Identification of operationally overlapping and independent cross-reactive neutralization regions on human rotavirus VP4

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Cross-reactive neutralization epitopes on VP4 of human rotavirus (HRV) were analysed by the use of VP4-specific neutralizing monoclonal antibodies (N-MAbs) and MAb-resistant mutants. Seven anti-VP4 N-MAbs obtained in this study by using HRV serotypes 1 and 3 as immunizing antigens showed a variety of cross-reactivity patterns to 20 HRV strains with different serotype specificity in neutralization tests and a broader cross-reactivity to them was found for four N-MAbs in an enzyme-linked immunosorbent assay. On the basis of the reactivity patterns against rotaviruses in neutralization tests, these seven N-MAbs were classified into four groups. Cross-neutralization tests using a total of 12 pairs of MAbs and resistant mutants, including five pairs which had been prepared previously, showed that VP4 of HRV (strain KU) contained two independent antigenic regions. One, region C1, was recognized by a single MAb (YO-2C2) and the other was made up of two antigenic regions (C2 and C3) which overlapped operationally. Identification of amino acid substitution sites on VP4 of representative mutants of HRV strain KU indicated that amino acid positions 385 or 392 and 428 or 433 were critical for the C2 and C3 regions, respectively. These results suggested that regions C2 and C3 exist as conformational antigenic sites.

Introduction

Establishment of the significance of group A rotaviruses as the major viral pathogen of infantile gastroenteritis world-wide (Kapikian & Chanock, 1985) has stimulated research for control and prevention of rotavirus infections (Kapikian et al., 1986; Estes et al., 1983). The outer capsid of rotavirus particles consists of two independent neutralizing antigens, VP4 and VP7, which are encoded by either gene segment 7, 8 or 9 depending on the strain (Kalica et al., 1981; Greenberg et al., 1983; Ward et al., 1988) and segment 4 (Mason et al., 1983), respectively. VP7 carries mainly serotype-specific neutralization antigens (Greenberg et al., 1981, 1983; Kalica et al., 1981; Taniguchi et al., 1985), although the serotype specificity of rotaviruses is defined by cross-neutralization tests using antisera prepared against the rotavirus virion (Wyatt et al., 1982). The other surface protein VP4, identified as haemagglutinin (Kalica et al., 1983), mediates penetration of the virion into cells (Kaljot et al., 1988) through the cleavage of VP4 into VP5* and VP8* in the presence of trypsin (Espejo et al., 1981; Estes et al., 1981; Offit et al., 1983) and is associated with virulence in mice and humans (Offit & Blavat, 1986; Flores et al., 1986). Neutralization antigens on VP4 generally include strain-specific antigens detectable on VP8* and inter-serotypic common antigens on VP5* (Kitaoka et al., 1986; Mackow et al., 1988b; Taniguchi et al., 1988b). Hence it is well known that cross-reactive neutralization observed between different serotypes usually is attributed to anti-VP4 antibody, though some antibodies directed to VP7 also manifest cross-reactivity (Coulson et al., 1986; Taniguchi et al., 1988a). Since the cross-reactivity due to VP4 is considered to play an important role in heterotypic cross-protection against rotavirus infections (Vesikari et al., 1984; Offit et al., 1986; Kapikian et al., 1986), analysis of cross-reactive neutralization epitopes on VP4 of human rotavirus (HRV) may contribute to the development of an effective rotavirus vaccine.

Functional and topographical analyses of neutralization epitopes on VP4 by using simian rotavirus strain SA11 (Burns et al., 1988) and rhesus rotavirus (RRV; Shaw et al., 1986) demonstrated the presence of plural neutralizing antigenic sites on the protein. Mackow et al. (1988b) identified six different neutralization regions and detected amino acid changes in neutralizing monoclonal antibody (N-MAb)-resistant mutants of RRV; amino acid substitutions corresponding to five regions were located on VP8*, and the remaining site involved in heterotypic neutralization was located on a limited area on VP5*. In HRV, Taniguchi et al. (1985) and Kitaoka et al. (1986) reported the production of cross-reactive and strain-specific N-MAbs to VP4, respectively. Further-
more, three distinct cross-reactive neutralization epitopes on VP4 were identified (Taniguchi et al., 1988a). However, further analysis is required to determine whether cross-reactive neutralization epitopes on VP4 are conformational as observed in VP7 (Dyall-Smith et al., 1986; Taniguchi et al., 1988a), and whether more antigenic sites involved in heterotypic neutralization exist on VP4. We have extended the study on the antigenic structure of HRV by preparing seven more cross-reactive neutralizing MAbs directed against VP4 of HRV and MAb-resistant mutants. The present study shows that operational overlapping exists among cross-reactive neutralization epitopes on VP4 of HRV.

**Methods**

Monoclonal antibodies. Two HRV strains, KU and S3, belonging to serotype 1 and 3, respectively, were used as immunizing antigens for BALB/c mice. These viruses were propagated in MA104 cells (Urasawa et al., 1981) and purified by metrizamide gradient centrifugation (Taniguchi et al., 1985). The fusion was performed as described previously (Taniguchi et al., 1983) except for the use of the mouse myeloma cell line PAI (Stocker et al., 1982) which was provided by the Japanese Cancer Research Resource Bank.

Determination of protein specificity of MAbs. In the first step, two gene reassortants, strain C15 with serotype 2 (strain HN126) specificity on VP7 and serotype 1 (strain Wa) specificity on VP4 (Urasawa et al., 1986) and strain C148 with serotype 3 (strain Yo) specificity on VP7 and serotype 4 (strain Hochi) specificity on VP4 (Urasawa et al., 1988) were used in neutralization tests for examining the protein specificity of MAbs.

Immunoprecipitation was also performed by using [35S]methionine-labelled KU virus, which is reactive with all N-MAbs produced in this study (Taniguchi et al., 1985).

Fluorescent focus (FF) neutralization test. An FF neutralization test was carried out to examine the reactivity patterns of the N-MAbs with numerous rotaviruses belonging to different serotypes. We employed the following HRVs in this study: (i) KU, Wa, S12, K8 and M37 (all serotype 1), (ii) S2, HN126, AK26, DS-1 and 1076 (all serotype 2), (iii) Yo, S3, AK35, P2, P and McN13 (all serotype 3), (iv) Hochi, Hosokawa and ST #3 (all serotype 4), (v) 69M (serotype 8) and (vi) W161 (serotype 9). Four of these strains (M37, 1076, McN13, and ST #3) had been isolated from neonates with asymptomatic infections (Hoshino et al., 1985; Gorziglia et al., 1988). We also used two simian rotaviruses (SA11 and RRV, both serotype 3) and one calf rotavirus (NCDV, serotype 6).

ELISA. The binding reactivity patterns of MAbs against a panel of rotavirus strains were evaluated by ELISA using the MAb as capture antibody as described previously (Urasawa et al., 1989).

N-MAb-resistant mutants. By using strain KU (serotype 1) as a parent strain, N-MAb-resistant mutants were selected and plaque-purified as described previously (Morita et al., 1988). For cross-neutralization tests between MAbs and mutants, we also employed five N-MAbs (KU-4D7, KU-6B11, YO-153, YO-2C2 and ST-1F2) and the resistant mutants of KU (V-KU-4D7, V-KU-6B11, V-YO-153, V-YO-2C2 and V-ST-1F2) selected by the respective MAbs prepared previously (Taniguchi et al., 1987).

**Results**

Hybridoma screening and determination of protein specificity

Seven hybridoma clones secreting cross-reactive N-MAbs were isolated from six separate fusion procedures. Four MAbs were considered to be directed at VP4 judging from their reaction patterns with the two gene reassortants and their parent strains. MAbs KU–10C, S3–2C and KU–12H had neutralization activity against reassortant strain C15 (VP4: Wa; VP7: HN126) without neutralizing activity against HN126 and KU–12H neutralized YO but not Hochi as well as YO-2C2 and ST-1F2. Immunoprecipitation analysis confirmed the VP4 specificity of all the seven cross-reactive MAbs as shown in Fig. 1.

Reactivity patterns of anti-VP4 N-MAbs as determined by neutralization test and ELISA

In FF neutralization tests, all the seven MAbs showed inter-serotypic cross-reactivity (Table 1). On the basis of the reactivity patterns of the N-MAbs to 20 HRV strains with different serotype specificity, these VP4-specific MAbs could be classified into four groups. Three MAbs, KU–10H, KU–2A and KU–7E (group 1), neutralized symptomatic HRV strains of serotypes 1 to 4 in addition to strain W161 (serotype 9). Group 2, defined by MAb, S3–5E, neutralized a subset of serotype 1 symptomatic strains (KU and S12) and all symptomatic strains tested belonging to serotypes 2 and 3 and strain W161. The MAbs of group 3 (KU–10C and S3–2C) exhibited neutralizing activity against serotypes 1 to 4 asymptomatic strains as well as symptomatic strains belonging to serotypes 1, 3 and 4 and strain W161. MAb KU–12H, classified as group 4, showed the least cross-reactivity in the MAbs obtained in this study and neutralized a subset of symptomatic serotype 1 strains (KU and Wa), serotype 3 strains (P and P2) and serotype 4 strains. Animal rotaviruses SA11, RRV and NCDV and two particular HRV strains, K8 (serotype 1) and 69M (serotype 8) were not neutralized by any MAb produced in this study (data not shown). On the basis of the results of FF neutralization tests with a number of rotavirus strains, five anti-VP4 MAbs prepared previously (Taniguchi et al., 1987) could be classified into the above mentioned group 1 (KU–4D7) or group 3 (KU–6B11,
Table 1. Reactivity patterns of cross-reactive N-MAbs against rotaviruses as determined by neutralization tests

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Rotavirus strain</th>
<th>Neutralizing titre* of monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KU-10H</td>
<td>KU-2A</td>
</tr>
<tr>
<td>Symptomatic strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>KU</td>
<td>≥25600</td>
</tr>
<tr>
<td>1</td>
<td>Wa</td>
<td>&gt;25600</td>
</tr>
<tr>
<td>1</td>
<td>S12</td>
<td>3200</td>
</tr>
<tr>
<td>2</td>
<td>S2</td>
<td>≥25600</td>
</tr>
<tr>
<td>2</td>
<td>AK26</td>
<td>12800</td>
</tr>
<tr>
<td>2</td>
<td>HN126</td>
<td>6400</td>
</tr>
<tr>
<td>2</td>
<td>DS-1</td>
<td>≥25600</td>
</tr>
<tr>
<td>3</td>
<td>VO</td>
<td>6400</td>
</tr>
<tr>
<td>3</td>
<td>S3</td>
<td>3200</td>
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<tr>
<td>3</td>
<td>AK35</td>
<td>3200</td>
</tr>
<tr>
<td>3</td>
<td>P2</td>
<td>12800</td>
</tr>
<tr>
<td>3</td>
<td>P</td>
<td>≥25600</td>
</tr>
<tr>
<td>4</td>
<td>Hochi</td>
<td>6400</td>
</tr>
<tr>
<td>4</td>
<td>Hoso-</td>
<td>kawa</td>
</tr>
<tr>
<td>9</td>
<td>W161</td>
<td>3200</td>
</tr>
<tr>
<td>Asymptomatic strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M37</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>10.76</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>McN13</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>ST #3</td>
<td>100</td>
</tr>
<tr>
<td>Reassortants</td>
<td>C15</td>
<td>3200</td>
</tr>
<tr>
<td></td>
<td>Cl48</td>
<td>3200</td>
</tr>
</tbody>
</table>

Functional groups of MAbs

* Neutralizing titre is expressed as the reciprocal of the highest dilution of ascitic fluid that reduced a fluorescent focus count by more than 60%. A neutralizing titre not less than one-eighth of the titre of the strain used for immunization (KU: MAbs KU-10H, KU-2A, KU-7E, KU-10C and KU-12H; S3: S3-5E and S3-2C) was regarded as indicating significant neutralization.

† Neutralizing titre of < 100.

‡ NT, Not tested.

YO-1S3, YO-2C2 and ST-1F2). These anti-VP4 MAbs also neutralized W161. However, with the exception of KU-6B11, they had no neutralizing activity to 69M or RRV (data not shown). KU-6B11 is considered to be a peculiar member of group 3 because it manifested neutralization activity against animal rotaviruses (SA11, RRV and NCDV) and 69M. As seen for the MAbs produced in the present study, none of the previous MAbs neutralized strain K8 (Taniguchi et al., 1987).

The binding reactivity patterns of the MAbs, except for KU-7E with too low an antibody titre, were examined by ELISA (Table 2). Four MAbs (KU-10H, KU-2A, S3-2C and KU-12H) exhibited a broader reactivity with rotavirus strains in ELISA than in neutralization tests, which was also observed previously for some MAbs employed in this study (KU-4D7, KU-6B11, YO-2C2 and YO-1S3). KU-2A and KU-10H MAbs of group 1 both reacted with all asymptomatic strains; another group 1 MAb KU-4D7 had a slight reactivity with M37 and ST #3 (data not shown). Group 2 MAb S3-5E and group 3 MAbs KU-10C and ST-1F2 showed the same reactivity patterns as those observed in neutralization tests. Group 3 MAbs S3-2C, KU-6B11, YO-2C2 and YO-1S3 reacted with all symptomatic and asymptomatic HRVs (except K8) including strains 69M and W161. Group 4 MAb KU-12H reacted with symptomatic HRVs of serotypes 1, 3 and 4, strain W161 and all asymptomatic HRVs. Only four MAbs, KU-6B11, YO-2C2, YO-1S3 and S3-2C reacted with strain 69M and simian rotaviruses SA11 and RRV, while KU-6B11 and YO-2C2 also reacted with NCDV (data not shown). However, no MAbs employed in this study reacted with symptomatic HRV strain K8 in ELISA (data not shown).

Epitope mapping

In order to construct an operational map of VP4, seven antigenic mutants resistant to the individual N-MAbs were selected from strain KU. Each mutant was
Fig. 1. Immunoprecipitation by MAbs of HRV (strain KU) proteins from [35S]methionine-labelled infected lysate. Lane 1, anti-HRV rabbit antiserum; lane 2, KU-10H; lane 3, KU-2A; lane 4, KU-7E; lane 5, KU-10C; lane 6, S3-2C; lane 7, S3-5E; lane 8, KU-12H; lane 9, normal culture fluid of myeloma cells (PAI). In lane 1, one of the two bands between VP2 and VP3 may be VP3, but the other band is thought to be an artefact due to the partial degradation from VP1, VP2 or VP3. Faint double bands found in lanes 5, 6 and 7 may have arisen from non-specific precipitation.

Table 2. Reactivity patterns of cross-reactive N-MAbs against rotaviruses as determined by ELISA

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Rotavirus strains</th>
<th>ELISA* test with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KU-10H (1)†</td>
<td>KU-2A (1)</td>
</tr>
<tr>
<td>Symptomatic strains</td>
<td></td>
<td>S3-5E (2)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>KU-10C (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S3-2C (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KU-12H (4)</td>
</tr>
<tr>
<td>1</td>
<td>KU</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Wa</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>S12</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>S2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AK26</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HN126</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>YO</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AK35</td>
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</tr>
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</tr>
<tr>
<td>4</td>
<td>Hochi</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Hoso-kawa</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>69M</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>Wi61</td>
<td>+</td>
</tr>
<tr>
<td>Asymptomatic strains</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>M37</td>
<td>+</td>
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<tr>
<td>2</td>
<td>10.76</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>McN13</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>ST #3</td>
<td>+</td>
</tr>
</tbody>
</table>

* Result is shown as + or -; +, a positive reaction in which the sum of the A492 × 1000 for two test wells minus the sum of the A492 × 1000 for two control wells is more than 300; -, value is less than 300.
† Number in parentheses indicates the functional group of the MAb as shown in Table 1.
designated by a V followed by the designation of the N-MAb used for its selection. Cross-neutralization tests were carried out by using a total of 12 pairs of MAbs and resistant mutants including five pairs which had been prepared previously (Taniguchi et al., 1988 b). The results are shown in Table 3. A mutant was regarded as resistant to a MAb when the neutralization titre of the MAb to the mutant was less than one-sixteenth the titre to the parent strain KU. Reciprocal (two-way) and non-reciprocal (one-way) reactivity patterns were found in pairwise combinations (Table 3). For example, non-reciprocal reactions were noted between KU–10C, KU–12H and S3–5E paired with S3–2C. Variant V–S3–2C was resistant to KU–10C, KU–12H and S3–5E antibodies as well as the homologous S3–2C antibody, while V–KU–10C, V–KU–12H and V–S3–5E were neutralized by S3–2C antibody. When reciprocal or non-reciprocal interaction was observed between two pairs of MAbs and the resistant mutant, the two epitopes recognized by the corresponding MAbs were constructed to overlap operationally. Thus, employing the procedure described by Yewdell & Gerhard (1981), we considered an operational map of cross-reactive neutralization epitopes on VP4 as shown schematically in Fig. 2. The degree of overlapping of circles representing each MAb epitope was determined by the operational relationship of epitopes interpreted from Table 3. For example, it can be understood from the results shown in Table 3 that the KU–2A epitope overlaps with the ST–1F2, KU–12H, S3–5E, KU–4D7, KU–7E and KU–10H epitopes. However, the KU–4D7, KU–7E and KU–10H epitopes do not overlap with the ST–1F2 and KU–12H epitopes, whereas the S3–5E epitope also overlaps with epitopes of KU–10C and S3–2C. In the epitope map constructed, therefore, the KU–2A epitope is included in the S3–5E epitope and overlaps with the KU–12H and ST–1F2 epitopes, but not with those of S3–2C and KU–10C. On the other hand, the KU–2A epitope includes epitopes for KU–4D7, KU–7E and KU–10H which do not overlap with epitopes for ST–1F2 and KU–12H (Fig. 2).
Table 4. Nucleotide and amino acid sequence changes found in antigenic mutants of strain KU selected with anti-VP4 N-MAbs

<table>
<thead>
<tr>
<th>Selecting MAb (functional group)</th>
<th>Antigenic mutant</th>
<th>Codon change</th>
<th>Amino acid change</th>
<th>Epitope region</th>
</tr>
</thead>
<tbody>
<tr>
<td>KU-2A (1)</td>
<td>V-KU-2A</td>
<td>AGT to CGT (1291)*</td>
<td>Ser to Arg (428)†</td>
<td>C3</td>
</tr>
<tr>
<td>KU-10H (1)</td>
<td>V-KU-10H</td>
<td>AGT to AGG (1293)</td>
<td>Ser to Arg (428)†</td>
<td>C3</td>
</tr>
<tr>
<td>KU-7E (1)</td>
<td>V-KU-7E</td>
<td>GAG to AAG (1306)</td>
<td>Glu to Lys (433)</td>
<td>C3</td>
</tr>
<tr>
<td>S3-5E (2)</td>
<td>V-S3-5E</td>
<td>GAG to GAT (1308)</td>
<td>Glu to Asp (433)</td>
<td>C3</td>
</tr>
<tr>
<td>KU-10C (3)</td>
<td>V-KU-10C</td>
<td>GCA to GTA (1184)</td>
<td>Ala to Val (392)</td>
<td>C2</td>
</tr>
<tr>
<td>KU-12H (4)</td>
<td>V-KU-12H</td>
<td>GAC to AAC (1162)</td>
<td>Asp to Asn (385)</td>
<td>C2</td>
</tr>
</tbody>
</table>

* Number in parentheses shows position of nucleotide substitution.
† Number in parentheses shows position of amino acid substitution.

The epitopes recognized by the 12 N-MAbs made up three antigenic regions, C1, C2 and C3. C1 represented the YO-2C2 epitope. The C3 region was recognized by the MAbs of functional groups 1 and 2, and the C2 region was recognized by MAbs of groups 3 and 4, except for YO-2C2. The C2 and C3 regions were closely related to each other and constituted a single antigenic site. By contrast, the C1 region had no operational overlapping with C2 or C3.

Identification of amino acid substitutions of antigenic mutants

In order to determine the location of the neutralization epitopes recognized by the anti-VP4 N-MAbs, the complete VP4 genes of antigenic mutant strains were sequenced. Table 4 shows the nucleotide changes and substitutions of the deduced amino acids detected in the six mutants examined. Two mutants, V-KU-7E and V-S3-5E, selected with the MAbs recognizing the C3 region, both sustained a base substitution in the codon for amino acid 433 although the resulting deduced amino acids were different from each other. V-KU-2A and V-KU-10H, selected by other MAbs recognizing C3, had identical amino acid changes at position 428. Amino acid substitutions at positions 392 and 385 were found in mutants V-KU-10C and V-KU-12H, respectively, which were selected by MAbs directed at the C2 region.

Discussion

The antigenic structure of rotavirus VP4 on simian SA11 (Burns et al., 1988) and RRV (Shaw et al., 1986) has been studied by competitive binding assays among MAbs, cross-neutralization tests between MAbs and resistant mutants and determination of the amino acid sequences of the mutants. However, the VP4 antigenic structure of the simian rotaviruses seems to be different from that of HRV since only a few anti-VP4 MAbs against simian rotavirus possess limited cross-reactivity with HRVs (Burns et al., 1988; Mackow et al., 1988b). By contrast, all of the anti-VP4 MAbs produced in the present study have extensive cross-reactivity among HRV strains. In addition, they are human-specific and KU-6B11 MAb is the only exception which neutralized SA11 and RRV. These observations are supported by VP4 amino acid sequence comparisons (Gorziglia et al., 1988; Nishikawa et al., 1988; Mackow et al., 1988b) in which the amino acid identity of VP4 between human and animal rotaviruses is low, while VP4 amino acid sequences are highly conserved among symptomatic HRVs. Therefore, HRV strains have been used to study the cross-reactive neutralization antigens on VP4.

In this study, we employed a total of 12 N-MAbs directed to VP4, but we could not obtain any serotype-specific or strain-specific N-MAbs directed against VP4. This result is reasonable, considering the sequence analysis of VP4 from numerous HRV strains with different serotype specificities. VP4 amino acid sequences of symptomatic HRV strains with serotypes 1, 3 and 4 are highly related (93-96% identity), whereas symptomatic serotype 2 strains possess VP4s only moderately related to symptomatic HRV strains with serotypes 1, 3 and 4 (89-90% identity; Gorziglia et al., 1988). Moreover, asymptomatic strains which possess the highly related VP4 amino acid sequence (95-97% homology) among four serotypes have VP4 sequences distinct from those of symptomatic rotaviruses (75-80% homology).
These sequence data reflect the reactivity patterns of 12 N-MAbs obtained in this and previous studies: four antibodies had neutralization activity with serotypes 1 to 4 symptomatic strains and six antibodies reacted with serotype 1, 3 and 4 symptomatic strains. By these serological and genetic analyses, antigenic specificity of VP4 is thought to be independent of that of VP7, although co-segregation between VP7 and VP4 has been detected in symptomatic serotype 2 HRV strains (Gorziglia et al., 1988; Taniguchi et al., 1987).

None of the 12 N-MAbs employed in this study reacted with strain K8 in neutralization tests or ELISA. This result implies that the VP4 antigenic structure of strain K8 is quite different from those of other HRV strains. Indeed, the amino acid sequence of the VP4 of strain K8 has a low identity (60% to 70%) to those of other representative HRV strains (Taniguchi et al., 1989). The preparation of N-MAbs directed to VP4 of K8 is in progress in our laboratory. Although VP4 sequence data for 69M are not yet available, it may also have a unique VP4 sequence since 69M was not reactive with any N-MAbs examined except KU-6B11. By contrast, WI61 reacted with all 12 N-MAbs. This suggests that WI61 (and probably other serotype 9 strains) has a VP4 structure antigenically and genetically similar to those of serotype 1, 3 and 4 strains.

Eight MAbs used in this study showed broader cross-reactivities in ELISA than those in neutralization tests. The difference in reactivity between ELISA and neutralization tests may be explained by the "two step theory" of virus neutralization, originally suggested by studies on adenovirus (Kjellén, 1962) and herpes simplex virus (Yoshino & Taniguchi, 1965). The process of virus neutralization is considered to be made up of at least two stages; an antibody binds to an epitope without causing virus neutralization in the initial stage, then the virus-antibody combination becomes irreversible in the next stage in which the virus is completely neutralized. It is conceivable that certain epitopes on some rotavirus strains may remain bound to an antibody with too weak a binding energy to lead to virus neutralization. This may explain the discrepant reactivity of some MAbs in the two tests.

We previously identified three independent cross-reactive neutralization epitopes on VP4 of HRV: epitope I defined by YO-2C2 antibody, epitope II defined by YO-1E6, ST-1F2 and KU-6B11 antibodies and epitope III defined by KU-4D7 antibody (see Introduction). In the present study using seven more N-MAbs and the corresponding resistant mutants, we confirmed the presence of the three antigenic regions (C1, C2 and C3) represented by the previously defined three independent epitopes. However, we discovered that regions C2 and C3 are closely related to each other, but that region C1 is independent. Furthermore, as seen in the operational map (Fig. 2), a general correlation was observed between the cross-reactivity patterns of MAbs and antigenic regions recognized by the MAbs; the MAbs belonging to functional group 1 recognized the C3 region, most of the MAbs of functional group 3 are directed to the C2 region and only MAb YO-2C2 recognized the independent antigenic region C1. MAb KU-12H which was found to recognize the C2 region had a moderately different cross-reactivity from other MAbs recognizing C2, but its reactivity in ELISA was the same as MAb KU-10C, one of the MAbs recognizing C2. However, MAb S3-5E which possessed a different cross-reactivity was found to be directed to the C3 region.

The above mentioned correlations of MAbs to epitope regions were further elucidated by the analysis of amino acid substitution sites of MAb escape mutants. Some of the amino acid positions critical to each epitope have previously been identified: amino acid residue 305 is critical to epitope I (included in the C1 region defined in this study), residue 392 or residue 439 to epitope II (included in the C2 region), and residue 433 to epitope III (included in the C3 region) (Taniguchi et al., 1988b). Of the six amino acid substitution sites that were detected in the present study, three positions were consistent with those previously identified; V-KU-10C sustained an amino acid change at position 392 (epitope II), V-KU-7E and V-S3-5E at position 433 (epitope III), although V-KU-10C and V-S3-5E had a substituted amino acid at the same position which was different from that in the previous report. However, an amino acid change on VP4 of V-KU-2A and V-KU-10H selected by MAbs KU-2A and KU-10H, respectively and recognizing the C3 region, was detected at position 428. This is a novel site, though it is close to another position of epitope III residue (433). Moreover, another new position, 385, was detected in the mutant V-KU-12H selected by MAb KU-12H recognizing the C2 region. These results indicate that amino acid positions 385 and 392 are critical for MAbs recognizing the C2 region and that positions 433 or 428 are critical for MAbs recognizing the C3 region on VP4 of HRV strain KU.

It was of interest that amino acid substitution sites 388 and 393 are detected in mutants of RRV (Mackow et al., 1988b). These positions correspond to positions 387 and 392, respectively on the VP4 sequence of HRV, because simian rotavirus possesses an extra amino acid at position 125 (Gorziglia et al., 1986). These findings suggest that amino acid residues 385 to 392 on VP4 are involved in cross-neutralization in both HRV and RRV. It is difficult to explain why different neutralization activities of MAbs ST-1F2, KU-12H and S3-5E were observed against two resistant mutants, V-KU-2A and
V-KU-10H (Table 3), which possessed the same amino acid change. There is a possibility that this finding is due to a conformational change of epitopes on VP4 caused by another amino acid change in the other surface protein VP7, although no evidence was published for the interaction between VP7 and VP4.

The evident operational overlapping between the C2 and C3 regions in spite of the distant location of the identified amino acid positions critical to these two epitope regions may be interpreted as follows; (i) the two antigenic regions are in close proximity on the folded VP4 or (ii) the amino acid change for escaping the recognition from a given N-MAb causes the allosteric effect which affects binding with another N-MAb. Similar observations have been shown in VP7 neutralization epitopes of RRV (Mackow et al., 1988a), SA11 (Dyall-Smith et al., 1986), and HRV (Morita et al., 1988). Morita et al. (1988) suggested the presence of at least five operationally related serotype 1-specific neutralization epitopes on VP7, which collectively form a large antigenic site. Amino acid positions critical for neutralization on VP7 of HRV strain KU were found in two variable regions which were located separately in the linear sequence. These were regions B (amino acids 87 to 101) and E (amino acids 208 to 221) (Taniguchi et al., 1988a). Thus, antigenic regions on VP4 and VP7 of HRV appear to be generally composed of multiple neutralization epitopes which are operationally related. However, in comparison to the VP7 epitopes, cross-reactive epitopes on VP4 may be less conformational because most N-MAbs to VP4 readily immunoprecipitated SDS-denatured VP4 and a synthetic peptide corresponding to one epitope (epitope I) can be recognized by the N-MAb YO-2C2 (Taniguchi et al., 1988b). Moreover, epitope I (amino acid 305) is located at some distance from epitope II and III and epitope region C1 has no operational overlapping with regions C2 or C3. These results suggest that out of the three cross-reactive neutralization epitope regions, C1 exists as a sequential antigenic site. These findings on the cross-reactive neutralization epitopes on VP4 may be significant for the development of a rotavirus vaccine.

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References


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