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Reactivity of Human Coronavirus OC43 and Neonatal Calf Diarrhoea Coronavirus Membrane-associated Antigens

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SUMMARY

Human embryonic lung fibroblast cultures and Vero cell cultures infected with cell culture-adapted strains of human coronavirus (HCV) OC43 or neonatal calf diarrhoea coronavirus (NCDCV) were shown to possess highly cross-reactive membrane-associated antigens (MAA) by the indirect fluorescent antibody technique (IFAMA). MAA appeared 3 h post-infection, concurrently with the appearance of cytoplasmic antigens. Electron microscopic observations of cell cultures infected with either coronavirus strain and labelled with the immunoperoxidase antibody (IPA) technique for MAA detection showed that MAA consisted mainly of a strongly labelled, discontinuous, brush-like layer of amorphous material, strictly associated with the infected cell membrane. By light microscopy, reactivity of MAA with homologous and heterologous immune serum was similar to that of antigens detected by IPA in ethanol-fixed infected cells. IPA and IFAMA, but not haemagglutination-inhibiting (HI) and neutralizing (Nt) antibody, were strongly decreased by absorption of immune sera with trypsin-treated glutaraldehyde-fixed cell cultures infected with homologous virus. MAA IgG antibodies were detected by IFAMA in both human and animal sera. Sera from infants showing an HI and Nt, but not an IPA, antibody response to HCV OC43 were also free of detectable IFAMA antibody to HCV OC43.

Human coronavirus (HCV) OC43 causes upper respiratory tract syndromes in humans (McIntosh *et al.*, 1967; Kaye *et al.*, 1971; Bradburne & Somerset, 1972); neonatal calf diarrhoea coronavirus (NCDCV) is a major cause of diarrhoea in newborn calves (Stair *et al.*, 1972; Mebus *et al.*, 1973). These two coronavirus strains have been shown to possess close antigenic relationships (Gerna *et al.*, 1981). Recently, Robb & Bond (1979) reported the expression of mouse hepatitis virus antigens in plasma membrane of infected cells, but the immunological reactivity of these antigens was not investigated. In this report, the presence of membrane-associated antigens (MAA) in HCV OC43- and NCDCV-infected cells was demonstrated, and their reactivity was investigated by light and immunoelectron microscopy using mouse immune sera.

HCV OC43 and NCDCV were adapted to growth in suckling mouse brain and then in human embryonic lung fibroblast (HELF) cell cultures as described previously (Gerna *et al.*, 1981). Subsequently, HELF-adapted strains were readily adapted to growth in Vero cells (American Type Culture Collection, Rockville, Md., U.S.A.). Cell cultures were repeatedly found to be mycoplasma-free by histochemical and cultural methods. HCV OC43 and NCDCV immune sera were obtained from weanling mice of the Swiss CD-1 strain, using suckling mouse brain-adapted strains, as described previously (Gerna *et al.*, 1981).

The fluorescent antibody technique for detection of MAA (IFAMA) was initially performed on HELF and Vero cell suspensions 24 to 72 h post-infection using mouse immune sera. Subsequently, Vero cell monolayers containing 20 to 60 plaques/microplate well were employed for determination of IFAMA-IgG antibody titres in human and animal sera. After 1 h of virus adsorption at 33 °C in a 5% CO_2 humidified atmosphere, the virus

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inoculum (0·05 ml/well) was replaced by the same plaquing medium (0·15 ml/well) used for the HCV OC43 plaque assay (Gerna *et al.*, 1980). After incubation at 33 °C for 48 and 72 h respectively, HCV OC43- and NCDCV-infected microplate cell cultures were stained for detection of MAA-IgG antibody. Cells were incubated with heat-inactivated test serum [diluted in Eagle's minimal essential medium (EMEM)] at 37 °C for 30 min and then washed three times with Hanks' balanced salt solution (HBSS). In some experiments this incubation was done at 4 °C in order to prevent redistribution of MAA. Cells were then fixed with ethanol at room temperature and washed again. Thereafter, the fluorescein-conjugated IgG fraction directed against the appropriate animal species (Cappel Laboratories, Cochranville, Pa., U.S.A.) was added, and incubation continued for an additional 30 min at 37 °C. In some experiments, results obtained in Vero cell cultures with the IFAMA-IgG test were compared with those achieved in HELF microplate cell monolayers using the immunoperoxidase antibody technique for detection of MAA-IgG antibody (IPAMA-IgG). The only differences in the IPAMA-IgG as compared to the IFAMA-IgG test were the use of a peroxidase-conjugated antiserum (Cappel Laboratories) and the addition, after the second step of the reaction, of the diaminobenzidine–hydrogen peroxide colour developing system. The specificity of both tests was checked by: (i) incubation of infected cells with serum diluent (EMEM) or non-immune serum, followed by addition of the appropriate anti-species conjugate; (ii) incubation of uninfected cells with immune serum and appropriate conjugate; (iii) incubation of HCV OC43- and NCDCV-infected cells first with human and bovine immune serum and, secondly, with homologous mouse immune serum followed by the addition of anti-mouse conjugate (blocking test).

The immunoperoxidase antibody (IPA) technique was used for the immunoelectron microscopic study of MAA. In some experiments, HELF and Vero living cell cultures infected with either coronavirus strain were processed 48 h post-infection for the IPA technique, before fixing and embedding. In other experiments, before IPA labelling, infected Vero cell cultures were treated 48 h post-infection with 0·25% trypsin for up to 60 min (to remove virus particles adhering to the cell membrane) and then fixed with 0·1% glutaraldehyde for 5 min (to prevent release of new virus particles). In either case, the IPA technique was performed as follows. In the first step of the reaction, cell cultures were incubated for 30 min at 4 °C (living cells) or 37 °C (fixed cells) with optimal dilutions of HCV OC43 and NCDCV mouse immune serum (containing 20 to 40 IPA-IgG antibody units); in the second step, peroxidase-conjugated anti-mouse IgG (Cappel Laboratories) at its optimal dilution (as determined by chequerboard titration) was added and incubation continued for 30 min, as in the first step. After addition of the diaminobenzidine–hydrogen peroxide colour developing system, cells were fixed with 2·5% glutaraldehyde, post-fixed with 1% OsO_4 in phosphate-buffered saline pH 7·4 and then embedded in Epon 812. Ultrathin sections were examined, directly or after staining with lead citrate and uranyl acetate, with Philips EM-201 and EM-301 electron microscopes.

Absorption of HCV OC43 and NCDCV immune sera (0·1 ml/490 cm^2 roller bottle) was done for 2 h at 37 °C and overnight at 4 °C using homologous virus-infected Vero cell cultures repeatedly washed with HBSS. In some other experiments, in order to obtain a differential absorption of antibody detected by different test systems, infected cell cultures were treated with trypsin and fixed with glutaraldehyde before absorption as described above.

The haemagglutination (HA) and haemagglutination-inhibition (HI) tests, the neutralizing (Nt) antibody assay and the immunoperoxidase test for virus-specific IgG (IPA-IgG) were performed as reported previously (Gerna *et al.*, 1981). Haemadsorption (HAd) was performed 48 h post-infection on HELF and Vero cell cultures at 4 °C using rat erythrocytes, as described previously (Gerna *et al.*, 1981). HAd was also performed on infected Vero cells every 10 min, following treatment with 0·25% trypsin for up to 60 min at room temperature.

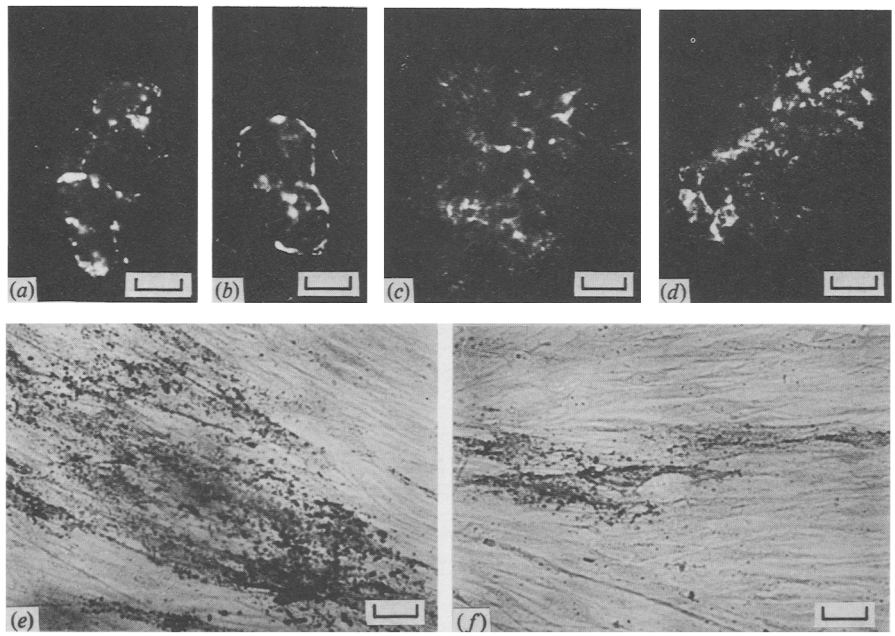

Fig. 1. Membrane-associated antigens (MAA), as stained by the IFAMA-IgG technique, in a Vero cell suspension infected with (*a*) HCV OC43 and (*b*) NCDCV, 48 h post-infection. (*c*) HCV OC43 and (*d*) NCDCV plaques on Vero cell monolayers 48 and 72 h post-infection respectively. Plaques of (*e*) HCV OC43 and (*f*) NCDCV MAA, as stained by the IPAMA-IgG technique, in HELF cell cultures 48 h post-infection are also shown. Bar markers represent: (*a*, *b*) 12·5 μm; (*c* to *f*) 50 μm.

In addition to mouse immune sera, the following groups of sera were tested for HCV OC43 and NCDCV antibodies by IFAMA-IgG: (i) 50 paired sera from infants and young children with acute upper respiratory tract infection or acute non-bacterial gastroenteritis; (ii) single sera from 32 calves; (iii) single sera from 42 African green monkeys. All sera were also tested by HI, Nt and IPA-IgG.

When stained 48 h post-infection by the IFAMA-IgG test using mouse immune sera, HCV OC43- and NCDCV-infected HELF and Vero cells showed a marked, often discontinuous, staining of their membranes with the presence of fine granules and clumps of labelled material (Fig. 1*a*, *b*). Staining of HCV OC43-infected cell cultures with HCV OC43-positive human sera and NCDCV-infected cells with NCDCV-positive bovine sera gave similar patterns. Incubation with serum from naturally infected hosts (humans and calves) prior to adddition of mouse antiserum to infected cell cultures, prevented the appearance of staining (blocking test). The first appearance of MAA was observed 3 h post-infection, concurrently with the appearance of cytoplasmic antigens. The amount of MAA progressively increased at subsequent times. Representative plaques of HCV OC43 and NCDCV MAA, as detected by IFAMA-IgG in Vero cells and by IPAMA-IgG in HELF cells, are shown in Fig. 1 (*c* to *f*). Immunoelectron microscopic observations of HCV OC43- and NCDCV-infected HELF and Vero cell cultures labelled with the IPA-IgG technique showed that staining of the infected membrane was due to the presence of a thick, often discontinuous, brush-like layer, closely associated with the cell membrane (Fig. 2*a*) and sometimes with single or variably grouped virus particles. When infected cells were incubated with immune serum at 37 °C, a marked redistribution of MAA was observed and labelled material appeared inside cytoplasmic vacuoles (Fig. 2*b*).

In HCV OC43- and NCDCV-infected cell cultures extensively treated with trypsin, HAd was abolished, while high HA activity was found in the trypsin solution used. However, even

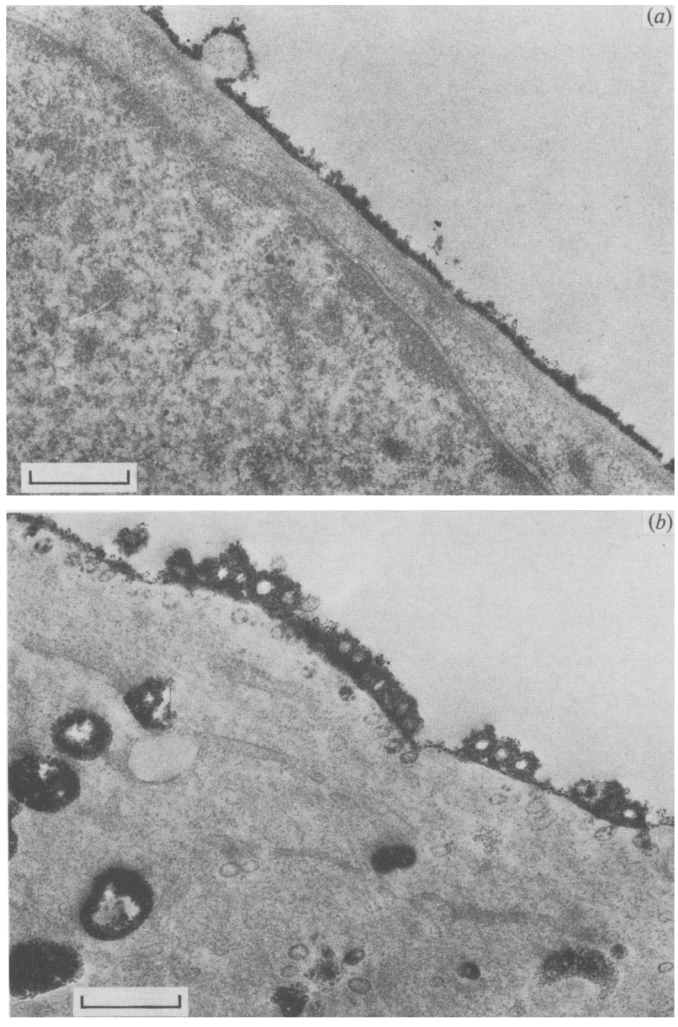

Fig. 2. **Immunoelectron** microscopy staining by the IPAMA technique of HELF cells infected with NCDCV 48 h post-infection at (*a*) 4 °C or (*b*) 37 °C. In (*b*), where several virus particles are adhering to the cell membrane, a marked redistribution of MAA was observed. Both bar markers represent 500 nm.

though fine granules of fluorescing material were greatly reduced in number, the intensity of IFAMA-IgG staining was not appreciably decreased. In addition, glutaraldehyde fixation following trypsin treatment did not modify the reactivity of HCV OC43 and NCDCV MAA, and immunoelectron microscopy showed distinct labelling of infected cell membrane in the absence of adhering virus particles.

The study of antigenic relationships between HCV OC43 and NCDCV MAA, showed that in HCV OC43 immune serum the IFAMA-IgG homologous titre (1 : 12800) was only a twofold dilution higher than heterologous titre, whereas in NCDCV immune serum homologous titre (1 : 25600) was eightfold higher. This degree of cross-reactivity of MAA appeared to be closely comparable to that shown for antigens detected by IPA-IgG in ethanol-fixed infected cells (Gerna *et al.*, 1981). The absorption of immune sera with living cells infected with homologous virus produced a $\geqslant$32-fold reduction in homologous and heterologous antibody titre, as detected by all tests employed. Whenever infected cells which

had been treated with trypsin and fixed with glutaraldehyde were used for serum absorption, a ⩾32-fold reduction in IFAMA-IgG and IPA-IgG antibody titre was observed, whereas the fall in HI and Nt antibody was within a twofold dilution.

Among paired sera tested from infants and young children, HCV OC43 and NCDCV IFAMA-IgG antibody titres consistently paralleled IPA-IgG titres, This finding was consistent in subjects showing either seroconversion or stable titres. Antibody to both HCV OC43 and NCDCV was detected by all tests, mostly in infants and children with upper respiratory tract infection. On the other hand, in some young patients with acute non-bacterial gastroenteritis, the appearance or presence of HCV OC43 (but not NCDCV) HI and Nt antibody was accompanied by absence of IFAMA-IgG and IPA-IgG antibody to either coronavirus strain. In all bovine sera tested, even though homologous titres by different tests were at least fourfold greater than heterologous titres, antibody was detected to both HCV OC43 and NCDCV by all tests. In African green monkey sera, IFAMA-IgG antibody and IPA-IgG antibody were consistently absent, even though both HCV OC43 and NCDCV HI and Nt antibody were present in 20 out of 42 animals.

This paper describes for the first time the presence of MAA in cells infected with either HCV OC43 or NCDCV. A similar finding has previously been reported only for two members of the hepatoencephalitis group of murine coronaviridae (Robb & Bond, 1979). Results reported in the present study show that MAA reactivity of both HCV OC43 and NCDCV is similar to that demonstrated for HCV OC43 and NCDCV antigens detected by the IPA-IgG technique (Gerna *et al.*, 1981). Three major structural proteins have recently been identified in coronavirions: a peplomeric glycoprotein, a membrane glycoprotein, and the virus nucleocapsid or core (Sturman *et al.*, 1980). For HCV 229E and HCV OC43, it has been shown that Nt and HA antigens are associated with virus surface projections (Schmidt & Kenny, 1981). Since reactivity of MAA has been shown here to be different from that of Nt and HA antigens, then core and/or virus membrane or non-structural antigens should be responsible for MAA reactivity.

The antigenic relationship between HCV OC43 and NCDCV, which has previously (Gerna *et al.*, 1981) been shown to be close for antigens detected by IPA-IgG and only slight for antigens reacting in HI and Nt tests, has been demonstrated in this report to be close also for MAA. These data seem to emphasize the possibility that, within the limits of the tests employed, circulating strains of human and animal coronaviruses may share some antigens reactive only in some test systems.

In conclusion, the expression in the plasma membranes of coronavirus-infected cells of antigens distinct from those eliciting Nt and HI antibodies appears to be peculiar. Although the nature of MAA remains to be determined, it is worthwhile to point out that recently, virus ribonucleoprotein has been detected on the membrane of cells infected with influenza (Virelizier *et al.*, 1977) and respiratory syncytial virus (Fernie *et al.*, 1981).

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REFERENCES

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.

FERNIE, B. F., FORD, E. C. & GERIN, J. L. (1981).The development of Balb/c cells persistently infected with respiratory syncytial virus: presence of ribonucleoprotein on the cell surface. *Proceedings of the Society for Experimental Biology and Medicine* **167**, 83–86.

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.

GERNA, G., CEREDA, P. M., REVELLO, M. G., CATTANEO, E., BATTAGLIA, M. & TORSELLINI GERNA, M. (1981). Antigenic and biological relationships between human coronavirus OC43 and neonatal calf diarrhoea coronavirus. *Journal of General Virology* **54**, 91–102.

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

McINTOSH, K., DEES, J. H., BECKER, W. B., KAPIKIAN, A. Z. & CHANOCK, R. M. (1967). 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.

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.

ROBB, J. A. & BOND, C. W. (1979). Pathogenic murine coronaviruses. I. Characterization of biological behavior 'in vitro' and virus-specific intracellular RNA of strongly neurotropic JMHV and weakly neurotropic A59V viruses. *Virology* **94**, 352–370.

SCHMIDT, O. W. & KENNY, G. E. (1981). Immunogenicity and antigenicity of human coronaviruses 229E and OC43. *Infection and Immunity* **32**, 1000–1006.

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.

STURMAN, L. S., HOLMES, K. W. & BEHNKE, J. (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *Journal of Virology* **33**, 449–462.

VIRELIZIER, J. L., ALLISON, A. C., OXFORD, J. S. & SCHILD, G. C. (1977). Early presence of ribonucleoprotein antigen on surface of influenza virus-infected cells. *Nature, London* **266**, 52–54.

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