PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 163, 360–366 (1980)

Human Coronavirus OC43 Serum Inhibitor and Neutralizing Antibody by a New Plaque-Reduction Assay[1] (40778)

GIUSEPPE GERNA, ERCOLE CATTANEO, PAOLO M. CEREDA, M. GRAZIA REVELO, AND GIORGIO ACHILLI

Virus Laboratory, Institute of Infectious Diseases, University of Pavia, 27100 Pavia, Italy

The determination of neutralizing antibody to human coronavirus OC43 in human sera has mostly been performed *in vivo* using the suckling mouse brain system (1–3). In cell cultures, a neutralization test based upon the evaluation of hemadsorption has been introduced (4), but hemadsorption of OC43-infected cell cultures has been shown to be a false hemadsorption or "pseudohemadsorption" (5).

It is well known that fetal calf serum (FCS) inhibits OC43 virus growth because of the presence of an inhibitor which does not appear to be an antibody (5). In the present report, the OC43 inhibiting activity of FCS has been utilized, as a plaquing factor, to set up a new plaque assay and a plaque-reduction neutralization test for determination of neutralizing antibody to coronavirus OC43 in human sera. Sera have been tested in parallel before and after treatment with phospholipase C (PLC) and neutralizing titers have been compared with those obtained by hemagglutination inhibition (HI) and the immunoperoxidase antibody technique (IPA) for immunoglobulin G (IgG) determination. Results show that human sera contain an OC43 inhibitor similar to that present in FCS, which is removed by PLC treatment. Therefore, human sera must be routinely treated before determining neutralizing antibody, in order to avoid false positive results. This conclusion applies to both OC43 antibody determination in seroepidemiological surveys and serodiagnosis of OC43 infections and renders previously reported results questionable (4, 6).

Materials and methods. Coronavirus OC43 strain and cell cultures. Suckling mouse brain-adapted strain of human coronavirus OC43 as well as mouse immune ascitic fluid was kindly supplied by both Dr. H. S. Kaye (Center for Disease Control, Respiratory Virology Unit, Atlanta, Ga.) and the National Institute of Allergy and Infectious Diseases (Bethesda, Md.). The adaptation of the virus to cell cultures has previously been reported in detail (7). Briefly, infected suckling mouse brain suspension was inoculated onto primary African green monkey kidney cell cultures (AGMK). Following incubation at 33° for 60 min, cell cultures were maintained with a serum-free medium consisting of equal parts of Eagle's minimum essential medium (EMEM) and Medium 199 (M199), both in Earle's base. Blind passages were performed every 10–12 days. Virus was initially detected in cell cultures at the sixth passage by the IPA technique (7). After 12 passages on AGMK, infected cell culture medium was inoculated onto several strains of human embryonic lung fibroblasts. The most suitable cell strain for coronavirus OC43 propagation was MA-321 (Microbiological Associates, Walkersville, Md.). Stock virus was prepared on MA-321 cell cultures, using serum-free EMEM-M199 as a maintenance medium and incubation at 33° for 60–72 hr. Titration of stock virus was performed initially by hemagglutination (7) and then by the plaque assay as reported below.

Plaque assay for OC43 quantitation using FCS. Initially, the OC43 inhibitor activity in FCS was quantitated by incubating twofold dilutions of several commercial lots of FCS with 100 $TCID_{50}$ of OC43 virus (as determined by hemagglutination) at 33° for 30 min and then inoculating mixtures onto

[1] This study was supported by Progetto Finalizzato Virus Grant 78.00365.84 from the Consiglio Nazionale delle Ricerche (CNR), Rome, Italy.

0037-9727/80/030360-07$01.00/0

replicate MA-321 microplate cell cultures maintained with serum-free EMEM-M199. Virus growth was then tested daily by hemagglutination for 7 days (7). In following experiments, MA-321 cell cultures infected with 100 $TCID_{50}$ of OC43 virus were fed, after virus adsorption, with medium EMEM-M199 supplemented with twofold dilutions of the same lots of FCS. After 3–5 days, a typical plaque appearance of cytopathic effect was observed in all cell cultures maintained with 10–20% FCS medium. The IPA technique (7) confirmed the plaque growth of the virus. Using 10% FCS maintenance medium, replicate MA-321 microplate cell cultures were then infected with 50 $TCID_{50}$ of OC43 virus and then fixed and stained by the IPA technique at 12-hr intervals for 4 days (10 replicates each time).

Following preliminary experiments, a plaquing medium consisting of EMEM-M199 supplemented with 10% of a selected lot of FCS was used for the plaque assay. For quantitation of OC43 stock virus, log virus dilutions were inoculated onto MA-321 replicate microplate cell cultures. After adsorption at 33° for 60 min, cell cultures were washed three times and fed with the plaquing medium. At 48 hr postinfection cell monolayers were washed, fixed with absolute ethanol, and stained by the IPA technique. This procedure was carried out using optimal dilutions of the reference mouse immune ascitic fluid in the first step and peroxidase-conjugated sheep anti-mouse IgG serum (Cappel Laboratories, Inc., Cochranville, Pa.) in the second step of the reaction. Histochemical detection of the enzyme was then obtained as previously reported (8). The number of plaques was easily counted using an inverted microscope.

Plaque-reduction assay (PRA). A plaque-reduction assay has been used for quantitation of both OC43 neutralizing antibody and inhibitor in bovine and human sera. The test was performed in microplates of MA-321 cell cultures. Serial twofold dilutions of each inactivated serum (56° for 30 min) in serum-free EMEM-M199 were mixed with equal volumes of virus suspension containing about 50 infectious virus particles/0.050 ml (as determined by the plaque assay). Virus controls contained equal volumes of medium and virus suspension. After incubation at 33° for 60 min, two replicate wells of confluent microplate MA-321 cell monolayers were inoculated (0.050 ml/well) with each mixture. Following adsorption at 33° for 60 min, cell cultures were washed, incubated at 33° with the plaquing medium for 48 hr, and then fixed and stained according to the procedure reported above for the plaque assay. The highest serum dilution reducing by 50% or more the number of plaques was considered to be the endpoint. Duplicate determinations gave in 95% of cases identical results. In a first group of experiments the PRA was used in order to partially characterize and study the treatment for removal of the OC43 inhibitor in a few reference OC43-negative and -positive bovine (fetal and adult) and human sera. These sera were tested before and after the following treatments: (i) precipitation of the γ-globulin fraction with a saturated solution of $(NH_4)_2SO_4$ according to a standard procedure (9); (ii) heating at 65° for 15 min; (iii) fractionation by sucrose density gradient centrifugation (10); (iv) since the effect of an infectious bronchitis virus (IBV) inhibitor has been shown to be reversed in animal sera by the presence of a 1.5% concentration of several sugars (11), sera were diluted in EMEM-M199 containing 1.5% D-glucose; (v) since nonspecific inhibitors of OC43 hemagglutination have recently been found in human sera and removed by treatment with PLC (12), the same treatment was applied to reference sera in order to suppress the OC43 inhibitor; after treatment, sera were dialyzed against phosphate-buffered saline (PBS) at 4° overnight and filtered through 0.22-μm Millipore membranes.

After initial tests on reference sera, it was decided to test all sera (human and bovine) in the PRA without prior treatment, in the presence of 1.5% D-glucose, and after PLC treatment.

HI test. Following treatment of sera with PLC (12), the HI test was performed according to the method of Kaye *et al.* (13), using a 0.5% suspension of chicken red

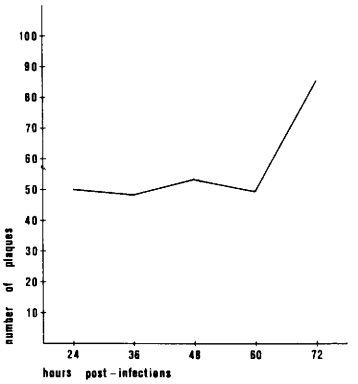

FIG. 1. Plaquing effect of 10% fetal calf serum in MA-321 cell cultures infected with 50 PFU of human coronavirus OC43. Figures are the average of three experiments performed at 1-month intervals in 10 replicates.

blood cells and OC43 hemagglutinating antigen prepared from OC43-infected MA-321 cell cultures (7).

IPA test. The IPA test was performed on microplate ethanol-fixed OC43-infected cell monolayers showing about 50% infected cells. Serial twofold dilutions of test sera were layered onto cell monolayers and incubated at 37° for 60 min. After three washings with PBS, cell cultures were covered with the optimal dilution of a peroxidase-conjugated goat anti-human (or rabbit anti-bovine) IgG serum (Cappel Laboratories). Virus–antibody reaction was then histochemically detected (8). Positive and negative bovine and human serum controls were included in each test. A blocking test, based upon adsorption of a reference positive serum with virus and negative IPA reaction following adsorption confirmed specificity of the test.

Sera examined. The following groups of bovine and human sera were tested by PRA, HI, and IPA-IgG tests: (i) 10 commercial lots of FCS obtained from different companies; (ii) individual sera from six young calves; (iii) single sera from 10 infants (6 months), 10 young children (2–5 years), and 12 young adults (13–20 years); (iv) seven paired sera from infants and children previously found to show seroconversion to OC43 virus.

Results. OC43 plaque assay. In initial experiments, when the OC43 growth was assayed by hemagglutination, all commercial lots of FCS tested were found to be able to block virus adsorption up to high serum dilutions. Subsequently FCS was shown to be able to "plaque" OC43 virus, when

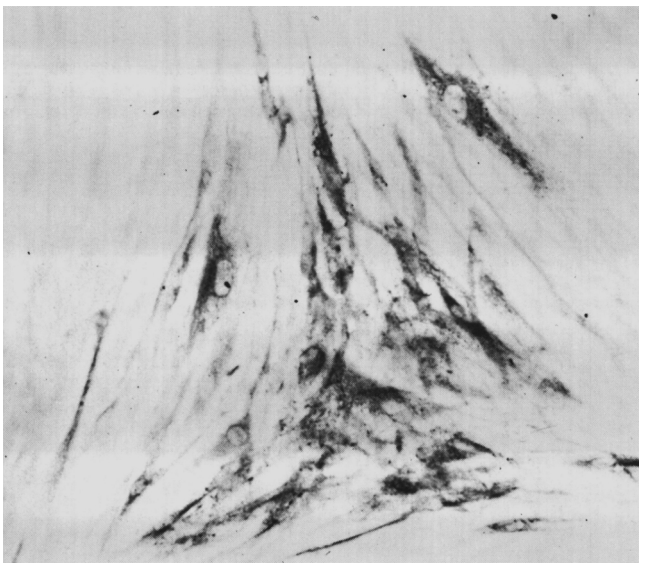

FIG. 2. A plaque of human coronavirus OC43 MA-321 infected cells, as obtained using a 10% fetal calf serum plaquing medium and stained 48 hr postinfection by the immunoperoxidase antibody technique. ×350.

added after virus adsorption to the maintenance medium at a concentration of 10–20%. Using 50 $TCID_{50}$ of OC43 stock virus suspension as inoculum and a 10% FCS plaquing medium, the number of plaques did not change more than 10% among different experiments up to 60 hr postinfection (Fig. 1). During this period the size of plaques changed from single infected cell foci (before 24 hr) to plaques of 10–20 infected cells at 48 hr (Fig. 2). At 60 hr some plaques tended to be confluent. At 72 hr new secondary plaques started appearing. Thus, it was decided to select 48 hr as the optimal time for plaque counting. The dose–response relationship in the plaque assay for OC43 virus quantitation is reported in Table I.

PRA for OC43 inhibitor studies. Quantitation of OC43 inhibitor in a lot of FCS and a human serum from a 6-month-old infant is reported in Fig. 3. The OC43 inhibitor activity was shown not to be associated with the γ-globulin fraction of bovine and human sera (free of OC43 antibody), as obtained by precipitation with $(NH_4)_2SO_4$ saturated solution. In addition, heating of sera at 65° for 15 min was found not to reduce or suppress the OC43 inhibitor activity. The serum fractionation by sucrose density gradient centrifugation showed that the OC43 inhibitor was present in a broad peak, corresponding to the IgG distribution, in both OC43-negative human and bovine sera as well as in FCS (Fig. 4).

The OC43 inhibitor appeared to be at least partially different from the IBV inhibitor found in animal sera by Lukert (11), since the titer was reduced (4- to 16-fold), but not suppressed, when the PRA was performed in the presence of a 1.5% D-glucose concentration. However, when sera were treated with PLC, the inhibitor activity appeared to be completely eliminated in human sera at a 1:10 dilution and in FCS at a 1:20 dilution.

TABLE I. HUMAN CORONAVIRUS OC43 STOCK VIRUS TITRATION BY THE PLAQUE ASSAY, USING 10% FETAL CALF SERUM

Virus dilution	No. plaques at 48 hr
10^{-1}	TMTC[a]
10^{-2}	TMTC
10^{-3}	45,54,52,48,50
10^{-4}	6,7,3,5,5
10^{-5}	0,0,1,0,1

[a] Too many to count.

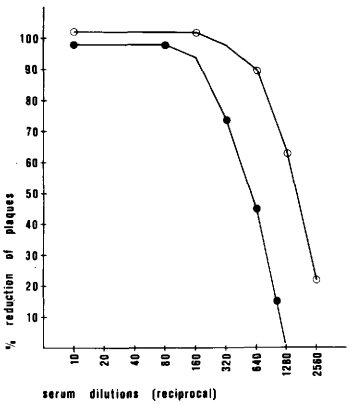

FIG. 3. Quantitation of the OC43 inhibitor in (○) a lot of fetal calf serum and (●) a human serum from a 6-month-old infant by the plaque-reduction assay.

PRA for OC43 neutralizing antibody determination in bovine and human sera. Results of testing of bovine and human sera by PRA (without treatment, in the presence of D-glucose, and after PLC treatment), HI, and IPA-IgG tests are reported in Fig. 5. In FCS, where OC43 antibody was never detected by HI and IPA-IgG tests, the OC43 inhibitor activity was fairly decreased in the presence of D-glucose and was almost completely suppressed after PLC treatment. However, 5 out of 10 lots of FCS tested still maintained a 1:10 inhibitor activity titer after PLC treatment. Similarly, in the 6-month-old infant group, the high OC43 in-

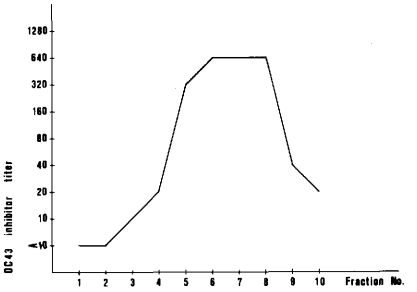

FIG. 4. Distribution of the OC43 inhibitor in fractions collected after sucrose density gradient centrifugation of a lot of fetal calf serum.

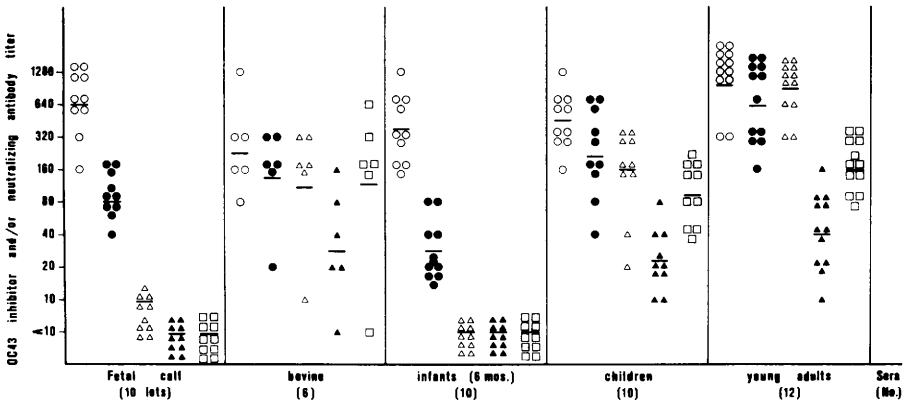

FIG. 5. In each column, horizontal lines indicate geometric mean titers of OC43 inhibitor and/or neutralizing activity in five groups of sera tested by plaque reduction (○) without prior treatment, (●) in the presence of 1.5% D-glucose, and (△) after phospholipase C treatment. Same sera were also tested by (▲) hemagglutination inhibition, and (□) the immunoperoxidase antibody technique for IgG determination.

hibitor titer was greatly reduced in the presence of D-glucose and entirely suppressed after PLC treatment. In infant sera the absence of OC43 antibody was also shown by HI and IPA-IgG tests.

In bovine and human sera positive for OC43 antibody (as shown by HI and IPA-IgG tests), the OC43 neutralizing antibody titer was usually not significantly modified by D-glucose and PLC treatment. However, in a few cases, the OC43 inhibitor titer appeared to be greater than the neutralizing antibody titer, as shown by the reduction in titer after PLC treatment and also in the presence of D-glucose. In a single bovine serum, negative for OC43 antibody as shown by HI and IPA-IgG tests, an inhibitor titer of 1:10 was still detected after PLC treatment.

PRA for serodiagnosis of human coronavirus OC43 infections. In seven cases of human coronavirus OC43 infection of infants and children, in which seroconversion had previously been detected by both HI and IPA-IgG tests, a fourfold or greater rise in specific OC43 antibody titer was also found by PRA (Table II). Four were cases of primary OC43 infection, as shown by the absence of specific antibody in the acute-phase serum, and three were cases of reinfection, as shown by the presence of antibody in the acute-phase serum by all three tests. The presence of OC43 neutralizing antibody in the acute-phase serum of a 4-month-old infant, in the absence of HI and IPA-IgG antibodies (Case No. 467), indicates that neutralizing antibody may appear earlier than HI and IPA-IgG antibodies or may be detected earlier by a more sensitive test. OC43 neutralizing antibody titers were usually higher than both HI and IPA-IgG titers.

Discussion. This paper describes for the first time three major findings: (i) a PRA for the determination of OC43 neutralizing antibody; (ii) the presence in human sera of an OC43 inhibitor similar to that found in FCS; (iii) a method for the removal of this inhibitor from human as well as bovine sera. Our PRA is the first reported for the determination of OC43 antibody, whereas a PRA for the determination of human coronavirus 229E antibody was reported several years ago (14). The OC43 PRA appears to be very sensitive for both neutralizing antibody (and inhibitor activity) determination and serodiagnosis of OC43 infections (and reinfections). The hemadsorption-neutralization procedure previously reported (4) appears to give unreliable results for at least two main reasons. First, hemadsorption of OC43-infected cells is a false hemadsorption (5). Second, the test did not require any prior treatment of sera and results were somewhat influenced by the presence of the

TABLE II. HUMAN CORONAVIRUS OC43 ANTIBODY TITERS BY THE PLAQUE-REDUCTION ASSAY (PRA), HEMAGGLUTINATION INHIBITION (HI), AND THE IMMUNOPEROXIDASE ANTIBODY (IPA) TECHNIQUE FOR IMMUNOGLOBULIN G (IgG) DETERMINATION, IN SEVEN CASES OF OC43 INFECTION IN INFANTS AND CHILDREN

Case No. and age	Interval between acute and convalescent serum (days)	OC43 antibody titer by			Type of OC43 infection
		PRA	HI	IPA-IgG	
338, 4 years	15	<10 80	<10 20	<10 40	Primary
467, 4 months	30	40 160	<10 20	<10 40	Primary
510, 9 months	28	<10 80	<10 20	<10 160	Primary
532, 4 years	13	<10 640	<10 80	<10 40	Primary
291, 12 years	?	40 160	10 40	10 40	Reinfection
440, 3 years	26	320 2560	20 160	40 640	Reinfection
768, 4 years	27	20 320	40 160	40 160	Reinfection

OC43 inhibitor described here. Similarly, neutralization tests carried out *in vivo* in suckling mice offer the same points of criticism (1–3).

Since an initially high-titer plaque-reduction activity was found in both FCS and sera from 6-month-old infants, the presence of an OC43 inhibitor was strongly suspected. In addition, in the same two groups of sera the presence of nonspecific inhibitors of OC43 hemagglutination was observed. This finding, well known for bovine sera (15), has only recently been reported for human sera (12). Since it had been shown that these inhibitors can be removed by the PLC treatment (12), it was decided to use the same treatment of sera for the PRA, in order to try to suppress the OC43 inhibitor. Results achieved (elimination of the inhibitor from human sera and its almost complete disappearance in FCS) indicate that the OC43 inhibitor is likely to be a lipoprotein. Furthermore, the distribution of the OC43 inhibitor following sucrose density gradient centrifugation shows that this lipoprotein is of the high-density class, and appears similar to some nonspecific inhibitors of rubella hemagglutination (16). In contrast to the IBV inhibitor previously reported (11), the OC43 inhibitor was not precipitated with the γ-globulin fraction, was not suppressed by heating at 65°, and was only partially reversed by D-glucose.

In conclusion, we believe that the PRA is a very sensitive test for OC43 neutralizing antibody determination and adds a new significant approach to the serodiagnosis of OC43 infections. However, before testing, sera must be routinely treated for elimination of the OC43 inhibitor. Finally, the OC43 PRA appears to be very useful for studies of antigenic relationships between human and animal coronaviruses.

The presence of antibody to neonatal calf diarrhea coronavirus (NCDCV) in human sera has sporadically been reported (17, 18) and the antigenic relationship between human coronavirus OC43 and NCDCV, already observed by immunofluorescence (19), is now under study in our laboratory.

Summary. A plaque-reduction assay for the determination of neutralizing antibody to human coronavirus OC43 has been set up, using as a plaquing factor the fetal calf serum containing an OC43 inhibitor. A similar inhibitor has also been found in human sera both negative and positive for OC43 antibody. The inhibitor can be removed by treatment of sera with phospholipase C and is likely to be a lipoprotein of the high-

density class. The new plaque-reduction assay appears to be a very sensitive test for both OC43 antibody determination and serodiagnosis of OC43 infections. It may also be very useful for studies of antigenic relationships between human and animal coronaviruses. However, before testing, sera must be routinely treated for removal of viral inhibitor.

We would like to thank Dr. Massimo Battaglia for his valuable suggestions, Joseph Strohofer for revision of English, and Giovanni Vercesi for having drawn graphs.

1. Kaye, H. S., and Dowdle, W. R., J. Infec. Dis. **119**, 282 (1969).
2. Bradburne, A. F., Arch. Gesamte Virusforsch. **31**, 352 (1970).
3. Kaye, H. S., Yarbrough, W. B., Reed, C. J., and Harrison, A. K., J. Infec. Dis. **135**, 201 (1977).
4. Kapikian, A. Z., James, H. D., Jr., Kelley, S. J., King, L. M., Vaughn, A. L., and Chanock, R. M., Proc. Soc. Exp. Biol. Med. **139**, 179 (1972).
5. Bucknall, R. A., Kalica, A. R., and Chanock, R. M., Proc. Soc. Exp. Biol. Med. **139**, 811 (1972).
6. Monto, A. S., and Rhodes, L. M., Proc. Soc. Exp. Biol. Med. **155**, 143 (1977).
7. Gerna, G., Cereda, P. M., Revello, M. G., Torsellini Gerna, M., and Costa, J., Microbiologica **2**, 331 (1979).
8. Gerna, G., Arch. Virol. **49**, 291 (1975).
9. Kawamura, A., Jr., "Fluorescent Antibody Techniques and Their Application," University Park Press, Baltimore (1969).
10. U.S. Department of Health, Education and Welfare, "Serodiagnosis of: Toxoplasmosis, Rubella, Cytomegalic Inclusion Disease, Herpes Simplex," pp. 101–110, Immunology Ser. No. 5. Center for Disease Control, Atlanta (1974).
11. Lukert, P. D., Arch. Gesamte Virusforsch. **40**, 93 (1973).
12. Hovi, T., Med. Microbiol. Immunol. **166**, 173 (1978).
13. Kaye, H. S., Marsh, H. B., and Dowdle, W. R., Amer. J. Epidemiol. **94**, 43 (1971).
14. Bradburne, A. F., and Tyrrel, D. A. J., Arch. Gesamte Virusforsch. **28**, 133 (1969).
15. Sharpee, R. L., Mebus, C. A., and Bass, E. P., Amer. J. Vet. Res. **37**, 1031 (1976).
16. Haukenes, G., Acta Pathol. Microbiol. Scand. Sect. B **81**, 719 (1973).
17. Sharpee, R., and Mebus, C. A., Lancet **1**, 639 (1975).
18. Kaye, H. S., Yarbrough, W. B., and Reed, C. J., Lancet **2**, 509 (1975).
19. Pedersen, N. C., Ward, J., and Mengeling, W. L., Arch. Virol. **58**, 45 (1978).

Received October 9, 1979. P.S.E.B.M. 1980, Vol. 163.