Host-mediated Selection of Influenza Virus Receptor Variants

SIALIC ACID-a2,6Gal-SPECIFIC CLONES OF A/DUCK/UKRAINE/1/63 REVERT TO SIALIC ACID-a2,3Gal-SPECIFIC WILD TYPE IN OVO*

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Human and animal influenza A isolates of the H3 serotype preferentially bind SAa2,6Gal or SAa2,3Gal linkages (where SA represents sialic acid), respectively, on cell-surface sialyloligosaccharides. Previously, we have demonstrated selection of SAa2,3Gal-specific receptor variants of several human viruses which differed from the parent viruses by a single amino acid at residue 226 of the hemagglutinin which is located in the receptor binding pocket (Rogers, G. N., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A., and Wiley, D. C. (1983) Nature 304, 76–78). In this report, the selection in the reverse direction was accomplished starting with a SAa2,3Gal-specific avian virus, A/duck/Ukraine/1/63 (H3N7), yielding SAa2,6Gal-specific variants that exhibit the receptor binding properties characteristic of the human isolates. Selection was again mediated at residue 226 of the hemagglutinin, in this case changing from Gln in the parent virus to Leu in the variants.

Although the SAa2,6Gal-specific avian virus variants were stable to passage in MDCK cells, they exhibited dramatic reversion to the SAa2,3Gal-specific phenotype of the parent virus during a single passage in chicken embryos. This was in contrast to the SAa2,6Gal-specific human virus isolates which were stable to passage in both hosts. The reversion of the avian virus variants in eggs provides compelling evidence for host-mediated selection of influenza virus receptor variants.

Influenza viruses exhibit considerable diversity in their ability to recognize specific sialyloligosaccharide structures as cell-surface receptor determinants (1–4), and it has become increasingly evident from laboratory models (5–8) that such specificity can provide the basis for changes in receptor binding that have been reported to occur during host adaptation (9–12). Indirect evidence that receptor binding properties may be important in the ecology of influenza viruses comes from the correlation between receptor specificity and species of origin observed for virus isolates bearing the H3 hemagglutinin (4). The human isolates of this serotype exhibit strong preferential binding to oligosaccharides terminating in SAa2,6Gal’ sequences, while avian and equine H3 isolates preferentially bind SAa2,3Gal sequences. Furthermore, binding of cell-surface receptors by the human H3 isolates is very sensitive to inhibition by a glycoprotein present in horse serum, αα-macroglobulin, whereas avian and equine isolates are not inhibited (7, 8). Thus, by growing human H3 isolates in the presence of horse serum, it has been possible to select variants with receptor properties that closely resemble those of avian and equine H3 isolates, i.e. that recognize oligosaccharides terminated by the SAa2,3Gal linkage and are insensitive to inhibition by equine αα-macroglobulin. Sequence analysis of the genes for the hemagglutinins of these viruses revealed that they differed by a single nucleotide resulting in an amino acid change at residue 226 from leucine in the parental (SAa2,6Gal-specific) phenotype to glutamine in the variant with the avian type (SAa2,3Gal-specific) receptor specificity (7). In the three-dimensional structure of the H3 hemagglutinin reported by Wilson et al. (13), it was determined that amino acid 226 is located in the receptor binding pocket in the distal globular region of the molecule. How the difference of a single amino acid at this site can mediate dramatic changes in receptor binding specificity is still not clear.

This report describes a novel approach to the selection of receptor variants from an avian influenza virus, A/duck/Ukraine/1/63 (H3N7), distinguished from the parental, SAa2,3Gal-specific, virus by their ability to bind SAa2,6Gal sequences of cell-surface oligosaccharides. As reported for receptor variants of the human H3 isolates (7), the hemagglutinins of these avian variants differ from the parent by a single amino acid, at residue 226, located in the receptor binding pocket. Unlike the human H3 isolates, however, the SAa2,6Gal-specific variants of A/duck/Ukraine/1/63 rapidly revert to the parental, SAa2,3Gal-specific, phenotype when grown in the egg allantois but not when grown in mammalian cell culture. While the mechanism for this receptor shift remains unclear, these results appear to present a very clear

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example of host-mediated selection based on receptor specificity.

MATERIALS AND METHODS

Cells—Medin-Darby canine kidney (MDCK) cells (ATCC CCL24) were passaged and maintained in Eagle's minimum essential medium supplemented with 5% fetal calf serum and 100 units/ml of penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. Growth—A/duck/Ukraine/1/63 (H3N7) was generously provided by Dr. Robert G. Webster, St. Jude Children's Research Hospital, Memphis, TN. Cloned variants of A/3/Indiana/102/72 (H3N2) were produced from a seed stock, also obtained from Dr. Webster, by passage in the absence (M1/5) or presence (M1/H8) of horse serum as previously described (8).

As standard procedure for growth in MDCK cell culture, monolayers were inoculated with virus, multiplicity of infection around 0.001, and incubated at 34 °C for 36-48 h in Eagle's minimum essential medium containing 2.5% bovine serum albumin and 20 μg/ml trypticase. At harvest, the medium was removed and centrifuged at 750 × g for 10 min to remove cell debris. Virus preparations were stored at −70 °C and assayed in parallel for hemagglutination activity and infectivity on MDCK cells in the presence of 20 μg/ml trypsin (14).

For growth of virus in ovo, 0.1 ml (10^6 pfu/ml) of MDCK cell lysates, diluted in phosphate-buffered saline containing antibiotics (15), was injected into the allantoic cavity of 10- to 11-day-old chicken embryos and incubated at 37 °C for 48 h. At harvest, eggs were cooled for 1 h at −20 °C. The allantoic fluid was collected and clarified at 7500 × g for 20 min. Virus was collected by centrifugation (5000 × g for 18 h), resuspended in phosphate-buffered saline, and stored at −25 °C.

Preparation of Derivatized Erythrocytes—The procedures for the enzymatic modification of human erythrocyte oligosaccharides have been previously described (4, 16). Briefly, native human erythrocytes were treated with Vibrio cholerae sialidase (GIBCO) to remove sialic acid and abolish viral adsorption and hemagglutination. The sialidic acid determinants were restored in a single, defined sequence by treatment of sialidase-treated (asialo) erythrocytes with CMP-[14C]sialic acid (New England Nuclear) and one of two highly purified mammalian sialyltransferases. The sialyltransferases employed in this study include the Galp1,GalNAc a2,3-sialyltransferase,1 which is presumably the predominant receptor on erythrocytes. Consequently, selection of SAa2,6Gal variants from SAa2,3Gal-specific avian isolates required a different approach. The procedure used, depicted in Fig. 1, involves selective adsorption and elution of the SAa2,3Gal-specific A/duck/Ukraine/1/63 with erythrocytes enzymatically modified to contain SAa2,6Gal sequences. First, virus was adsorbed to cells under conditions of large viral excess as previously described (7). The bound virus was eluted from cells by addition of C. perfringens sialidase (250 milliliters/ml) and incubation at 37 °C for 3 h. The virus released from erythrocytes was then grown in MDCK cell culture to amplify variants. After four such cycles, the eluted virus was plaqued on MDCK cells without intermediate amplification. Primary clones were initially screened for sensitivity to inhibition of growth in MDCK cells in the presence of horse serum and, finally, for receptor specificity by adsorption to derivatized erythrocytes. Of 64 primary clones, 5 were found to be specific for SAa2,6Gal linkages.

The receptor binding characteristics of five wild type clones and five cloned variants from A/duck/Ukraine/1/63 were compared to the parent virus, and the results are shown in Table I. Each of the virus preparations was examined for its ability to agglutinate native, sialidase-treated (asialo) and enzymatically resialylated erythrocytes containing SAa2,3Gal or SAa2,6Gal sequences and for sensitivity to equine α2-macroglobulin, the major glycoprotein inhibitor of horse serum. The parent strain of A/duck/Ukraine/1/63-agglutin-
ated erythrocytes derivatized to contain the SAα2,3Gal linkage did not agglutinate SAα2,6Gal-derivatized cells and was not sensitive to hemagglutination inhibition by equine α-M-macroglobulin. The five wild type clones exhibited receptor binding properties identical to those of the parent strain. In contrast, five variants agglutinated cells modified to contain SAα2,6Gal linkages did not agglutinate SAα2,3Gal-derivatized cells and were sensitive to hemagglutination inhibition by equine α-M-macroglobulin. The receptor binding properties of these variants of A/duck/Ukraine/1/63, therefore, are very similar to those previously associated with the corresponding receptor type isolated for the human H3 isolates (4, 7, 8).

The complete amino acid sequences of the HAI region of the hemagglutinins expressed by the parent strain and representative SAα2,3Gal- and SAα2,6Gal-specific clones were deduced from the nucleotide sequences of their RNA genes as previously described (7). The only amino acid sequence changes detected were at residue 226 as a result of changes in the triplet nucleotides 754-756 from CAG (glutamine, Gln) to CTG (leucine, Leu).

Receptor variants of A/duck/Ukraine/1/63 were obtained as described in the text, plaque-purified three times, and grown in MDCK cell culture. Hemagglutination titrations were performed with MDCK cell lysates after removal of cell debris by low speed centrifugation (700 × g for 10 min).

Human erythrocytes were either unmodified (Native), treated with V. cholerae sialidase (asialo), or sialidase-treated cells resialylated with CMP-sialic acid and purified sialyltransferases as described under “Materials and Methods.” The sialyloligosaccharide structures examined are SAα2,3Galβ1,3GalNAc (SAα2,3Gal) and SAα2,6Galβ1,4GalNAc (SAα2,6Gal) commonly found as terminal sequences on O- and N-linked oligosaccharides, respectively. HA titers were determined as described and are expressed as the reciprocal of the greatest dilution of virus that produced agglutination (0 = <2).

Hemagglutination inhibition by purified, heat-inactivated (30 min at 56 °C) equine α-M-macroglobulin (α-M) was performed as described. The initial concentration of inhibitor (3 mg/ml) was roughly the same concentration found in unfractionated serum (22). Results are expressed as the reciprocal of the highest dilution of α-M-macroglobulin causing inhibition of native erythrocyte agglutination by 4 hemagglutinating units of virus.

Deduced from the nucleotide sequences of the HAI region of the hemagglutinin genes. Nucleotide sequences were determined using the dideoxynucleotide chain termination method with a primer extension system containing vinylpyridine or 5'-32P-labeled synthetic oligonucleotide primers (7). The only amino acid sequence changes detected were at residue 226 as a result of changes in the triplet nucleotides 754–756 from CAG (glutamine, Gln) to CTG (leucine, Leu).
**Table II**

Receptor specificities of Ukraine variants after passage in the egg allantois and MDCK cell culture

| Host     | Infectious dose* | pfu | HA titer|
|----------|------------------|-----|---------|
| MDCK*    |                   |     |         |
| 10^3     | 4,096            | 6,3 |         |
| 10^4     | 4,096            | 6,2 |         |
| 10^5     | 4,096            | 6,0 |         |
| EGG*     |                   |     |         |
| 10^3     | 1,286            | 66  |         |
| 10^4     | 6,248            | 6,0 |         |

*a Virus seed stocks were prepared by growth of plaque-purified virus (4 successive plaque to plaque passages) in MDCK cell cultures. Infectious titers (plaque-forming units/ml) of the cell lysates were determined as described under “Materials and Methods.” These preparations had log pfu/HA ratios of 6.3 (UK19), 6.2 (UK25), 6.0 (UK49), and 5.7 (UK49). Receptor specificity was confirmed by hemagglutination titrations with derivatized erythrocytes, and results are shown in Table I.

*b Hemagglutination titers toward resialylated SAA2,3Gal (a2,3) and SAA2,6Gal (a2,6) erythrocytes were assessed for each virus preparation as described in Table I. All preparations were concentrated 100-fold prior to assay.

*c Seed stocks were diluted as shown and grown on MDCK monolayers in the presence of trypsin (20 pg/ml) at 34°C. Virus was harvested at optimum times for each infectious dose, as determined by visual assessment of the cytopathic effect.

*d Appropriate dilutions of virus seed stocks were injected (0.2 ml) into the allantoic cavity of 10-day-old chicken embryos and incubated 20 h at 37°C.

**Table III**

Time course of adaptation by Ukraine variants during growth in ovo

| Variant* | 1 day | 2 day | 3 day | 4 day |
|----------|------|------|------|------|
|          | a2,3 | a2,6 | a2,3 | a2,6 |
|          | a2,3 | a2,6 | a2,3 | a2,6 |
|          | a2,3 | a2,6 | a2,3 | a2,6 |

*a The allantoic cavity of 10-day-old chicken embryos were injected (0.1 ml) with 10 pfu/egg of virus and incubated at 37°C.

*b Hemagglutination titers of SAA2,3Gal (a2,3) or SAA2,6Gal (a2,6)-derivatized cells by concentrates (100 ×) of egg grown virus was examined as described in Table I. Results are expressed as reciprocals of the highest dilution of virus that resulted in agglutination (0 = <2).

At the lower infectious doses (10–100 pfu/egg), the SAA2,6Gal clones grew poorly in eggs, and of the virus that was present (after only 1 day of growth), there was no evidence for the specificity changes that occurred at the higher doses (Table II). However, incubation of eggs inoculated with SAA2,6Gal-specific variants at 10 pfu/egg for 2, 3, and 4 days also revealed evidence of selection on the basis of receptor binding (Table III). The same pattern of adaptation by the SAA2,6Gal-specific variants was also observed when plaque eluates were used as the source of inoculum (data not shown). These observations suggested that the wild type virus with the SAA2,3Gal-binding phenotype grew more rapidly in ovo than the SAA2,6Gal variants (Tables II and III) and that selection could occur simply by the wild type virus outgrowing and variants.

**Table IV**

Comparison of receptor specificities for human and avian receptor variants bearing the H3 hemagglutinin after growth in ovo

| Original specificity | Parent virus | Clone | Host* | Specificity* |
|---------------------|--------------|-------|-------|--------------|
| SAA2,3Gal           |              |       |       |              |
| Duck                | UK6          | MDCK  | 64    |              |
|                    | Egg          | 1024  | 0     |              |
| SAA2,6Gal           |              |       |       |              |
| Duck                | UK3          | MDCK  | 0     | 128          |
|                    | Egg          | 1024  | 0     | 128          |

*a Virus (MDCK cell lysates) was grown either in MDCK cells or 10-day fertile eggs as described under “Materials and Methods.”

*b Receptor specificity was determined by agglutination of derivatized erythrocytes as described in the legend to Table I.
Hemagglutination assays were performed as described under which is a potent inhibitor of virus bearing SAa2,6Gal-specific variants can also be made. Using the H3 virus A/duck/Ukraine/l/63 which recognize sialic acid in different linkages obtained by a procedure, fundamentally different from previous selection procedures, involving multiple cycles of adsorption to and elution from enzymatically modified erythrocytes. Analysis by thin layer chromatography showed). Thus, while differential binding of human and avian hemagglutinins can be demonstrated. We have reported previously that influenza viruses of the H3 subtype which recognize sialic acid in different linkages can be obtained by passaging wild type virus in the presence of inhibitors of agglutination (7, 8). Specifically, Hong Kong influenza viruses isolated from humans and grown in the egg show restricted binding of receptors present in the egg, the apparent restricted binding of the SAa2,6Gal-specific A/duck/Ukraine/l/63 implies that a component of the receptor molecule in addition to sialic acid linkage is involved in determining the precise specificity of the virus-receptor interaction. This conclusion is supported by our observation that the SAa2,6Gal-specific isolates of A/Memphis/102/72 and A/duck/Ukraine/l/63 differ in their ability to agglutinate derivatized erythrocytes containing N-glycolyneramic acid. The molecular basis of this additional specificity is unknown. However, by comparing the amino acid sequences of the SAa2,6Gal-specific hemagglutinins from human and avian isolates and examining the locations of differing residues in the three-dimensional structure of the X-31 hemagglutinin (13), it is apparent that the amino acid differences at positions 137, 155, 158, 189, 193, 227, and 228 in or near the receptor pocket could individually or in combination impose the additional receptor requirements.

Finally, the observation that cell-surface receptors can mediate selection of receptor variants provides a partial explanation for the apparent changes in receptor binding properties which can occur during adaptation of influenza viruses to growth in different hosts. It is in this regard particularly relevant to the suggestion that an avian virus antigenically related to A/duck/Ukraine/l/63 was the progenitor of the virus responsible for the Hong Kong influenza pandemic of 1968 (20, 21).

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**Note Added in Proof**—A recent report relevant to work presented here has examined reassortant viruses of A/duck/Ukraine/l/63 containing a human virus H3 hemagglutinin (27). Results suggest that changes in amino acids 226 and 228 can alter the tissue tropism of the virus in ducks.

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**Previous finding**—that residue 226 is a component of the sialic acid receptor binding pocket and emphasize its importance in determining the receptor specificity of the H3 hemagglutinin molecule.
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