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Antigenic and Biological Relationships between Human Coronavirus OC43 and Neonatal Calf Diarrhoea Coronavirus

By G. GERNA,* P. M. CEREDA, M. GRAZIA REVELLO, E. CATTANEO, M. BATTAGLIA AND MARIA TORSELLINI GERNA
Virus Laboratory, Institute of Infectious Diseases, University of Pavia, 27100 Pavia, Italy

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SUMMARY

Monospecific antisera were prepared in mice to human coronavirus OC43 and neonatal calf diarrhoea coronavirus (NCDCV) which had been previously adapted to growth in suckling mouse brain. Brain suspension from infected suckling mice was used as immunogen. The antigenic relationship between OC43 and NCDCV was studied by the indirect immunoperoxidase antibody technique, by the haemagglutination-inhibition (HI) test and a new infectious centre-reduction neutralization test. In mouse immune sera, a two-way cross-reaction between OC43 and NCDCV was detected. However, the antigenic relationship appeared to be closer for internal (as shown by immunoperoxidase staining) as compared to surface antigens (as shown by HI and neutralization). In primary infections of natural hosts there was a high degree of cross-reactivity between the two coronavirus strains for both surface and internal antigens, and homologous and heterologous titres were consistently within an eightfold dilution difference by all tests. Most human adults and calves had antibody to both OC43 and NCDCV and geometric mean titres of homologous antibody were higher than titres of heterologous antibody. Although OC43 and NCDCV share antigenic determinants, they possessed several different biological properties, including plaque morphology by the infectious centre assay, agglutination of 1-day-old chick erythrocytes and resistance of haemagglutinin to physical and chemical treatments.

INTRODUCTION

Human coronavirus OC43 is a well known cause of upper respiratory tract syndromes (McIntosh *et al.*, 1967*a*, *b*, 1970; Kaye *et al.*, 1971; Hendley *et al.*, 1972; Bradburne & Somerset, 1972). Coronaviruses have been implicated in the aetiology of human gastroenteritis (Caul & Clarke, 1975; Caul *et al.*, 1975; Caul & Egglestone, 1977; Lennartz *et al.*, 1978). Neonatal calf diarrhoea coronavirus (NCDCV) is an important cause of diarrhoea in newborn calves (Stair *et al.*, 1972; Mebus *et al.*, 1973), but antiviral activity to NCDCV has been found in human sera, suggesting a possible role of a related coronavirus in gastroenteritis of humans (Sharpee & Mebus, 1975; Kaye *et al.*, 1975). In addition, a close antigenic relationship between OC43 and NCDCV (both adapted to growth in suckling mouse brain) has been detected using complement-fixation, haemagglutination-inhibition (HI) and neutralization tests (Kaye *et al.*, 1975). More recently, however, non-specific inhibitors of OC43 haemagglutination (Hovi, 1978) and infectivity (Gerna *et al.*, 1980) have been described. Similar inhibitors of NCDCV are demonstrated in this report in human and animal sera.

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In view of the presence of these inhibitors, the antigenic relationship between OC43 and NCDCV has been studied by HI and a new infectious centre-reduction neutralization test after inhibitors were removed with phospholipase C, and by the indirect immunoperoxidase antibody technique for IgG determination. The neutralization test was performed using a liquid overlay medium containing foetal calf serum (FCS), which inhibits OC43 (Gerna *et al.*, 1980). In addition, several biological properties of the two coronavirus strains, grown in the same human cell type, have been compared.

METHODS

Viruses. Suckling mouse brain (SMB)-adapted strain of human coronavirus OC43 at the 18th passage and mouse immune ascitic fluid, were kindly supplied by Dr H. S. Kaye (Center for Disease Control, Respiratory Virology Unit, Atlanta, Ga., U.S.A.). Virus was subsequently passaged in our laboratory by the intracerebral (i.c.) route in 3-day-old suckling mice of the Swiss CD-1 strain (obtained from Charles River Italia S.p.A., Calco, Italy) which are said to be free of mouse hepatitis virus (MHV) and other endemic viruses of mice, such as lymphocytic choriomeningitis virus, reovirus type 3 and parainfluenza virus type 1. Inoculated mice developed encephalitis and died 48 to 72 h after inoculation.

NCDCV seed virus as a bovine embryonic kidney infected cell suspension and bovine NCDCV antisera from experimentally infected gnotobiotic animals were obtained from the Institute of Agriculture and Natural Resources, University of Nebraska (Lincoln, Ne., U.S.A.). Virus was adapted to SMB by i.c. inoculation in 1-day-old suckling mice. Symptoms of encephalitis were observed at 72 h, and animals died at 96 to 120 h. The second passage was done in the same way using a 10% SMB suspension, and animals died at 84 to 96 h. The following two passages were performed in 3-day-old suckling mice and death occurred at 48 to 72 h.

Production of antisera. OC43 and NCDCV antigens were prepared as a 10% suspension of infected mouse brain in phosphate-buffered saline (PBS). A 10% suspension of normal mouse brain was used as control antigen. Monospecific immune sera were prepared in 4-week-old weanling mice (monitored by serological testing for MHV and the other endemic viruses of mice reported above) by four 0·5 ml intraperitoneal inoculations of antigen each 1 week apart in complete Freund's adjuvant (Kaye *et al.*, 1977). Mice were bled 7 days after the final inoculation, and ascitic fluids were collected. Similarly, negative control sera were prepared from mice inoculated with control antigen.

Cell cultures. The MA-321 strain (Microbiological Associates, Walkersville, Md., U.S.A.) of human embryonic lung fibroblast cell cultures at the 20 to 30th passage was used for propagation of both OC43 and NCDCV, preparation of haemagglutinating antigen and performance of haemadsorption, neutralization and immunoperoxidase tests. Eagle's minimum essential medium (EMEM) was supplemented with 10% FCS when used as a growth medium, and with 2% FCS when used as a maintenance medium. Cell cultures were free of mycoplasma by histochemical and cultural methods.

Virus propagation in cell cultures. Human coronavirus OC43 from SMB suspension was adapted to African green monkey kidney cells (monitored for SV-5 and other endemic simian viruses) and then to MA-321 cell cultures, according to a previously described procedure (Gerna *et al.*, 1979). MA-321 cell monolayers were washed three times with Hanks' balanced salt solution (HBSS) and then inoculated with seed virus for 60 min at 33 °C and then a serum-free maintenance medium consisting of equal parts of medium 199 (M199) and EMEM was added. Virus was grown for 48 to 60 h using infected culture medium as inoculum. The 45 to 55th passage of OC43 (stocks stored at −80 °C) was used in all experiments.

Following two initial passages on primary bovine kidney, NCDCV was easily adapted to MA-321 cell cultures as described for OC43, but was passed at intervals of 60 to 72 h. NCDCV passages 10 to 20 (stored at −80 °C) were used for all experiments. Controls

(cytopathic effect, haemadsorption, electron microscopy, serological tests) performed to detect contamination of virus by most common endemic viruses of monkey and mouse were consistently negative.

The identity of MA-321-adapted strains of OC43 and NCDCV was checked by reference to the original SMB-adapted viruses, and the reference sera (OC43 mouse immune ascitic fluid and NCDCV bovine antiserum) by neutralization and HI. In addition, the two coronavirus strains were monitored for cross-contamination by testing weekly some of their differential biological properties, such as haemagglutination (HA) with 1-day-old chick erythrocytes, plaque morphology and haemagglutinin sensitivity to treatment with chemical and physical agents (see below).

Virus infectivity titration. Titration of both viruses was performed in MA-321 cell cultures grown in microplates. Cell monolayers were washed three times with HBSS and then inoculated with serial 10-fold virus dilutions (10 replicates for each virus dilution) in serum-free EMEM–M199 at 33 °C in a humidified 5% CO_2 chamber for 1 to 6 days. Viral infectivity was determined by HA (Gerna *et al.*, 1979) or by immunoperoxidase, as described herein. Titres were calculated according to the method of Reed & Muench (1938).

Haemagglutination (HA). Both coronaviruses, grown in MA-321 cell cultures, were tested by the microtitre method for HA against rat (Sprague–Dawley and Wistar), grivet, human 'O', 1-day-old chick and chicken erythrocytes at 4 °C, room temperature and 37 °C. A spectrophotometrically standardized 0·4% suspension of mammalian and a 0·5% suspension of 1-day-old chick and chicken red blood cells in PBS, were used for the HA reaction (Kaye & Dowdle, 1969). After initial experiments, the HA reaction for both viruses was routinely performed at room temperature using chicken erythrocytes.

Nature of the haemagglutinin. The stability of the haemagglutinin of virus suspensions from infected MA-321 cell cultures was tested at 56 °C for 30 min, and by treatment with Tween 80–ether (Norrby, 1962) or 0·1% glutaraldehyde (Zaia & Oxman, 1977).

Haemadsorption. OC43- and NCDCV-infected and uninfected MA-321 cell monolayers were tested using chicken and rat erythrocytes. After three washings with HBSS, each tube culture received 0·8 ml HBSS and 0·2 ml erythrocyte suspension. Following incubation at 4 or 25 °C for 30 min, cell cultures were observed for evidence of haemadsorption (HAd). In some experiments, cell monolayers were fixed with acetone, absolute ethanol or 0·1% glutaraldehyde before testing for HAd.

Haemagglutination-inhibition (HI). Non-specific inhibitors of OC43 (Hovi, 1978) and NCDCV (Sharpee *et al.*, 1976) HA were first removed by treatment with phospholipase C (PLC; type I, Sigma) as described by Haukenes & Blom (1975). Briefly, equal volumes of serum and 0·2% (w/v) PLC in PBS were incubated for 1 h at 37 °C. PLC was then inactivated by adding 2 vol. 0·2% (w/v) 1,10-phenanthroline (Sigma) and incubating the mixture at 4 °C for 22 h. Sera were then absorbed with 50% chicken erythrocyte suspension and diluted 1:0 with PBS. The HI test was performed as described by Kaye *et al.* (1971) with fluids from virus-infected MA-321 cells.

Infectious centre and infectious centre-reduction neutralization assays. A new plaque assay was used for OC43 (Gerna *et al.*, 1980). For quantification of NCDCV, which does not form plaques, but only single enlarged infected cells, a new infectious centre assay was used. For both assays, a liquid medium consisting of EMEM–M199 supplemented with 10% FCS was employed. FCS contains an OC43 viral inhibitor (Gerna *et al.*, 1980), which also inhibits NCDCV and allows virus to spread only by cell-to-cell contact. Tenfold virus dilutions were inoculated on to MA-321 replicate microtitre plate cell cultures at 33 °C for 60 min, cell cultures were washed with HBSS and fed with medium. At 48 h p.i., cell monolayers were washed, fixed with absolute ethanol and stained by immunoperoxidase. This procedure was carried out using optimum dilutions (containing eight immunoperoxidase antibody units) of

mouse anti-OC43 or anti-NCDCV antiserum in the first step of the reaction. The optimum dilution of a peroxidase-conjugated sheep anti-mouse IgG (Cappel Laboratories, Cochranville, Pa., U.S.A.) was used in the second step of the reaction. Peroxidase was then detected as described by Graham & Karnovsky (1966). Infectious centres were counted using an inverted microscope.

For neutralization (Nt) test, treatment of sera with PLC was necessary to avoid false positive results (Gerna *et al.*, 1980). The treatment was done as reported for the HI test, and was followed by overnight dialysis against PBS at 4 °C and filtration through 0·22 μm Millipore membranes. Serial twofold dilutions of each inactivated serum (56 °C for 30 min) in serum-free EMEM–M199 were mixed with equal volumes of virus suspension containing about 50 infectious units of virus/0·05 ml, as determined by the infectious centre assay. After incubation at 33 °C for 60 min, two replicate wells of confluent MA-321 cell monolayers were inoculated (0·05 ml/well) with each mixture. Following adsorption at 33 °C for 60 min, cell cultures were washed, incubated with the plaquing medium for 48 h, and then fixed and stained as reported above for the infectious centre assay. The highest serum dilution reducing the number of infectious centres by 50% or more was considered to be the endpoint. Duplicate determinations gave identical results in more than 95% of cases.

Immunoperoxidase antibody technique for IgG determination. The immunoperoxidase test for OC43 and NCDCV IgG determination (IPA-IgG) was performed on ethanol-fixed MA-321 cell monolayers grown in microtitre plates in which about 50% of the cells were infected. Serial twofold dilutions of test sera were layered on to cell monolayers and incubated at 37 °C for 60 min. After three washings with PBS, cell cultures were covered with the optimum dilution (as determined by checkerboard titration) of a peroxidase-conjugated goat anti-human, rabbit anti-bovine or sheep anti-mouse IgG (Cappel Laboratories). Virus–antibody reaction was then histochemically detected using the diaminobenzidine–H_2O_2 colour developing system. Positive and negative serum controls were included in each test.

Sera examined. In addition to mouse antisera, the following groups of sera were tested for OC43 and NCDCV antibodies by HI, Nt and IPA-IgG tests: (i) 11 commercial lots of FCS obtained from different companies; (ii) individual sera from 31 calves; (iii) single sera from 34 children (2 to 12 years old); (iv) single sera from young adults (13 to 20 years old); (v) five paired sera from infants and children previously found to show seroconversion to OC43, and two paired sera (pre-serum and immune serum) from newborn calves experimentally infected with NCDCV. For HI and Nt tests, all sera were treated with PLC; for comparison, some of them were also tested without prior PLC treatment.

RESULTS

OC43 and NCDCV growth on MA-321 cell cultures

Both coronaviruses adapted to growth on MA-321 cell cultures reached peak titres of infectivity at 48 to 72 h post-infection (p.i.). HA titres reached a peak at 48 h for OC43, and at 72 h for NCDCV and remained at a stable level until 144 h p.i. (Fig. 1). C.p.e. of either virus did not appear until 4 to 5 days p.i., unless high passage cells (over 30 to 35) were employed. Cells became refractile, oval or rounded, with a cytoplasm rich in microvacuoles. Complete degeneration of cell monolayers was observed 6 to 10 days p.i. with both viruses.

Patterns of IPA-IgG staining of OC43- and NCDCV-infected cells

When stained by the IPA-IgG technique, both OC43- and NCDCV-infected MA-321 cells showed a diffuse cytoplasmic staining with a darker perinuclear area. When monolayers infected with 10^2 to 10^3 $TCID_{50}$ of either virus were maintained in serum-free medium, a diffuse distribution of single infected cells was observed 48 h p.i. When the same amount of

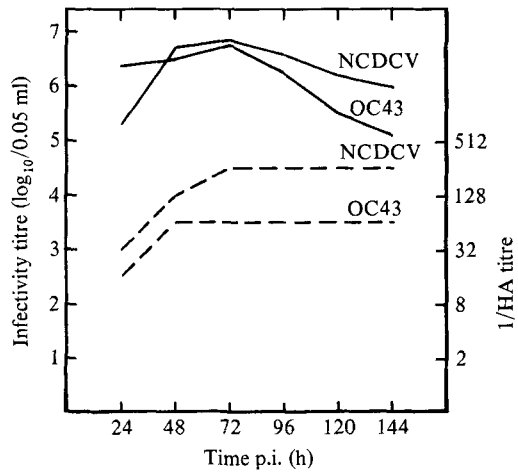

Fig. 1. Infectivity (——) and haemagglutination (HA; — — —) titres of extracellular MA-321 cell culture-adapted strains of human coronavirus OC43 and neonatal calf diarrhoea coronavirus (NCDCV).

virus was grown in the presence of 10% FCS, the IPA-IgG technique showed large plaques in OC43-infected cell cultures, but only single enlarged infected cells in NCDCV-infected cultures (Fig. 2). However, a linear dose-response relationship was consistently observed with both OC43 and NCDCV. At 96 h p.i., OC43 plaques tended to become confluent, and single infected cells between plaques started appearing, whereas NCDCV-infected cells in the presence of FCS still remained single, irregular in shape and largely swollen (Fig. 3).

Haemagglutination

For both OC43 and NCDCV, chicken and rat (Sprague–Dawley and Wistar) erythrocytes showed optimum patterns of agglutination at all three temperatures tested. Human 'O', grivet and 1-day-old chick erythrocytes showed more distinct patterns of complete HA at 4 °C, but titres were within a twofold dilution difference at room temperature. Both viruses agglutinated all species of erythrocytes (at different titres) at room temperature, except that OC43 did not agglutinate 1-day-old chick cells (Table 1). Great variations in the sensitivity to agglutination were observed among individuals of the same species and strain. In about 10% of cases, human 'O', grivet and 1-day-old chick erythrocytes were not agglutinated by either virus.

Nature of the haemagglutinin

Treatment with Tween 80 plus ether, 0·1% glutaraldehyde and heat (56 °C for 30 min) completely inactivated OC43 haemagglutinin, but had no effect on NCDCV. Ether alone destroyed the haemagglutinin of both coronavirus strains.

Haemadsorption

HAd with chicken and rat erythrocytes was stronger in NCDCV-infected than in OC43-infected cell cultures 48 h p.i. at either 4 or 25 °C. Prefixation with glutaraldehyde completely abolished HAd in OC43-infected cells, but did not modify HAd in NCDCV-infected cell cultures. Prefixation with acetone and absolute ethanol completely prevented HAd in cell cultures infected with either coronavirus strain.

Serological tests on human and calf sera

Results reported in Table 2 show that all sera found to be negative by IPA-IgG became negative after PLC treatment by both HI and Nt. Calf sera were diluted from 1:20 in the Nt

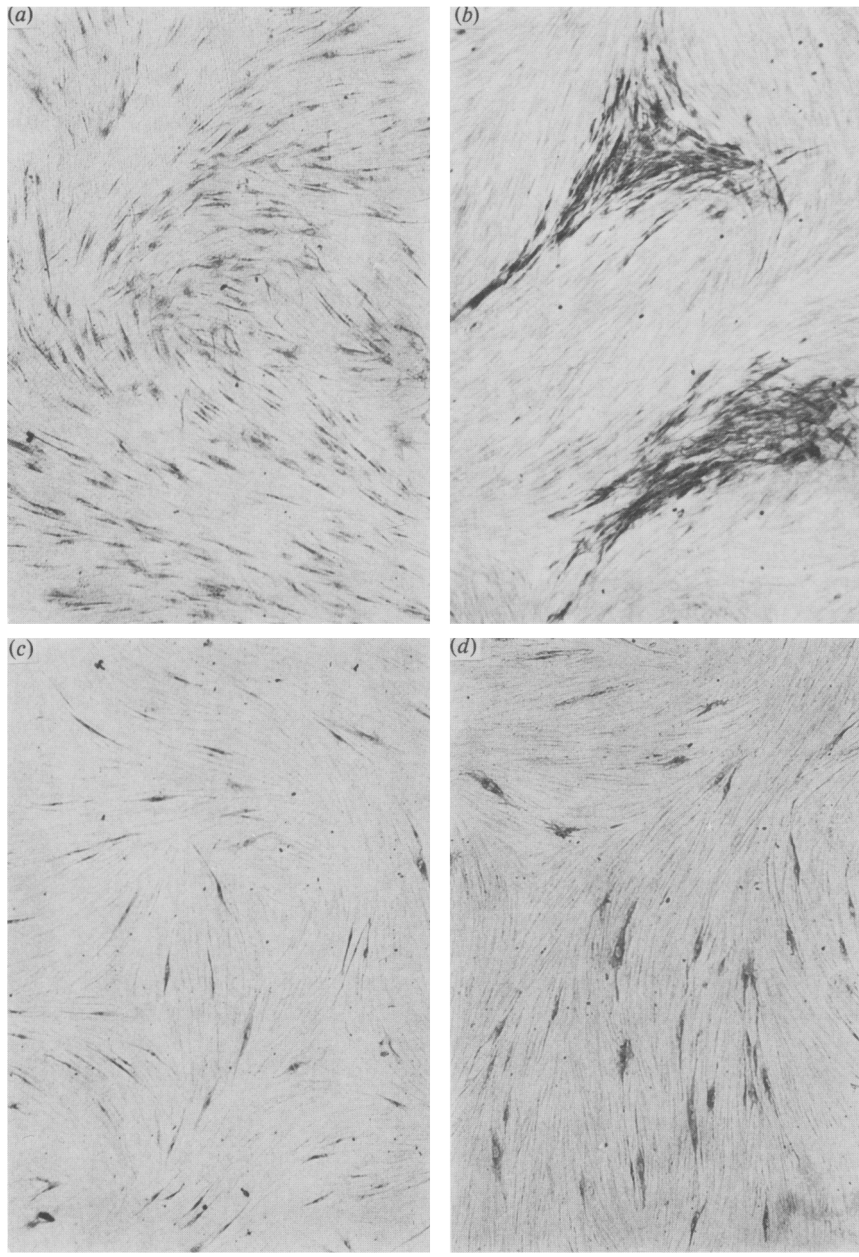

Fig. 2. MA-321 cell cultures infected with (*a*, *b*) human coronavirus OC43 and (*c*, *d*) NCDCV, and stained by the immunoperoxidase antibody technqiue for IgG determination 48 h p.i. (*a*) OC43-infected cells in serum-free medium, and (*b*) in 10% FCS medium; (*c*) NCDCV-infected cells in serum-free medium, and (*d*) in 10% FCS medium.

test to avoid residual inhibitors. In positive sera, the HI and Nt antibody titres were sometimes reduced after PLC treatment. Antibody to homologous virus was consistently associated with the presence of antibody to heterologous virus in both calf and human sera.

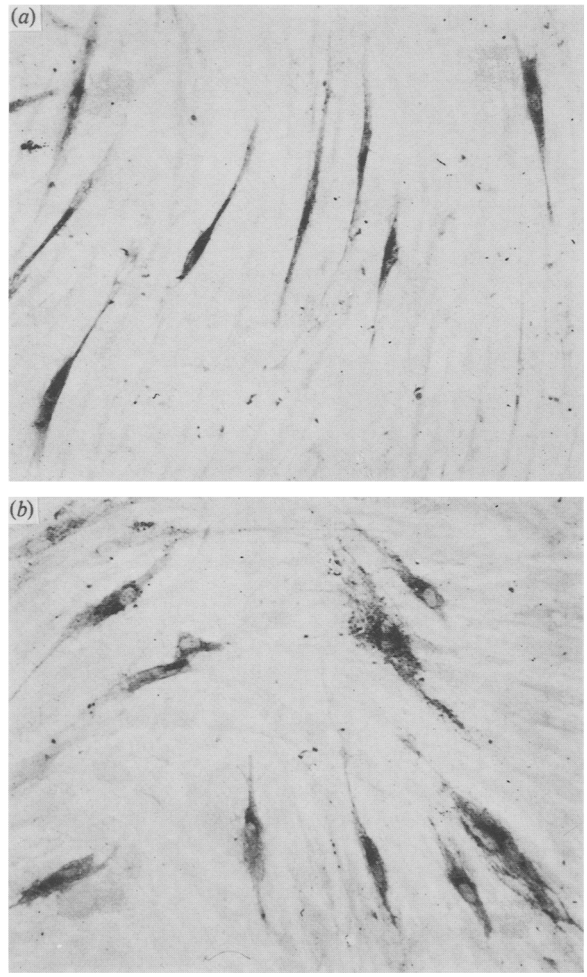

Fig. 3. MA-321 cell cultures infected with NCDCV and stained by the IPA-IgG technique 96 h p.i. (*a*) NCDCV-infected cells in serum-free medium, and (*b*) in 10% FCS medium.

Table 1. *Human coronavirus OC43 and neonatal calf diarrhoea coronavirus (NCDCV) haemagglutination titres at room temperature*

	Haemagglutination titre*	
Source of erythrocytes	OC43	NCDCV
Human 'O'†	16 (<1–32)‡	32 (<1–64)
Grivet†	16 (<1–32)	32 (<1–64)
Rat (Sprague–Dawley)	32 (16–64)	128 (64–256)
Rat (Wistar)	64 (32–128)	256 (128–512)
Chicken	64 (8–128)	128 (16–256)
1-day-old chick†	<1 (<1–1)	32 (<1–64)

* Mammalian and fowl erythrocytes were used as a 0·4% and 0·5% suspension respectively.

† Erythrocytes from this animal species may show more distinct patterns of complete agglutination when incubated at 4 °C.

‡ HA titres are expressed as the reciprocal of the highest dilution of antigen showing complete (4+) or nearly complete (3+) haemagglutination, and are listed as the medians (range).

Table 2. *Antibody titres to human coronavirus OC43 and NCDCV in human and calf sera by haemagglutination-inhibition (HI), neutralization test (Nt) and the immunoperoxidase antibody technique for IgG (IPA-IgG) determination*

		Antibody titre to indicated virus by									
		HI				Nt				IPA-IgG	
		OC43		NCDCV		OC43		NCDCV			
Study group	Serum no.	NT*	T*	NT	T	NT	T	NT	T	OC43	NCDCV
Foetal calf sera	1	<20	<20	<20	<20	160	<20	160	<20	<10	<10
	2	<20	<20	20	<20	320	<20	320	<20	<10	<10
	3	20	<20	40	<20	1280	<20	1280	<20	<10	<10
	4	20	<20	40	<20	1280	<20	1280	<20	<10	<10
Calves	3	40	10	80	80	>640	>640	>640	>640	80	320
	7	<20	<20	20	<20	80	<20	80	<20	<10	<10
	21	160	40	160	160	640	640	>640	>640	320	5120
	23	160	160	320	320	640	640	>640	>640	160	2560
Children	996	<10	<10	<10	<10	160	<10	80	<10	<10	<10
	1299	10	<10	10	<10	640	<10	640	<10	<10	<10
	1242	40	20	20	10	640	160	640	80	40	20
	1263	20	<10	10	<10	320	<10	80	<10	<10	<10
Young adults	1096	80	40	40	10	>640	640	320	320	160	80
	1124	80	80	160	40	>640	>640	>640	>640	320	320
	1272	160	160	40	20	>640	>640	160	160	320	320
	1304	40	40	40	10	>640	>640	320	320	160	160

* NT, Not treated; T, treated with phospholipase C.

Table 3. *Antigenic relationships between human coronavirus OC43 and NCDCV by HI, Nt and IPA-IgG using specific mouse immune sera*

	Geometric mean titres† to indicated virus by									
	HI				Nt				IPA-IgG	
	OC43		NCDCV		OC43		NCDCV			
Immune serum*	NT‡	T‡	NT	T	NT	T	NT	T	OC43	NCDCV
OC43	2032	2153	320	34	57926	57926	1810	226	10240	5120
NCDCV	226	28	3620	3620	1810	113	57926	57926	2153	17212

* Ten animals were experimentally infected with OC43 and 10 with NCDCV. Pre-immunization sera had no detectable antibody to OC43 and NCDCV after PLC treatment.

† Titres were calculated on five replicates from two experiments.

‡ NT, Not treated; T, treated with phospholipase C.

Antigenic relationship

The antigenic relationship between human coronavirus OC43 and NCDCV, using monospecific mouse antisera, is reported in Table 3. In OC43 immune serum the antibody titre to homologous virus was ⩾64-fold higher than titre to heterologous virus by both HI and Nt tests, whereas homologous and heterologous titres by IPA-IgG were within a twofold difference. In NCDCV immune serum, the homologous titre was also ⩾64-fold higher than heterologous titre by HI and Nt tests, but an eightfold dilution difference was observed by the IPA-IgG test. Monospecific immune ascitic fluids gave similar results to immune sera. Following removal of non-specific viral inhibitors by PLC treatment, heterologous HI and Nt titres appeared to be reduced (8- to 16-fold), whereas homologous titres, as well as IPA-IgG titres, were not affected.

Table 4. *Antibody response to human coronavirus OC43 and NCDCV by HI, Nt and IPA-IgG in humans with OC43 primary infection, and in newborn calves experimentally infected with NCDCV*

Study group	Age (years)	Interval between acute and convalescent serum (days)	Antibody titre to indicated virus by HI* OC43	HI* NCDCV	Nt* OC43	Nt* NCDCV	IPA-IgG OC43	IPA-IgG NCDCV
Children	4	30	<10	<10	40	10	<10	<10
			20	<10	160	40	40	20
	9	28	<10	<10	<10	<10	<10	<10
			20	<10	160	40	160	80
	11	19	<10	<10	80	20	<10	<10
			40	<10	640	160	160	80
	4	15	<10	<10	<10	<10	<10	<10
			20	<10	160	40	40	20
	4	13	<10	<10	<10	<10	<10	<10
			80	20	640	160	40	20
Calves	Newborn	30	<20	<20	<20	<20	<10	<10
			40	80	80	640	80	640
	Newborn	30	<20	<20	<20	<20	<10	<10
			40	40	160	640	80	640

* All sera were pretreated with phospholipase C.

Table 5. *Antibody titres to human coronavirus OC43 and NCDCV by HI, Nt and IPA-IgG in bovine and human sera*

Study group (no. of sera)	Geometric mean titres* to indicated virus by HI† OC43	HI† NCDCV	Nt† OC43	Nt† NCDCV	IPA-IgG OC43	IPA-IgG NCDCV
Foetal calf sera (11)	<20	<20	<20	<20	<10	<10
Calves (31)	52	160	209	915	117	1119
		(17)‡		(30)		(28)
Children (34)	14	6	63	36	35	23
	(9)		(7)		(5)	
Adults (12)	40	9	806	226	169	120
	(10)		(6)		(0)	

* Titres <1:10 and <1:20 were assigned values of 5 and 10 for calculation of geometric mean titres, which are expressed as reciprocals.

† All sera were pretreated with phospholipase C.

‡ Numbers in parentheses represent sera with homologous titre at least fourfold greater than heterologous titre. In no serum was the heterologous titre found to be more than twofold greater than homologous antibody titre.

Antibody response

In five cases of primary OC43 infection in infants and young children, a seroconversion to OC43 virus was detected by all three tests, whereas a seroconversion to NCDCV was detected in all patients by Nt and IPA-IgG, and only in one case by HI (Table 4). Two newborn calves experimentally infected with NCDCV seroconverted to both coronavirus strains by all tests. However, a ⩾fourfold difference was observed between homologous and heterologous titre by HI and Nt in children, and by Nt and IPA-IgG in newborn calves.

A similar difference between homologous and heterologous titre was observed in two groups of sera collected from calves and adult humans (Table 5). In human sera, the

Table 6. *Prevalence of antibody to human coronavirus OC43 and NCDCV by HI, Nt and IPA-IgG in normal human and bovine populations**

Study group (no. of sera)	No. positive (%) to indicated virus by					
	HI		Nt		IPA-IgG	
	OC43	NCDCV	OC43	NCDCV	OC43	NCDCV
Foetal calf sera (11)	0	0	0	0	0	0
Calves (31)	30	30	30	30	30	30
	(97)	(97)	(97)	(97)	(97)	(97)
Children (34)	24	9	25	25	25	24
	(71)	(26)	(74)	(74)	(74)	(71)
Adults (12)	12	6	12	12	12	12
	(100)	(50)	(100)	(100)	(100)	(100)

* All sera were pretreated with phospholipase C. Foetal calf and calf sera were considered to be positive when titre was $\geqslant$1:20 in the HI and Nt tests. The same sera in the IPA-IgG test, as well as human sera in all three tests, were considered to be positive when titre was $\geqslant$1:10.

reciprocal geometric mean titres (GMT) to OC43 were $\geqslant$fourfold greater than titres to NCDCV by HI and Nt, but almost identical by IPA-IgG. In calf sera, GMT to NCDCV were $\geqslant$fourfold greater than titres to OC43 by both Nt and IPA-IgG.

In calves and adults, the prevalence of antibody to both OC43 and NCDCV was close to 100% using Nt and IPA-IgG tests (Table 6). By the same two tests, antibody to both viruses was also observed in children's sera, although the prevalence was lower. The same finding was observed with the HI test in calf sera, whereas in human sera the prevalence of homologous antibody was much higher, compared to that of heterologous HI antibody (i.e. almost 100% of adults possessed HI antibody against OC43, but only 50% showed HI antibody to NCDCV).

DISCUSSION

In the present study, analysis of the antigenic relationship between OC43 and NCDCV, using specific HI and Nt tests and the IPA-IgG technique, revealed a close two-way cross-reactivity only for internal antigens (as shown by IPA-IgG); in fact, in mouse antisera heterologous HI and Nt titres were drastically reduced by PLC treatment. Previous reports have shown the presence in humans of antibody to animal coronaviruses (Hartley *et al.*, 1964; Miller & Yates, 1968) and the existence of serological cross-reactions among and between human and animal coronavirus strains (McIntosh *et al.*, 1967*a*, *b*; Kaye & Dowdle, 1969; Bradburne, 1970; Pedersen *et al.*, 1978). The detection in human sera of antibody to NCDCV suggested the existence of an enteric coronavirus in man (Sharpee & Mebus, 1975). In addition, a close antigenic relationship between OC43 and NCDCV, along with the finding of seroconversion to both viruses in a group of children with upper respiratory illness, has been reported (Kaye *et al.*, 1975). However, in these studies the HI test was performed without appropriate treatment of sera for removal of non-specific inhibitors (Hovi, 1978). Furthermore, the neutralization test was done without treatment of sera for removal of the coronavirus inhibitor, which has recently been shown to grossly interfere with test results in our laboratory (Gerna *et al.*, 1980). We believe that the reported close antigenic relationship would have appeared less close by eliminating non-specific viral inhibitors. Previously reported antigenic relationships between OC43 and coronavirus strains of other animal species, such as haemagglutinating encephalomyelitis virus of swine (Kaye *et al.*, 1977), might be reconsidered by testing treated immune sera.

On the other hand, the antibody response of natural hosts (humans for OC43 and calves for NCDCV) to infection by homologous virus showed a degree of cross-reactivity between

homologous and heterologous surface antigens (as shown by HI and Nt) higher than that observed in immunized mice. The higher specificity of mouse antisera, as compared to convalescent sera from children and calves, may be due to a difference in the antibody response among different animal species, or to a difference between coronavirus strains used for immunization and those infecting natural hosts. In our study, primary infections of children may have been caused by a circulating strain only partially related to OC43, but experimental infections of newborn calves were obtained using the same strain employed for mouse immunization. Thus, we believe that the different degree of specificity of the antibody response between immunized mice and infected natural hosts depends primarily on the animal species studied. In addition, whether the route of antigen administration may have been responsible for the different degree of specificity remains to be determined.

The high degree of cross-reactivity between the two coronavirus strains observed in most infected natural hosts has not been detected in some infants with non-bacterial gastroenteritis (G. Gerna *et al.*, unpublished observations). These cases showed seroconversion to OC43 by HI and Nt, but not by IPA-IgG, in the absence of any significant antibody rise to NCDCV. The different reactivity of convalescent-phase sera to surface and internal antigens of OC43 suggests a possible role of an as yet unknown OC43-related enteric coronavirus in some cases of infantile diarrhoea.

Although OC43 and NCDCV share some antigens, they can be readily differentiated on the basis of several biological properties, although why cell-to-cell propagation is possible for OC43 virus, but not for NCDCV, remains to be determined. The agglutination by NCDCV of erythrocytes of selected 1-day-old chicks was found in this study to be a good and rapid method for differentiating NCDCV from OC43. Individual variability in sensitivity of erythrocytes of several animal species to agglutination by both OC43 and NCDCV confirmed previous data for NCDCV (Sato *et al.*, 1977). NCDCV and OC43 can be further differentiated as OC43 haemagglutinin is more heat-labile and sensitive to Tween 80–ether and glutaraldehyde treatment than NCDCV haemagglutinin. Only HAd of OC43-infected cells was blocked by previous aldehyde fixation.

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REFERENCES

BRADBURNE, A. F. (1970). Antigenic relationships amongst coronaviruses. *Archiv für die gesamte Virusforschung* **31**, 352–364.

BRADBURNE, A. F. & SOMERSET, B. A. (1972). Coronavirus antibody titres in sera of healthy adults and experimentally infected volunteers. *Journal of Hygiene* **70**, 235–244.

CAUL, E. O. & CLARKE, S. K. R. (1975). Coronavirus propagated from patient with non-bacterial gastroenteritis. *Lancet* ii, 953–954.

CAUL, E. O. & EGGLESTONE, S. I. (1977). Further studies on human enteric coronaviruses. *Archives of Virology* **54**, 107–117.

CAUL, E. O., PAVER, W. K. & CLARKE, S. K. R. (1975). Coronavirus particles in faeces from patients with gastroenteritis. *Lancet* i, 1192.

GERNA, G., CEREDA, P. M., REVELLO, M. G., TORSELLINI GERNA, M. & COSTA, J. (1979). A rapid microneutralization test for antibody determination and serodiagnosis of human coronavirus OC43 infections. *Microbiologica* **2**, 331–344.

GERNA, G., CATTANEO, E., CEREDA, P. M., REVELLO, M. G. & ACHILLI, G. (1980). Human coronavirus OC43 serum inhibitor and neutralizing antibody by a new plaque-reduction assay. *Proceedings of the Society for Experimental Biology and Medicine* **163**, 360–366.

GRAHAM, R. C. & KARNOVSKY, M. J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubule of mouse kidney: ultrastructural cytochemistry by a new technique. *Journal of Histochemistry and Cytochemistry* **14**, 291–306.

HARTLEY, J. W., ROWE, W. P., BLOOM, H. H. & TURNER, H. C. (1964). Antibodies to mouse hepatitis viruses in human sera. *Proceedings of the Society for Experimental Biology and Medicine* **115**, 414–418.

HAUKENES, G. & BLOM, H. (1975). False positive rubella virus haemagglutination inhibition reactions: occurrence and disclosure. *Medical Microbiology and Immunology* **161**, 99–106.

HENDLEY, J. O., FISHBURNE, H. B. & GWALTNEY, J. M., JR (1972). Coronavirus infections in working adults. Eight-year study with 229E and OC43. *American Review of Respiratory Diseases* **105**, 805–811.

HOVI, T. (1978). Nonspecific inhibitors of coronavirus OC43 haemagglutination in human sera. *Medical Microbiology and Immunology* **166**, 173–176.

KAYE, H. S. & DOWDLE, W. R. (1969). Some characteristics of hemagglutination of certain strains of 'IBV-like' virus. *Journal of Infectious Diseases* **120**, 576–581.

KAYE, H. S., MARSH, H. B. & DOWDLE, W. R. (1971). Seroepidemiologic survey of coronavirus (strain OC43) related infections in a children's population. *American Journal of Epidemiology* **94**, 43–49.

KAYE, H. S., YARBROUGH, W. B. & REED, C. J. (1975). Calf diarrhoea coronavirus. *Lancet* ii, 509.

KAYE, H. S., YARBROUGH, W. B., REED, C. J. & HARRISON, A. K. (1977). Antigenic relationship between human coronavirus strain OC43 and hemagglutinating encephalomyelitis virus strain 67N of swine: antibody responses in human and animal sera. *Journal of Infectious Diseases* **135**, 201–209.

LENNARTZ, H., MAAS, G. & SARATEANU, D. E. (1978). Diagnosis of gastroenteritis in young children by the use of electronmicroscope and serological methods. In *Abstracts of the Fourth International Congress for Virology, The Hague*, p. 455. Wageningen: Centre for Agricultural Publishing and Documentation.

MCINTOSH, K., BECKER, W. B. & CHANOCK, R. M. (1967*a*). Growth in suckling-mouse brain of 'IBV-like' viruses from patients with upper respiratory tract disease. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 2268–2273.

MCINTOSH, K., DEES, J. H., BECKER, W. B., KAPIKIAN, A. Z. & CHANOCK, R. M. (1967*b*). Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proceedings of the National Academy of Sciences of the United States of America* **57**, 933–940.

MCINTOSH, K., KAPIKIAN, A. Z., TURNER, H. C., HARTLEY, J. W., PARROTT, R. H. & CHANOCK, R. M. (1970). Seroepidemiologic studies of coronavirus infection in adults and children. *American Journal of Epidemiology* **91**, 585–592.

MEBUS, C. A., STAIR, E. L., RHODES, M. B. & TWIEHAUS, M. J. (1973). Neonatal calf diarrhea: propagation, attenuation, and characteristics of a coronavirus-like agent. *American Journal of Veterinary Research* **34**, 145–150.

MILLER, L. T. & YATES, V. J. (1968). Neutralization of infectious bronchitis virus by human sera. *American Journal of Epidemiology* **88**, 406–409.

NORRBY, E. (1962). Hemagglutination by measles virus. A simple procedure for production of high potency antigen for hemagglutination-inhibition (HI) tests. *Proceedings of the Society for Experimental Biology and Medicine* **111**, 814–818.

PEDERSEN, N. C., WARD, J. & MENGELING, W. L. (1978). Antigenic relationship of the feline infectious peritonitis virus to coronaviruses of other species. *Archives of Virology* **58**, 45–53.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent end points. *American Journal of Hygiene*, **27**, 493–497.

SATO, K., INABA, Y., KUROGI, H., TAKAHASHI, E., SATODA, K., OMORI, T. & MATUMOTO, M. (1977). Hemagglutination by calf diarrhea coronavirus. *Veterinary Microbiology* **2**, 83–87.

SHARPEE, R. L. & MEBUS, C. A. (1975). Rotaviruses of man and animals. *Lancet* i, 639.

SHARPEE, R. L., MEBUS, C. A. & BASS, E. P. (1976). Characterization of a calf diarrheal coronavirus. *American Journal of Veterinary Research* **37**, 1031–1041.

STAIR, E. L., RHODES, M. B., WHITE, R. G. & MEBUS, C. A. (1972). Neonatal calf diarrhea: purification and electron microscopy of a coronavirus-like agent. *American Journal of Veterinary Research* **33**, 1147–1156.

ZAIA, J. A. & OXMAN, M. N. (1977). Antibody to varicella-zoster virus induced membrane antigen: immunofluorescence assay using monodisperse glutaraldehyde-fixed target cells. *Journal of Infectious Diseases* **136**, 519–530.

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