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IMMUNOLOGICAL STUDIES ON THE INFECTIOUS CANINE HEPATITIS VIRUS

II. HEMAGGLUTININ

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INTRODUCTION

A previous publication³⁾ has described the properties of the complement fixing antigen (CFA) of infectious canine hepatitis (ICH) virus, and demonstrated that it is composed of soluble and viral antigens.

The discovery of the hemagglutinating properties of certain groups of viruses, such as the myxoviruses and the arbor group, has facilitated the delineation of relationship between the members of these groups, and has in addition provided techniques by which these viruses and their respective antibodies can be easily and rapidly identified.

The purpose of this communication is to report that, under certain conditions, tissue culture fluids containing ICH virus would agglutinate guinea pig and human erythrocytes.

MATERIALS AND METHODS

Virus strains ICH virus strain employed, namely "MATSUDA" was isolated directly on cultures of dog kidney epithelial cells from naturally infected dog liver suspensions in this laboratory. The second strain "Woc-4" of Dr. POPPENSIEK was kindly provided by Prof. OCHI of the laboratory of veterinary bacteriology, Tokyo University, Tokyo. These viruses were maintained by serial transfers in dog kidney tissue cultures, infected material being stored between passages in deep freezer at -20°C .

Tissue culture Monolayer cultures of dog kidney epithelial cells were prepared according to the method described by YOUNGNER. Dog kidney epithelial cell sheets were usually employed both for propagation and for titration of ICH viruses.

Titration experiments Virus material was diluted in steps of 1.0 log in Hanks' balanced salt solution and 0.1 ml of the dilution was inoculated into each of 3 to 5 tubes containing preformed sheets of dog kidney cells. The 50% end point (TCD_{50}) was calculated by the method of BEHRENS-KÄRBER.

Hemagglutination test Hemagglutination titrations were carried out in a manner similar

to that used with influenza virus⁴). Blood samples from humans and guinea pigs were collected and stored in Alsever's solution at approximately 4°C. Before use the erythrocytes were washed with reaction mixture three times and then prepared as a 0.25% suspension. Tests for hemagglutination were carried out in tubes (12×75 mm) by the addition of 0.5 ml of erythrocyte suspension to 0.5 ml amounts of serial twofold dilutions of the virus stock in diluent. After the tubes were shaken, the erythrocytes were allowed to settle. The first tube showed agglutination of approximately 50% the cells, as determined by the SALK's pattern of sedimentation.

Complement fixation test The technique employed has been described in detail previously³). Tests were performed using 2 units of complement, and overnight fixation at 4°C. The antigen was tested in twofold dilutions, starting at 1/2 against 4~8 antibody units of hyperimmune dog sera.

RESULTS

1. Ability to agglutinate erythrocytes from different animal species

The species of erythrocytes agglutinated with "MATSUDA" strain of ICH virus and the hemagglutinin titres (HATs) are shown in table 1. Of among erythrocytes from a number of domestic animals, only those of the human and guinea pig were specifically agglutinated under certain conditions. There appeared to be no gross intraspecies variation, just as in the human blood group.

TABLE 1. *Relationship between Sorts of Erythrocytes and HAT*

| SORTS OF ERYTHROCYTES | HAT |
|-----------------------|------|
| Human O-Group | 1024 |
| A-Group | 1024 |
| B-Group | 1024 |
| AB-Group | 1024 |
| Guinea pigs | 1024 |
| Sheep | < 20 |
| Rabbits | sp |
| Domestic fowls | 40 |
| Horses | < 20 |
| Cows | < 20 |
| Dogs | sp |
| Mice | 32* |

sp) spontaneous agglutination

*) agglutinated nonspecifically with normal tissue culture fluid

With fowl cells there were marked differences in cell susceptibility to hemagglutination dependent upon environmental factors and they indicated lower titre than human cells. The erythrocytes from rabbits and dogs were agglutinated spontaneously in repeated experiments.

2. Environmental factors influencing hemagglutination

a. Incubation temperature and concentration of erythrocytes As shown in table 2, incubation temperature and concentration of erythrocytes were important factors influencing the HAT. Table 2(a) shows the results of three titrations which were the same in all respects except for the temperature at which the mixtures were stood. In the incubator at 37°C the titre recorded was 512, whereas at room temperature and at 4°C as well the observed end points were both 256. At 37°C the reaction turned negative by virtue of the rate at which hemagglutinin became dissociated from the erythrocytes. At the temperatures lower than 37°C a longer time was required for complete sedimentation of the erythrocytes because of the slower rate of settling.

Another factor that influences the reaction was the concentration of the erythrocytes in the mixture. Identical serial dilutions of tissue culture fluid containing ICH virus were mixed with erythrocyte suspensions of varying concentration. It was observed that the titre of the

TABLE 2.

(a) *Effect of Temperature in Titration of Agglutinins*

| TEMP. °C | HAT | INCUBATION TIME (hrs) |
|----------|-----|-----------------------|
| 37 | 512 | 1 |
| 15~18 | 256 | 2 |
| 4 | 256 | 2 |

(b) *Variation in HAT in Relation to Quantity of Erythrocytes*

| FINAL CONC. OF ERYTHROCYTES (%) | HAT |
|---------------------------------|-----|
| 1 | 16 |
| 0.5 | 256 |
| 0.25 | 512 |
| 0.125 | 512 |

same fluid was appreciably higher when a lower concentration of erythrocytes was used (Table 2 (b)). While it was clear that the greatest sensitivity for practical purposes was obtained with the use of the most diluted erythrocyte suspensions, the minimum quantity of erythrocytes necessary to produce an easily visible reaction limits the dilution of erythrocyte suspension that may be employed. The optimum concentration seems to be 0.5 ml of a 0.25% suspension of human erythrocytes mixed with an equal volume of each serial dilution of virus.

b. pH requirement for reaction It was found that the pH value was the determining factor in hemagglutination with this virus. The erythrocytes were washed in 0.85% NaCl solution and diluted to a concentration of

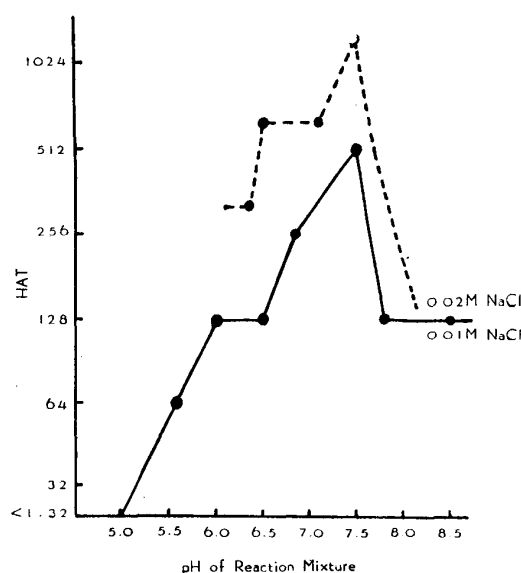


FIGURE 1. *Effect of pH of Reaction Mixture on HAT*

0.25% in reaction mixture (0.25 M dextrose plus 0.02 or 0.01 M NaCl solution) with appropriate M/15 phosphate buffers. The virus preparations were diluted in twofold steps in corresponding phosphate buffered mixtures. As shown in figure 1, optimum conditions were between pH 6.0 and 7.8. Maximum titre was obtained at pH 7.5. On the basis of the results of these and other similar experiments reaction mixture buffered at pH 7.5 was employed in subsequent experiments.

c. Ionic environment In 0.25 M dextrose solution, ICH virus did not agglutinate human erythrocytes. Then, this fact was studied with additions of various concentrations of NaCl to 0.25 M dextrose solution buffered at pH 7.5 with M/15 phosphate buffer solution. NaCl concentration of diluent varied from 0.5 to 0.01 M. Dextrose was not added when 0.15 M or more were used. Doubling serial dilutions of virus suspension were made in these diluents. An equal volume of human erythrocytes suspended in similar molarities of NaCl was added to the corresponding series of tubes and the results recorded. The results showed that hemagglutination occurred in NaCl concentration of from 0.3 to 0.01 M but with concentrations of 0.15 to 0.05 M it was agglutinated spontaneously. This spontaneous agglutination could be prevented by addition of inactivated rabbit serum, chicken blood plasma or skim milk. Reduction of the titre, however, was observed as shown in table 3. The results indicated that optimum NaCl concentration for hemagglutination may be 0.02 M in ICH viruses.

TABLE 3. *Relationship between HAT and NaCl Concentration*

| MOLARITY OF NaCl | 0.5 | 0.3 | 0.2 | 0.15 | 0.1 | 0.05 | 0.02 | 0.01 |
|---|-----|-----|-----|------|-----|------|------|------|
| 0.25 M Dextrose buffered at pH 7.5 (PBD) } | <32 | 256 | 256 | sp | sp | sp | 512 | 256 |
| Inactivated rabbit serum added to PBD (fin. conc. 0.1%) } | * | * | 32 | 32 | 128 | 256 | * | * |
| Chicken blood plasma added to PBD (fin. conc. 0.1%) } | * | * | 128 | 128 | 256 | 256 | * | * |
| Skim milk added to PBD (fin. conc. 1%) } | * | * | <32 | <32 | 32 | 128 | * | * |

*) Not tested

d. Components of reaction mixture Many workers have prepared various reaction mixtures for hemagglutination to prevent spontaneous agglutination and to enhance the hemagglutinating activity. As shown in table 4, seven kinds of reaction mixture were prepared; titration was conducted in these diluents. Maximum titre of hemagglutination was obtained by conducting the titrations in phosphate buffer saline solution buffered at pH 7.5 while lower titres were obtained in other diluents. Hemagglutination also occurred in unbuffered physiological saline but its titres varied with the age of the preparation and various other factors. Calcium chloride solution prepared according to SHINGU could not be used for hemagglutination because spontaneous agglutination occurred in the diluent.

TABLE 4. *Results of Hemagglutination Tests performed in Various Reaction Mixtures*

| REACTION MIXTURE TESTED | HAT |
|--|------|
| Physiological saline solution (PSS) | 256 |
| Inactivated rabbit serum added to PSS (fin. conc. 0.1%) | < 32 |
| Chicken blood plasma added to PSS (fin. conc. 0.1%) | 128 |
| Sodium citrate added to PSS (fin. conc. 0.1%) | 128 |
| Skim milk added to PSS (fin. conc. 1%) | 32 |
| Phosphate buffered saline (pH: 7.5) | 512 |
| 1.5% Calcium chloride solution | sp |

3. Heat stability of hemagglutinin, CFA and infectious unit

The tissue culture fluids infected with strain "MATSUDA" were heated for different durations of time; samples were withdrawn at various for use in the test. Titrations of the hemagglutinin, CFA and infectivity were carried out. As shown in table 5, initial HAT was retained at 56°C for 5 minutes, but at 10 minutes the titre fell extremely; it disappeared at 65°C for 30 minutes.

TABLE 5. *Heat Stability of ICH Virus Hemagglutinin and Other Components*

| TEMP. (°C) | MINUTES | HAT | CFAT** | TCD ₅₀ ** |
|------------|---------|------|--------|----------------------|
| 0 | 0 | 1024 | 256 | 6.0 |
| 56 | 5 | 1024 | 256 | 5.5 |
| " | 10 | 256 | 256 | < 1.0 |
| " | 30 | 256 | 256 | < 1.0 |
| " | 180 | 32 | 256 | < 1.0 |
| 60 | 30 | 128 | 128 | < 1.0 |
| 65 | " | < 2 | 64 | < 1.0 |
| 70 | " | < 2 | 32 | < 1.0 |
| 75 | " | < 2 | < 2 | < 1.0 |
| 80 | " | < 2 | < 2 | < 1.0 |
| 100 | 5 | < 2 | < 2 | < 1.0 |

*) complement fixing antigen titre

**) 1/log 50% tissue culture infectious doses

As recorded elsewhere³⁾, the CFA resisted 70°C for 30 minutes, but exhibited gradual titre deterioration parallel to the rise of temperature. By heating at 70°C for 30 minutes titre

lowered to 1/8 compared with that of nontreated; it was almost eliminated at 75°C for 30 minutes. On the other hand, there was marked abolishment of infectivity to tissue cultures at 56°C for 10 minutes. From the above described results, the ICH virus hemagglutinin considered to be a comparatively heat stable component of the viral particle; the heat stability of CFA is stronger than that of hemagglutinin.

4. Adsorption and elution of ICH virus

When mixture of human erythrocytes and ICH virus was held at 4°C the virus was adsorbed rapidly by the erythrocytes and sedimented with them on light centrifugation. When the sedimented erythrocytes were washed once in chilled saline, resuspended in original volume of phosphate buffer saline buffered at pH 7.5 and warmed for 2 hours at 37°C, elution of the virus occurred. Results of a typical experiment are shown in table 6, which indicates that infectious unit and hemagglutinin could be adsorbed upon human erythrocytes under above conditions, subsequent sedimentation of the latter removing both components from the fluid phase without reducing the concentration of CFA. Elution of both adsorbed components occurred upon warming the suspension to 37°C, respectable recovery resulting from this treatment.

TABLE 6. *Adsorption of ICH Virus on Human Erythrocytes*

| FRACTION TESTED | CONC. OF ERYTH. USED FOR ADSORP. | HAT | CFAT | TCD ₅₀ |
|--|-------------------------------------|-----|------|-------------------|
| Starting material | | 128 | 32 | 4.5 |
| Residual fluid following adsorption | { Packed cell 50% 20% | < 4 | 32 | 1.8 |
| | | < 4 | 32 | 1.5 |
| | | < 4 | 32 | 1.1 |
| Elution product | { Packed cell 50% 20% | < 4 | < 4 | 0.5 |
| | | 32 | < 4 | 2.5 |
| | | 32 | < 4 | 2.8 |

The optimum proportion between hemagglutinin and the human erythrocytes was required for removal of the hemagglutinin and infectious unit. The efficiency of adsorption and elution was highest when 20% suspension of human erythrocytes was used.

DISCUSSION

FASTIER¹⁾ first reported that under specific conditions of temperature and pH, the tissue culture fluid containing ICH virus agglutinates fowl erythrocytes.

In a previous paper³⁾ it was reported that these infected tissue culture fluids have been found to possess high CFA titres which composed of S and V. Subsequent studies on the immunological activity of ICH virus were conducted; they demonstrated its hemagglutinating capacity to human and guinea pig erythrocytes. This phenomenon differs in several respects from the corresponding

phenomenon exhibited by the myxovirus group. Considering firstly the relationship of many environmental factors to virus hemagglutination titre it has been shown that the reaction occurred in a narrow pH zone and range of electrolyte concentration.

FASTIER^{1,2)} stated that only fowl erythrocytes were specifically agglutinated by ICH virus; the present writers have demonstrated that erythrocytes from human and guinea pigs were also more sensitive than fowls to the virus. These characteristics of ICH virus hemagglutinin seem to be similar to those exhibited by the arbor group.

In heat stability of ICH virus component, three components, namely, infectivity, hemagglutinin and CFA seem to be different respectively. CFA is the most stable among these components. On the other hand, removal of ICH virus hemagglutinin from infected tissue culture fluids by adsorption experiment resulted in a concomitant decrease in infectivity and HAT without materially affecting the CFA titre. This complement fixing activity might be caused by soluble antigen.

From the above stated results, ICH virus hemagglutinin may be a comparatively heat stable component of the virus particle itself and not separable therefrom. Furthermore, adsorption experiments showed that the chief component of residual fluid may be a soluble antigen which has no relation to hemagglutinin.

Hemagglutination inhibition tests were performed to determine the specificity of hemagglutinin. Non-specific inhibition in dilutions lower than 1:40~1:80 was observed when eight or less agglutinating doses of virus were used. Furthermore, considerable amount of hemagglutinin is recognized in some of the normal dog sera. A further report on the removal of these nonspecific substances will be published elsewhere.

SUMMARY

Experiments on the hemagglutination reaction by ICH virus were performed. The results are summarized as follows:

- 1) Two strains of ICH virus, "MATSUDA" and "Woc-4", agglutinated erythrocytes of human and guinea pigs under certain conditions.

- 2) HAT was affected by many environmental factors such as pH and electrolyte concentration of the reaction mixture, incubation temperature, concentration of erythrocytes and so on. Optimum conditions for the reaction were determined in respect to these factors.

- 3) Heat stability of infecting virus particle, CFA and hemagglutinin was studied and the relationship among these three components was discussed on the basis of the results of adsorption and elution experiments.

4) Normal dog sera were found to contain different amounts of normal inhibitor and hemagglutinin. Some methods to remove these substances are now under investigation.

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