THE JOURNAL OF INFECTIOUS DISEASES • VOL. 120, NO. 5 • NOVEMBER 1969
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Some Characteristics of Hemagglutination of Certain Strains of "IBV-like" Virus

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In recent years a "new" group of viruses has been recognized as a cause of respiratory illness in man [1]. Prototype strains have been designated as B814, 229E, and "IBV-like" virus [2–4]. These viruses have been shown by negative-contrast electron microscopy to resemble morphologically avian infectious bronchitis virus (IBV) and mouse hepatitis virus (MHV) [5, 6]. This characteristic structural resemblance and other shared properties of these viruses have caused certain virologists to propose the name *coronavirus* for this previously unrecognized group [7].

Although hemagglutinins have been detected by investigators using avian IBV treated by different methods [8, 9], the virus does not agglutinate erythrocytes under "normal" conditions [10]. So far hemagglutination has not been recorded for either the human or murine strains [7].

The property of hemagglutination of 2 strains of "IBV-like" virus that had been adapted to growth in suckling mouse brain forms the basis of this report.

Material and Methods

Viruses. "IBV-like" viral strains OC 38 (HET_4SM_7) and OC 43 (HET_5SM_7) and control mouse brain harvests (SM_7) were received from Dr. Kenneth McIntosh, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, Maryland. Both viruses had been isolated in organ cultures of human embryonic trachea from patients with respiratory illness [4] and adapted to growth in suckling mouse brain [11].

Received for publication June 26, 1969.

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Viral and control harvests were further passaged in our laboratory by intracerebral inoculation of 0.02 ml of undiluted brain suspension into 3-day-old Swiss white mice (ICR) from colonies of mice free of MHV. Infected mice showing typical encephalitic symptoms [11], usually within 48–60 hr, were pooled by strain and frozen at −70 C. Dead mice were discarded. Frozen mice were thawed at room temperature, and brains were harvested by transoccipital needle puncture and syringe aspiration. Ten percent suspensions of brain were made in tryptose phosphate broth (TPB) containing 0.5% gelatin. Suspensions of brain were stored at −70 C. Control mice were treated in the same manner.

Production of antigen. Antigens were prepared by making a 10% suspension of infected and normal mouse brain in phosphate buffered saline (PBS, *p*H 7.2) and veronal buffered diluent (VBD, *p*H 7.3), then clarified by refrigerated centrifugation at 600 *g* for 20 min. The antigens were stored at −20 C and −70 C.

Antisera. Mouse antisera to strain OC 43, control mouse sera, and paired sera from 2 patients from whom "IBV-like" viruses were isolated were received from Dr. McIntosh and Dr. Albert Kapikian, Laboratory of Viral Diseases, NIAID. Immune sera were prepared in this laboratory by intraperitoneal and intracerebral inoculation of weanling mice according to the method of McIntosh et al. [11]. Mice were bled 28 and 35 days after inoculation. Paired sera from children participating in a longitudinal survey of respiratory disease [12] were also selected for study.

Serologic tests. Complement fixation (CF) tests were performed with the use of the Laboratory Branch Complement Fixation (LBCF) test as adapted to the microtiter technique [13]. Neutralization (N) testing was carried out in 3-day-old suckling mice inoculated intracerebrally with

approximately 100 LD_{50} of virus per 0.02 ml and equal volumes of antisera diluted in PBS. Hemagglutination-inhibition (HI) tests were performed with the microtiter technique [14] and PBS diluent. All sera were inactivated at 56 C for 30 min.

Results

Hemagglutination (HA). Harvests from the ninth passage of both "IBV-like" strains in suckling mouse brain were tested for HA with a variety of species of erythrocytes at 4 C, room temperature, and 37 C. Chicken, mouse, and rat cells were agglutinated by both OC 38 and OC 43 at all 3 temperatures (table 1). Rhesus and guinea pig cells failed to agglutinate under test conditions. Human "O" and vervet cells were agglutinated only at 4 C. Rat and mouse erythrocytes appeared to be more sensitive to agglutination than those of the other species, particularly at room temperature and 37 C.

No spontaneous elution from any of the erythrocytes was observed for periods up to 24 hr at optimal HA temperature. However, elution occurred with human "O" and vervet erythrocytes after the agglutinated cells were transferred from 4 C to room temperature. Resuspension and reincubation of these cells at 4 C yielded the original HA titer.

Nature of the hemagglutinin. A standard titration of receptor-destroying enzyme (RDE) was performed to investigate the possible role of neuraminic acid in the agglutination of erythrocytes by OC 38 and OC 43 [15]. Chicken cells treated with a 1:128 dilution of RDE failed to agglutinate in the presence of a standard myxovirus control (influenza A/PR/8/34). Agglutination of the same treated chicken cells by the "IBV-like" viruses was unaffected even at the lowest dilution of RDE used (1:2).

Treatment with trypsin [10], ether [16], and Tween 80 plus ether [17] completely destroyed the HA activity of the brain antigen (table 2). Heating at 56 C for 30 min considerably reduced the activity, but multiple freezing and thawing cycles had no noticeable effect. Sonic oscillation increased HA titers only slightly. Any treatment that caused a decrease in the HA titer also produced a corresponding decrease in infectivity.

The HA antigen titers of both viruses were stable after 8 weeks of storage at —20 C or —70 C, whereas they completely disappeared after storage at 4 C for the same period of time. HA titers of antigens held at room temperature were lost after 4 weeks.

Relation of HA and CF activity to infectivity. Litters of 3-day-old mice (8 per litter) were inoculated intracerebrally with mouse brain suspensions of OC 38 containing approximately 100 suckling mice LD_{50} of virus per 0.02 ml. Two litters of mice were sacrificed by freezing at —70 C at 12-hr intervals up to and including the typical encephalitic syndrome. Dead mice were excluded from the experiment. Brains of the mice harvested at each period of time were divided into 3 pools, and 10% suspensions were made in TPB for determination of infectivity, in PBS for determination of HA activity, and in VBD for titration of CF antigen. Assays of infectivity on each sample were performed by intracerebral inoculation of 3-day-old mice. Estimates of LD_{50} were

Table 1. Agglutination titers of various erythrocyte species by strains of "IBV-like" virus at different temperatures

Erythrocyte species*	OC 38, 4 C	OC 38, Room temperature	OC 38, 37 C	OC 43, 4 C	OC 43, Room temperature	OC 43, 37 C
Rhesus	<10	<10	<10	<10	<10	<10
Guinea pig	<10	<10	<10	<10	<10	<10
Human "O"	80	<10	<10	320	<10	<10
Vervet	80	<10	<10	320	<10	<10
Chicken	80	80	80	320	320	320
Rat	160	320	320	640	1280	1280
Mouse	160	320	320	640	1280	1280

* All erythrocytes made up as 0.4% suspensions in PBS with exception of chicken, 0.5%.

Table 2. HA titers and infectivity of strains of "IBV-like" virus under various physical and chemical treatments

Type of treatment	HA titer*		Infectivity for suckling mice
	OC 38	OC 43	
None	160	320	yes
Ether	<10	<10	no
Tween 80	160	320	yes
Tween 80 and ether	<10	<10	no
Trypsin	<10	<10	no
Sonic oscillation† (5–10–15 min)	320	640	yes
Heat (56 C for 30 min)	10	20	no
Freezing and thawing (3 cycle—70 C)	160	320	yes

* 0.5% chicken erythrocytes in PBS.
† Raytheon Sonic Oscillator, 250 watt, 10 kc.

calculated by the method of Karber [18]. CF titrations were performed by stepwise dilutions of antigen against a constant optimal dilution of antiserum ("straight-line determination").

Infectivity increased rapidly from an LD_{50} of $10^{1.6}$ at 24 hr to an LD_{50} of $10^{7.3}$ at 60 hr (figure 1). A plateau of 10^7–10^8 $\log_{10}$ LD_{50} was maintained between 60–96 hr. HA and CF antigen titers remained at < 8 until 48 hr after inoculation. A rapid increase of the titers of both antigens was seen at 48–60 hr, corresponding to an increase of infectivity of $10^{5.6}$–$10^{7.3}$ LD_{50}. CF antigen titers remained between 1:32 and 1:64 from 60 to 96 hr. HA antigen titers increased 4-fold from 1:64 to 1:256 between 72 and 84

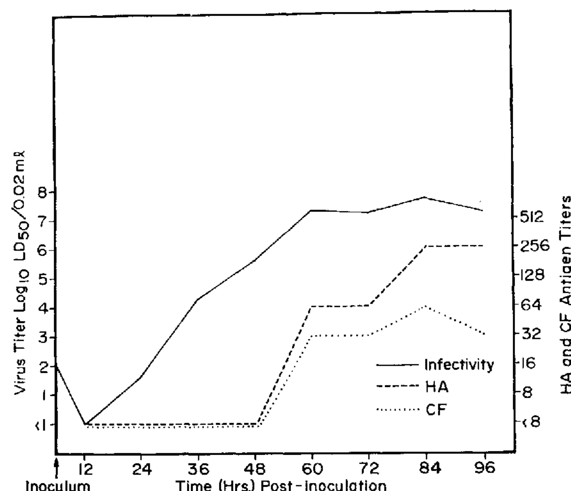

Figure 1. Growth curve of "IBV-like" virus (OC 38).

hr and remained at that level until termination of the experiment.

Specificity. Antigens and antisera prepared from both strains of virus were examined by CF, HI, and N tests against a variety of viruses (table 3). No antigenic relationships were established with any of the viruses studied except a one-way cross by HI and CF between "IBV-like" viral antigens and mouse hepatitis polyvalent antisera (table 4) as previously described [11]. Nonspecific inhibition of chicken cell hemagglutinins was

Table 3. Viruses for which reciprocal tests with "IBV-like" viral strains and antisera were negative

Reagent	Test performed
Influenza:	
A/Swine/1976/31	HI, N
A/PR/8/34	HI, N
A1/FM/1/47	HI, N
A2/Poly. antisera	HI
A/Soluble	CF
A/Equi 1/Prague/56	N
A/Equi 2/Miami/1/63	N
B/Lee/40	HI, N
B/Poly. antisera	HI
B/Soluble	CF
C/Taylor/1233/47	HI, CF, N
Parainfluenza:	
1 (HA2)	HI, CF, N
1 (Sendai)	HI, N
2 (Greer)	HI, CF, N
2 (SV-5)	HI, CF, N
2 (SV-41)	HI, CF
3 (HA1)	HI, CF, N
3 (SF4)	HI, N
4A	HI, N
4B	HI, N
mumps virus	HI, CF, N
Newcastle disease virus	HI, CF, N
measles virus	CF, N
resp. syncytial virus	CF, N
adenovirus	CF, N
psittacosis	CF
herpesvirus	CF
Mycoplasma pneumoniae	CF, N
rubella virus	CF
mouse adenovirus	CF
lymphocytic choriomeningitis virus	CF
vaccinia	CF
reovirus type 3	HI
pneumo. virus of mice	HI
GD-7	HI
polyomavirus	HI
"K" virus	HI

NOTE.—Neutralization testing was performed against antisera only with strains of "IBV-like" virus.

Table 4. Homologous and heterologous antibody responses in sera from mice immunized with "IBV-like" and MHV strains

	Antigens						
	OC 38			OC 43			MHV*
Antiserum	HI	N	CF	HI	N	CF	CF
OC 38 Lot 2	160	160	64	160	80	64	ND†
OC 38 Lot 3	80	80	32	80	160	32	<10
OC 43 Lot 2	160	160	64	320	320	128	ND
OC 43 Lot 3	2560	>1280	128	5120	>1280	128	<10
OC 43 (NIH)	640	ND	128	640	ND	128	ND
MHV‡	20	ND	80	10	ND	80	320
Normal mouse (RVU)	<10	<10	<8	<10	<10	<8	<10
Normal mouse (NIH)	<10	<10	<8	<10	<10	<8	ND

* MHV polyvalent antigen, V-1, Viral Immunoserology Unit (NCDC).
† Not done.
‡ MHV polyvalent antisera, no. 30008, Microbiological Associates.

not encountered with any of the animal and human sera employed, which suggests that no special methods for serum treatment are required.

Purity. Strains OC 38 and OC 43 having infectivity titers greater than 10^7 LD_{50} in suckling mice failed to produce a cytopathic effect, chicken cell hemagglutinin, or positive guinea pig hemadsorption after 2 passages in primary rhesus monkey kidney or WI 38 tissue cultures. Embryonated hens' eggs 7–10 days old, were inoculated amniotically and allantoically. No HA was detected in the 3–5-day harvest fluids. Blood agar, mycoplasma media, Sabouraud's, and sodium thioglycollate broth were inoculated with suspensions of mouse brain infected with "IBV-like" virus. No bacterial, mycoplasmal, or fungal contaminants were observed.

Sensitivity. Various lots of mouse sera immune to OC 38, OC 43, and MHV were tested by HI and neutralization and/or CF against homologous and heterologous antigens, and the results were compared (table 4). OC 38 and OC 43 were indistinguishable by all 3 tests. Titers of HI antibody closely paralleled those found by neutralization; titers of CF antibody were lower. MHV polyvalent antiserum reacted with "IBV-like" antigens both by CF and HI; titers were significantly less by HI than by CF. Reciprocal CF tests showed only a one-way cross between MHV and the "IBV-like" viruses. In the absence of a hemagglutinin for MHV, the existence of the one-way cross could not be confirmed by HI.

The superior sensitivity of the HI test over the CF was apparent with human sera as well (table 5). Paired sera from children with respiratory disease previously unassociated with an etiologic agent were screened by HI and CF. Of the 16 pairs demonstrating a 4-fold or greater antibody rise by either test, all were positive by HI and only 7 were positive by CF. The specificity of the HI reactions was also confirmed by diagnostic rises in paired sera, furnished by Dr. Kapikian,

Table 5. HI and CF antibody titers to "IBV-like" virus in 18 paired human sera

	HI		CF	
Serum no.	Acute	Convalescent	Acute	Convalescent
807	20	320	<8	64
822	<10	40	<8	<8
825	10	40	<8	<8
837	<10	20	<8	<8
844	10	40	<8	<8
853	20	80	<8	16
860	10	40	<8	<8
877	<10	20	<8	16
882	<10	20	<8	<8
919	<10	20	<8	<8
956	<10	80	<8	16
960	<10	40	<8	16
975	<10	40	<8	<8
967	10	40	<8	16
981	<10	20	<8	16
977	<10	20	<8	<8
689*	<10	160	<8	32
712*	20	160	8	64
No. with 4-fold or greater rises	18		9	
Geometric mean titers	<10	44	<8	10

* Sera from patients who yielded isolants of "IBV-like" virus, Kapikian (NIH).

from patients from whom "IBV-like" viruses had been isolated.

Discussion

Strains of "IBV-like" viruses that have been adapted to suckling mouse brain were shown to agglutinate some erythrocyte species; this represents the first time a member of the coronavirus group has demonstrated this ability without certain manipulations.

Hemagglutination by the "IBV-like" viruses was not observed in earlier studies with this group [3], and the present finding probably results from further adaptation of the virus to growth in suckling mouse brain and production of higher titers of antigen. Studies of the growth curve of OC 38 revealed that the activity of HA was not detected until infectivity rose from $10^{5.6}$–$10^{7.3}$ LD_{50}.

The mechanism of hemagglutination by the "IBV-like" viruses remains to be determined, but preliminary evidence suggests that it differs from the myxoviruses in several respects. Unlike certain myxoviruses, spontaneous elution of the hemagglutinin at optimal temperature of incubation was not observed. Although elution from human "O" and vervet cells occurred with increased temperature, the original HA titer could be restored by reincubation at 4 C. HA apparently involves receptor sites on the erythrocyte that are different from those required by the myxoviruses. Treatment of erythrocytes with neuraminidase failed to inhibit agglutination by the "IBV-like" viruses. Although treatment with trypsin, ether, and Tween-80 and ether often enhances the activity of HA of certain myxoviruses, the same treatment destroyed all hemagglutinins of "IBV-like" viruses. In general, any treatment that caused a loss in infectivity resulted in a corresponding loss in HA titer.

The HI test was specific; no cross-reactions were detected with the other 23 hemagglutinating viruses tested. Time-consuming procedures for treatment of serum were unnecessary because nonspecific inhibitors were not encountered when heat-treated sera from a variety of sources were used. The lack of serologic distinction by HI between strains of "IBV-like" virus and the "one-way cross" between these antigens and MHV polyvalent antisera corroborate earlier findings by CF and neutralization tests [11]. In general, the titers of HI antibody paralleled results of neutralization test.

The HI test was also sensitive; titers were generally higher by HI than by CF. The HI test detected twice as many rises of antibody in paired sera from patients with respiratory disease than did the CF. The sensitivity of the HI test might be further improved by using erythrocytes from rats or mice, but certain disadvantages are inherent. Rat and mouse cells are more difficult to obtain and often yield erratic HA patterns. Also, the use of chicken erythrocytes eliminates the procedure of removing nonspecific agglutinins by serum adsorption.

The advantages of the HI test for identification of viruses, antigenic characterization, sero-diagnosis, sero-surveys, and other laboratory procedures are well recognized, and the test should prove to be a useful tool for the study of "IBV-like" viruses as well.

Summary

The ability of strains of "IBV-like" viruses, adapted to suckling mouse brain, to agglutinate certain species of erythrocytes was demonstrated. The mechanism of hemagglutination by the "IBV-like" viruses is unlike that of the myxoviruses in several respects, notably in that neuraminic acid receptors are not involved. The hemagglutination-inhibition (HI) tests with chicken erythrocytes were specific, and hemagglutination was not inhibited by antisera to a wide variety of hemagglutinating viruses. The identity of strains OC 38 and OC 43 and the one-way relationship of those strains with mouse hepatitis virus previously observed by neutralization and complement fixation (CF) tests were confirmed. The HI test was also sensitive; titers were generally higher, and twice as many positive serologic conversions in patients with respiratory disease were detected by HI as by CF. The HI test should prove to be a useful tool for the study of "IBV-like" viruses.

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