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Demonstration of an Immunodominant Neutralization Site by Analysis of Antigenic Variants of SA11 Rotavirus

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Serotype-specific monoclonal antibodies were used to select mutants of SA11 rotavirus that were resistant to neutralization. The antigenic characteristics of these mutants were studied with a panel of monoclonal antibodies. We isolated one type of mutant which showed a dramatic increase (greater than 10-fold) in resistance to neutralization by hyperimmune antiserum, and this together with other data indicates the presence on the rotavirus major outer shell glycoprotein of an immunodominant antigenic site involved in virus neutralization. The mutants were also useful in classifying neutralizing monoclonal antibodies.

Two proteins present on the outer shell of the rotavirus capsid have been shown to elicit antibodies capable of neutralizing virus infectivity (1, 6, 11). For SA11 rotavirus these proteins are p84 and gp34, which are the products of gene segments 4 and 9, respectively (8, 11). By far the most important of the two, and the one that determines the virus serotype, is the major outer shell glycoprotein, gp34 (3, 7, 8). The nature of the antigenic sites on this protein is therefore of particular interest in the development of rotavirus vaccines. Although they are difficult to produce, neutralizing monoclonal antibodies (mAbs) to three serotype 3 viruses have been described previously (2, 12), and Sonza et al. have reported the existence of one major antigenic epitope as well as another less important epitope on the major outer shell glycoprotein of SA11 (12). In the present study we have extended these findings by producing and analyzing mutants of SA11 which are resistant to neutralizing mAbs.

SA11 (i.e., type 3). These mAbs react with the major outer shell glycoprotein and have been shown by extensive testing to be serotype specific (2). SA11 rotavirus was grown in MA104 cells as previously described (4), except that for this work several virus stocks were used. These stocks were produced by inoculating cells in 500-ml roller bottles at a very low multiplicity of infection, and the harvest from each bottle was kept as a separately numbered stock. Since each had arisen from a small starting population (approximately 100 PFU), it was expected that the relative frequencies of mutations in the virus population of each stock would differ, thus favoring the isolation of different types of resistant mutants. Selection of mutants resistant to neutralizaing mAbs was performed as follows. Virus stocks were sonicated briefly (10 s) and trypsin activated (2 μg of porcine trypsin per ml, type IX; Sigma Chemical Co.) at 37°C for 15 to 30 min. Fetal calf serum was added to 2% (vol/vol) to

TABLE 1. Characteristics of neutralizing mAbs to SA11 and RV-3 rotaviruses used to select resistant mutants[a]

mAb	N_t[b]		EIA[c]		Competition binding[d]			
	SA11	RV-3	SA11	RV-3	A10/N3	A11/M9	B8/X1	A6/H1
A10/N3	10^6	ND[e]	2×10^6	ND	+	−	−	+
A11/M9	10^5	ND	2×10^6	ND	+	+	+	−
B8/X1	10^6	ND	10^6	ND	+	+	+	+
RV-3:1	940,000	235,000	650,000	200,000	ND	ND	ND	ND
RV-3:2	12,000	235,000	8,000	200,000	ND	ND	ND	ND

[a] Data from references 2 and 12. SA11 and RV-3 mAbs were both ascites fluids.
[b] Reciprocal titers determined in immunofluorescence neutralization tests (2, 12).
[c] Enzyme immunoassay titers as described previously (2, 12).
[d] Summary of competition-binding tests from reference 12; +, >70% competition of 1/50 dilution of competing ascites fluid; −, <10% competition under the same conditions.
[e] ND, Not done.

The neutralizing mAbs A10/N3, A11/M9, and B8/X1, which are directed against the major outer shell protein of SA11, have been described previously (12). We also used two neutralizing mAbs (RV-3:1 and RV-3:2) produced against RV-3, a human rotavirus of the same serotype as

inhibit further trypsin activity, and the activated virus was serially diluted, mixed with an equal volume (500 μl) of a constant dilution of mAb (to give approximately 50 to 500 times the dose for 50% neutralization as determined in neutralization titrations) and incubated at 37°C for 1 h. Aliquots of virus-antibody mixture were assayed for plaque-forming virus as described previously (11), except that mAb was also present in the agarose overlay. After 3 to 5 days,

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TABLE 2. Neutralization titers of mAbs to rotavirus mutants

Virus	No. of isolates analyzed	Titer for antibody: αSA11[a]	A10/N3[b]	RV-3:1	A11/M9	B8/X1	RV-3:2
SA11		409,600	409,600	409,600	1,638,400	409,600	6,400
VA10/N3[c]	6	1,600	≤400	≤400	1,638,400	400	6,400
VRV-3:1	2	1,600	400	≤400	100,000	≤400	ND[e]
VA11/M9	6	409,600	1,000,000	1,000,000	400	1,000,000	6,400
VB8/X1	2	409,600	409,600	≤400	409,600	≤400	5,000
VRV-3:2	4	409,600	1,638,400	1,638,400	409,600	1,638,400	≤400
VA11/RV-3:1/a	2	25,600	≤400	≤400	≤400	≤400	6,400
VA11/RV-3:1/b	2	409,600	1,638,400	≤400	≤400	1,638,400	5,000

[a] Hyperimmune rabbit anti-SA11 antiserum.
[b] Monclonal antibodies were all ascites fluids.
[c] Antigenic mutants of SA11 virus are prefixed with the letter V followed by the mAb(s) used to select them.
[e] ND, Not done.
[d] Underlines indicate significantly reduced titers.

visible plaques were picked off and virus was grown up once. These preparations were again neutralized and plaque titrated, and virus grown from the resulting plaques were analyzed by reaction in neutralization tests (5, 13) against a panel of mAbs, as well as hyperimmune (rabbit) anti-SA11 antiserum (1).

Table 1 summarizes some of the known characteristics of the mAbs used in this study to select resistant mutants. They were chosen on the basis of their different serologic or competition binding properties or both, although all are serotype specific.

Each of these mAbs was used to select resistant mutants which were then analyzed in neutralization tests against all five mAbs (Table 2). The frequencies at which mutants were isolated varied between 10^{-4} and 10^{-5} (data not shown), which is in agreement with mutation frequencies observed in other RNA viruses (10). It was found that the isolation frequency was markedly affected by the dilution of mAb used, so that at low dilutions very few or no mutants could be detected. For example, B8/X1-resistant mutants (VB8/X1) were readily isolated at a 10^{-4} dilution of B8/X1 (frequency of 0.6×10^{-4}) but could not be isolated at all if the antibody dilution was decreased to 10^{-3} (frequency of $<10^{-7}$). Once isolated, mutants were found to be highly resistant to their selecting mAb with titers of ≤400 (Table 2), and some mutants (e.g., VB8/X1) were found to show concomitant resistances to other mAbs. Two groups (VA10/N3 and VRV-3:1) also displayed a marked resistance to polyclonal anti-SA11 antiserum, which was unexpected. Another interesting feature of these results was that while attempts were made to isolate different kinds of resistant mutants, we found that each mAb selected mutants which behaved identically in these tests. For example, the six A10/N3 mutants analyzed had identical neutralization titers against all antisera (Table 2).

It is evident from Table 2 that not only are mAbs useful in analyzing the antigenic structure of resistant mutants, but conversely the mutants are equally as useful in characterizing mAbs. For example, mAbs A10/N3 and RV-3:1 both select mutants with the same resistance pattern (VA10/N3, VRV-3:1), and in fact these have identical mutations (to be published elsewhere), but the binding sites of these mAbs are not identical since they react differently to B8/X1-resistant mutants (VB8/X1; Table 2).

To further explore the potential of mAb resistant mutants, we took a mutant resistant to A11/M9 (VA11/M9) and (since it was sensitive to the other mAbs) selected for RV-3:1-resistant mutants. The resulting "double mutants" were characterized as above, and the results are shown at the bottom of Table 2. Two groups of mutants were observed (VA11/RV-3:1/a and VA11/RV-3:1/b). Group a mutants displayed a resistance pattern which was simply the sum of the resistances shown by VA11/M9 and VRV-3:1 mutants. However, group b mutants had a different pattern: they were resistant only to their selecting mAbs, which indicates that the mutation selected by RV-3:1 in group b mutants differs from that in group a mutants. When the group b double mutants are included, all five mAbs can be clearly distinguished (Table 2), confirming that they all have different binding sites.

The most striking finding of this study is the resistance to hyperimmune antiserum observed in A10/N3- and RV-3:1-

TABLE 3. Neutralization titers[a] of SA11, RV-3, and VA10/N3 with a range of hyperimmune antirotaviral antisera

Antiserum[b]	Serotype	Titer for virus: SA11	VA10/N3	RV-3	Wa	RV-5	ST-3
SA11	3	409,000	1,600	—[c]	200	—	—
RV-3	3	188,000	19,000	320,000	—	—	—
Wa	1	320	150	2,000	50,000	—	—
RV-4	1	240	150	—	50,000	—	—
RV-5	2	2,200	<100	2,400	—	64,000	—
ST-3	4	170	150	305	—	—	35,000

[a] Immunofluorescence neutralization tests were performed as described in reference 2.
[b] Hyperimmune rabbit (SA11, RV-3, Wa) or mouse (RV-4, RV-5, ST-3) antisera to the stated rotavirus. Further information concerning these antisera and viruses can be found in reference 2.
[c] —, Not tested.

resistant mutants. We therefore analyzed the antigenicity of one A10/N3 mutant further by using sera raised against rotaviruses belonging to all four at the known human serotypes (Table 3). Antiserum to RV-3 (a type 3 virus) also showed a significant (10-fold) reduction in neutralization titer against the mutant than against SA11. In addition, the low titers against heterotypic antisera confirmed that the VA10/N3 mutant had not converted to another human serotype.

This dramatic change in antigenicity could be interpreted in two ways: (i) the mutation causes a drastic alteration in conformation of the major outer shell glycoprotein, which thereby inhibits the binding of a large range of neutralizing antibodies directed to many different regions of the molecule, or (ii) the mutation alters only a very local region of the molecule, but this inhibits the binding of neutralizing antibodies, most of which are directed against a single (i.e., immunodominant) antigenic site. Given the structural function of this protein and the many conformational constraints imposed on it in forming the outer shell of the virus capsid, the first possibility appears unlikely. The second model is more probable and is supported by recent work with influenza virus, when it was demonstrated that a mAb-selected mutation in the viral hemagglutinin affected only the region immediately surrounding the substituted amino acid (9). This model is consistent with the competition binding data reported earlier (12), which agree with the assumption of one major antigenic site encompassing both the major and minor epitopes previously proposed, since competition occurred between members of the two groups (A and B) of neutralizing mAbs.

The resistance patterns of A10/N3-, RV-3:1-, and B8/X1-selected mutants suggest that their mutations lie within the same antigenic site, and although the A11/M9-resistant mutants (VA11/M9) appeared distinct, the competition binding results given in Table 1 indicate that mAb A11/M9 binds to a site close to those of A10/N3 and B8/X1. RV-3:2-selected mutants were antigenically distinct from all the other mutants, and in the absence of other data it is not possible to judge whether RV-3:2 binds to a site close to any of the other mAbs. Interestingly, its neutralization titer against SA11 was much lower than its homologous titer against RV-3 (Table 1).

The facts that mutants of SA11 virus could be used to analyze mAbs induced against a human type 3 virus (RV-3) and that the serotype-specific antigens on both human and animal serotype 3 strains appear to be closely related demonstrates the general application of the method. It would be extremely useful to have standard panels of resistant mutants from viruses representing each serotype for the analysis and comparison of mAbs produced in different laboratories.

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LITERATURE CITED

1. **Bastardo, J. W., J. L. McKimm-Breschkin, S. Sonza, L. D. Mercer, and I. H. Holmes.** 1981. Preparation and characterization of antisera to electrophoretically purified SA11 virus polypeptides. Infect. Immun. **34**:641–647.
2. **Coulson, B. S., K. J. Fowler, R. F. Bishop, and R. G. H. Cotton.** 1985. Neutralizing monoclonal antibodies to human rotavirus and indications of antigenic drift among strains from neonates. J. Virol. **54**:14–20.
3. **Dyall-Smith, M. L., A. A. Azad, and I. H. Holmes.** 1983. Gene mapping of rotavirus double-stranded RNA segments by Northern blot hybridization: application to segments 7, 8, and 9. J. Virol. **46**:317–320.
4. **Dyall-Smith, M. L., and I. H. Holmes.** 1981. Gene-coding assignments of rotavirus double-stranded RNA segments 10 and 11. J. Virol. **38**:1099–1103.
5. **Dyall-Smith, M. L., and I. H. Holmes.** 1984. Sequence homology between human and animal rotavirus serotype-specific glycoproteins. Nucleic Acids Res. **12**:3973–3982.
6. **Greenberg, H. B., J. Valdesuso, K. Van Wyke, K. Midthun, M. Walsh, V. McAuliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino.** 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J. Virol. **47**:267–275.
7. **Kalica, A. R., H. B. Greenberg, R. G. Wyatt, J. Flores, M. M. Sereno, A. Z. Kapikian, and K. M. Chanock.** 1981. Genes of human (Wa strain) and bovine (strain UK) rotaviruses that code for neutralization and subgroup antigens. Virology **112**:385–390.
8. **Kantharidis, P., M. L. Dyall-Smith, and I. H. Holmes.** 1983. Completion of the gene coding assignments of SA11 rotavirus: gene products of segments 7, 8, and 9. J. Virol. **48**:330–334.
9. **Knossow, M., R. S. Daniels, A. R. Douglas, J. J. Skehel, and D. C. Wiley.** 1984. Three-dimensional structure of an antigenic mutant of the influenza virus haemagglutinin. Nature (London) **311**:678–680.
10. **Minor, P. D., G. C. Schild, J. Brotman, D. M. A. Evans, M. Ferguson, P. Reeve, M. Spitz, G. Stanway, A. J. Cann, R. Hauptmann, L. D. Clarke, R. C. Mountford, and J. W. Almond.** 1983. Location and primary structure of a major antigenic site for poliovirus neutralization. Nature (London) **301**:674–679.
11. **Smith, M. L., I. Lazdins, and I. H. Holmes.** 1980. Coding assignments of double-stranded RNA segments of SA11 rotavirus established by in vitro translation. J. Virol. **33**:976–982.
12. **Sonza, S., A. M. Breschkin, and I. H. Holmes.** 1984. The major surface glycoprotein of simian rotavirus (SA11) contains distinct epitopes. Virology **134**:318–327.
13. **Thouless, M. E., G. M. Beards, and T. H. Flewett.** 1982. Serotyping and subgrouping of rotavirus strains by the ELISA test. Arch. Virol. **73**:219–230.