

Characterization of the sialic acid binding activity of transmissible gastroenteritis coronavirus by analysis of haemagglutination-deficient mutants

C. Krempl,¹ M.-L. Ballesteros,² G. Zimmer,^{1,3} L. Enjuanes,² H.-D. Klenk¹ and G. Herrler^{1,3}

¹ Institut für Virologie, Philipps-Universität Marburg, Robert-Koch-Str. 7, 35037 Marburg, Germany

² Centro Nacional de Biotecnología, Department of Cell and Molecular Biology, CSIC Campus Universidad Autónoma de Madrid, Canto Blanco, 28049 Madrid, Spain

³ Institut für Virologie, Tierärztliche Hochschule Hannover, Bünteweg 17, 30559 Hannover, Germany

Transmissible gastroenteritis coronavirus (TGEV) agglutinates erythrocytes of several species by virtue of sialic acid binding activity of the surface protein S. We have isolated and characterized five haemagglutination-defective (HAD) mutants. In contrast to the parental virus, the mutants were unable to bind to porcine submandibular mucin, a substrate rich in sialic acid. Each of the mutants was found to contain a single point mutation in the S protein (Cys 155Phe, Met 195Val, Arg 196Ser, Asp 208Asn or Leu 209Pro), indicating that these amino acids are affecting the sialic acid binding site. In four of the HAD mutants a nearby antigenic site is affected in addition to the sialic acid binding site, as indicated by reactivity with monoclonal antibodies. The parental virus was found to have an increased resistance to the detergent octylglucoside compared to the HAD mutants. This effect depended on cellular sialoglycoconjugates bound to the virion. If the binding of sialylated macromolecules was prevented by neuraminidase treatment, the parental virus was as sensitive to octylglucoside as were the HAD mutants. We discuss the possibility that the sialic acid binding activity helps TGEV to resist detergent-like substances encountered during the gastrointestinal passage and thus facilitates the infection of the intestinal epithelium. An alternative function of the sialic acid binding activity – accessory binding to intestinal tissues – is also discussed.

Introduction

Transmissible gastroenteritis virus (TGEV) is a prototype enteropathogenic coronavirus that causes fatal diarrhoea in newborn piglets (Pensaert *et al.*, 1993). The nucleocapsid of TGEV, containing the positive-stranded RNA genome and the N protein, is surrounded by a core shell which is protected by a lipid envelope (Risco *et al.*, 1996). There are three proteins inserted into the viral membrane: the S (220 kDa), the M (29–36 kDa) and a minor E (10 kDa) protein. The S protein, which is the main inducer of neutralizing antibodies, plays a crucial role in the initial stage of the infection. By interacting with porcine aminopeptidase N, the cellular receptor for TGEV (Delmas *et al.*, 1992), the S protein mediates binding of the virus to the cell surface (Suñé *et al.*, 1990). Presumably, it is also

involved in the fusion between the viral and cellular membranes. In addition, the S protein has haemagglutinating activity (Noda *et al.*, 1987, 1988). The agglutination of erythrocytes by TGEV does not involve aminopeptidase N. The binding of TGEV to erythrocytes is mediated by sialic acid binding activity of the S protein. Among the different types of sialic acid, TGEV has a preference for *N*-glycolylneuraminic acid (Schultze *et al.*, 1996). The sensitivity to different monoclonal antibodies (MAbs) indicates that the binding site for aminopeptidase N and the binding site for sialic acid are located on different portions of the S protein (Schultze *et al.*, 1996). Recent studies with mutants of TGEV indicated that residues within a short stretch of amino acids (145–155) are important for the recognition of sialic acids (Krempl *et al.*, 1997). The mutants had been selected for resistance to a MAb (Delmas *et al.*, 1986). Interestingly, the point mutations that were responsible for the lack of antibody reactivity also resulted in the concomitant loss of both the haemagglutinating

Author for correspondence: Georg Herrler (at address 3).
Fax +49 511 953 8898. e-mail herrler@viro.tiho-hannover.de

activity and the enteropathogenicity (Bernard & Laude, 1995; Kreml *et al.*, 1997). These results not only indicated that the respective amino acids are located at or close to the sialic acid binding site, but also they suggested that the sialic acid binding activity is a pathogenicity factor of TGEV. This conclusion is in agreement with data reported for a porcine respiratory coronavirus (PRCV) that is closely related to TGEV. This virus replicates with high efficiency in the respiratory tract, but with very low efficiency in the gut (Cox *et al.*, 1990). Like the mutants mentioned above, PRCV has no haemagglutinating activity. The lack of sialic acid binding activity is explained by a large deletion in the S gene that results in a truncated spike protein. A stretch of 224 amino acids that is present near the N terminus of the TGEV S protein (starting at position 21 of the unprocessed protein) is missing in the corresponding protein of PRCV (Rasschaert *et al.*, 1990; Sánchez *et al.*, 1992). The point mutations that resulted in the loss of the haemagglutinating activity and the enteropathogenicity are also located in that portion of the S protein that is present in the TGEV S but absent from the PRCV S protein (Bernard & Laude, 1995; Kreml *et al.*, 1997).

How the sialic acid binding activity might contribute to the enteropathogenicity of TGEV is not known. Both PRCV and the haemagglutination-deficient (HAD) mutants can replicate in cultured cells, suggesting that the sialic acid binding activity is important only in the context of the intestinal infection. To get further information about the sialic acid binding site and the sialic acid binding activity we have isolated HAD mutants. Here we report amino acids that are important for the sialic acid binding activity. Furthermore, we show that the sialic acid binding activity increases the resistance of TGEV to the detergent octylglucoside.

Methods

■ **Virus.** The Purdue strain of TGEV (PUR46-MAD) was used throughout this study (Sánchez *et al.*, 1990). The isolation of the HAD mutants has been described elsewhere (Kreml *et al.*, 1998). Stock virus was propagated in swine testicular (ST) cells. After incubation for 20–24 h at 37 °C, the supernatant was harvested, clarified by low speed centrifugation and, after addition of 1% foetal calf serum (FCS), stored at –80 °C.

■ **Cells.** ST cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS (McClurkin *et al.*, 1966).

■ **Sedimentation and purification of TGEV and TGEV mutants.** Confluent monolayers of ST cells were infected with stock virus (PUR46 or HAD mutants) at an m.o.i. of 0.1. After incubation for 2 days at 37 °C in medium without FCS, the supernatant was clarified by centrifugation for 10 min at 1400 g. Virus was sedimented by ultracentrifugation at 140 000 g for 1 h. The virus pellet was resuspended in PBS and treated with neuraminidase as described below. For purification, the virus was layered onto a 20–60% sucrose gradient (w/w, in PBS), and centrifuged for 4 h at 150 000 g. The virus band was harvested and, following dilution with PBS, sedimented by centrifugation for 1 h at 150 000 g. Purified virus was stored at –20 °C.

■ **Neuraminidase treatment of virus.** The supernatant of infected cells was incubated with neuraminidase from *Vibrio cholerae* (Behringwerke) at a final concentration of 40 mU/ml. As a control a sample was treated with an equal volume of PBS. Following incubation for 30 min at 37 °C, the samples were cooled on ice and used for haemagglutination assays.

Virus that had been sedimented was resuspended in 200 µl PBS per tube and treated with 50 mU/ml *V. cholerae* neuraminidase for 30 min at 37 °C. Following enzyme treatment, virus was purified by sucrose gradient centrifugation as described above.

■ **Neuraminidase treatment of cells.** Confluent monolayers of ST cells were washed twice with PBS and incubated with neuraminidase from *Clostridium perfringens* (type X, Sigma) for 1 h at 37 °C. An enzyme activity of 25 mU/cm² of cell monolayer was used. Following incubation, the neuraminidase was removed by three washings with PBS and the cells were infected with TGEV.

■ **Stability assays.** Confluent ST cells were infected at an m.o.i. of 1. The supernatant containing virus but no FCS was harvested 18–20 h post-infection, clarified by low speed centrifugation and used for the following experiments.

To analyse the effect of low pH, virus was diluted 1:10 with McIlvaine's buffer (0.1 M citric acid and 0.2 M Na₂HPO₄ were mixed to get the desired pH) of defined pH values. Following incubation for 15 min at room temperature, the pH was neutralized by dilution of the samples in HEPES (25 mM)-buffered DMEM (Gibco)–10% FCS. Serial tenfold dilutions were analysed for infectivity by plaque assay.

The sensitivity of the virions to protease treatment was examined by mixing the supernatant with an equal volume of PBS containing different concentrations of trypsin (TPCK-trypsin, Sigma). After incubation for 1 h at 37 °C, a serial tenfold dilution in DMEM–10% FCS was prepared. To inactivate trypsin, the medium for the first dilution was supplemented with soybean trypsin inhibitor (Sigma) to a final concentration of 25 µg/ml, a concentration high enough to inhibit the highest amount of trypsin used.

To examine the effect of detergent on the stability of TGEV, the supernatant of infected cells was diluted with an equal volume of PBS containing different amounts of octylglucoside (n-octyl α -D-glucopyranoside, Sigma). Following incubation for 15 min at room temperature, a serial tenfold dilution of the samples in PBS was prepared. The residual infectivity of the virus was determined by plaque assay.

■ **Virus binding to mucin.** Wild-type virus and mutants of TGEV were analysed for binding to immobilized porcine submandibular mucin (PSM). PSM (kindly provided by R. Schauer, Kiel, Germany) was immobilized on microtitre plates (MikroELISA, Dynatech) by adding 100 µl of a mucin solution (1 mg/ml PBS) to each well. After incubation overnight at 4 °C, the mucin solution was removed and nonspecific binding sites were blocked with 'blocking reagent' (Boehringer Mannheim). Immobilized PSM was incubated with serial twofold dilutions of neuraminidase-treated purified virus. Bound virus was detected with MAb 6A.C3 directed against antigenic site A of the S protein (Gebauer *et al.*, 1991). After incubation with horseradish peroxidase-conjugated anti-mouse IgG (Amersham), the peroxidase substrate ABTS (Kirkegaard & Perry Laboratories) was added according to the instructions of the manufacturer. At the end of the colour reaction, the absorbance was determined at 405 nm.

■ **Western blotting.** Viral proteins were separated by SDS-PAGE under nonreducing conditions and blotted to nitrocellulose using a semi-dry Western blot method as described by Schultze *et al.* (1991). After blocking of nonspecific binding sites by incubation with 10% nonfat dry

milk in PBS, the immobilized TGEV S protein was incubated with different MAbs. Bound antibodies were detected with biotinylated sheep anti-mouse IgG (Amersham) and streptavidin-biotinylated horseradish peroxidase complex (Amersham).

For detection of proteins in polyacrylamide gels, a silver stain reagent (BIO-RAD) was used according to the instructions of the manufacturer.

■ **Haemagglutination assay.** This was performed in microtitre plates (U form). Serial twofold dilutions of neuraminidase-treated or untreated virus suspensions were prepared in PBS. An equal volume (50 µl) of chicken erythrocytes in PBS (0.5%) was added to each dilution. After incubation for 1 h at 4 °C, the haemagglutination titre was determined. The reciprocal value of the highest virus dilution causing complete haemagglutination was used as a measure of the haemagglutinating activity expressed as HA units/ml.

■ **Plaque assay.** Confluent monolayers of ST cells grown in 6-well tissue culture plates or 60 mm tissue culture dishes were infected with 250 µl or 500 µl, respectively, of a serial tenfold dilution of the virus suspension analysed. After incubation for 1 h at 37 °C, cells were overlaid with 0.7% Sea-Plaque agarose in DMEM–2% FCS and incubated at 37 °C. At 2 days post-infection, plaques were visualized by staining with 0.0125% neutral red in 0.8% agar.

■ **HPLC analysis of sialic acids.** For HPLC analysis, sialic acids were released from glycoconjugates and derivatized with 1,2-diamino-4,5-methylendioxybenzyl to generate a fluorescent compound (Hara *et al.*, 1989; Schauer, 1982). Analysis was carried out by separation of sialic acids on a C18 reversed phase column (4 × 250 mm; grain size 4 µm; size of pores 100 Å; Merck) with acetonitrile–methanol–H₂O (9:7:84; v/v/v) as mobile phase. Samples of 10 µl were applied to the column and eluted at a flow rate of 1 ml/min for 20 min (L-6200A HPLC-pump, Merck). Sialic acids were detected with a fluorescence detector operating at 375 nm for excitation and 445 nm for emission.

■ **Sequencing of the HAD mutants.** A cDNA fragment corresponding to the first 1300 nucleotides of the S gene was amplified by using RT-PCR and sequenced by oligodeoxynucleotide primer extension and dideoxynucleotide chain termination procedures (Sanger *et al.*, 1977; Zimmern & Kaesberg, 1978). Sequence data were assembled and analysed using software of the Wisconsin Package version 9.0, Genetics Computer Group, Madison, Wisconsin, USA.

Results

Sequence analysis of mutants

We have recently isolated five mutants of TGEV that were selected for the inability to bind to bovine erythrocytes

(Krempf *et al.*, 1998). Bovine erythrocytes were chosen because they contain a high proportion (> 90%) of their surface-bound sialic acids in the form of *N*-glycolylneuraminic acid (Reuter *et al.*, 1988), which is recognized by TGEV with higher efficiency than is *N*-acetylneuraminic acid, the most common type of sialic acid (Schultze *et al.*, 1996). Mutants unable to bind to bovine erythrocytes were expected to have a defect in the sialic acid binding activity. The five plaque-purified mutants were designated HAD mutants 2, 3, 4, 7 and 29. In order to characterize the mutants in more detail, a sequence analysis was performed. A stretch of the S gene ranging from nucleotide – 69 (69 nucleotides upstream of the open reading frame) to nucleotide 1344 was sequenced. This stretch included the region of the S gene that is deleted in the corresponding gene of PRCV and that encodes a crucial part of the sialic acid binding site. The result is summarized in Table 1. Each of the mutants HAD2, HAD3, HAD4, HAD7 and HAD29 had a single point mutation resulting in an amino acid exchange at residues 155, 195, 196, 208 or 209, respectively, of the mature S protein. These mutations are located within that stretch of the S gene that is deleted in the corresponding gene of PRCV.

Reactivity with MAbs

Since MAbs against site B (Gebauer *et al.*, 1991) inhibit haemagglutination, the binding of these MAbs to the S protein of the HAD mutants was examined in a Western blot. Fig. 1 shows the S proteins of the HAD mutants and wild-type virus after incubation with site B-specific MAbs 1D.B12, 1B.H11 or 8F.B3 (Gebauer *et al.*, 1991). Silver staining of the polyacrylamide gel and staining of immobilized S proteins on nitrocellulose with MAb 1D.E7 (directed against site A) were used to visualize the blotting efficiency. The results showed that the S proteins of the HAD mutants were recognized by site B MAbs to a different extent. HAD2 was recognized by all site B MAbs analysed. The S proteins of HAD3 and HAD29 gave a positive reaction only with two of the MAbs, 1B.H11 and 8F.B3. The lowest reactivity was obtained with the HAD4 and HAD7 mutants, the S protein of which was recognized only by MAb 8F.B3. Incubation of MAb 8F.B3 with the S protein of HAD7 resulted in a weak band. This may in part be due to a

Table 1. Haemagglutination-deficient (HAD) mutants of TGEV

Mutant	Nucleotide exchange	Amino acid exchange	Position (amino acid)	HA activity (HA units/ml)	Infectivity (p.f.u./ml)
HAD2	ATG → GTG	Met → Val	195	< 2	2.1 × 10 ⁸
HAD3	CTT → CCT	Leu → Pro	209	< 2	5.1 × 10 ⁸
HAD4	GAC → AAC	Asp → Asn	208	< 2	5.0 × 10 ⁸
HAD7	TGT → TTT	Cys → Phe	155	< 2	2.9 × 10 ⁸
HAD29	CGT → AGT	Arg → Ser	196	< 2	1.3 × 10 ⁸
PUR46	–	–	–	512	1.5 × 10 ⁸



Fig. 1. Recognition of TGEV mutants HAD 2, 3, 4, 7 and 29 and wild-type virus (Pur) by different MAbs against the S protein in a Western blot. Only the S protein is shown. Specificity of the MAbs: 1D.E7: site A (b); 1D.B12, 1B.H11, 8F.B3: site B. Silver staining and detection by MAb 1D.E7 were used to visualize the amount of S protein applied.

lower amount of protein present on the blot because incubation with site A antibody (1D.E7) also resulted in a somewhat weaker band compared to the other HAD mutants. Our finding that the amino acid exchanges in the S proteins of the HAD mutants did not abolish the reactivity with MAb 8F.B3 is consistent with a previous finding that this antibody had no, or only a slight, haemagglutination-inhibition activity (Schultze *et al.*, 1996).

Interaction with mucins

A haemagglutination assay is a convenient but not a sensitive method for determining the sialic acid binding activity. Therefore, we applied an ELISA-type assay to determine virus binding to porcine mucin from *Glandula submandibularis* (PSM). As shown by HPLC analysis (Fig. 2B), the sialic acids of PSM predominantly (> 90%) consist of *N*-glycolylneuraminic acid (Neu5Gc). PSM was immobilized on microtitre plates and incubated with different dilutions of purified neuraminidase-treated virus preparations of PUR46,

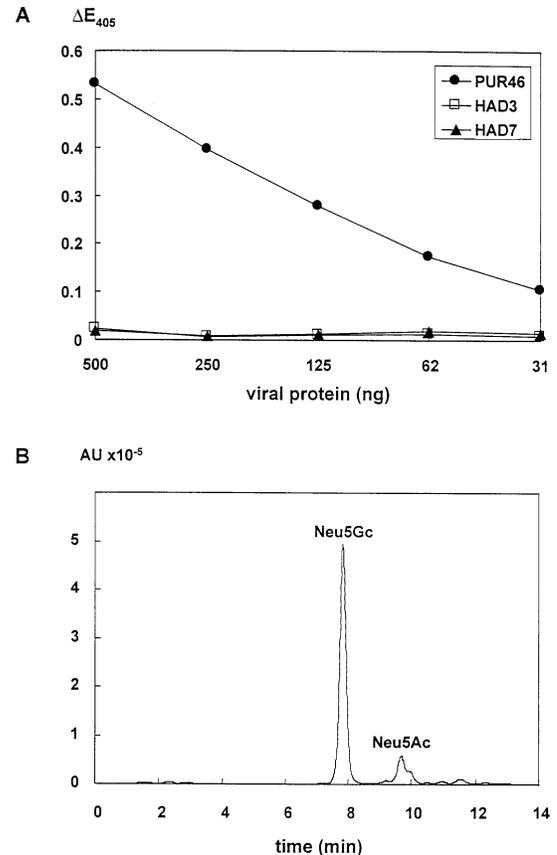


Fig. 2. (A) Binding of PUR46 wild-type, HAD3 and HAD7 to mucin from porcine *Glandula submandibularis*. The mucin was immobilized on ELISA plates (100 µg per well) and incubated with different amounts of *Vibrio cholerae* neuraminidase-treated virus. Bound viruses were detected with MAb 6A.C3 (site A-specific) and a peroxidase-conjugated antibody against mouse IgG. The amount of bound enzyme was determined by measuring the absorbance of substrate at 405 nm. (B) Sialic acids of porcine submandibular mucin (PSM) determined by HPLC analysis. The peaks for *N*-glycolylneuraminic acid (Neu5Gc) and *N*-acetylneuraminic acid (Neu5Ac) are indicated.

HAD3 or HAD7. The viral protein content was determined to ensure that equivalent amounts of virions were applied for each of the three viruses. Bound virus was detected with the site A-specific MAb 6A.C3 (Gebauer *et al.*, 1991) and a peroxidase-conjugated antibody against mouse IgG. As can be seen in Fig. 2(A), the wild-type virus PUR46 bound with high affinity to PSM. An amount of viral protein as low as 31 ng was sufficient for a positive enzyme reaction. On the other hand, no binding of HAD3 and HAD7 was detectable even at the highest concentration of virus examined (500 ng). Thus, HAD3 and HAD7 are not only haemagglutination-deficient but also deficient in binding to PSM.

Resistance to octylglucoside

As TGEV infects pigs via the gastrointestinal tract, it is expected to be resistant to low pH, to proteolytic enzymes and to detergent-like bile salts. We were interested to know

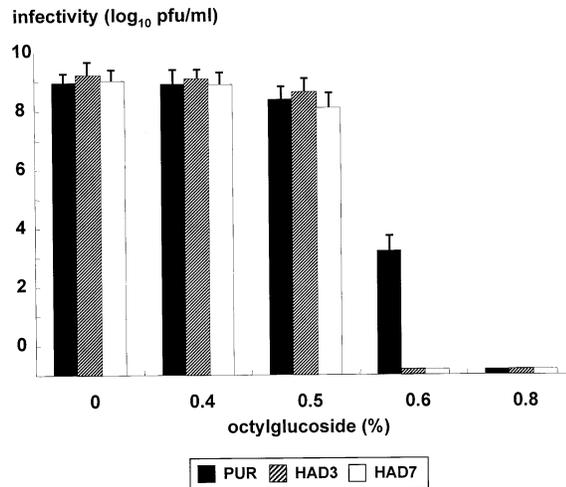


Fig. 3. Stability of PUR46 wild-type (black bars), HAD3 (hatched bars) and HAD7 (white bars) at different concentrations of octylglucoside. After incubation with detergent, the residual infectivity was determined by a plaque assay. Shown are the mean values of five experiments.

whether the sialic acid binding activity affects the sensitivity of TGEV to such agents. Therefore, PUR46 and two of the mutants, HAD3 and HAD7, were examined to see whether infectivity is reduced by treatment of the virions with low pH buffers, with different concentrations of trypsin or with different concentrations of the detergent octylglucoside. As far as acidic conditions are concerned, the mutants were found to be as resistant as was the parental strain. Incubation for 30 min at a pH of 3.0 did not decrease infectivity (not shown). A pH of 2.2 caused a 10^3 -fold reduction of the titre for all three viruses. Neither did PUR46 and the two mutants differ from each other in their sensitivity to trypsin treatment. Incubation with 1 mg of enzyme/ml resulted in a 10^3 -fold reduction of infectivity. Increasing the concentration of trypsin to 2 mg/ml did not result in a further decrease of infectivity (not shown).

A difference between PUR46 and the two HAD mutants was observed when the viruses were analysed for their sensitivity to the action of octylglucoside (Fig. 3). Up to a concentration of 0.5%, the detergent had no effect on the infectivity of the three viruses. At a concentration of 0.6% both HAD mutants were completely inactivated. In the case of PUR46 the titre was reduced from 9×10^8 p.f.u./ml to 2×10^3 p.f.u./ml. A further increase of the concentration of octylglucoside resulted in a complete inactivation of PUR46 also. This result shows that PUR46 is somewhat less sensitive to the action of octylglucoside than are the HAD mutants.

TGE virions released from infected cells show a transient haemagglutination activity that disappears later in infection. Sialoglycoconjugates derived from the cell surface appear to interact with the sialic acid binding site and prevent binding to erythrocytes. Sialylated macromolecules bound to the virus surface might increase virus stability. We were interested in knowing whether this mechanism may explain the increased resistance of PUR46 to octylglucoside. Binding of cellular

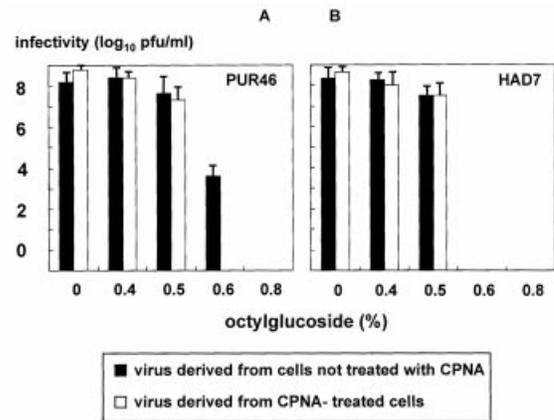


Fig. 4. Effect of octylglucoside on TGEV grown in untreated or desialylated ST cells. (A) PUR46; (B) HAD7. Prior to infection, cells were incubated in the absence (black bars) or presence (white bars) of neuraminidase from *Clostridium perfringens*. Virus released from the cells was treated with octylglucoside as indicated and the residual infectivity was determined.

sialoglycoconjugates to TGE virions can be abolished by neuraminidase treatment of cells prior to infection. Therefore, we compared virus derived from untreated cells and virus derived from neuraminidase-treated cells for sensitivity to octylglucoside. As shown in Fig. 4(A), TGEV grown in desialylated cells (white bars), i.e. without surface-bound sialoglycoconjugates, were completely inactivated after treatment with 0.6% octylglucoside. On the other hand, PUR46 grown in untreated cells (dark bars), i.e. with surface-bound sialoglycoconjugates, retained a residual infectivity of about 10^4 p.f.u./ml after incubation with the same concentration of octylglucoside. HAD7 was completely inactivated by treatment with 0.6% detergent and this effect was independent of pretreatment of cells with neuraminidase (Fig. 4B). This result indicates that sialoglycoconjugates bound to the virus surface may increase the resistance of TGEV to the detergent octylglucoside.

Discussion

A previous analysis of MAb-resistant mutants has shown that Pro145, Cys147 and Cys155 of the mature S protein are important for the ability of TGEV to agglutinate erythrocytes (Krempel *et al.*, 1997). The mutants described here were selected for their inability to agglutinate erythrocytes. Interestingly, one of these HAD mutants (HAD7) also had a mutation at amino acid 155, showing the importance of this residue for the haemagglutinating activity of the S protein. Sequence analysis of the other HAD mutants indicated that Met195, Arg196, Asp208 and Leu209 are also essential for this activity. As only part of the S gene has been sequenced it has not been formally excluded that a mutation at the C-terminal portion of the S protein may be responsible for the loss of the haemagglutination activity. However, we feel that this is rather unlikely, because the mutations detected are all within a

narrow stretch of amino acids that has been implicated in the sialic acid binding activity by analysis of other mutants (Krempl *et al.*, 1997) and by inhibition studies with MAbs (Schultze *et al.*, 1996).

In molecular terms, TGEV-induced haemagglutination is the interaction of the viral S protein with sialic acid residues on the surface of erythrocytes. The above-mentioned amino acids obviously are important for the sialic acid binding activity of TGEV. This is evident not only in the haemagglutinating activity but also in the binding to PSM. The amino acid substitutions in the HAD mutants are all located in a region of the TGEV S protein that is missing in the corresponding protein of PRCV. As PRCV lacks sialic acid binding activity, our data substantiate the concept that the sialic acid binding site is located in that stretch of amino acids present only in TGEV. Cys155, Met195, Arg196, Asp208 and Leu209 may either directly interact with sialic acid or play a structural role by forming the shape of the sialic acid binding site.

Amino acids 5–228 of the mature TGEV S protein, which are missing in the European isolates of PRCV, harbour an antigenic site. Compared to the sialic acid binding site, the antigenic site B (Gebauer *et al.*, 1991) appears to have a more N-terminal location. MAb 1D.B12 recognizes an epitope involving Ser128 and Trp81 of the mature S protein (residues 144 and 97, respectively, when a signal peptide of 16 amino acids is included in the numbering) (Gebauer *et al.*, 1991). This epitope is affected most by the mutations in the sialic acid binding site, because among the five HAD mutants only HAD2 (Met195Val) is recognized by MAb 1D.B12. MAb 1B.H11 recognizes an epitope involving Leu60 and Trp81 of the mature protein. This epitope is less affected by mutations in the sialic acid binding site than the former one, because only two HAD mutants (HAD4 and HAD7) are not recognized by MAb 1B.H11. These results suggest that the amino acid substitutions in the HAD mutants affect the conformation of those parts of the S protein that are in close proximity to the sialic acid binding site. In this respect, the epitope recognized by MAb 1D.B12 is located closer to the sialic acid binding site than is the epitope recognized by MAb 1B.H11. Because of these considerations it appears likely that at least four of the amino acids replaced in the HAD mutants (Cys155, Arg196, Asp208 and Leu209) play a structural role. They may be important in the formation of the sialic acid binding site rather than directly interacting with the sialic acid. An exception may be Met195. Substitution of a valine in the HAD2 mutant abolished the sialic acid binding activity but did not affect the binding of MAbs. The methionine at this position may directly interact with the sialic acid.

The HAD mutants not only provide information about the sialic acid binding site, they can also be helpful in analysing the importance of the sialic acid binding activity. The available data indicate that this activity contributes to the enteropathogenicity of TGEV. The strongest evidence comes from the finding that point mutations at positions 145, 147 or 155 result

in the loss of both the haemagglutinating activity and the enteropathogenicity. As the ability of TGEV to bind to sialoglycoconjugates is dispensable for replication in cell culture, the sialic acid binding activity may play a role only in the context of an intestinal infection. It has been suggested that binding of sialylated macromolecules to the virus surface may increase the stability of TGE virions and thus facilitate survival in the gastrointestinal tract (Schultze *et al.*, 1996). Detrimental factors encountered during the gastrointestinal passage include low pH, proteases and detergent-like bile salts. Cell culture-grown TGEV interacts with sialoglycoconjugates derived from the cell surface. This interaction can be abolished either by incubating the virions with neuraminidase or by treating the cells with this enzyme prior to virus infection. Comparison of parental TGEV containing surface-bound cellular sialoglycoconjugates with HAD mutants did not reveal any difference in the resistance to trypsin or to low pH conditions. However, there was a difference when the resistance to the detergent octylglucoside was analysed. While the HAD mutants were completely inactivated at 0.6% octylglucoside, there was a residual infectivity of 10^3 – 10^4 p.f.u./ml in the case of the parental virus. This difference was reproducible and depended on the sialoglycoconjugates bound to the virus surface. When binding of these sialylated cellular compounds was abolished by neuraminidase treatment of virions, the parental virus behaved like the mutants, i.e. it was completely inactivated at 0.6% octylglucoside. An increased resistance to detergent-like substances would explain the unique position of coronaviruses among enteropathogenic viruses. Most enteric viruses that infect the organism via the gastrointestinal route are non-enveloped viruses; coronaviruses, however, contain a lipid envelope (Saif, 1990). It has been proposed that the presence of detergent-like bile salts in the intestinal lumen may be more favourable for nonenveloped viruses (Morrison & Fields, 1991). The increase in the resistance to octylglucoside that we observed may appear not very dramatic and therefore not sufficient to provide a survival advantage in the intestine. However, it is possible that within the lumen of the intestine with a high content of organic substances the difference in the resistance to bile salts may be more pronounced than under our experimental setting. It is also possible that sialoglycoconjugates from the intestine, e.g. mucins, provide better protection than sialylated macromolecules derived from cultured cells.

An increase in virus stability is not the only way that the sialic acid binding activity might contribute to the enteropathogenicity of TGEV. Under cell culture conditions, binding to the cellular receptor aminopeptidase N is sufficient to initiate infection. In the intestine, the epithelium is covered by mucins and access to the target cells may be more difficult. Under these conditions, an additional binding activity could help to overcome these difficulties. In a recent study on recombinants between an enterotropic and a respiratory variant of TGEV, it has been shown that a factor that maps around amino acid 203

of the mature protein is important for TGEV infection of the gut (Ballesteros *et al.*, 1997; Sánchez *et al.*, 1999). It has been suggested that this factor may be the binding to a coreceptor. Coreceptors have been shown to be important for a number of viruses, including human immunodeficiency virus (Weiss & Clapham, 1996), herpes simplex virus (Montgomery *et al.*, 1996), adenoviruses and coxsackieviruses (Bergelson *et al.*, 1997). In these cases, the coreceptor is required for the viruses in a postattachment step in order to get across the barrier of the cellular membrane. Whether TGEV requires a specific coreceptor on intestinal cells for the penetration step remains to be analysed. With respect to the sialic acid binding activity it may be more likely that binding to *N*-glycolylneuraminic acid reinforces the attachment process and thus facilitates the infection of intestinal cells by TGEV.

It should be noted that we do not claim that the sialic acid binding activity is the only factor determining the enteropathogenicity of TGEV. We expect that there are more determinants that are critical for the ability of TGEV to cause an intestinal disease. They may be responsible either for the enterotropism, i.e. the ability to get to the enteric tract and infect the intestinal cells, or for the severity of the pathogenic effects, i.e. the detrimental effect on the epithelial cells and on the functional integrity of the intestine. Loss of either of these factors may reduce the enteropathogenicity, e.g. resulting in a longer incubation time, in less severe symptoms or in a disease restricted to colostrum-deprived piglets. Such variations in the enteropathogenicity of TGEV have been reported and are observed when enteropathogenic field strains are adapted to growth in cell culture. Therefore, it may appear to be best to study the enteropathogenicity of TGEV using field strains. Many approaches to the analysis of the pathogenicity factors, e.g. the selection of mutants, depend on cell cultures. However, initial titres of field strains grown in cell culture are low and adaptive changes may occur even after one passage in cultured cells. For this reason, many aspects of pathogenicity can only be studied with cell culture-adapted virus. As shown by Bernard & Laude (1995), infection by the high passage strain Purdue-115 results in intestinal infection and in the death of piglets, demonstrating that this strain of TGEV is still enteropathogenic. Strains like Purdue-115 have retained at least some of the pathogenicity factors and can be used to analyse these determinants in more detail. The sialic acid binding activity is retained in cell culture-adapted strains of TGEV and so far it is the only measurable biological activity that has been linked with the enteropathogenicity of TGEV. Mutants like those described in this paper should help to clarify how the binding to sialic acids contributes to the pathogenic potential of TGE.

We are thankful to Roland Schauer for providing porcine submandibular mucin. This work was done by C.K. in partial fulfilment of the requirements for the Dr. Rer. Physiol. degree at Philipps-Universität Marburg, Fb 20. Financial support was provided by Deutsche Forschungs-

gemeinschaft (He1168/10-1 and SFB 280) and Deutscher Akademischer Austauschdienst (322-ai-e-dr) to G.H. L.E. was supported by grants from CICYT, Spain and from the European Union (Biotech and FAIR) and by the Spanish-German exchange program 'Acciones Integradas Hispano-Alemanas'. C.K. received a fellowship from 'Hessische Graduiertenförderung'. M.-L.B. received a fellowship from the 'Consejo Superior de Investigaciones Científicas' (CSIC) of Spain.

References

- Ballesteros, M. L., Sánchez, C. M. & Enjuanes, L. (1997). Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. *Virology* **227**, 378–388.
- Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. & Finberg, R. W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320–1323.
- Bernard, S. & Laude, H. (1995). Site-specific alteration of transmissible gastroenteritis virus spike protein results in markedly reduced pathogenicity. *Journal of General Virology* **76**, 2235–2241.
- Cox, E., Pensaert, M. B., Callebaut, P. & van Deun, K. (1990). Intestinal replication of a porcine respiratory coronavirus closely related antigenically to the enteric transmissible gastroenteritis. *Veterinary Microbiology* **23**, 237–243.
- Delmas, B., Gelfi, J. & Laude, H. (1986). Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer glycoprotein. *Journal of General Virology* **67**, 1405–1418.
- Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L. K., Sjöström, H., Noren, O. & Laude, H. (1992). Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV. *Nature* **357**, 417–420.
- Gebauer, F., Posthumus, W. P., Correa, I., Suñé, C., Smerdou, C., Sánchez, C. M., Lenstra, J. A., Meloen, R. H. & Enjuanes, L. (1991). Residues involved in the antigenic sites of transmissible gastroenteritis coronavirus S glycoprotein. *Virology* **183**, 225–238.
- Hara, S., Yamaguchi, M., Furuhashi, K., Ogura, H. & Nakamura, M. (1989). Determination of mono-*O*-acetylated *N*-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. *Analytical Biochemistry* **179**, 162–166.
- Krempl, C., Schultze, B., Laude, H. & Herrler, G. (1997). Point mutations in the S protein connect the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus. *Journal of Virology* **71**, 3285–3287.
- Krempl, C., Ballesteros, M. L., Enjuanes, L. & Herrler, G. (1998). Isolation of haemagglutination-defective mutants for the analysis of the sialic acid binding activity of transmissible gastroenteritis virus. *Advances in Experimental Medicine and Biology* **440**, 563–568.
- McClurkin, A. W. & Norman, J. O. (1966). Studies on transmissible gastroenteritis of swine. II. Selected characteristics of a cytopathogenic virus common to five isolates from transmissible gastroenteritis. *Canadian Journal of Comparative Veterinary Science* **30**, 190–198.
- Montgomery, R. I., Warner, M. S., Lum, B. J. & Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**, 427–436.
- Morrison, L. A. & Fields, B. N. (1991). Parallel mechanisms in neuropathogenesis of enteric virus infections. *Journal of Virology* **65**, 2767–2772.
- Noda, M., Yamashita, H., Koide, F., Kadoi, K., Omori, T., Asagi, M. & Inaba, Y. (1987). Hemagglutination with transmissible gastroenteritis virus. *Archives of Virology* **96**, 109–115.

- Noda, M., Koide, F., Asagi, M. & Inaba, Y. (1988).** Physicochemical properties of transmissible gastroenteritis virus hemagglutinin. *Archives of Virology* **99**, 163–172.
- Pensaert, M., Callebaut, P. & Cox, E. (1993).** Enteric coronaviruses of animals. In *Viral Infections of the Gastrointestinal Tract*, pp. 627–696. Edited by A. Z. Kapikian. New York: Marcel Dekker.
- Rasschaert, D., Duarte, M. & Laude, H. (1990).** Porcine respiratory coronavirus differs from transmissible gastroenteritis virus by a few genomic deletions. *Journal of General Virology* **71**, 2599–2607.
- Reuter, G., Stoll, S., Kamerling, J. P., Vliegenthart, J. F. G. & Schauer, R. (1988).** Sialic acids on erythrocytes and in blood plasma of mammals. In *Sialic Acids 1988: Proceedings of the Japanese–German Symposium on Sialic Acids*, pp. 88–89. Edited by R. Schauer & T. Yamakawa. Kiel, Germany: Kieler Verlag Wissenschaft und Bildung.
- Risco, C., Antón, I. M., Enjuanes, L. & Carrascosa, J. L. (1996).** The transmissible gastroenteritis coronavirus contains a spherical core shell consisting of M and N proteins. *Journal of Virology* **70**, 4774–4777.
- Saif, L. J. (1990).** Comparative aspects of enteric viral infections. In *Viral Diarrheas of Man and Animals*, pp. 9–31. Edited by L. J. Saif & K. W. Theil. Boca Raton, Florida: CRC Press.
- Sánchez, C. M., Jiménez, G., Laviada, M. D., Correa, I., Suñé, C., María, J. B., Gebauer, F., Smerdou, C., Callebaut, P., Escribano, J. M. & Enjuanes, L. (1990).** Antigenic homology among coronaviruses related to transmissible gastroenteritis virus. *Virology* **174**, 410–417.
- Sánchez, C. M., Gebauer, F., Suñé, C., Mendez, A., Dopazo, J. & Enjuanes, L. (1992).** Genetic evolution and tropism of transmissible gastroenteritis coronaviruses. *Virology* **190**, 92–105.
- Sánchez, C. M., Izeta, A., Sanchez-Morgado, J. M., Alonso, S., Sola, I., Balasch, M., Plana-Duran, J. & Enjuanes, L. (1999).** Targeted recombination demonstrates that the spike gene of transmissible gastroenteritis coronavirus is a determinant of its enteric tropism and virulence. *Journal of Virology* **73**, 7606–7618.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463–5467.
- Schauer, R. (1982).** Chemistry, metabolism, and biological functions of sialic acids. *Advances in Carbohydrate Chemistry and Biochemistry* **40**, 131–234.
- Schultze, B., Gross, H.-J., Brossmer, R. & Herrler, G. (1991).** The S protein of bovine coronavirus is a hemagglutinin recognizing 9-*O*-acetylated sialic acid as a receptor determinant. *Journal of Virology* **65**, 6232–6237.
- Schultze, B., Kreml, C., Ballesteros, M. L., Shaw, L., Schauer, R., Enjuanes, L. & Herrler, G. (1996).** Transmissible gastroenteritis coronavirus, but not the related porcine respiratory coronavirus, has a sialic acid (*N*-glycolylneuraminic acid) binding activity. *Journal of Virology* **70**, 5634–5637.
- Suñé, C., Jiménez, G., Correa, I., Bullido, M. J., Gebauer, F., Smerdou, C. & Enjuanes, L. (1990).** Mechanisms of transmissible gastroenteritis coronavirus neutralization. *Virology* **177**, 559–569.
- Weiss, R. A. & Clapham, P. R. (1996).** Hot fusion of HIV. *Nature* **381**, 647–648.
- Zimmern, D. & Kaesberg, P. (1978).** 3′-Terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **75**, 4257–4261.

Received 4 August 1999; Accepted 15 October 1999