, and the sera were gathered from several of them, because the serum from a mouse was so little. But when considering individuality in mice, it is desirable to test the complement-fixing antibodies of every individual. For this purpose, I designed a micro-method for complement fixation test by means of a glass capillary pipette. And by this method, I investigated what influence *Freund's* adjuvant had on complement-fixing antibody productions in each mouse, immunized with Japanese B encephalitis virus⁵⁾.

Materials and Methods.

1. Virus strain: — *Nakayama* strain of Japanese B encephali-

tis virus was used, which was shared through the kindness of Prof Ando of Japanese National Institute of Health.

2. Vaccine:— prepared from mice's brains infected with Japanese B encephalitis. These were emulsified to 1/10 with 0.85% NaCl solution, and inactivated by adding formalin in a proportion of 0.2%.

3. Vaccine-adjuvant mixture:— was prepared by mixing 1 part of vaccine to 1 part of sterile anhydrous lanoline and 2 parts of sterile paraffin-oil.

4. Complement fixation test:— was carried out by the following new micro-method.

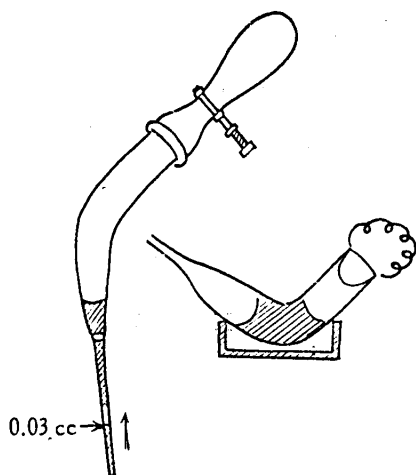
Antigen, complement solution and sensitized sheep red cells suspension were prepared by the method described by Ando⁵⁾, without essential modification.

0.15–0.2 cc of blood was obtained by amputating the mouse's tail. This blood was put into a glass micro-test tube with a interior diameter of 0.5 cm. Bleeding from the tail was stopped by scorching it on fire. The test tube was plugged with cotton, and kept in a ice-box over night. About 0.05 cc of serum was obtained after centrifugation, and was diluted 4 times with saline solution. This may conveniently be done by means of a small capillary pipette, fitted with a rubber teat pinched with Hoffman's cock, and marked near the point corresponding to the desired volume. The serum was taken up by this pipette and allowed to run slightly up the tube so as to admit an air bubble to serve as an index, then the pipette was dipped into saline solution, and a similar volume was admitted three times. The serum and saline solution were then blown out into the micro-test tube, and mixed by drawing it in and out of the pipette. The test tube was plugged with cotton, and heated at 57°C for 30 minutes in a water-bath. A part of this serum was again diluted successively 2 times with saline solution by means of a capillary pipette marked about 0.05 cc, and with concaved slide glasses. The diluted serum must be used rapidly for the following test to avoid evaporation.

Complement fixation test was carried out by means of capillary pipettes like Figure 1, which were bended in a obtuse angle at its waist, and marked at about 0.03 cc. The previously stated diluted serum, two units of complement and antigen were sucked up one after another into this pipette by the same method as used in diluting the serum. The tip of the pipette must be cleaned with

ganze every time a solution is taken in. The capillary pipette was then laid on the rack, and after the rubber teat was taken off, it was well shaken to mix the three components. The air-bubbles could be blown out by a blast into a capillary pipette by means of

Figure 1.



another capillary pipette. The pipette was kept in a ice box between 2-8°C for 18-20 hours. And then sensitized sheep red cells suspension was taken up twice to the mark and mixed. After the pipette was left in an incubator at 30°C for half an hour, the results were read.

Since results obtained with undiluted antisera, with sera diluted 1:2 are questionable, the dilutions used in my test were over 1:4. Only 2, 3 or 4 plus fixations were considered significant.

Results.

I. Examination of the micro-method for complement fixation test.

The new micro-complement fixation method, using egg-albumin and its antibody made in the rabbit, was tested and compared with the ordinary method described by Ando⁵⁾. The results were as in Table I. Both methods will always give the same results when performed carefully.

Table I. Complement fixation test of Anti-egg-albumin rabbit serum with homologous antigen.

Method	Antiserum dilutions					Antigen cont.	Serum cont.
	1:8	1:16	1:32	1:64	1:128	1:4	1:4
Ordinary method	4	4	4	2	0	0	0
New micro-method	4	4	4	2	0	0	0

Dilution of antigen is 1:100. 4 = no hemolysis, 0 = complete hemolysis.

II. The complement fixation tests in mice, which received repeated injections of Japanese B encephalitis vaccine.

10 mice were injected intraperitoneally with 0.2 cc of injected mice brain vaccine every five day for ten times. 10 days after the last injection, bloods were taken from their tails, and tested according to the method described beforehand. The results are as in Table II. Complement fixation titers of mice showed a difference between 1:8 to 1:128 inspite of giving them a similar immunization dosage. No reactions were seen with normal brain antigen.

Table II. Complement fixation test in mice immunized with Japanese encephalitis virus.

No.	Weight	Antiserum dilutions						N	S	Titer
		1:4	1:8	1:16	1:32	1:64	1:128			
1	20 g	4	4	4	4	4	3	0	0	1:128
2	20.5 g	4	4	4	4	4	2	0	0	1:128
3	18 g	4	4	4	4	3	1	0	0	1:64
4	17.5 g	4	4	4	4	3	1	0	0	1:64
5	16.5 g	4	4	4	3	1	0	0	0	1:32
6	22 g	4	4	4	3	0	0	0	0	1:32
7	23 g	4	4	4	2	0	0	0	0	1:32
8	20 g	4	4	3	1	0	0	0	0	1:32
9	19 g	4	4	1	0	0	0	0	0	1:8
10	21 g	4	3	0	0	0	0	0	0	1:8

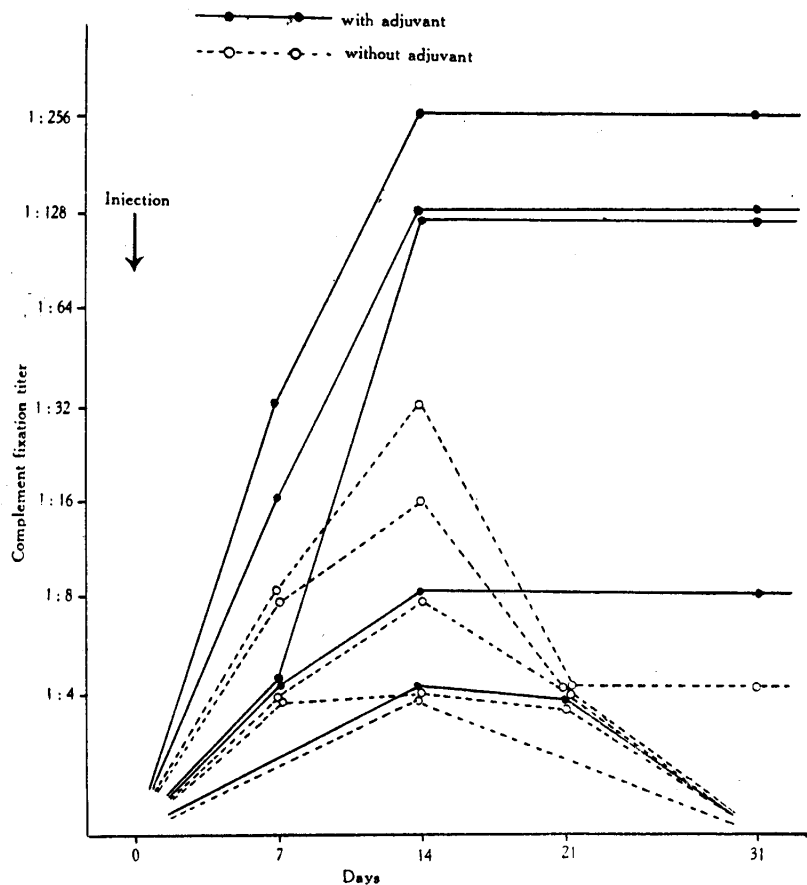
N: Normal mouse brain cont., S: Serum cont.

III. The progreess of complement-fixing antibody production in the mouse, when injected with Japanese B encephalitis vaccine incorporated with adjuvant.

10 mice weighing between 15 g to 20 g were divided into two equal groups, I and II. Group I received a single subcutaneous injection of 1 cc vaccine-adjuvant mixture (containing 0.25 cc of vaccine). Group II was injected 1 cc of vaccine diluted 2 times with saline solution as a control. Mice were bleed every week after injection, and the complement fixation titers were measured. The results are as in Figure II. The individuality of the mouse is

seen in this case, too. But in general, the antibody titers in group I reached higher levels, and were more sustained than those in group II, where the antibodies disappeared in a month after injection.

Figure II. Complement fixation test in mice immunized with Japanese encephalitis virus.



Discussion.

To study the complement fixation test in a mouse, it is necessary to perform a test with very little serum. If a mouse could be bled about 1/100 of their body weight without evil effect to life, only about 0.15 cc blood could be taken from a mouse weighing 15 g, and from this blood about 0.05 cc of serum could be obtained. And by the usual complement fixation method, this serum must be highly diluted for test. But by the above mentioned new micro-

method, the complement fixation test could be carried out with only 4 times dilution of the serum. I designed this method by a hint taken from the technics used in *Wright's* opsonin test⁷⁾, and bended the capillary pipette at its waist so that the reaction could be performed in the pipette. This method could be applied to human beings, when it is difficult to obtain large quantity of blood from the vein, and only a small amount of blood is obtainable from the ear-lobe.

By using this method, I tested the so-called hyperimmune serum taken from a mouse, injected with Japanese B encephalitis virus, and found that these immune sera had a considerable individuality in their complement fixation titers.

By this method, I also succeeded in investigating the progress of immunity in a mouse injected with a mixture of Japanese B encephalitis vaccine and adjuvant. In 1942 *Freund* and *McDermott*⁵⁾ demonstrated that an adjuvant containing mineral oil, emulsifying agents and killed acid-fast organismus, when mixed with horse serum, was especially effective in increasing the immune response of rabbits to this antigen. The antibody response to a variety of antigen has been potentiated in this way⁸⁾. And on Japanese encephalitis, *Warren* and *Hough*⁹⁾ carried out the protection test against infection in mice injected with a mixture of *Falva*, mineral oil and vaccine made from infected mice brain, and reported that in this case the minimal immunizing dosis could be decreased to 1/3 as compared with the control which had no adjuvant. Accordingly, I selected anhydrous lanoline and paraffin-oil as adjuvant, and found that this adjuvant was effective in mice to potent complement-fixing antibody productions against Japanese encephalitis virus.

Summary.

- 1) I designed a new micro-method for complement fixation test by means of a capillary pipette.
- 2) By this method, the complement-fixing antibodies in an individual mouse could be tested without taking its life.
- 3) The complement fixation titers in mice immunized with Japanese B encephalitis had a considerable individuality.
- 4) An adjuvant containing anhydrous lanoline and paraffin-oil, when mixed with Japanese B encephalitis vaccine, was effective

to potent complement-fixing antibody productions in mice to this antigen.

In conclusion, I wish to express my sincere thanks to Prof. *M. Ogata* for his kind advice and suggestions during this investigation.

References.

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