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Enzyme-linked Immunosorbent Assay for Coronaviruses HCV 229E and MHV 3

By CORNELIS A. KRAAIJEVELD, M. HILARY MADGE AND MALCOLM R. MACNAUGHTON
Division of Communicable Diseases, Clinical Research Centre, Harrow, Middlesex HA1 3UJ, U.K.

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SUMMARY

The antigenic relationship between human coronavirus strain 229E (HCV 229E) and mouse hepatitis virus strain 3 (MHV 3) was studied by means of the indirect form of enzyme-linked immunosorbent assay (ELISA). A cross-reaction was found with hyperimmune rabbit sera between HCV 229E and MHV 3 which may be due to the adherence of bovine serum components from tissue culture media, which were present on virus particles even after extensive purification. No cross-reaction was observed with immune sera absorbed with bovine serum, or with HCV 229E grown in tissue culture without serum. This indirect ELISA with HCV 229E may prove to be useful for studies with human sera.

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) was first described by Engvall & Perlmann (1971) and van Weemen & Schuurs (1971). Since then variations of the test have found wide application in the detection of antibodies and small amounts of antigen (Bidwell *et al.* 1977). In diagnostic serology, enzyme-immunoassays are highly sensitive and relatively easy to perform and seem to be especially suited to large-scale epidemiological surveys. However, the high sensitivity of ELISA means that the specificity of the antigens used is of major importance.

Detection of antibodies against coronaviruses by neutralization, complement fixation, immunofluorescence or immunodiffusion is cumbersome and generally not very reliable (McIntosh, 1974). Therefore, we attempted to see if an ELISA technique with human coronavirus strain 299E (HCV 299E) and mouse hepatitis virus strain 3 (MHV 3) could be used as a sensitive, specific test for detecting antibodies in animal hyperimmune sera.

Bradburne (1970) found a cross-reaction between HCV 229E and MHV 3 with hyperimmune animal sera in complement fixation and immunodiffusion tests. Cross-reactions between these viruses were not found by other workers (McIntosh *et al.* 1969; Pedersen *et al.* 1978). Antibodies reacting with MHV have been detected in human sera and are probably responses to related human coronavirus antigens (Hartley *et al.* 1964; McIntosh, 1974).

In this study we present evidence showing that cross-reactions between HCV 229E and MHV 3 may be caused by antigenically related serum components from tissue culture media adhering to both viruses.

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METHODS

Preparation of virus antigens. The virus strains used and the preparation of purified virus suspensions for biochemical and electron microscopic analysis have been described previously (Macnaughton & Madge, 1978; Macnaughton *et al.* 1978). HCV 229E was grown in monolayer cultures of human cells of the MRC continuous (MRCc) line at 33 °C for 32 h in Eagle's BME with 2% newborn calf serum (Flow Laboratories, Irvine, Scotland) or with L15 medium with 2% rabbit serum – these were called antigens HCV 229E (N) and HCV 229E (R) respectively. One batch of virus was grown without serum in the tissue culture medium. MHV 3 was grown in confluent monolayers of secondary mouse embryonic fibroblasts at 37 °C for 72 h in Eagle's MEM with 2% foetal calf serum (Flow Laboratories) – this was called antigen MHV 3 (F). Cell cultures containing HCV 229E and MHV 3 particles were frozen and thawed three times.

All the purification steps were performed at 0 to 4 °C. The virus suspensions of freeze-thawed cells were clarified at 2000 *g* for 30 min and then pelleted at 75000 *g* for 1 h. Each pellet was resuspended in 1 ml of Dulbecco's phosphate-buffered saline 'A' (PBSA), overlaid on to a linear 25 to 55% (w/w) sucrose gradient in PBSA and centrifuged at 90000 *g* for 16 h. Virus peaks of density 1·18 g/ml were collected, diluted in PBSA and layered on to another linear 25 to 55% (w/w) sucrose gradient in PBSA and centrifuged at 90000 *g* for 16 h. The number of virus particles in peak fractions at 1·18 g/ml were determined by electron microscopy. Virus suspensions for use in ELISA contained about 10^{10} particles/ml and were stored in small portions at −70 °C.

In a few experiments sonicated virus was used as an antigen. Immediately before use a virus suspension was diluted 1:100 in PBSA to a vol. of 2·5 ml. The virus suspension was sonicated with a Dawe Soniprobe Type 7532A at 65 W for periods of up to 90 s.

Preparation of disrupted cells. Coronaviruses mature on membranes of internal vesicles (Tyrrell *et al.* 1978). Membrane lipids are incorporated in the virus particle and may be immunogenic as suggested by Berry & Almeida (1968) for glyco-lipids of IBV strains. Therefore, uninfected cells were sonicated to expose internal cellular antigens. Confluent monolayers of secondary mouse embryo fibroblasts and MRCc cells were freeze-thawed three times and then washed three times with PBSA. The freeze-thawed cells were sonicated in 2·5 ml PBSA with a Dawe Soniprobe for 30 s at 65 W. Sonicated cell material was used as a coating antigen and for absorbing animal immune sera.

Preparation of animal antisera. Immune sera against HCV 229E and MHV 3 were prepared in adult New Zealand white rabbits. A 0·5 ml amount of purified coronavirus at a concentration of 10^{10} virus particles/ml was mixed with an equal volume of Freund's complete adjuvant (FCA) and injected intracutaneously at up to 20 different places in the shaven back of rabbits. Two days before immunization animals received 0·5 ml *Bordetella pertussis* vaccine (The Lister Institute of Preventive Medicine, Elstree, U.K.) intracutaneously as an additional adjuvant. Each animal was bled before and 6 weeks after immunization. The sera were stored at −20 °C. The code used for the different antisera is as follows: serum from a rabbit immunized with HCV 229E grown with newborn calf serum, anti-HCV 229E (N); serum from a rabbit immunized with HCV 229E grown with rabbit serum, anti-HCV 229E (R); serum from a rabbit immunized with MHV 3 grown with foetal calf serum, anti-MHV 3 (F).

Absorption of hyperimmune animal sera with bovine tissue culture sera. Equal volumes of bovine tissue culture sera were mixed with hyperimmune animal sera and incubated for 18 h at 4 °C. Mixtures were stored at −20 °C. A dilution of 1:50 of the serum mixture was equivalent to a dilution of 1:100 for immune and control sera.

ELISA procedure. A modification of the method described by Voller *et al.* (1976) was used,

Table 1. *ELISA of HCV 229E (N)* against dilutions of anti-HCV 229E (N) and anti-MHV 3 (F)*†

Serum dilution	Anti-HCV 229E (N)		Anti-MHV 3 (F)	
	Immune	Pre-immune	Immune	Pre-immune
1:300	1·68	0·01	0·30	0·03
1:1000	0·94	0·00	0·14	0·00
1:3000	0·55	0·00	0·05	0·00
1:10000	0·18	0·00	0·01	0·01

* HCV 229E (N) dilution 1:400.
† Data are given as absorbance values at 405 nm after 30 min.

Flat-bottomed wells in polystyrene microtitre plates (Cooke Microtitre M29 AR, Dynatech Ltd., Billingshurst, Sussex, U.K.) were coated with antigen by the addition of 0·2 ml of the antigen, diluted to optimal concentration, in 0·1 M-carbonate–bicarbonate buffer, pH 9·6, and incubated overnight at room temperature. Before coating, the plates were washed four times in PBSA. After incubation they were washed four times with PBS containing 0·05 % Tween 20 and 0·02 % sodium azide (PBST) and shaken dry. Coated plates were immediately used.

Test sera were diluted in PBST and 0·2 ml of each dilution was added to duplicate wells. The sera were incubated with the antigen for 3 h at room temperature. After incubation the plates were washed four times in PBST and shaken dry. Conjugate (see below) was then added in 0·2 ml quantities and left overnight at room temperature. The plates were covered with parafilm for all incubations. After four further washes in PBST, 0·2 ml of the substrate (see below) was added to each well. The enzyme-mediated reaction was stopped after 30 min by the addition of 0·05 ml of 3 M-NaOH.

The absorbance value of the fluid was measured at 405 nm in a Unicam SP 500 spectrophotometer. Residual substrate solution was used as a blank. A serum was considered positive for specific antibodies when the ratio between the absorbance value of immune serum and the absorbance value of pre-immune serum of the same dilution was 2·0 or greater.

Phosphatase substrate. This was a 0·1 % solution of *p*-nitrophenyl phosphate in 10 % (w/v) diethanolamine buffer, pH 9·8, with 0·02 % sodium azide and 0·01 % $MgCl_2.6H_2O$.

Conjugate. The conjugate was a commercial goat anti-rabbit IgG antiserum labelled with alkaline phosphatase (Miles Laboratories Ltd., Stoke Poges, Berks., U.K.). The dilutions of 1:400 to 1:800 recommended by the manufacturer, proved to be the most useful.

RESULTS

Antigen concentration for the indirect ELISA

Optimal dilutions of all virus antigens for the indirect enzyme-immunoassay were determined by chequerboard titrations. Antigen dilutions of 1:50 and 1:150 gave high absorbance values with the positive serum although there were significant background reactions with control serum. When the virus suspension was diluted to 1:500 the absorbance values were still high for immune serum and acceptably low values were found for the control or pre-immune serum. In all cases antigen dilutions of 1:300 to 1:600 proved to be satisfactory.

Cross-reaction in the indirect ELISA between HCV 229E and MHV 3

Antisera prepared in rabbits to HCV 229E (N) and MHV 3 (F) were tested against HCV 229E (N) for homologous and heterologous antibodies. As shown in Table 1 there was a

Table 2. *ELISA of MHV 3 (F)* against dilutions of anti-MHV 3 (F) and anti-HCV 229E (N)†*

	Anti-MHV 3 (F)		Anti-HCV 229E (N)	
Serum dilution	Immune	Pre-immune	Immune	Pre-immune
1:300	1·14	0·11	0·96	0·12
1:1000	1·07	0·03	0·41	0·04
1:3000	0·45	0·01	0·12	0·01
1:10000	0·22	0·01	0·04	0·01

* MHV 3 (F) dilution 1:600.
† Data are given as absorbance values at 405 nm after 30 min.

Table 3. *ELISA of newborn and foetal calf serum against dilutions of anti-MHV 3 (F) and anti-HCV 229E (N)**

	Newborn calf serum† against			
	Anti-MHV 3 (F)		Anti-HCV 229E (N)	
Serum dilutions	Immune	Pre-immune	Immune	Pre-immune
1:200	1·30	0·01	> 2·00	0·00
1:2000	0·13	0·00	0·77	0·00
	Foetal calf serum‡ against			
	Anti-MHV 3 (F)		Anti-HCV 229E (N)	
	Immune	Pre-immune	Immune	Pre-immune
1:200	1·03	0·01	0·78	0·05
1:2000	0·11	0·00	0·15	0·01

* Data are given as absorbance values at 405 nm after 30 min.
† Newborn calf serum dilution 1:1000.
‡ Foetal calf serum dilution 1:1000.

strong reaction with the homologous antigen against anti-HCV 229E (N). Antibodies were still detected at the lowest dilution tested, 1:10000. In this and most other experiments very low or even negative absorbance values were measured for pre-immune sera. Cross-reacting antibodies were found with anti-MHV 3 (F) serum (see also Table 4). A 1:100 dilution of anti-MHV 3 contains about the same amount of antibodies to HCV 229E as a 1:3000 dilution of the homologous serum, assuming that absorbance values in the same experiment were representative and comparable for a serum's content of specific antibodies.

Table 2 shows the reaction of MHV 3 (F) against anti-MHV 3 (F) and anti-HCV 229E (N). The highest absorbance values were found with anti-MHV 3 (F) although there were also cross-reactions with anti-HCV 229E (N).

Detection of serum components on HCV 229E and MHV 3 using ELISA

No positive reactions were found with sonicated cell suspensions used as coating antigens in chequerboard titrations with anti-MHV 3 (F) and anti-HCV 229E (N). Furthermore, anti-MHV 3 (F) and anti-HCV 229E (N) absorbed with sonicated cells and tested for antibodies against whole virus suspensions showed no significant differences in absorbance values from unabsorbed immune sera.

Table 3 shows the reaction of foetal and newborn calf serum, used as antigens, against anti-HCV 229E (N) and anti-MHV 3 (F). Although positive reactions were obtained with both immune sera, anti-HCV 229E (N) reacted much more strongly against newborn calf serum than foetal calf serum. The reaction of both sera against anti-MHV 3 (F) was similar. These results suggest that bovine serum components from tissue culture media adhere to

Table 4. *ELISA of HCV 229E (N) and MHV 3 (F) against dilutions of anti-HCV 229E (N) and anti-MHV 3 (F)**

		HCV 229E (N)† against			
		Anti-HCV 229E (N)		Anti-MHV 3 (F)	
Serum dilutions	Absorption with newborn calf serum	Immune	Pre-immune	Immune	Pre-immune
1:100	Not absorbed	1·90	0·17	0·80	0·16
	Absorbed	0·30	0·16	0·17	0·14
1:300	Not absorbed	1·65	0·15	0·53	0·12
	Absorbed	0·21	0·13	0·13	0·12
		MHV 3 (F)‡ against			
		Anti-HCV 229E (N)		Anti-MHV 3 (F)	
	Absorption with foetal calf serum	Immune	Pre-immune	Immune	Pre-immune
1:100	Not absorbed	0·30	0·19	1·11	0·24
	Absorbed	0·14	0·17	0·94	0·22
1:300	Not absorbed	0·20	0·15	0·89	0·16
	Absorbed	0·07	0·12	0·70	0·15

* Data are given as absorbance values at 405 nm after 30 min.
† HCV 229E (N) dilution 1:400.
‡ MHV 3 (F) dilution 1:500.

Table 5. *ELISA of HCV 229E (R)* against dilutions of anti-HCV 229E (R) and anti-MHV 3 (F)†*

	Anti-HCV 229E (R)		Anti-MHV 3 (F)	
Serum dilution	Immune	Pre-immune	Immune	Pre-immune
1:100	1·27	0·10	0·15	0·09
1:300	1·04	0·07	0·09	0·07
1:1000	0·88	0·04	0·12	0·05
1:3000	0·69	0·03	0·02	0·03

* HCV 229E (R) dilution 1:300.
† Data are given as absorbance values at 405 nm after 30 min.

both MHV 3 and HCV 229E and that these serum components are highly immunogenic and may be responsible for the cross-reactions observed between these viruses.

Table 4 shows the effect on the reaction of absorbing immune and pre-immune sera with newborn and foetal calf serum. The cross-reaction of HCV 229E (N) against anti-MHV 3 (F) was abolished after absorbing anti-MHV 3 (F) with newborn calf serum, while the reaction of MHV 3 against anti-MHV 3 (F) was only slightly reduced after absorbing anti-MHV 3 (F) with foetal calf serum. However, the low cross-reaction of MHV 3 (F) against anti-HCV 229E (N) completely disappeared after absorption of anti-HCV 229E (N) with foetal calf serum. Furthermore, the absorption of anti-HCV 229E (N) with newborn calf serum removed all significant activity against HCV 229E (N). This result varied with different HCV 229E (N) preparations and its significance is discussed later.

The indirect ELISA with HCV 229E grown with rabbit serum

Serum used from a rabbit immunized with HCV 229E (R) had a titre of more than 1:3000 against HCV 229E (R) (Table 5). This reaction could not be absorbed with either rabbit serum or sonicated washed MRCc cells. However, there was a strong reaction of newborn calf serum against anti-HCV 229E (R) which could be completely absorbed out with

Table 6. *ELISA of HCV 229E grown without serum* against dilutions of anti-HCV 229E (R) and anti-MHV 3 (F)*†

Serum dilution	Anti-HCV 229E (R)		Anti-MHV 3 (F)	
	Immune	Pre-immune	Immune	Pre-immune
1:100	1·10	0·16	0·18	0·16
1:300	0·86	0·13	0·18	0·13
1:1000	0·58	0·12	0·17	0·14
1:3000	0·52	0·09	0·17	0·11

* HCV 229E grown without serum dilution 1:100.
† Data given as absorbance values at 405 nm after 30 min.

newborn calf serum. The reaction of HCV 229E (N) against anti-HCV 229E (R) was partially absorbed with newborn calf serum. The absorbance left was probably caused by specific antiviral antibodies. However, it was not possible to absorb the reaction of HCV 229E (R) against either anti-HCV 229E (R) or anti-HCV 229E (N) with newborn calf serum. Thus, these absorbance values probably represent specific antibodies against virus antigens.

Cross-reaction with anti-MHV 3 (F) against HCV 229E (R) and HCV 229E grown without serum

Table 5 shows that there is no significant reaction of HCV 229E (R) with anti-MHV 3 (F). HCV 229E grown without serum in tissue culture medium was a satisfactory antigen for indirect ELISA, producing a titre of more than 1:3000 with anti-HCV 229E (R) (Table 6). No significant reaction was observed between HCV 229E grown without serum and anti-MHV 3 (F).

DISCUSSION

Cross-reactions occurred between MHV 3 (F) and anti-HCV 229E (N), and HCV 229E (N) and anti-MHV 3 (F) (Table 1 and 2) which could be completely absorbed with calf serum (Table 4). These results suggest that the cross-reactions were due to serum components. No cross-reaction was found with MHV 3 (F) against HCV 229E grown with rabbit serum or with no serum (Table 5 and 6). In Table 4 the reaction of HCV 229E (N) against anti-HCV 229E (N) was almost completely removed after absorption with newborn calf serum. The removal of this reaction with newborn calf serum varied considerably with different HCV 229E (N) preparations and may have been due to the degree of masking of the virus antigens, as more of the reaction remained with the same anti-HCV 229E (N) and some other HCV 229E (N) preparations. Similar results were observed with different MHV 3 (F) preparations. It is of interest that bovine serum components adhere to both HCV 229E and MHV 3 even after elaborate purification. As sucrose-gradient centrifugation favours selection of whole particles, it would appear that these serum components attached to the surface structures of these virus particles. The bovine serum components proved to be highly immunogenic in rabbits which may be due to their position on the virus particles and the mixture of these particles with FCA. We do not know at present which serum components bind to the virus particles.

Serum and other components may cause problems with other serological techniques. The cross-reaction observed by Bradburne (1970) between MHV 3 and HCV 229E could be due to serum or cellular components adhering to the viruses. Furthermore, Bradburne & Tyrrell (1971) reported that bovine serum was highly anti-complementary and they obtained better results with foetal calf serum or no serum in the tissue culture media, in agreement with our ELISA results.

The serum components attached to HCV 229E and MHV 3 may also affect the haemagglutination and neutralization of these viruses. The coronaviruses, avian infectious bronchitis virus (IBV) and HCV strain OC43 agglutinate red blood cells (McIntosh, 1974; Bingham *et al.* 1975), and anti-host cell antibody can neutralize IBV (Berry & Almeida, 1968). Haemagglutination has not been found with HCV 229E and MHV 3 (Bradburne & Tyrrell, 1971). The lack of haemagglutination by HCV 229E and MHV 3 may be due to a masking of the relevant virus structures by serum components.

We have found a satisfactory antigen for detection of HCV 229E antibodies in hyperimmune sera of animals and, probably, in human sera. Further studies are in progress to use these HCV 229E antigens with human sera.

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