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# Multiplication and Antibody Formation of Japanese Encephalitis Virus in Snakes*

## 1. Antibody responses to the virus and serum

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**ABSTRACT**

Japanese encephalitis (JE) shows its explosive epidemicity in the temperate zone of Asia but little is known on the overwintering mechanism. One of the hypotheses on the overwintering mechanism is that the virus overwinters in the hibernating animals. There has been no report on the proliferation of JE virus (JEV) or antibody formation in the snakes. The purpose of this experiment is to explore the mutual relationship between JEV and snake and to clarify whether JEV proliferates and induce antibody formation in snakes. Three species of non-poisonous common snakes were employed. Precipitation test was carried out after injecting calf serum and, HI and neutralization tests were done by injecting JEV into the snakes. The gamma globulin fraction of pre- and post-injection serum were compared by paper chromatography. According to the results, precipitating antibody reaction to calf serum could be observed only at 4°C. It was failed to demonstrate HI antibody formation but neutralizing antibody could be detected in one of the 9 snakes. Although antibody could not be detected in test-tube, the result of paper chromatography shows the remarkable increase of gamma globulin fraction after the injection. Above results are strongly indicating the antibody formation in the snakes.

## INTRODUCTION

Japanese encephalitis (JE) shows its explosive epidemicity during every summer in the temperate zone of Asia, and in Korea thousands of JE cases have been reported every year since the prevalence in 1947.

But little is known on the maintenance of JE virus (JEV) during the cold winter of this endemic area. There are many hypotheses on the overwintering mechanisms of JEV, one of which is that the virus overwinters in the body of hibernating cold-blooded animals.

In 1960, Thomas and Eklund (1960) reported that Western equine encephalitis virus (WEEV) proliferates in the snakes and it can be transmitted to susceptible animals by mosquito-bites after overwintering under artificial conditions. In 1964, Gebhardt et al (1964) isolated WEEV in the wild snakes collected in the early spring. Burton et al (1966) reported that WEEV was isolated from the snakes and frogs, and they detected neutralizing antibody in the frogs.

There has been no report on the proliferation of JEV or antibody formation in cold-blooded animals.

The author reported (Lee 1966) on the presence of hemagglutnation inhibition (HI) anti-

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body to JEV in the wild-snakes collected from 1964 till 1966 and found that some of the snakes collected in 1967 have neutralizing antibodies to JEV. But HI antibody or neutralizing antibody could not be detected in frogs until now. JEV was isolated by the principal investigator (Lee 1967) in one of 314 snakes collected in 1966, and one of 535 snakes collected in 1967.

The purpose of this experiment is to determine 1) antibody response and 2) whether JEV can proliferate in snakes.

As a preliminary experiment, we studied whether antibody can be produced in the snakes and some part of the results are reported hereafter.

## MATERIALS and METHODS

**Snakes:**

The employed snakes are the following 3 species of non-poisonous common snakes known in Korea.

1) Natrix tigrina lateralis Berthold
2) Elaphe rufodorsata Cantor
3) Elaphe schrenckii Strauch

**Antigens:**

1) JEV M5/596 strain (Lee and Scherer 1961) of 12th suckling mouse passage and Nakayama strain (Lee and Scherer 1961) of 9th suckling mouse passage. The $LD_{50}$ of the viruses were $10^{6}$/0.03 ml and $10^{6.4}$/0.03 ml, respectively.
2) Calf serum.
3) Bovine albumin crystalized (Difco).

A known amount of suckling mouse brain virus, 5% bovine albumin or calf serum was injected respectively into the dorsal portion of snakes intradermally or subcutaneously at various intervals. At the beginning of the experiment, plasma of the snakes was obtained by centrifuging the blood collected by snipping the snake-tail, but recently, blood is collected by cardiac-puncture with 26 gauze needle. The composition of pre-injection plasma and post-injection plasma were also compared each other simultaneously.

**HI test to JEV:**

Buescher et al's (1959) method was employed.

**Precipitation Test:**

Equal amount of the antigen was added into 2 fold dilution of plasma contained in 10×75mm test-tube and incubated at 36°C for 1 hour. The mixture was stored at room-temperature and 4°C separately, and precipitation reaction was observed daily for 2 weeks.

**Plaque neutralization Test:**

Plaque neutralizing antibody was tested with chick embroyo cell culture (Lee 1966).

**Paper chromatography:**

The method of paper chromatography used in this experiment was based on the method of Mellor (1957).

**Table 1.** Precipitin Formation in Snakes to Bovine Albumin and Calf Sreum

| Snake species | Snake No. | Dose and route of inoculation on days | | | | | | | | | Precipitin formation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 21 | 28 | 36 | 42 | 50 | 55 | 64 | 72 | |
| Natrix tiagrina lateralis Berthold | 201 | 5% B.A.[1] 0.1 ml I.D. | 5% B.A. 0.1 ml I.M. | 5% B.A. 0.15 ml S.C. | CaS[2] 0.5 ml S.C. | CaS 1.0 ml S.C. | CaS 1.0 ml S.C. | CaS 1.0 ml S.C. | CaS 2.0 ml S.C. | Total bleeding | + |
| 〃 | 203 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | + |
| 〃 | 206 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | − |
| 〃 | 214 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | − |
| 〃 | 220 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | − |
| 〃 | 221 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | − |
| Elaphe schrenckii Strauch | 205 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | − |
| 〃 | 215 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | − |

1. B.A. =Bovine albumin  2. CaS=Calf serum

## RESULTS

### Precipitating-antibody formation to bovine serum in snakes

Table 1 shows the injected amounts of bovine albumin and bovine serum, intervals between each injection and the results of antibody response. Into 8 snakes, injections were done initially with long intervals between each injection and later with short intervals. Total blood was collected at 72nd day and plasma was separated.

Table 2 and 3 shows the results of precipitating antibody formation to calf serum. No reaction was visible at room temperature but at 4°C there was precipitation since 4th day, even though it showed very low titer, and on 7th day, relatively strong precipitation reaction was seen. But the other 6 samples showed no demonstrable reaction. And no reaction could be observed when bovine albumin was used as antigen.

According to the result of analysis of plasmas of the day 0 and 72nd day by paper chromatography, remarkable increase of gamma globulin fraction was observed after antigen injection into snake No. 203, as shown in Table 4. There was some degree of changes in other snakes but it was not remarkable.

**Table 2.** Precipitation Test of 72nd Day Snake Plasma (No. 201) Immunized with Calf Serum

| Dilution of plasma | 1 : 2<br>0. 2 ml | 1 : 4<br>0. 2 ml | 1 : 8<br>0. 2 ml | Antibody control<br>1:2 0. 2 ml | Antigen control<br>0 |
|---|---|---|---|---|---|
| Antigen CaS[1] | 0. 2 ml | 0. 2 ml | 0. 2 ml | 0 | 0. 2ml |
| Mixed in 37°C water bath for 60 minutes and then incubated at 4°C | | | | | |
| day 1 | − | − | − | − | − |
| day 2 | − | − | − | − | − |
| day 3 | − | − | − | − | − |
| day 4 | + | − | − | − | − |
| day 5 | + | − | − | − | − |
| day 6 | ++ | − | − | − | − |
| day 7 | +++ | − | − | − | − |

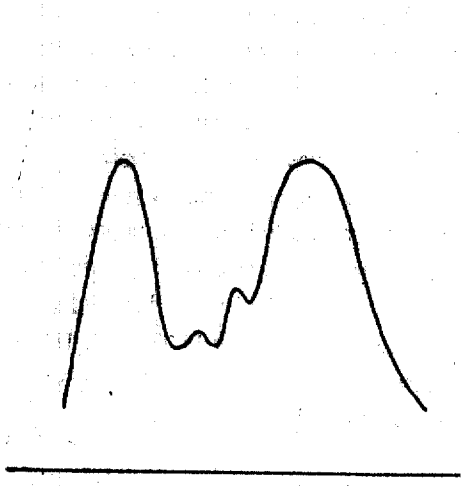
1. CaS=Calf serum

**Table 3.** Precipitation Test of 72nd Day Snake Plasma (No. 203) Immunized with Calf Serum

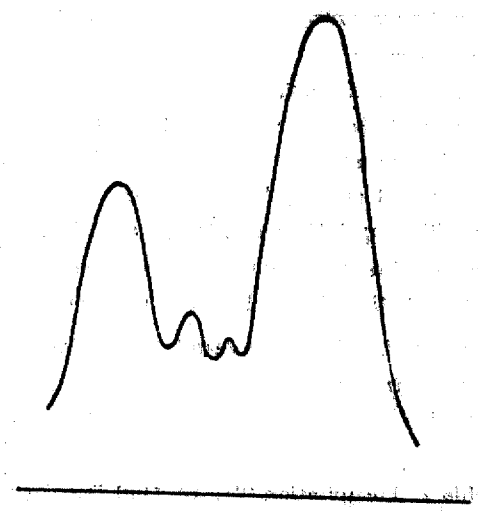
| Dilution of plasma | 1:2<br>0. 2 ml | 1:4<br>0. 2 ml | 1:8<br>0. 2 ml | Antibody control<br>1:2 0. 2 ml | Antigen control<br>0 |
|---|---|---|---|---|---|
| Antigen CaS[1] | 0. 2 ml | 0. 2 ml | 0. 2 ml | 0 | 0. 2 ml |
| Mixed in 37°C water bath for 60 minutes and then incubated at 4°C | | | | | |
| day 1 | − | − | − | − | − |
| day 2 | − | − | − | − | − |
| day 3 | − | − | − | − | − |
| day 4 | + | − | − | − | − |
| day 5 | ++ | − | − | − | − |
| day 6 | +++ | − | − | − | − |
| day 7 | +++ | − | − | − | − |

1. CaS=Calf Serum

**Table 4.** Paper Chromatographic Pattern of Snake Plasma (No. 203)



The day 0 plasma



72nd Day Plasma Immunized with Bovine Serum

**Antibody responses to JEV in snakes**

As shown in Table 5, JEV M5/596 and Nakayama strain were inoculated at various intervals into 9 snakes which were containing no HI antibody to JEV. Total blood was collected at 55th day to detect HI antibody and neutralizing antibody formation, and analysis of gamma globulin fraction was done by paper chromatography.

The results show that snake No. 216 and 218 showed titer 1:10. Significant titer of plaque neutralizing antibody was observed only in snake No. 244.

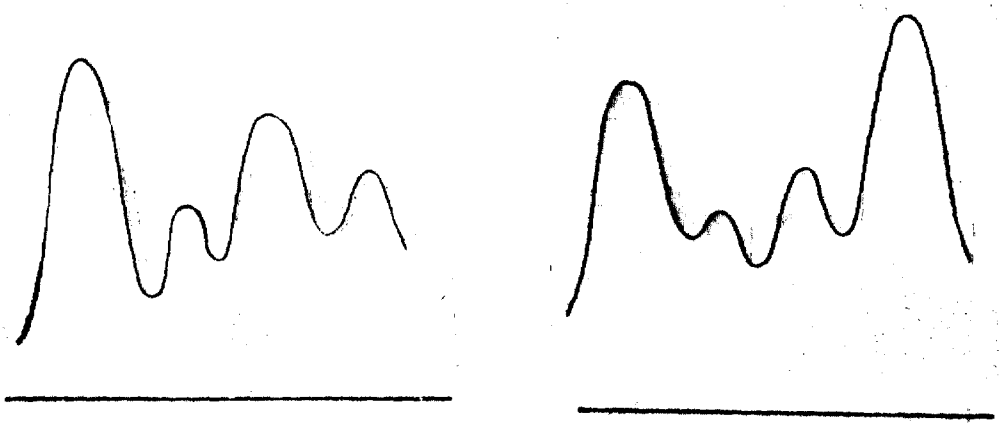
According to the paper chromatography as shown in Table 6, snake No. 218 showed marked increase of gamma globulin fraction at 55th day, comparing with the day 0. But other snakes did not show similar changes.

Table 7 shows the result of precipitation reaction. Grossly observable precipitation reaction began to appear since 9th day at 4°C with the snake plasma No. 79.

**Table 5.** Antibody Responses to Japanese Encephalitis Virus in Snakes

| Snake species | Snake No. | Dose and route of virus inoculation on day | | | | | HI titer on day | | Neutralization No. of plaques on day | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 28 | 35 | 42 | 50 | 0 | 55 | 0 | 55 |
| Natrix tigrina lateralis Berthold | 204 | JEV M5/596 0.1 ml $10^{-4}$SMB I.D. | JEV M5/596 0.1 ml $10^{-3}$SMB I.D. | JEV M5/596 0.1 ml $10^{-2}$SMB I.D. | JEV M5/596 0.3 ml $10^{-1}$SBM I.D. | JEV M5/596 0.2 ml $10^{-1}$SMB I.D. | 10 | 10 | 190 | 151 |
| 〃 | 216 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 | 10 | | |
| 〃 | 217 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 | <10 | 120 | 120 |
| 〃 | 218 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 | 10 | 136 | 132 |
| 〃 | 227 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 | <10 | 120 | 120 |
| 〃 | 209 | JEV Nakayama 0.1 ml $10^{-4}$SMB I.D. | JEV Nakayama 0.1 ml $10^{-3}$SMB I.D. | JEV Nakayama 0.1 ml $10^{-2}$SMB I.D. | JEV Nakayama 0.3 ml $10^{-1}$SMB I.D. | JEV Nakayama 0.2 ml $10^{-1}$SMB I.D. | <10 | <10 | 151 | 118 |
| 〃 | 210 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 | <10 | | |
| 〃 | 212 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 | <10 | 145 | 93 |
| 〃 | 244 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 | <10 | 100 | 40 |

Table 6. Paper Chromatographic Pattern of Snake Plasma (No. 218)



The Day 0 Plasma

55th Day Plasma Immunized with JEV M5/596 Strain

Table 7. Precipitation Test of 77th Day Snake Plasma (No. 79) after Immunization with JEV M5/596

| Dil. of plasma | 1:2 0.2 ml | 1:4 0.2 ml | 1:8 0.2 ml | 1:16 0.2 ml | Antigen control 0 | Serum control 1:2 0.2 ml |
|---|---|---|---|---|---|---|
| JEV $10^{-2.5}$ SMB | 0.2 ml | 0.2 ml | 0.2 ml | 0.2 ml | 0.2 ml | 0 |
| Incubated at 4°C | | | | | | |
| day 1 | − | − | − | − | − | − |
| day 2 | − | − | − | − | − | − |
| day 3 | − | − | − | − | − | − |
| day 4 | − | − | − | − | − | − |
| day 5 | + | + | + | + | − | − |
| day 6 | + | + | + | + | − | − |
| day 7 | + | + | + | + | − | − |

**Attempt to demonstrate anamnestic HI antibody response to JEV in snakes**

As shown in Table 8 large amount of virus was inoculated into 9 snakes containing HI antibody at various intervals. HI antibody test was carried out with the serum separated from the blood collected on 77th day.

According to the result, no anamnestic response could be observed, but there was remarkable decrease of antibody titer during the period.

## DISCUSSION

The plasma of about 40% of Korean non-poisonous common snakes collected during the past 3 years was found to have HI antibody to JEV (Lee 1967). Recent study showed that there was plaque neutralizing antibody in the plasma. And only small number of snakes were found to have both HI antibody and neutralizing antibody (Lee 1967). The problem calling our attention is whether this antibody is specific to JEV or not.

It has been used to extract the serum twice with aceton for HI test according to the Buescher et al's method (1959), however, it was found that there was increase of HI antibody titer of the snake plasma when extracted the plasma with aceton 1 or 2 times more. Therefore, it seems

**Table 8.** Attempt to Demonstrate Anamnestic HI Antibody Response to JEV in Snakes (E. rufodorsata Cantor)

| Snake No. | HI titer day 0 | Dose and route of JEV M5/596 inoculation on day | | | | | | | HI titer on day 77 |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 24 | 37 | 42 | 49 | 57 | 61 | |
| 78 | 40 | 0.1 ml $10^{-1}$SMB I.D. | 0.1 ml $10^{-1}$SMB I.D. | 0.2 ml $10^{-1}$SMB I.D. | 0.2 ml $10^{-1}$SMB I.D. | 0.2 ml $10^{-1}$SMB I.D. | 0.2 ml $10^{-1}$SMB I.D. | 0.2 ml $10^{-1}$SMB I.D. | 10 |
| 79 | 80 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 10 |
| 81 | 40 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 20 |
| 96 | 160 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 10 |
| 104 | 10 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 10 |
| 115 | 20 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 10 |
| 122 | 40 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 10 |
| 154 | <10 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 |
| 155 | 40 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 | <10 |

that the antibody is specific to JEV.

Bovine albumin and bovine serum were injected into the snakes and antibody formation to this antigen was tested at first. As shown in the result, precipitating antibody could be detected in 2 snake-sera out of 8 snakes immunized, but it was different from the phenomenon of warm-blooded animal serum in that the precipitation reaction in snake sera occurred at 4°C. It indicates that the mechanism of antibody formation in cold-blooded animals is cold type, quite different from that of warm-blooded animals.

According to the result of paper chromatography, there was remarkable increase in gamma-globulin fraction after the immunization, and this result is thought to indicate the presence of antibody formation in the snakes.

Large amount of JEV was inoculated into the snakes, free of HI antibody to JEV. And HI tests were carried out with the plasma, but the plasma from two out of the 9 snakes showed the titer, 1:10. Significant titer of plaque neutralizing antibody was observed in the plasma of only 1 out of the 9 snakes inoculated.

The result of paper chromatography shown in Table 6 shows that there was remarkable increase of gamma-globulin fraction in the plasma collected on 55th day from the snakes inoculated with JEV. This result indicates that there was antibody formation to JEV.

Large amount of JEV was injected into the 9 snakes containing HI antibody to JEV, and HI test was done with the plasma of 77th day. There was no observable anamnestic HI antibody response but decrease of the titer. This result is quite different from the result of experiment with monkey (Lee and Scherer 1961). As shown in Table 8, snake No. 79 showed remarkable precipitation reaction at 4°C, but no other snakes showed such a clear result.

Our experimental condition was different from the natural condition, because the snakes were bred at room temperature and it is thought that the method employed in this experiment is not suitable because the virus employed is not a local strain, but laboratory adapted Nakayama and M5/596 strain of JEV originally isolated in Japan.

Moreover, it has been known that the temperature gives great influence on the function and formation of enzymes in warm-blooded animals. Therefore, any special discussion or conclusion can not be made with the result of our limited preliminary experiments.

Considering above irregular results in the snakes no clear conclusion can be made now

and more critical further experiments are now required. At the beginning of the experiment, virus test could not be done because aseptic collection of snake blood was so difficult. These days snake blood is collected by cardiac puncture and the experiment of virus proliferation and antibody formation are simultaneously carried out.

## SUMMARY


1) The result reported here is the result of a preliminary experiment, and in future experiment local strain of JEV will be desirable.
2) Precipitating antibody to bovine serum was detected in some of the snake plasmas only at 4°C. Paper chromatography showed remarkable increase of gamma-globulin fraction in some of the snakes after immunization.
3) HI test with the plasma from the virus inoculated snakes which did not contain HI antibody previously was negative. However, one of the experimental snakes showed neutralizing antibody formation and increase of gamma-globulin fraction.
4) Anamnestic antibody response was tested in the plasma from the snakes which contained HI antibody. No anamnestic response was observed, whereas, there was remarkable decrease of HI antibody titer. But precipitating antibody was detected in one out of 8 snakes.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