Carbohydrate masking of an antigenic epitope of influenza virus haemagglutinin independent of oligosaccharide size

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Comparison of the haemagglutinins (HA) of the pathogenic avian influenza viruses A/FPV/Dutch/27 (H7N7) and A/FPV/Rostock/34 (H7N1) revealed 94.7% nucleotide and 93.8% amino acid sequence homologies. Six of the seven N-glycosidic oligosaccharides of the Rostock HA are at the same positions as the six carbohydrates of the Dutch strain. The additional oligosaccharide side chain of the Rostock strain, which is of the complex type, is attached to asparagine$_{149}$ in antigenic epitope B. The accessibility of this antigenic epitope has been analysed by using rabbit antisera raised against synthetic peptides comprising amino acids 143–162. The carbohydrates of the HA of the Rostock strain have been modified (i) to truncated cores by expression in insect cells using a baculovirus vector, (ii) to oligomannosidic side chains by growth in the presence of the trimming inhibitor methyldeoxynojirimycin and (iii) to a single N-acetylglucosamine residue by removal of the oligomannosidic sugar with endo-β-N-acetylglucosaminidase H. Neither the authentic nor the modified oligosaccharides allowed antibody binding, as indicated by enzyme-linked immunosorbent assay (ELISA) and Western blot analyses. Reactivity was observed, however, after complete removal of the carbohydrate from HA of the Rostock strain by digestion with peptide-N-glycosidase F. HA of the Dutch strain was reactive without prior peptide-N-glycosidase F treatment. These results demonstrate that a single N-acetylglucosamine at asparagine$_{149}$ is sufficient to prevent recognition of the peptide epitope.

Key words: carbohydrate-mediated antigenic epitope masking/ influenza virus glycoprotein/N-linked oligosaccharides

Introduction

Glycosylated proteins are standard constituents on the surface of most enveloped viruses and are essential for the initiation of infection, i.e. for receptor binding and for fusion of viral envelopes with cellular membranes (Klenk and Rott, 1988). They are also the major surface antigens eliciting neutralizing antibodies. With most enveloped viruses, the carbohydrates are N-glycosidically bound to asparagine and the structures of the carbohydrates are different at each attachment site. Evidence has been obtained that carbohydrates are important for the proper folding and correct oligomerization of polypeptides,

which are both prerequisites for the transport of the glycoproteins from the rough endoplasmic reticulum to the place of virus budding (Gibson et al., 1980; Olden et al., 1985; Deom et al., 1986; Gething et al., 1986; Schuy et al., 1986; Pitta et al., 1989; Vidal et al., 1989; Garten et al., 1992). Studies on influenza virus haemagglutinin (HA) have shown that carbohydrates may also modulate glycoprotein functions by interfering with proteolytic activation (Kawaoka et al., 1984; Ohuchi et al., 1989) and with receptor binding (Robertson et al., 1985). Because the synthesis of N-glycosidic carbohydrate side chains by cellular enzymes follows the general pathway for this class of glycans (Kornfeld and Kornfeld, 1985), the oligosaccharides are not recognized as foreign by the immune system and are therefore not antigenic determinants by themselves, but may influence antigenicity by altering polypeptide folding or by modulating the accessibility of polypeptide epitopes to antibodies. Thus, the removal of carbohydrate residues from the sugar side chains can change the antigenic reactivity of viral glycoproteins (Van Eldik et al., 1978; Kaluza et al., 1980; Heinz et al., 1984). Furthermore, escape mutants of influenza virus have been obtained under the pressure of a monoclonal antibody that had lost an oligosaccharide in the respective antigenic epitope (Skehel et al., 1984), and the appearance or disappearance of oligosaccharide side chains has been found to occur naturally during antigenic drift of influenza viruses (Seidel et al., 1991). The acquisition of new carbohydrate side chains may therefore provide a mechanism by which the virus escapes host defence.

Since it is well known that the structures of oligosaccharide side chains of viral glycoproteins vary, depending on the glycosylation site on the polypeptide (Keil et al., 1985) and on the host cell, it was of interest to find out whether the structure and, in particular, the size of an oligosaccharide have an influence on antigenic epitope masking. We have analysed these questions in the present study on the HA of fowl plague virus (FPV). The structure of the HA oligosaccharides of the Rostock variant of this virus had been elucidated previously, and it was known that one of these side chains was attached to antigenic epitope B (Keil et al., 1985). This oligosaccharide is missing in the Dutch variant of FPV and we have analysed here the reactivity of epitope B with specific peptide antisera depending on the presence of the side chain. We have also modified the oligosaccharide by using a trimming inhibitor, by expressing the HA in insect cells and by using endoglycosidases. We have found that the antisera reacted with the epitope only when it was completely devoid of carbohydrate.

Results

Comparison of the HA of the FPV strains Dutch and Rostock

Sequence analysis reveals that the HA gene of FPV strain Dutch has the same length of 1742 nucleotides as found with FPV strain Rostock (Porter et al., 1979; Garten et al., 1985).

The HA of both strains differ in 86 nucleotides: 51 of these exchanges are silent mutations and 35 nucleotide exchanges result in 29 exchanged amino acids, six of which are double mutations (Figure 1).

The number and positions of all glycosylation sites are conserved, except for the attachment site at asparagine$_{149}$, which cannot be glycosylated with the Dutch strain because serine at position 151 has been replaced by alanine (Figure 1). This site is localized on a loop forming antigenic epitope B on the tip of the HA molecule (Figure 8). That all attachment sites on the HA of the Dutch strain are glycosylated has been shown by the controlled removal of the oligosaccharides by endoglucosaminidase H (endo H). To obtain uniform oligosaccharides, virus was grown in the presence of the trimming inhibitor N-methyl-1-deoxynojirimycin (MdN) prior to enzyme incubation (Figure 2). Five distinct HA$_1$ (large subunit of influenza virus HA) and three HA$_2$ (small subunit of influenza virus HA) bands were found after labelling with [^{35}S]methionine, indicating that HA$_1$ and HA$_2$ have four and two side chains, respectively. Figure 2 shows also that, when grown in the absence of the trimming inhibitor, HA$_1$ is largely insensitive to endo H treatment, whereas HA$_2$ has only one endo H-sensitive oligosaccharide. These results are similar to those obtained with the Rostock strain, except for an additional endo H-resistant oligosaccharide present on Rostock HA$_1$. Thus, it could be demonstrated that the Dutch HA differs in its glycosylation pattern from the Rostock HA mainly by the absence of the complex oligosaccharide at asparagine$_{149}$.

The oligosaccharide at asparagine$_{149}$ prevents antibody binding at antigenic epitope B

Since asparagine$_{149}$ is located in antigenic epitope B, it was of interest to find out if the differential glycosylation observed in this position with strains Rostock and Dutch had an influence on the reactivity of the epitope with specific antibodies. For this purpose, we have raised antisera in rabbits against peptides homologous to amino acids 143−162 of both strains. These sequences, differing only in two positions, one of which is essential for the glycosylation site (Figure 1), form a linear epitope at the tip of the HA spike (Figure 3).

Prior to examining the binding abilities of the anti-peptide antisera to native virions, it was necessary to demonstrate that the two rabbits immunized with either Dutch or Rostock peptide 143−162 coupled to keyhole limpet haemocyanin (KLH) produced a specific anti-peptide response. Antisera obtained from the two rabbits bound both peptides at comparable levels, whereas little binding to a control peptide (insulin) was observed, as determined by enzyme-linked immunosorbent assay (ELISA) (Figure 4B). The titre of the anti-Dutch peptide antiserum was ~1:50 000, expressed as the dilution required to give 50% maximum optical density reading at 492 nm, and that of the anti-Rostock peptide antiserum 1:6000, irrespective of the peptide used for coating the plates. Thus, both antisera developed a specific anti-peptide response and the differences within the amino acid sequences did not influence antigenic reactivities.

When the peptide antisera were tested for their binding capacities to virions, they were coated to the solid phase under mild conditions to ascertain that the HA is present in undenatured form. Although the response of the antisera to virus was less efficient than their response to the peptide antigens, the results show that both antisera recognize virions of the Dutch strain much better than virions of the Rostock strain (Figure 4A).

We then analysed the reactivity of the anti-Dutch peptide antiserum with both HA in an immunoblot assay, and assayed the effect of glycosidase-mediated carbohydrate removal from HA on immune recognition. As shown in Figure 5, the HA$_1$ subunit of the Dutch strain was immunostained, whereas the anti-peptide antiserum failed to recognize that of the Rostock strain. However, after complete removal of the oligosaccharide by treatment with peptide N-glycosidase F (glyco F), HA$_1$ of the Rostock strain could be detected in the immune reaction. On the other hand, apart from reducing the electrophoretic mobility, deglycosylation had no effect on the immune reactivity of Dutch HA$_1$. As indicated by the immunoblot with anti-virus antiserum also shown in Figure 5, equivalent amounts of HA$_1$ of both strains have been used in this experiment. These observations indicate that the complex oligosaccharide attached to asparagine$_{149}$ prevents the binding of site-specific antibodies to the HA.

Epitope shielding by modified oligosaccharide side chains

The complex oligosaccharides found on HA from vertebrate hosts are replaced by small truncated side chains of the structures Man$_3$GlcNAc$_2$ and Man$_3$[Fuc]GlcNAc$_2$ when the HA is expressed in insect cells (Kuroda *et al.*, 1990). To test the effect of a trimannosyl core at asparagine$_{149}$ on the binding of the anti-peptide antisera, we have expressed the HA of the Rostock strain in SF9 cells using a baculovirus vector. Comparative immunoblots of Rostock HA obtained from insect cells, and from Rostock and Dutch HA originating from chick embryo cells (CEC), are shown in Figure 6.

The results show that the immunoreactivity of the Rostock strain is enhanced after carbohydrate removal by glyco F, regardless of whether the HA has been derived from SF9 cells or CEC. In contrast, Dutch HA$_1$ obtained from CEC reacted with the antiserum without prior glyco F treatment. Thus, the presumptive trimannosyl core (Kuroda *et al.*, 1990) interferes with antiserum binding at asparagine$_{149}$, as does the complex oligosaccharide side chain.

When FPV is grown in the presence of the trimming inhibitor (MdN), HA is assembled into trimers, transported to the cell surface and incorporated into virus particles, but its carbohydrate side chains persist in the unprocessed oligomannosidic form (Romero *et al.*, 1983). These oligosaccharides, among which the structures Man$_{6-9}$ GlcNAc$_2$ prevail, are susceptible to cleavage by endo H which leaves the proximal N-acetylglucosamine residue bound to the polypeptide. It was therefore possible to analyse the effects of oligomannosidic side chains and of a single N-acetylglucosamine on epitope shielding.

FPV strains Dutch and Rostock were grown in CEC in the absence or presence of the trimming inhibitor. Purified virus grown in the presence of MdN was treated with endo H and, for complete deglycosylation, virus grown in the absence of MdN was incubated with glyco F. Enzyme-treated or untreated virus samples were subjected to SDS−PAGE, and HA was visualized by immunoblotting with anti-Dutch peptide antiserum (Figure 7). The results obtained with Rostock strain show that anti-peptide antiserum recognized HA grown in the presence of the trimming inhibitor MdN neither before nor after endo H treatment, but did recognize HA after complete carbohydrate removal by glyco F. In contrast, irrespective of
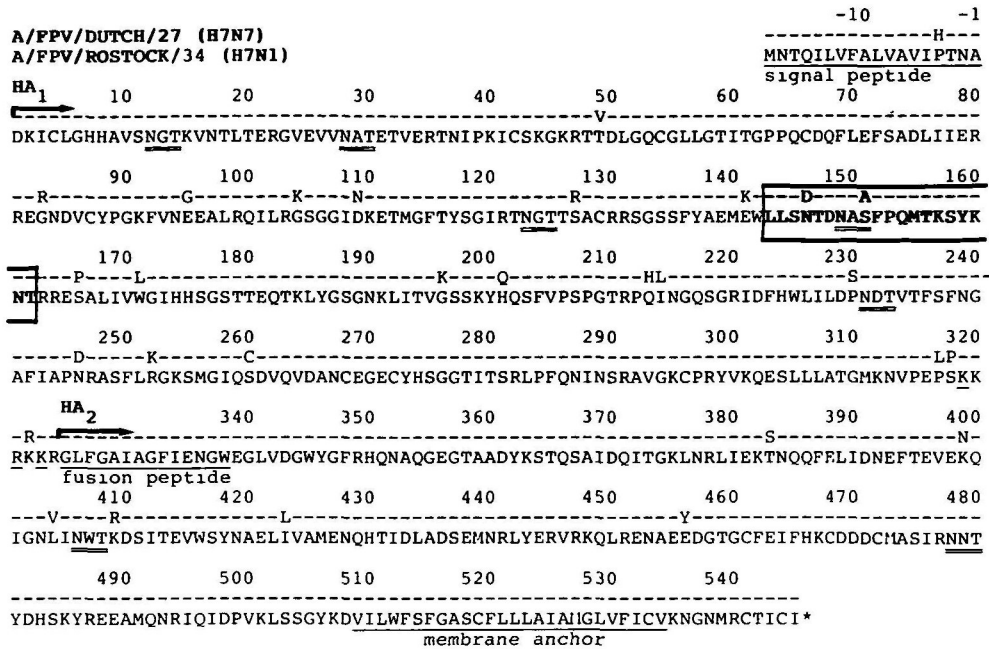
H7 HEMAGGLUTININS OF THE INFLUENZA VIRUS STRAINS

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                                                      -10        -1
                                                 --------------H---
A/FPV/DUTCH/27 (H7N7)                            MNTQILVFALVAVIPTNA
A/FPV/ROSTOCK/34 (H7N1)                          signal peptide
HA                                                          70        80
   1      10        20        30        40        50        60        70        80
  ──►──────────────────────────────────────────V─────────────────────────────
  DKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPKICSKGKRTTDLGQCGLLGTITGPPQCDQFLEFSADLIIER

          90        100       110       120       130       140       150       160
  --R-----------G---------K----N---------------R------------K--┌──-D---A---------┐
  REGNDVCYPGKFVNEEALRQILRGSGGIDKETMGFTYSGIRTNGTTSACRRSGSSFYAEMEWLLSNTDNASPPQMTKSYK

          170       180       190       200       210       220       230       240
  ┌─┤---P----L----------------------K----Q----------HL---------------S----------
  NTRRESALIVWGIHHSGSTTEQTKLYGSGNKLITVGSSKYHQSFVPSPGTRPQINGQSGRIDFHWLILDPNDTVTFSFNG

          250       260       270       280       290       300       310       320
  -----D-----K-------C----------------------------------------------------LP--
  AFIAPNRASFLRGKSMGIQSDVQVDANCEGECYHSGGTITSRLPFQNINSRAVGKCPRYVKQESLLLATGMKNVPEPSKK

      HA
        2     340       350       360       370       380       390       400
  -R--──►─────────────────────────────────────────────────S---------------N-
  RKKRGLFGAIAGFIENGWEGLVDGWYGFRHQNAQGEGTAADYKSTQSAIDQITGKLNRLIEKTNQQFFLIDNEFTEVEKQ
      fusion peptide
          410       420       430       440       450       460       470       480
  ---V----R------------L------------------------------Y------------------------
  IGNLINWTKDSITEVWSYNAELIVAMENQHTIDLADSEMNRLYERVRKQLRENAEEDGTGCFEIFHKCDDDCMASIRNNT

          490       500       510       520       530       540
  --------------------------------------------------------------------
  YDHSKYREEAMQNRIQIDPVKLSSGYKDVILWFSFGASCFLLLAIAIIGLVFICVKNGNMRCTICI*
                              membrane anchor
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Fig. 1. Amino acid sequences of the HA of the strains A/FPV/Rostock/34 (H7N1) and A/FPV/Dutch/27 (H7N7). The hydrophobic domains (signal, fusion and membrane anchor peptides) are underlined, the attachment sites for N-glycosylated carbohydrates are indicated by double lines and the amino acids connecting HA₁ with HA₂ by a dashed line. The sequences of the synthetic peptides used for immunization are framed.
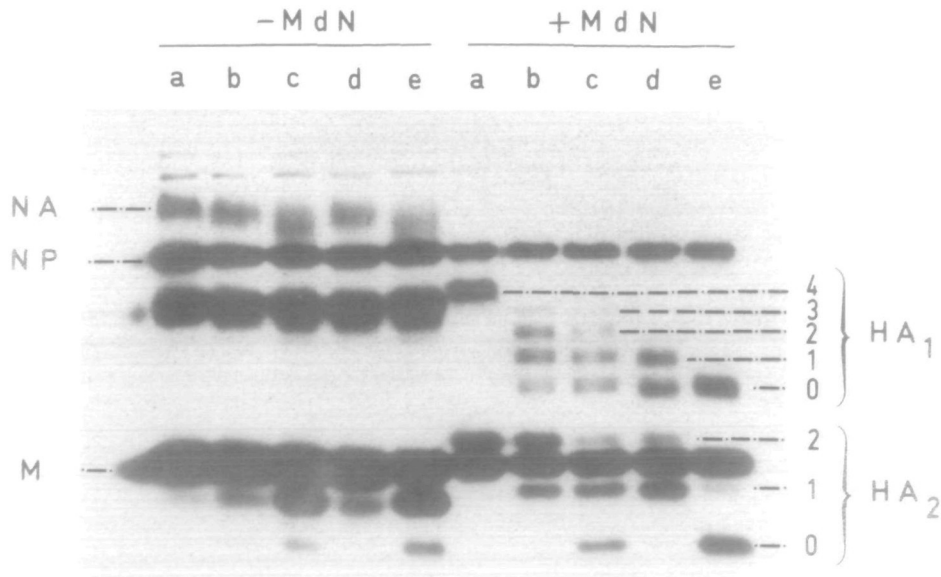

Fig. 2. Controlled removal of oligosaccharides from the HA of strain A/FPV/Dutch/27. Virus labelled with [35S]methionine was grown in the absence (−MdN) or in the presence (+MdN) of the trimming inhibitor. After purification, native or denatured (96°C, 2 min, 0.2% SDS and 1% mercaptoethanol) virus was incubated with endo H. (a) Control virus not incubated with endo H. (b) Virus incubated with endo H for 15 min. (c) Denatured virus incubated with endo H for 15 min. (d) Native virus incubated with endo H for 3 h. (e) Denatured virus incubated with endo H for 3 h. The viral proteins were separated by SDS−PAGE and analysed by fluorography. The HA bands (HA₁ and HA₂) with the number of carbohydrate side chains are indicated. Neuraminidase (NA), nucleoprotein (NP) and matrix protein (M) of influenza virus are also shown.
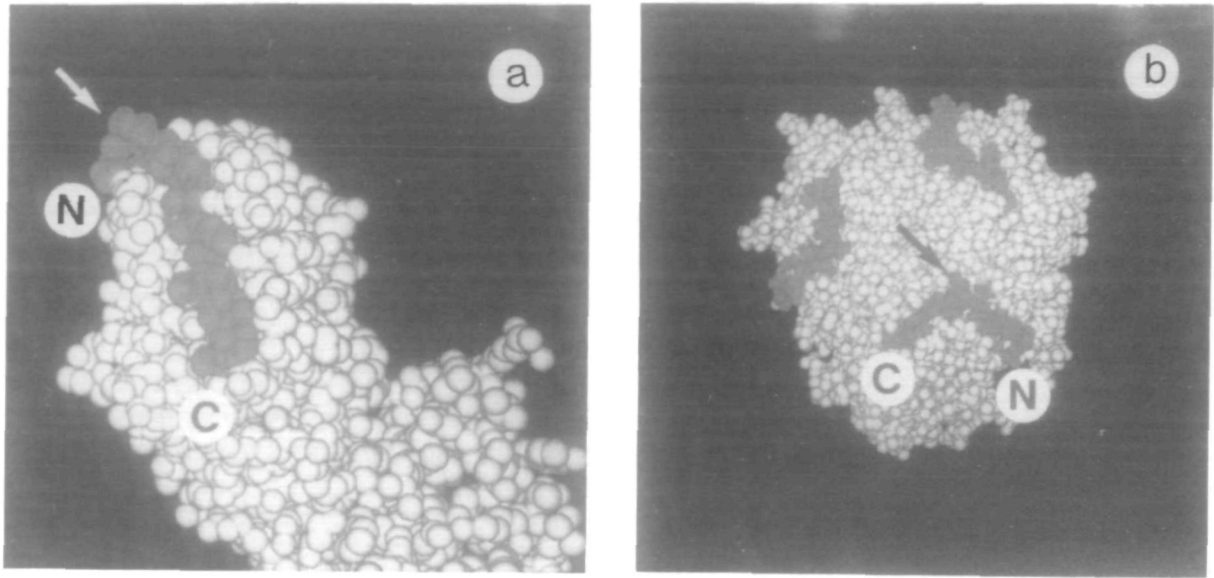
Fig. 3. The location of the peptide segment 143—162 within the globular domain of the HA. (a) HA monomer, (b) HA trimer. The oligosaccharide attachment site at asparagine$_{149}$ in the HA of strain Rostock (arrow) and the N- and C-terminal orientation of the peptide segment used for immunization (dark atoms) are indicated.
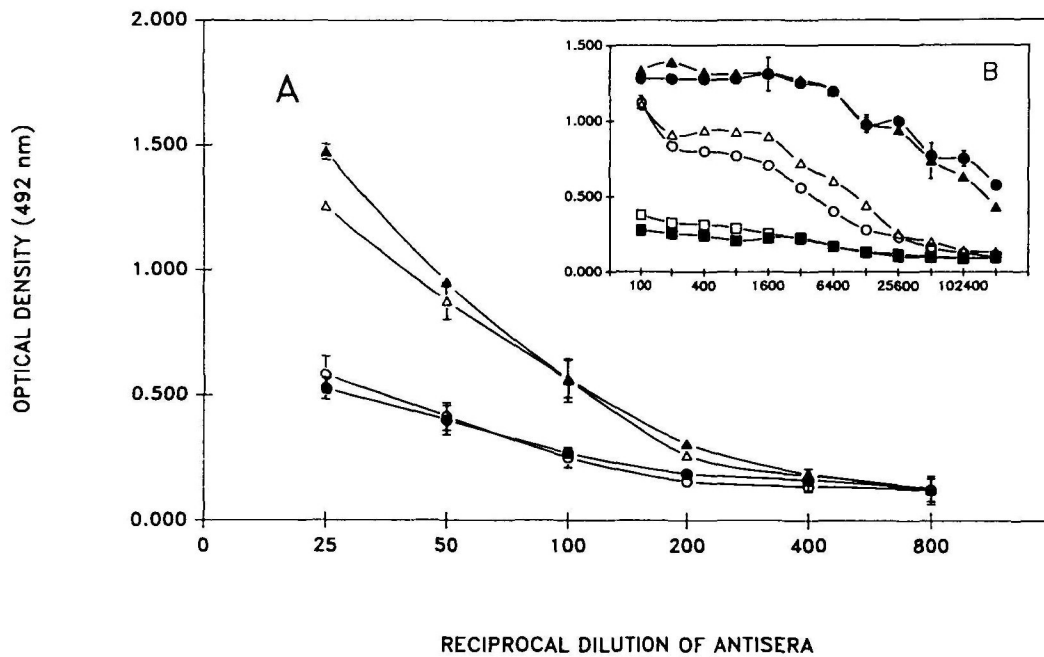

RECIPROCAL DILUTION OF ANTISERA

Fig. 4. Specificity of peptide antisera. Antisera raised in rabbits against synthetic peptides linked to KLH were analysed at the dilutions indicated in an ELISA for their reactivity with virions and peptides. (A) Reactivity with virions. FPV/Dutch virions (triangles) and FPV/Rostock virions (circles) were used as antigens. The binding curves of the antisera raised against the Dutch peptide (filled symbols) and against the Rostock peptide (open symbols) are shown. When preimmune sera were used, only background binding was observed at dilutions of 1:25 (data not shown). (B) Reactivity with peptides. The following peptides were used to coat the solid phase: Dutch peptide (triangles), Rostock peptide (circles) and insulin (rectangles) as control. The binding curves of the antisera raised against the Dutch peptide (filled symbols) and against the Rostock peptide (open symbols) are shown.
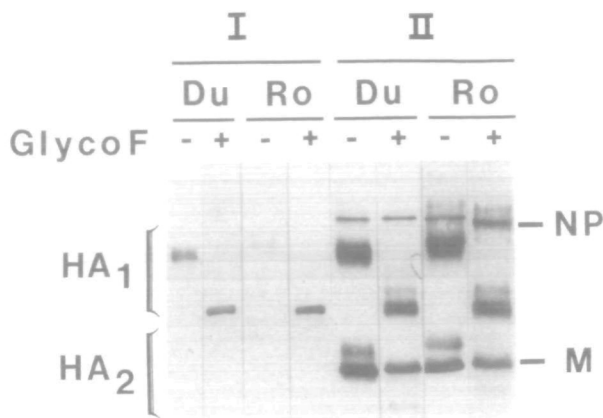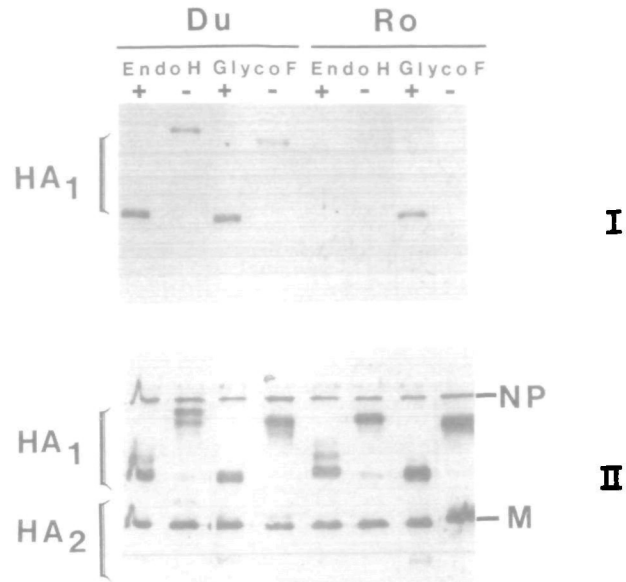
Fig. 5. Effect of HA deglycosylation with glyco F on the reactivity with the anti-Dutch peptide antiserum. Strains A/FPV/Rostock/34 (Ro) and A/FPV/Dutch/27 (Du) were grown in embryonated eggs. The immune reactivity was assessed against purified virus, which was either deglycosylated (+) by glyco F or untreated (−), subjected to SDS−PAGE and subsequently blotted onto nitrocellulose. I Reactivity of anti-Dutch-peptide antiserum with HA_1. II Reactivity of antiserum raised against virus A/FPV/Rostock/34 with HA_1, HA_2, NP and M.
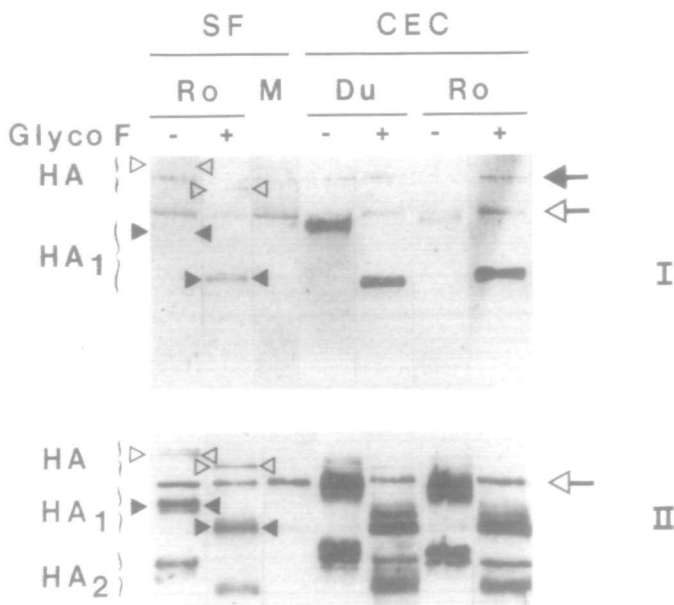

Fig. 6. Reactivity of anti-Dutch peptide antiserum with HA expressed in insect cells. Rostock HA (Ro) expressed in *S.frugiperda* (SF) cells, and HA of strains Rostock (Ro) and Dutch (Du) obtained from fowl plague virions, grown in CEC, were immunoprecipitated using the monoclonal antibody HC2 before treatment with glyco F. Treated (+) and untreated (−) samples of immunoprecipitated HA were subjected to SDS−PAGE, transferred to nitrocellulose and incubated with anti-Dutch peptide antiserum (I) or antiserum raised against influenza virus A/FPV/Rostock/34 (II).
SF cells infected with wild-type baculovirus AcNPV are also shown (M). Uncleaved HA is indicated by open triangles, subunit HA_1 by filled-in triangles. Two non-specific bands are marked by open and filled arrows.

treatment with MdN, endo H or glyco F, HA of the Dutch strain was always recognized. Similar results were obtained when an anti-Rostock antiserum was used for immunoblotting (data not shown). A control blot with anti-FPV antiserum also shown in Figure 7 demonstrates that equal amounts of viral

Fig. 7. Effect of HA deglycosylation with endo H on the reactivity with the anti-Dutch peptide antiserum. Strains A/FPV/Rostock/34 (Ro) and A/FPV/Dutch/27 (Du) were grown in CEC in the presence and absence of the trimming inhibitor MdN. Purified virus grown in the presence of MdN was incubated with (+) or without (−) endo H. Purified virus grown in the absence of MdN was incubated with (+) or without (−) glyco F. Samples were subsequently analysed with the anti-peptide antiserum (I) and as a control with antibodies directed against influenza virus FPV/Rostock/34 (II).

proteins had been used in all lanes. These data indicate that a single *N*-acetylglucosamine residue at asparagine$_{149}$ prevents the binding of the anti-peptide antibodies, as is the case with carbohydrates of the complex and the oligomannosidic type, and with the truncated oligosaccharide cores obtained in invertebrate cells.

Discussion

The HA of the Dutch and Rostock strains of FPV are closely related in their structural and biological properties. They have 94.7% homology at the nucleotide level and 93.8% homology at the amino acid level. Homology includes glycosylation sites at asparagine residues 12, 28, 123, 231 and 478, which all contain complex oligosaccharides, as well as an attachment site at asparagine$_{406}$ that is utilized by an oligomannosidic side chain. The Rostock HA has a seventh glycosylation site at asparagine$_{149}$ which is occupied by a complex side chain. This oligosaccharide is missing in the Dutch strain, in which the consensus sequence Asn-Ala-Ser has been altered to Asn-Ala-Ala. As pointed out above, it has been shown before that variable oligosaccharides, such as the one at asparagine$_{149}$, mask antigenic peptide epitopes and thus enable the virus to escape immune defence by specific antibodies (Alexander and Elder, 1984; Skehel *et al.*, 1984; Caust *et al.*, 1987; Davis *et al.*, 1987). This concept is supported by the observation that the epitope B-specific antisera used in the present study recognize the HA of the Dutch, but not of the Rostock strain. It was also of interest to find out if structure and size of the oligo-saccharide have an effect on its masking capacity. We have therefore extended our studies by modifying the oligosaccharide
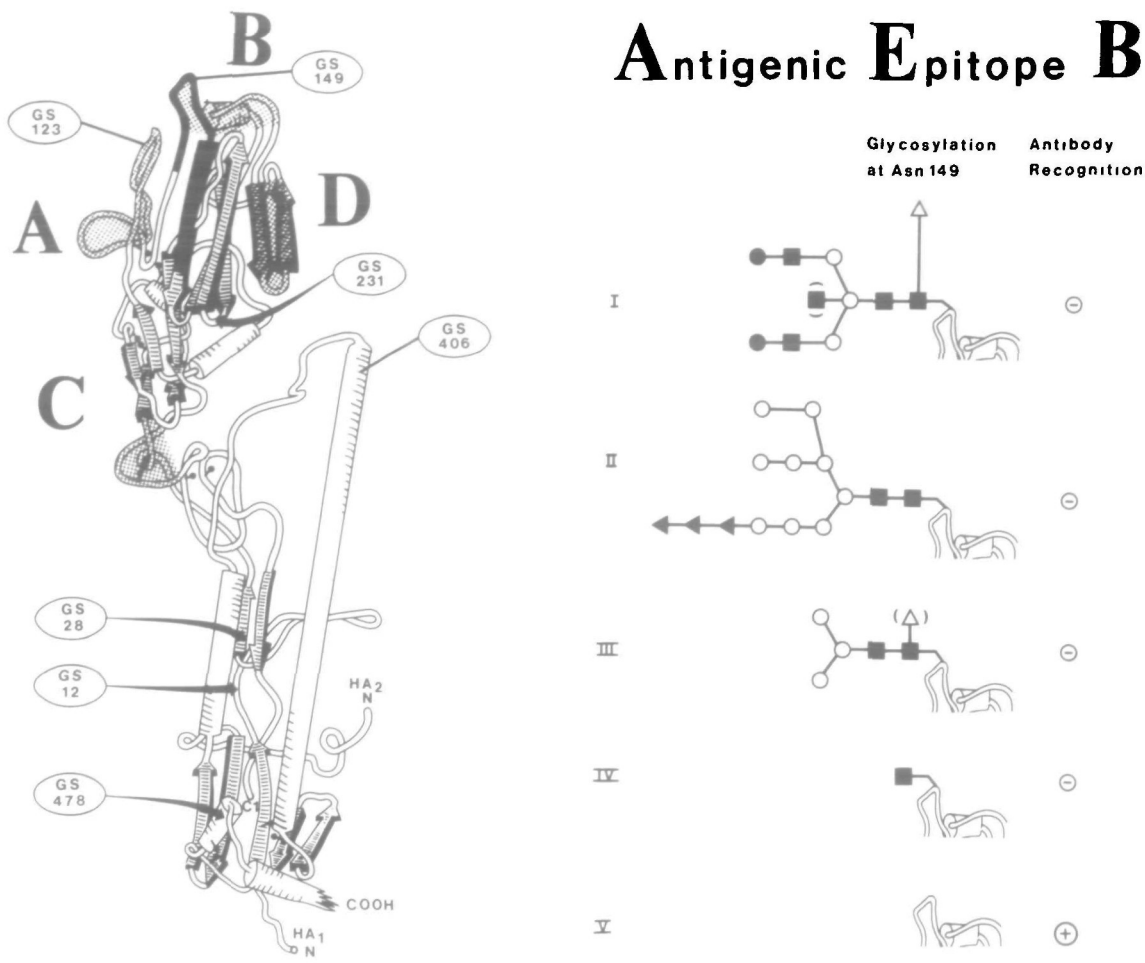
Fig. 8. Influence of different oligosaccharide structures on antibody binding to antigenic epitope B. The three-dimensional structure of the H3 HA molecule with the antigenic epitopes A, B, C and D (Wiley *et al.*, 1981; Wilson *et al.*, 1981) and the glycosylation sites (GS) as determined for the HA of A/FPV/Rostock/34 (H7N1) (Keil *et al.*, 1985) are shown on the left. The peptide segment to which the antisera are directed is drawn in black. Recognition of antigenic epitope B by the site-specific antisera, as depending on the structure of the oligosaccharide attached to asparagine$_{149}$, is shown on the right. The following oligosaccharide side chains have been analysed: I, complex biantennary oligosaccharide present on Rostock HA derived from chick embryo cells; II, oligomannosidic found on Rostock HA grown in the presence of the trimming inhibitor MdN; III, truncated oligosaccharide present on Rostock HA expressed in insect cells; IV, *N*-acetylglucosamine retained after endo H treatment of structure III; V, complete absence of carbohydrate observed on Rostock HA after glyco F treatment or on Dutch HA; (■) *N*-acetylglucosamine; (○) mannose; (●) galactose; (△) fucose; (▲) glucose.

at position asparagine$_{149}$ of the Rostock HA. The results, which are summarized in Figure 8, indicate that the structure of the oligosaccharide is not important and that a single *N*-acetylglucosamine residue is sufficient for antigen masking.

Antisera directed against epitope B were prepared by immunizing rabbits with a site-specific synthetic peptide. It was known from a previous study, employing antisera against a series of subtype H3-specific peptides, that the sole requirement for the generation of anti-peptide antibodies reactive with native molecules is the presentation of the peptide on the surface of the proteins (Green *et al.*, 1982). As shown in Figure 3, peptide 143–162 fulfils this requirement. The model also reveals that this peptide has a linear configuration on the HA surface. It is therefore not surprising that antibodies raised against both synthetic peptides are also recognized by native HA. Furthermore, it is reasonable to assume that unfolding of the HA, occurring during Western blot analysis, does not drastically alter the antigenicity of this epitope. Since the same results were obtained with antisera raised against the Dutch and Rostock specific peptides, it is clear that only the variations in glycosyl-

ation, but not the amino acid exchanges at positions 146 and 151, account for the differential reactivities of the antisera with both HA.

Because viruses use the glycosylation machinery of the cell for carbohydrate synthesis, the glycosylation patterns of viral glycoproteins are determined to a large extent by the host. Such host-specific changes in glycosylation are quite dramatic when a glycoprotein of vertebrate origin is expressed in insect cells by a baculovirus vector. Under these conditions, the complex oligosaccharides present on the FPV HA derived from vertebrate cells are replaced by truncated side chains (Kuroda *et al.*, 1990). Because of the reduced size of these side chains, only 10% of the HA surface is covered with carbohydrate, as compared to 20% on HA derived from mammalian cells. It was therefore interesting to see in the present study that, at least for epitope B, antibody accessibility was not enhanced by reduction of oligosaccharide size. Even more surprisingly, antibody reactivity was abolished by a single glucosamine residue. An explanation for these unexpected results may be provided by the information available on the chemistry of antibody binding.

The main factors supporting the antigenicity of a polypeptide are high mobility, low packing density, convex shape, and significant negative electrostatic potential at the molecular surface (Geysen et al., 1987). Our data suggest that two of these factors, the electrostatic potential and the flexibility of the polypeptide chain, are affected by the variations in glycosylation. The immunogenic loop of epitope B of the Rostock HA contains three charged amino acids. Two of them are localized close to the C-terminus of the synthetic peptide and may play a minor role in eliciting an immune response because the peptide was C-terminally coupled to the carrier protein for immunization, whereas the majority of antibodies obtained from rabbits are assumed to be directed against the N-termini of synthetic peptides (Dyrberg and Oldstone, 1986). However, the third charged amino acid, aspartate$_{148}$, appears to be directly affected by the carbohydrate. Since it is immediately adjacent to the glycosylation site at asparagine$_{149}$, it is not difficult to understand that a truncated oligosaccharide core or even a single N-acetylglucosamine is sufficient to mask the charged residue. Only a complete lack of carbohydrate would then allow antibody—antigen complex formation at an increased rate by promoting facilitated diffusion and stabilizing a final antigen—antibody contact. The second factor interfering with the antigenicity of the underlying peptide appears to be the relative rigidity of the trimannosyl core and of the single N-acetylglucosamine residue, which are both less flexible than the ordinary biantennary complex oligosaccharide (Montreuil, 1984). Rigidity of the carbohydrate might then prevent side chain rearrangement of the local amino acids which is assumed to be necessary for antibody binding to a protein. Finally, when compared to the regular biantennary complex oligosaccharide, the partially fucosylated trimannosyl core has an increased hydrophobicity (Montreuil, 1984). Hydrophobicity, well known to be incompatible with antigenicity, may therefore be another factor contributing to diminished antibody binding. Thus, there are at least three mechanisms that could explain why carbohydrate side chains retain their capacity to mask antigenic epitopes even when they are drastically reduced in size. It therefore appears that steric hindrance may not be the only mechanism by which carbohydrates modulate the accessibility of a peptide epitope to antibody, but that they also accomplish this by imposing conformational changes on the protein structure.

Materials and methods

Propagation and purification of virus

The Dutch and Rostock strains of FPV [A/FPV/Dutch/27(H7N7) and A/FPV/Rostock/34 (H7N1)] were grown in 11-day-old embryonated hen eggs and subsequently purified by sucrose gradient centrifugation after adsorption to and elution from chicken erythrocytes. In some experiments, virus was propagated in primary chick fibroblasts in the absence or presence of MdN (2 mM) (Rott et al., 1984) and purified by equilibrium centrifugation in sucrose density gradients. Virus was metabolically radiolabelled with [^{35}S]methionine (10 μCi/ml). The recombinant Autographa californica nuclear polyhedrosis virus (AcNPV-HA) was propagated in Spodoptera frugiperda (SF9) cells (Kuroda et al., 1986).

Preparation of viral RNA and nucleotide sequencing

Viral RNA was obtained from egg-grown FPV, Dutch strain. The HA gene was analysed by the primer extension method (Daniels et al., 1983; Schuy et al., 1986) using synthetic oligonucleotide primers complementary to the nucleotide sequences 4–17, 205–215, 427–437, 573–583, 721–730, 996–1005, 1315–1323, 1444–1457 and 1597–1607 of the HA gene of FPV (Porter et al., 1979; Garten et al., 1985). Computer-assisted sequence analyses were performed using the microGenie system (Beckmann, Palo Alto, CA).

Synthetic peptide antigens and anti-peptide antibodies

Peptides comprising amino acids 143–162 of the FPV HA were synthesized by the solid phase method (Merrifield, 1963). Peptide (15 mg) was incubated with 15 mg of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Sigma, Deisenhofen, FRG) for 20 min at 0°C in 200 μl 1 mM HCl (pH 3.0). KLH (30 mg; Calbiochem, Frankfurt/Main, FRG) solubilized in water was mixed with the activated peptide in a final volume of 1 ml, adjusted to pH 9.0 by the addition of triethylamine and the mixture was stirred at 20°C for 3 h. The C-terminal KLH-coupled peptide was desalted by chromatography through a prepacked Sephadex G-25 column (Pharmacia, Freiburg, FRG) which was equilibrated with phosphate-buffered saline (PBS). Amounts of 5 mg KLH—peptide conjugates were mixed with complete Freund's adjuvant and s.c. injected into rabbits. Injections were repeated twice at intervals of 4 weeks. The rabbits were bled 4 weeks after the last booster injection.

ELISA

Virus was plated at 100 μl/well of 0.5 μg protein/ml in PBS (calcium and magnesium free) and incubated overnight at 4°C. Using radioactively labelled virions, equal amounts (~65%) of both strains were found to be absorbed to the plastic. Synthetic peptides, as solid-phase substrates, were prepared by drying 0.5 μg peptide in distilled water on 96-well plates (Nunc Immulon, Wiesbaden, FRG) overnight at 37°C (Vaux et al., 1988). Non-specific sites were blocked by preincubation with PBS containing 0.05% Tween 20 and 0.2% bovine serum albumin (BSA) for 1 h at 37°C. For detection, biotin—donkey antibody to rabbit Ig and avidin-conjugated horseradish peroxidase (Amersham-Buchler, Braunschweig, FRG), each at dilutions of 1:600 in blocking solution, were used. Peroxidase activity was determined with o-phenylenediamine (Abbott, Wiesbaden, FRG) as substrate. The binding of the rabbit antisera was quantitated spectrophotometrically at 495 nm.

Digestion with endo H and glyco F

For controlled removal of oligosaccharides, labelled virus samples [20 haemagglutinating units (HAU)] were incubated with endo H [(EC 3 2.1.96), Streptomyces plicatus recombinant from Escherichia coli] (0.5 mU) (Boehringer Mannheim, FRG) in 20 μl Tris–HCl buffer (0.1 M, pH 6.8) at 37°C or had been denatured by boiling for 2 min in the presence of 0.2% SDS and 1% mercaptoethanol prior to enzyme incubation (Rott et al., 1984). For the other deglycosylation experiments, virus samples or immunoprecipitated HA (Kuroda et al., 1989) were suspended in 20 μl sodium acetate buffer (0.1 M, pH 7.0; 50 mM EDTA) containing 0.1% SDS, 0.5% mercaptoethanol and 0.5% n-octylglycoside. After boiling for 5 min, samples were incubated for 15 min at 37°C with endo H (0 2 mU) and glyco F (40 mU) [(EC 3.2.2.18), from Flavobacteriun meningosepticum] (Boehringer Mannheim, FRG), respectively. The virus proteins of the immunoprecipitated samples were clarified by centrifugation prior to enzyme incubation.

Immunoblotting

Samples were analysed on 12% gels by SDS–PAGE (Laemmli, 1970) and subsequently blotted onto nitrocellulose (Kyhse-Andersen, 1984). The blots were visualized with peptide or virus-specific rabbit antisera which were used at a dilution of 1:500. After washing, the blots were incubated with biotinylated donkey antibody to rabbit immunoglobulins (Amersham-Buchler, Braunschweig, FRG). After incubation with streptavidin—peroxidase complex (Amersham-Buchler), the blots were developed with 4-chloro-1-naphthol (Sigma, Deisenhofen, FRG) and H$_2$O$_2$.

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Abbreviations

CEC, chick embryo cells; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; endo H, endo-β-N-acetylglucosaminidase H; FPV, fowl plague virus; glyco F, peptide N-glycosidase F; HA$_1$, haemagglutinin of influenza virus, large subunit; HA$_2$, haemagglutinin of

influenza virus, small subunit; HAU, haemagglutinating units; KLH, keyhole limpet haemocyanin; M, matrix protein of influenza virus; MdN, methyldeoxynojirimycin; NP, nucleoprotein of influenza virus; PBS, phosphate-buffered saline; SF9, *Spodoptera frugiperda* cell line 9.

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