IMMUNOLOGICAL INVESTIGATIONS, 22(2), 95-103 (1993)

HLA CLASS I ANTIGEN SERVES AS A RECEPTOR FOR HUMAN CORONAVIRUS OC43

Arlene R. Collins

Department of Microbiology

School of Medicine, State University of New York at Buffalo

Buffalo, New York 14214

Human coronaviruses are associated with acute respiratory and enteric disease in man as such their target cells are probably the epithelial cells lining the respiratory and enteric tract. Attachment of virus to specific receptors on the cell surface is a major determinant of virus tropism in pathogenesis (1). Recently, aminopeptidase-N was identified as a cell receptor for the 229e coronavirus (2). Cell receptor(s) for OC43 coronavirus have not been identified. However, it is of pathologic significance that OC43 virus shares DNA sequence homology with the two coronavirus isolates, SK and SD, from the brain of patients with multiple sclerosis (MS) (3). Probing MS and control brain with probes specific for human, murine, porcine and bovine coronavirus by in situ hybridization resulted in the detection of coronavirus RNA in 12 of 22 MS brain samples; five of which were positive with the OC43 probe (4).

A study of virus-ligand interactions of OC43 with human rhabdomyosarcoma (RD) cells, which are highly susceptible to virus infection, was undertaken to identify possible cell receptors. The binding of virus collected from the supernatant of infected cells to cell proteins immobilized on nitrocellulose paper was used to screen for virus-ligand interactions. The next step was the identification or development of antibodies to each of the ligands, and to test their ability to blockade receptor activity by culturing infected cells in medium containing the ligand antibodies and measuring the effect on virus yield. The preliminary experiments reported here reveal an interesting observation of strong affinity of OC43 virus for the HLA class I antigen.

Viruses and Cells

Human coronavirus OC43 and 229e were plaque purified in MRC-5, human diploid lung cells (Viro Med, Minnetonka, MN, 55343). Stock virus was maintained by passage in MRC-5 cells. Human rhabdomyosarcoma (RD) cells obtained from R. Crowell, Hanneman Medical College, Philadelphia, PA. Monkey kidney, CV-1 cells were obtained from R. Hughes, RPMI, Buffalo, NY.

For plaque assays virus suspensions were diluted in EMEM and duplicate volumes of 0.2ml were allowed to adsorb to MRC-5 cells in 24 well trays

(Costar, Cambridge, MA) for 1h at 37°C. The monolayers were then overlayed with 0.5ml of 0.5% agarose with EMEM plus 0.2% serum. Cultures were stained with neutral red and plaques were counted after 4 days at 33°C. The titer was determined as the average number of plaques per dilution times the dilution.

Growth and Purification of Virus

HCV-OC43 was propagated in RD cells. Supernatant virus was harvested after 4-5 days incubation at 33°C, clarified by low speed centrifugation (1000xg, 10 min) and sedimented by ultracentrifugation at 100,000 xg for 4hr in a Beckman T35 rotor. The pellet was resuspended at 10x concentration in TMEN buffer (50mM tris-maleate, 1m MEDTA, 100mM NaCl pH 6.5). For virus purification, concentrated virus was layered onto a sucrose gradient (20-60% w/w in TMEN) and centrifuged in an SW41 rotor at 60,000 xg for 4h. The virus band (~ 3cm from the bottom) was collected, diluted in phosphate buffered saline (PBS, 0.1M pH 7.2) and sedimented under the same conditions. The pellet was resuspended in PBS and stored at -70°C.

Antibody Serum Production

The procedure of Knudsen (5) was followed. Briefly, the 90kRD protein separated by sodium dodecyl sulfate - 10% polyacrylamide gel electrophoresis SDS-PAGE was eluted from the gel by transfer to nitrocellulose paper. The protein-bearing nitrocellulose was solubilized with dimethyl sulfoxide and used as an immunogen in rabbits. An equal volume of Freund's adjuvant, either complete (first injection) or incomplete (subsequent injections) was added to the mixture which was then injected subcutaneously in four sites in a rabbit. A total of four injections was given at two week intervals. Antisera were tested by a Western blot assay and in a receptor blocking assay as described.

Monoclonal Antibodies

Monoclonal anti-Human HLA class I-ABC, clone W6/321, mouse antibody was obtained as ascites fluid (10mg/ml) from Dr. T. Shanahan, Tissue Typing Laboratory, Erie County Medical Center, Buffalo, NY, 14214. It was originally obtained from C-six Diagnostics, Inc., Mequon, WI 53092. Mouse monoclonal anti-coronavirus antibody to 110/120KD peplomer of bovine coronavirus (BCV), clone Bio-09, was purchased from Biosoft, AMAC Inc., Westbrook, ME 04092.

Virus-Immunoblot Assay

Virus: 100,000 xg pellet from supernatant medium of infected RD cells, preclarified at 1000xg for 10 min.

Substrate: Cells grown in T75 flasks (Costar) were washed twice with rinse buffer (20nM Tris-HCl pH 9.0, 137mM NaCl, 1mM $CaCl_2$ and 0.5M $MgCl_2$) and solubilized on ice for 20 min in 1ml ice cold lysis buffer (rinse buffer with 1% v/v NP_{40}, 10% v/v aprotinin). Insoluble cell debris was pelleted at 1000xg for 10 min.

Preparation of solid phase substrate: Virus in 2 x sample buffer, L + H molecular weight markers (BioRad, Richmond, CA 94804) in 2 x sample buffer were boiled 5 min and loaded along with cell lysates in lysis buffer (30-45μg protein per lane) onto 10% SDS gels .75mm thick. Proteins were separated at 15 mamps per slab for 1-2 h and immediately transferred onto Immobilon-P (Millipore, Bedford, MA 01730) membranes for 25-30 min in Transfer buffer (0.1M Tris, 0.192M glycine pH 8.4, 20% methanol, 0.1% SDS) at 100mA constant current. Membranes containing transferred proteins were soaked in 5% bovine serum albumin in TBS buffer (0.01M Tris buffer pH 7.5, 0.15M NaCl) at 37°C for 30 min and washed three times in TBS with 0.1% bovine serum albumin (TBS-BSA).

Solid Phase binding assay

The interaction of OC43 with cell membrane proteins was performed as follows: OC43 virus (512 HAU) in 5.0ml TBS-BSA was incubated with solid phase substrate at 25°C for 1hr with gentle shaking. To detect bound virus the membranes were washed three times with TBS-BSA and then incubated with a 1:200 dilution of rabbit anti-OC43 polyclonal antibody (prepared by immunization with gradient purified virus from infected suckling mouse brain) for 1h at 25°C. Virus-bound antibody was visualized by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG 1:1000 dilution (Bio Rad) and developed in TBS buffer containing 0.5mg/ml 4-chloro-1-naphthol and 0.03% H_2O_2.

Receptor Blocking Assay

RD cells, grown in 24-well culture trays (1.4 x 10^5 cells per well), were incubated for 1h at 37°C with 0.5ml of EMEM medium containing heat inactivated antibody diluted twofold beginning at 1:100. The antibody was removed and the cells were infected with virus at a multiplicity of 1 in 0.2ml of inoculum for 1h at 37°C. After washing the monolayers twice to remove unbound virus, the cells were incubated for 24h at 37°C in EMEM medium containing the antibody. Cells were then scraped into the medium and frozen at -70°C for titration by plaque assay. Receptor blockade was expressed as percent inhibition of virus yield and was determined by calculating the difference in virus yield between the virus titer in the presence of each antibody dilution and the virus titer of the untreated control. When preimmune rabbit

serum was tested in the receptor blocking assay, 50% inhibition was given by the 1:50 dilution but <10% inhibition at the twofold greater dilution.

RESULTS

Multiple RD cell proteins bound OC43 virus in a virus immunoblot assay comparing RD cell membranes and CV-1 cell membranes (FIGURE 1). In the RD membrane preparation, the virus-binding bands were observed at approximately 90kd, 45kd and 36kd. Some binding activity is shown with the CV-1 membrane preparations but this was also visible in the control incubated with rabbit antibody alone. In order to characterize these virus-binding proteins in RD cells as cell surface receptors, antibodies that would specifically block each virus-protein interaction were sought. For the 90kRD cell protein, a rabbit antibody was produced by immunization with the protein transferred to nitrocellulose. A monoclonal antibody, W6/32, to HLA class I heavy chain was found to be reactive with the 45kRD cell protein. The 36kRD cell protein is uncharacterized, as yet. Binding of virus to specific cell proteins was repeatable. Six virus and three RD membrane preparations have been tested.

Identification of the RD cell proteins by specific antibodies

RD cell proteins were separated on SDS-PAGE gels and eluted from the gel by transfer to Immobilon membranes. After blocking non-specific sites on the membrane by incubation with 5% bovine serum albumin, the membranes were reacted with test antibodies at 1:100, 1:200 and 1:400 dilution along with pre-immune rabbit serum. One lane of RD cell proteins was reacted with the second antibody (goat anti-rabbit or goat anti-mouse conjugated to horseradish peroxidase, 1:1000 dilution) alone. The rabbit antibody to 90kRD bound to a single protein as shown by the band in the 90,000 molecular weight region of the gel (FIGURE 2a). Pre-immune rabbit serum at 1:100 dilution was negative. The W6/32, anti-Human HLA class I antibody bound to a single RD cell protein shown by a band at 45,000kD, the approximate molecular weight of the HLA class I heavy chain. No bands were visible in the CV-1 cell control lane (FIGURE 2b). Another protein was identified on RD cell membrane substrate which was not a virus-binding band. This was the carcinoembryonic antigen (data not shown).

Identification of multiple virus receptors by receptor blockade

It was then important to identify those virus-binding reactions that could lead to infection of RD cells. Using RD cell monolayers as target cells, the ability of antibodies to 90kRD, HLA class I (W6/32) and BCV 110/120kD peplomer to block OC43 virus infection when present in the supernatant medium was compared. Serial dilutions of antibodies were

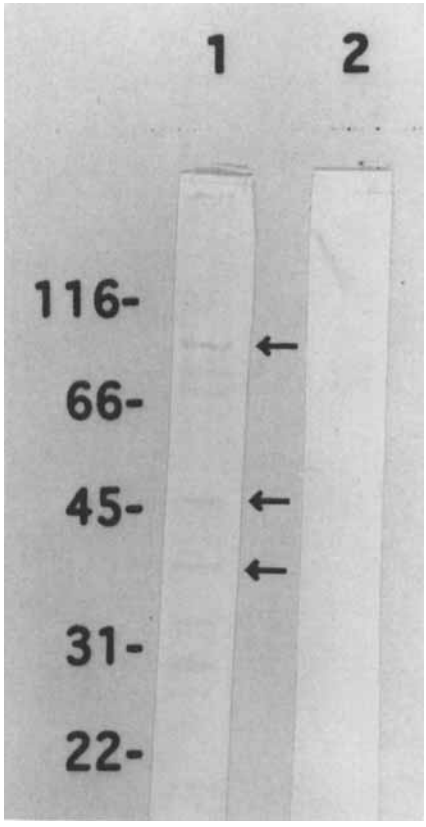

FIGURE 1

Binding of OC43 to RD cell proteins. Solubilized RD (lane 1) and CV-1 (lane 2) cell proteins after SDS-PAGE were transferred to Immobilon and reacted with OC43 virions. Virus-binding was detected by rabbit anti-OC43 antibody, followed by goat anti-rabbit-HRP conjugate and the reaction was developed with 4 chloro-1-naphthol. The virus-binding bands are at 90kD, 45kD and 36kD as indicated by the arrows.

incubated with the RD cells for 1hr prior to infection and for 24hr after infection and the effect on virus yield was determined by comparing virus plaque titers in samples with antibody to the virus titer of an untreated control (FIGURE 3). Rabbit antibody to the 90kRD protein blocked the production of infectious virus in these experiments. The highest concentration (1:100 dilution) gave incomplete protection from virus infection showing a 96% reduction in the total yield. The preimmune rabbit

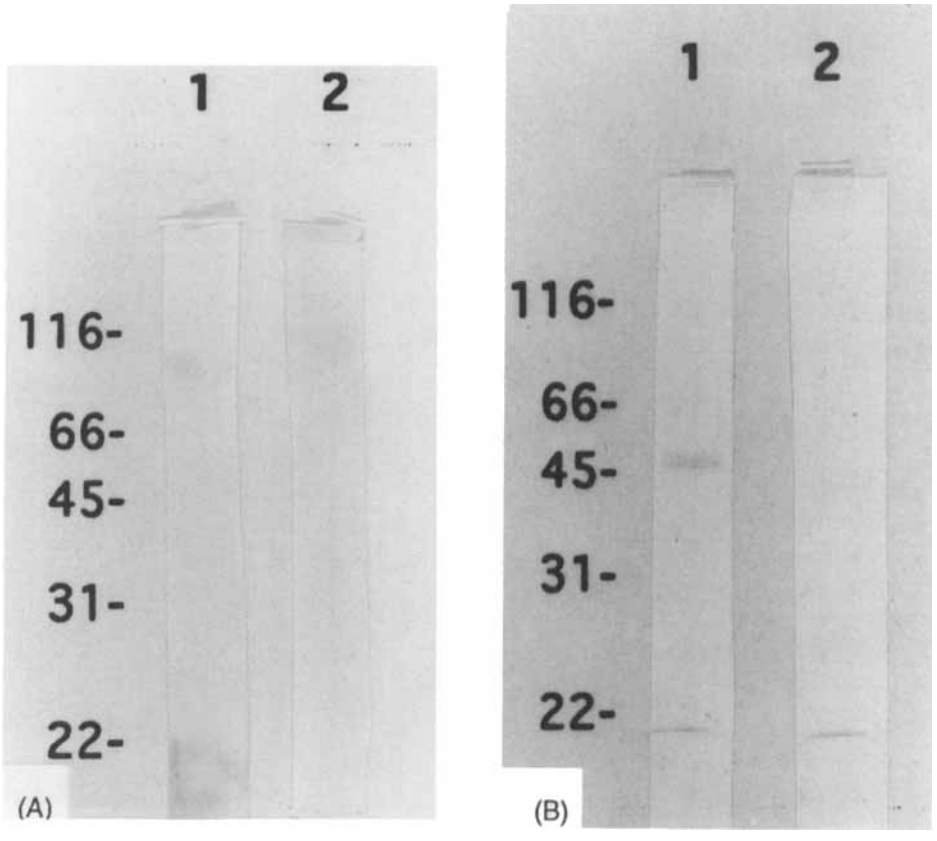

FIGURE 2

Binding of antibodies to RD cell receptor proteins. Solubilized cell proteins (lane 1 RD cells, lane 2 CV-1 cells), after SDS-PAGE, were transferred to Immobilon and reacted with (A) 90kRD cell antibody or (B) monoclonal anti HLA-class 1 antibody (10mg/ml). Binding of primary antibody was detected with goat anti-mouse or rabbit-HRP conjugate and the reaction was developed with 4 chloro-1-naphthol. The 90kD and 45kD bands are indicated by arrows.

serum gave less than 10% inhibition at this concentration. W6/32 monoclonal antibody was very effective in blocking infectious virus production. At 1:400 dilution of antibody, 25ug/ml, less than 10^2 plaque forming units of virus was synthesized, indicating that no virus was detectable within the sensitivity of the plaque assay. Monoclonal antibody to the BCV 110/120kD peplomer gave a 40% reduction in OC43 virus yield at the 1:100 dilution.

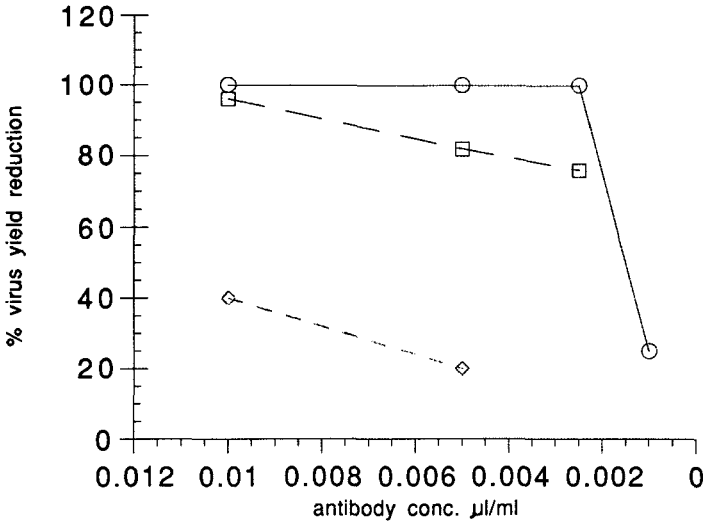

FIGURE 3

Inhibition of OC43 virus replication by anti-receptor antibody. RD cell monolayers were treated for 1hr with serial dilutions of antibody, infected with OC43 virus at a multiplicity of 1 for 1hr at 37°C, washed twice and reincubated for 24hr at 33°C with medium containing antibody. The total virus yield was then determined by plaque assay and the % yield reduction due to antibody was calculated by comparison to the virus controls (without antibody). ○——○ anti HLA, □ —— —□ anti 90 kRD, ◇—— —— ——◇ anti BCV, 110/120 kD.

Using the 229e virus, a human coronavirus belonging to another group, W6/32 antibody reduced the virus yield by less than 10% at the 1:100 dilution, indicating that HLA class I antigen was not a receptor for the 229e virus.

DISCUSSION

The preliminary results presented here indicate that multiple receptors for OC43 coronavirus are found on RD cells. At least three bands (at 90kd, 45kd and 36kd molecular weight) were seen by virus-immunoblotting of RD cell proteins. While membrane proteins were thought to be the ligand in this assay, the possibility that cytoplasmic proteins bind to the virus was not excluded. Virus binding to intracellular proteins would be also of interest since such interactions could lead to alterations in cell function. Another method was sought to identify those proteins likely to be on the cell surface.

The receptor blockade test employed antibodies of known specificity to identify the cell proteins with the receptor activity. The ability of receptor antibody to block infection is indirect evidence of receptor activity. Introduction of the receptor molecule into previously negative cells would be direct proof. However blocking infectivity by antibody gives preliminary evidence of receptor activity. Previous studies with human coxsackie B-3, rhinoviruses 14 and murine coronavirus, A59 have shown that antibodies to a specific receptor reduced viral replication and modulated disease (1,6,7).

The experiments reported here indicate that two receptor specificities, 90kRD and HLA class I, are involved in OC43 infection of RD cells. HLA class I antigen appears to be an essential component of the virus attachment complex since monoclonal antibody W6/32 completely blocked infectivity. Previously, monoclonal antibody W6/32 was used to show that measles virus-specific T cell clones were HLA class II restricted and not HLA class I (8). HLA class I is a member of the immunoglobulin gene superfamily which includes carcinoembryonic antigen, the receptor for mouse hepatitis coronavirus (MHV). The MHV receptor plays an important role in the pathogenesis of MHV infections. SJL/J mice which lack a functional virus receptor are resistant to MHV infection and develop chronic demyelination (9). MHV and OC43 belong to the same group. It would be reasonable to expect that two viruses in the same group use receptors which are structurally similar. MHC class 1 (HLA) molecules are highly polymorphic and function to provide a target for cytotoxic T cell receptor. In the process of antigen presentation, viral proteins are degraded and antigenic fragments in association with HLA are targets for immune recognition by cytotoxic T cells. Utilization of HLA antigen as a virus receptor would involve a different mechanism than MHC restriction which is the normal function of HLA. The relatively large mass of individual virions and the mobility of the HLA antigens in the membrane suggested that multiple HLA receptor sites may be occupied by the virus and may undergo conformational changes in the process making available other receptors embedded further in the plasma membrane followed by endocytosis. Further studies with monoclonal HLA class I antibody are underway to detect these complexes.

Rabbit antibody raised to the 90kRD cell protein incompletely blocked virus infection. The reasons may be that the antigen used for immunization lacked conformational domains present in the native molecule or that more than one receptor is involved. Purification of the 90kRD protein and its use in receptor competition experiments may clarify this issue. The involvement of more than one cell surface protein as receptor is of considerable interest

since the process of virus attachment and penetration for this virus may be quite complex. Human coronavirus OC43 is an enveloped virus containing two types of surface peplomers; a spike protein which recognizes 9-0-acetylated sialic acid as a receptor determinant and the hemagglutinin-esterase which is a less efficient hemagglutin and has esterase activity capable of cleaving the 9-0-acetyl bond which comprises the receptor determinant (10). More importantly, the ability to blockade virus receptors by specific peptides is a strategy to prevent coronavirus disease. Therefore further work on the characterization of these interactions is necessary.

ACKNOWLEDGEMENTS

This work was supported by grant PP 0170 from the National Multiple Sclerosis Society to A. Collins.

REFERENCES

1. F.G. Hayden, J.M. Gwaltney and R.J. Colonno, Antiviral Res. 9, 233-247 (1988).
2. C.L. Yeager, R.A. Ashmun, R.K. Williams, C.B. Cardellichio, L.H. Shapiro, A.T. Look and K.V. Holmes, Nature 357, 420-422 (1992).
3. S.R. Weiss, Virology 126, 669-677 (1983).
4. R.S. Murray, B. Brown, D. Brian and G.F. Cabirac, Ann. Neurol. 31, 525-533 (1992).
5. K. Knudsen, Anal. Biochem. 147, 285-288 (1985).
6. A.H. Weller, K. Simpson, M. Herzum, N. van Houten and S.A. Huber, J. Immunol. 143, 1843-1850 (1989).
7. A.L. Smith, C.B. Cardellichio, D.F. Winograd, M.S. deSouza, S.W. Barthold and K.V. Holmes, J. Infect. Dis. 163, 879-882 (1991).
8. S. Jacobson, T. Nepom, J.R. Richert and H.F. McFarland, J. Exp. Med. 161, 263-268 (1985).
9. R. Williams, G-S. Jiang and K.V. Holmes, Proc. Natl. Acad. Sci. USA, 88, 5533-5536 (1991).
10. B. Schultze, H-J. Gross, R. Bossmer and G. Herrler, J. Virol. 65, 6232-6237 (1991).