The Outer Capsid Glycoprotein VP7 of Simian Rotavirus SA11 Contains Two Distinct Neutralization Epitopes

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SUMMARY

Seven neutralizing monoclonal antibodies (MAbs) to the rotavirus simian agent 11 were produced. Although displaying variable degrees of haemagglutination-inhibiting activity, they were shown by radioimmunoprecipitation and Western blot analyses to react with the major outer capsid glycoprotein (VP7). In competition binding assays, MAbs defined two distinct VP7 epitopes, which appeared to be close to each other or partially overlapping. In addition, MAbs of the two epitope groups enhanced binding of a broadly reactive, non-neutralizing, MAb specific for rotavirus group antigen.

Rotaviruses are a major cause of acute gastroenteritis in most animal species, including humans. Their virions are composed of an outer and an inner capsid enclosing 11 segments of double-stranded RNA. Genetic reassortment occurs at a high frequency during co-infection, and analysis of reassortant viruses has provided much information on the functional properties of rotavirus gene products. Thus, gene 4 has been shown to code for the outer capsid glycoprotein VP3, the haemagglutinin, responsible for trypsin enhancement of infectivity and for restriction of rotavirus growth in cell culture (Greenberg et al., 1983a; Kalica et al., 1983). In addition, rotavirus gene 4 has recently been shown to play an important role in virulence and pathogenicity (Flores et al., 1986; Gorziglia et al., 1986; Offit et al., 1986). Gene 6 has been shown to code for VP6 protein, specifying rotavirus group and subgroup antigens, and gene 8 or 9 for the major neutralization antigen on VP7 glycoprotein (Greenberg et al., 1983a, c; Kalica et al., 1981). In parallel, monoclonal antibodies (MAbs) have been shown to exhibit both haemagglutination-inhibiting (HI) and neutralizing (Nt) activity when directed to VP3 (Greenberg et al., 1983c; Taniguchi et al., 1985), group- or subgroup-specific reactivity when reactive with VP6 (Greenberg et al., 1983b), and serotype-specific reactivity when directed to VP7 (Greenberg et al., 1983c; Sonza et al., 1983; Coulson et al., 1985; Taniguchi et al., 1985). More recently, it has been shown that two serotype specificities, represented by epitopes on VP3 and VP7, are typical of each rotavirus strain and may segregate independently in nature (Hoshino et al., 1985; Offit & Blavat, 1986). Consequently, in the future, the serotype specificity of both VP3 and VP7 should be designated for each rotavirus strain.

The simian rotavirus simian agent 11 (SA11) (a serotype 3 rotavirus) has been reported to possess a single major neutralization epitope on the VP7 glycoprotein (Sonza et al., 1984). From a series of hybridoma fusions we obtained seven MAbs reacting with VP7 and Nt, at high titres, SA11 and serotype 3 human rotavirus (HRV) strains. When analysed in ELISA competitive binding assays (CBAs), they showed non-reciprocal interactions but appeared to belong to two
distinct epitope groups. In addition, Nt MAbs of both epitope groups differentially enhanced binding of a broadly reactive, non-neutralizing, MAb specific for the rotavirus group antigen. Thus, MAbs appeared to be directed against two neutralization epitopes which are likely to occur close together in one major neutralization domain on the VP7 glycoprotein of SA11.

The following cell culture-adapted viruses were used in the present study: HRV strains Wa (serotype 1), DS1 (serotype 2) and ST3 (serotype 4) (provided by Dr R. G. Wyatt and Dr Y. Hoshino, NIAID, Bethesda, Md., U.S.A.); HRV strain YO (serotype 3) (provided by Dr S. Urasawa, Sapporo Medical College, Sapporo, Japan); SA11 (serotype 3) (provided by Dr S. Kalter, Southwest Foundation for Animal Research, San Antonio, Tx., U.S.A.). MA104 cells were grown in Eagle's MEM (Gibco) supplemented with 10% foetal calf serum (FCS). All strains were propagated in roller tubes and bottles of MA104 cultures in serum-free MEM containing 1 μg of tissue culture grade trypsin per ml, as previously described (Gerna et al., 1984).

Eight-week-old female BALB/c mice were first immunized intraperitoneally each with 200 μg protein of partially purified virus in complete Freund's adjuvant. Virus was concentrated from cell culture fluids and purified by sedimentation through 40% sucrose as previously described for the preparation of rotavirus antisera (Gerna et al., 1984). Thirty days later virus was administered intraperitoneally in incomplete Freund's adjuvant. After an additional 30 days, mice were boosted by intraperitoneal inoculation of the same amount of virus in phosphate-buffered saline. Three days after the final booster, mice were sacrificed and their spleens were removed for fusion with NS-1 myeloma cells. Fusion, using 45% (w/v) polyethylene glycol 4000 (Merck), as well as subsequent steps, were done according to Greenberg et al. (1983c). Cells were finally suspended in HAT medium supplemented with 10% FCS and 2% human endothelial culture supernatant (Astaldi et al., 1980), plated in 96-well or 24-well Costar plates and refed after 7 days with fresh HAT medium. When hybridomas occupied at least one-fifth of the well areas, supernatants were screened for immunological reactivity. Hybridomas were cloned twice by limiting dilution and then expanded in flasks, to obtain enough supernatant for testing and enough cells for inoculating pristane-primed mice to induce ascites. The immunoglobulin isotypes of MAbs were determined by ELISA (Mouse-Typer sub-isotyping kit, Bio-Rad). MAbs were screened by the neutralization assay, using stationary MA104 cultures grown in microtitre plates (Gerna et al., 1984). The neutralization titre was expressed as the reciprocal of the highest dilution giving at least 50% reduction in the number of infected cells, as compared to the virus control wells (approx. 200 f.f.u./0.1 ml).

The antibody reactivity of MAbs was also examined by ELISA, HI assay, Western blotting (WB) and radioimmunoprecipitation (RIP). Two ELISA systems were initially used for testing MAbs. In the first one, partially purified virus (about 1 μg per well) was passively bound to the solid phase and tested against dilutions of ascitic fluids. In the second assay, which is referred to as double-antibody sandwich ELISA, rotaviruses of different serotypes were bound to microtitre plate wells precoated with homologous polyclonal antibodies (diluted 1:10000) from rabbit antiserum prepared as previously described (Gerna et al., 1984). In both systems reactivity was finally detected by using peroxidase-conjugated goat anti-mouse Ig (Cappel Laboratories). After preliminary experiments, it was observed that specific ELISA reactivity of Nt MAbs appeared to be properly evaluated only by indirect double-antibody sandwich ELISA, which was then used in subsequent testing. Haemagglutination and HI assays were performed using human O erythrocytes (Kalica et al., 1978).

SDS-PAGE in reducing conditions was performed as reported by Laemmli (1970). For analysis of unreduced proteins, samples were solubilized in Laemmli sample buffer without 2-mercaptoethanol. For WB, proteins were transferred from gels onto 0.2 μm pore size nitrocellulose paper essentially as described by Towbin et al. (1979). Immunological detection of blotted proteins was performed by the indirect immunoperoxidase antibody (IPA) technique or by the avidin–biotin peroxidase complex (ABC) procedure (Vectastain, Vector Laboratories, Burlingame, Ca., U.S.A.). RIP was done as described by Coulson et al. (1986), except that roller tubes of MA104 cells, infected with trypsin-treated SA11, HRV YO or HRV Wa were used for the preparation of radiolabelled cell lysate. The antibody avidities of MAbs were also
Table 1. Nt and HI activity of MAbs directed against SA11 rotavirus

<table>
<thead>
<tr>
<th>MAb</th>
<th>Ig class</th>
<th>SA11 (3)</th>
<th>ST3 (4)</th>
<th>SA11 (3)</th>
<th>ST3 (4)</th>
<th>HI titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A2</td>
<td>A</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>4 × 10^4</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
</tr>
<tr>
<td>5A10</td>
<td>G2a</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>2 × 10^4</td>
<td>&lt;10^2</td>
<td>10^240</td>
</tr>
<tr>
<td>3C3</td>
<td>G2b</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>2 × 10^4</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
</tr>
<tr>
<td>4C3</td>
<td>G2b</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>160</td>
</tr>
<tr>
<td>3A6</td>
<td>G2b</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>1 × 10^4</td>
<td>&lt;10^2</td>
<td>160</td>
</tr>
<tr>
<td>5D6</td>
<td>G2b</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>1 × 10^4</td>
<td>&lt;10^2</td>
<td>40</td>
</tr>
<tr>
<td>4A4</td>
<td>G2b</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>2560</td>
</tr>
<tr>
<td>3C4†</td>
<td>G2a</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>&lt;10^2</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

* Titres expressed as reciprocals.
† Rotavirus group-specific MAb.
VP3

VP6

VP7

Fig. 1. Reactivity of MAbs by RIP with \[^{35}S\]methionine-labelled SA11-infected cell lysate. Lane 1, infected cell lysate only; lane 2, polyclonal antiserum directed to SA11 rotavirus; lane 3, MAb 3C3; lane 4, 4C3; lane 5, 5D6; lane 6, 3A6; lane 7, 5A10; lane 8, 4A4; lane 9, 2A2; lane 10, 3C4 (group-specific). Molecular weights of marker proteins (phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrase) are shown on the right.

5A10 and the other (group B) including 3A6, 4C3, 4A4, 3C3 and probably 5D6. Group A MAbs competed partially with labelled group B MAbs, whereas group B MAbs totally blocked the binding of group A MAbs. In addition, the VP6-specific MAb, 3C4, displayed a moderate degree of competition with labelled group A MAbs and a low level of competition with group B MAbs. On the other hand, binding of labelled 3C4 was significantly increased by all Nt MAbs. In particular, group A MAbs displayed a high enhancing effect (82 to 100%), whereas enhancement by those in group B was lower. Antibody avidity differed greatly and did not correlate with epitope group, Nt or HI activity (data not shown). For example, 5A10 and 2A2 (group A) showed the highest and the lowest avidity, respectively, while intermediate values were found for the other MAbs examined.

In the present study, seven MAbs with high Nt activity were selected from MAbs screened only by neutralization assay. Thus, weakly Nt hybridomas, as well as non-neutralizing MAbs with rotavirus group-specific reactivity, were left out by the screening procedure. When Nt reactivity was tested in hybridoma supernatants and in mouse ascitic fluids, all seven Nt MAbs were found to be specific for serotype 3 rotavirus. The only variation within Nt reactivity to serotype 3 rotavirus was that titres to human strains (heterospecific titres) were about 10-fold lower than homospecific titres. The same level of specific reactivity was detected by indirect double-antibody sandwich ELISA, which gave antibody titres about 10-fold higher than corresponding Nt titres. However, when virus was bound directly to the solid phase, some cross-reactivity with heterotypes was consistently found. This lack of specificity may be a result of damage to virions and of conformational changes of VP7 on the virus surface due to direct binding to solid phase. One of the seven Nt MAbs was found to be in the IgA class. This finding, which, surprisingly, is not very unusual for rotavirus (Coulson et al., 1986; Heath et al., 1986), may be due to the prolonged immunization period of mice.

As expected on the basis of the high Nt activity and serotype-specificity, all seven MAbs were found to react by RIP with VP7 of SA11 and HRV YO, but not with the heterotypes. Some difficulty was encountered in detecting the specific polypeptide reactivity of MAbs by the WB technique using IPA, but all seven Nt MAbs reacted with unreduced VP7 when the highly amplifying ABC procedure (Hsu et al., 1981) was used for immunostaining. Reactivity of MAbs with VP7 separated under reducing conditions could not be detected by either technique. This confirms that the Nt epitopes on rotavirus VP7 are sensitive to reducing agents, as previously reported by others (Coulson et al., 1985, 1986; Sabara et al., 1985). The immunological reactivity
Table 2. Summary of competitive binding assays of peroxidase-labelled MAbs with ascitic fluids

<table>
<thead>
<tr>
<th>Labelled MAb</th>
<th>2A2</th>
<th>5A10</th>
<th>3C3</th>
<th>4C3</th>
<th>3A6</th>
<th>5D6</th>
<th>4A4</th>
<th>3C4</th>
<th>Absorbance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A2</td>
<td>12 (90)</td>
<td>13 (89)</td>
<td>10 (92)</td>
<td>5 (96)</td>
<td>2 (98)</td>
<td>12 (90)</td>
<td>0 (100)</td>
<td>63 (46)</td>
<td>115</td>
</tr>
<tr>
<td>5A10</td>
<td>17 (86)</td>
<td>15 (88)</td>
<td>20 (84)</td>
<td>8 (94)</td>
<td>0 (100)</td>
<td>11 (91)</td>
<td>0 (100)</td>
<td>68 (45)</td>
<td>123</td>
</tr>
<tr>
<td>3C3</td>
<td>48 (63)</td>
<td>62 (52)</td>
<td>20 (85)</td>
<td>16 (88)</td>
<td>3 (98)</td>
<td>21 (84)</td>
<td>1 (99)</td>
<td>96 (25)</td>
<td>128</td>
</tr>
<tr>
<td>4C3</td>
<td>49 (64)</td>
<td>48 (64)</td>
<td>21 (85)</td>
<td>13 (91)</td>
<td>6 (96)</td>
<td>20 (85)</td>
<td>1 (99)</td>
<td>101 (25)</td>
<td>134</td>
</tr>
<tr>
<td>3A6</td>
<td>63 (58)</td>
<td>61 (59)</td>
<td>23 (85)</td>
<td>18 (88)</td>
<td>11 (93)</td>
<td>22 (86)</td>
<td>2 (99)</td>
<td>123 (17)</td>
<td>148</td>
</tr>
<tr>
<td>4A4</td>
<td>45 (65)</td>
<td>45 (65)</td>
<td>20 (85)</td>
<td>12 (91)</td>
<td>5 (96)</td>
<td>20 (85)</td>
<td>1 (99)</td>
<td>100 (22)</td>
<td>128</td>
</tr>
<tr>
<td>3C4</td>
<td>200 (+100)$</td>
<td>182 (+82)</td>
<td>164 (+64)</td>
<td>148 (+48)</td>
<td>141 (+41)</td>
<td>129 (+29)</td>
<td>128 (+28)</td>
<td>6 (94)</td>
<td>100</td>
</tr>
</tbody>
</table>

* Absorbance (x 100) by competitive antibody, values in parentheses represent percent inhibition.
† Rotavirus group-specific MAb.
‡ Absence of competitor antibody.
§ + in parentheses indicates percent increase in absorbance (enhanced binding).
of the group-specific MAb with VP6 was readily detected by RIP and WB. We also examined by
WB several rotavirus group- and subgroup-specific MAbs produced in our laboratory, and
found all of them reactive with VP6 under reducing as well as non-reducing conditions
(unpublished observations). As a conclusion, we suggest that both RIP and WB using the ABC
immunostaining procedure should be considered methods of choice for studying the molecular
reactivity of rotavirus Nt MAbs.

In CBAs the seven highly Nt MAbs could be divided into two groups: group A containing
2A2 and 5A10 which moderately inhibited binding of labelled group B members and group B
containing the other five MAbs which competed strongly with labelled group A MAbs. Group A
MAbs were the only ones that, in WB, were found to react with SA11 VP7 not only by the highly
amplifying ABC technique but also by IPA. Both group A and B MAbs showed variable degrees
of HI activity. The HI activity of VP7-specific MAbs is not a consequence of direct
immunological interaction with viral haemagglutinin, but it is probably due to steric hindrance
(Greenberg et al., 1983c; Coulson et al., 1985), the degree of which might not necessarily
correlate with the binding site of the MAbs on the VP7 molecule. In conclusion, our MAbs
identified two distinct neutralization epitopes of VP7, which, as suggested by the finding of non-
reciprocal interactions, should be close to each other or even partially overlapping. Non-
reciprocal interactions between group A and B MAbs in CBAs might be due to steric hindrance,
or to binding of antibody at the most external region of a cleft in VP7, thus preventing reactivity
of antibodies directed to other epitopes within the cleft region (Shaw et al., 1986). Our data are
compatible with the presence of a single major, immunodominant neutralization site on VP7 of
SA11 (Sonza et al., 1984; Lazdins et al., 1985) Dyall-Smith et al. (1986) sequenced the VP7 genes
from SA11 mutants resistant to Nt MAbs and identified three regions (A, B and C) of which two
(A and C) appeared to be in close proximity on the native VP7 glycoprotein. They found that
mutations in both A and C regions could be selected by a single MAb and concluded that these
two regions probably form one antigenic site with different epitopes. Moreover, Shaw et al.
(1986), using MAbs and viral antigenic variants, recently provided evidence for the existence of
at least two regions on each of VP3 and VP7 of rhesus rotavirus, which are involved in virus
neutralization.

An interesting finding emerging from our CBAs was the low degree of competition between
the anti-VP6 MAb 3C4 and labelled MAbs of both epitope groups. It has been shown, using
peroxidase-catalysed iodination of bovine rotavirus particles, that inner capsid proteins, mostly
VP2 but, to a lesser extent, also VP6, are exposed on the virus surface (Novo & Esparza, 1981).
In our study, exposure of VP6 might have been increased by treatment of virus with trypsin,
which produces a cleavage not only of VP3, but also of VP2, an inner capsid polypeptide
covering most of VP6 in rotavirus particles (Estes et al., 1981). The presence of VP6 on the virus
surface would cause the anti-VP6 MAb to react with double-shelled rotavirus particles. Thus,
the competition between 3C4 and labelled Nt MAbs could be interpreted as due to a steric
hindrance phenomenon. In our opinion, the weak Nt activity previously reported for antisera
raised to purified VP6 of rotavirus (Bastardo et al., 1981; Sabara et al., 1985) could be
interpreted in the same way. On the other hand, although the enhanced binding of labelled anti-
VP6 MAb induced by all Nt MAbs is puzzling, it might be at least partially due to further
exposure of VP6 on the rotavirus surface, as a consequence of the interaction between VP7 and
Nt MAbs of both epitope groups. Such findings, which, to our knowledge, have not previously
been described for rotavirus, further support the existence of two close or partially overlapping,
but distinct, neutralization epitopes on VP7 of SA11. To clarify these points further,
comparative testing of different sets of VP7 serotype-specific MAbs obtained by different
groups is required.

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