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Original article

Infection of the tracheal epithelium by infectious bronchitis virus is sialic acid dependent

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Abstract

Avian Infectious bronchitis virus (IBV) is a coronavirus that infects chickens via the respiratory epithelium as primary target cells. The binding of coronaviruses to the cell surface is mediated by the viral surface protein S. Recently we demonstrated that α2,3-linked sialic acid serves as a receptor determinant for IBV on Vero cells and primary chicken embryo kidney cells. Here we analyze the importance of the sialic acid binding activity for the infection of tracheal organ cultures (TOCs) by different IBV strains. Our results show that α2,3-linked sialic acid also serves as a receptor determinant on chicken TOCs. Infection of TOCs by IBV results in ciliostasis. Desialylation induced by neuraminidase treatment of tracheal organ cultures prior to infection by IBV delayed the ciliostatic effect or resulted in partial loss of ciliary activity. This effect was observed with both respiratory and nephropathogenic strains. Inhibition of ciliostasis was also observed when TOCs were pretreated with an α2,3-specific neuraminidase. Analysis of the tracheal epithelium for reactivity with lectins revealed that the susceptible cells in the epithelium abundantly express α2,3-linked sialic acid. These results indicate that α2,3-linked sialic acid plays an important role for infection of the respiratory epithelium by IBV.

Keywords: Avian infectious bronchitis virus; Coronavirus; Sialic acid; Tracheal organ culture

1. Introduction

Infectious bronchitis is one of the most significant diseases of chickens in the commercial poultry industry. The etiologic agent is *Infectious bronchitis virus* (IBV), a member of the family *Coronaviridae* [1]. The pathology of the disease associated with IBV infection can show different characteristics depending on the tissue where virus replication occurs. IBV enters its host via the oro/oculo-nasal route and the first site of replication is the respiratory epithelium. From there the virus infection can spread to several organs, including kidney and reproductive tract (reviewed in [2]). Secondary pathogens can complicate the disease resulting in increased morbidity and mortality.

The tissue tropism of viruses may be determined by several factors including the distribution of the cellular receptor for the virus. In the case of coronaviruses, the surface protein S is responsible for attachment to cells. The S protein has not only receptor-binding activity, it also mediates the fusion of the viral lipid envelope with the cellular membrane. For several coronaviruses specific proteins have been described that serve as cellular receptors for the initiation of infection. Angiotensin-converting enzyme 2 has been identified as a receptor for SARS coronavirus [3,4] and several group 1 coronaviruses including transmissible gastroenteritis virus require aminopeptidase N to enter their host cells [5,6]. A protein receptor has not been identified so far for IBV. Recently we demonstrated that α2,3-linked sialic acids serve as receptor determinants on Vero-cells and primary chicken embryo kidney cells [7]. Here we show that sialic acids also play a role in the infection of the avian respiratory epithelium by IBV.

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2. Materials and methods

2.1. Preparation of TOCs

Tracheas were prepared from 20-day-old SPF chicken embryos (Lohmann, Cuxhaven, Germany) and, after removing connective tissue, were cut manually into approximately 1 mm thick rings by using a microtome blade. Individual rings were transferred into 5 ml tubes (Sarstedt, Nümbrecht, Germany) containing 0.5 ml of Medium 199 with Hanks salts (Biochrom, Berlin, Germany) and incubated at 37 °C on a rotator. The next day, TOCs were screened for 100% ciliary activity (see Section 2.5).

2.2. Viruses

Stock virus of the IBV strains M41 and B1648 were obtained by inoculating embryonated SPF chicken eggs. Following incubation at 37 °C, the allantoic fluid was collected, clarified by low speed centrifugation and stored at −80 °C. Strain Beaudette of IBV was propagated in Vero cells. Supernatants of infected cell cultures were harvested, clarified by low speed centrifugation and stored at −80 °C. All IBV strains were kindly provided by Dave Cavanagh, Institute for Animal Health, Compton, UK. *Avian metapneumovirus* subtype A, employed as a control virus, was kindly provided by Silke Rautenschlein, University of Veterinary Medicine, Hannover, Germany.

2.3. Neuraminidase treatment and virus infection of TOCs

TOCs were washed with PBS prior to addition of neuraminidase from *Clostridium perfringens* (Type 6) or *Streptococcus pneumoniae* (Sigma–Aldrich, St. Louis, MO, USA) using MES (2[*N*-morpholino]ethanesulfonic acid) buffer as a diluant. If not otherwise indicated TOCs were incubated with 50 mU neuraminidase per ring. After incubation at 37 °C for 1 h, the TOCs were washed three times with PBS and infected by IBV-Beaudette (10^4 pfu/ring) or any of the other viruses for 1 h at 37 °C. Following three washes with PBS, the TOCs were incubated with medium at 37 °C on a rotator. For all experiments groups of four TOCs were used to estimate the mean ciliary activity. All experiments were performed in triplicate.

2.4. Neuraminidase treatment

Each ring of TOC was suspended in 100 µl medium 199 containing 50 mU neuraminidase of *Clostridium perfringens* and in some cases 2.5 mg of the neuraminidase inhibitor DANA (2,3-didehydro-2-deoxyneuraminic acid). After incubation for 1 h at 37 °C TOCs were washed and infected by the Beaudette strain with 10^4 pfu/ring.

2.5. Ciliary activity assay

TOCs were analyzed daily with a microscope to estimate the ciliary activity. Rings were virtually divided into 10 parts and each part was monitored for ciliary movement. Only rings with a starting ciliary activity of 100% were used for the experiments.

2.6. Infectivity

Groups of five TOCs were infected in triplicate by 2×10^4 pfu of IBV. At 24 h post infection, the supernatants were collected and titrated on primary chick kidney cells as described previously [7]. Plaques were visualized by immunofluorescence using a polyclonal anti-IBV serum raised in rabbits.

2.7. Preparation of cryosections

Tracheas were prepared from three-week old SPF chickens (Lohmann, Cuxhaven, Germany). They were cut into rings approximately 1 cm thick. The rings were washed with PBS and either infected by IBV-Beaudette (1×10^5 pfu/ring) for 1 h at 37 °C. or subjected to neuraminidase treatment and lectin staining. Infected TOCs were incubated in Medium 199 (Biochrom, Berlin, Germany) at 37 °C. After 24 h, they were mounted on small filter papers with tissue freezing medium (Jung, Heidelberg, Germany) and frozen in liquid nitrogen. Neuraminidase treatment was performed by incubation with 500 µl Medium199 containing 200 mU neuraminidase from *Clostridium perfringens* for 1 h at 37 °C, before freezing in liquid nitrogen. The frozen organs were stored at −20 °C until they were cut with a cryostat.

2.8. Immunofluorescence analysis

Cryosections were fixed with ice-cold acetone for 10 min followed by air drying for another 10 min. The antibodies or lectins were diluted in 1% bovine serum albumin and sections were incubated with antibodies or lectins for 1 h at room temperature in an incubation chamber. After three washing steps with PBS, the sections were incubated with appropriate second antibodies for 1 h at room temperature in the dark. Virus antigen was stained with polyclonal anti-IBV Beaudette serum raised in rabbits. Sialic acids were detected with lectins from *Maackia amurensis* (binding to α2,3 linked sialic acids) or *Sambucus nigra* agglutinin (binding to α2,6 linked sialic acids) labeled with digoxigenin (DIG Glycan Differentiation kit, Roche, Basel, Switzerland). Mucus producing goblet cells were stained with anti MUC-5AC antibody (Acris, Hiddenhausen, Germany). Bound antibodies or lectins were visualized by FITC- and Cy3-labeled anti-rabbit (Sigma–Aldrich), anti-mouse (Acris) or anti-digoxigenin antibodies (Roche). Cilia were detected by CY3 labeled anti-β-tubulin antibody (Sigma–Aldrich). Fluorescence microscopy was performed with a Leica inverted-2 confocal microscope.

3. Results

3.1. Effect of neuraminidase treatment on the infection of tracheal organ cultures (TOCs) by IBV

After having shown recently that sialic acid serves as a receptor determinant for IBV on cultured cells, we were

interested to find out whether this type of sugar is also important for an infection in vivo. To address this question we chose TOCs as a model for the upper respiratory epithelium. The trachea of chicken embryos was cut into pieces about 1 mm thick. Tracheal rings chosen for this analysis showed ciliary activity along the whole contact area of the epithelium with the lumen of the trachea, i.e. 360° of the tracheal ring. Infection of TOCs by IBV results in ciliostasis that can easily be detected microscopically. As shown in Fig. 1, infection by IBV-Beaudette reduced the portion of the epithelium with ciliary activity. At 2 days post infection (d.p.i.), complete ciliostasis was observed at this experimental setting. To analyze the importance of sialic acids we treated TOCs with neuraminidase from *Clostridium perfringens*, which releases the sialic acids from the cell surface. As a result desialylated TOCs were less sensitive to the ciliostatic effect of the IBV infection. At 5 d.p.i., about 25% of the epithelium still showed ciliary activity. This effect is accounted for by the neuraminidase itself rather than by a contaminant enzyme, because in the presence of a neuraminidase inhibitor the enzyme was unable to protect the tracheal epithelium from the ciliostatic effect of the IBV infection (Fig. 1).

The infection of cultured cells by IBV was found to be dependent on α2,3-linked sialic acid. To find out whether there is also a linkage specificity in the infection of the tracheal epithelium, TOCs were treated with neuraminidase from *Streptococcus pneumoniae*, which has a high preference for cleaving α2,3-linked sialic acid. As shown in Fig. 2, incubation with this enzyme protected the epithelial cells from the ciliostatic effect of the IBV infection in the same way as was observed with the neuraminidase from *Clostridium perfringens* (Fig. 1). From this result we conclude that α2,3-linked sialic acid serves as a receptor determinant for the infection of avian tracheal epithelial cells by the Beaudette strain of IBV.

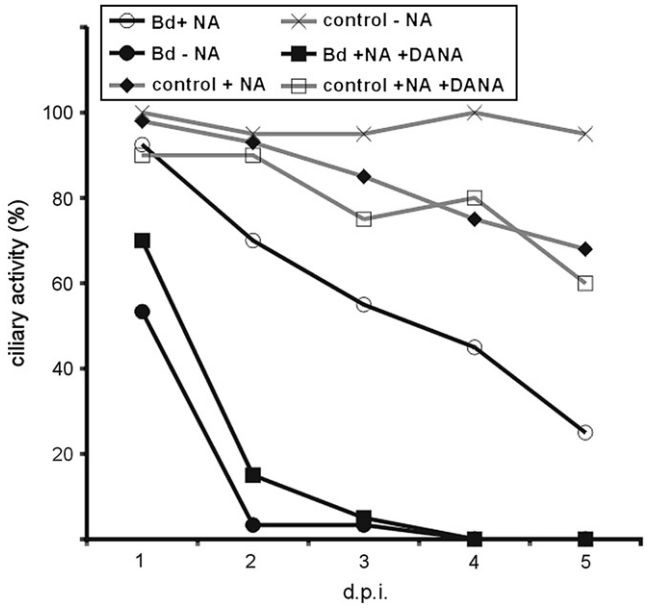

Fig. 1. Effect of neuraminidase treatment on the infection of TOC by IBV. TOC were incubated in the absence (−NA) or presence (+NA) of neuraminidase. In two samples the enzyme activity was prevented by addition of the inhibitor DANA. Following neuraminidase treatment, the cultures were infected by IBV-Beaudette. The effect of the infection was estimated at different days post infection (d.p.i.), by determining the loss of the ciliary activity.

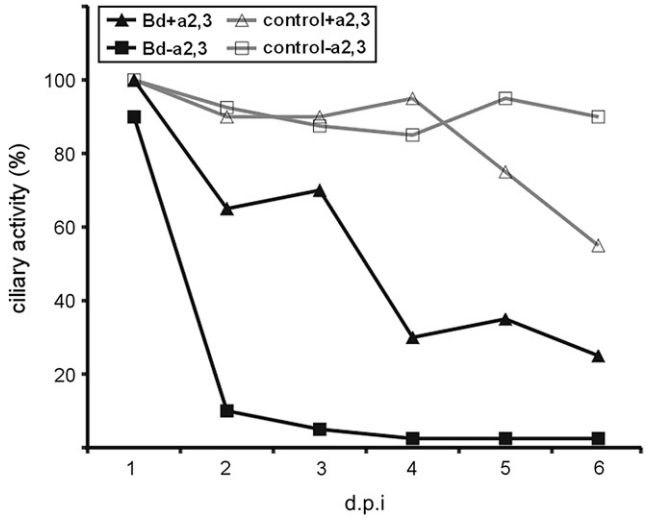

Fig. 2. Effect of α2,3-specific neuraminidase on the infection of TOC by IBV. Tracheal rings were incubated with neuraminidase from *Streptococcus pneumoniae* prior to infection IBV-Beaudette. The effect of the virus infection was estimated by determining the loss of ciliary activity.

3.2. Strain-dependent differences

The Beaudette strain of IBV has been adapted to grow in cultured cells of non-avian origin, such as Vero cells. To demonstrate that the sialic acid-dependent infection of tracheal epithelial cells is a general feature of IBV, we included two other strains. The M41 strain causes respiratory disease, whereas the B1648 strain has a nephropathogenic potential [8]. After application of an infectious dose of 10^4 pfu per tracheal ring, the IBV strains caused ciliostasis by day 3 (M41), 4 (B1648) or 5 (Beaudette) after infection (Fig. 3A–C). Strain M41 was more pathogenic than the other two strains resulting in a loss of ciliary activity at 2 d.p.i. on 80% of the epithelium in the microscopic field (Fig. 3C). A comparable reduction was observed with the two other strains only 3 days following infection of TOCs (Fig. 3A and B). For all three strains, a protective effect of neuraminidase treatment was found. In the case of M41, ciliostasis was delayed for 1 day; in the case of the other two strains, the ciliary activity was retained in about 60% of the desialylated epithelium even on day 5 following infection. Ciliostasis was also found after infection by *Avian metapneumovirus*. However, in contrast to IBV infection, neuraminidase treatment did not prevent the ciliostatic effect of the metapneumovirus infection. This result confirms that the sialic acid dependence of the TOC infection is a characteristic feature of IBV.

In the experiments described above, the ciliary activity was used to monitor the course of infection. To determine the effect of neuraminidase treatment on virus production, the amount of infectious virus released from the epithelial cells was measured. Groups of five TOC rings were treated with neuraminidase prior to infection by either of the IBV strains,

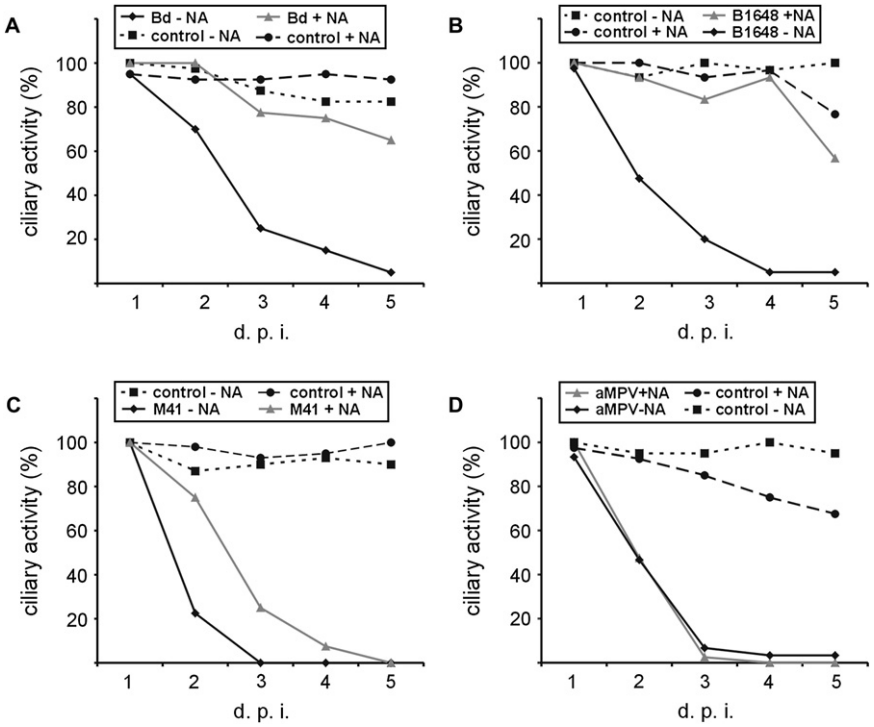

Fig. 3. Infection of tracheal organ cultures by different IBV strains. TOC were incubated in the absence (−NA) or presence (+NA) of neuraminidase prior to mock-infection (control) or infection by IBV strains Beaudette (Bd), B1648 or M41 (panels A–C) or *Avian metapneumovirus* (panel D). The effect of the virus infection was estimated by determining the loss of ciliary activity.

Beaudette and M41. In parallel, TOC were infected that had been incubated in the absence of neuraminidase. At 24 h p.i. the supernatants were collected and titrated on primary chicken kidney cells. With both virus strains, desialylation resulted in a decrease of the virus titer by about 60% (Fig. 4). There was a clear difference in the amount of virus released from infected TOCs. For the Beaudette strain, the titer of infectious virus in the supernatant was 50–100 fold higher than that determined for strain M41.

3.3. Infection of different cell types in the chicken tracheal epithelium

The respiratory epithelium is the primary target for an IBV infection. Cryosections of TOC were prepared and ciliated cells were visualized by staining with antibodies directed against β-tubulin. Mucus-producing goblet cells were stained with an antibody recognizing MUC5AC. As shown in Fig. 5 (top two rows), both ciliated and mucus-producing cells are infected by IBV. Cryosections were also stained for sialic acid expression using the lectin MAA which binds to α2,3-linked sialic acids and SNA which recognizes α2,6-linked sialic acids. The epithelial cell layer lining the surface of the trachea shows bright fluorescence after staining with MAA, indicating that these cells abundantly express α2,3-linked sialic acid. They co-localize with IBV-infected cells (Fig 5, third row). This result is consistent with a role of α2,3-linked sialic acid as a receptor determinant for IBV. Staining with SNA indicates that basal cell layers of the tracheal epithelium express α2,6-linked sialic acid. These cells are not infected by IBV (Fig. 5, bottom row).

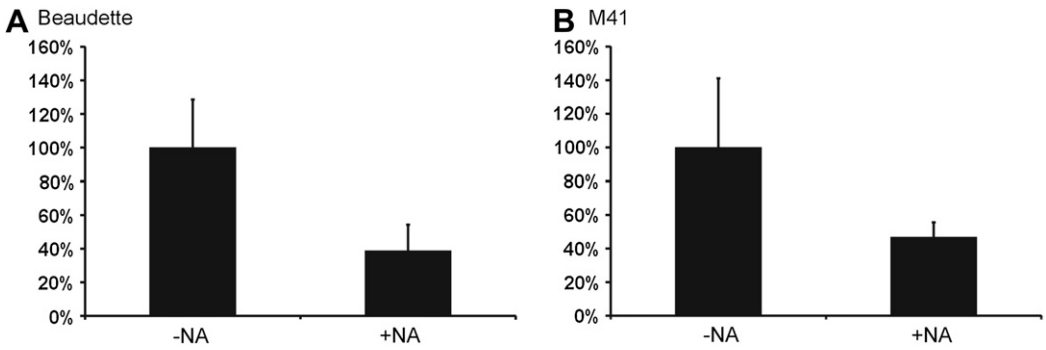

Fig. 4. Effect of pretreatment with neuraminidase on the virus release from IBV-infected TOC. TOC were incubated in the absence (−NA) or presence (+NA) of neuraminidase from *Clostridium perfringens* and then infected by either of two IBV strains, Beaudette or M41. At 24 h post infection, infectious virus in the supernatants was titrated by plaque assay on primary chicken kidney cells.

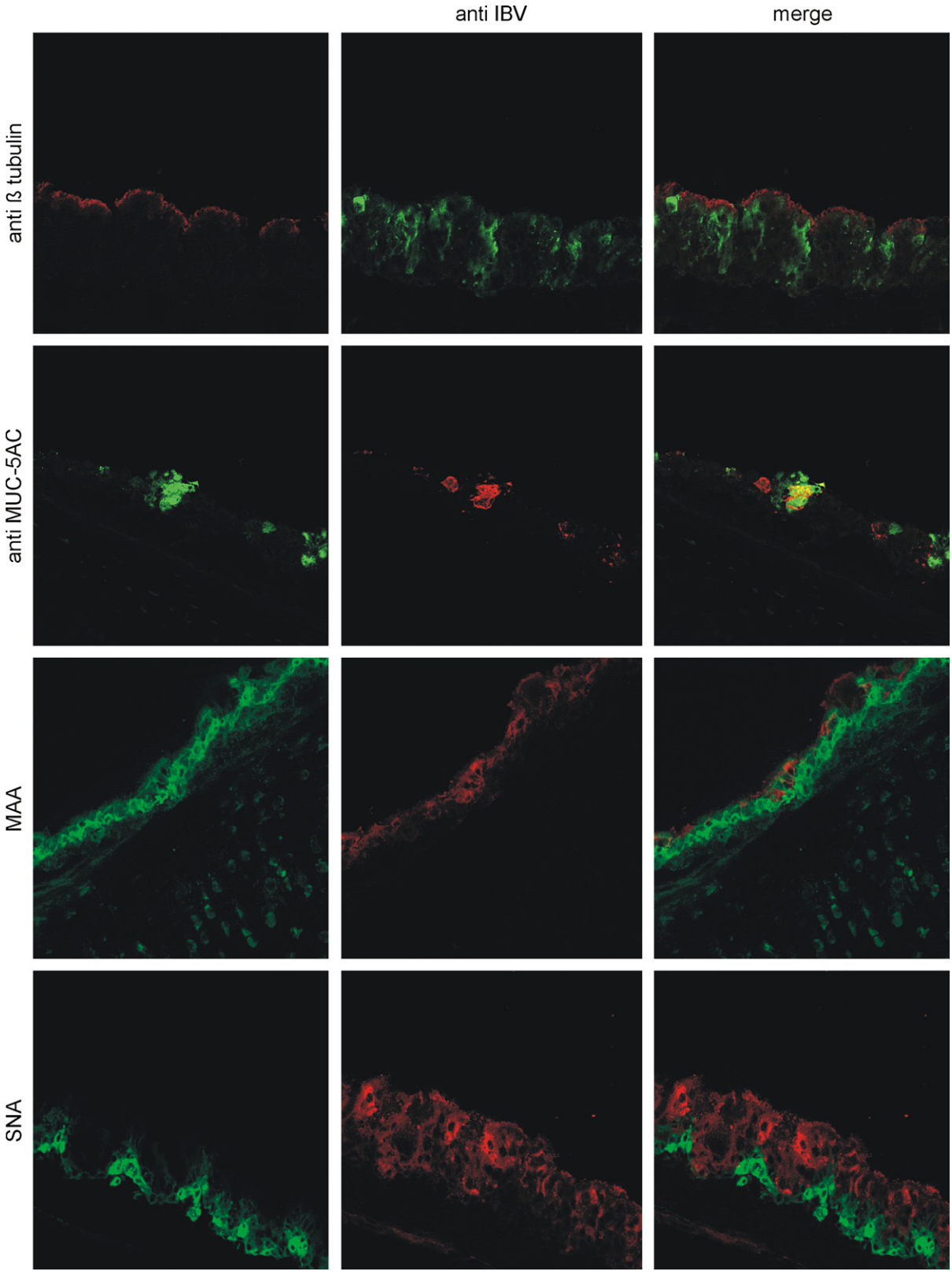

Fig. 5. Immunofluorescence analysis of IBV-infected TOC. Cryosections were prepared from infected TOC and stained for virus antigen (green fluorescence in the top row and red fluorescence in rows 2–4). In addition, the samples were stained for the presence of ciliated cells (top row, anti-β-tubulin), of mucin-producing cells (second row, anti-MUC-5AC), cells expressing α2,3-linked sialic acids (third row, MAA), and cells expressing α2,6-linked sialic acids (bottom row, SNA).

3.4. Effect of neuraminidase treatment on the staining by the lectin MAA

To visualize the effect of neuraminidase treatment, we prepared cryosections of neuraminidase-treated tracheal rings and stained the sections with the MAA lectin. As shown in Fig. 6, neuraminidase treatment resulted in a reduced reactivity of MAA with the apical membrane of the epithelial cells. The residual fluorescent signal indicates that the neuraminidase did not release all sialic acids from the epithelial cells.

4. Discussion

The binding to receptors on the cell surface is an important determinant of cell tropism and viral pathogenesis. Recently we demonstrated that α2,3-linked sialic acid serves as a receptor determinant for IBV on cultured cells [7]. This binding activity of IBV has been shown for strains that only grow in avian cells as well as for the Beaudette strain that has been adapted to grow also in cultured cells of mammalian origin. For the latter strain it has been recently reported that it also recognizes glycosaminoglycans of the heparan sulfate type [9]. The acquisition of this additional binding activity may have allowed the adaptation to new host cells as has been shown for other viruses, e.g. foot and mouth disease virus [10] and tick-borne encephalitis virus [11]. By contrast, the sialic acid binding activity has been detected on all IBV strains analyzed including respiratory and nephropathogenic variants. Here we have demonstrated that this also holds true for epithelial cells of the trachea, which are among the primary target cells for this virus.

The importance of sialic acid for IBV infection was evident from the delayed appearance of ciliostasis on desialylated TOCs. For the M41 strain, the ciliostatic effect was delayed only by 1 day, whereas for the two other strains analyzed, some ciliary activity was retained even 5 days after infection. This difference in the protective effect of neuraminidase treatment is probably accounted for by the difference in the cytopathogenicity of these viruses. This may also explain the larger amount of virus that is released from TOCs infected by IBV-Beaudette compared to M41-infected tracheal rings. Despite these differences, the relative effect of desialylation was similar, i.e. the amount of released virus was reduced by about 60%. The partial protection of TOCs from IBV infection may be attributable to the difficulties in removing all sialic acid residues from the cell surface of the tracheal epithelium. Staining by MAA revealed that some sialic acids are still present on the apical membrane of the epithelial cells after neuraminidase treatment. Whether this low level of sialic acid by itself is sufficient for a low level of infection is not known. An alternative explanation is that binding to surface-bound sialic acids is only a first attachment step in the infection cycle that precedes the binding to a second receptor, though such a receptor has not been identified for IBV so far. In favor of the latter explanation is the fact that IBV recognizes sialic acid with lower affinity when compared to influenza virus and Sendai virus [7]. Because of the higher affinity for sialic acids, a second receptor may be dispensable for influenza viruses; however, it may require the presence of a neuraminidase (receptor-destroying enzyme) to allow passage through the mucus layer covering the respiratory epithelium [12] and to enable virus release from infected cells [13]. On the other hand, a receptor-destroying enzyme may be dispensable for IBV because of the lower affinity for sialic acids.

So far, two groups of coronaviruses with sialic acid binding activity have been described. On one hand, there is bovine coronavirus and related viruses that resemble influenza C virus, because they recognize *N*-acetyl-9-*O*-acetylneuraminic acid and contain an acetylesterase that acts as a receptor-destroying enzyme comparable to the neuraminidase of influenza A and B viruses [14,15]. These viruses depend on sialic acid for infection of cells. On the other hand, there is transmissible gastroenteritis virus, a porcine coronavirus. This virus uses aminopeptidase N as a cellular receptor and it does not require sialic acid for successful infection of cultured cells. However, binding to sialylated surface components may increase the efficiency of infection, because mutants lacking the sialic acid binding activity have lost the enteropathogenicity [16,17]. IBV takes an intermediate position; sialic acid is important for infection of cultured cells but it lacks a receptor-destroying enzyme. As mentioned above, IBV may—in addition to sialylated surface molecules—bind to a specific receptor similar to the interaction of TGEV with aminopeptidase N. Though it

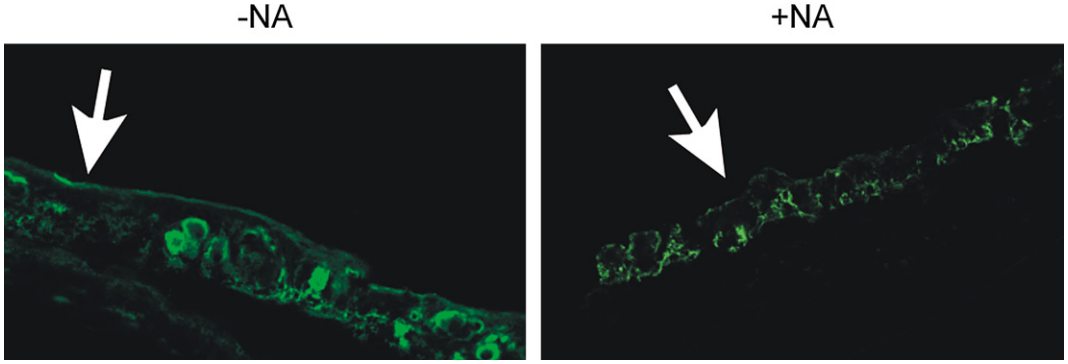

Fig. 6. Effect of neuraminidase treatment on the staining of the tracheal epithelium by the lectin MAA. TOCs were incubated in the presence (+NA) or absence (−NA) of neuraminidase. After incubation, cryosections were prepared and subjected to MAA staining. Arrows point to the apical membrane of the tracheal epithelium. It should be noted that the enzyme had access only to the apical side of the epithelium.

has been suggested that feline aminopeptidase N may be used by IBV to infect cells [18], recently it has been shown that aminopeptidase N does not serve as a receptor for IBV [19].

Our analysis of TOC revealed that both ciliated and mucus-producing cells are susceptible to infection. From this result we conclude that in vivo both cell types can act as primary target cells for IBV. The expression of α2,3-linked sialic acids and the absence of α2,6-linked sialic acids is consistent with the preference of IBV for the former linkage type ([7] and this report). In this respect IBV appears to have developed a similar strategy to avian influenza viruses which also use α2,3-linked sialic on the cell surface to initiate an infection. However, there are also major differences between avian influenza and avian coronaviruses. A major determinant of the pathogenicity of influenza viruses is the proteolytic cleavage of the hemagglutinin. The hemagglutinins of highly pathogenic viruses have a multibasic cleavage site that is susceptible to furin-like enzymes present in many cell types. Viruses of low pathogenicity become fusion-active by a protease that is secreted by respiratory cells. The S protein of all IBV strains has a multibasic cleavage site and is cleaved in all cell types into the subunits S1 and S2. Therefore, proteolytical activation of S is not a major determinant of the pathogenicity of IBV. Why some IBV strains are predominantly respiratory pathogens whereas others affect other organs to cause disease, e.g. the renal system or the reproductive tract, is not known. If there exists a second receptor for this virus as discussed above, such a protein may be responsible for the host specificity as well as the tissue or organ tropism of IBV. Future work should clarify whether a second receptor exists. Furthermore, the sialoglyconjugates used for primary attachment should be analyzed. The available lectins and neuraminidases only allow a differentiation between α2,3- and α2,6-linked sialic acids. Different oligosaccharides exist that contain α2,3-linked sialic acid, and the sialoglycoconjugates recognized by IBV may be different from those that are preferred by avian influenza viruses. Such differences may also contribute to a different course of infection. Expression of α2,3-linked sialic acid on epithelial cells in chickens has been analyzed for the trachea and the intestine [20,21]. As far as other target organs of IBV are concerned, e.g. kidney and the reproductive system, α2,3-linked sialic acid has been shown to be present on primary kidney cells [7]. It will be interesting in the future to find out whether infection of the reproductive system is also mediated by sialic acid.

Acknowledgments

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