

Characterization of the Hemagglutinin Receptor Specificity and Neuraminidase Substrate Specificity of Clinical Isolates of Human Influenza A Viruses

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Six clinical isolates of influenza A viruses were examined for hemagglutinin receptor specificity and neuraminidase substrate specificity. All of the viral isolates minimally passaged in mammalian cells demonstrated preferential agglutination of human erythrocytes enzymatically modified to contain NeuAc alpha2,6Gal sequences, with no agglutination of cells bearing NeuAc alpha2,3Gal sequences. This finding is consistent with the hemagglutination receptor specificity previously demonstrated for laboratory strains of influenza A viruses. The neuraminidase substrate specificities of the clinical isolates examined were also identical to that described for the N2 neuraminidase of recent laboratory strains of human influenza viruses. The H3N2 viruses all displayed the ability to release sialic acid from both alpha2,3 and alpha2,6 linkages. In addition, two clinical isolates of H1N1 viruses also demonstrated this dual neuraminidase substrate specificity, a characteristic which has not been previously described for the N1 neuraminidase. These results demonstrate that complementary hemagglutinin and neuraminidase specificities are found in recent isolates of both H1N1 and H3N2 influenza viruses.

Key words: influenza virus - clinical isolates - hemagglutinin - neuraminidase - specificity

Influenza viruses express two envelope glycoproteins, the hemagglutinin and the neuraminidase. Both of these viral glycoproteins recognize the sugar sialic acid (neuraminic acid or NeuAc). The hemagglutinin mediates viral attachment to host cells by binding to sialic acid residues on host cell glycoproteins (Paulson 1985). The neuraminidase hydrolyses sialic acid residues from host cell glycoproteins, and has been postulated to facilitate release of budding virus from cells and to prevent virus entrapment in respiratory tract mucus (Baum & Paulson 1991). Both the hemagglutinin and the neuraminidase have been shown to demonstrate preferential recognition of sialic acid in specific linkages to adjacent sugar residues. The H1, H2 and H3 hemagglutinins of human influenza A viruses preferentially bind to the NeuAc alpha2,6Gal sequence, while the hemagglutinins of avian and equine influenza A virus strains preferentially bind the Neu alpha2,3Gal sequence (Rogers & Paulson 1983, Rogers & D'

Souza 1989, Paulson & Webster, unpublished results). It has been proposed that the shift in hemagglutinin receptor specificity from NeuAc alpha2,3Gal to NeuAc alpha2,6Gal sequences is the result of selection pressure exerted by human host cells. The ciliated epithelial cells of the human respiratory tract contain primarily NeuAc alpha2,6Gal sequences (Couceiro et al. 1993), so that viruses which can recognize and bind to this sequence may have a selective advantage in humans.

Similarly, the N2 neuraminidase of human influenza A viruses has been reported to discriminate between sialic acid in alpha2,3 and alpha2,6 linkages. Previous studies have shown that the N2 neuraminidase of an H2N2 strain had exclusive substrate specificity for the NeuAc alpha2,3Gal sequence (Drzeniek 1973, Carrol et al. 1981). However, it has been recently found that the N2 neuraminidase has undergone a drift in specificity, so that more recent human isolates can hydrolyse both the NeuAc alpha2,3Gal and the NeuAc alpha2,6Gal sequences (Baum & Paulson 1991). The acquisition of a neuraminidase specificity which matches the hemagglutinin specificity may provide a selective advantage to the virus, by facilitating release of progeny virus from infected cells expressing NeuAc alpha2,6Gal sequences which can bind the hemagglutinin.

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Six clinical isolates of human influenza A viruses have been examined for hemagglutinin and neuraminidase specificity. The isolates all demonstrated a hemagglutinin receptor specificity for NeuAc alpha2,6Gal sequences. Four of the clinical isolates with an N2 neuraminidase hydrolyzed substrates containing both the NeuAc alpha2,3Gal and NeuAc alpha2,6Gal linkages, as has been found in later N2 laboratory strains. In addition, two clinical isolates with an N1 neuraminidase also demonstrated dual neuraminidase specificity, a feature which has not been described previously in H1N1 strains.

MATERIALS AND METHODS

Viruses - Nasopharyngeal aspirates from patients with symptoms of upper respiratory tract infection were inoculated onto monolayers of Madin Darby Canine Kidney (MDCK) epithelial cells. Virus was harvested, partially purified and assayed for hemagglutination activity as previously described (Rogers et al. 1983b). The samples were classified as influenza A virus, H1N1 and H3N2, by hemagglutination inhibition and neuraminidase inhibition assays, using standard antisera (Center for Disease Control, Atlanta, USA).

M1/5 and M1/5HS8 clonal isolates of influenza virus A/Memphis/102/72 (H3N2) which have well characterized receptor-binding and sialidase specificities (Rogers et al. 1983b, Baum & Paulson 1991) were used as controls for hemagglutination receptor specificity and neuraminidase specificity assays.

Hemagglutination receptor specificity assay - Human erythrocytes were enzymatically derivatized to contain either the NeuAc alpha2,6Gal or NeuAc alpha2,3Gal linkages as previously described (Carrol et al. 1981). This assay takes advantage of the ability of various sialyltransferase enzymes to add sialic acid (NeuAc) to cell surface glycoproteins in a single defined linkage. Briefly, type A erythrocytes were desialylated by incubation with *Vibrio cholerae* neuraminidase and resialylated with CMP-[¹⁴C] NeuAc and either the Galβ1,4GlcNAc alpha2,6 sialyltransferase or the Galβ1,4(3)GlcNAc alpha2,3 sialyltransferase to create the NeuAc alpha2,6Galβ1,4GlcNAc or NeuAc alpha2,3Galβ1,4GlcNAc sequences, respectively. Asialo erythrocytes were with *V. cholerae* neuraminidase and not resialylated. Derivatized erythrocytes contained 141-161 nmol NeuAc/ml packed cells (NeuAc alpha2,3Gal) and 35-50 nmol NeuAc/ml packed cells (NeuAc alpha2,6Gal).

Hemagglutination assays were performed with native, asialo and derivatized erythrocytes (Carrol

et al. 1981, Rogers & Paulson 1983, Paulson 1985, Rogers & D' Souza 1989). The assays were performed in a microtiter system (Cooke Engineering Co.). Serial two-fold dilutions of virus (25 μl) were mixed with 25 μl of a 1.5% suspension of erythrocytes. Hemagglutination titers were read at 1 hr and were expressed as the reciprocal of the maximum dilution of virus that caused complete agglutination.

Neuraminidase specificity assay - The neuraminidase activities of the samples were measured using native alpha 1 acid glycoprotein (alpha 1 AGP, Sigma), which contains both NeuAc alpha2,3Gal and NeuAc alpha2,6Gal sequences, as a standard substrate (Paulson et al. 1982). Reaction mixtures (100 μl) contained 0,5 mg alpha 1 AGP (195 nmol NeuAc), 0,25 mg bovine serum albumin (BSA, Sigma) and 5 μl of virus concentrate in 0,1 M sodium cacodylate, pH 6.5. Samples were incubated for 30 min at 37°C, and sialic acid release was quantitated using the thiobarbituric acid assay, which detects free, but not bound sialic acid (Warren 1959). One unit of neuraminidase activity was defined as the amount of enzyme which caused hydrolysis of one umol NeuAc per minute at 37°C.

To determine the neuraminidase substrate specificity, 3' sialyllactose (NeuAc alpha2,3Galβ1,4GlcNAc: 3'-SL) and 6' sialyllactose (NeuAc alpha2,6Galβ1,4GlcNAc: 6'-SL) were used as substrates (BioCarb Chemicals). Reaction mixtures (100 μl) contained 0,5 mg 3'-SL or 6'-SL, 0,25 mg BSA and concentrated virus (0,12 mU neuraminidase activity in the standard assay) in 0,1 M sodium cacodylate, pH 6.5. Assay mixtures were incubated for 30 min at 37°C, and sialic acid release was quantitated using the thiobarbituric acid assay. Neuraminidase activity with the specific substrates was defined as described above.

RESULTS

Hemagglutination receptor specificity of clinical isolates of influenza A viruses - Six clinical isolates of influenza A viruses were examined for the ability to agglutinate the derivatized erythrocytes prepared as described in Materials and Methods. These included two H1N1 and H3N2 influenza A viruses. As shown in Table I, all of the virus strains agglutinated only the erythrocytes containing the NeuAc alpha2,6Gal sequences, while no agglutination of erythrocytes bearing the NeuAc alpha2,3Gal sequences was observed. This difference was not merely due to differential resialylation of the two types of erythrocytes, since the cells containing the NeuAc alpha2,3Gal linkages actually incorporated more sialic acid than

TABLE I

Hemagglutinin receptor specificities of clinical isolates of influenza A virus determined with derivatized erythrocytes

Erythrocyte preparation	HA titer of virus samples ^a						Control M1/5	Control M1/5HS8
	1 H1N1	2 H1N1	3 H3N2	4 H3N2	5 H3N2	6 H3N2		
Native	80	20	40	40	8	32	10,240	10,240
Asialo	0	0	0	0	0	0	0	0
NeuAc alpha2,6Gal	80	40	40	16	16	32	10,240	0
NeuAc alpha2,3Gal	0	0	0	0	0	0	0	5,120

^a: hemagglutinin titers were determined as described in Methods using a 1.5% suspension of each of the erythrocyte preparations, and are expressed as the reciprocal of the highest titer. M1/5 and M1/5HS8 cloned samples of influenza A viruses showing known hemagglutinin receptor specificities for NeuAc alpha2,6Gal and NeuAc alpha2,3Gal linkages were used as controls. The virus samples were typed with standard antisera, and designed as similar to the following standard strains of influenza A virus: 1: A/Chile /1/83; 2: A/England/333/60/85; 3: A/Mississippi/1/85; 4: A/Leningrad/360/5/86; 5: A/Wellington/4/85; 6: A/Capetown/2/85.

the cells with the NeuAc alpha2,6Gal linkages. The agglutination titers of all the viruses with the derivatized cells bearing the NeuAc alpha2,6Gal sequences were virtually identical to the titers observed with native erythrocytes. As expected, no agglutination of the asialo erythrocytes was detected. These data indicate that the clinical isolates of human influenza A viruses demonstrated the same pattern of hemagglutinin receptor specificity as has been described for laboratory strains of influenza A viruses.

Neuraminidase substrate specificity of influenza A viruses - To examine the neuraminidase substrate specificities of the clinical isolates, commercially available sialyllactose containing sialic acid in either the alpha2,6 or alpha2,3 linkage (6'SL or 3'SL) were used. A standard aliquot of virus suspension was incubated with the two substrates and released sialic acid was quantitated by a colorimetric assay which detects only free sialic acid (Warren 1959). This method has an advantage over our previously published technique (Baum & Paulson 1991) of not requiring enzymatic derivatization of glycoprotein substrates with purified sialyltransferase enzymes, as well as eliminating the use of a radioisotopic detection system.

The two H1N1 and four H3N2 virus strains were assayed using this method. As shown in Table II, all six strains released approximately equal amounts of sialic acid from the alpha2,3 and alpha2,6 linkages. The dual substrate specificity of the four H3N2 virus strains was identical to that seen previously with later N2 strains, those isolated after 1972 (Baum & Paulson 1991). This specificity differs from that of earlier N2 neuraminidases in H2N2 virus stains, in which

preferential cleavage of the NeuAc alpha2,3Gal sequence had been demonstrated (Drzeniek 1973, Carrol et al. 1981).

No previous studies have examined the neuraminidase substrate specificity of the N1 neuraminidase. Like the N2 neuraminidases examined here, the two H1N1 strains cleaved sialic acid from both types of substrates with equal efficiency. However, the amount of sialic acid released was slightly less than that seen with N2 viruses.

DISCUSSION

It was been proposed that the recognition of specific sialic acid linkages by influenza virus hemagglutinin and neuraminidase results from selection pressures on virus populations exerted by host cell sialyloligosaccharides (Rogers & Paulson 1983, Rogers et al. 1983b). In this report, six clinical human influenza A virus isolates were analyzed and it was found that all of the strains preferentially agglutinated erythrocytes bearing the NeuAc alpha2,6Gal sequence. This finding is consistent with previous analyses of laboratory strains of influenza A viruses, which has demonstrated that virtually all human virus strains preferentially bind the NeuAc alpha2,6Gal sequence, in contrast to avian and equine strains, which preferentially bind the NeuAc alpha2,3Gal sequence. Although avian strains are thought to be the progenitors of human strains of influenza virus, Rogers and Paulson (1983) have shown that a single amino acid substitution, leucine to glutamine at amino acid 226, alters the hemagglutinin receptor specificity from human type to avian type (Weis et al. 1988, Rogers et al. 1983a), suggesting that a small number of muta-

TABLE II

Neuraminidase activity of the clinical isolates of influenza A virus on 3' sialyllactose and 6' sialyllactose

Substrate	% NeuAc released by virus samples ^a							
	1 H1N1	2 H1N1	3 H3N2	4 H3N2	5 H3N2	6 H3N2	Control M1/5	Control M1/5HS8
NeuAc alpha2,3Galβ1,4GlcNAc	6	6	16	9	12	12	10	50
NeuAc alpha2,6Galβ1,4GlcNAc	5	7	7	7	7	10	12	24

^a: samples contained 0.12 mU of viral neuraminidase activity, and were added to reaction mixtures containing either 3'-SL or 6'-SL. samples were incubated for 30 min at 37°C and the percentage NeuAc released was determined using the thiobarbituric acid assay to measure free sialic acid. M1/5 and M1/5HS8 cloned samples showing known neuraminidase specificity for NeuAc alpha2,3Galβ1,4GlcNAc and NeuAc alpha2,6Galβ1,4GlcNAc sequences were used. The virus samples were typed with standard antisera, and designed as similar to the following standard strains of influenza virus: 1: A/Chile/1/83; 2: A/England/333/60/85; 3: A/Mississippi/85; 4: A/Leningrad/360/5/86; 5: A/Wellington/4/85; 6: A/Capetown/2/85.

tional events may allow transformation of an avian-specific to a human pathogen. Thus, the predominance of NeuAc alpha2,6Gal sequences on ciliated epithelial cells of human trachea (Couceiro et al. 1993) may select for those viruses which can effectively bind this sequence and establish an infection in these cells.

It is important to note that all the isolates examined here were minimally passaged in MDCK cells, in order to obtain adequate amounts of virus to analyze. A number of reports have shown that growth of influenza virus in MDCK cells does not alter the sequence or the receptor specificity of the hemagglutinin (Robertson et al. 1990, 1991). In contrast, passage of virus in eggs has been demonstrated to alter the viral hemagglutinin, and, in some cases, to select for binding to the NeuAc alpha2,3Gal sequence (Rogers et al. 1983b, Robertson et al. 1990, 1991). Since all the isolates were grown only in MDCK cells, it is likely that our results reflect the hemagglutinin receptor specificity of the virus in the original clinical samples.

The neuraminidase substrate specificity of the influenza A viruses was also examined. All of the virus strains demonstrated approximately equal hydrolysis of 6'SL and 3'SL. Dual neuraminidase substrate specificity has recently been described for N2 strains isolated after 1972. However, the dual neuraminidase substrate specificity has not previously been demonstrated for the N1 neuraminidase. An examination of additional recent H1N1 isolates will determine whether the ability to cleave both the alpha2,3 and alpha2,6 linkages is a common feature of the N1 neuraminidase. It will also be of interest to examine H1N1 strains isolated between 1948 and 1956, to determine whether this char-

acteristic existed in earlier N1 strains, or, like the N2 neuraminidase, has only recently been acquired.

We have proposed that the ability to cleave the NeuAc alpha2,6Gal linkage facilitates release of progeny virus from host cells, and allows more rapid dissemination of virus to adjacent uninfected cells (Baum & Paulson 1991). The retention of the ability to cleave the NeuAc alpha2,3Gal sequence may be related to another proposed function of the neuraminidase, to release virus from entrapment in respiratory mucus, which is rich in NeuAc alpha2,3Gal sequences (Breg et al. 1987). The presence in the N1 neuraminidase of dual substrate specificity, which we have previously demonstrated in later N2 viruses, suggest that a broadening of neuraminidase substrate specificity provides a selective advantage to human influenza A viruses.

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