Hemadsorption by Coronavirus Strain OC43 (36105)

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Coronaviruses appear to be important etiologic agents of adult upper respiratory tract illnesses (1–3). However, study of these viruses has been hampered by the lack of convenient and suitable methods for their propagation. Nine of the 23 coronavirus strains recovered to date were originally isolated in human embryonic tracheal (HET) organ cultures only, whereas the remaining 14, all of which were similar to a prototype strain designated 229E, were isolated with difficulty in monolayer tissue cultures (2, 4-9).

B814 virus, the first organ culture (OC) strain, two other OC strains, LP and EVS, and strain 229E were subsequently recovered in L132 cell monolayers from original nasal washings obtained from volunteers inoculated with these agents (8, 10). More recently, OC43 virus (and OC38 virus, an identical agent) both of which were originally recovered in OC and later adapted to grow in suckling mice, were not only shown to directly hemagglutinate various erythrocytes, but were also adapted to grow in monolayer monkey kidney cell cultures (11–13). Stimulated by the hemagglutination and tissue culture adaptation findings, we carried out hemadsorption studies with OC43 virus-infected monolayer cultures. The results of these studies are described below.

Materials and Methods. Tissue cultures. Tube cultures of human diploid cell strain (HDCS) WI38, primary human embryonic kidney (HEK), semicontinuous human embryonic intestine (MA177), primary rhesus monkey kidney (MK), and a continuous *Cercopithecus aethiops* monkey kidney cell line designated BS-C-1 were purchased from commercial sources (2, 14). Cultures were washed twice with Hanks' balanced salt solution (BSS) before the addition of maintenance medium consisting of equal parts of Eagle's minimum essential medium in Earle's BSS and medium 199, 100 units of penicillin/ml and 100 mg of streptomycin/ml. Serum was not used in the maintenance medium.

Infectivity titrations. Tenfold dilutions of virus were made in Hanks' BSS containing 0.5% gelatin, 100 units of penicillin/ml and 100 μg streptomycin/ml. Two-tenths milliliter of the appropriate virus dilutions was inoculated into each tube culture; the cultures were examined for hemadsorption as described below at varying intervals under the low power of a light microscope. If cultures were to be examined again for hemadsorption at a later date, the maintenance medium containing red blood cells was decanted and the monolayer was washed twice with Hanks' BSS before the addition of new maintenance medium.

Serology. All sera were inactivated at 56° for 30 min. In the neutralization test, equal volumes of virus and the appropriate serum dilution were incubated at room temperature for 2 hr. Two-tenths milliliter of the mixture was inoculated into each of two tube cultures. Five days after stationary incubation at 34–35°, 0.2 ml of a 0.4% suspension of rat red blood cells was added directly into the maintenance medium of tube cultures at each tenfold dilution of the simultaneous titration to determine if 32–320 TCD_{50} of virus were present; red blood cells were similarly added to one of the control tubes. If sufficient virus was present, and in addition, the control tubes did not exhibit hemadsorption, rat red blood cells were added to all cultures and the tubes were incubated at room temperature for approximately 45 min in a stationary position with the cell monolayer dependent.

Immediately before examining the cell sheet for hemadsorption, the fluid in each tube was gently tilted so that the maintenance medium and red blood cells were set in motion over the cell sheet, thereby dislodging erythrocytes adhering nonspecifically.

The complement-fixation (CF) test was performed by a modification of the Bengston technique using microtiter plates, 1.7 units of complement and overnight incubation at 4° (15, 16).

The hemagglutination-inhibition (HI) test was performed in microtiter plates with incubation of 0.025 ml virus–0.025 ml serum mixtures at room temperature for 1 hr followed by addition of 0.025 ml of 1.5% adult chicken red blood cells and incubation at 4°; the test was read in approximately 2–3 hr after addition of erythrocytes (12, 16).

Viruses and sera. The BS-C-1 cell culture grown OC43 and OC 38 virus suspensions used in this study had been previously passaged in HET organ cultures, suckling mice and primary monkey kidney cell cultures in the order indicated, and were available in this laboratory from previous studies by Bručková *et al.* or were derived from such harvests (13). Unless otherwise noted, such OC43 and OC38 virus suspensions were used in this study. HET OC grown B814 virus was derived from an OC strain of B814 virus kindly supplied by Dr. D. A. J. Tyrrell (4); HET OC grown OC38, OC43, OC16, OC37, OC44, and OC48 viruses were available in this laboratory from previous studies by McIntosh *et al.*, or were derived from such harvests (6). 229E virus grown in HDCS WI38 cultures was kindly supplied by Dr. Dorothy Hamre and tissue culture pools used in this study were derived from this strain (5). Mouse hepatitis virus (MHV) strain A-59 adapted to grow in HDCS W138 cultures was kindly supplied by Dr. Janet W. Hartley of the National Institutes of Health, Bethesda, MD. Hyperimmune sera to OC43 and OC38 viruses prepared in suckling mice were available in this laboratory from previous studies by McIntosh *et al.* (11). Hyperimmune serum to 229E virus was prepared in guinea pigs inoculated intramuscularly on three separate occasions with virus emulsified with incomplete Freund's adjuvant. Polyvalent MHV serum (strains A-59, MHV-1, MHV-S, and JHM) and its appropriate control were kindly supplied by Dr. John C. Parker of Microbiological Associates, Inc. Hyperimmune serum to MHV strain A-59 prepared in weanling mice from strains derived from a mouse adapted A-59 strain, kindly supplied by Dr. Janet W. Hartley, was available in this laboratory from previous studies by Dr. Marie Bručková. Initially the weanling mice were inoculated intraperitoneally with inactivated (56° for 30 min) virus emulsified with Freund's adjuvant; subsequent booster inoculations of either live or inactivated A-59 strains were administered by this same route without adjuvant. Human sera were obtained from National Institutes of Health (NIH) employees engaged in a volunteer study of acute respiratory illnesses; acute sera were obtained during the early phase of illness and convalescent sera approximately 3 to 4 weeks later (2).

Erythrocytes. With the exception of human "O" and guinea pig erythrocytes which were available at the NIH, all erythrocytes were purchased from commercial sources. The red blood cells were routinely suspended in Alsever's solution after collection and subsequently washed three times in dextrose glucose veronal buffer solution and on the day of use suspended in 0.85% saline to a 0.4% concentration.

Results. Erythrocyte spectrum of hemadsorption. Erythrocytes from 5 animal species and man were studied for hemadsorption in OC43 virus-infected monolayer BS-C-1 tube cultures 3, 6, and 12 days following infection. At 3 days, hemadsorption was readily observed with rat and mouse cells; chicken cells hemadsorbed minimally, and human "O," vervet monkey, and guinea pig cells not at all. By 6 days after infection, the amount of hemadsorption increased further with both rat and mouse red blood cells, whereas adult chicken cells hemadsorbed minimally, vervet monkey and human O cells minimally and inconsistently, and guinea pig cells not at all (Table I). The incubation temperature following the addition of rat or mouse erythrocytes did not markedly affect the degree of

TABLE I. Degree of Hemadsorption of Various Erythrocytes at Different Temperatures in OC43 Virus-Infected BS-C-1 Cell Cultures 6 Days After Inoculation.[a]

Type of erythrocyte	Log_{10} dilutions of OC43 virus inoculated into BS-C-1 cultures											
	10^{-1}			10^{-2}			10^{-3}			10^{-4}		
	4°[b]	21°[b]	37°[b]	4°[b]	21°[b]	37°[b]	4°[b]	21°[b]	37°[b]	4°[b]	21°[b]	37°[b]
Rat	3+	2–3+	3–4+	3+	1–2+	2–3+	1+	0–1+	2+[c]	0–1+[c]	0	0–1+[c]
Mouse	3+	1–2+	3+	2+	1–2+[d]	2+	1–2+[c]	0–1+[d]	1+	0–1+[c]	0–1+[c]	±
Chick	0	0–1+	1+	0	0–1+	0–1+	0	0	0–1+	0	0	0–1+[c]
Human "O"	±	0–1+[c]	0	0	0–1+[c]	—	0	0–1+[c]	—	0	0–1+[c]	—
Vervet monkey	0	0	—	0	1+	—	0	1+[c]	—	0	1+	—
Guinea pig	0	0	0	0	0	0	0	0	0	0	0	0

[a] 0 = none, 1+ = 25%, 2+ = 50%, 3+ = 75%, 4+ = 100% of cell sheet demonstrated hemadsorption (2 tubes inoculated/dilution); — = unable to interpret degree of hemadsorption since one of two control cultures was hemadsorption positive; ± = minor degree of hemadsorption which may have been nonspecific.

[b] Incubation temp. (centigrade).

[c] Only one of two tubes hemadsorption positive.

[d] Only one tube available at this dilution.

TABLE II. Degree of Hemadsorption of Various Erythrocytes at Different Temperatures in OC43 Virus-Infected BS-C-1 Cells 12 Days After Inoculation.[a]

Type of erythrocyte	Log_{10} dilutions of OC43 virus inoculated into BS-C-1 cultures											
	10^{-1}			10^{-2}			10^{-3}			10^{-4}		
	4°[b]	21°[b]	37°[b]	4°[b]	21°[b]	37°[b]	4°[b]	21°[b]	37°[b]	4°[b]	21°[b]	37°[b]
Rat	3–4+	HA[c]	HA	3+	HA	HA	3+	HA	HA[d]	3–4+[d]	0	HA[d]
Mouse	HA	HA	HA	HA	HA[e]	HA[e]	NT	HA[e]	HA	HA[e]	HA[f]	NT
Chick	0	0	0	0	0	0	0	0	0	0	0	0
Human "O"	0	1+	0	0	0	0	0	1+[d]	0	0	0	0
Vervet monkey	0	0	0	0	0	0	0	0	0	0	0	0
Guinea pig	0	0	0	0	0	0	0	0	0	0	0	0

[a] 0 = none, 1+ = 25%, 2+ = 50%, 3+ = 75%, 4+ = 100% of cell sheet demonstrated hemadsorption (2 tubes inoculated/dilution); HA = gross hemagglutination observed (in some tubes hemadsorption occurred concurrently also); NT = not tested.

[b] Incubation temp. (centigrade).

[c] Only one tube positive for HA, other tube hemadsorption positive (4+) only after maintenance medium was discarded and cell sheet was washed twice prior to re-adding of erythrocytes.

[d] Only one of 2 tubes positive.

[e] Only one tube available at this dilution.

[f] One tube positive for HA, other tube demonstrated slight degree of hemadsorption.

hemadsorption. By 12 days after infection, most tubes which were hemadsorption positive at 6 days exhibited gross hemagglutination following the addition of rat or mouse erythrocytes to the maintenance medium (Table II). The removal of maintenance medium and washing of the cell sheet twice before addition of erythrocytes resulted in marked hemadsorption patterns in tubes which had shown hemagglutination previously. Although mouse cells hemadsorbed as well as rat cells, the latter were used in subsequent experiments.

In further experiments, hemadsorption was also demonstrated in OC43 virus-infected HEK, HDCS WI38, and rhesus MK cell cultures. OC38 virus-infected BS-C-1 cells hemadsorbed rat erythrocytes also. Attempts to isolate OC43 and OC38 viruses from the original nasal washings yielding these agents, from OC43 and OC38 organ culture harvests, and from a limited number of original specimens using the technique of hemadsorption were unsuccessful in HDCS WI38, rhesus MK, and BS-C-1 monolayer cultures as were attempts to hemadsorb 229E, B814, OC16, OC37, OC44, and OC48 viruses in these same culture systems.

Pattern of hemadsorption. Most hemadsorption studies were performed in BS-C-1 cell cultures. In early stages of infection in such cultures, clusters of red blood cells arranged in ovals, rosettes, and triangular shapes adhered to 10–25% of the cell sheet. Later, the entire cell monolayer was covered with firmly adsorbed red blood cells. The hemadsorption reaction in the BS-C-1 cultures was quite stable as the red cells remained attached following shaking and repeated washing of the monolayer. Hemadsorption appeared from several days to 1 week before cytopathogenic effect (CPE); the CPE in HDCS WI38 cultures resembled that of the 229E virus in cultures of HDCS WI38, and in human embryonic intestine (HEI or MA177) cell cultures, whereas, in HEK cells, it resembled that of OC43 virus in BS-C-1 cell cultures (2, 5, 13). Nonspecific hemadsorption was observed frequently with HEK cell cultures and occasionally in BS-C-1 cells; an estimate of the frequency of nonspecific hemadsorption in rhesus MK and HDCS WI38 cultures could not be made since tests were not routinely performed in these cell cultures. Occasionally certain lots of BS-C-1 cultures infected with OC43 virus did not hemadsorb satisfactorily.

Specificity of hemadsorption. Prevention of hemadsorption by specific OC43 hyperimmune antisera was demonstrated in conventional neutralization tests using the complete absence of hemadsorption as the measure of neutralization. Table III shows the neutralizing antibody titers of OC43, MHV, 229E, and normal mouse sera as determined by this method. It is of interest that the previously described relationship between MHV and OC43 viruses was not demonstrated in the test shown (17); however, in some other neutralization-hemadsorption tests, the MHV A-59 serum (but not the polyvalent MHV serum) inhibited OC43 virus hemadsorption at low dilutions.

TABLE III. Reciprocal of Neutralizing Antibody (NAB) Titer to Coronavirus Strain OC43 in Various Sera as Measured by Neutralization-Hemadsorption Technique in BS-C-1 Cell Cultures.

Serum vs indicated agent (and animal species in which prepared)	Reciprocal of NAB titer vs 320 TCD_{50} of OC43 virus
OC43 (mouse—lot 1347)	640
OC43 (mouse—lot 1571)	640
OC38 (mouse)	640
Normal (mouse)	<5
MHV-A-59 (mouse)	<5[a]
Polyvalent MHV (mouse)	<5[b]
229E (guinea pig)	<5[c]

[a] Homologous NAB titer 1:320 vs 32 TCD_{50} in HDCS WI38 cultures.

[b] NAB titer vs MHV strain A-59 1:320 vs 32 TCD_{50} in HDCS WI38 cultures.

[c] Homologous NAB titer 1:160 vs 100 TCD_{50} in HDCS WI38 cultures.

Hemadsorption as indicator system in neutralization tests with human sera. Paired sera from individuals with previously recognized OC43 or 229E virus infections were tested in OC43 virus neutralization tests using absence of hemadsorption as the indicator of neutralization (Table IV). Two individuals with significant CF antibody rises to 229E virus (one

of whom yielded 229E virus) did not develop significant OC43 virus neutralizing antibody rises, whereas 4 of 7 with such CF antibody rises to OC43 virus did. One individual in the latter group also yielded OC43 virus (see Table IV). The presence of neutralizing antibody in acute phase sera in all individuals was noteworthy. The test was simple to read since hemadsorption rather than CPE was employed as the indicator system of virus growth; in addition, hemadsorption appeared up to 1 week before CPE, permitting more rapid completion of the test. Lack of reproducibility similar to that observed in other neutralization tests occurred occasionally. It was of interest that only 3 of 7 patients with a significant CF antibody rise to OC43 virus developed fourfold or greater increases in serum antibody in the hemagglutination-inhibition test, and these 3 individuals also had a significant rise in neutralization-hemadsorption antibody (Table IV).

Discussion. Studies with the known human coronaviruses have been hampered by the lack of simple and satisfactory methods of propagating these agents. However, progress in this area has been achieved recently: viruses similar to 229E, the prototype of the initial tissue culture strain, were successfully isolated in human embryonic intestine monolayer cultures from patients with naturally occurring upper respiratory illnesses; B814 virus, the first OC strain, 2 other OC strains LP and EVS, and 229E virus, were recovered in L132 cell monolayers from nasal washings of patients challenged with these agents. In addition, OC43 (and OC38) viruses which were originally recovered in OC and later

TABLE IV. Hemagglutination-Inhibition (HI), Neutralizing, and Complement-Fixing (CF) Antibody Responses to OC43 Virus in Patients with Previously Recognized OC43 or 229E Virus Infection.[a]

Patient no.	Coronavirus strain recovered	Serum tested	Reciprocal of antibody titer vs OC43 virus: CF	HI (vs 8 units)	Neutralization[b] (vs 320 TCD_{50})	229E virus: CF
648	None	Acute	*8*	*<4*	*32*	8
		Convalescent	*>32*	*64*	*256*	8
689	None	Acute	*4*	*4*	*8*	16
		Convalescent	*16*	*16*	*>256*	32
690	OC43	Acute	*4*	8	*16*	—
		Convalescent	*16*	4	*128*	<4
712	None	Acute	*8*	*4*	*32*	4
		Convalescent	*32*	*32*	*256*	4
636	None	Acute	*4*	4	64	<4
		Convalescent	*16*	<4	32	<4
698	None	Acute	*4*	4	16	16
		Convalescent	*16*	8	32	16
725	None	Acute	*8*	8	16	—
		Convalescent	*32*	8	32	<4
844	229E	Acute	8	4	16	*<4*[c]
		Convalescent	8	4	8	*16*[c]
884	None	Acute	16	16	256	*<4*[d]
		Convalescent	16	8	256	*32*[d]

[a] Previously recognized by one or a combination of the following studies: virus isolation; ≥fourfold CF antibody rise; ≥fourfold neutralizing antibody (NAB) rise.

[b] Absence of hemadsorption used as index of virus neutralization.

[c] NAB titer of 1:4 in acute phase serum and 1:16 in convalescent phase serum vs TCD_{50} of 229E virus.

[d] Not tested for NAB vs 229E virus.

adapted to grow in suckling mice were not only shown to directly hemagglutinate various erythrocytes but were also adapted to grow in monkey kidney monolayers (2, 8, 10, 12, 13). In this paper we have described the hemadsorption of both rat and mouse erythrocytes to various monolayer tissue cultures infected with OC43 virus.

Hemadsorption with rat cells was found to be a simple system for performing neutralization tests with OC43 virus. The neutralization-hemadsorption technique offers several distinct advantages over the conventional neutralization test in which CPE is used as the indicator system since (i) hemadsorption patterns are much easier to read than the characteristic CPE of this virus; and (ii) the hemadsorption patterns appear up to 1 week before the characteristic CPE permitting more rapid completion of the test, a factor which may be of more than practical importance in a serum neutralization end point test with low titered sera. Although only 4 of 7 individuals with a CF antibody rise to OC43 virus demonstrated fourfold or greater serum antibody rises by the neutralization-hemadsorption test, the relative sensitivity of the latter test system cannot be determined since only two OC43 or "OC43-like" strains have been recovered to date (OC43 [from patient 690] and OC38 [from patient 664]) (6, 11, 17). In addition, the specificity of the CF technique in OC43 virus infection remains to be determined. It was of interest, however, that only 3 of the 7 patients with CF evidence of OC43 virus infection developed fourfold or greater increases in serum antibody by the HI test but these 3 individuals also had rises by the neutralization-hemadsorption technique. These limited data would suggest that OC43 antigen may react more broadly with CF antibody induced by other coronaviruses thereby accounting for the greater reactivity (and lesser specificity) of complement-fixation in comparison to the HI and neutralization techniques in these limited studies of adults with respiratory illnesses. Kaye and Dowdle (12) found that the HI test was considerably more sensitive than the complement-fixation technique in detecting antibody rises to OC38 and OC43 antigens in children with respiratory illnesses. Whether this contrasting experience in the sensitivity of the HI and complement-fixation techniques reflects differences in previous exposure to coronaviruses in these different age groups or is a result of the small number of patients studied remains to be determined. It was also noteworthy that all 4 individuals with neutralizing antibody rises to OC43 virus possessed detectable neutralizing antibody (1:8–1:32) in acute phase sera. Further studies should reveal whether or not the pattern of OC43 virus infection in adults is similar to that of the respiratory syncytial and parainfluenza type 3 viruses, both of which in adults characteristically produce reinfection (18–20).

Additional data on the sensitivity and specificity of various coronavirus test systems in adults was recently reported by Bradburne (21). He found that only 5 of 14 volunteers developing "colds" following OC43 virus challenge had fourfold or greater HI rises to OC43 virus but 2 of the 14 developed neutralizing antibody rises to either LP or 229E viruses; none of 18 volunteers administered 229E or LP viruses developed rising CF titers to OC43 virus but 4 of the 18 developed such titers to MHV_3 virus. In addition, 10 of 70 volunteers infected with various coronaviruses, excepting OC38 and OC43, developed fourfold or greater HI antibody rises to OC43 virus. With the introduction of the OC43 neutralization-hemadsorption test it will be possible to obtain additional information on the sensitivity and specificity of the various test systems studied above.

Attempts to isolate OC43 and OC38 viruses from the original nasal washings yielding these agents, from OC43 and OC38 organ culture harvests, and from a limited number of original specimens in various tissue cultures using the technique of hemadsorption were unsuccessful as were attempts to hemadsorb B814, 229E, OC16, OC37, OC44, and OC48 viruses. However, with hemadsorption as the initial indicator system, BS-C-1 cell culture grown OC43 virus, (which had been previously passaged in HET organ cultures, suckling mice, and primary monkey kidney

cell cultures in the order indicated), was successfully adapted for the first time to HDCS WI38 and HEK cell cultures; the subsequent CPE in HDCS WI 38 cultures resembled that of 229 E virus in the same cell system and in human embryonic intestine cell cultures, whereas in HEK cells it resembled that of OC43 virus in BS-C-1 cell cultures (2, 5, 13).

Summary. OC43 virus-infected BS-C-1, rhesus MK, HEK, and HDCS WI38 cell cultures were found to hemadsorb rat and mouse erythrocytes. The hemadsorption technique provided a simple method for performing OC43 virus neutralization tests with both human and animal sera.

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