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Antigenic Relationships amongst Coronaviruses

By

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Summary

Several serological interrelationships between various members of the coronavirus group have been revealed in neutralization, complement fixation, and gel-diffusion tests, using human and hyperimmune animal sera. Several members of this group of human and animal pathogens are shown to cross-react in one or more type of test, but one member, avian infectious bronchitis virus, was shown to be unrelated. Mouse hepatitis virus (MHV_3) was found to be antigenically related to a number of human types of coronavirus. Difficulties were encountered in the investigation of paired human sera in demonstrating the specificity of antibody rises, placing doubt on the values of some serological studies. The significance of these interrelationships is discussed in the light of other investigations.

1. Introduction

A new group of viruses has been isolated from cases of upper respiratory disease in man. These viruses have a common morphology with that of the viruses of avian infectious bronchitis (Berry *et al.*, 1964) and mouse hepatitis (Tyrrell and Almeida, 1967). These agents are ether and acid-labile, probably contain RNA and are composed of pleomorphic bodies 80—150 mμ in diameter which, by negative staining, are seen to be surrounded by a fringe of club-shaped projections about 15 mμ deep. The resemblance of this fringe to a crown has resulted in the name "Coronaviruses" being proposed for the group (Nature, 1968).

Many isolations have been made of avian infectious bronchitis (AIB) and serologically these fall into several serological types on the basis of neutralization tests (Estola, 1966). There are at least three serological types of mouse hepatitis virus (MHV) (Gledhill, 1961). Some of the isolations of coronaviruses from man have been made in human embryo diploid lung cell strains (HDCS). These isolates have all cross-reacted completely in neutralization tests with antisera to the prototype strain 229E isolated by Hamre in 1962 (Hamre and Procknow, 1966). Other viruses of this group which are pathogenic to man have been isolated in

organ cultures of human embryo nasal and tracheal epithelium. These isolates comprise at least three, and probably more, distinct serological types as judged by neutralization tests using convalescent human sera (BRADBURNE and TYRRELL, 1969; MCINTOSH *et al.*, 1969).

This work describes the detection of several serological relationships between the viruses isolated from man, and those of mouse hepatitis and avian infectious bronchitis. The majority of these results were obtained before similar studies were reported by MCINTOSH *et al.* (1969); furthermore, different reagents were used and several extra cross-reactions were detected.

2. Materials and Methods

2.1. Tissue Cultures

These were all propagated in this laboratory. Roller tube neutralization tests were performed in monolayer cultures of human embryo diploid lung cells (HDCS). These were either a strain derived at the Unit, or the WI-38 cell strain.

Plaque neutralization tests were made using monolayers of L132 cells in 50 mm plastic Petri dishes (Sterilin). The L132 cell line is a continuous epitheloid cell line which was derived from human embryo lung (DAVIS, 1960) and has been shown to be sensitive to several respiratory viruses, including 4 strains of coronaviruses (BRADBURNE, 1969). All cultures were maintained at 33°C after inoculation.

2.2. Viruses

The 229E isolate of Dr. D. Hamre was propagated in WI-38 and L132 cell cultures. The OC38 and OC43 virus strains had been isolated by McIntosh in human tracheal organ cultures (MCINTOSH *et al.*, 1967) and had been adapted to infect suckling mice by the intracerebral route (MCINTOSH, BECKER and CHANOCK, 1967). The virus was propagated by intracerebral inoculation of 2 to 5 day-old mice (Porton Albino strain). Mouse hepatitis virus (strain MHV_3) was obtained from the late Dr. A. Gledhill. It was cultivated in weanling mice, in the brains of suckling mice or in primary mouse macrophage cultures obtained by peritoneal lavage after stimulation of the animals with starch broth. B814 und LP viruses (TYRRELL and BYNOE, 1965; TYRRELL, BYNOE and HOORN, 1968) were used as pools prepared from infected L132 cells. Avian infectious bronchitis virus (Beaudette strain) was obtained from Mr. R. Henry and was used as infectious suckling mouse brain, or allantoic fluid obtained after the inoculation of 10-day embryonated eggs.

2.3. Antisera

Volunteers were inoculated with coronavirus isolates which had been made from man. The inocula used were either infectious organ culture fluids, or nasal washings taken in broth saline. Paired serum samples were taken from these persons, one before inoculation, and one about three weeks later. Hyperimmune animal sera were raised either in rabbits, by intramuscular injection with incomplete Freund's adjuvant followed by intravenous challenge with the same antigen, or in mice by the production of immune ascitic fluid by the method of Sommerville (SOMMERVILLE, 1967).

Wherever possible, the mice were immunised with suspensions of infected mouse brain to avoid the production of anti-tissue antibody. If this was not possible then animals were inoculated with tissue culture pools of virus prepared from monolayers which had been washed free of serum and then infected. When cytopathic effects appeared in the cultures they were frozen and thawed, concentrated 50-fold by dialysis against polyethylene glycol 'PEG 6000', and clarified by centrifugation. Immune mouse sera against MHV_1 and MHV_3 were prepared by inoculating weanling mice with 0.5—2 MLD_{50} of virus; survivors were exsanguinated four weeks later. The Porton albino mice used had no antibody against MHV_1 or MHV_3 prior to immunization and were highly susceptible to both pathogens.

Sera were inactivated at 56°C for 30 minutes before neutralization tests and complement fixation tests with hyperimmune ascitic fluids. Human and rabbit sera, when used in complement fixation tests, were inactivated at 65°C for 30 minutes to remove any anti-complementary activity.

2.4. Neutralization Tests

Preparations of virus of known titre were diluted in a special diluent containing 50% nutrient broth and 50% Hanks' BSS and a final 0.1% bovine plasma albumin. The diluted suspensions were centrifuged at 3000 *g* for 20 minutes and stored at −70°C. For tissue culture neutralization tests approximately 200 TCD_{50}/ml (in HDCS) or p.f.u./ml (in L132 cells) of the appropriate virus were mixed with equal volumes of dilutions of the inactivated sera. These were maintained at room temperature for 2 hours and then 0.2 ml aliquots were inoculated into roller tube cultures of HDCS cells (WI-38). The tubes were examined for cytopathic effects at 5 and 7 days.

Plaque reduction tests were performed in monolayers of L132 cells in 50 mm Petri dishes. These were inoculated with 0.2 ml of the virus-serum mixture, absorbed for 3 hours at 30°C, and then overlaid with a medium described by BRADBURNE and TYRRELL (1969). Plaques were counted after 6 days incubation at 33°C.

Neutralization tests with MHV_3 virus were performed in a similar manner but the virus was inoculated into cultures of primary mouse peritoneal macrophages. For infectious bronchitis virus (IBV Beaudette) the test system used was primary chick embryo kidney prepared by the method of ESTOLA (1966). Neutralization tests with the OC43 virus were carried out in suckling mice by intracerebral inoculations of the virus-serum mixtures.

For each virus-serum mixture 3 plates of 3 tubes were used in tissue culture neutralization tests, and at least 5 mice in mouse neutralization tests. Neutralization end-points in plaque reduction tests were determined as the dilution of serum which would just produce a 50% reduction in the control plaque count. For tube neutralization tests, end-points were determined by the method of REED and MUENCH (1938).

2.5. Haemagglutination-Inhibition Tests

KAYE and DOWDLE (1969) have reported that high-titre preparations of OC43 and OC38-infected mouse brain agglutinate human '0', rat, mouse and chicken red cells at various temperatures. This was confirmed using antigens made by homogenising infected mouse brain at a 10% concentration in phosphate buffered saline (pH 7.1) and clarifying by centrifugation at 3000 *g* for 10 minutes. Although rat and mouse cells were 4 to 8-fold more sensitive in the haemagglutination test, they often did not settle satisfactorily and therefore 0.5% chicken erythrocytes were used. In haemagglutination-inhibition (HI) tests 4 agglutinating doses of virus were used and the test was performed in microtitre plates using 0.025 ml drop-volumes. Antisera were inactivated at 56°C for 30 minutes and reacted with virus for 1 hour at room temperature. Chicken erythrocytes were then added and allowed to settle for a further 50 minutes; the tests were read immediately.

Attempts were made to detect haemagglutination by other coronavirus antigens, including extracts of mouse brain infected with MHV_1, MHV_3 and AIB viruses. These were all unsuccessful.

2.6. Complement Fixation Tests

These were carried out by the microtitre method of Takatsy (SEVER, 1962) using 0.025 ml volumes. Specific fixation was only revealed when low doses of complement were used and in most tests between 1.5 and 1.75 100% haemolytic units of complement were employed, using overnight fixation at +4°C. The complement dose was determined by preparing close-range dilutions of the solution of complement in triplicate and estimating these in parallel with the rest of the test.

Where possible (for MHV_3, AIB, OC43) 20% suspensions of infected suckling mouse brain were used as CF antigens. These were clarified by centrifugation at 3000 *g*. Antigens for those coronaviruses which could only be cultivated in tissue culture,

were partially purified by gel filtration. Tissue culture pools of virus, prepared in serum-free medium were homogenised, concentrated 10-fold by dialysis against PEG 6000, clarified by centrifugation, and filtered on a column of Sephadex G-200 gel. Material which had been excluded by the gel was concentrated by dialysis and frozen at −20°C until use.

2.7. Gel-diffusion Tests

These were done by the micro-method of Crowle using 1% Agar No. 1 (Oxoid) in phosphate buffered saline (pH 7.1) containing 1% phenol and 0.1% sodium azide. Tests were set up on microscope slides using "Perspex" templates. The reagents were allowed to diffuse for 48–96 hours at room temperature. Then the templates were removed and the slides were washed overnight in PBS and photographed by dark-ground illumination.

3. Results

3.1. Neutralization Tests

Paired sera taken from volunteers who had been infected with the LP or the 229E virus showed significant rises in neutralizing antibody against both viruses by both roller-tube and plaque-reduction tests. An antibody response to the

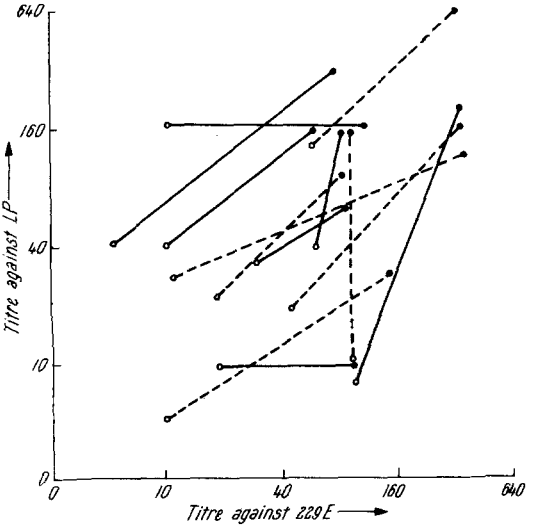

Fig. 1. The correlation between antibody rises to 229E and to LP viruses in paired sera taken from volunteers showing rising antibody titres to the virus with which they were infected. Volunteers given ——— 229E, - - - - - LP, Preserum O, Postserum ●

homologous virus was usually accompanied by a rise in titre to the heterologous virus (see Fig. 1). Antibody rises detected after infection of volunteers with the B814 virus or with OC43 virus only related to the homologous virus. These results are expressed in Table 1. It should be emphasized that only a limited number of sera were available from volunteers with B814 and OC43 infections and were not screened for antibody rises against the infecting strain because of difficulties in manipulating the viruses. Five and 6 pairs of sera showing greater than 4-fold rises in antibody titre to the 229E and LP viruses, respectively, were also tested against MHV_3 and AIB; no rises in neutralizing antibody directed against MHV_3 were detected and none of the sera developed any antibody to AIB even at a serum dilution of 1 in 5.

The immune response detected against 229E and LP viruses in these volunteer sera were not necessarily greatest against the homologous virus as shown in

Fig. 1. One cannot be sure whether antibody rises detected in human volunteers are the result of the virus infection given experimentally. They may be influenced by previous infections with related viruses. To clarify this situation, hyperimmune ascitic fluids from mice exposed to only one coronavirus were studied. Where possible, plaque reduction tests were employed as these had proved to be a more sensitive assay than the neutralization of cytopathic effects in roller tube cultures. The results are shown in Table 2.

Sera directed against LP virus neutralized 229E but antiserum to 229E virus almost failed to neutralize LP in tube neutralization tests. Mouse ascites stimulated by OC43 virus neutralized the 229E virus, but to a 10- to 20-fold lower dilution than OC43. Ascitic fluid from mice hyperimmunized with 229E virus did not neutralize the effects of OC43 virus in suckling mice, but it neutralized LP plaques repeatedly to between 1/5th and 1/10th of the homologous titre. Hyperimmune ascitic fluids against B814, MHV_3 and AIB (Beaudette) viruses did not neutralize OC43, 229E or LP viruses.

Table 1. *Rising Neutralizing Antibody Titres to 229E and LP Viruses after Infection of Volunteers with Various Coronaviruses*

Volunteers given:	No. of sera showing 4-fold or greater rises against:		Number tested (pairs)
	229E	LP	
229E[1]	18	16	18
LP[1]	20	20	20
OC43[2]	1	1	14
B814[2]	0	0	10

Of 14 paired sera from volunteers given OC43 virus, 5 had 4-fold or greater rises in HI antibody titres to OC43.

Of 10 paired sera from volunteers given B814 virus 3 had 4-fold rises in neutralizing antibody titres to B814.

[1] Sera selected because they showed significant rises to the homologous virus.

[2] Sera selected from volunteers with colds.

The cross reactions were further confirmed by performing kinetic neutralization tests and determining the neutralization constants (k) for each hyperimmune serum (diluted and inactivated 1 in 5 in saline) against LP and 229E viruses. There was only a small non-neutralizable fraction (0.01—0.5%), of virus. The "k" values indicate that an antiserum to LP virus neutralizes LP and 229E at roughly the same rate, while antisera against the 229E virus neutralize LP less rapidly than 229E. These results (Table 3) are thus very similar to previous tests.

The effects of complement on the plaque-reduction assay for the titration of antisera to the 229E and LP viruses were investigated as complement can have a marked effect on the neutralization of some enveloped viruses. Virus-serum mixtures were made in a diluent containing a final 25% of fresh rabbit serum. This diluent had no inhibitory effect on the viruses during the two-hour reaction period, as compared with dilutions made in a medium containing 25% heated rabbit serum. Complement was found neither to increase nor decrease the titres

of antisera obtained against 229E and LP viruses in its absence. Furthermore, heterologous reactions were no more prominent than in the absence of complement.

Various anti-human sera were tested against these two viruses in the same system, as anti-host antisera have been shown to neutralise infectious bronchitis virus (BERRY and ALMEIDA, 1968). Of 5 such sera tested, only one serum had any activity. This serum had been prepared in rabbits by the method already described. The antigens used for intramuscular injection were uninfected HDCS lung cells, and the intravenous injection was of an extract of normal human embryo lung. 229E was neutralised, but only when complement (rabbit serum) was present; LP was unaffected. The titre against 229E was 10; the cytotoxic titre to L132 cells was 1 in 3 and the agglutinin titre for human erythrocytes was 2560.

Table 2. *Reciprocal Neutralization Titres of Antisera Directed against Various Coronaviruses*

Serum against	Virus used in neutralization test					
	229E (PR)	LP (PR)	B814	OC43 (SM)	MHV_3 (SM)	AIB (PR)
229E	*1600*	80	5	5	5	5
LP	10240	*5120*	5	5	5	5
B814	5	5	*640*	5	5	5
OC43	160	5	ND	*5120*	5	5
MHV	5	5	ND	5	*1000*	5
AIB	5	5	ND	5	5	*40*

PR = 50% plaque reduction titre. SM = 50% end-point titre in suckling mice. All tests used between 20 and 100 infectious units of virus.

3.2. Haemagglutination Inhibition

KAYE and DOWDLE (1969) report that OC38 and OC43 mouse-brain antigens react with polyvalent antiserum to mouse hepatitis virus in the HI test. This cross reaction has been confirmed in these studies when mouse antisera to mouse hepatitis viruses MHV_1 and MHV_3 viruses were tested by HI; these had HI titres of 40 and 10, respectively. These sera both had titres of greater than 5000 in mouse neutralization tests. Hyperimmune antisera to other coronaviruses failed to give any reaction at dilutions of 1 in 2.5 or greater.

Paired sera which had been taken from volunteers before and after infection with various coronaviruses, except OC38 and OC43, were tested by HI against OC43. Of 70 such sera 10 (14%) had 4-fold or greater rises by HI.

3.3. Complement Fixation Tests

At an early stage in the investigation it was found that the results of complement fixation tests using coronavirus antigens were seldom constant or repeatable. This non-reproducibility has been reported by others (MCINTOSH *et al.*, 1969). The titres of the antisera used were found to be exceptionally dependent upon the exact quantity of complement used during the overnight fixation. Using the standard quantity of complement (two haemolytic units) only high

titre hyperimmune sera and potent antigens gave complete fixation. Using lower doses of complement the titres of some, but not all, sera increased considerably. Therefore all the tests quoted have been repeated to confirm the results, with the dose of complement usually kept at 1.75 units.

The dependence of such tests on the quantity of complement used was demonstrated by using a single antigen (MHV_3-infected mouse brain) with five different antisera at various dilutions of complement. The results of this are shown in Fig. 2. It can be seen that, apart from the serum resulting from a normal infection with MHV_3, the sera do not have a uniform response over the complement range of 2 to 1.25 units and show maxima and minima in their reactions. This variation in complement-fixing equivalence is reproducible in terms of the maxima and minima shown but not in the actual titres obtained, probably because of the difficulty in the exact determination of the dose of complement. At about 1.5 units of complement, most of the sera are showing their maximum reactivity.

Table 3. *Neutralization Constants (23° C) for Hyperimmune Anti-coronavirus Antisera against 229E and LP Viruses*

Hyperimmune serum used	Virus used	
	229E	LP
Anti-229E	5.75	0.28
Anti-LP	7.22	4.60
Anti-B814	0.07	0.08
Anti-OC43 (1)	0.83	0.05
(2)	0.14	0.05
Anti-MHV_3	0.03	0.05
Anti-AIB	0.01	0.05

However, this dose of complement is lower than the optimum for reactions with LP and 229E antigens, which is about 1.8 units; for OC43 the optimum is about 1.65 units. Thus it is very difficult to use a number of coronavirus antigens with a single dose of complement. So, although tedious, the best results were obtained when three-dimensional complement fixation tests were employed.

Using crude tissue culture fluids as antigens, very few volunteer convalescent sera showed detectable levels of complement fixation. Therefore tissue culture fluids were concentrated 10- to 20-fold by dialysis, but the resulting fluids were often anti-complementary. It was found that the anti-complementary activity was removed by filtration on "Sephadex" G-200 gel, as described in "Materials and Methods". Using these antigens, significant antibody rises to LP were found in 8 of 20 serum pairs taken from volunteers who had colds induced by the 229E or LP viruses. As with neutralizing antibody rises, infection with either of these viruses produced a rise in complement-fixing antibody to both. Of 18 serum pairs taken from volunteers infected with 229E or LP viruses, none had rising CF titres to OC43, but 4 pairs showed rises to MHV_3.

Hyperimmune ascitic fluids produced against those coronaviruses which could be grown only in human tissue culture cells contained anti-human activity. How-

ever, these hyperimmune ascitic fluids had at least 4-fold higher titres against antigens prepared from infected L132 cells than against uninfected tissue culture antigens. Ascitic fluids and antisera prepared in mice using infected mouse brain inocula (for the AIB, MHV_3 and OC43 viruses) did not fix complement, at dilutions of 1/10 or greater, with normal mouse brain or with uninfected L132 cell antigens.

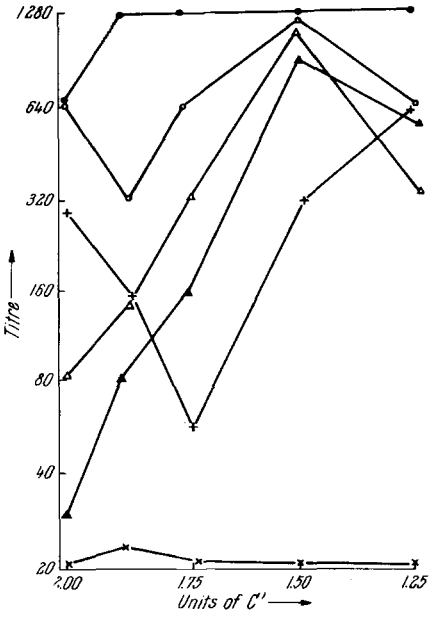

Fig. 2. The effect of the dose of complement (as 100 % haemolytic units) on the reaction of coronavirus antisera with an MHV_3 mouse-brain antigen

Ascites of serum against	Animal	Resulting from
MHV_2	Mouse	Hyperimmunization
MHV_3	Mouse	Experimental infection
MHV_1	Mouse	Experimental infection
229 E	Mouse	Hyperimmunization
Normal mouse brain	Mouse	Hyperimmunization
LP	Human	Experimental infection

Chequer-board complement fixation tests were performed on the same day; all the hyperimmune sera were set up against all the antigens and several cross-reactions were revealed. These are shown in Table 4. Chequer-board titrations were necessary because prozones occurred when high-titre sera were tested. These reactions were reproduced in repeated tests, but the titres obtained were not identical.

Homologous mixtures usually fixed complement well, but heterologous reactions often used less than 1 unit of complement; that is "4+ fixation" was never observed.

Antisera to LP, 229 E and B 814 viruses fixed complement with OC43 antigens, and antiserum to the OC43 virus fixed complement with antigens from these

viruses. The MHV_3 virus also showed reciprocal cross-reaction with 229E, LP and OC43 viruses using the hyperimmune ascites, and the MHV_3 antigen also fixed complement with the B814 ascitic fluid.

3.4. Gel-diffusion Tests

Precipitin lines were detected with human sera and coronavirus antigens prepared from tissue cultures only when the latter were concentrated 10- to 20-fold. When such antigens were reacted with convalescent sera from volunteers who had been given 229E virus, 2 precipitin lines against the 229E and LP antigens were regularly seen. These lines were fully developed after about 48 hours and there were reactions of identity between the lines produced by the two antigens. However, sera taken from volunteers after infections with LP virus produced

Table 4. *Cross Reactions between Coronaviruses Detected by Complement Fixation*

Mouse ascites against	Antigens (produced in L132 cells)				Antigens (produced in mice)			
	229E	LP	B814	Normal L132	OC43	MHV_3	AIB	Normal brain
229E[1]	*640*	NR	NR	60	40	40	<10	<10
LP	NR	*1280*	NR	640	40	15	<10	<10
B814	NR	NR	*640*	60	30	15	<10	<10
OC43	20	40	60	<10	*1280*	40	<10	<10
MHV_3	80	20	30	<10	20	*640*	10	<10
AIB	<10	<10	<10	<10	<10	<10	*60*	<10
Normal mouse serum	<10	<10	<10	<10	<10	<10	<10	<10

The reciprocals of titres obtained in complement fixation tests using hyperimmune mouse ascitic fluids and various coronavirus antisera. In all tests 1.5—1.75 units of complement were used, and 3—6 units of antigen.

[1] Ascites produced by inoculating mice with virus grown in L132 cells; therefore anti-human reactions may be involved in the complement fixation with antigens produced in these cells. NR = results not reliable because of anti-tissue reactions.

3 lines of precipitation with LP antigens, two of which were identical with lines produced with 229E antigens. The third line, produced against the LP and not 229E antigens was, like the others, seldom produced by volunteer sera taken prior to infection, but was occasionally produced by convalescent sera taken after a 229E virus infection. The B814 virus antigen seldom produced lines of precipitation with human sera, even with convalescent sera from volunteers who developed severe colds after inoculation with this virus. In the reactions that were observed, only single lines were detected.

The results with animal antisera to 229E, LP and B814 viruses and their homologous antigens were not amenable to a reliable interpretation because of the profusion of anti-tissue lines which appeared. On the other hand, hyperimmune virus-specific mouse ascites produced against AIB (1 serum), MHV_3 (3 sera) and OC43 (3 sera) gave clear lines in the homologous reactions, as seen in Fig. 3. Using these sera, the cross-reactions observed by neutralization and complement fixation tests were corroborated. It is interesting to note that very few of the heterologous lines produced showed reactions of identity with homolog-

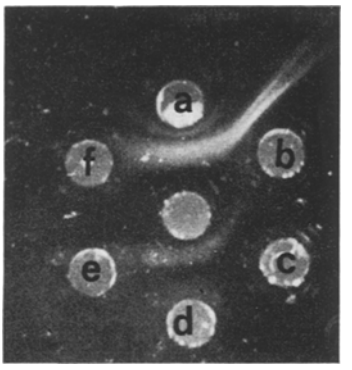

Fig. 3a

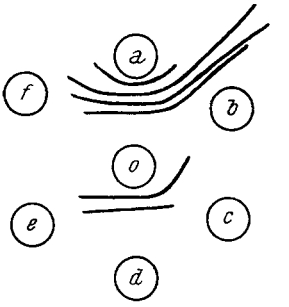

Fig. 3b

Fig. 3. Results of an immunodiffusion test with coronavirus antigens and hyperimmune mouse ascitic fluids.

Well b – ascitic fluid produced by hyperimmunising adult mice with ether-treated OC43 antigen.
o – ascitic fluid produced in mice after recovery from an infection with OC43 virus.
f – control ascitic fluid.
a – OC43 mouse-brain antigen.
c – normal L132 cell antigen.
d – LP antigen produced in L132 cells.
e – normal mouse brain antigen.

The line drawing is a representation of the lines seen on the original immunodiffusion plate; not all these lines are visible or separately resolved in the photograph

Table 5. *Cross-reactions Detected between Coronaviruses by Immunodiffusion. The Numbers of Precipitin Lines Obtained in the Reactions between Various Coronavirus Antigens and Antisera*

Serum or ascites	Antigens					
	229E	LP	B814	OC43	MHV_3	AIB
Hyperimmune 229E	—	—	—	0	1	0
Human after 229E	2	2 (show identity)	0	0	0	0
Hyperimmune LP	—	—	—	2	1	0
Human after LP	2	3 (show identity)	0	0	0	0
Hyperimmune B814	—	—	1	0	0	0
Human after B814	0	0	1	0	0	0
Hyperimmune OC43 (specific)	0	1	0	4	0	0
Hyperimmune MHV_3 (specific)	1	0	0	0	2	0
Hyperimmune AIB (specific)	0	0	0	0	0	2

— = not included because of the presence of strong lines against tissue culture components. Except where indicated, reactions of complete or partial identity were not observed.

ous lines for the same virus. The reasons for this are not known; however, the lines were not produced against control antigens, or with control sera.

A summary of the immuno-diffusion results is seen in Table 5. Precipitin lines were observed with some human sera against strong OC43 mouse-brain antigens, but human sera were never shown to react with MHV_3 or AIB antigens. This may reflect the fact that comparatively weak antigens were made with these two viruses and that they gave only two precipitin lines against the homologous hyper-immune sera, although three precipitin lines were obtained with AIB by TEVETHIA and CUNNINGHAM (1968).

Table 6. *Summary of Antigenic Cross-reactions amongst Coronaviruses*

Virus or antigen:	229E	LP	B814	OC43	MHV_3	AIB
Sera against:						
229E	H	+ (n, c, g)	—	—	+ (c, g)	—
LP	+ (n, c, g)	H	—	—	—	—
B814	—	—	H	—	+ (c)	—
OC43	+ (n, c)	+ (c, g)	—	H	+ (c)	—
MHV_3	+ (c, g)	+ (c, g)	—	+ (c)	H	—
AIB	—	—	—	—	—	H

H = homologous reaction, n = neutralization, c = complement fixation, g = gel-diffusion.

4. Discussion

These studies have demonstrated that viruses which were grouped together as "coronaviruses" because they were morphologically similar show several serological similarities as well. A summary of the cross-reactions detected in these studies is shown in Table 6. The cross-reactions found have not revealed common or group antigens as are found with the influenza viruses, but rather haphazard inter-relationships like those found between the different parainfluenza viruses, mumps and Newcastle disease virus. As might be expected, the less specific serological tests of immuno-diffusion and complement fixation produced more evidence of cross-reaction than did neutralization and HI tests.

Some of these results confirm other studies on antigenic relationships amongst coronaviruses (MCINTOSH *et al.*, 1969; KAYE and DOWDLE, 1969) particularly in that avian infectious bronchitis virus does not seem to be related to the others by any of the tests used.

The viruses tested here showed some anomalies in their neutralization by antisera. Neutralization of a virus may involve several immunological reactions and not enough is known about the neutralization of coronaviruses. Complement or fresh rabbit serum will enhance the neutralization of, for example, rubella (NEVA and WELLER, 1964) and respiratory syncytial virus (STOTT *et al.*, 1967); nevertheless

the human coronaviruses were not neutralized by antiviral antisera to any greater degree when complement was added to the system. This contrasts with the neutralization of avian infectious bronchitis virus in which complement increases the neutralization index of some sera by up to three orders of magnitude (BERRY and ALMEIDA, 1968). This virus is also neutralized by anti-host cell antisera which in the presence of complement (unheated guinea-pig serum) produce 80 Å holes in the virus envelope. These holes are also produced by heterotypic (rabbit) but not by homotypic (chicken) antisera. Anti-human antisera did not produce any significant neutralization of either the 229E or LP viruses and complement holes are produced in the envelope of 229E virus by both homotypic and heterotypic (human) antisera (ALMEIDA and BRADBURNE, unpublished data). This suggests that these two viruses differ somewhat in assembly from infectious bronchitis virus, though such differences have not been detected in electron-microscopic studies by other workers (BECKER *et al.*, 1967). This could be due, amongst other things, to differences in surface structure or in susceptibility to complement enzymes.

The complement fixation and haemagglutination-inhibition results suggest that MHV possesses several antigens which it shares with one or other of the human strains. This work shows that the 229E and LP viruses are closely related but distinguishable serologically and that they cross-react with MHV_3; also OC43 antisera can neutralize 229E. This does not support the suggestion by MCINTOSH *et al.* (1969) that 229E-like viruses form one uniform group serologically, and that the OC43—38 group are a more heterogeneous collection, being serologically distinct from 229E and characterized by their cross-reactions with MHV strains.

The use of human sera in coronavirus serology has been shown to be of only limited value in differentiating between infections of the various human coronaviruses because of the heterologous rises which can be found in these sera. A specific antibody response probably only occurs on the first exposure to a member of the coronavirus group, and there is evidence that this response is poor, and may be boosted by later immunological stimulation.

Of the two animal coronaviruses, infectious bronchitis virus seems to be antigenically far more distant from the human coronaviruses than mouse hepatitis virus (MHV_3). This fits well with the observations of HARTLEY *et al.* (1964) that human sera contain antibody to MHV which may rise during an infection, while MILLER and YATES (1968) detected no antibody, in human sera, to infectious bronchitis virus except in persons who were in contact with poultry.

Studies on the antigenic structure of the coronaviruses are continuing so that the antigens responsible for these cross-reactions may be isolated.

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