Operational and Topological Analyses of Antigenic Sites on Influenza C Virus Glycoprotein and Their Dependence on Glycosylation

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SUMMARY

In our previous study, seven monoclonal antibodies specific for influenza C virus glycoprotein (gp88) were prepared and tentatively classified into two groups: group A (J14, J9, Q5, K16) has neutralization activity whereas group B (S16, J6, J15) does not. These antibodies were used to analyse the antigenic structure of gp88 and to examine the effect of glycosylation on the antigenicity of the glycoprotein. Operational analysis with a panel of antigenic variants selected with each of the group A antibodies identified two non-overlapping antigenic sites on the gp88 molecules, site A-1 recognized by J14, J9 and Q5 and site A-2 by K16. Sites A-1 and A-2 were shown, however, to be topographically overlapping by competitive binding assays. Competitive binding analysis with group B antibodies identified two additional non-overlapping antigenic sites, site B-1 recognized by S16 and site B-2 by J6 and J15. It was found in radioimmunoprecipitation experiments that antibodies to sites B-1 and B-2 were reactive not only with gp88 but with its non-glycosylated form (T76) synthesized in the presence of tunicamycin. Antibodies to sites A-1 and A-2, in contrast, immunoprecipitated the T76 polypeptide in only trace amounts or not at all. Additionally, Western blot analysis showed that denatured gp88 blotted on nitrocellulose was reactive with antibodies to sites B-1 and B-2 but not with those to sites A-1 and A-2. These observations suggest that glycosylation of gp88 selectively influences the integrity of antigenic sites A-1 and A-2 which are composed of conformation-dependent epitopes.

INTRODUCTION

Influenza C virus, like influenza A and B viruses, has three biological activities, haemagglutinating (HA) activity, receptor-destroying enzyme (RDE) and virus-mediated membrane fusion. It is well established that influenza A and B viruses possess two distinct surface glycoproteins, haemagglutinin and neuraminidase, the three biological activities being segregated on these two glycoproteins. In contrast, only one single glycoprotein spike, designated gp88, has been detected in influenza C virions (Herrler et al., 1979, 1981), and it is generally agreed that all three biological activities reside in this glycoprotein. A stretch of hydrophobic amino acid residues, homologous in its sequence to the N-terminal portion of HA2 of influenza A and B viruses, was shown to exist at the N terminus of a smaller subunit (gp30) of gp88 and it was suggested that this is involved in membrane fusion (Herrler et al., 1981; Pfeifer & Compans, 1984; Nakada et al., 1984). No information is available, however, about the location of HA and RDE activities in the gp88 molecule.

Influenza C virus causes a mild upper respiratory illness in humans (Katagiri et al., 1983) and shows a world-wide distribution (Chakraverty, 1978; Jennings, 1968; O'Callaghan et al., 1980; Homma et al., 1982). Previous seroepidemiological studies suggested that influenza C virus is antigenically much more stable than influenza A and B viruses (Chakraverty, 1978; Meier-Ewert et al., 1981; Kawamura et al., 1986). Sequence analysis as well as oligonucleotide mapping
of the RNA genomes of various influenza C isolates also suggested less variation than has been observed for the other types of influenza virus (Meier-Ewert et al., 1981; Guo & Desselberger, 1984; Buonagurio et al., 1985; Kawamura et al., 1986). The reason for the marked antigenic and genetic stability of influenza C virus is not understood as yet.

To obtain more detailed information about the topographical location of the biologically active sites of gp88 and about the nature of antigenic variation of this glycoprotein, we have used seven monoclonal antibodies previously prepared against the gp88 glycoprotein of the C/Ann Arbor/1/50 strain. On the basis of their reactivity patterns in haemagglutination inhibition (HI), haemolysis inhibition (HLI) and neutralization tests, these antibodies were tentatively classified into two groups, A and B (Sugawara et al., 1986). Four antibodies of group A (J14, J9, Q5, K16) all possessed HI, HLI and neutralization activities whereas three antibodies of group B (S16, J6, J15) had none of these activities. Among the antibodies of group A, K16 was unique in that its HI activity was extremely low while the HLI and neutralization activities were comparable with those of the other antibodies. One of the aims of the present study was to delineate the antigenic sites recognized by these antibodies on the gp88 molecule. For this purpose, antigenic variants selected with group A monoclonal antibodies were examined for their reactivity with each of seven antibodies in HI and ELISA, and topological relationships among the antigenic sites were analysed by means of competitive binding assays.

In our previous study, a non-glycosylated precursor of gp88 was identified in infected cells treated with tunicamycin (TM) and was designated T76 (Hongo et al., 1986a). A comparison of the relative antigenicity of gp88 and T76 of the C/Ann Arbor/1/50 strain with three monoclonal antibodies (Q5, S16, J6) demonstrated that glycosylation was essential for the formation of the epitope recognized by one of the antibodies, Q5 (Hongo et al., 1986b). In this study, to extend these observations, we analysed the antigenic properties of the gp88 and T76 proteins obtained from each of 14 different influenza C strains by radioimmunoprecipitation (RIP) using all seven monoclonal antibodies described above. The results reveal that carbohydrate addition is specifically required for the integrity of the epitopes recognized by group A antibodies.

METHODS

Cells and viruses. The MDCK line of canine kidney cells and the LLC-MK2 line of monkey kidney cells were grown in Eagle's MEM containing 10% bovine serum. Stocks of the C/Ann Arbor/1/50 strain as well as of representative influenza C isolates (Table 4) were grown in the amniotic cavity of embryonated hen's eggs as described previously (Yokota et al., 1983). Growth of influenza C virions in MDCK cells and their purification are described elsewhere (Sugawara et al., 1981). For purification of egg-grown virions, the amniotic harvests were clarified by low speed centrifugation, and the virions were pelleted at 33000 r.p.m. for 60 min in an International SB-283 rotor. The pellets were suspended in phosphate-buffered saline (PBS) lacking Ca2+ and Mg2+ pH 7.2 and then purified further by banding in a potassium tartrate gradient as described by Compans et al. (1970).

Monoclonal antibodies. The preparation of monoclonal antibodies against the gp88 glycoprotein of the C/Ann Arbor/1/50 strain grown in MDCK cells has been described previously (Hongo et al., 1986b).

Selection of antigenic variants and estimation of frequency of the occurrence of antigenic variants. Serial 10-fold dilutions of the parent virus (10^4 p.f.u./ml), cloned by plaquing on LLC-MK2 cells and then grown in embryonated eggs, were mixed with an equal volume of 1:10 dilution of the ascites containing monoclonal antibody. After incubation for 30 min at room temperature, the mixture was inoculated onto a monolayer of LLC-MK2 cells in 35 mm Petri dishes, and the virus that escaped neutralization was allowed to grow under the agar overlay medium as described by Nerome et al. (1979). Ten or 11 days later the plaques were picked, treated again with 1:10 diluted ascitic fluid and then plaqued again on LLC-MK2 cells. These viruses were used for analysis after growth in embryonated eggs. The viruses cloned in this way were all found to have greatly decreased reactivity with the monoclonal antibody used for selection, confirming that they are antigenic variants. The frequency of the occurrence of antigenic variants was determined by comparing the plaque titre of parent virus treated with individual monoclonal antibodies with that of the virus treated with control ascitic fluid.

Serological assays. HI titrations were performed as described previously (Katagiri et al., 1983). ELISA was done according to the method of Kida et al. (1982), using either purified MDCK-grown virions (1 µg/well) or purified egg-grown virions (2.5 µg/well) to coat the wells of immunoplates (Nunc).

Competitive binding assay. The method of Kimura-Kuroda & Yasui (1983) was followed. Briefly, monoclonal antibodies were partially purified from ascitic fluids by salting out with saturated ammonium sulphate and were then conjugated with horseradish peroxidase as described by Wilson & Nakane (1978). Fifty µl of serial fourfold
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Dilutions of partially purified competitor antibody was added to wells coated with purified MDCK-grown virions, and the plates were incubated for 2 h at room temperature. After washing four times with PBS containing 0.05% Tween 20 (PBS-T), 50 μl of peroxidase-labelled antibody (1-0 to 1.5 \( A_{405} \) units) in PBS-T plus 0.5% bovine serum albumin was added to each well and allowed to react at room temperature for 2 h. The wells were again washed four times with PBS-T and further incubated with 100 μl of substrate solution [0.05 m-citrate buffer pH 4.0, 0.008% \( H_2O_2 \), 40 mM-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt] for 1 h at room temperature. The green colour that developed was measured by absorbance at 405 nm in a multichannel photometer.

RIP. Confluent monolayers of MDCK cells infected with influenza C virus were labelled with \(^{35}S\)methionine and then subjected to immunoprecipitation according to the procedures described previously (Sugawara et al., 1986). The immunoprecipitates obtained were analysed by SDS-PAGE followed by fluorography as described elsewhere (Hongo et al., 1986a).

Western blotting. Purified C/Ann Arbor/1/50 virions grown in MDCK cells were boiled for 3 min in 0.0625 M-Tris-HCl pH 6.8 containing 2% SDS and 5% 2-mercaptoethanol and then subjected to SDS-PAGE. After electrophoresis, viral proteins were transferred to a nitrocellulose filter by the method of Towbin et al. (1979). To prevent non-specific binding, the filter was soaked in TNA buffer (0.01 M-Tris-HCl pH 7.4, 0.9% NaCl, 4% egg albumin) at 40 °C for 90 min, and was cut into strips. Each strip was incubated at 4 °C overnight with monoclonal antibody or rabbit antiviral serum diluted appropriately in TNA buffer. The strips were then washed with TNA buffer lacking egg albumin, and further incubated at 4 °C overnight with anti-mouse IgG goat serum (Tago, Burlingame, Ca., U.S.A.) or anti-rabbit IgG goat serum (Miles Laboratories), each of which had been conjugated to horseradish peroxidase. After extensive washing, the strips were developed by incubation with the substrate solution consisting of 0.05% \( o \)-phenylenediamine, 0.03% \( H_2O_2 \), 0.01 M-Tris-HCl pH 7.5.

RESULTS

Frequency of selection of antigenic variants

A parental virus (JM7-3), obtained by two successive plaque purifications of the stock of the C/Ann Arbor/1/50 strain, was treated with an excess of each group A monoclonal antibody and antigenic variants were isolated by plaquing on LLC-MK2 cells. As shown in Table 1, the frequency of variant isolation was in the range of 10\(^{-3.3}\) to 10\(^{-6.9}\) with monoclonal antibodies J14, Q5 and K16, but was below the limit of detection with J9. J9-resistant variants could be obtained, however, at a frequency of 10\(^{-5.5}\) when the parent virus treated with this antibody was inoculated into the amniotic cavity of embryonated eggs.

Operational mapping of gp88

To delineate antigenic sites on the gp88 molecule of the C/Ann Arbor/1/50 strain, a panel of antigenic variants isolated as above was examined for reactivity with each of seven monoclonal antibodies in HI (Table 1) and ELISA (Table 2). It should be mentioned here that the HI activity of K16 varied from experiment to experiment and was sometimes undetectable, and that the reactivity patterns of antigenic variants selected with a given monoclonal antibody were virtually identical except for two variants selected with Q5 which displayed significant differences in ELISA.

The reactivity patterns of group A monoclonal antibodies were distinguishable from one another, indicating that they recognize different epitopes of the gp88 molecule. It was found, however, that Q5 reacted with the variants selected with either J14 or J9 to a lower extent than with the parent virus, and that J14 and J9 showed lower ELISA titres against one of two variants selected with Q5. These observations suggest that the epitopes defined by J14 and J9, although they are separate from each other, are both linked to that recognized by Q5. In contrast, no overlap was seen between the epitope recognized by K16 and that recognized by J14, J9 or Q5. Thus two non-overlapping antigenic sites were defined by neutralizing group A monoclonal antibodies, one recognized by J14, J9 and Q5 (site A-1), and the other by K16 (site A-2). Monoclonal antibodies of group B showed no HI activity against either the parent virus or any of the antigenic variants (data not shown) but were found in ELISA to bind to all of these viruses (data not shown), suggesting that the antigenic sites recognized by non-neutralizing group B antibodies are discrete from antigenic sites A-1 and A-2.
Table 1. Reactivity of group A monoclonal antibodies with antigenic variants of C/Ann Arbor/1/50 in HI tests

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Frequency (log_{10}) of variants</th>
<th>J14†</th>
<th>J9</th>
<th>Q5</th>
<th>K16</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM7-3 (parent) V1 to V11</td>
<td>2560000 &lt;400</td>
<td>2560000</td>
<td>1280000</td>
<td>640000</td>
<td>640000</td>
</tr>
<tr>
<td>J14</td>
<td>-6.9</td>
<td>2560000</td>
<td>640000</td>
<td>1280000</td>
<td>640000</td>
</tr>
<tr>
<td>J9</td>
<td>&lt;-7.9 (-5.5)*</td>
<td>1280000</td>
<td>640000</td>
<td>400</td>
<td>320000</td>
</tr>
<tr>
<td>Q5</td>
<td>-5.2</td>
<td>10240</td>
<td>640</td>
<td>80</td>
<td>&lt;40</td>
</tr>
<tr>
<td>K16</td>
<td>-3.3</td>
<td>40</td>
<td>&lt;40</td>
<td>40</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

* Embryonated hen’s eggs were used for isolation of antigenic variants.
† Monoclonal antibody used for selection of variants.
‡ Antigenic variant number.

Table 2. Reactivity of group A monoclonal antibodies with antigenic variants of C/Ann Arbor/1/50 in ELISA

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>J14†</th>
<th>J9</th>
<th>Q5</th>
<th>K16</th>
</tr>
</thead>
<tbody>
<tr>
<td>J14</td>
<td>-‡</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>J9</td>
<td>+</td>
<td>-</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Q5</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Monoclonal antibody used for the selection of variants.
† Antigenic variant number.
‡ +, Titre with variant was identical to that with parental virus; ±, titre with variant was 10- to 100-fold less than that with parental virus; -, titre with variant was 100-fold less than that with parental virus.

Table 3. Summary of competitive binding assays

<table>
<thead>
<tr>
<th>Antibody group</th>
<th>Antigenic site</th>
<th>Unlabelled competitor</th>
<th>J14</th>
<th>J9</th>
<th>Q5</th>
<th>K16</th>
<th>S16</th>
<th>J6</th>
<th>J15</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A-1</td>
<td>J14</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>A-2</td>
<td>J14</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>J9</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q5</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K16</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>J16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* +, >75% competition at 1 mg/ml of unlabelled competitor; ±, 50% to 75% competition; -, <50% competition.

Topological mapping of gp88

To investigate the spatial distribution of the operationally defined epitopes on gp88, competitive binding assays were carried out. The results are illustrated in Fig. 1. Fig. 1(a) shows that unlabelled group A antibodies, with the exception of K16, competed to various degrees with the peroxidase-labelled antibody J14 whereas no group B antibody did. Fig. 1(b) demonstrates that the binding of labelled S16 to gp88 was almost completely blocked by unlabelled homologous antibody but not at all by the others. The data obtained in this way for seven monoclonal antibodies are summarized in Table 3.
Monoclonal antibodies J14, J9 and Q5, all of which define antigenic site A-1, competed efficiently with one another, confirming the overlapping nature of the epitopes recognized by these antibodies. It was also observed that antibody K16, which defines antigenic site A-2, competed significantly with the other group A antibodies directed to antigenic site A-1 though the competition with either J14 or Q5 was not reciprocal. These results suggest that antigenic sites A-1 and A-2, though operationally distinct, overlap topographically. Monoclonal antibodies of group B did not show significant levels of competition with any group A antibody, confirming that antigenic sites recognized by group B antibodies are remote from both antigenic sites A-1 and A-2. Table 3 also suggests that the epitopes recognized by the three antibodies of group B can be divided into two non-overlapping antigenic sites, one recognized by S16 (site B-1) and the other by J6 and J15 (site B-2).

Analysis of antigenic variants with polyclonal sera

Antigenic variants of the C/Ann Arbor/1/50 strain selected with monoclonal antibodies were examined for their antigenicity by HI tests using three different polyclonal anti-C/Ann Arbor/1/50 sera prepared in rabbit, chicken and dog. Of 23 variants analysed, none could be distinguished from the parent virus by any of the sera used (data not shown), suggesting that antigenic changes that occurred in these variants are not sufficient to provide an epidemiological advantage to influenza C virus.

Glycosylation dependence of individual antigenic determinants

To determine the requirement for glycosylation in the formation of each antigenic determinant, the antigenicity of gp88 and T76 of the C/Ann Arbor/1/50 strain was compared by RIP using the seven monoclonal antibodies characterized above. The gp88 glycoprotein synthesized in the absence of TM was precipitated by any of the antibodies tested (Fig. 2a).
Fig. 2. Reactivity of monoclonal antibodies with the non-glycosylated form or the denatured form of gp88. C/Ann Arbor/1/50 strain-infected MDCK cells were labelled with \( ^{35} \)S)methionine in the absence (a) or presence (b) of TM and then immunoprecipitated with either rabbit antiviral serum (lanes 2) or each of the monoclonal antibodies: lanes 3, Q5; lanes 4, S16; lanes 5, J6; lanes 6, J14; lanes 7, J9; lanes 8, K16; lanes 9, J15. As described in detail elsewhere (Sugawara et al., 1986), Protein A–Sepharose 4B beads (Pharmacia) pretreated with anti-mouse IgG (Miles-Yeda, Rehovot, Israel) were used for RIP with monoclonal antibodies J14, J9, K16 and J15. For RIP with the other antibodies, the beads were used without pretreatment. RIP was also performed with control ascitic fluids using Protein A–Sepharose beads with (lanes 10) or without (lanes 1) pretreatment with anti-mouse IgG. The NP protein was sometimes non-specifically precipitated. T80 is a host cell protein whose synthesis is enhanced in the presence of TM (Hongo et al., 1986a). (c) Following gel electrophoresis of purified C/Ann Arbor/1/50 virions grown in MDCK cells, viral proteins were transferred to a nitrocellulose filter, and then allowed to react with rabbit antiviral serum (lane 1), control ascites fluid (lane 2) or individual monoclonal antibodies: lane 3, J14; lane 4, K16; lane 5, Q5; lane 6, J9; lane 7, S16; lane 8, J6; lane 9, J15. Antiviral serum and control ascitic fluid were used at dilutions of 1:1000 and 1:100, respectively. The ascitic fluids containing monoclonal antibodies were used at 1:1000, except for J15 which was diluted 1:100.
Three antibodies of group B were also able to precipitate the T76 polypeptide synthesized in the presence of TM (Fig. 2b). In contrast, three monoclonal antibodies in group A (Q5, J9, K16) did not precipitate T76 at all. Although J14 of this group was found to react with both gp88 and T76, the latter polypeptide was precipitated by this antibody in much smaller amounts than by the former. These observations suggest that the binding of group A antibodies to gp88 depends on glycosylation whereas that of group B does not. To confirm this idea, we repeated similar experiments with a variety of influenza C isolates listed in Table 4. It was evident that group B antibodies were all reactive with both glycosylated and non-glycosylated forms of gp88 irrespective of the virus strain used. The only exception was that S16 precipitated neither gp88 nor T76 of the C/Aichi/1/81 strain. Three monoclonal antibodies of group A (J9, Q5, K16), on the other hand, did not precipitate T76 from any of the strains tested. Although J14 was found to bind to the T76 proteins of several strains such as C/Aomori/74, C/Kanagawa/1/76, C/Miyagi/77, C/Kanagawa/1/81 and C/Aichi/1/81, this antibody failed to react with the protein of any other strain. From these observations, we suggest that glycosylation of gp88 is important for the formation of antigenic sites A-1 and A-2 but not for that of antigenic sites B-1 and B-2.

The reactivity of monoclonal antibodies with gp88 of the homologous strain C/Ann Arbor/1/50 was further investigated by Western blot analysis. The results shown in Fig. 2(c) indicate that the denatured gp88 blotted on the nitrocellulose membranes was reactive with all of the group B antibodies but not with any of the group A antibodies, suggesting that heat denaturation of gp88 in the presence of SDS and 2-mercaptoethanol destroyed antigenic sites A-1 and A-2 without affecting antigenic sites B-1 and B-2. To confirm that the sites A-1 and A-2 are more sensitive to conformational changes than the sites B-1 and B-2, C/Ann Arbor/1/50 virions grown in MDCK cells were treated with 1% SDS at 37 °C for 10 min and then tested for reactivity with monoclonal antibodies by ELISA. Fig. 3 shows that SDS treatment of influenza C virions under these conditions markedly reduced the reactivity of gp88 with monoclonal antibodies of group A without significantly influencing the reactivity with those of group B. It is concluded, therefore, that antigenic sites A-1 and A-2 are highly susceptible to denaturation of gp88 and thus are conformation-dependent.
DISCUSSION

In the present study, seven monoclonal antibodies prepared against the gp88 molecule of influenza C virus were used to estimate the frequency of spontaneously occurring antigenic variants, to construct an antigenic map and to determine the glycosylation dependence of each antigenic determinant. Previous serological studies showed that influenza C strains isolated over a long period in different parts of the world were highly cross-reactive in HI tests with polyclonal antiviral sera (Chakraverty, 1978; Meier-Ewert et al., 1981; Kawamura et al., 1986), suggesting that the antigenicity of influenza C virus is much less variable than that of the other types of influenza virus. The frequency of antigenic variants in cloned preparations of the C/Ann Arbor/1/50 strain was estimated, however, to range between $10^{-3.3}$ and $10^{-6.9}$, which was similar to the frequency found with influenza A virus (Webster & Laver, 1980; Webster et al., 1982) and was 10- to 10000-fold higher than that found with influenza B virus (Webster & Berton, 1981; Berton & Webster, 1985). It appears, therefore, that the striking stability of influenza C virus antigenicity can not be explained simply by a low capacity to produce antigenic variants. Portner et al. (1980) have also observed with Sendai virus and vesicular stomatitis virus, both of which are antigenically stable in nature, that antigenic variants arise in cloned virus populations at the same frequency as that found in the influenza A virus population.

Antigenic variants resistant to antibody J9 could be isolated at a frequency of $10^{-5.5}$ when the mixture of cloned parent virus and this antibody was inoculated into embryonated eggs but not at all when it was inoculated onto LLC-MK$_2$ cells (Table 1). This observation could be accounted for if the mutations that occurred in such variants caused a loss in their capacity to grow in LLC-MK$_2$ cells. Experiments are in progress to confirm this possibility.

Two non-overlapping antigenic sites, A-1 and A-2, were delineated on gp88 by operational analysis with four monoclonal antibodies of group A. We observed previously that antibodies...
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J14, J9 and Q5, all of which define antigenic site A-1, inhibited haemagglutination efficiently whereas antibody K16, which defines antigenic site A-2, did so only poorly (Sugawara et al., 1986), suggesting that site A-1 is closer to the receptor-binding site than site A-2. Among the epitopes located in site A-1 the J14-recognizing one may be the closest. Our preliminary studies have shown that chicken erythrocytes treated with a small amount of the parent virus at 37 °C for 2 h, though no longer agglutinated by the parent virus itself, could still be agglutinated with all of the antigenic variants selected with J14 (K. Nakamura et al., unpublished observations), which suggests that amino acid substitution(s) in the region corresponding to the epitope recognized by this antibody may cause a change in the specificity of receptor recognition by influenza C virus.

Two additional non-overlapping antigenic sites (B-1 and B-2) were identified in the competitive binding analysis by three monoclonal antibodies of group B. The biological significance of these antigenic sites is not clear since no group B antibody inhibited haemagglutination, haemolysis or infectivity (Sugawara et al., 1986). We observed, however, that monoclonal antibodies S16 and J6, which define site B-1 and site B-2, respectively, were able to bind to the gp88 protein on intact virions though antibody J15, directed to site B-2, was not (data not shown), raising the possibility that the antibodies to antigenic sites B-1 and B-2 may be involved in protective immunity in vivo through activation of the complement system, enhancement of the phagocytic activity of mononuclear cells and so on.

The non-glycosylated precursor of gp88 synthesized in the presence of TM could be immunoprecipitated with any of the monoclonal antibodies to sites B-1 and B-2 but not with most of the antibodies to sites A-1 and A-2. The possibility that the latter group may be directed against carbohydrate moieties appears unlikely since they did not immunoprecipitate any of the cellular glycoproteins (see Fig. 2), and their reactivity with gp88 was largely dependent on the virus strain (see Table 4). It was impressive that glycosylation-dependent epitopes on gp88 were coincident with biologically active ones that are responsible for the induction of virus-neutralizing antibodies. Bruck et al. (1984) also demonstrated that carbohydrate addition was essential to the integrity of the neutralizing antibody-induced epitopes of bovine leukaemia virus (BLV) envelope glycoproteins. Therefore these two viruses may be unable to elicit neutralizing antibodies if glycosylation is prevented. To draw a firm conclusion, however, non-glycosylated forms of these viral glycoproteins must be directly examined for their ability to produce virus-neutralizing antibodies in immunized animals. Bruck et al. (1984) have previously shown that all three carbohydrate-dependent epitopes of gp51 of BLV display antigenic differences among various BLV isolates and that only one of five carbohydrate-independent epitopes shows such differences. We also observed that glycosylation-dependent antigenic sites of gp88 (sites A-1 and A-2) underwent considerable antigenic changes while glycosylation-independent ones (sites B-1 and B-2) were conserved among various influenza C isolates (see Table 4). These results sound reasonable since in both cases glycosylation-dependent epitopes are involved in the production of neutralizing antibodies and thus would be expected to be exposed to antibody pressure in infected hosts.

Recently, Long et al. (1986) have reported that five monoclonal antibodies against the fusion (F) protein of Newcastle disease virus are all reactive with native F but are unreactive with both the non-glycosylated form of F and denatured F in Western blots. They have also shown that seven antibodies against the haemagglutinin–neuraminidase (HN) protein, deficient in reactivity with non-glycosylated HN, are unreactive with heat-denatured HN transferred to nitrocellulose membranes. It was found in this study that monoclonal antibodies of group A, directed to glycosylation-dependent antigenic sites, did not bind to the denatured form of gp88. These observations are compatible with the idea that there is a correlation between conformational epitopes and glycosylation-dependent ones.

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