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ISOLATION OF HEMAGGLUTINATION-DEFECTIVE MUTANTS FOR THE ANALYSIS OF THE SIALIC ACID BINDING ACTIVITY OF TRANSMISSIBLE GASTROENTERITIS VIRUS

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1. ABSTRACT

The surface protein S of transmissible gastroenteritis virus (TGEV) has a sialic acid binding activity that enables the virus to agglutinate erythrocytes. A protocol is described that has been successfully applied to the isolation of hemgglutination-defective mutants. The potential of these mutants for the characterization of the sialic acid-binding site and the function of the binding activity is discussed.

2. INTRODUCTION

The ability to recognize sialic acid is shared by more viruses than any other of the viral binding acitivities elucidated so far. In addition to several nonenveloped viruses (rotaviruses, reoviruses, encephalomyocarditis virus, polyomavirus) a number of enveloped viruses attach to sialic acid residues present on glycoproteins or glycolipids (reviewed by Herrler et al., 1995). Most information is available about the sialic acid binding activity of enveloped viruses. Influenza viruses, several members of the paramyxovirus family, as well as bovine coronavirus and antigenically related human and porcine coronaviruses use

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sialic acids on the cell surface as receptor determinants for attachment to cells. In the case of erythrocytes virus binding results in a hemagglutination reaction that serves as a convenient assay for the viral sialic acid binding activity. In the case of cultured cells, binding to sialylated surface glycoconjugates is the initial stage of the virus infection. As shown in Fig. 1, viruses may differentiate between different types of sialic acid. Influenza A and B as well as the paramyxoviruses have a preference for N-acetylneuraminic acid. Influenza C virus and bovine coronavirus recognize N-acetyl-9-O-acetylneuraminic acid. In addition, these viruses may have a preference for a certain linkage type between sialic acid and the neighboring sugar (e.g. α2,3 or α2,6-linked to galactose). Efficient virus binding also requires a multivalent virus-cell interaction. Therefore, only a limited number of surface sialoglycoconjugates appear to be suitable as virus receptors. In the case of MDCK cells, a single surface glycoprotein has been shown to be the major protein recognized by influenza C virus and bovine coronavirus (Zimmer et al., 1995; Schultze et al., 1996). A hall-

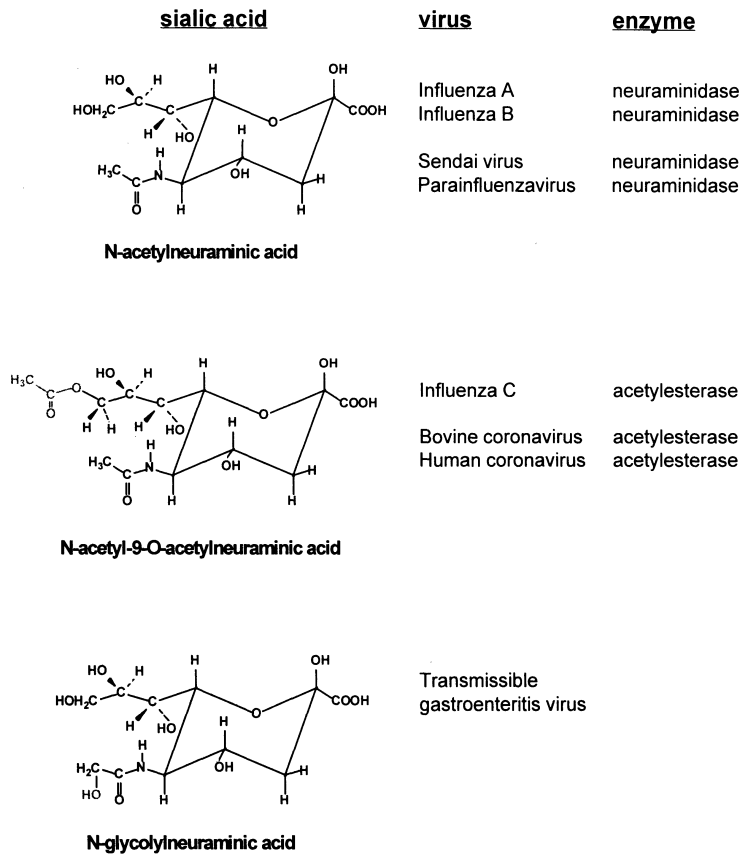

Figure 1. Different types of sialic acid that function as receptor-determinants for enveloped viruses. The viruses that recognize each of these sialic acids are indicated. Several of these viruses contain a receptor-destroying enzyme. On the right it is indicated whether the enzyme is a neuraminidase or an acetylesterase.

mark of enveloped viruses that use sialic acid to initiate infection is the presence of a receptor-destroying enzyme on the virion surface (Fig. 1). This enzyme is a neuraminidase in the case of influenza A and B viruses as well as of paramyxoviruses (Sendai virus and parainfluenza viruses). With viruses that recognize 9-O-acetylated sialic acids—influenza C virus and bovine coronavirus—the receptor-destroying enzyme is an acetylesterase releasing the acetyl group from position C-9 of sialic acid. It is assumed that these viruses require a receptor-destroying enzyme to prevent interactions with sialoglycoconjugates that are unfavorable for infection. Available evidence indicates that lack of such an enzyme results in the fromation of virus aggregates on the surface of infected cells and thus affects the spread of virus to other cells (Liu et al., 1995; Höfling et al., 1996).

Coronaviruses are exceptional, because within one genus they contain both viruses with and viruses without a receptor-destroying enzyme. While viruses of one genetic lineage (bovine coronavirus, human coronavirus OC43, and porcine hemagglutinating encephalomyelitis virus) contain an acetylesterase (Vlasak et al., 1988; Schultze et al., 1990), two other genetic lineages lack such an enzyme. The latter two genetic groups are represented by porcine transmissible gastroenteritis virus (TGEV) and avian infectious bronchitis virus (IBV). Though both TGEV and IBV do not contain a receptor-destroying enzyme, they nevertheless are able to recognize sialic acid (Schultze et al. 1992, 1996). The sialic acid binding activity of these two viruses can also be assayed in a hemaglutination assay. However, in the course of infection there is usually only a transient hemagglutinating activity detectable in the cell supernatant (Schultze et al., 1996). The disappearance of the hemagglutinating activity late in infection is due to competitive inhibitors that interact with the sialic acid binding site and prevent the virus from agglutinating erythrocytes. The hemagglutinating activity of viruses with a receptor-destroying enzyme is usually observed throughout the infectious cycle, because these viruses are able to inactivate potential competitive inhibitors by the viral acetylesterase or neuraminidase, respectively. In order to reproducibly detect hemagglutinating activity with TGEV, competitive inhibitors have to be inactiviated by the addition of an exogenous receptor-destroying enzyme. Neuraminidase has been shown to be effective in this respect (Schultze et al., 1992,1996). Following neuraminidase treatment of the cell supernatant of infected cells, the hemagglutinating activity of TGEV is no longer a transient event but is as stable as that of influenza viruses or bovine coronavirus.

Results obtained with mutants of TGEV indicate that point mutations in the surface protein S of TGEV may result in the loss of both the hemagglutinating activity and the enteropathogenicity (Krempl et al., 1997). These findings correlate the sialic acid binding activity with the enteropathogenicity of TGEV.

3. EXPERIMENTAL OUTLINE

In order to strengthen the experimental data that correlate the sialic acid binding activity of TGEV with the enteropathogenicity of this virus, it is desirable to isolate hemagglutination-defective mutants of TGEV. For this purpose we have applied a selection procedure that is schematically shown in Fig. 2. In a first step a preparation of TGEV was treated with neuraminidase (150 mU of the *Vibrio cholerae*-enzyme in a final volume of 3 ml PBS, 30 min, 37°C) in order to inactivate potential inhibitors that may block the sialic acid binding site. In this way virions are obtained that show optimal hemagglutinating activity (Schultze et al., 1996). Neuraminidase was removed by ultracentrifugation (150.000xg, 1 h, 4°C). The virus sediment was resuspended in 3 ml of PBS and filtrated to

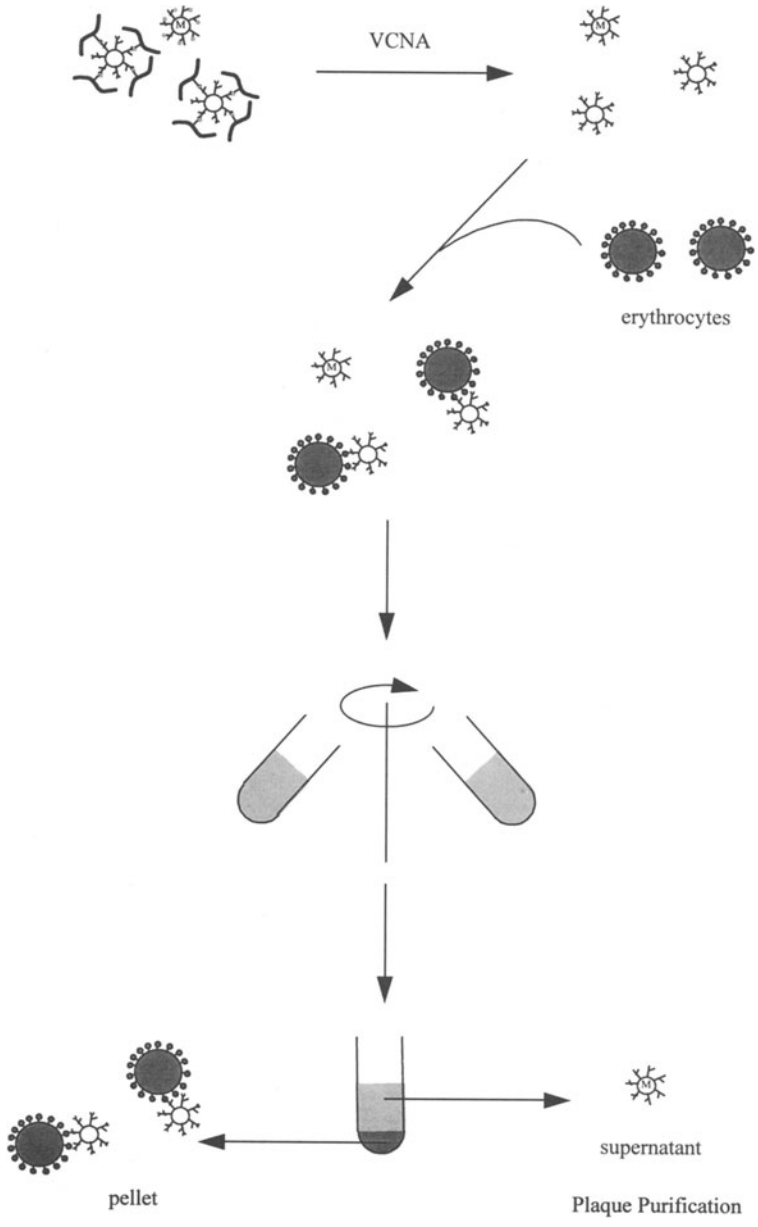

Figure 2. Protocol for the selection of hemagglutination-defective mutants of TGEV. Virus was treated with neuraminidase from *Vibrio cholerae* to inactivate competive inhibitors (sialoglycoconjugates) that may block the sialic acid-binding site of the S protein. The virus was then incubated with glutaraldehyde-fixed erythrocytes. Virions with intact sialic acid-binding site will bind to the erythrocytes and can be sedimented by low speed centrifugation while hemagglutination-defective mutants (designated M) will remain in the supernatant.

Table 1. Infectivity and hemagglutinating activity of TGEV after different rounds of the selection procedure

	Virus grown in ST cells	
Step of the selection procedure	Infectivity PFU/ml	HA-activity HAU/ml
Before selection	3.2×10^8	1024
After first round	1.5×10^7	8
After second round	4.2×10^8	< 2
After third round	3.0×10^8	< 2

remove microbial contaminants. To the sterile virus suspension, glutaraldehyde-fixed bovine erythrocytes were added to a final concentration of 2.5%. The latter cells are more sensitive to the hemagglutinating activity of TGEV than the erythrocytes from other species tested (Schultze et al., 1996). This suspension was incubated for 1 h on ice to allow the virions to attach to the erythrocytes. Following centrifugation at 2.500xg for 10 min, the supernatant was subjected to two further rounds of incubation with bovine erythrocytes. The supernatant derived from the third batch of erythrocytes was expected to be enriched in natural variants of TGEV that were defective in the sialic acid-binding activity. It was used to infect swine testicular (ST) cells. At about 18 h.p.i., when a cytopathic effect was detectable, virions were harvested. The virus was subjected to the selection procedure (neuraminidase treatment, three incubation rounds with bovine eryhtrocytes, growth in ST cells) two more times. Finally, individual viruses were isolated by two rounds of plaque purification. Stock virus was grown in ST cells and analyzed for hemagglutination activity as described previously (Schultze et al., 1996).

4. RESULTS

The selection protocol described above was applied to the PUR46-MAD (Sanchez et al, 1990) strain of TGEV. After the first round of the selection procedure (neuraminidase treatment, 3 incubations with bovine erythrocytes), the final supernatant was grown in ST cells. As shown in Table 1, virus recovered from the infected cells had a reduced hemagglutinating activity compared to the parental virus. This virus was again subjected to the selection protocol. This time, virus was recovered that had the same infectivity as the original virus with no hemagglutinating activity detectable. After having run through the selection procedure for a third time, virus was plaque-purified. Eight individual plaques were picked to grow stock virus. All of them were devoid of hemagglutinating activity.

5. DISCUSSION

Hemagglutination-defective mutants are interesting in several respects. It is expected that most of these viruses differ from the parental virus by mutations affecting the sialic acid binding site. Comparison of the sequences of the respective S genes should reveal amino acids that are involved in the receptor-binding site. The available evidence indicates that the binding site or an essential part of it is located between amino acids 20 and 245 (Schultze et al., 1996). This stretch of amino acids is missing in the S gene of a re-

lated virus, porcine respiratory coronavirus (PRCV), that is unable to agglutinate erythrocytes. The portion of the TGEV S protein that is deleted in the corresponding PRCV protein contains an antigenic site and monoclonal antibodies directed to this epitope have been found to inhibit the hemagglutinating activity of TGEV very efficiently. Several antibody-resistant variants have been reported to lack hemagglutinating activity (Krempl et al., 1997). These variants differed from the parental virus by point mutations or a deletion of four amino acids. All changes were located between amino acids 145 and 155. These amino acids appear to be located at or close to the sialic acid binding site of TGEV. Sequence analysis of the hemagglutination-defective viruses described above should help to further identify amino acids of the S protein that are involved in the sialic acid binding site of TGEV.

Hemagglutination-defective viruses may also be useful in elucidating the functional role of the sialic acid binding site. One concept is that binding of sialylated macromolecules to the virion surface increases the resistance of TGEV to detrimental agents such as detergents and proteases (Schultze et al., 1996; Krempl et al., 1997). In this way the sialic acid binding activity may help TGEV to survive the gastrointestinal passage. In future work we will analyze whether the hemagglutination-defective virions are more sensitive to the action of detergents and proteases than is the parental virus.

REFERENCES

Herrler, G., Hausmann, J., and Klenk, H.-D., 1995, Sialic acid as receptor determinant of ortho- and paramyxoviruses, in *Biology of the Sialic Acids* (A. Rosenberg , ed.), Plenum Press, New York pp. 315–336.

Höfling, K., Brossmer, R., Klenk, H.-D. and Herrler, G., 1996; Transfer of an esterase-resistant receptor analog to the surface of influenza C virions results in reduced infectivity due to aggregate formation, *Virology* **218**: 127–133.

Krempl, C, Laude, H., and Herrler, G., 1997, Point mutations in the S protein connect the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus, *J. Virol.* **71**: 3285–3287.

Liu, C., Eichelberger, M.C., Compans, R.W. and Air, G.M., 1995, Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding, *J. Virol.* **69**:1099–1106.

Sanchez, C. M., Jimenez, G., Laviada, M. D., Correa, I., Sune. C., Bullido, M. J., Gebauer, F., Smerdou, C., Callebaut, P., Escribano, J. M., and Enjuanes, L., 1990, Antigenic homology among coronaviruses related to transmissible gastroenteritis virus, *Virology* **174**: 410–417.

Schultze, B., Groß, H.J., Brossmer, R., Klenk, H.-D. and Herrler, G. 1990, Hemagglutinating encephalomyelitis virus attaches to N-acetyl-9-O-acetylneuraminic acid-containing receptors: comparison with bovine coronavirus and influenza C virus, *Virus Res.* **16**: 185–194.

Schultze, B., Cavanagh, D. and Herrler, G. 1992, Neuraminidase treatment of avian infectious bronchitis virus reveals a hemagglutinating activity that is dependent on sialic acid containing receptors on erythrocytes, *Virology* **189**: 792–794.

Schultze, B., Krempl, C., Shaw, L., Enjuanes, L. and Herrler, G., 1996, Transmissible gastroenteritis coronavirus but not the related porcine respiratory coronavirus has a sialic acid (N-glycolylneuraminic acid) binding activity, *J. Virol.* **70**: 5634–5637.

Schultze, B., Zimmer, G. and Herrler, G., 1996, Virus entry into a polarized epithelial cell line (MDCK): similarities and dissimilarities between influenza C virus and bovine coronavirus, *J. Gen. Virol.* **77**: 2507–2514.

Vlasak, R., Luytjes, W., Spaan, W. and Palese, P., 1988, Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses, *Proc. Natl. Acad. Sci. U.S.A.* **85**: 4526–4529.

Zimmer, G., Klenk, H.-D. and Herrler, G., 1995; Identification in a MDCK cell line of a 40 kDa cell surface sialoglycoprotein with the characteristics of a major influenza C virus receptor, *J .Biol. Chem.* **270**: 17815–17822.