

Identification of Cross-Reactive and Serotype 2-Specific Neutralization Epitopes on VP3 of Human Rotavirus

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The group A rotaviruses are composed of at least seven serotypes. Serotype specificity is defined mainly by an outer capsid protein, VP7. In contrast, the other surface protein, VP3 (775 amino acids), appears to be associated with both serotype-specific and heterotypic immunity. To identify the cross-reactive and serotype-specific neutralization epitopes on VP3 of human rotavirus, we sequenced the VP3 gene of antigenic mutants resistant to each of seven anti-VP3 neutralizing monoclonal antibodies (N-MAbs) which exhibited heterotypic or serotype 2-specific reactivity, and we defined three distinct neutralization epitopes on VP3. The mutants sustained single amino acid substitutions at position 305, 392, 433, or 439. Amino acid position 305 was critical to epitope I, whereas amino acid position 433 was critical to epitope III. In contrast, epitope II appeared to be more dependent upon conformation and protein folding because both amino acid positions 392 and 439 appeared to be critical. These four positions clustered in a relatively limited area of VP5, the larger of the two cleavage products of VP3. At the positions where amino acid substitutions occurred, there was a correlation between amino acid sequence homology among different serotypes and the reactivity patterns of various viruses with the N-MAbs used for selection of mutants. A synthetic peptide (amino acids 296 to 313) which included the sequence of epitope I reacted with its corresponding N-MAb, suggesting that the region contains a sequential antigenic determinant. These data may prove useful in current efforts to develop vaccines against human rotavirus infection.

Human rotavirus (HRV) is the major etiologic agent of severe gastroenteritis in infants and young children worldwide (3, 5, 15). HRV is particularly important in less-developed countries where diarrhea is a major cause of death, and HRV is known to be associated with severe episodes of diarrhea which, if untreated, can be fatal (2). Thus, the need for an effective vaccine is quite clear.

Rotaviruses (RVs) have two outer capsid proteins, VP3 and VP7, which are involved in neutralization (9, 11, 24). Independent segregation of the neutralization specificities of VP3 and VP7 was demonstrated previously by analysis of RV reassortants (11). Furthermore, these two proteins are independently capable of inducing a protective immune response in experimental animals which confers resistance against RV challenge (19, 21).

Extensive cross-neutralization tests have identified at least seven serotypes among group A RV (12). This serotype specificity is defined mainly by an outer capsid protein, VP7, which is encoded by RNA segment 8 or 9 (13, 22). In contrast, there is also the following evidence that one or more cross-reactive neutralization antigenic sites are present among heterotypic RVs. (i) Hyperimmune or convalescent-phase serum from animals or humans immunized with some RV strains often neutralizes RVs belonging to one or more different serotypes, although the heterotypic titers are low (1, 5, 29). (ii) Heterotypic cross-protection against diarrhea can be induced by active or passive immunization (16, 27, 30). Hence, it would be of interest to define the structure of the cross-reactive antigenic site(s) since this may allow us to formulate a broadly reactive protective RV vaccine. Recent

studies have begun to elucidate the role of VP3 in heterotypic reactivity. Recently, a number of anti-VP3 neutralizing monoclonal antibodies (N-MAbs) have been produced which react with heterotypic virulent HRVs or only with virulent HRV serotype 2 strains (23, 24). In addition, a relatively high degree of homology of VP3 amino acid sequence has been demonstrated among different HRV strains belonging to serotypes 1, 3, and 4 (7, 8; M. Gorziglia et al., submitted for publication). These various observations suggest that VP3 may be responsible for some of the heterotypic immunity or cross-protection previously described.

We previously prepared (i) six anti-VP3 N-MAbs capable of neutralizing virulent HRV strains belonging to serotypes 1 through 4 or serotypes 1, 3, and 4 and (ii) antigenic mutants resistant to each of the six N-MAbs (23). The pattern of neutralization of the antibody-selected mutants by the panel of N-MAbs suggested the presence of three distinct cross-reactive neutralization epitopes on HRV VP3 (23). In the present study, we identified the location of cross-reactive and serotype 2-specific neutralization epitopes on the HRV VP3 by sequence analysis of the VP3 gene of antigenic mutants selected with N-MAbs which exhibited different reactivity patterns with HRV strains.

MATERIALS AND METHODS

Virus. Strain KU (serotype 1) and DS-1 (serotype 2) of HRV and strain MMU18006 of rhesus RV (RRV) (serotype 3) were used to produce the antigenic mutants. KU antigenic mutants were selected with each of six anti-VP3 cross-reactive N-MAbs (KU-4D7, KU-6B11, YO-1E6, YO-1S3, YO-2C2, and ST-1F2) and characterized serologically (23). We used several tubes for each selection as described previously (23) and isolated two or three independently selected clones for each mutant. A DS-1 antigenic mutant

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resistant to serotype 2-specific anti-VP3 N-MAb (S2-2F2) and an RRV mutant resistant to KU-6B11 antibody were also selected as described previously (23, 24). Each mutant was designated by a V followed by the designation of the N-MAb used for its selection. The viruses were propagated in MA-104 cells in the presence of trypsin (1 μ g/ml) and harvested 2 or 3 days after infection.

Neutralization test. The following 20 HRV strains were used to examine the neutralizing reactivity of the N-MABs: Wa, KU, K8, S12, and M37 (serotype 1); DS-1, HN-126, S2, P1, AK26, and 1076 (serotype 2); P, YO, S3, P2, AK35, and McN13 (serotype 3); and Hochi, Hosokawa, and Saint Thomas 3 (ST-3; serotype 4). Four of these strains, M37, 1076, McN13, and ST-3, were recovered from neonates undergoing asymptomatic infection. A fluorescent-focus neutralization test or plaque neutralization test was performed as described previously (12, 26).

Preparation of viral mRNA. Single-shelled particles obtained by treatment of concentrated virions with EDTA were purified by CsCl gradient centrifugation. Viral mRNA was synthesized *in vitro* by the endogenous transcriptase in viral cores and was freed of double-stranded RNA by precipitation with 2 M lithium chloride (6).

Nucleotide sequence analysis. Oligonucleotide primers complementary to the mRNA of gene 4 of Wa or RV-5 virus (14) at intervals of 200 to 300 nucleotides were synthesized on an Applied Biosystems synthesizer. These primers were used to sequence mRNA by reverse transcription in the presence of dideoxynucleotides as previously described (8). Briefly, a mixture of the primer and RV mRNA was heated at 95°C for 1 min and allowed to anneal at 42°C for 4 min. Primer extension was performed in a set of four reverse transcription reactions containing 3 μ M dATP; 50 μ M each dCTP, dGTP, and dTTP; 1 U of reverse transcriptase (Molecular Genetics Resources); 1.5 μ Ci of [α -³²P]dATP; and the appropriate dideoxynucleoside triphosphate. Reaction mixtures were incubated at 42 to 45°C for 30 min, combined with 10 μ l of sequencing gel sample buffer, heated at 95°C for 3 min, and analyzed on 6% sequencing gels. The 3'-terminal 80 nucleotides of the viral RNA were determined by using denatured double-stranded RNA.

Preparations of synthetic peptides. The synthetic peptide and the peptide-keyhole limpet hemocyanin conjugate were prepared as previously described (18).

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed as follows. Polyvinyl microtiter plates were coated with free synthetic peptide (5 μ g per well) and incubated overnight at 37°C. After being washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST), the wells were coated with 10% fetal calf serum and subsequently washed thoroughly. Samples (50 μ l) of various dilutions of ascitic fluid were then placed in the wells and incubated for 2 h at 37°C. After being washed with PBST, the samples were incubated for a further 2 h with alkaline-phosphatase-conjugated goat anti-mouse immunoglobulin G. Finally, 100 μ l of a *p*-nitrophenylphosphoric acid disodium salt solution was added. Values over 300, when expressed as the sum of the $A_{405} \times 1,000$ for two wells, were considered positive.

RESULTS

Identification of cross-reactive neutralization epitopes on VP3 of HRV. The complete nucleotide sequence of the VP3 gene of the KU strain (serotype 1) was determined by primer extension. The gene contained 2,359 base pairs with 5'- and

3'-noncoding regions of 9 and 25 nucleotides, respectively. The gene contained an open reading frame of 2,325 bases capable of coding for a protein of 775 amino acids. Figure 1 shows the nucleotide sequence and the deduced amino acid sequence of the VP3 gene of strain KU.

Previous analysis of the six N-MABs in neutralization tests with antigenic mutants selected with these N-MABs identified three distinct cross-reactive neutralization epitopes on VP3 (23): epitope I (defined by YO-2C2 antibody), epitope II (defined by KU-6B11, YO-1E6, YO-1S3, and ST-1F2 antibodies), and epitope III (defined by KU-4D7 antibody). We sought to determine the location of the cross-reactive neutralization epitopes on VP3 by sequencing the VP3 gene of the six antigenic mutants. Only a single nucleotide base change was found on the entire VP3 gene in each mutant. Table 1 shows the nucleotide and deduced amino acid sequence changes in the mutants. V-YO-2C2 (epitope I) or V-KU-4D7 (epitope III) sustained a base substitution in the codon for amino acid 305 (Leu to Pro) or 433 (Glu to Lys), respectively. Three mutants, V-KU-6B11, V-YO-1S3, and V-ST-1F2, selected by N-MABs recognizing epitope II had an identical amino acid change at position 392 (Ala to Thr). However, V-YO-1E6, which was also resistant to the N-MAB directed against epitope II, sustained an amino acid substitution at a different position (amino acid 439; Leu to Ser). In addition, an RRV mutant selected with KU-6B11 antibody (epitope II) had a single base substitution (AGA to GGA) in the codon for amino acid 441 (Arg to Gly). Two or three clones independently selected with the same N-MABs were sequenced, and the same amino acid substitution was found at the same position in all clones derived from each mutant. Thus, each of the six mutants sustained only one amino acid substitution, and these substitutions were clustered in a relatively limited area of VP5, which is the larger of the two cleavage products of VP3.

The amino acid sequence of the VP3 gene of virulent and asymptomatic HRV strains of serotypes 1, 2, 3, and 4 was recently determined (8; Gorziglia et al., submitted). These sequences were analyzed in the context of the amino acid substitutions identified in the various N-MAB escape mutants and the cross-reactivity patterns of the N-MABs with virulent and asymptomatic HRV strains of different serotypes. Sequence of the various HRV strains of serotype 1, 2, 3, or 4 in the regions identified as critical to epitopes I, II, or III exhibited a correlation to the reactivity of these viruses with N-MABs.

Thus, amino acid Leu at position 305 appears to be critical to epitope I because N-MAB YO-2C2 selected an escape mutant of KU with an amino acid substitution at this position. Leu is conserved in each of the other HRVs which react with this N-MAB (i.e., virulent HRV serotypes 1, 3, and 4 and asymptomatic HRV serotypes 1 through 4), whereas the two virulent HRV serotype 2 viruses which do not react with this N-MAB possess another amino acid at position 305 (Fig. 2). Similarly amino acids Ala at position 392 and Leu at position 439, which appear to be critical to epitope II, are conserved among HRVs (strains of virulent serotypes 1, 3, and 4 and avirulent serotypes 1 through 4) reactive with the N-MABs used to select epitope II escape mutants, whereas the two virulent HRV serotype 2 viruses and one virulent HRV serotype 4 strain (VA70) which do not react with these N-MABs exhibit divergence of sequence in one or both of these two positions. Finally, amino acid 433 (Glu), which is critical to epitope III (as indicated by a sole amino acid substitution at this site in an epitope III escape mutant V-KU-4D7), is present on virulent HRV serotype 1,

12 24 36 48 60 72 84 96 108 120
 GGC TAT AAA ATG GCT TCG CTC ATT TAT AGA CAG CTT CTC ACT AAT TCA TAT TCA GTA GAT TTA CAT GAT GAA ATA GAG CAA ATT GGG TCA GAA AAA ACT CAA AAC GTA ACT GTA AAT CCA
 MET Ala Ser Leu Ile Tyr Arg Gln Leu Leu Thr Asn Ser Tyr Ser Val Asp Leu His Asp Glu Ile Glu Gln Ile Gly Ser Glu Lys Thr Gln Asn Val Thr Val Asn Pro

132 144 156 168 180 192 204 216 228 240
 GGT CCA TTT GCC CAA ACT AGA TAT GCT CCA GTA AAT TGG GGT CAT GGA GAG ATA AAT GAT TCA ACC ACA GTA GAA CCA ATT TTA GAT GGT CCT TAT CAG CCT ACT ACA TTT AAA CCA CTT
 Gly Pro Phe Ala Gln Thr Arg Tyr Ala Pro Val Asn Trp Gly His Gly Glu Ile Asn Asp Ser Thr Thr Val Glu Pro Ile Leu Asp Gly Pro Tyr Gln Pro Thr Thr Phe Lys Pro Leu

252 264 276 288 300 312 324 336 348 360
 ACT GAT TAT TGG ATA CTT ATT AAC TCA AAT ACA AAT GGA GTG GTA TAC GAG AGT ACG AAT AAT AGT GAC TTT TGG ACT GCA GTA GTT GCT ATT GAA CCG CAC GTT ATC CAA GTA GAT AGA
 Thr Asp Tyr Trp Ile Leu Ile Asn Ser Asn Thr Asn Gly Val Val Tyr Glu Ser Thr Asn Asn Ser Asp Phe Trp Thr Ala Val Val Ala Ile Glu Pro His Val Ile Gln Val Asp Arg

372 384 396 408 420 432 444 456 468 480
 CAA TAT ACT GTA TTT GGT GAA AAT AAA CAA TTT AAT GTA AGA AAT GAT TCA GAT AAA TGG AAG TTT TTA GAA ATG TTT AGA GGC AGT AGT CAA AAT GAA TTT TAT AAT AGA CGT ACA CTA
 Gln Tyr Thr Val Phe Gly Glu Asn Lys Gln Phe Asn Val Arg Asn Asp Ser Asp Lys Trp Lys Phe Leu Glu MET Phe Arg Gly Ser Ser Glu Asn Glu Phe Tyr Asn Arg Thr Leu

492 504 516 528 540 552 564 576- 588 600
 ACT TCT GAT ACT AAA CTC GTA GGA ATA TTA AAA TAT GGT GGA AGG ATA TGG ACA TTT CAT GGT GAA ACA CCG AGA GCT ACT ACT GAT AGT TCA AAT ACT CCA AAT TTA AAC GAT ATA TCA
 Thr Ser Asp Thr Lys Leu Val Gly Ile Leu Lys Tyr Gly Gly Arg Ile Trp Thr Phe His Gly Glu Thr Pro Arg Ala Thr Thr Asp Ser Ser Asn Thr Ala Asn Leu Asn Asp Ile Ser

612 624 636 648 660 672 684 696 708 720
 ATT ATA ATA CAT TCA GAA TTT TAT ATT ATC CCA AGG TCC CAA GAA TCT AAG TGT AAT GAA TAT ATT AAC AAT GGT TTG CCA CCA ATT CAA AAT ACT AGA AAT GTA GTA CCA TTA TCA TTA
 Ile Ile Ile His Ser Glu Phe Tyr Ile Ile Pro Arg Ser Gln Glu Ser Lys Cys Asn Glu Tyr Ile Asn Asn Gly Leu Pro Ile Gln Asn Thr Arg Asn Val Val Pro Leu Ser Leu

732 744 756 768 780 792 804 816 828 840
 TCA TCT AGA TCC ATA CAG TAT AAA AGA GCA CAA GTT AAT GAA GAT ATT ACA ATT TCA AAA ACC TCA TTA TGG AAA GAA ATG CAA TGT AAT AGG GAT ATT ATA ATT AGA TTT AAA TTT GGT
 Ser Ser Arg Ser Ile Gln Tyr Lys Arg Ala Gln Val Asn Glu Asp Ile Thr Ile Ser Lys Thr Ser Leu Trp Lys Glu MET Gln Cys Asn Arg Asp Ile Ile Ile Arg Phe Lys Phe Gly

852 864 876 888 900 912 924 936 948 960
 AAT AGT ATT GTA AAA CTG GGG GGA CTA GGT TAT AAA TGG TCC GAA ATA TCA TAT AAA GCA GCA AAT TAT CAA TAT AAT TAT CTA CGT GAT GGC GAA CAA GTA ACT GCA CAT ACT ACT TGC
 Asn Ser Ile Val Lys Leu Gly Glu Leu Tyr Lys Trp Ser Glu Ile Ser Tyr Lys Ala Ala Asn Tyr Gln Tyr Asn Tyr Leu Arg Asp Gly Glu Gln Val Thr Ala His Thr Thr Cys

972 984 996 1008 1020 1032 1044 1056 1068 1080
 TCA GTA AAT GGA GTA AAT AAT TTT AGC TAC AAC GGA GGA TCT TTA CCT ACT GAT TTT AGT GTC TCA AGG TAT GAA GTT ATT AAA GAA AAT TCT TAT GTA TAT GTA GAT TAT TGG GAT GAT
 Ser Val Asn Gly Val Asn Asn Phe Ser Tyr Asn Gly Gly Ser Leu Pro Thr Asp Phe Ser Val Ser Arg Tyr Glu Val Ile Lys Glu Asn Ser Tyr Val Tyr Val Asp Tyr Trp Asp Asp

1092 1104 1116 1128 1140 1152 1164 1176 1188 1200
 TCA AAA GCA TTT AGA AAT ATG GTA TAT GTC AGA TCA TTA GCA GCT AAT TTG AAC TCA GTG AAA TGT ACA GGT GGA AGT TAT GAC TTT AGT ATA CCT GTA GGT GCA TGG CCA GTC ATG AAT
 Ser Lys Ala Phe Arg Asn MET Val Tyr Val Arg Ser Leu Ala Ala Asn Leu Asn Ser Val Lys Cys Thr Gly Ser Tyr Asp Phe Ser Ile Pro Val Gly Ala Trp Pro Val MET Asn

1212 1224 1236 1248 1260 1272 1284 1296 1308 1320
 GGT GGC GCT GTT TCG TTG CAT TTT GCT GGA GTT ACA TTA TCT ACG CAA TTC ACA GAT TTC GTA TCA TTG AAT TCA CTA CGA TTT AGA TTT AGT TTG ACA GTG GAT GAG CCA TCT TTT TCA
 Gly Gly Ala Val Ser Leu His Phe Ala Gly Val Thr Leu Ser Thr Gln Phe Thr Asp Phe Val Ser Leu Asn Ser Leu Arg Phe Arg Phe Ser Leu Thr Val Asp Glu Pro Ser Phe Ser

1332 1344 1356 1368 1380 1392 1404 1416 1428 1440
 ATA TTG ACA ACA CGT ACG GTG AAT TTG TAC GGA TTA CCA GCT GCA AAT CCA AAT AAT GGA AAT GAA TAC TAT GAA ATA TCA GGA AGG TTT TCG CTC ATT TCT TTA GTT CCA ACT AAT GAT
 Ile Leu Arg Thr Arg Thr Val Asn Leu Tyr Gly Leu Pro Ala Ala Asn Pro Asn Asn Gly Asn Glu Tyr Tyr Glu Ile Ser Gly Arg Phe Ser Leu Ile Ser Leu Val Pro Thr Asn Asp

1452 1464 1476 1488 1500 1512 1524 1536 1548 1560
 GAT TAT CAG ACT CCA ATT ATG AAT TCA GTA ACA GTA AGA CAA GAT TTA GAA CGT CAA CTT ACT GAT TTA CGA GAG GAA TTT AAT TCA TTA TCA CAA GAA ATA GCT ATG TCA CAA TTA ATT
 Asp Tyr Gln Thr Pro Ile MET Asn Ser Val Thr Val Arg Gln Asp Leu Glu Arg Gln Leu Thr Asp Leu Arg Glu Glu Phe Asn Ser Leu Ser Gln Glu Ile Ala MET Ser Gln Leu Ile

1572 1584 1596 1608 1620 1632 1644 1656 1668 1680
 GAT TTA GCG TTA TTA CCT TTA GAT ATG TTT TCT ATG TTT TCG GAA TTA AAA AGT ACA ATT GAT TTG ACT AAA TCA ATG GCA ACT AGT GTA ATG AAA AAA TTT AGA AAA TCA AAA TTA GCT
 Asp Leu Ala Leu Leu Pro Leu Asp MET Phe Ser MET Phe Ser Glu Leu Lys Ser Thr Ile Asp Leu Thr Lys Ser MET Ala Thr Ser Val MET Lys Lys Phe Arg Lys Ser Lys Leu Ala

1692 1704 1716 1728 1740 1752 1764 1776 1788 1800
 ACA TCA ATT TCA GAA ATG ACT CAT TCA TTG TCA GAC GCA CCA TCA TCA GCA TCA AGA AGC GTT TCT ATC AGA TCG AAT ATA TCC ACA ATT TCG AAT TCG AAT GTT TCA AAT GAT GTA
 Thr Ser Ile Ser Glu MET Thr His Ser Leu Ser Asp Ala Ala Ser Ser Ala Ser Arg Ser Val Ser Ile Arg Ser Asn Ile Ser Thr Ile Ser Asn Trp Thr Asn Val Ser Asn Asp Val

1812 1824 1836 1848 1860 1872 1884 1896 1908 1920
 TCA AAT GTG ACT AAT TCG TTG AGT GAT ATT TCA ACA CAA ACG TCT ACA ATC AGT AAG AAC CTT AGA TTA AAA GAA ATG ATT ACT CAA ACT GAA GGA ATG AGT TTT GAT GAT ATT TCA GCG
 Ser Asn Val Thr Asn Ser Leu Ser Asp Ile Ser Thr Gln Thr Ser Thr Ile Ser Lys Asn Leu Arg Leu Lys Glu MET Ile Thr Gln Thr Glu Gly MET Ser Phe Asp Asp Ile Ser Ala

1932 1944 1956 1968 1980 1992 2004 2016 2028 2040
 GCA GTA TTA AAA ACA AAA ATA GAT ATG TCT ACT CAA ATT GGA AAG AAT ACT TTA CCC GAT ATA GTC ACA GAG GCA TCT GAG AAA TTT ATT CCA AAA CGA TCG TAT CGA ATA TTG AAA GAT
 Ala Val Leu Lys Thr Lys Ile Asp MET Ser Thr Gln Ile Gly Lys Asn Thr Leu Pro Asp Ile Val Thr Glu Ala Ser Glu Lys Phe Ile Pro Lys Arg Ser Tyr Arg Ile Leu Lys Asp

2052 2064 2076 2088 2100 2112 2124 2136 2148 2160
 GAT GAA GTA ATG GAA ATT AAT ACT GAA GGG AAA GTC TTT GCA TAT AAA ATC GAC ACA CTT AAT GAA GTG CCA TTT GAT GTA AAT AAA TTT GCT GAA CTT GTA ACA AAT TCT CCA GTT ATA
 Asp Glu Val MET Glu Ile Asn Thr Glu Gly Lys Val Phe Ala Tyr Lys Ile Asp Thr Leu Asn Glu Val Pro Phe Asp Val Asn Lys Phe Ala Glu Leu Val Thr Asn Ser Pro Val Ile

2172 2184 2196 2208 2220 2232 2244 2256 2268 2280
 TCA GCA ATA ATC GAT TTT AAA ACA TTA AAA AAT TTG AAT GAT AAT TAT GGA ATT ACT CGA ATA GAA GCA TTA AAT TTA ATT AAA TCG AAT CCA AAT GTA TTA CGT AAT TTC ATT AAC CAA
 Ser Ala Ile Ile Asp Phe Lys Thr Leu Lys Asn Leu Asn Asp Asn Tyr Gly Ile Thr Arg Ile Glu Ala Leu Asn Leu Ile Lys Ser Asn Pro Asn Val Leu Arg Asn Phe Ile Asn Gln

2292 2304 2316 2328 2340 2352
 AAT AAT CCA ATT ATA AGG AAT AGA ATT GAA CAG CTA ATT CTA CAA TGT AAA TTG TGA GAA CGC TAT TGA GGA TGT GAC C
 Asn Asn Pro Ile Ile Arg Asn Arg Ile Glu Gln Leu Ile Leu Gln Cys Lys Leu

FIG. 1. Complete nucleotide sequence and deduced amino acid sequence of the VP3 gene of strain KU. The numbers above the sequence refer to the nucleotide positions. The amino acid residues which changed in the antigenic mutants are boxed. Cleavage sites are shown by arrows. The amino acid sequence corresponding to the region from which peptides were synthesized is underlined.

TABLE 1. Nucleotide and amino acid sequence changes found in the antigenic mutants selected with anti-VP3 N-MAbs

Selecting N-MAb		Neutralizing reactivity of N-MAbs ^a		Antigenic mutant		Codon change	Amino acid change	Epitope group ^b
Immunizing virus (serotype)	Designation	Virulent HRV	Asymptomatic HRV	Parent virus	Designation			
YO (3)	YO-2C2	Serotypes 1, 3, 4	Serotypes 1, 2, 3, 4	KU	V-YO-2C2	CTA to CCA (923) ^c	Leu to Pro (305) ^d	I
KU (1)	KU-6B11	Serotypes 1, 3, 4	Serotypes 1, 2, 3, 4	KU	V-KU-6B11	GCA to ACA (1183)	Ala to Thr (392)	II
YO (3)	YO-1S3	Serotypes 1, 3, 4	Serotypes 1, 2, 3, 4	KU	V-YO-1S3	GCA to ACA (1183)	Ala to Thr (392)	II
ST-3 (4)	ST-1F2	Serotypes 1, 3, 4	Serotypes 1, 2, 3, 4	KU	V-ST-1F2	GCA to ACA (1183)	Ala to Thr (392)	II
KU (1)	KU-4D7	Serotypes 1, 2, 3, 4	None	KU	V-KU-4D7	GAG to AAG (1306)	Glu to Lys (433)	III
YO (3)	YO-1E6	Serotypes 1, 3, 4 ^e	Serotypes 1, 2, 3, 4	KU	V-YO-1E6	TTG to TCG (1325)	Leu to Ser (439)	II
S2 (2)	S2-2F2	Serotype 2	None	DS-1	V-S2-2F2	AAA to GAA (1183)	Lys to Glu (392)	II
KU (1)	KU-6B11	Serotypes 1, 3, 4	Serotypes 1, 2, 3, 4	RRV	V-KU-6B11 (RRV)	AGA to GGA (1330)	Arg to Gly (441)	II

^a Neutralizing reactivity of N-MAbs was determined by fluorescent-focus reduction or plaque reduction assay against 16 virulent and 4 asymptomatic HRV strains.

^b Epitope group was defined by cross-neutralization tests between N-MAbs and their antigenic mutants.

^c Number in parentheses shows position of nucleotide substitution.

^d Number in parentheses shows position of amino acid substitution.

^e YO-1E6 antibody neutralized two serotype 4 virulent HRV strains (Hochi and Hosokawa) but not one strain (VA70).

2, 3, and 4 strains that react with the epitope III N-MAb KU-4D7. It should be noted that the asymptomatic HRV strains of serotype 1 through 4 which do not react with this N-MAb also possess the conserved Glu at position 433; however, these viruses exhibit sequence divergence at position 432, which may explain their lack of reactivity.

Antigenicity of synthetic peptides which correspond to putative epitopes. The preceding observations are compatible with the view that the region in which amino acid substitutions occurred in the escape mutants is the actual binding site of the antibody. This hypothesis was evaluated by preparing three synthetic peptides corresponding to the regions around the position where each mutant had sustained its amino acid substitution and examining the reactivity of the N-MAbs with the peptides by ELISA (Table 2). N-MAb YO-2C2, which defines epitope I, exhibited specific binding to synthetic peptide 1 (amino acids 296 to 313) which included amino acid 305, which was previously shown to be critical to epitope I. However, the other two peptides were not recognized by their corresponding N-MAbs. As a positive control reaction, guinea pig antisera to the synthetic peptides were used in the ELISA. They reacted specifically with the corresponding peptides.

Identification of a serotype 2-specific neutralization epitope on VP3 of HRV. We previously obtained several anti-VP3 serotype 2-specific N-MAbs by using strain S2 (serotype 2) as the immunizing antigen (24). One (S2-2F2 antibody) of these N-MAbs was used for selecting an escape mutant from strain DS-1 (serotype 2). Sequence analysis of the VP3 gene of the parent DS-1 and its escape mutant V-S2-2F2 showed that the latter had undergone a single amino acid substitution at position 392 (Lys to Glu). Lysine or glycine was present at position 392 of the two serotype 2 virulent strains, but a different amino acid (Ala) was conserved in the corresponding position in the virulent serotype 1, 3, and 4 strains, as well as in the asymptomatic serotype 1 through 4 strains.

DISCUSSION

At least four serotypes of HRV have been recognized by cross-neutralization tests (12, 25, 28). Serotype specificity is determined mainly by epitopes on the outer capsid glycoprotein VP7 (13). On the other hand, cross-reactive antigenic sites which react with antibodies induced by heterotypic HRV strains have been described. VP3, the other surface protein of RV, appears to be responsible for some of this

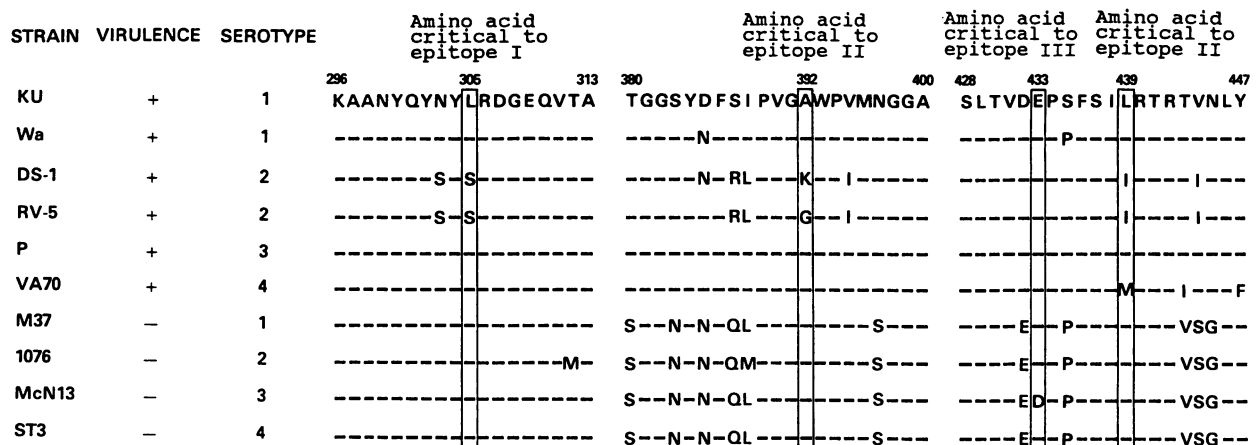


FIG. 2. Comparison of amino acid sequences among serotype 1 through 4 HRV strains in the region in which amino acid substitutions were found in the antigenic mutants. The entire VP3 amino acid sequence of RV-5 has been previously described (14). The entire VP3 amino acid sequence of the other strains will be described elsewhere (Gorziglia et al., submitted). Virulence refers to the status of the individual from whom the HRV strain was isolated.

TABLE 2. Reactivity of synthetic peptides with anti-VP3 cross-reactive N-MAbs

Antibody	ELISA titer ^c		
	Peptide 1 (296-313) ^b	Peptide 2 (383-400)	Peptide 3 (428-445)
Ascitic fluids			
YO-2C2	3,200	<200	<200
KU-6B11	<200	<200	<200
YO-1S3	<200	<200	<200
ST-1F2	<200	<200	<200
KU-4D7	<200	<200	<200
YO-1E6	<200	<200	<200
Guinea pig sera ^c			
Anti-peptide 1			
Pre-immune	<200	<200	<200
Hyperimmune	6,400	<200	<200
Anti-peptide 3			
Pre-immune	<200	<200	<200
Hyperimmune	<200	<200	1,600

^a ELISA was performed as described in Materials and Methods.

^b Amino acid sequence number of VP3 for which peptides were synthesized.

^c Guinea pigs were immunized intramuscularly with 50 µg of peptide-keyhole limpet hemocyanin conjugate emulsified in complete Freund adjuvant. Four boosters with the same amount of conjugate in incomplete Freund adjuvant were given at intervals of 2 weeks.

heterotypic cross-reactivity. Thus, VP3 N-MAbs induced by a virulent strain of serotype 1, serotype 3, or serotype 4 also react with the other two serotypes (23). In addition, VP3 N-MAbs specific for strains of serotype 1, 3, or 4 virulent HRV have not been isolated to date. However, several serotype 2-specific VP3 N-MAbs have been produced (24). The latter observation was not unexpected because the amino acid sequence of virulent serotype 1, 3, and 4 HRV VP3 is highly conserved and quite distinct from that of virulent serotype 2 HRV. Additionally, the VP3 sequence of the DS-1 and RV-5 strains of serotype 2 is highly conserved (8, 14; Gorziglia et al., submitted). Thus, by serological characterization and amino acid sequence analysis, it is implied that serotypic diversity of VP3 among serotype 1, 3, and 4 HRV strains is quite limited.

In this study, sequence analysis of antigenic mutants selected with the various cross-reactive or serotype 2-specific N-MAbs allowed us to identify cross-reactive and serotype 2-specific epitopes on VP3. The highly hydrophobic region which includes amino acid residue 392 appears to be an especially immunodominant epitope. Thus, four of the seven HRV VP3 antigenic mutants selected with a panel of seven N-MAbs sustained a single amino acid substitution at position 392. Furthermore, each of seven antigenic mutants of simian RV SA-11 selected with SA-11 hyperimmune serum sustained a substitution of amino acid 393 (which corresponds to amino acid 392 of HRV) (8; Gorziglia et al., submitted). Antigenic mutants selected with other N-MAbs also had a single amino acid substitution, but in these instances the substitution was located at position 305, 433, or 439. Thus, in the seven antigenic mutants, a single amino acid substitution occurred and was located in the N-terminal half of VP5 which is the larger cleavage product of VP3. This suggests that this region contains the major antigenic sites involved in cross-reactive or serotype 2-specific neutralization. It is of interest that the epitope II including residue 392 is a neutralization epitope cross-reactive to serotype 1, 3, and 4 strains and, at the same time, specific to serotype 2

strains. Since the residue 305, which is critical to epitope I, is conserved among only serotypes 1, 3, and 4, epitope I also might be a site specific to serotype 2 as well as cross-reactive to serotypes 1, 3, and 4. For clarifying more the location of the serotype 2-specific neutralization epitopes, the sequence analysis of the antigenic mutants selected with each of several other serotype 2-specific N-MAbs is in progress.

The shorter trypsin cleavage product (VP8) of VP3 may also have neutralization epitopes, especially strain-specific neutralization epitopes, since the sequences in the region of amino acids 70 to 202 are highly variable among different RV strains and since several amino acid substitutions in this region of VP8 have been detected in SA-11 mutants selected in the presence of hyperimmune SA-11 antiserum (8, 14; Gorziglia et al., submitted). Also, several N-MAbs which have hemagglutination inhibition activity and which react with VP8 by Western blot (immunoblot) have been produced (17).

There was a correlation between the epitope specificity of the cross-reactive antibodies used to select mutants and the position at which an amino acid substitution occurred. Mutant V-YO-2C2 selected with YO-2C2 antibody (epitope I) had an amino acid substitution at residue 305, whereas mutant V-KU-4D7 selected with KU-4D7 (epitope III) antibody had an amino acid substitution at position 433. Three mutants, V-KU-6B11, V-YO-1S3, and V-ST-1F2, selected with KU-6B11, YO-1S3, or ST-1F2 antibody (epitope II) had an amino acid substitution at residue 392. However, V-YO-1E6 selected with YO-1E6 antibody which was also directed against epitope II had an amino acid substitution at position 439. Furthermore, an RRV antigenic mutant selected with KU-6B11 antibody had an amino acid substitution at position 441 (corresponding to position 440 of HRV). This suggests that amino acid residues in the vicinity of positions 392 and 439 are in close proximity on the folded molecule and that they contribute to a single antigenic region. This might explain why two of the synthetic peptides (corresponding to amino acids 383 to 400 and 428 to 445) were not recognized by the corresponding N-MAbs. Similar observations have been made regarding the major antigenic sites on RV VP7; two regions (amino acids 87 to 96 and 211 to 223) seem to be in close proximity on the native protein, although they are widely spaced on the linear molecule (4). Synthetic peptides corresponding to each of these two regions did not elicit neutralizing antibodies (10).

At positions where amino acid substitutions occurred in the antigenic mutants, there was a correlation between conservation of sequence among different HRVs and the reactivity of these viruses with antibodies used for selection of mutants (Table 1 and Fig. 2). Also, the synthetic peptide corresponding to the YO-2C2 epitope (amino acids 296 to 313) reacted with the YO-2C2 monoclonal antibody. Furthermore, all the VP3 N-MAbs readily immunoprecipitated VP3, in contrast to the failure of most VP7 N-MAbs to immunoprecipitate VP7 (23, 24). These findings suggest that some VP3 neutralization epitopes, i.e., the YO-2C2 epitope, are sequential or linear. As the YO-2C2 epitope is located in a highly hydrophilic region (data not shown), this particular peptide may be a candidate for a synthetic vaccine.

VP3 appears to play a major role in RV virulence (20). This view is supported by the observation that the VP3 genes of virulent HRV strains are highly conserved and distinct from the VP3 genes of asymptomatic HRV strains which also exhibit a high level of conservation of VP3 gene sequence. It should be noted that all the cross-reactive N-MAbs used in this study, except the KU-4D7 antibody,

neutralized the four asymptomatic HRV strains tested, which included a representative of each of the four HRV serotypes (Table 1). This finding indicates that asymptomatic viruses carry some cross-reactive neutralization epitopes on VP3 which are similar to those of virulent viruses. Consequently, asymptomatic strains or reassortants with a VP3 gene from an asymptomatic strain might be desirable candidates for inclusion in an RV vaccine.

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