We have previously reported that the binding properties of the hemagglutinin (HA) of the WSN-F strain of influenza A are affected by the cells in which the virus is grown (Crecelius et al., 1984). At 37°C, chick embryo fibroblast-grown F virus has a greater affinity for host cells than does the same virus grown in Madin-Darby bovine kidney (MDBK) cells. In an attempt to explain this host-determined property, we have characterized the carbohydrate put onto the viral HA by these two cells. Experiments using tunicamycin indicate that the HA made by MDBK cells contains about 4000 daltons of carbohydrate in excess of that on the HA from chick embryo fibroblast. Serial lectin affinity chromatography of the asparagine-linked oligosaccharides on the HA subunits, HA1 and HA2, detected a number of host-dependent differences in the complex oligosaccharides. Both HA1 and HA2 from MDBK cells contained more highly branched (i.e. tri- and tetraantennary) complex oligosaccharides than did the subunits from chick embryo fibroblasts. In addition, the HA subunits from the two sources differed in the amount of galactose-containing "bisected" complex oligosaccharides and in the presence of certain fucosylated triantennary oligosaccharides. Profiles of the asparagine-linked oligosaccharides from the host cells did not show these differences, indicating that the HA subunit profiles were not necessarily representative of the structures found on the cellular glycoproteins. The data support the conclusion that bulky oligosaccharides on the MDBK-HA subunits of WSN-F reduce the affinity of the virus for cellular receptors.

The hemagglutinin (HA) is the major glycoprotein on the surface of the influenza viruses. It is responsible for the attachment and entry of the virus into host cells and is the antigen to which neutralizing antibodies are produced. Through its antigenic variability, the HA is responsible for the continued appearance in the human population of epidemic strains of influenza A virus.

The biologically active HA is a trimer of identical glycoproteins each of which is cleaved into two subunits, designated HA1 and HA2 (1,2). Carbohydrate side chains are attached to both subunits through asparagine linkages (N-linked) (3) and are of the complex, high mannose, or hybrid types (4, 6). These oligosaccharides can vary in number and structure depending on the virus strain and the host cell. Strain-specific variations in the number and type of carbohydrates result from differences in the primary structure of the HA protein (6, 7). Host-specific variations in the size of the HA oligosaccharides (4, 6) are assumed to be due to differences in the ability of various cells to process the oligosaccharide side chains. However, the extent to which the host cell determines specific aspects of the oligosaccharide structures of the influenza HA and how these structures influence the biological activities of this glycoprotein are unknown.

This study was undertaken to document host-determined differences in carbohydrate composition of the HA using a strain of virus known to have host cell and erythrocyte binding properties which are influenced by the cells in which it is grown (8). This virus, the F H A variant of the WSN strain of influenza A, grows well in CEF but poorly in MDBK cells. In addition, binding studies have indicated that the F virus from CEF cells (FCEF) has a greater affinity for both MDBK and CEF cells than does the F virus from MDBK cells (FFMK). These differences between FCEF and FMMK have been shown to be phenotypic rather than genetic; growth of both FCEF and FMMK in the opposite host cell for one passage changes the size of the HA and the binding properties of the viruses to those expected from the particular host in which the virus is last grown (8, 9). Thus, we are confident that we are examining host-determined differences in the glycosylation of the same HA polypeptide sequence. By using serial lectin affinity chromatography (10), we have obtained information about the structure of the oligosaccharides put onto the two viral HAs by the two host cells and have identified host-dependent differences in oligosaccharide composition which could be responsible for the differences in binding properties. In addition, we present evidence that the N-linked oligosaccharides on the virus are not necessarily representative of those found on the host cell glycoproteins.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

The lectin affinity analysis presented here clearly demonstrates a number of differences between the complex oligosaccharides from the two viral HA subunits.
Oligosaccharide Composition of an Influenza Virus HA

...charide side chains put onto the HA subunits by two host cells. First, the MDBK-HA subunits carry tri- and tetra- 
tennary oligosaccharides more frequently than do the CEF-HA polypeptides. Second, galactose-containing "bisected" 
complex oligosaccharides are more abundant on CEF-HA subunits than on MDBK-HA subunits. Third, certain fucosyl-
ated triantennary oligosaccharides are found on the HA subunits from CEF but not from MDBK cells. Finally, gly-
copeptides from MDBK-HA subunits contain a class of mole-
cules which interact with leukoagglutinating phytohemagglu-
tinin-agarose with high affinity. These are not found on CEF-HA subunits.

Our data indicate that MDBK-HA contains about 4000 daltons of carbohydrate in excess of that on CEF-HA, ap-
proximately 3000 of which are in the HA3 subunit. This can be due in part to the large number of tri-
and tetraantennary oligosaccharides on the MBDK-HA. However, this extra car-
bohydrate is about 20 monosaccharides distributed over five 
complex oligosaccharides on each MBDK-HA monomer, sug-
gests that additional sugar residues are attached to the outer 
galactoses of many of the complex carbohydrates synthesized 
by MBDK cells. The data presented here do not permit us to 
make comparisons of the number of additional outer sugar 
residues between the two glycoprotein preparations. How-

ever, other lines of evidence indicate that MBDK-grown 
virions have relatively few unsubstituted galactose residues 
as compared to CEF-grown virions. Influenza virions do not 
contain sialic acid, but these residues can be added to terminal 
galactoses on the HAs by incubating the virions with sialyl-
transferase and CMP-sialic acid (18). When this was done, 
CEF-HA was sialylated at about 20 times the rate of MBDK-
HA, even though the lectin profiles presented here indicate 
that the HA subunits from the two sources contain compar-
able amounts of oligosaccharides with outer galactoses. The 
data suggest that terminal galactose residues are in abundance 
on CEF-HA whereas the outer galactoses on MBDK-HA are 
predominantly substituted with additional sugars. These ad-
ditional sugar residues together with the highly branched 
oligosaccharides on the MBDK-HA would easily account for 
its large size. It is interesting in this regard that tri-
and tetraantennary oligosaccharides from a mouse lymphoma cell 
line frequently have an outer sequence composed of the re-
petating disaccharide [Galβ1,4GlcNAcβ1,3] (19).

When considering the ways in which these host-related 
differences in HA oligosaccharides could explain the binding 
properties of this virus, the three-dimensional structure of the 
HA (20) prompts the following speculation. An MBDK-spe-
cified oligosaccharide at the distal end of the HA might be 
expected to restrict access to the receptor-binding pocket 
located in that area and thereby reduce the affinity of the 
HA for cellular receptor. Alternatively, the size of the 
oligosaccharides on specific asparagine residues may be 
important in determining both the conformation and the sta-

bility of the HA. Since oligosaccharides cover approximately 
20% of the surface of the HA trimer (20), carbohydrate-
protein interactions within the subunits and at subunit 
faces could be critical to the proper folding of the nascent 
HA at the end of this paper. Miniprint is easily read with the aid of 
a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 8650 Rockville Pike, Bethesda, MA 20814. Request the HA document No. 85M-1406, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

...poly peptide and assembly of the biologically active trimer. It is also possible that the affinity of the HA for cellular recep-
tors could be modulated by specific outer sugar sequences or 
by modifications such as the sulfation of specific complex 
oligosaccharides (21). These possibilities, although not ruled 
out for the virus strain used here, seem unlikely. Neither attachment of sialic acid residues to the HA (18) nor removal 
of terminal sugars from the HA (22) destroys the infectivity of 
these particles. In addition, although the amount of sulfate on 
the HA can be determined by the host, the hemagglutini-
gating activity of the virus appears to be independent of the 
degree of sulfation (23).

The mechanisms proposed here are similar to those sug-
gested by Zhu and Laine (24) to explain their observations 
that placental fibronectin which contains high molecular 
weight polylactosamine carbohydrates has a lower binding 
affinity for gelatin than those forms which contain smaller 
N-linked complex carbohydrates. In addition, oligosaccharide 
size has been shown to affect the folding and conformation of 
the G protein of the San Juan strain of vesicular stomatitis 
virus (25).

This study also shows that although the host clearly deter-
mines some of the characteristics of the HA oligosaccharides, 
the HA glycoform profiles are not simple replicas of the 
cellular lectin profiles. For example, fucosylated trianten-
nary structures are present on CEF-HA subunits and are 
averently absent from the CEF glycoproteins. Whether these 
differences result from control of glycosylation by the primary 
structure of the HA protein or from selection of a subset of 
glycosylated HA molecules during assembly of the virions is 
not known.

The work presented here indicates that interaction between 
the host cell glycosylation system and the HA polypeptide 
sequence can be important in determining host range. We are 
presently investigating a mutant of the FsK virus which has 
increased host cell binding activity (8) in order to identify 
molecular changes which can broaden the host range of the 
influenza viruses.

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**Oligosaccharide Composition of an Influenza Virus HA**

**SUPPLEMENTARY MATERIAL TO:**

**OLIGOSACCHARIDE COMPOSITION OF AN INFECTIOUS VIRUS HOST-DETERMINED BINDING PROPERTIES**

**By** Carl M. Dean and Irene T. Schulze

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**EXPERIMENTAL PROCEDURES**

**Materials.** G-25 Sephadex and ConA-Sepharose were obtained from Pharmacia Fine Chemicals; lentil lectin-agarose, EPHA-agarose and L-PHA-agarose from E-Y Laboratories; 20 mM Tris, 100 mM NaCl, and 2% Triton X-100, from Sigma Chemical Co.; 0.1 M a-methyl-D-mannoside, from Calbiochem-Novabiochem Corp.; 2-2' Man(1→6)Man from Worthington Biochemical Corp.; and EN3Hance from New England Nuclear.

**Cell and Virus Growth.** MDBK cells and CEF were grown as previously described (8). Radiolabeled cells were obtained from cultures grown to confluency over a period of 2-3 days in MEM containing 10% fetal calf serum (8). A CEF-grown stock of the A/Puerto Rico/8/34 (H1N1) virus was used in all experiments. Radiolabeled viruses were purified from infected cell cultures grown in the presence of 2 µCi of [3H]deoxymannose as previously described (8).

**Glycoproteins and unpurified HA.** Infected cell monolayers were pulse-labeled at 6 hours post-infection for 1 hour at 37°C in MEM containing one-fourth the normal concentration of methionine, 2% dialyzed fetal calf serum and 50 µCi of [3H]deoxymannose at 30°C. Following a chase period of 15 minutes at 37°C in MEM containing 2% fetal calf serum, the cells were washed three times in ice-cold PBS and lysed at 4°C for 10 minutes in 0.1 M Tris-HCl (pH 7.0), 0.15 M NaCl, 0.5% Triton X-100, and 1% NP-40. Nucleotides were removed by centrifugation at 22,000 rpm for 5 minutes and the supernatant fraction saved for electrophoresis. When Tunicamycin (2 µg/ml) was used to inhibit glycosylation, it was added at 3 hours post-infection and was present throughout the chase-period. The HA was immunoprecipitated from cell lysates as previously described (11) using anti-HA monoclonal antibodies obtained from Dr. Walter Gerhard (Wistar Institute, Philadelphia, PA). 

**SOS-PAGE.** The positions of the glycoproteins were determined using serial lectin affinity chromatography to identify the minimal structural features required for the interaction of the cellular glycoproteins but not in the HA glycoproteins, do not affect the elution profiles. As effect on the lectin profiles of modifications such as sulfation or phosphorylation of complex oligosaccharides has not been documented.

**Preparation and fractionation of Glycopeptides.** [3H]Mannose-labeled, purified virus was used to test for lectin affinity chromatography was subjected to tryptic treatment (10) for 30 minutes at 37°C. This treatment removes tryptic affinity chromatography profiles similar to HA, HA, and HA subunits were then separated on SDSPAGE and dried in contact with Kodak XAR-5 film at -70°C. Fluorographs were used as templates to locate HA, and radioactive HA glycoproteins were excised from the gel and eluted in 1 ml of 0.1 M Tris-HCl (pH 8.0), containing 10 µg/ml pronase, 10 µg/ml CaCl2, and 10% Triton X-100. This procedure allowed us to fractionate up to 120 µg of TSP. Radioactivity from all columns was quantitated by fluorography in EN3Hance dried and placed in contact with Kodak XAR-5 film at -70°C. Fluorographs were used as templates to locate HA, and radioactive HA glycoproteins were excised from the gel and eluted in 1 ml of 0.1 M Tris-HCl (pH 8.0), containing 10 µg/ml pronase, 10 µg/ml CaCl2, and 10% Triton X-100. This procedure allowed us to fractionate up to 120 µg of TSP. Radioactivity from all columns was quantitated by fluorography.
When the complex glycopeptides in pool I and II from each HA source were further fractionated, the lectin profiles (Fig. 3) were highly similar to those obtained from their respective HA subunits (Fig. 2). Thus, the host-dependent differences observed with HA were again observed with NG despite the difference in the number of glycosylation sites of the two subunits.

We also generated lectin affinity profiles of the N-linked oligosaccharides obtained from both cell sources. A comparison of these lectin profiles (Fig. 4) with those from the HA subunits (Figs. 2 and 3) indicated that the N-linked complex oligosaccharides in the HA subunits were not necessarily representative of those found on the host cell glycoproteins. First, the lectin-agarose (L-PHA-agarose) in contrast to ConA-Sepharose, did not reflect that found on the glycoproteins from either cell. Essentially all of the biantennary glycopeptides in each HA subunit were found to bind to lentil lectin-agarose (L-PHA-agarose), with essentially all of the glycopeptides in pool IA bound to lentil lectin-agarose (L-PHA-agarose) but not to ConA-Sepharose. This is consistent with the N-linked complex glycoprotein structures from the cell surface glycoproteins, which lack outer galactose residues and are always found on HA subunits (Figs. 1g, 2h, and 3h). Second, fucosylated glycopeptides were found to be virtually absent from the HA subunits than on the glycoproteins from either cell. Essentially all of the biantennary glycopeptides from MDBK- and CEF-HA subunits contained high levels of fucose (36% and 31%), while only a minor fraction of these structures from the cellular glycoproteins was fucosylated (Figs. 2c and 3c). In addition, CEF-HA subunits contained fucosylated trisaccharide glycopeptides (Figs. 2i and 3i) which were essentially absent from CEF glycoproteins (Fig. 4i). Finally, since the majority (95%) of the trisaccharide and tetrasaccharide glycopeptides from the CEF-HA subunits interacted with L-PHA-agarose, they contain outer galactose and human N-acetylgalactosamine at position C-2 and C-6. Glycopeptides containing these two structural features are much less abundant on the CEF glycoproteins. This difference, although less pronounced, was also observed between the HA subunits and cellular glycoproteins from MDBK cells.

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