Binding of Rabbit Hemorrhagic Disease Virus to Antigens of the ABH Histo-Blood Group Family

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Rabbit hemorrhagic disease virus (RHDV) is a noncultivable calicivirus that infects rabbits and causes epidemics of an acute fatal hepatitis. The disease is characterized by high morbidity and mortality rates for adult animals. Death is the result of a widespread circulation dysfunction associated with disseminated intravascular coagulation and necrotizing hepatitis lesions (14, 24). Large quantities of virus particles are found in several organs, especially the liver, which is considered the major site of virus replication (6, 14, 19, 27). The viral genome consists of a single-stranded RNA of nearly 7.5 kb, packaged in a small icosahedral capsid (3, 15). The capsid protein has an estimated molecular mass of 60 kDa (VP60) (16), and expression of the corresponding cDNA in insect cells infected with a recombinant baculovirus yields a protein that spontaneously assembles into virus-like particles (VLPs). These VLPs are completely lack ABH antigens, were not agglutinated. Native viral particles from extracts of infected rabbit liver as well as virus-like particles from the recombinant virus capsid protein specifically bound to synthetic A and H type 2 blood group oligosaccharides. Both types of particles could attach to adult rabbit epithelial cells of the upper respiratory and digestive tracts. This binding paralleled that of anti-H type 2 blood group reagents and was inhibited by the H type 2-specific lectin UEA-I and polyacrylamide-conjugated H type 2 trisaccharide. Young rabbit tissues were almost devoid of A and H type 2 antigens, and only very weak binding of virus particles could be observed on these tissues.

NOTES

RHDV hemagglutinating activity depends on the presence of ABH blood group antigens. RHDV agglutinates human red blood cells but not erythrocytes from rabbits or other mammals (2, 7). A distinctive characteristic of human erythrocytes is the presence of a complex ABH blood group system consisting of three antigens: A, B, and H. The ABH antigens are expressed on the surface of red blood cells and are involved in various physiological functions. The ABH antigens are also expressed on epithelial cells of the respiratory and digestive tracts, and they play a role in the development of the immune system. The presence of ABH antigens on epithelial cells has been associated with the entry of RHDV into the host cell, suggesting that the virus may use these antigens as receptors.

The ability of RHDV to agglutinate human erythrocytes and to attach to rabbit epithelial cells of the upper respiratory and digestive tracts was shown to depend on the presence of ABH blood group antigens. Indeed, agglutination was inhibited by saliva from secretor individuals but not from nonsecretors, the latter being devoid of H antigen. In addition, erythrocytes of the rare Bombay phenotype, which completely lack ABH antigens, were not agglutinated. Native viral particles from extracts of infected rabbit liver as well as virus-like particles from the recombinant virus capsid protein specifically bound to synthetic A and H type 2 blood group oligosaccharides. Both types of particles could attach to adult rabbit epithelial cells of the upper respiratory and digestive tracts. This binding paralleled that of anti-H type 2 blood group reagents and was inhibited by the H type 2-specific lectin UEA-I and polyacrylamide-conjugated H type 2 trisaccharide. Young rabbit tissues were almost devoid of A and H type 2 antigens, and only very weak binding of virus particles could be observed on these tissues.

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presence of ABH antigens. Those from other mammals are devoid of such antigens (21). This prompted us to test the hemagglutinating activity of RHDV on human red blood cells, which have either low or no expression of ABH antigens. To this end, the liver of one adult New Zealand rabbit dead after an experimental infection with RHDV strain VHD L4/90-10 (kindly supplied by IFFA Laboratory, Lyon, France) was used as a source of the virus and prepared as previously described (26). A liver extract from a noninfected rabbit was used as a negative control. Human red blood cells, phenotyped for ABH and Lewis antigens, and saliva were obtained from the Blood Transfusion Center (Nantes, France). The hemagglutination assay was carried out in microtiteration plates with V-bottomed wells with serial dilutions from 12.5% (wt/vol) liver suspensions as previously described (26). As shown in Fig. 1A, the virus-containing liver preparation strongly agglutinated human adult red blood cells irrespective of their ABO phenotype. However, cord blood cells, which present only small amounts of ABH epitopes compared to adults cells (4), as well as erythrocytes of the rare Bombay phenotype, which are completely devoid of such epitopes, were not agglutinated at all. Bombay individuals lack ABH epitopes because of inactivating mutations in the gene (FUT2) encoding the α,1,2-fucosyltransferase responsible for the synthesis of the H antigen on erythrocytes (8, 12). To confirm that the presence of the H antigen was required for agglutination to occur, a hemagglutination inhibition test was performed as previously described (26), using saliva from O blood group individuals of either the secretor [O,Le(a−, b−)] or the nonsecretor [O,Le(a+, b−)] phenotype. The former possess large amounts of H antigen in their saliva, of which the latter are devoid. This absence of antigen in the saliva of about 20% of Europeans is due to inactivating mutations in the FUT2 gene, which encodes an α,1,2-fucosyltransferase responsible for the synthesis of the H antigen in saliva (9, 12). As depicted in Fig. 1B, the H antigen-containing saliva from a secretor strongly abolished agglutination, whereas saliva from a nonsecretor did not.

**RHDV binds to A and H type 2 antigens.** In order to define more precisely the specificity of the virus for antigens of the ABH family, liver extracts containing virus particles were incubated on a set of immobilized synthetic oligosaccharides. These oligosaccharides, coupled to a silica solid support (SYNSORB), were obtained from Chembiomed Ltd. and from R. U. Lemieux (Edmonton, Alberta, Canada). A total of 100 µl of a 1/50 dilution (in phosphate-buffered saline [PBS]) of the viral suspension (25% [wt/vol]) were incubated for 1 h at 37°C under gentle agitation on 10 mg of wet SYNSORB. After adsorption, supernatants were recovered and tested for the presence of viral particles by capture enzyme-linked immunosorbent assay (ELISA). For this experiment, Nunc immunoplates were coated with purified chicken anti-RHDV (AFSSA, Ploufragan, France) by an overnight incubation at 37°C in a wet atm-
were incubated for 1 h at 37°C. RHDV binding was detected restricted to epithelial cells. 

incubating a liver extract from an uninfected rabbit followed by 10C5 (control). Intensity of labeling was scored from very strong (+ + + +) to completely negative (−). +++/+−, areas of heterogeneous labeling. Only extremely weak binding of RHDV could be observed on human stomach and small intestine. Labeling was always restricted to epithelial cells.

Labeling was restricted to biliary ducts.

RHDV could bind to tissues from two young (6 weeks old) and adult rabbit tracheae.

The virus also strongly agglutinates human erythrocytes with an A phenotype. Although it could not be directly tested, recognition of the B type 2 antigen is likely, since the virus strongly agglutinates human erythrocytes with a B phenotype.

**RHDV binds to histo-blood group antigens of rabbit epithelial cells.** ABH histo-blood group antigen expression is not restricted to erythrocytes. Instead, they are widely distributed among tissue types. They have been found on epithelial cells of the digestive tracts of all terrestrial vertebrates tested. However, their appearance on red blood cells seems to be a recent event in phylogenetic terms, since expression on this cell type is restricted to anthropoid apes. We therefore tested whether RHDV could bind to tissues from two young (6 weeks old) and two adult rabbits, fixed in 95% ethanol and paraffin embedded. Sections were incubated with 200 μl of either RHDV or control liver extracts diluted 1/10 or with VLPs at 5 μg/ml. After washings in PBS, RHDV or VLP binding was detected using MAb 10C5 followed by biotinylated secondary antibody (Vector Labs, Burlingame, Calif.) and peroxidase-conjugated avidin (Vector). Reactions were revealed with 3-amino-9-ethylcarbazole. Counterstaining was performed with Harris hematoxylin. The presence of histo-blood group antigens on these tissues was determined using the anti-H MAb 7 E11, specific for H
type 2 determinants (data not shown), and the anti-A blood group antigen 2A-1#8, specific for all types of A structures: A types 1, 2, 3, 4, ALe\(^b\), and ALe\(^c\) (13). Their binding was detected as described above for antibody 10C5. The presence of H type 2 antigen was also detected using peroxidase-labeled UEA-I lectin (Sigma) at 1 \(\mu\)g/ml. No binding of either native RHDV or VLPs was observed on liver, kidney, heart, or spleen. In contrast, a clear labeling of epithelial cells was detected in the trachea, large bronchi of the lung, concha nasalis, tonsils, or small intestine. Strikingly, a parallel labeling was observed with the anti-H MAb 7 E11 and with peroxidase-labeled UEA-I lectin in all these tissues. A parallel labeling was also observed using the anti-A MAb, with the exception of biliary ducts, which were weakly stained (Table 2). Since both MAb 7 E11 and UEA-I specifically recognize the H type 2 antigen, the parallel labeling suggested that RHDV and VLPs could bind to epithelial cells via this antigen. To test this possibility, competitions of RHDV and of VLPs binding on the epithelial cells of the trachea were carried out using unlabeled UEA-I or PAA neoglycoconjugates. To this end, RHDV liver extracts or VLPs were coincubated with either unconjugated UEA-I lectin at 40 \(\mu\)g/ml or PAA neoglycoconjugates at 50 \(\mu\)g/ml. Binding was revealed as described above using MAb 10C5. A near-complete inhibition of the virus binding was obtained by coincubation with UEA-I. Similarly, attachment of VLPs was almost completely inhibited by the H type 2 neoglycoconjugate but not by the Le\(^c\) neoglycoconjugate used as a control (Table 3 and Fig. 3A, B, C, and D). Taken together, these results indicate that binding of native RHDV or VLPs to rabbit epithelial cells depends on the recognition of A or H type 2 antigens.

Absence of RHDV binding to young rabbit epithelial cells

FIG. 3. Histochemical staining of rabbit tracheae. Adult rabbit trachea sections (A through D) were incubated with either native RHDV particles from liver extract (A and B) or VLPs (C and D). RHDV liver extract or VLPs were coincubated with either the UEA-I lectin (B), Le\(^c\)-polyacrylamide conjugate structure 15 (C), or the H type 2-polyacrylamide conjugate (D). Binding of peroxidase-labeled UEA-I lectin to epithelial cells from adult and 6-week-old rabbits is shown in panels E and F, respectively.
correlates with low expression of A and H antigens. Under natural conditions, adult rabbits are highly susceptible to infection. However, young animals are not, and susceptibility progressively increases from 1 to 3 months of age (18, 27). We therefore tested whether native RHDV or VLPs would bind to young rabbit epithelial cells as strongly as they did to adult epithelial cells. As shown in Table 3, it was observed that almost no binding was detectable on the tracheae of 6-week-old rabbits. Likewise, epithelial cells from these young animals did not express detectable amounts of A histo-blood group antigen and expressed much smaller amounts of H type 2 antigen as deduced from the weak labeling given by MAb 7 E11 and UEA-I (Table 3 and Fig. 3E and F).

In order to determine whether the A and H antigens appear on rabbit epithelial cells, their expression was tested weekly by immunofluorescence on buccal epithelial cells. These cells from four rabbits aged from 3 to 12 weeks and from four adults were collected using cotton swabs. After recovery in PBS, cells were labeled with the anti-A MAb diluted 1/2 followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulins (Sigma) or with FITC-conjugated UEA-I at 20 μg/ml. No histo-blood group A antigen expression was detected until the eighth week, when it appeared on a subset of cells. Expression on young rabbit cells increased until 12 weeks, when it was almost as high as on cells from adults. In contrast, the H antigen was detected from the third week on. However, the staining of cells from the younger animals was clearly weaker than that of cells from adults, with full expression being reached at 10 weeks.

The mechanism by which RHDV infects rabbits is unknown at present. We were unable to detect attachment of either native or recombinant virus particles to liver sections. Yet large numbers of viral particles can be isolated from the livers of infected animals, and their presence within hepatocytes has been detected by immunostaining and in situ hybridization (5, 22). Moreover, a recent report describes in vitro infection of rabbit hepatocytes by RHDV (10). Yet ABH antigens were not detected on rabbit hepatocytes, a situation similar to that found in humans. It is thus unlikely that RHDV uses histo-blood group antigens as receptors on hepatocytes. Nevertheless, upper respiratory and digestive tract epithelial cells are likely the first to encounter virus particles at the time of infection. These cells could be a primary site of viral replication. This is in agreement with the fact that tracheitis is a frequent early sign of the disease (6, 24, 27). We were able to observe that both native and recombinant virus particles can attach to them through recognition of A or H type 2 antigens. In addition, very little binding was observed on tracheae from young animals, which turned out to express only small amounts of the antigens compared to adults. This correlates with the very low infectivity of RHDV in young rabbits. These observations suggest, yet do not prove, that the histo-blood group-specific lectin activity of RHDV could participate in the infectious process, and these observations warrant further study.

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REFERENCES