ANTIGENIC RELATIONSHIP AMONG STRAINS OF INFECTIOUS CANINE HEPATITIS VIRUS

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INTRODUCTION

The infectious canine hepatitis (ICH) virus is a member of the adenovirus group (KAPSENBERG, 1959). The adenovirus group is divided, according to natural hosts, into 6 subgroups represented by the human, simian, bovine, canine, murine, and avian adenoviruses (PEREIRA et al., 1963). Several of these subgroups could be further divided into a number of types on the basis of type-specific serological tests. The human adenovirus was also classified into 4 groups according to their hemagglutinating characteristics (ROSEN, 1960). However, in ICH virus (canine adenovirus), only one serological type has been recognized (PEREIRA et al., 1963).

Although many strains of ICH virus have been isolated in various parts of the world, there have been no reports regarding detailed information about their antigenic relationships.

As described in a previous report (KINJO & YANAGAWA, 1967), we found that there were some differences between strains of ICH virus following multiplication in HeLa cell cultures. This fact prompted us to compare the strains serologically.

This report deals with the results of cross-neutralization and -hemagglutination inhibition tests among strains of ICH virus.

MATERIALS AND METHODS

Virus strains The strains of ICH virus used and their sources are given in table 1.

The first 11 strains in the table were obtained through the courtesy of Dr. MOTOHASHI, Nippon Institute for Biological Science, Tokyo. The strains Woc-4 and D-43 were provided by the laboratory of Veterinary Bacteriology, University of Tokyo, and the strain Matsuda was originally isolated in this laboratory (OSAMURA et al., 1957).

Antisera For antisera production guinea-pigs were inoculated by 2 intraperitoneal injections with 2 ml of undiluted virus derived from infected DKC using strains N-II, Otaru, P2, Winthrop, and FD. Two animals were used for each strain. The virus titer of the inocula was 10^5 $TCID_{50}$/ml or greater. The immunizing inoculations were given twice at a 5-day interval, and 2 to 3 weeks after the second inoculation the animals were exanguinated

TABLE 1 *Designation and source of ICH virus strains*

DESIGNATION	AREA ISOLATED	SOURCE	COMMENT
N-II	Japan	Dr. MOTOHASHI of N.I.B.S.*1	4 DKC*2 passages
N-III	"	"	"
N-III E	"	"	Repeated passages in DKC-CEC of the strain N-III
Nakano	"	"	4 DKC passages
Otaru	"	"	14 DKC passages
N-IV	"	"	5 DKC passages
FS	U.S.A.	Dr. FIELDSTEEL	about 56 DKC passages
P2	"	Dr. LEADER	about 22 "
Winthrop	"	Winthrop Vaccine virus	3 DKC passages at N.I.B.S.
FD	"	Fort Dodge Vaccine virus	"
C-I	"	—	Strain Cornell-I
Woc-4	"	Dr. POPPENSIEK	—
D-43	Japan	Dr. OCHI, Univ. of Tokyo	—
Matsuda	"	Dr. OSAMURA	about 10 DKC passages

*1 N.I.B.S.: Nippon Institute of Biological Science
*2 DKC: Dog kidney culture

by cardiac puncture. Two sera obtained from animals immunized with same strains were pooled.

Three rabbits were given an intravenous injection of 2 ml of undiluted infected tissue culture fluids of strains FD, Woc-4 and D-43, respectively, and 10 days later they received an additional 2.5 ml by the same route. The sera were collected 3 weeks after the second injection.

All the sera were inactivated by heating at 56°C for 30 min and then stored at −20°C without a preservative.

Tissue cultures and media Secondary cultures of dog kidney cells (DKC) were used in all the work described here. The methods employed in preparing the DKC and media were the same as those used previously (KINJO & YANAGAWA, 1967).

Neutralization tests Neutralization tests were carried out in tube cultures of DKC. Four-fold serial dilutions of heat-inactivated sera were prepared in Hanks balanced salt solution, and each dilution was mixed with equal volumes of virus, diluted to contain approximately 100 $TCID_{50}$ per 0.1 ml. The virus-serum mixtures were incubated for 1 hr at 37°C, and the 0.2 ml of the mixture was used to inoculate each of 3 tubes per dilution. We allowed 2 hr at 37°C for adsorption, then we added 0.5 ml of maintenance medium to the inoculated DKC tubes. The cell cultures were read 7 days later.

Cross-neutralization tests with 2 or more strains and corresponding antisera were carried out simultaneously.

Hemagglutination (HA) and hemagglutination inhibition (HI) tests The HA test was done in a manner similar to the technique described previously (KINJO & YANAGAWA, 1967). The HI test, similar to that described by HIRST (1942), was carried out as follows. Two-fold serial dilutions of inactivated serum were made in physiological saline. To 0.25 ml of each dilution was added 0.25 ml of infected tissue culture fluid, diluted so as to contain 4 HA units of virus in each tube. To this mixture we then added 0.5 ml of a 0.3% suspension of human red blood cells (RBC). Readings were taken after 2 hr incubation at room temperature, and the end point was taken as the highest dilution of serum which completely inhibited RBC agglutination. Cross-HI tests with 2 or more strains and corresponding antisera were carried out simultaneously.

Antigenic relationship The antigenic relationships were expressed as neutralizing- and HI-titer ratios between 2 virus strains with the formula suggested by ARCHETTI & HORSFALL (1950): $r=\sqrt{r_1 \times r_2}$. The ratio, r_1 is the titer of serum 1 versus virus 2 divided by the titer of serum 1 versus virus 1; the ratio, r_2 is the titer of serum 2 versus virus 1 divided by the titer of serum 2 versus virus 2. By definition, homologous titer ratio is always 1. The degree of relationship is estimated as follows: Values of r of 0.5 or less maybe considered significant. The value $r=0.5$ indicates a 50% relationship, $r=0.25$ is a 25% relationship, etc.

RESULTS

1 Cross-neutralization tests among strains of ICH virus

The antigenic relationship between strains of ICH virus were obtained by cross-neutralization tests of 14 strains and 8 antisera. The results were summarized in table 2.

All titer ratios (r_1 & r_2) obtained were more than 0.5. The lowest (0.58) and relatively low titer ratios were found between the Nakano strain and its heterologous antisera. As we did not prepare the antiserum against Nakano strain, the titer ratios of reverse combinations could not be shown. But from the other data presented here, we could expect to obtain similar relationships with them. The "r" values were calculated from 8 strains whose corresponding antisera were prepared.

The smallest value of "r" in table 2 was 0.78, found between strains P2 and D-43. This value indicates no evidence of antigenic difference between them. Therefore, the results obtained by neutralization tests showed that there is a close antigenic relationship between those strains of ICH virus tested.

2 Hemagglutinating properties of ICH virus strains

Our trials to classify ICH virus by HA properties were also conducted using several kinds of erythrocytes, such as those from human, dog, mouse, guinea-pig, rabbit, hamster, and chicken.

The HA tests were performed both at room temperature and 4°C.

All strains tested agglutinated human erythrocytes at room temperature and 4°C, and those from the guinea-pig and chicken at 4°C. Rabbit and dog erythrocytes were aggluti-

TABLE 2 *Cross-neutralization tests among strains of ICH virus*

SERA	VIRUS STRAINS													
	N-II	Otaru	FS	P2	Winthrop	FD	Woc-4	D-43	N-III	N-III E	Nakano	C-I	N-IV	Matsuda
Neutralizing antibody titer														
N-II	**6.5**	6.5	7.2	7.5	6.2	6.5	7.5	7.5	5.5	8.2	5.2	6.5	6.2	6.5
Otaru	5.2	**5.5**	5.5	6.2	5.5	5.5	5.5	5.5	4.5	6.5	3.5	5.2	6.2	4.8
FS	5.5	5.2	**5.5**	5.5	5.5	6.5	5.5	6.2	4.5	5.5	5.2	6.2	5.5	6.5
P2	4.2	4.5	4.5	**5.5**	3.8	4.5	4.5	3.5	4.2	6.2	3.2	3.5	3.5	4.2
Winthrop	3.5	4.5	3.5	4.5	**4.5**	4.5	4.2	4.5	3.2	4.5	2.8	4.5	4.2	4.5
FD	5.5	5.5	6.5	6.2	6.5	**6.5**	6.2	6.8	5.2	5.5	5.2	5.5	6.8	5.2
Woc-4	6.5	6.2	6.2	7.5	6.2	7.2	**7.5**	6.8	5.5	5.5	4.5	6.5	6.5	5.5
D-43	5.5	6.5	6.5	7.2	6.2	6.5	7.5	**7.5**	5.5	5.5	4.5	6.5	5.8	6.2
Titer ratio (r_1 & r_2)														
N-II	**1.00**	1.00	1.11	1.15	0.95	1.00	1.15	1.15	0.84	1.26	0.80	1.00	0.95	1.00
Otaru	0.95	**1.00**	1.00	1.13	1.00	1.00	1.00	1.00	0.80	1.18	0.64	0.95	1.13	0.88
FS	1.00	0.94	**1.00**	1.00	1.00	1.18	1.00	1.12	0.80	1.00	0.95	1.13	1.00	1.18
P2	0.76	0.82	0.82	**1.00**	0.69	0.82	0.82	0.64	0.77	0.95	0.58	0.64	0.64	0.77
Winthrop	0.78	1.00	0.78	1.00	**1.00**	1.00	0.93	1.00	0.71	1.00	0.62	1.00	0.93	1.00
FD	0.84	0.84	1.00	0.95	1.00	**1.00**	0.95	1.05	0.80	0.84	0.80	0.84	1.05	0.80
Woc-4	0.87	0.83	0.83	1.00	0.83	0.96	**1.00**	0.91	0.73	0.73	0.60	0.87	0.87	0.73
D-43	0.74	0.87	0.87	0.96	0.83	0.87	1.00	**1.00**	0.73	0.73	0.60	0.87	0.77	0.83
Antigenic relation ($r=\sqrt{r_1 \times r_2}$)														
Virus strains: N-II	1.00													
Otaru	0.97	1.00												
FS	1.05	0.97	1.00											
P2	0.93	0.96	0.90	1.00										
Winthrop	0.86	1.00	0.88	0.83	1.00									
FD	0.91	0.99	1.08	0.88	1.00	1.00								
Woc-4	1.00	0.91	0.91	0.90	0.88	0.95	1.00							
D-43	0.92	0.93	0.98	0.78	0.93	0.95	0.95	1.00						

Neutralizing antibody titer was expressed as log 4 of reciprocal of the highest serum dilution showing 50 % neutralization of virus infectivity.

For r, r_1 and r_2 see «materials and methods» in the text.

Gothic letter means homologous antibody titer or titer ratio.

nated spontaneously. The erythrocytes from the mouse and hamster were not agglutinated by any strains.

The HA titers were compared next by using 3 kinds of erythrocytes (human, guinea-pig, and chicken) and these are given in figure 1.

Generally, the HA titer was high when human and guinea-pig erythrocytes were used, regardless of the reaction temperature. The erythrocytes from the chicken were agglutinated clearly and rapidly by every strain but their HA titers were low.

There appeared to be no differences in the HA characteristics of the strains of ICH

FIGURE 1 *Comparison of HA reaction of the ICH virus strains using 3 kinds of erythrocytes*

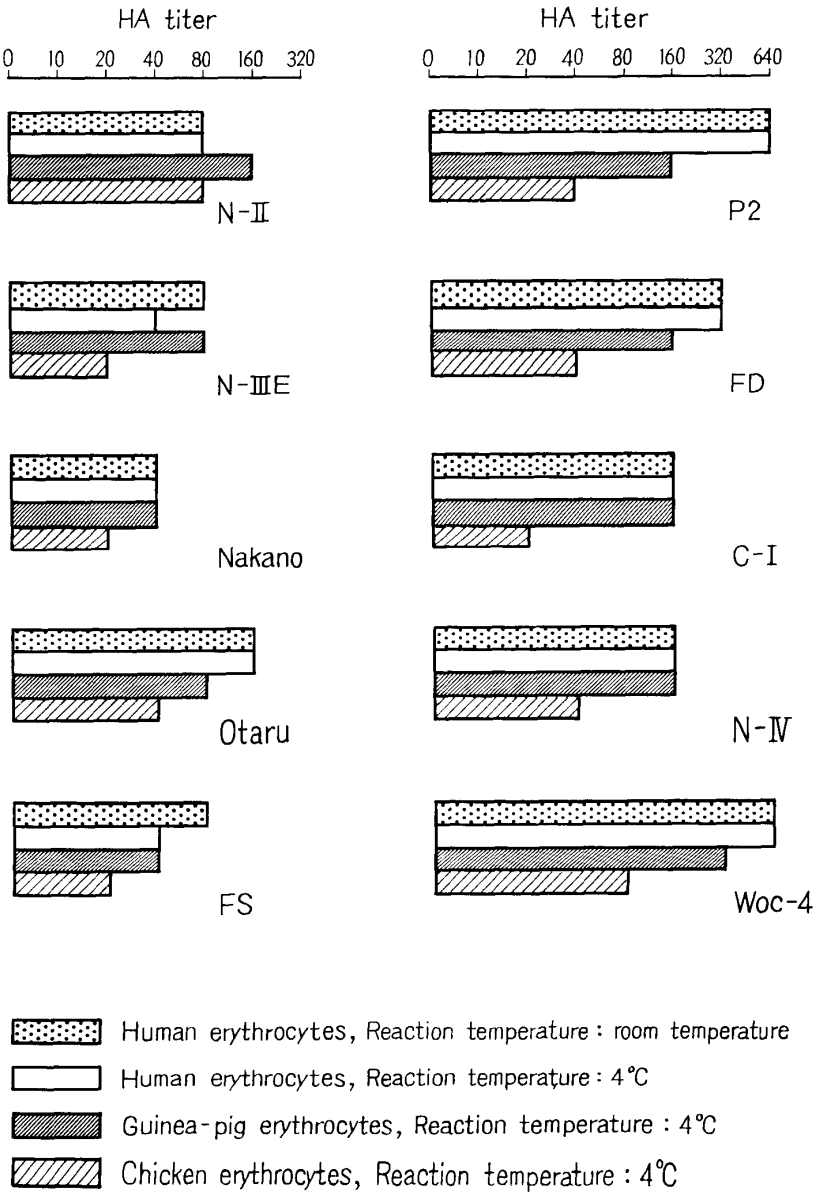

TABLE 3 *Cross-hemagglutination inhibition tests among strains of ICH virus*

SERA	VIRUS STRAINS													
	N-II	Otaru	FS	P2	Winthrop	FD	Woc-4	D-43	N-III	N-III E	Nakano	C-I	N-IV	Matsuda
	HI antibody titer													
N-II	**11**	12	10	11	10	13	12	10	9	10	11	11	11	10
Otaru	9	**9**	9	9	9	11	10	9	9	9	11	9	9	8
FS	11	10	**9**	10	9	11	11	10	10	10	10	10	10	10
P2	9	9	9	**8**	8	10	9	8	8	9	9	9	9	8
Winthrop	7	8	6	8	**9**	9	8	8	7	7	8	8	7	8
FD	9	9	8	9	8	**10**	9	9	9	9	10	9	9	9
	Titer ratio (r_1 & r_2)													
N-II	**1.00**	1.09	0.91	1.00	0.91	1.18	1.09	0.91	0.82	0.91	1.00	1.00	1.00	0.91
Otaru	1.00	**1.00**	1.00	1.00	1.00	1.22	1.11	1.00	1.00	1.00	1.22	1.00	1.00	0.88
FS	1.22	1.11	**1.00**	1.11	1.00	1.22	1.22	1.11	1.11	1.11	1.11	1.11	1.11	1.11
P2	1.12	1.12	1.12	**1.00**	1.00	1.25	1.12	1.00	1.00	1.12	1.12	1.12	1.12	1.00
Winthrop	0.77	0.88	0.75	0.88	**1.00**	1.00	0.88	0.88	0.77	0.77	0.88	0.88	0.77	0.88
FD	0.90	0.90	0.80	0.90	0.80	**1.00**	0.90	0.90	0.90	0.90	1.00	0.90	0.90	0.90
	Antigenic relation ($r=\sqrt{r_1 \times r_2}$)													
Virus strains N-II	1.00													
Otaru	1.04	1.00												
FS	1.05	1.05	1.00											
P2	1.05	1.05	1.11	1.00										
Winthrop	0.84	0.94	0.86	0.93	1.00									
FD	1.03	1.04	0.99	1.06	0.89	1.00								

HI antibody titer was expressed as log 2 of reciprocal of the highest serum dilution causing complete inhibition of hemagglutination.

For r_1 and r_2 see «materials and methods» in the text.

Gothic letter means homologous antibody titer or titer ratio.

virus tested under the above experimental conditions.

3 Cross-hemagglutination inhibition tests among strains of ICH virus

Two rabbit antisera (anti-Woc-4 & -D-43) showed a nonspecific hemagglutination with human RBC, and were omitted from use in the following tests.

Cross-HI tests were performed with 14 strains of virus and 6 guinea-pig antisera types. Table 3 illustrates the HI titers, titer ratios, antigenic relationships between strains of ICH virus.

The HI antibody titers varied from 6 to 13 log 2. However, even the smallest, titer ratios showed 0.75 suggesting a high relationship.

The "*r*" values which we were able to obtain from 6 strains showed 0.84 or greater indicating close antigenic relationships between them.

The results obtained by the cross-HI titers indicated that there were no significant differences in antigenic characteristics of the strains tested.

DISCUSSION

Our primary interest is whether or not the ICH virus has oncogenic properties.

The human adenovirus has been classified by neutralization tests into more than 30 serotypes. Oncogenicities of the human adenovirus have been demonstrated in a few serotypes, which are readily distinguishable by the HA test (ROSEN, 1960). From these findings it seemed necessary for us to clarify the antigenic relationships between strains of ICH virus before such experiments could be started.

From this view point we first attempted to compare the hemagglutinating properties of 14 ICH virus strains, 6 isolates were from U.S.A. and 8 from Japan.

Hemagglutination ability was demonstrated in all strains tested with human, guinea-pig, and chicken erythrocytes, and no agglutination occurred with the erythrocytes from other 4 kinds of animals.

The ability of each strain to agglutinate human, guinea-pig and chicken erythrocytes indicated no differences in the HA characteristics of the strains tested (fig. 1).

Cross-HI and -serum neutralization tests which have been very widely used for antigenic analysis were next conducted, and antigenic relationships among ICH virus strains were expressed by the formula suggested by ARCHETTI & HORSFALL (1950).

The lowest "*r*" value of 0.84 and 0.78 in the cross-HI and -neutralization tests respectively suggested fairly close antigenic relationships among the strains tested (tabs. 2 & 3).

Although the object of this study was to find the serological differences between the ICH virus strains, the results presented here indicated the opposite.

That the strains studied here were found to have a marked antigenic similarity by the conventional serological test procedures.

MOTOHASHI (1965) observed differences of plaque formation in DKC and of multiplication in chick embryo cell cultures. The authors found that there were some differences in ICH virus strains upon multiplication in HeLa cell cultures (KINJO & YANAGAWA, 1967). Both these observations suggest that there are differences in biological properties of strains of ICH virus.

Kinetic studies of the serum neutralization of polioviruses, by McBRIDE (1959) suggest that late antisera is somewhat less specific than early antisera, for the detection of antigenic differences. RAFAJKO & YOUNG (1965) classified strains of adenovirus type 12 into 2 serological intratypic groups by cross-neutralization tests using early antiserum.

From the above findings, it appears that the ICH virus strains could likewise be differentiated by the use of early antisera. But no information is at hand to allow us to say like that. Further experiments along this line will be necessary.

SUMMARY

An attempt was made to serological classify the infectious canine hepatitis (ICH) virus using 14 strains isolated from U.S.A. and Japan.

Human, guinea-pig and chicken erythrocytes were agglutinated by all strains tested and no differences in hemagglutination characteristics were demonstrated.

No or spontaneous agglutinations were observed with red blood cells from dog, mouse, rabbit and hamster.

Conventional cross-hemagglutination inhibition and -serum neutralization tests were conducted and the "*r*" values of the antigenic relationships were calculated by the formula of ARCHETTI & HORSFALL (1950). The lowest "*r*" values found were 0.84 and 0.78 respectively suggesting a fairly close antigenic relationship between the strains tested.

It appeared from the 14 strains studied that the ICH virus, consists of only one serotype.

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