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Transmissible Gastroenteritis Coronavirus, but Not the Related Porcine Respiratory Coronavirus, Has a Sialic Acid (*N*-Glycolylneuraminic Acid) Binding Activity†

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The hemagglutinating activity of transmissible gastroenteritis virus (TGEV), an enteric porcine coronavirus, was analyzed and found to be dependent on the presence of α-2,3-linked sialic acid on the erythrocyte surface. *N*-Glycolylneuraminic acid was recognized more efficiently by TGEV than was *N*-acetylneuraminic acid. For an efficient hemagglutination reaction the virions had to be treated with sialidase. This result suggests that the sialic acid binding site is blocked by virus-associated competitive inhibitors. Porcine respiratory coronavirus (PRCV), which is serologically related to TGEV but not enteropathogenic, was found to be unable to agglutinate erythrocytes. Incubation with sialidase did not induce a hemagglutinating activity of PRCV, indicating that the lack of this activity is an intrinsic property of the virus and not due to the presence of competitive inhibitors. Only monoclonal antibodies to an antigenic site that is absent from the S protein of PRCV were able to prevent TGEV from agglutinating erythrocytes. The epitope recognized by these antibodies is located within a stretch of 224 amino acids that is missing in the S protein of PRCV. Our results indicate that the sialic acid binding activity is also located in that portion of the S protein. The presence of a hemagglutinating activity in TGEV and its absence in PRCV open the possibility that the sialic acid binding activity contributes to the enterotropism of TGEV.

Porcine transmissible gastroenteritis virus (TGEV) is a prototype enteropathogenic coronavirus (17, 21). It affects pigs of all ages, but infection is most severe in newborn piglets, resulting in a fatal diarrhea. The positive-stranded RNA genome of TGEV that is associated with the nucleocapsid protein is surrounded by a lipid envelope (for a review, see reference 9). There are three proteins inserted into the viral membrane: the S (220-kDa), the M (29- to 36-kDa), and the minor sM (10-kDa) protein. The main target of the immune response to TGEV is the S protein, which contains four antigenic sites (4, 6), named C, B, D, and A (from the amino terminus to the carboxy terminus). This surface glycoprotein plays a crucial role in the initial stage of the infection. By specific interaction with porcine aminopeptidase N, the cellular receptor for TGEV, the S protein mediates the binding of the virus to the cell surface (7, 29). It is presumably also involved in the fusion between the viral and the cellular membrane. In addition, it is responsible for the hemagglutinating activity that has been demonstrated for several strains of TGEV (15, 16). Among coronaviruses, bovine coronavirus (BCV) and serologically related viruses are best known as hemagglutinating viruses. Their ability to agglutinate erythrocytes is due to a receptor-binding activity that specifically recognizes *N*-acetyl-9-*O*-acetylneuraminic acid on surface glycoconjugates (26, 30). The same receptor determinant is used by BCV to infect cells (27). The sialic acid binding activity is primarily a function of the surface glycoprotein S (25). Another surface protein of BCV, the HE protein, only has a weak hemagglutinating activity (12, 25, 28). The HE protein is able to inactivate receptors for BCV because of an acetylesterase activity that releases the 9-*O*-acetyl group from sialic acid. An HE gene is present only in the genome of the BCV serogroup. Coronaviruses that belong to antigenic clusters different from BCV lack a receptor-destroying enzyme. These viruses have been reported to be either poor hemagglutinins or devoid of hemagglutinating activity (2, 15). They include avian infectious bronchitis virus (IBV) and TGEV. We have shown that IBV can be converted into a potent hemagglutinating agent by treatment of virions with sialidase (24). The receptor determinant on erythrocytes recognized by IBV is α-2,3-linked sialic acid. These findings indicated that coronaviruses that lack a receptor-destroying activity may also have a sialic acid binding activity. It appears that sialic acids from viral or cellular glycoconjugates occupy the binding site of IBV and prevent the virus from agglutinating erythrocytes. Treatment with sialidase inactivates such competitive inhibitors on the viral surface and allows the virus to interact with sialic acids on the erythrocyte surface.

In the winter of 1983-1984, a new coronavirus emerged, porcine respiratory coronavirus (PRCV) (18). This virus replicates with high efficiency in the respiratory tract but with very low efficiency or not at all in the gut (5). In serological tests, PRCV was found to be closely related to TGEV (3, 23). Analysis of the nucleotide sequences of both viruses revealed an overall homology of 96% (19, 23, 31). The main differences found between the two viruses were three deletions in the genome of PRCV (19, 23, 31). Two minor deletions render the open reading frame of a nonstructural protein nonfunctional. A large deletion in the S gene results in a truncated spike

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TABLE 1. Comparison of Neu5Ac and Neu5Gc as receptor determinants for the agglutination of 1-day-old chicken erythrocytes by sialidase-treated TGEV and fowl plague virus

Erythrocytes	CMP-sialic acid amt (nmol)	HA activity (U/ml) of:	
		Treated TGEV	Fowl plague virus
Control	0	1,024	512
Asialo	0	<2	<2
Resialylated			
Neu5Ac	400	<2	512
	800	2	512
Neu5Gc	40	<2	<2
	100	2	256
	200	128	512
	400	512	512

protein lacking 224 amino acids that are present near the N terminus of the TGEV S protein of European PRCV strains (starting at position 21 of the unprocessed protein). Slight variations in the size and the position of the deletion have been reported for American isolates (reviewed in reference 9). The deletion within the spike protein of PRCV is responsible for the loss of two antigenic sites (C and B), as indicated by the lack of reactivity with monoclonal antibodies (22) and by sequence data (23). Another two (A and D) of the four antigenic sites that have been assigned to the S protein of TGEV (10) are not affected by the deletion and explain the serological relatedness between PRCV and TGEV.

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The Purdue strain (PUR46-MAD) of TGEV (22) used in this study was propagated in LLC-PK1 (pig kidney) cells and harvested 2 days after infection. Virus was sedimented from the clarified supernatant of infected cells (48 h postinfection [p.i.]) by centrifugation for 1 h at 110,000 × *g*. The virus pellet was suspended in 200 μl of phosphate-buffered saline (PBS), layered onto a 10 to 60% (wt/wt, in PBS) sucrose gradient, and centrifuged for 2 h at 130,000 × *g*. The virus band was harvested and, following dilution with PBS, sedimented by centrifugation for 1 h at 150,000 × *g*. For sialidase treatment, virus was incubated at 37°C for 30 min with the enzyme from *Vibrio cholerae* (50 mU/ml). TGEV was found to have the same hemagglutination (HA) properties that have previously been reported for IBV (24): (i) sialidase treatment of purified virions induced the hemagglutinating activity, (ii) sialidase treatment of erythrocytes rendered the cells resistant to agglutination by TGEV, and (iii) the susceptibility of asialo cells to agglutination was restored, when the cells were resialylated to contain α-2,3-linked *N*-acetylneuraminic acid (Neu5Ac). To observe agglutination of resialylated erythrocytes, the substrate for the sialyltransferase had to be supplied in a relatively high level (1 μmol or more). As TGEV is a porcine virus and *N*-glycolylneuraminic acid (Neu5Gc) is a common type of sialic acid of porcine cells, we analyzed whether Neu5Gc is recognized by TGEV more efficiently than is Neu5Ac. A 10% suspension of chicken erythrocytes was incubated for 30 min at 37°C with sialidase from *V. cholerae* (20 mU/ml). Asialo cells were washed and resuspended in PBS to a final concentration of 20%. For resialylation, 200 μl of the sialidase-treated cells was incubated with α-2,3-sialyltransferase (5.2 mU; Boehringer Mannheim) and the amount of CMP-activated Neu5Ac (Sigma) or Neu5Gc indicated in Table 1. CMP-Neu5Gc was prepared as described by Lepers et al. (13). After 2 h at 37°C, cells were washed and used as a 1% suspension to determine the HA titer of a sialidase-treated suspension of TGEV (Table 1). Erythrocytes that had been modified to contain Neu5Ac were agglutinated very efficiently by an influenza A virus (fowl plaque virus FPV/Rostock/34, kindly provided by H.-D. Klenk, Marburg, Germany). TGEV, however, was unable to agglutinate these erythrocytes, even when CMP-Neu5Ac was used in a level as high as 800 nmol. Erythrocytes that had been modified to contain Neu5Gc were agglutinated by TGEV with higher efficiency. Sialylation of the cells in the presence of as little as 200 nmol of CMP-Neu5Gc was sufficient to observe HA. The minimum amount of activated Neu5Gc required for agglutination by fowl plaque virus was only twofold lower. This result indicates that Neu5Gc is preferred by TGEV as a receptor determinant over Neu5Ac.

The virus used for the experiments described above was harvested 2 days after infection. When TGEV was analyzed 24 h p.i., it was noticed that the virus was able to agglutinate chicken erythrocytes even if it had not been treated with sialidase. Therefore, the HA activity was determined during the time course of an infection. As shown in Fig. 1, HA activity was not detectable before 16 h p.i. and maximum values were measured with untreated virus at 24 h p.i. The HA activity was not fully developed, because enzyme treatment increased the HA titer by a factor of about 10. Later in infection the HA activity of the supernatant decreased and was no longer detectable after 60 h p.i. This result indicates that the loss of the HA activity of TGEV is a late event. The transient HA activity after infection by TGEV was observed not only with LLC-PK1 cells (Fig. 1) but also with ST (swine testis) cells (not shown). The sialidase treatment of TGEV did not affect the infectivity of the virus.

In order to analyze whether PRCV is able to agglutinate erythrocytes, the BEL85 strain (22) was concentrated from the cell supernatant and purified by sucrose gradient centrifugation. For comparison, TGEV was prepared in the same way. The identities of both viruses were confirmed by Western blot (immunoblot) analysis. The S proteins of both TGEV and PRCV were recognized by monoclonal antibody 6A.C3, whereas 1D.B12 reacted only with the S protein of TGEV (not shown). The latter monoclonal antibody recognizes an anti-

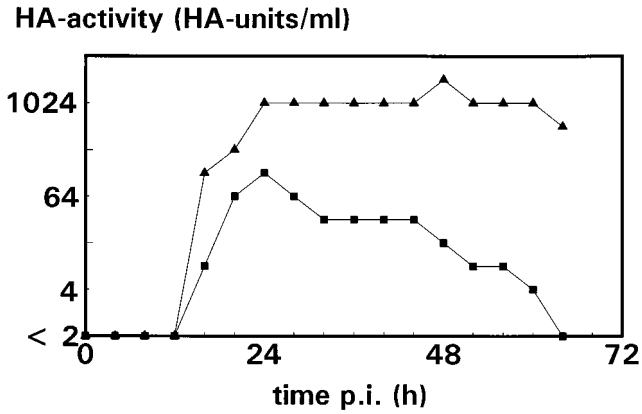


FIG. 1. Hemagglutinating activity of TGEV released from LLC-PK1 cells at different times p.i. Cells were infected by TGEV and incubated with minimum essential medium in the absence of serum. At the times indicated the supernatant was analyzed for hemagglutinating activity. Prior to the HA assay with chicken erythrocytes (26), the supernatant (100-μl aliquots) was incubated in the presence (triangles) or absence (squares) of sialidase (4 mU, 30 min, 37°C) from *V. cholerae* (Behringwerke, Marburg, Germany).

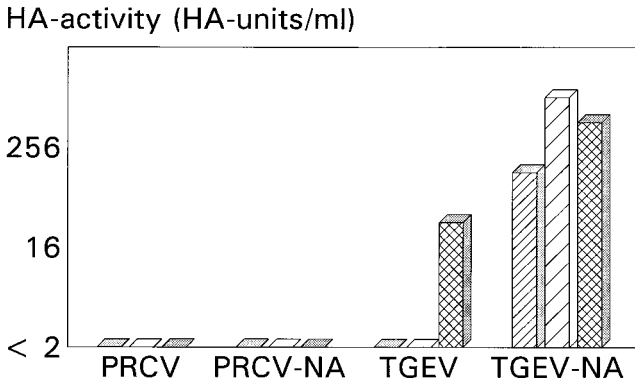
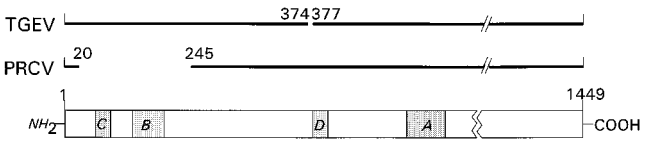


FIG. 2. Ability of TGEV and PRCV to agglutinate erythrocytes from different species. Purified preparations of TGEV and PRCV were used for HA assays with porcine (narrowly hatched), chicken (widely hatched), and bovine (cross-hatched) erythrocytes. The hemagglutinating activity was determined with untreated (TGEV and PRCV) and sialidase-treated (TGEV-NA and PRCV-NA) virus. To prolong the storage time, bovine and porcine erythrocytes were fixed with glutardialdehyde (0.1%, 24 h, 4°C).



MoAb	Site	HI-units/ml
1C.C12	A	32
1D.E7	A	16
6A.C3	A	16
1D.B12	B	1024
1B.H11	B	512
8F.B3	B	32
5B.H1	C	16
1D.G3	D	16

FIG. 3. Ability of monoclonal antibodies to prevent sialidase-treated TGEV from agglutinating chicken erythrocytes. Monoclonal antibodies (MoAb) directed to site A, B, C, or D were analyzed for HA inhibition (HI) activity. The locations of the antigenic sites on the S protein are shown at the top. The schematic representation of the S proteins of TGEV and PRCV indicates the large deletion in the surface protein of PRCV.

genic site that is present in the spike protein of TGEV but absent from the corresponding protein of PRCV (22) (see Fig. 3). Western blot analysis with the former antibody recognizing antigenic site A, which is present in both TGEV and PRCV, indicated that comparable amounts of protein were present in both virus preparations. However, only TGEV was able to agglutinate chicken erythrocytes (512 HA units/ml). No hemagglutinating activity (<2 HA units/ml) was detectable with PRCV, although it had been pretreated with sialidase in the same way as TGEV (data not shown).

To confirm the lack of hemagglutinating activity, erythrocytes from different species were included in this analysis. As shown in Fig. 2, the inability of PRCV to induce HA was not restricted to avian erythrocytes but was also observed with porcine and bovine cells. These cells were chosen because they have been reported to contain 90% or more of their sialic acids in the form of Neu5Gc (20). Bovine erythrocytes were agglutinated by TGEV to some extent even prior to sialidase treatment of the virus. This finding suggests that on bovine erythrocytes, Neu5Gc is present in a favorable spatial arrangement making the interaction with TGEV less susceptible to competitive inhibitors, as has been shown for influenza viruses (reviewed in reference 11). Despite their sensitivity to HA by TGEV, bovine erythrocytes were resistant to agglutination by PRCV. PRCV harvested earlier in infection was also unable to agglutinate erythrocytes (not shown), indicating that in contrast to TGEV (see above), there is no transient HA activity measurable during the infectious cycle. These results indicate that the lack of hemagglutinating activity in PRCV is an intrinsic property of the virus and not due to the presence of competitive inhibitors.

In order to locate the sialic acid binding site on the S protein of TGEV, we analyzed a panel of S protein-specific monoclonal antibodies for HA inhibition activity. Serial twofold dilutions of the different monoclonal antibodies were prepared with PBS in microtiter plates. Each dilution (25 μl) was mixed with an equal volume of a purified preparation of sialidase-treated TGEV. The virus had been diluted with PBS to have a hemagglutinating activity of 16 HA units/ml. After addition of 50 μl of 0.5% chicken erythrocytes (in PBS), the plate was incubated for 1 h at 4°C. The reciprocal value of the maximum dilution that caused complete HA inhibition was used as a measure of the inhibitory activity (HA inhibition units per milliliter) of the monoclonal antibodies. Antibodies directed against antigenic site A, C, or D did not prevent TGEV from agglutinating erythrocytes (Fig. 3). Three antibodies directed against site B, 1D.B12 (assigned to amino acids 76 to 97), 1B.H11 (assigned to amino acids 97 to 144), and 8F.B3 (10), have been analyzed. Two of the antibodies (1D.B12 and 1B.H11) efficiently inhibited the HA activity of TGEV. The HA inhibition observed with antibody 8F.B3 was only slightly above background values. This result indicates that the sialic acid binding site is located within or close to antigenic site B. It should be noted that the designation of antigenic sites is that proposed by Correa et al. (4). Other authors have used different designations. Site B shown in Fig. 3 corresponds to site D described by Delmas et al. (6). This epitope is absent from the S protein of PRCV because of a deletion of 224 amino acids (Fig. 3). We conclude from our results that the sialic acid binding site of TGEV or a crucial part of it is located within this stretch of amino acids (residues 21 to 244 of the unprocessed protein), close to residues 76 to 144.

The deletion in the S protein is the most prominent difference between TGEV and PRCV and is therefore likely to contribute to the differences in tropism and pathogenicity between these two viruses. It is tempting to speculate that the ability to bind to sialylated glycoproteins is important for the enterotropism of TGEV. Prior to our finding that TGEV possesses a sialic acid binding activity, it was proposed that infection in the gut may require an additional receptor binding activity or another factor mapping around amino acid 219 of the S protein (23). In this view, the sialic acid binding activity might allow a more efficient binding to intestinal cells and thus improve the infection. For this effect, competitive inhibitors would not have to be inactivated by pretreatment with sialidase, because (i) untreated virus has a transient hemagglutinating activity and (ii) the sialic acids on intestinal cells may be present in a favorable arrangement similar to that on bovine erythrocytes. Recently mutants of TGEV that had lost enteropathogenicity have been described (1). The mutants had been selected for resistance to a neutralizing monoclonal antibody. The antibody was directed against an antigenic site that corresponds to site B in Fig. 3. Several mutants differed from

the wild-type virus only by point mutations within that portion of the S protein that is missing in the PRCV glycoprotein (residues 145 to 155). This finding shows that this part of the surface protein is important for enteropathogenicity. It will be interesting to analyze these mutants for hemagglutinating activity. At present the importance of the sialic acid binding activity is not known. Both PRCV and TGEV replicate very well in cell cultures with aminopeptidase as a receptor (7, 8). Therefore, the sialic acid binding activity appears to be dispensable for virus growth in cell culture. It may, however, play a role for the infection of animals. Most enteropathogenic viruses of mammals are nonenveloped viruses (14, 21). Coronaviruses contain a lipid envelope and can obviously cope with the unfavorable conditions in the alimentary tract (low pH, proteases, and bile salts). The sialic acid binding activity may increase the stability of the virions by binding to sialoglycoconjugates. An increased stability would help the virus to survive the passage through the gastrointestinal tract. In future studies we will address the question of the importance of the sialic acid binding activity for the enterotropism of TGEV.

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