Journal of General Virology (1997), **78**, 567–570. Printed in Great Britain SHORT COMMUNICATION

Inactivation of inhibitors by the receptor-destroying enzyme of influenza C virus

Katja Höfling, Hans-Dieter Klenk and Georg Herrler

Institut für Virologie, Philipps-Universität Marburg, Robert-Koch-Str. 17, D-35037 Marburg, Germany

The importance of the receptor-destroying enzyme of influenza C virus for inactivation of inhibitors was analysed. Using three different inhibitors (rat serum, bovine submandibulary mucin and bovine brain gangliosides) inhibition of virus infection was observed only at an inhibitor concentration that was about 100-fold higher than the maximum concentration of inhibitor that could be inactivated by the receptor-destroying enzyme of a given amount of virus. From our data and other observations we conclude that the receptor-destroying enzyme is not required to inactivate inhibitors.

Among enveloped viruses, members of various families are able to use sialic acid as a receptor determinant for attachment to cells. Interestingly, all these viruses contain a receptor-destroying enzyme. Viruses of the genus *Paramyxovirus* as well as influenza A and B viruses recognize *N*-acetylneuraminic acid (Neu5Ac) and inactivate their receptors by a sialidase (neuraminidase) that releases terminal sialic acid from glycoconjugates (Klenk *et al.*, 1955). Influenza C virus and several coronaviruses require a different type of sialic acid for binding to cells, *N*-acetyl-9-*O*-acetylneuraminic acid (Herrler & Klenk, 1987*b*; Rogers *et al.*, 1986; Vlasak *et al.*, 1988*a*; Schultze & Herrler, 1992). Their receptor-destroying enzyme is an acetylesterase that releases the 9-*O*-acetyl group from the receptor determinant (Herrler *et al.*, 1985*c*; Vlasak *et al.*, 1988*a*).

The importance of these enzymes is not completely clear. In the case of influenza A viruses evidence has been presented indicating a role in virus maturation by preventing the formation of virus aggregates and by facilitating the release of virions from the infected cell (Liu *et al.*, 1995). Some evidence also suggests a role of the receptor-destroying enzyme during the initiation of infection either by promoting fusion activity (Huang *et al.*, 1980, 1985) or by releasing sialic acids from oligosaccharides near the receptor-binding site that may interfere with the binding to cellular receptors (Ohuchi *et al.*, 1995). In the case of influenza C virus, the importance of the receptor-destroying enzyme has been studied using sialic acid analogues. Some of these artificial sialic acids can function as receptor determinants but are resistant to the receptor-destroying enzyme (Herrler *et al.*, 1992). With this approach, it has been shown that the acetylesterase is required to keep the virus surface free of receptor determinants, which otherwise would result in a decrease of the infectious virus titre due to aggregate formation (Höfling *et al.*, 1996). Studies with sialic acid analogues as well as with enzyme inhibitors have indicated that the acetylesterase of influenza C virus and bovine coronavirus may also play a role during virus entry (Vlasak *et al.*, 1988*b*, 1989; Brossmer *et al.*, 1993; Strobl & Vlasak, 1993).

In addition to the role during virus entry and virus maturation, it has been suggested that the receptor-destroying enzyme may be important for the *in vivo* infection (for a review see Herrler *et al.*, 1995). The primary target cells of the viruses mentioned above are the epithelial cells of the respiratory tract. Some coronaviruses may proceed to the epithelium of the intestinal tract. Both the respiratory and the intestinal epithelium are covered by a layer of mucous substances. Mucins are very rich in sialic acid and are known to be haemagglutination-inhibitors for viruses that interact with sialic acids on erythrocytes. The inhibitory activity of mucins might prevent viruses from binding to sialylated receptors on cells of the respiratory or intestinal epithelium. It has been proposed that the receptor-destroying activity enables viruses to inactivate these competitive inhibitors thus allowing access to the target cells. However, no experimental evidence has been provided to substantiate this possibility.

We have tested this concept for influenza C virus by comparing the amount of inhibitor that is required for inhibition of virus infection with the amount that can be inactivated by the receptor-destroying enzyme. Three different inhibitors were included in this study. Rat serum has long been known to be a very potent haemagglutination-inhibitor. Most of the inhibitory activity is accounted for by α1-macroglobulin and murinoglobulin (Herrler *et al.*, 1985*b*; Kitame *et al.*, 1985). Another inhibitor is bovine submaxillary mucin (BSM), which is a rich source of 9-*O*-acetylated sialic acid and has been reported to prevent influenza C virus from agglutinating erythrocytes (Herrler *et al.*, 1985*c*). Glycolipids were also included in this study. Bovine brain gangliosides (BBG) can

Author for correspondence: Georg Herrler.
Fax +49 6421 285482. e-mail herrler@mailer.uni-marburg.de

0001-4380 © 1997 SGM

Table 1. Haemagglutination activity of influenza C virus after incubation at 4 °C or 37 °C with different dilutions of three haemagglutination-inhibitors: rat serum, bovine submandibulary mucin or bovine brain gangliosides (BBG)

Inhibitor dilution	Haemagglutinating activity (HA units/ml) of influenza C virus preincubated with:					
	Rat serum		Mucin (10 mg/ml)		BBG (5 mg/ml)	
	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C
Undiluted	< 2	< 2	< 2	< 2	< 2	< 2
1:10	< 2	< 2	< 2	< 2	4	8
1:100	< 2	< 2	< 2	2	16	16
1:1000	< 2	16	< 2	16	16	16
No inhibitor	16	16	16	16	16	16

Table 2. Virus yield after infection of MDCK I cells by influenza C virus that had been preincubated at 4 °C or 37 °C with different dilutions of three inhibitors: rat serum, bovine submandibulary mucin or bovine brain gangliosides (BBG)

Inhibitor dilution	Virus yield (HA units/ml) after infection with influenza C virus preincubated with:					
	Rat serum		Mucin (10 mg/ml)		BBG (5 mg/ml)	
	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C
Undiluted	< 2	< 2	< 2	< 2	< 2	< 2
1:10	8	8	16	16	32	32
1:100	32	32	32	64	64	64
1:1000	64	64	64	64	64	64
No inhibitor	128	128	128	128	128	128

function as receptors for influenza C virus when they are incorporated into the plasma membrane (Herrler & Klenk, 1987*b*). When they are present in liposomes, they act as haemagglutination-inhibitors (Herrler & Klenk, 1987*a*). Among these inhibitors, only mucins might have relevance for natural infections with influenza C virus. However, human mucins from the respiratory tract have not been analysed for haemagglutination-inhibition activity. The ability of the receptor-destroying enzyme of influenza C virus to inactivate the three inhibitors mentioned above was first analysed in a haemagglutination assay. Influenza C virus strain Johannesburg/1/66 was grown in 8-day-old embryonated chicken eggs. After incubation for 3 days at 33 °C, the allantoic fluid was harvested, clarified by low speed centrifugation (3700 ***g***, 20 min, 4 °C) and stored at −80 °C. The amount of virus was determined by haemagglutination titration in a microtitre assay as previously described (Herrler *et al*., 1985*a*). The reciprocal value of the highest virus dilution causing complete agglutination of chicken erythrocytes was used as a measure of the haemagglutinating activity expressed in HA units/ml. A virus suspension containing 16 HA units/ml was incubated with different dilutions of either of the three inhibitors for 20 min at 4 °C. Half of the sample was then transferred to a 37 °C water-bath, whereas the other half was kept at 4 °C. After an incubation time of 1 h, the samples were put on ice and the HA titre was determined. As shown in Table 1, rat serum was a very efficient haemagglutination-inhibitor; a 1:1000 dilution completely prevented influenza C virus from agglutinating the cells. Incubation at 37 °C inactivated the inhibitors present in the 1:1000 dilution as shown by the positive agglutination reaction (16 HA units/ml). The 1:100 dilution resulted in a complete inhibition of haemagglutination irrespective of the incubation temperature indicating that the viral acetylesterase was unable to inactivate the amount of inhibitors present in this dilution. With BSM, complete inhibition was obtained with as little as 10 μg/ml. At that concentration, the inhibitory activity was lost after incubation at 37 °C. The virus was, however, unable to inactivate BSM at a tenfold higher concentration (100 μg/ml). Liposomes containing BBG at a concentration of 5 mg/ml completely prevented influenza C virus from agglutinating erythrocytes. In a 1:10 dilution the liposomes caused a partial inhibition (4 compared to 16 HA units/ml of the control virus). At the amount of virus used for this experiment, influenza C virus was unable to inactivate the glycolipid inhibitors except for a slight effect on the 1:10 diluted sample resulting in an increase of the HA titre from 4 to 8 HA units/ml. This is in accordance with a previous report which showed that 9-*O*-acetylated sialic acids present on gangliosides are only poor substrates for the acetylesterase of influenza C virus (Schauer *et al*., 1988).

The three haemagglutination-inhibitors were also analysed for their ability to prevent influenza C virus infection. In order to obtain a high sensitivity of our assay, the amount of influenza C virus was lowered to 2 HA units/ml. The virus was incubated with different dilutions of any of the three inhibitors described above. After incubation at 4 °C or 37 °C, respectively, an aliquot of 0·2 ml was used without further dilution to infect a confluent monolayer of MDCK I cells in a six-well plate. Following an adsorption time of 30 min at room temperature, the virus was removed and the cells were washed. After incubation for 36 h at 33 °C with minimal essential medium, the virus yield in the cell supernatant was determined by haemagglutination titration. As shown in Table 2, a 1:10 dilution of rat serum resulted in a more than 10-fold reduction of the virus yield and complete inhibition of the influenza C virus infection was observed with undiluted serum. A 1:100 or

1:1000 dilution only had a minor effect on the release of virus into the supernatant. A similar profile was obtained with BSM. At a concentration of 10 mg/ml, the mucin caused complete inhibition of virus infection. A concentration of 1 mg/ml of BSM resulted in an eightfold reduction of the virus yield. Further tenfold dilutions of the mucin had only minor effects on the influenza C virus infection. With BBG, no virus release was detectable when a liposome preparation was used that contained BBG at a concentration of 5 mg/ml. In no case was a significant increase of the virus yield observed when the mixture of virus and inhibitor was incubated at 37 °C prior to infection of cells. This result indicates that at the amount of virus used the acetylesterase was unable to inactivate any of the three inhibitors to a significant extent. This result is not surprising in the case of the gangliosides, because they are less efficient inhibitors and had already been shown to be resistant to inactivation in the haemagglutination-assay (Table 1). In the case of the potent haemagglutination-inhibitors (rat serum and BSM) a much higher concentration of inhibitor is required to prevent infection than is needed for haemagglutination-inhibition. For example a 1:1000 dilution of rat serum completely prevented haemagglutination by influenza C virus, while complete inhibition of infection required undiluted serum. Obviously, the haemagglutination reaction is much more sensitive to inhibitors than is virus infection. For agglutination of erythrocytes to occur, viruses not only have to bind to cells, they also have to cross-link the cells in such a way that a lattice-work of erythrocytes is formed. In order to prevent this delicate virus-cell interaction it is probably sufficient to block a proportion of the virus receptor-binding sites. In the case of cultured cells, infection can be initiated by a single virion. Thus, to prevent a virus from infecting a cell, all or almost all attachment proteins on the virus surface have to be blocked, which requires a very high concentration of the competitive inhibitor. The results from Tables 1 and 2 show that the amount of inhibitor that is required to prevent a given amount of influenza C virus from infecting cells cannot be inactivated by the receptor-destroying enzyme of that virus in a reasonable time. This finding argues against the suggestion that the acetylesterase is required in natural infections to inactivate inhibitors. This conclusion is supported by the fact that several viruses that recognize sialic acid lack a receptor-destroying enzyme. Polyoma virus (Fried *et al*., 1981), rotaviruses (Yolken *et al*., 1987), reoviruses (Paul *et al*., 1989) and encephalomyocarditis virus (Burness & Pardoe, 1981) have been reported to use *N*-acetylneuraminic acid as a receptor determinant for binding to cells, but none of them contains a neuraminidase or an acetylesterase. Though these viruses are unable to inactivate potential inhibitors, they successfully infect man or animals. Thus, soluble sialoglycoconjugates as potential inhibitors are not really a problem for viruses that use sialic acid as a receptor-determinant. The viruses mentioned above are nonenveloped viruses. Receptor-destroying enzymes appear to be necessary only for enveloped viruses that recognize sialic acid. In contrast to nonenveloped viruses, they contain glycoproteins that are inserted into the lipid envelope. As described in the introductory section, sialic acid present on these surface proteins may interfere with both virus entry and virus maturation. Therefore, the function of the receptor-destroying enzyme is to keep the virus surface free from receptor determinants but not to inactivate soluble inhibitors.

This work was conducted by K.H. in partial fulfilment of the requirements for the Dr med. degree from FB21, Philipps-Universität Marburg. Financial support was provided by Deutsche Forschungsgemeinschaft (SFB 286).

References

Brossmer, R., Isecke, R. & Herrler, G. (1993). A sialic acid analogue acting as a receptor determinant for binding but not for infection by influenza C virus. *FEBS Letters* **323**, 96–98.

Burness, A. T. H. & Pardoe, I. U. (1981). Effect of enzymes on the attachment of influenza and encephalomyocarditis viruses to erythrocytes. *Journal of General Virology* **55**, 275–288.

Fried, H., Cahan, L. D. & Paulson, J. C. (1981). Polyoma virus recognizes specific sialyloligosaccharide receptors on host cells. *Virology* **109**, 188–192.

Herrler, G. & Klenk, H.-D. (1987*a*). Restoration of receptors for influenza C virus on chicken erythrocytes by incorporation of bovine brain gangliosides. In *The Biology of Negative Strand Viruses*, pp. 63–67. Edited by B. W. J. Mahy & D. Kolakofsky. Amsterdam: Elsevier.

Herrler, G. & Klenk, H. D. (1987*b*). The surface receptor is a major determinant of the cell tropism of influenza C virus. *Virology* **159**, 102–108.

Herrler, G., Rott, R. & Klenk, H.-D. (1985*a*). Neuraminic acid is involved in the binding of influenza C virus to erythrocytes. *Virology* **141**, 144–147.

Herrler, G., Geyer, R., Muller, H. P., Stirm, S. & Klenk, H. D. (1985*b*). Rat alpha 1 macroglobulin inhibits hemagglutination by influenza C virus. *Virus Research* **2**, 183–192.

Herrler, G., Rott, R., Klenk, H. D., Muller, H. P., Shukla, A. K. & Schauer, R. (1985*c*). The receptor-destroying enzyme of influenza C virus is neuraminate-O-acetylesterase. *EMBO Journal* **4**, 1503–1506.

Herrler, G., Gross, H. J., Imhof, A., Brossmer, R., Milks, G. & Paulson, J. C. (1992). A synthetic sialic acid analogue is recognized by influenza C virus as a receptor determinant but is resistant to the receptor-destroying enzyme. *Journal of Biological Chemistry* **267**, 12501–12505.

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

Höfling, K., Brossmer, R., Klenk, H.-D. & 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.

Huang, R. T. C., Rott, R., Wahn, K., Klenk, H.-D. & Kohama, T. (1980). Function of neuraminidase in membrane fusion induced by myxoviruses. *Virology* **107**, 313–319.

Huang, R. T. C., Dietsch, E. & Rott, R. (1985). Further studies on the role of neuraminidase and the mechanism of low pH dependence in influenza virus-induced membrane fusion. *Journal of General Virology* **66**, 295–301.

Kitame, F., Nakamura, K., Saito, A., Sinohara, H. & Homma, M. (1985). Isolation and characterization of influenza C virus inhibitor in rat serum. *Virus Research* **3**, 231–244.

Klenk, E., Faillard, H. & Lempfrid, H. (1955). Über die enzymatische Wirkung von Influenzavirus. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **301**, 235–246.

Liu, C., Eichelberger, M. C., Compans, R. W. & Air, G. M. (1995). Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. *Journal of Virology* **69**, 1099–1106.

Ohuchi, M., Feldmann, A., Ohuchi, R. & Klenk, H.-D. (1995). Neuraminidase is essential for fowl plague virus hemagglutinin to show hemagglutinating activity. *Virology* **212**, 77–83.

Paul, R. W., Choi, A. H. C. & Lee, P. W. K. (1989). The a-anomeric form of sialic acid is the minimal receptor determinant recognized by reovirus. *Virology* **172**, 382–385.

Rogers, G. N., Herrler, G., Paulson, J. C. & Klenk, H.-D. (1986). Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. *Journal of Biological Chemistry* **261**, 5947–5951.

Schauer, R., Reuter, G., Stoll, S., Posadas del Rio, F., Herrler, G. & Klenk, H.-D. (1988). Isolation and characterization of sialate 9(4)-O-acetylesterase from influenza C virus. *Biological Chemistry Hoppe-Seyler* **369**, 1121–1130.

Schultze, B. & Herrler, G. (1992). Bovine coronavirus uses *N*-acetyl-9-*O*-acetylneuraminic acid as a receptor determinant to initiate the infection of cultured cells. *Journal of General Virology* **73**, 901–906.

Strobl, B. & Vlasak, R. (1993). The receptor-destroying enzyme of influenza C virus is required for entry into target cells. *Virology* **192**, 679–682.

Vlasak, R., Luytjes, W., Spaan, W. & Palese, P. (1988*a*). Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. *Proceedings of the National Academy of Sciences, USA* **85**, 4526–4529.

Vlasak, R., Luytjes, W., Leider, J., Spaan, W. & Palese, P. (1988*b*). The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetylesterase activity. *Journal of Virology* **62**, 4686–4690.

Vlasak, R., Muster, T., Lauro, A. M., Powers, J. C. & Palese, P. (1989). Influenza C virus esterase: analysis of catalytic site, inhibition, and possible function. *Journal of Virology* **63**, 2056–2062.

Yolken, R. H., Willoughby, R., Wee, S. B., Miskuff, R. & Vonderfecht, S. (1987). Sialic acid glycoproteins inhibit in vitro and in vivo replication of rotaviruses. *Journal of Clinical Investigation* **79**, 148–154.

Received 30 August 1996; Accepted 14 November 1996