Studies on Cytopathogenic Viruses from Cats with Respiratory Infections

IV. Properties of Hemagglutinin in Feline Herpesvirus Suspensions and Receptors on Feline Erythrocytes

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Abstract. Biochemical properties of feline herpesvirus (FHV) hemagglutinin obtained from infected feline lung cell (FLC) cultures and receptors on feline erythrocytes were investigated. Generally, a close parallelism was shown to exist between the infectivity and hemagglutination (HA) activity of fresh and crude FHV suspensions. When FHV suspension was held at 37°C, its HA activity was slightly enhanced and maintained, while its infectivity was reduced. The HA titer of the virus fluid supplemented with polyoxyethylene sorbitan mono-oleate (Tween 80) ahead of ethyl ether treatment was 2 to 4 times higher than that of the virus fluid without Tween 80. Hemagglutinin was proved to be associated with the viral envelope. Viral hemagglutinin obtained by Tween 80-ether treatment possessed the character of a glycoprotein. A large quantity of hemagglutinin which was not always considered to be derived from the mature virion was also detected in the infected FLC. The agglutinability of the feline erythrocyte was inactivated by the trypsin treatment. Discussion was made on the HA reaction of FHV in comparison with that of equine abortion and measles viruses.

There have been few reports on the hemagglutination (HA) of the herpesvirus group [2-4, 11]. Only one report describes the biochemical properties of equine abortion virus hemagglutinin [3]. The authors already carried out a supplementary examination to confirm the HA of feline herpesvirus (FHV) and found that the HA titer of the virus was enhanced by treatment with ethyl ether [5].

The effect of ether-treatment to enhance the HA activity of the virus has been well known in the study of the measles virus [9, 10].

The present report describes a more

efficient treatment of the virus for the hemagglutination-inhibition test, biochemical properties of FHV hemagglutinin, and the receptor of hemagglutinin on feline erythrocytes.

Materials and Methods

Cell culture and media

The lined feline lung cell (FLC) culture [5] was used for the virus propagation and titration. A cell sheet was formed 4 days after seeding with $10^{5.0}$ cells per milliliter. The growth medium used was Eagle's minimum essential medium (Eagle's MEM) (Wellcome Laboratories) supplemented with 10% tryptose phosphate broth and 10% inactivated calf serum. The maintenance medium used was Eagle's MEM with no supplements.

ネコの呼吸器疾患より分離した細胞変性ウイルスに関する研究 IV. ネコヘルペスウイルス血球凝集素および、そのレセプターの性状: 望月雅美・小西信一郎・尾形 学 (東京大学農学部家畜微生物学教室)

Infectivity titration

Serial tenfold dilutions were prepared from the test material. Two-tenths ml of aliquot was inoculated into three or four tubes per dilution. Seven days after inoculation, the end point was determined by the appearance of the cytopathic effect (CPE). The titer was expressed as the 50% tissue culture infective dose per 0.2 ml ($TCID_{50}/0.2\text{ ml}$) calculated by the method of Reed and Muench [12].

Virus strain and preparation of virus material

The C7301 strain of feline herpesvirus (FHV) [5] was used. On the 4th day after seeding with 100 ml of cell suspension in the 1,500 ml cell culture bottles, they were inoculated with 5 ml of virus inoculum containing about $10^{5.0}TCID_{50}/0.2\text{ ml}$. The bottles were frozen about 48 hours later, when CPE was at a maximum. After thawing, the fluid was centrifuged at 1,600 g for 15 minutes, and the supernatant used as the virus material.

Hemagglutination (HA) test

The HA test was performed by the tube method using phosphate-buffered saline solution (PBSS; 0.01 M, pH 7.5). To 0.5 ml each of serial twofold dilutions of the test material was added the same volume of 0.5% feline erythrocyte suspension. After 3 hours at 37°C, HA titer was recorded as the reciprocal of the highest dilution that caused a complete agglutination.

The calcium borate-buffered saline solution was also used in the neuraminidase treatment of the feline and chicken erythrocytes.

Concentration of virus material

The virus material was passed through the membrane filter (Millipore Corp.) 450 nm in pore size. After filtration the virus fluid was concentrated by the ultrafiltration (Sartorius collodion bag which have a cut off of approximately 25,000 daltons; Brinkman Instrument Inc.). It was usually concentrated to such amount as corresponding to one-hundredth of the original volume.

Fractionation of FHV by Nonidet P-40 (NP-40) treatment

The crude virus concentrate was treated with NP-40 (Iwaki Kagaku Yakuhin Co., Ltd.) to remove envelope material from the virus. This treatment was performed by the method described by Abo-deely et al. [1], Klingeborn and Dinter [3] and Scheid et al. [13].

Zonal distribution of infectivity and HA activity after ultracentrifugation

A 5 ml crude virus concentrate was ultracentri-

fuged in an RPS65T rotor (Hitachi 65P; Hitachi Ltd.) at 201,500 g (R_{max}) for 3 hours. The resulting pellet was resuspended in 1 ml of PBSS, and the supernatant divided into 4 fractions by a density gradient fractionator (Hitachi Ltd.). Both infectivity and HA titer of every fraction were determined.

Thermal stability of infectivity and HA activity of FHV

The growth curve of the FHV C7301 strain in FLC culture was already presented [5]. In it, the infectivity and HA activity of the virus in the later stage of growth were related so characteristically with each other that their thermal stability at 37°C was examined further in detail. The virus fluid, which possessed an infective titer of $5 \times 10^{6.2} TCID_{50}/\text{ml}$ and of an HA titer of 1:2 (2 HAU/ml), was held at 37°C, and its infectivity and HA activity were determined at regular intervals.

Hemagglutinin production in FLC culture inoculated with FHV

Nine of the 200 ml cell culture bottles were inoculated with 2 ml ($10^{5.0}TCID_{50}/0.2\text{ ml}$) of the virus inoculum 4 days after cell seeding with 20 ml of cell suspension. Intra- and extracellular hemagglutinins were determined at regular intervals. The cells were detached from the glass surface by the rubber policeman and centrifuged at 1,600 g for 10 minutes. The resulting supernatant was examined as the extracellular material. The pellet was washed 3 times and resuspended in 1 ml of PBSS. After 3 times of freezing and thawing, it was dispersed by gentle sonication. The resulting suspension was examined as the intracellular material.

Preparation of viral hemagglutinin

The virus material was supplemented with polyoxyethylene sorbitan mono-oleate (Tween 80; Sigma Chemical Company) at a concentration of 0.25 v/v %. After dissolved completely by shaking, it was mixed with peroxide-free ethyl ether (Showa Ether Company) at a concentration of 20 v/v % at 4°C for 2 hours by a magnetic stirrer. The ether was removed thoroughly from the solution by decanting and cautious bubbling of nitrogen gas. Hemagglutinin possessing 32 to 64 HAU/ml was always prepared by this procedure. It was concentrated by ultrafiltration as occasion demanded.

Biochemical reagents and enzymes

The following reagents were used in the experiments: trypsin, trypsin-inhibitor and cholera filtrate (crude neuraminidase) (Sigma Chemical Company); dithiothreitol (DTT), potassium periodate (KIO_4),

2-mercaptoethanol (2-ME) and α -amylase (Wako Pure Chemical Industries, Ltd.); ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific Company); β -glucosidase (P-L Biochemicals, Inc.); sodium deoxycholate (DOC) (Difco Laboratories); equine influenza virus (A/equi-2/Tokyo/71) hemagglutinin (Nippon Institute for Biological Science).

Treatment of viral hemagglutinin and feline erythrocytes with various reagents

Hemagglutinin obtained by Tween 80-ether treatment and erythrocytes collected from conventional cats were used. The procedures and conditions of treatment used were the same as described by Klingeborn and Dinter [3], except some modifications shown in Tables 3 and 4. The reagents used were eliminated from the treated-hemagglutinin suspension by forced dialysis using a collodion bag or by ultracentrifugation in the RPS65T rotor (Hitachi 65P) at 358,200 g (R_{max}) for 6 hours. Controls using PBSS, instead of the reagents, were always set up.

Results

Hemagglutinin production by FHV in FLC culture

As shown in Table 1, there was an inverse relationship between the viral inoculum dose and the HA titer appearing in a crude virus material obtained after infection. The HA titer did not vary after Tween 80-ether treatment when the viral inoculum dose was less than $5 \times 10^{5.0}$ TCID₅₀/ml.

As illustrated in Fig. 1, hemagglutinin

Fig. 1. Hemagglutinin production in FLC cultures inoculated with the FHV C7301 strain*

Remarks.

*: Two ml ($10^{5.0}$ TCID₅₀/0.2 ml) of the virus were inoculated.

**: Hemagglutination (HA) titer was expressed as the highest dilution showing complete agglutination.

***: Infective titer was expressed as the index number of TCID₅₀/0.2 ml.

—●—: HA titer of cell material containing intracellular virus.

—○—: HA titer of extracellular virus.

production was first recognized in the cell 6 hours after inoculation and increased in quantity thereafter. The quantity of intracellular virion reached a maximum 24 hours after inoculation. Extracellular hemagglutinin increased with the advance in virus release, which was first detected 48 hours after inoculation.

Table 1. Relationship between the inoculum and the production of hemagglutinin in FLC culture

Experiment No.	Inoculum (TCID ₅₀ /ml)	Harvesting time (hours after inoculation)	Infectivity (TCID ₅₀ /ml)	Hemagglutination titer (HAU/ml)	
				Crude	After tween-ether treatment
1	$5 \times 10^{6.0}$	30	$5 \times 10^{7.2}$	—*	8
	$5 \times 10^{5.0}$	48	NT**	2	32
2	$5 \times 10^{7.2}$	28	$5 \times 10^{5.2}$	—	4
	$5 \times 10^{5.2}$	46	$5 \times 10^{5.8}$	2	32
	$5 \times 10^{3.2}$	75	$5 \times 10^{5.8}$	4	32

Remarks.

*: Negative, but partial agglutination was observed.

**: Not tested.

Infectivity and HA activity of FHV

As illustrated in Fig. 2, there was generally a parallel relationship between infectivity and HA activity of FHV concentrate. There was, however, a variation of HA titer in the virus fluid which had an infective titer of $5 \times 10^{6.0}$ to $10^{7.0}$ TCID₅₀/ml.

As illustrated in Fig. 3, infectivity decreased abruptly after 24 hours and was lost

Fig. 2. Relationship between infectivity and hemagglutinating (HA) activity of an FHV C7301 strain concentrate

Remarks.

● : Plotted from six determinations.

Fig. 3. Thermal stability of infectivity and hemagglutinability of the FHV C7301 strain at 37°C

Remarks.

—●— : Infective titer expressed as TCID₅₀/ml.
 —○— : Hemagglutination (HA) titer expressed as the highest dilution showing complete agglutination (HAU/ml).

Fig. 4. Distribution of infectious and hemagglutinating activity after ultracentrifugation at 201,500 g for 3 hours

Remarks.

▨ : Infective titer expressed as TCID₅₀/ml.
 □ : Hemagglutination (HA) titer expressed as the highest dilution showing complete agglutination (HAU/ml).

eventually after 48 hours when exposed to 37°C. On the other hand, HA activity rose slightly after 8 hours and remained in this state throughout an observation period of 80 hours.

Fig. 4 presents the zonal distribution of infectivity and HA activity after ultracentrifugation. The scales of infectivity and HA activity employed in it were determined from the given in Fig. 2. As illustrated in Fig. 4, the pellet had an infective titer of $5 \times 10^{9.0}$ TCID₅₀/ml, but contained 32 HAU/ml. Fraction I contained 16 HAU/ml, which was very high, as compared with its infective titer. The relationship between infectivity and HA titer in fractions II, III and IV resembled the data shown in Fig. 2.

The HA activity of deenveloped virus and envelope materials is shown in Table 2. The envelope material had HA activity in both experiments.

Effect of Tween 80 on hemagglutinin preparation

Hemolysis was observed when the virus material was supplemented with Tween 80 to a concentration of more than 0.25 v/v % ahead of ether treatment. When the concentration of Tween 80 was less than 0.25 v/v %, slight hemolysis was found at 37°C more than 4 hours after the onset of reaction. The feline erythrocyte control failed to develop any positive pattern even 3 hours after setting at 37°C. Consequently, Tween 80 was used at a concentration of 0.25 v/v % in the present study.

The supplementation with Tween 80 alone had no effect on HA titer. When it

was followed by treatment with ether, the HA titer became two to four times higher than that determined after treatment with ether alone.

Effects of various agents on the hemagglutinating activity of viral hemagglutinin obtained by Tween 80-ether treatment

As shown in Table 3, viral hemagglutinin was influenced by trypsin (proteolytic enzyme), KIO₄ (oxidizing agent), DTT (disulfide-bond reducing agent), DOC (anionic detergent), α -amylase and β -glucosidase (carbohydrases), formaldehyde and heating (at 56°C for 30 minutes).

The crude concentrate of FHV had no hemolytic activity when incubated at 37°C for 24 hours with an equal volume of 0.5% erythrocyte suspension.

Effects of various agents on the agglutinability of feline erythrocytes by viral hemagglutinin at pH 7.5

As shown in Table 4, trypsin alone destroyed the receptor of hemagglutinin on the erythrocyte.

Table 2. Hemagglutinating activity of purified deenveloped virus and envelope materials

Experiment No.	Hemagglutination titer (HAU/ml)	
	Deenveloped virus material	Envelope material
1	—*	16
2	—	4

Remarks.

*: Hemagglutination was completely negative.
In experiments 1 and 2, original material possessed 32 and 16HAU/ml, respectively.

Table 3. Effects of various agents on hemagglutinating activity of feline herpesvirus hemagglutinin obtained by Tween 80-ether treatment

Agent	Condition of treatment*	Final concentration*	% Reduction of activity**
Trypsin	37°C, 60 min, pH 7.4	0.2 %	≥87.5
KIO ₄	37°C, 60 min, pH 7.4	0.25%	≥84.4
DTT	30°C, 60 min, pH 7.8	0.002M	≥84.4
DOC	37°C, 60 min, pH 7.4	0.5 %	≥81.3
Formaldehyde	37°C, 60 min, pH 7.2	0.2 %	25.0
2-ME	37°C, 60 min, pH 7.4	5×10 ⁻³ M	0
EDTA	37°C, 60 min, pH 7.4	5×10 ⁻² M	0
α -Amylase	37°C, 60 min, pH 7.4	0.4 %	≥92.7
β -Glucosidase	37°C, 60 min, pH 7.4	2.0 %	63.5
Heating	56°C, 30 min, pH 7.5		100

Remarks.

*: Condition of treatment and final concentration of chemical agents were the same as used by Klingeborn and Dinter, 1973, except for formaldehyde treatment.

** : Mean value of 2 experiments.

Table 4. Effects of various agents on agglutinability of feline erythrocytes by feline herpesvirus hemagglutinin at pH 7.5

Agent	Condition of treatment*	Final concentration*	% Reduction of activity**
Trypsin	37°C, 60 min	0.2 %	≥92.3
KIO ₄	37°C, 30 min	5×10 ⁻⁴ M	0
DTT	37°C, 60 min	0.004M	0
DOC	20°C, 60 min	0.025%	0
2-ME	37°C, 60 min	5×10 ⁻³ M	0
EDTA	37°C, 60 min	5×10 ⁻² M	0
α-Amylase	37°C, 60 min	0.5 %	0
β-Glucosidase	37°C, 60 min	2.0 %	0
Neuraminidase***	37°C, 60 min	25 %	0

Remarks.

* : Condition of treatment and final concentration of chemical agents the same as used by Klingeborn and Dinter, 1973, except for DOC and neuraminidase treatments and pH value.

** : Mean value of 2 experiments.

*** : Commercial cholera filtrate (lyophilized *Vibrio* culture fluid) which had 97% reduction of agglutinability of chicken erythrocytes by equine influenzavirus (A/Equi-2/Tokyo/71 strain) hemagglutinin at fourfold dilution.

Discussion

There has been only one report on the properties of herpesvirus hemagglutinin, which was found in equine abortion virus (EAV) by Klingeborn and Dinter [3]. EAV hemagglutinin appears to be an integral part of the viral envelope and a protein as required to show whether it is a glycoprotein. In the present study it seemed that the HA of FHV might resemble that of both EAV [3] and measles virus [8-10]. FHV differed, however, from EAV in the following points: (1) FHV hemagglutinin was considered to be a glycoprotein. (2) The receptor on the erythrocyte was destroyed only by trypsin treatment. (3) Infectivity was proportional to HA activity in FHV, but not so distinctly as in EAV. (4) The HA activity of FHV was enhanced by incubation at 37°C and treatment with ether or Tween 80-ether as that of measles virus. It could be regarded that the HA activity of FHV was closer to that of measles virus than to that of EAV.

In the present study FHV hemagglutinin

could be separated into two types (small and large), though not so clearly as measles virus hemagglutinin mentioned by Norrby [10]. It could be supposed, however, that another type of hemagglutinin which did not seem to be associated with any infective virus (mature virus) might exist. FHV hemagglutinin was considered to be a part of the infective and empty viral envelope. It must have been separated from the envelope by the Tween 80-ether treatment and/or the spontaneous dissociation, with which the HA titer was enhanced consequently.

Hemadsorption (HAd) phenomenon was reported to have been observed in the FHV-infected cell culture by Gillespie et al. [2]. The present authors also observed it (unpublished data). In FHV, which is a DNA-type virus, the nucleocapsids are usually considered to be matured by envelopment at the site of the nuclear membrane. So that the usual interpretation of HAd is not practicable. Nii et al. [7] reported the complex antigenic alterations induced in cells by infection with herpes simplex virus

(HSV), using the ferritin-conjugated antibody technique. In the cells infected with HSV, not only nuclear membrane but also cell membrane altered antigenically and structurally to such extent as to be the envelope of the virus. EAV hemagglutinin was reported to exist on the viral envelope [3]. FHV hemagglutinin was known to be in the same condition in the present study. Therefore, the HA phenomenon of FHV will be interpreted by these facts.

It was reported that HA could not be observed when FHV was propagated in Crandell's stable feline kidney cell line [2]. This is probably because the virus yield in the cell line may have been small. No samples of feline erythrocytes were discarded from the HA test because of indistinct agglutinability.

On the sero-diagnosis of FHV infection, the authors have reported the hemagglutination-inhibition test with viral hemagglutinin obtained by ether treatment [6]. The procedure used in the present study is believed to be more efficient for the preparation of viral hemagglutinin than that used in the last. As a source of hemagglutinin with high titer, solubilized infected cell material is also convenient.

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