NOTE *Virology*

Attachment and Infection to MA104 Cells of Avian Rotaviruses Require the Presence of Sialic Acid on the Cell Surface

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ABSTRACT. To determine the characters of receptors on target cells for avian rotaviruses, the receptors on MA104 cells for the pigeon rotavirus PO-13, the turkey rotaviruses Ty-1 and Ty-3, and the chicken rotavirus Ch-1 were analyzed. Pretreatment of MA104 cells with neuraminidase greatly reduced the infection by all of the four avian rotavirus strains. Binding of the cell-attachment protein, purified VP8 expressed in bacteria, of strain PO-13 to MA104 cells was also inhibited by pretreatment of cells with neuraminidase. These findings suggest that avian rotaviruses primarily utilize sialic acid-containing molecules as receptors on MA 104 cells.
KEY WORDS: rotavirus, sialic acid, VP8.

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Group A rotaviruses, members of the *Reoviridae* family, are the most common cause of gastroenteritis in young children and animals, including many mammalian and avian species [5]. Two surface proteins, VP4 and VP7, are present on the outer capsid of rotaviruses. They have independent neutralization antigens and define P (for protease-sensitive) and G (for glycoprotein) types, respectively. In the presence of trypsin, VP4 is cleaved into two polypeptides,VP5 and VP8. This proteolytic cleavage is associated with an increase in infectivity [12]. The attachment of the virus to cell surface receptors is mediated by VP4 [14]. There is evidence that rotaviruses have multiple plasma membrane receptors, including sialic acid (SA) [6], integrins [4] or other membrane proteins [13]. Some animal rotaviruses can bind to the cell either through interactions mediated by VP8 or VP5 via SA-containing and SA-independent cell surface receptors, respectively [7, 24]. Human strains appear to use an SA-independent route [6], and an $\alpha 2\beta 1$ integrin-binding motif (DGE) present in VP5 at amino acids 308–310 may function as the receptor-binding site [23].

Rotaviruses have also been isolated from several avian species [15, 16]. Previous studies have suggested that avian rotaviruses separated from mammalian rotaviruses early during evolution [10, 11]. The bovine rotavirus 993/83 was isolated in Germany from the feces of a calf suffering from diarrhea [1]. This virus is more similar to avian rotaviruses than to mammalian rotaviruses in terms of genetic and antigenic properties [1, 2, 21]. Furthermore, a pigeon rotavirus PO-13 was found to be infectious and to have a level of virulence similar to that of the monkey rotavirus SA11 in a suckling ddY mouse model [20]. These observations suggest that avian rotaviruses play a role as cross-species pathogens between avian and mammalian species. However, it is not known whether avian rotaviruses can enter cells and infect animals by the same mechanisms as those by which mammalian rotaviruses cause infection. To investigate the involvement of SA on the cell surface, we tested four avian rotavirus strains.

The avian rotavirus strain PO-13 (G7, P[17]) was isolated from a pigeon in Japan [16] and was passaged 12 times in MA104 cells. Turkey rotavirus strains Ty-3 (G7, P[17]) and Ty-1 (G7, P[17]) and a chicken rotavirus, strain Ch-1 (G7, P[17]), isolated using chicken embryo fibroblast cells and/or chick kidney cells in the United Kingdom [15], were provided by McNulty, Veterinary Research Laboratories, Belfast, United Kingdom, and were passaged several times in MA104 cells in our laboratory. For this study, all of the avian rotaviruses, a simian rotavirus strain SA11 (G3, P[2]) and a human rotavirus strain Wa (G1, P1A[8]) were grown in MA104 cells as described previously [16].

Infectivity assays were carried out to determine whether avian rotaviruses are SA-dependent or -indipendent. Monolayers of MA104 cells in 24-well plates were treated with 100 mU/m*l* of neuraminidase from *Arthrobacter (A.) ureafaciens* (Nacalai Tesque, Kyoto, Japan) or *Clostridium (C.) perfringens* (Sigma Chemical Co., MO, U.S.A.). After treatment with the enzyme at 37°C for 1 hr, the cells were washed with Hanks' solution three times and inoculated with approximately 200 focus-forming units (ffu) of trypsin-activated viruses. Following incubation with the viruses for 1 hr on ice, each monolayer was washed with Hanks' solution three times and covered with 1 m*l* per well of the overlay medium consisting Eagle's MEM supplemented with 0.5% methyl cellulose, 2% fetal calf serum and antibiotics. The cells were incubated for 24 hr at 37°C and fixed with 2% paraformaldehyde for 1 hr and methanol for 5 min. The cells infected with rotaviruses were detected using ABC staining (Vector Laboratories, CA, U.S.A.) with monoclonal antibody P3-1 against VP6 of strain PO-13 [17]. Titers were expressed as ffu by counting the number of stained infectious foci. Infectivity in the neuraminidase-treated cells was expressed as a percentage of the infectivity titers in control cells.

A previous study has shown that treatment of MA-104 cells with neuraminidase reduced the infectivity of the simian rotavirus strain SA11 but had no effect on the infectivity

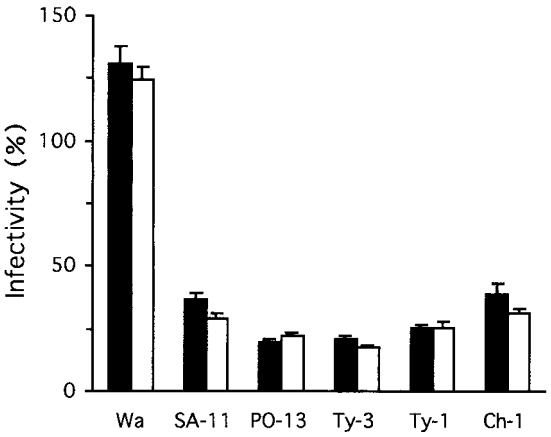

Fig. 1. Infectivity of rotaviruses in MA104 cells treated with neuraminidase from *A. ureafaciens* (closed bars) or *C. perfringens* (open bars). Arithmetic mean ± standard error from three replicate experiments is shown.

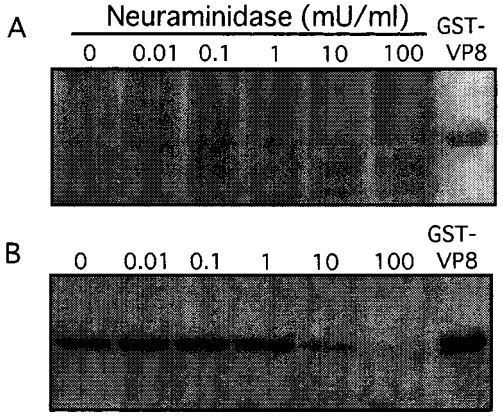

Fig. 2. Effects of neuraminidase treatment of MA104 cells on GST-VP8 binding. MA104 cells in 24-well plates were treated with 0.01 to 100 mU/m*l* of neuraminidase from *C. perfringens*. After treatment at 37°C for 1 hr, the cells were incubated with GST-VP8 for 1 hr on ice, washed three times, and lysed with 0.05 m*l*/well of lysis buffer. Five microliters of each lysate and 5 μg of the purified GST-VP8 were used for SDS-PAGE (A) and Western blot analysis with anti-PO-13 rabbit serum (B).

of the human strain Wa [6]. Figure 1 also shows that strain SA11 was sensitive to treatment with both neuraminidases and that strain Wa was resistant to the treatment. The infectivity titers of the four avian rotaviruses were reduced to 61–80% and to 69–83% of pretreatment levels by treatment with neuraminidases from *A. reafaciens* and *C. perfringens*, respectively. These results show that the avian rotaviruses used in this study are SA-dependent. However, it has been reported that the turkey rotavirus strain Ty-1 does not require SA molecules for efficient infectivity [3]. At present, we do not have an explanation for this discrepancy, but the Ty-1 strain used in this study was provided directly by a researcher who had isolated this strain and passaged it for a limited number of times. Strain Ty-1 was also confirmed to be an avian rotavirus by sequencing its VP6, VP8 and NSP4 genes [9, 19, 21]. The character of MA104 cells used in this study might be different from that of MA104 cells in their experiments, since it has been suggested that the distinction between neuraminidase-sensitive and -insensitive strains may be influenced by the cell type used to carry out the assays [14].

It has been reported that recombinant VP8 protein, produced in bacteria as a fusion product with glutathione S-transferase (GST-VP8), was found to bind to MA104 cells in a specific and saturable manner and that it was capable of inhibiting the binding of a homologous virus when it was preincubated with MA104 cells [24]. To confirm the specificity of binding of an avian rotavirus to sialic acid on the cell surface, we prepared GST-VP8 of strain PO-13 and carried out a binding assay using purified GST-VP8. Genomic dsRNA of strain PO-13 was extracted from the partially purified virion using ISOGEN (Nippon Gene, Japan) as described by the supplier. The cDNA of PO-13 VP8 (nucleotides 1 to 715 of VP4 gene) gene was produced from the extracted dsRNA by reverse transcription-polymerase chain reaction with a pair of oligonucleotide primers, 5' CGGATCCATGGCTTCTCTCGTATATAGACA 3' and 5' AGAATTCGCACTGATCGCTCAACTGGCATT 3', which had additional recognition sequences for the restriction endonucleases *Bam*HI and *Eco*RI (underlined), respectively. The cDNA of PO-13 VP8 gene was cloned into the *Bam*HI and *Eco*RI sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, NJ, U.S.A.). The resultant fusion protein, GST-VP8, contained 226 amino acids from the GST protein, a thrombin recognition site, 5 amino acids resulting from translation of part of the vector poly linker, and 239 amino acids of PO-13 VP8, resulting in a fusion protein of approximately 54 kDa. The preparation of the purified GST-VP8 was performed as described previously [18]. Monolayers of MA104 cells in 24-well plates were treated with 0.01 to 100 mU/m*l* of neuraminidase from *C. perfringens* as described above. After washing the monolayers, 0.5 mg/m*l* of GST-VP8 was applied to them. They were incubated on ice for 1 hr and washed with Hanks' solution three times. The cells were lysed with lysis buffer containing 20 mM CHAPS (Dojin-kagaku, Japan) as described previously [22], and the lysates were subjected to SDS-PAGE and Western blot analyses with anti-PO-13 rabbit serum. These analyses were performed as described previously [8].

The quantity and quality of proteins in MA104 cells were not changed by treatment with neuraminidase from *C. perfringens* (Fig. 2A). The binding of purified GST-VP8 of strain PO-13 to MA104 cells, as measured by this direct assay, was inhibited by neuraminidase treatment in a dose-dependent manner (Fig. 2B). These results indicate that avian rotaviruses can bind to SA residues on the cell surface through their VP8s.

Our results show that the cell attachment and infectivity of avian rotaviruses used in this study are SA-dependent. These findings suggest that avian rotaviruses primarily utilize SA-containing molecules as receptors on MA 104 cells.

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