Serotypic Analysis of VP3 and VP7 Neutralization Escape Mutants of Rhesus Rotavirus

ROBERT D. SHAW,^{1*} ERICH R. MACKOW,² MICHAEL L. DYALL-SMITH,³ IEVA LAZDINS,³
IAN H. HOLMES,³ AND HARRY B. GREENBERG²

Department of Medicine, Stanford University School of Medicine, and the Palo Alto Veterans Administration Medical Center, Palo Alto, California 94304²; Department of Medicine, School of Microbiology, University of Melbourne, Parkville, Australia³; and State University of New York at Stony Brook and the Northport Veterans Administration Medical Center, Northport, New York 11768¹

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Neutralization escape mutants of simian rotaviruses (rhesus rotavirus and SA11) were tested in hemagglutination inhibition and neutralization assays against hyperimmune and infection sera to determine if mutation in an immunodominant epitope could enable neutralization escape. An SA11 mutant with a new glycosylation site at amino acid 211 of VP7 was shown to escape neutralization by hyperimmune but not infection sera.

Advances have been made recently in the production and characterization of monoclonal antibodies (MAbs) directed at the two outer capsid rotavirus proteins (VP3 and the glycoprotein VP7). Antibodies to these proteins are responsible for viral neutralization in vitro and disease protection in vivo (3, 5, 9, 10, 12-16). Competitive binding and neutralization studies using these MAbs against simian and human rotaviruses have identified several distinct regions on both surface proteins that are capable of mediating viral neutralization. Selection and characterization of neutralization-resistant viral variants have enabled more precise antigenic mapping. The characterization of variant viruses in functional assays and the sequencing of variant viral genomes have located the point mutations which allowed escape from neutralization and prevented binding of neutralizing antibodies (4, 8, 13; E. Mackow, R. Shaw, S. Matsui, P. Vo, D. Benfield, H. Greenberg, submitted for publication). These studies have confirmed the presence of several regions on VP3 and VP7 that are capable of mediating the neutralization of rotaviruses of one or, in some cases, several serotypes. The antigenic regions which are known to mediate serotype-specific neutralization have been mapped to two principal areas on VP7 (4, 13; Mackow et al., submitted). On VP3, six distinct regions which mediate homotypic and heterotypic neutralization have recently been located (8).

The selection of a MAb that is specific for a particular neutralization site is not sufficient evidence to prove that such an antigenic site is strongly immunogenic during hyperimmunization or infection. To determine whether the immune response to a particular neutralization site was dominant in the overall humoral neutralizing response to the rotavirus (that is, if a site is immunodominant), we used a collection of variant rhesus rotaviruses (RRV) in hemagglutination inhibition (HI) and neutralization assays with several anti-RRV serum samples. These variants possessed single amino acid substitutions in either VP3 or VP7 that allowed them to be selected from the parental RRV strain in the presence of a neutralizing MAb (8, 13; Mackow et al., submitted). The ability of a particular variant to escape more than 90% of the neutralizing or hemagglutination-inhibiting effect of hyperimmune or infection sera was considered to

identify the altered region as one which stimulated an immunodominant antibody response.

RRV parenteral hyperimmunization serum samples from five guinea pigs and 10 BALB/c mice were prepared (Table 1). Parenteral hyperimmunizations were performed by inoculating animals with gradient-purified double-shelled virus and Freund adjuvant twice at a 1-month interval and then 1 month later with antigen in saline. Animals were bled 10 days after the third immunization. Six individual mouse infection samples were taken from mice orally infected with RRV (Table 1). Infection serum samples were produced by orally infecting 4-week-old BALB/c mice with approximately 10^7 PFU of RRV. The inoculation was repeated 1 month later, and the animals were bled 10 days afterward. SA11 infection serum samples were made by the oral administration of 10^6 PFU of SA11 rotavirus to a pool of CD1 outbred adult mice. The virus was administered three times at 2-week intervals; serum samples were obtained 4 weeks after the final immunization.

The variant RRV library included viruses that escaped neutralization by VP3- or VP7-specific neutralizing MAbs (8, 13). The RRV antisera were tested in HI assays against each of the variants and the parental RRV. The HI assay was performed as previously described, with careful attention given to standardization of each variant hemagglutination antigen (13). Table 1 summarizes the results. A mutation in an immunodominant region was not identified in any variant. Three test serum samples (guinea pigs 962 and 964 and mouse pool 4/15) demonstrated small (fourfold) reductions in HI titers against the viral variants that were selected by the serotype-specific neutralizing MAbs 159 and 4F8. The mutations that characterized each of these variants were located at amino acids 94 and 97, respectively. However, the double variant 159/5H3, with mutations at amino acids 94 and 212, did not escape the HI effects of any of the sera. Similar results were obtained when selected variants were tested against hyperimmune sera in plaque reduction neutralization assays (Table 2). These data suggest that the epitopes of RRV defined by this library of VP3- and VP7-specific neutralizing MAbs are not immunodominant in the response to hyperimmunization or infection.

The failure to identify an immunodominant region on VP7 seems to conflict with recent studies which report that a serotype-specific neutralizing region on VP7 was responsible

* Corresponding author.

TABLE 1. Differences in HI titers of hyperimmune or infection sera against RRV variants compared with titers against RRV^a

N-MAbs ^b for selection of viral variants	Difference in titers for sera from:											
	Hyperimmunized guinea pigs					Hyperimmunized mice 4/15	Orally immunized mice					
	962	963	964	965	0		1	2	3	989	993	995
VP7												
4G2	0	1	1	1	0	-1	0	0	0	0	0	-1
4C3	0	0	0	0	0	0	-1	0	-1	0	0	-1
4F8	1	0	0	1	0	-2	0	0	0	0	0	-1
96	0	0	-1	0	0	0	0	0	0	0	0	0
159	-2	-1	-2	-1	-1	0	-1	0	-1	0	0	0
159/5H3	0	1	1	1	1	0	0	0	0	0	0	0
4F5	-1	1	0	0	0	0	0	0	-1	0	0	0
3	0	1	0	1	1	0	0	0	-1	0	0	0
57-8	-1	0	-1	0	0	0	0	0	-1	0	0	0
VP3												
2G3	1	1	1	1	0	-1	1	0	0	0	0	-1
23	2	2	2	2	2	-1	2	0	2	0	0	0
KU6B11	1	1	1	2	1	0	0	0	0	0	0	0
2G4/1A9/7A12	0	0	1	1	0	0	0	0	0	0	0	0
2G4	0	0	0	0	-1	0	-1	0	-1	0	0	0
7A12	1	0	1	1	0	0	0	0	-1	0	0	0
5D9	0	0	0	NT ^c	0	0	-1	0	NT	0	0	0
5C4	0	1	1	1	0	0	1	0	1	0	0	0
A15	0	0	0	0	0	0	0	0	-1	0	0	0
M2	-1	-1	-1	0	-1	0	0	0	-1	0	0	0

^a The comparison of titers indicated represents the log₂ HI titer against the variant minus the log₂ HI titer against RRV. Negative numbers indicate resistance to neutralization. Values -4 or greater may be significant. The actual anti-RRV HI titers of the indicated sera are as follows: guinea pigs 962, 963, 964, and 965, 1:12,800; guinea pig 0, 1:25,600; mice 4/15 (a pool of serum from 10 mice), 1:6,400; orally infected mice 1 to 3, 1:800; mice 989, 993, and 995, 1:1,600.

^b N-MAbs, Neutralizing MABs. The VP7 viral variants (except the double mutant 159/5H3) had single point mutations at amino acids 94, 95, 96, 97, 99, 211, and 212 (Mackow et al., submitted). The VP3 viral variants had single point mutations at amino acids 89, 100, 114, 135, 150, 173, 188, 388, and 393 (8). The locations of the mutations in variants 23 and 2G3 are unknown.

^c NT, Not tested.

for eliciting 95 to 99% of the neutralizing immune response in a rabbit hyperimmunized with serotype 3 simian rotavirus SA11 and 90% of the response in a rabbit hyperimmunized with another serotype 3 virus, RV-3 (2, 3, 7). These previous findings were based on the reductions in neutralizing titers that were observed when sera were tested against a variant SA11 virus (A10/N3). Variant A10/N3 has been found to have an Asp-to-Asn mutation at amino acid 211 which, unlike mutations in any of the RRV variants tested, formed and utilized a new glycosylation site at amino acid 211 (2, 4). RRV variant 3 has been found to have a new glycosylation site at amino acid 99 which is also utilized (Mackow et al.,

submitted), but as the data in Tables 1 and 2 indicate, this variant did not escape HI or neutralization by any antisera. However, RRV variants 96 and 159/5H3 possess mutations at amino acid 211 and 212, respectively (Mackow et al., submitted) but these mutations do not result in new glycosylation sites, nor do they provide escape from either HI or neutralization by hyperimmune sera (Table 2).

SA11 variant A10/N3 was previously reported to be dramatically resistant to anti-SA11 hyperimmune serum made in a rabbit (7). We performed plaque reduction neutralization assays to confirm the reported escape from neutralization of SA11 variant A10/N3. Selected RRV variants were included

TABLE 2. Comparisons of plaque reduction neutralization titers of hyperimmune and infection sera against viral variants and the parental virus strain^a

Serum	Titers against:		Degree of escape for:						
	RRV	SA11	RRV-derived variants						SA11-derived variant A10/N3
			4F8	GOT	96	159	3	159/5H3	
4/15 ^b	51,200	≥25,600	-1	0	0	0	0	-1	-4
962 ^c	512,000	512,000	0	0	0	0	-1	-1	-4
964 ^c	512,000	512,000	-1	0	0	0	-1	-1	-6
14B1 ^d		800							-1
14A2 ^d		200							0
14B3 ^d		400							0

^a The comparison of titers indicated represents the log₂ plaque reduction neutralization titer against the variant minus the log₂ titer against the parental strain (RRV for the RRV-derived variants 4F8, GOT, 96, 159, 3, and 159/5H3; SA11 for variants A10/N3 and clone 28). Negative values of ≤-4 indicate significant escape from the neutralization effects of the indicated serum (≥90% plaque reduction). See the text for localization of the mutation sites of each of these variants.

^b Hyperimmune anti-RRV (pooled) sera made in mice.

^c Guinea pig hyperimmune anti-RRV sera.

^d Infection serum obtained from mice orally inoculated with SA11 twice.

to verify the HI results and to provide a comparison with the SA11 variant. The results of the neutralization assays with the RRV variants confirmed the HI results of Table 1; no RRV variant significantly escaped the effects of hyperimmune or infection sera (Table 2). However, substantial (>90%) decreases were noted in neutralization titers of both mouse and guinea pig hyperimmunization sera against A10/N3. The titers of the mouse RRV infection sera against SA11 were inadequate for evaluation of this serum against the variant (Table 2). However, we tested SA11 mouse infection sera in plaque reduction neutralization assays against wild-type SA11 and SA11 mutant A10/N3. Surprisingly, there was no significant reduction in the neutralization titers of the three infection sera against the glycosylated mutant (Table 2).

Our studies have confirmed and extended those of Caust et al. (2) and Lazdins et al. (7), who originally identified the neutralization resistance of SA11 variant A10/N3 to hyperimmunization sera. While none of the individual epitopes on VP3 or VP7 defined by neutralizing MAbs were themselves immunodominant in this study, the addition of a carbohydrate chain to a new glycosylation site at amino acid 211 resulted in at least 16- to 64-fold reductions in neutralization titers of guinea pig and mouse hyperimmune RRV sera. This implies that most (perhaps 90 to 98%) of the RRV-specific neutralizing immunoglobulin molecules present in these sera were directed at regions of the virus made inaccessible or unrecognizable by the addition of the carbohydrate chain at VP7 amino acid 211. The addition of a carbohydrate chain at VP7 amino acid 99 (RRV variant 3) had no effect, nor did a mutation at amino acid 211 or 212 that did not result in the addition of a new carbohydrate chain (RRV variants 96 and 159/5H3). However, the addition of a glycosylation site at VP7 amino acid 211 had no effect on the neutralization titers of mouse infection sera. The sensitivity of SA11 variant A10/N3 to the neutralizing effects of infection sera may have been the result of antibodies directed at VP3, as the neutralization activities of two VP3-specific MAbs were unaffected (data not shown). This finding demonstrates the marked differences that can characterize the immune responses to parental hyperimmunization in comparison with responses to intestinal infection.

The antigenic alteration invoked by glycosylation at VP7 amino acid 211 could have been caused by steric interference from the carbohydrate chain. It is interesting to note, however, that MAbs which formed bonds at the amino acid 211 site did not compete effectively with MAbs directed at amino acid sites 94 to 99 when tested in competitive binding assays, even though the immunoglobulin G molecules are much larger than the carbohydrate side chain (13). It is also possible that the mutation at amino acid 211 and the carbohydrate addition had an effect on protein conformation that altered antigenic recognition of a large number of neutralizing epitopes, an effect that is not seen as a result of only a single amino acid change.

Many neutralizing MAbs specific for VP7, such as MAbs 159, 4F8, and 3 used in this study (4; Mackow et al., submitted), are directed at amino acid regions 94 to 99. These MAbs have been found to predominantly recognize the native antigen conformations presented on the intact virus. This conclusion is based on reported difficulties in the isolation of VP7 from infected cell lysates by immunoprecipitation with MAbs (13), the loss of these neutralization epitopes after outer capsid disruption by calcium chelation, the failure of these antibodies to detect VP7 in Western blots (immunoblots; R. Shaw and H. Greenberg, unpublished

data), and the difficulties in antigenically simulating these regions with synthetic peptides (6) or expressed proteins (1). The importance of conformationally sensitive epitopes in the human immune response to rotavirus has been demonstrated in a study using an epitope-specific competitive enzyme-linked immunosorbent assay, in which a substantial immune response to the neutralizing region of VP7 reactive with MAb 4F8 was identified (11). Therefore, any antigenic modification that permits a profound escape from neutralization must functionally disable antibodies directed at the serotype-specific region of amino acids 94 to 99. If one assumes that this functional alteration is accomplished by the prevention of antibody binding, the ability of glycosylation at amino acid 211 to facilitate escape from neutralization implies the possibility that these two areas of VP7 that mediate serotype-specific neutralization (amino acids 94 to 99 and 211) are physically approximated in the intact virion. The same conclusion has been reached in other studies of rotavirus variants (4).

The relative immunogenicities of VP3 and VP7 are important factors in vaccine design. Many studies have demonstrated that VP3, as well as VP7, plays an important role in *in vitro* viral neutralization and *in vivo* protection in the heterologous mouse model (9, 10). The presence of neutralizing VP3 antibodies has been demonstrated in the sera of children orally vaccinated with simian RRV (11). Antibodies to VP3 may constitute a very large element of the adult human neutralization antibody repertoire induced by infection with the human rotavirus CYN (17). The reason for the differences in the immunogenicity of the two rotavirus-neutralizing proteins based on the route of administration is unknown, but differences in antigen processing and presentation to the immune system after systemic immunization with adjuvant may be markedly different from the analogous process that occurs in the intestine in the presence of active viral replication, cell lysis, and the influences of gut-associated lymphoid tissues. These possibilities suggest that the mode of antigen presentation is an important determinant of the immune response to rotavirus and perhaps other viral intestinal infections.

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