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Cross-Linking of Rotavirus Outer Capsid Protein VP7 by Antibodies or Disulfides Inhibits Viral Entry

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Antibodies that neutralize rotavirus infection target outer coat proteins VP4 and VP7 and inhibit viral entry. The structure of a VP7-Fab complex (S. T. Aoki, et al., Science 324:1444–1447, 2009) led us to reclassify epitopes into two binding regions at inter- and intrasubunit boundaries of the calcium-dependent trimer. It further led us to show that antibodies binding at the intersubunit boundary inhibit uncoating of the virion outer layer. We have now tested representative antibodies for each of the defined structural epitope regions and find that antibodies recognizing epitopes in either binding region neutralize by cross-linking VP7 trimers. Antibodies that bind at the intersubunit junction neutralize as monovalent Fabs, while those that bind at the intrasubunit region require divalency. The VP7 structure has also allowed us to design a disulfide cross-linked VP7 mutant which recoats double-layered particles (DLPs) as efficiently as does wild-type VP7 but which yields particles defective in cell entry as determined both by lack of infectivity and by loss of α-sarcin toxicity in the presence of recoated particles. We conclude that dissociation of the VP7 trimer is an essential step in viral penetration into cells.

Rotaviruses cause a large proportion of the worldwide cases of dehydrating childhood diarrhea, accounting for over 400,000 deaths annually (47). Their nonenveloped virions enclose an 11-segment, double-stranded RNA genome (19). The infectious particles (triple-layered particles [TLPs]) have three icosahedrally organized protein shells (38, 48, 52, 60). The outermost layer, which comprises two proteins, is an essentially continuous shell of trimeric VP7 arranged in a T=13 icosahedral lattice with 60 spikelike projections of VP4. Neutralizing antibodies recognize epitopes on either of these proteins.

VP4 is the viral attachment protein (23). Trypsin activates it by a cleavage that yields two fragments, VP8* and VP5* (4, 18, 20). Uncleaved VP4 incorporates into assembling virions as a trimmer. The trypsin-activated spike bears two lectinlike VP8* attachment domains at its tip, supported by three VP5*s; the spike appears to have lost the attachment domain of the third VP8* (52). The VP7 layer anchors the VP4 spikes onto the underlying double-layered particle (DLP) and allows proper proteolytic cleavage of VP4 to prime it for membrane disruption (2, 56). During cell entry, the VP7-VP4 outer layer uncoats, releasing the intact DLP into the cytosol. VP4 mediates cell binding (23) and membrane disruption (8, 35), but the final steps of DLP delivery require uncoating of VP7 (7, 39). Full uncoating of the outer layer is also necessary to activate the DLP (5, 27, 36), which contains multiple copies of the viral polymerase complex that synthesizes and extrudes mRNA transcribed from each of the 11 genome segments.

Protection against rotavirus infection is mediated primarily by the adaptive immune system, and a heterotypic response is important for broad protective immunity (21, 25, 28). Antibodies directed against VP4 and VP7 define the 14 P and 14 G group A rotavirus serotypes; reassortment generates a large number of P-G combinations (19, 51). Neutralizing antibodies that recognize epitopes on VP8* inhibit infection by blocking attachment (49). Some of those directed against epitopes on VP5* may have a similar mechanism (through steric interference); others probably prevent membrane disruption by interfering with a VP5* conformational rearrangement required for DLP penetration. We consider here the mechanism(s) of neutralization by antibodies directed against VP7. VP7 epitopes map to the outer surface of the VP7 trimer (1).

We confirm here that VP7 antibodies reduce viral infectivity by cross-linking VP7 subunits on the outer layer of the virion, as initially demonstrated by Ludert et al. (40), thereby interfering with the required uncoating step (7, 39). We extend these findings by demonstrating that cross-linking can be effected either by a divalent antibody or by a monovalent Fab with a footprint that extends across an intersubunit contact. We further show that the introduction of an intersubunit disulfide has a similar inhibitory effect and that VP7 uncoating is necessary for the penetration activity of VP5*.

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MATERIALS AND METHODS

Rotavirus and recombinant protein purification. MA104 cells were grown in M199 medium (Invitrogen) supplemented with 25% fetal bovine serum (FBS; Clone Fabs, Inc.) and 10 mM HEPES, pH 7.3, 2 mM MgCl₂ and 100 units/ml penicillin. For rotavirus DLP/TLP production, cells were grown in 10-stack cell culture chambers (Corning), and confluent monolayers were infected with rhesus rotavirus (RRV; G3 serotype, PSB3[3]) at a multiplicity of infection (MOI) of 0.1 focus-forming unit (FFU) in M199 medium supplemented with 1 μg/ml porcine pancreatic trypsin (Worthington Biochemical). Cell culture medium was collected after 24 to 36 h, when cell adherence was less than 5%. TLPs or DLPs were purified by freeze-thawing, ultracentrifugation pelleting, pre-110000 g extraction, and a cesium chloride gradient as previously described (56).

Recombinant RRV VP4 and VP7, including mutant VP7, were expressed and purified from Sf9 cells infected with a baculovirus vector as previously described (11, 12). Briefly, wild-type RRV VP4 was purified from VP4-expressing baculovirus-infected cell pellets by ion exchange and size exclusion chromatography (Bio-Beads S1) and a diisulfide mutant (VP7-S-S) was purified from infected insect cell medium by concanavalin A lectin affinity and a monoclonal antibody (Fab) antibody column, which is specific to the VP7 trimmer. Elution of VP7 wild-type protein from the antibody column was achieved by ionic exchange with EDTA. VP7 S-S was eluted by EDTA with similar efficiency to the elution of the wild-type protein. The ratio of trimer to dimer and monomer species on nondenaturing Coomassie blue SDS-PAGE gels could be increased by prolonged incubation (>48 h) at 4°C or by long-term storage at −80°C.

Antibody and Fab production. Mouse hybridomas expressing MAb 159, 4C3, or 5H3 were adopted gradually over several weeks from RPMI supplemented with 10% FBS, 2 mM MgCl₂, and 100 units/ml penicillin to serum-free hybridoma growth medium (Invitrogen). RV4:3 mouse ascites fluid was bound to a protein G column and eluted with 100 mM glycine, pH 3.0, into 1 M HEPES, pH 7.3. Antibody preparations were determined by Bradford assay (Bio-Rad), pooled, and dialyzed into 20 mM HEPES, pH 7.3, and 100 mM NaCl. The sample was separated on a Sephacryl S200 gel filtration column (GE Healthcare), and the antibody peak collected, concentrated, and stored at −80°C. To produce Fab, purified monoclonal antibodies were incubated with papain (1 mg/ml; Worthington Biochemical) for 2 to 4 h, and Fe fragments were removed with protein A resin (Pierce). Uncloned MAbs were separated from Fabs by S200 gel filtration. Final concentrations of MAbs and Fabs were determined by Bradford assay.

Antibody neutralization infectious focus assay. TLPs purified on a CCl₃ gradient were primed with trypsin (5 μg/ml; Worthington Biochemical) for 1 h at 37°C, followed by trypsin inactivation with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich). Virus was 10-fold serially diluted (6 logs) and incubated with antibody or Fab for 1 h at 37°C. Medium without MAb or Fab was used as a negative control. For RV3:4 Fab inhibition rescue experiments, virus dilutions were further incubated at 37°C in the presence of 100 μg/ml; porcine pancreatic trypsin (Worthington Biochemical) for 1 h at 37°C. To serve as a background control. Bound VP7 was detected with a primary guinea pig anti-RRV hyperimmune serum and secondary goat anti-guinea pig horse-radish peroxidase-conjugated antibody (Kirkegaard & Perry Laboratories, Inc.) diluted in PBS, 0.5% Tween 20, 2% (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich). Serial dilutions of either wild-type VP7 or the disulfide mutant (VP7 S-S) were added to each well at specific concentrations and incubated for 1 h at room temperature (~22°C). Wells were also incubated with buffer alone to serve as a background control. Bound VP7 was detected with a primary guinea pig anti-RRV hyperimmune serum and secondary goat anti-guinea pig horse-radish peroxidase-conjugated antibody (Kirkegaard & Perry Laboratories, Inc.) diluted in PBS, 0.5% Tween 20, 2% BSA for 1 h at room temperature. Between each incubation step, plates were washed with either PBS containing 0.5% Tween 20 or PBS containing 0.5% Tween 20 and 0.9 mM CaCl₂, as performed previously (9, 10). Tetramethylbenzidine (TMB; Pierce) was used to assay for the presence of peroxidase. The reactions were stopped with 2 M sulfuric acid, and wells read for the absorbance of 492 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Biotech). Data were normalized to background, and standard deviations (error bars) determined from triplicate wells. Experiments were repeated at least three times with three different VP7 S-S preparations and showed similar results.

Recoating reaction. Previous publications describe the reassortment of infectious virus particles in greater detail (56). Briefly, recombinant VP4 was added to purified DLPs in at least a 5-fold excess for complete occupancy in buffer adjusted to pH 5.2 with sodium acetate. After a 1-h incubation at room temperature (~22°C), mutant or wild-type VP7 was added in at least a 2-fold molar excess for complete occupancy in buffer supplemented with calcium. Titers of recoating reactions were determined by infectious focus assay as described above. Proper assembly was also assessed by a protease protection assay as described previously (56). Briefly, samples were incubated in the presence or absence of trypsin (5 μg/ml; Worthington Biochemical) for 1 h at 37°C. Trypsinized samples were also treated with or without 5 mM EDTA. Samples were inactivated with 1 mM PMSF, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (PVDF; Millipore) by electroblotting. VP4 was detected by MAb HS2 (46) under reducing conditions. Immunoblots were developed by chemiluminescence after incubation with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Kirkegaard & Perry Laboratories, Inc.).

α-Sarcin coentry assay. The α-sarcin coentry assay was performed as previously described (35). Briefly, MA104 cells in medium lacking cysteine or methionine were infected with TLPs, DLPs, or recoated particles (~5 × 10⁴ cells/well) in the presence or absence of α-sarcin (100 μg/ml; Sigma-Aldrich). Cells were washed and transferred to Dulbecco’s modified Eagle’s medium (DMEM) containing 5% labeled cysteine and methionine (1 μCi/ml EasyTag EXPRESS™ protein labeling mix; PerkinElmer). Samples were washed and precipitated with the addition of 10% trichloroacetic acid to wells. Wells were then washed with ethanol, air dried, and dissolved overnight with 0.5% SDS in 0.1 M NaOH. Sample lysates were analyzed by liquid scintillation counting in Opti-Fluor scintillation fluid (PerkinElmer). The results were normalized to the label incorporation in uninjected cells not treated with α-sarcin.
RESULTS

VP7 neutralization epitopes. VP7 is a $\text{Ca}^{2+}$-stabilized, trimeric glycoprotein. The crystal structure of rhesus rotavirus VP7 (RRV, G3 serotype) complexed with the Fab of MAb 4F8 (1) shows that two $\text{Ca}^{2+}$-binding sites at the subunit interface hold the trimer together. Positions of mutations that confer resistance to neutralization by various monoclonal antibodies map to two regions of the outward-facing surface of the protein (Fig. 1). One of these regions spans the $\text{Ca}^{2+}$-stabilized, inter-subunit contact; the other spans an intrasubunit junction between two compact domains. We have designated these regions 7-1 and 7-2, respectively, dividing the former into 7-1a and 7-1b, depending on which side of the subunit boundary the surface residues fall (Fig. 1). Each of these regions includes several epitopes, mapped by neutralization escape mutations and binding competition before the structure was known.

Neutralization by antibodies that bind in region 7-1. The Fab in the crystal structure contacts residues in both 7-1a and 7-1b and should therefore stabilize the trimer. We postulated that neutralization is a consequence of this stabilization and suggested that at suitable concentrations, the isolated Fabs of MAbs that bind in this way would block infectivity. Indeed, the Fab of MAb 4F8 did neutralize infectious virus, although at higher concentrations than does the intact antibody (1). We attributed this difference to the divalency of the latter and its consequent higher avidity. We have extended our experimental test of this prediction, as shown in Fig. 2, for three additional 7-1 antibodies, MAbs 159, 4C3, and 5H3, which all neutralize G3 rotavirus strains (Fig. 2). Escape mutations to 159 and 4C3 map to residue 96 in region 7-1a (45) (Fig. 2A and B), and 5H3 escape mutants map to residue 211 in 7-1b (41) (Fig. 2C). We generated the corresponding Fab fragments by papain digestion and showed that they all neutralize virus (Fig. 2). The difference in effective concentration between intact antibody and antibody fragment is approximately 3- to 70-fold, which we again attribute to avidity differences between a divalent MAb and monovalent Fab. Neutralization by the monovalent fragments implies that, like 4F8, these antibodies bridge the VP7 subunit interface. We suggest that most neutralizing antibodies that map to region 7-1 will have this property.

Neutralization by antibodies that bind in region 7-2. Binding of monovalent Fab to region 7-2 is not expected to stabilize the VP7 trimer, as the sites are far from a subunit contact. MAbs that map to this region are less common than those that map to 7-1. We obtained frozen ascites fluid from mice producing antibody RV34, for which escape mutations map to residues 148 and 264 (37), purified the antibody by protein G binding and size exclusion

FIG. 1. Two views of VP7 (1). On the left, a view from the outside the virion, along the 3-fold axis. The three subunits are shown as molecular surfaces, shaded different intensities of gray. Side chains in epitope regions 7-1a, 7-1b, and 7-2 are in red, pink, and blue, respectively. On the right, a view tangential to the virion.

FIG. 2. Neutralization curves for antibodies with escape mutations in region 7-1. For each of the three monoclonal antibodies indicated, infectivity is shown for a concentration series of antibody or Fab. MAbs 159 (A) and 4C3 (B) select escape mutations at position 96 in region 7-1a, and MAb 5H3 (C) at position 211 in 7-1b. Error bars show standard deviations for three measurements from independent experiments. Infectivity below a 3-log reduction in titer is not reported.
the gel; ethidium bromide staining detected coated or uncoated associated and free VP7 (Fig. 4). We labeled recombinant VP7 coat of viral particles at a low calcium concentration by assaying whether VP7-targeted neutralizing antibodies stabilize the outer VP7 efficiently recoats DLPs in the presence of sufficient calcium essential, although it enhances potency.

The latter group. Because each Fab arm of antibodies in the former group makes contact across a subunit boundary, divalency is not necessary for antibodies in the former group directed against 7-2 epitopes neutralize by preventing dissociation of a Fab-saturated particle on VP4-mediated virus binding and entry (Fig. 3A). This result supports the hypothesis that neutralization requires VP7 cross-linking, either by contact of a single Fab across a subunit interface or by divalent interaction of an intact antibody with two VP7 polypeptide chains. We tested this idea by binding Fab RV3:4 and then adding an anti-Fab antibody (Fig. 3B)—an approach first used to show that neutralizing antibodies stabilize poliovirus capsids by cross-linking sites on their surface (17). The secondary antibody restored effective neutralization by Fab RV3:4. Samples without secondary antibody were not affected by the presence of the Fab, and virus incubated with anti-Fab antibody alone was likewise unaffected. We conclude that, like antibodies directed against epitopes in region 7-1, those directed against 7-2 epitopes neutralize by preventing dissociation of the VP7 trimer. Divalent is necessary for antibodies in the latter group. Because each Fab arm of antibodies in the former group makes contact across a subunit boundary, divalency is not essential, although it enhances potency.

** Antibody stabilization of VP7-coated DLPs.** Recombinant VP7 efficiently recoats DLPs in the presence of sufficient calcium and uncoats in the absence of sufficient calcium (56). We tested whether VP7-targeted neutralizing antibodies stabilize the outer coat of viral particles at a low calcium concentration by assaying uncoating, using agarose gel electrophoresis to separate particle-associated and free VP7 (Fig. 4). We labeled recombinant VP7 with a 488 fluorescent dye to allow observation of its position in the gel; ethidium bromide staining detected coated or uncoated DLPs. We adjusted the free calcium concentration in samples before electrophoresis with Ca-EGTA buffers (24, 50). Fluorescently labeled VP7 (VP7-488) assembled onto DLPs supports full infectivity (A. H. Abdelhakim and S. C. Harrison, unpublished data). The electrophoretic analysis showed that VP7 uncoated from particles after incubation at free calcium concentrations of 21 nM or lower (Fig. 4A). This threshold is similar to the concentration previously reported for RRV uncoating, based on other techniques (40). VP7-coated DLPs migrate more rapidly in the gel than uncoated DLPs, probably because of the negative charge of VP7. Incubation of VP7-coated DLPs with region 7-1 MAb 4F8 or with its Fab prevents uncoating in a dose-dependent manner (Fig. 4B). 4F8 MAb-bound particles are retained in the sample wells, even at low calcium concentrations, probably because of increased virus-particle radius and antibody-mediated aggregation. 4F8 Fab-bound particles enter the gel, and DLP and VP7 fluorescence still migrate together in low calcium at the higher Fab concentrations tested. Region 7-2 MAb RV3:4 also blocks VP7 dissociation at low free calcium concentrations, preventing complete conversion to DLPs at lower antibody dilutions. RV3:4 Fabs do not prevent dissociation of VP7 from DLPs, however, at any concentration tested (Fig. 4C). In the presence of calcium, MAb/Fab-treated viral particles migrated more slowly in the gel than did untreated VP7-coated DLPs, showing that the concentration and affinity of the Fab were adequate to achieve high occupancy. These results support a general cross-linking neutralization mechanism, in which both 7-1-specific and 7-2-specific MAbs prevent loss of the VP7 outer coat but only 7-1-specific Fabs can stabilize the outer capsid.

**Disulfide cross-linking of VP7 trimers.** Can a mutationally introduced cross-link mimic the effect of a neutralizing antibody? Guided by the crystal structure, we introduced apposing cysteines at the subunit interface (Fig. 5A and B). Of the three constructs tested, the T276C-Q305C mutant gave the best results, yielding soluble, trimeric protein secreted from recombinant baculovirus-infected insect cells, as judged initially by reducing and nonreducing SDS-PAGE (Fig. 5C) and immunoblot with a VP7 specific antibody (data not shown). We purified this disulfide variant (VP7 S-S) with the same protocol used to prepare wild-type recombinant protein (12), concanavalin A affinity chromatography followed by immunoaffinity purification with an immobilized, VP7 trimer-specific antibody (159). Elution of VP7 S-S from the antibody column could be accomplished with similar efficiency by either calcium chelation (with EDTA) or a chaotropic concentration of magnesium (data not shown). Elution with EDTA shows that loss of Ca$^{2+}$ reduces antibody affinity, probably because the single disulfide does not tether the interface as rigidly as do the two Ca$^{2+}$ ions, particularly for the trimeric species in which the disulfide has formed at only two of the three interfaces. Wild-type VP7 trimer and VP7 S-S eluted in essentially the same volume by size exclusion chromatography (data not shown). Nonreduced samples separated by SDS-PAGE and detected by Coomassie blue staining showed a predominant band at 100 kDa, which we interpret as cross-linked trimer (Fig. 5C). Minor bands at 55 and 65 kDa were also observed and most likely represent a small percentage of incorrectly formed, cross-linked dimeric species. Samples boiled in the presence of reducing agent (β-mercaptoethanol) migrated, like wild-type recombinant VP7, with an apparent molecular mass of ~35 kDa (Fig. 5C).
The VP7-directed, nonneutralizing MAb 60 binds the VP7 subunit in monomers or trimers and does not require Ca\textsuperscript{2+} to detect VP7 in an ELISA (Fig. 6A). The trimer-dependent MAbs 159 and 4F8, which map to region 7-1, will detect wild-type VP7 only when Ca\textsuperscript{2+} is present in both the wash and incubation steps (9, 10) (Fig. 6A and B). VP7 S-S yields a strong ELISA signal with all three antibodies, regardless of whether Ca\textsuperscript{2+} is included in the assay (Fig. 6C and D). The neutralizing MAb 7A12, which binds VP4 (53), was used as a negative control in these experiments. Thus, the addition of an intersubunit disulfide link preserves 7-1 epitopes, even when Ca\textsuperscript{2+} is depleted from the medium. Although binding of VP7 S-S to MAb 159 is weaker under these conditions, as shown by EDTA elution of the disulfide-linked trimer from the 159 antibody column, the affinity is sufficient for detection in the ELISA format.

Recoating with VP7 S-S. Recombinant VP7 coats DLPs completely and efficiently (2, 56). The incorporation of VP4 in its correct conformation requires a VP4 excess, the addition of VP7 before VP7, and careful adjustment of conditions (2, 35, 56). Selective trypsin sensitivity of VP4 is an indicator of proper recoating, as VP7 protects VP4 from nonspecific trypsinic digestion and restricts cleavage to specific sites between VP8\textsuperscript{*} and VP5\textsuperscript{*} (2, 4, 18, 20, 35, 56). The data in Fig. 7A show that VP7 S-S protects VP4 in this way. VP5\textsuperscript{*} bands appear at comparable levels following the addition of trypsin to particles recoated with wild-type VP7 and with VP7 S-S. The addition of EDTA before trypsinization eliminates the VP5\textsuperscript{*} band in both samples. These results imply that VP7 S-S recoats properly with VP4 onto DLPs but that, despite covalent cross-linking, VP7 S-S depends on calcium for tight binding. Our preparations of VP7 S-S were purified by elution with EDTA from the Ca\textsuperscript{2+}-dependent antibody, MAb 159, which binds region 7-1. While stably trimeric, even in the absence of Ca\textsuperscript{2+} (Fig. 6), VP7 S-S retains enough flexibility at the subunit interface that the withdrawal of Ca\textsuperscript{2+} loosens the contact, reducing affinity both for 159 and for the DLP.

The levels of protection of VP4 from nonspecific proteolytic digestion correlate with the infectious titers of recoated wild-type particles (35, 56). We prepared particles recoated with wild-type VP7 and VP7 S-S that gave comparable levels of VP4 protection. Particles recoated with VP7 S-S had a 20-fold lower infectious titer than those recoated with wild-type VP7 (Fig. 7B). We used an α-sarcin coentry assay (7, 35, 39) to determine that the presence of the engineered disulfide indeed inhibited penetration (Fig. 7C). The toxin, which inactivates ribosomes,
requires a “helper” (e.g., a virus) to gain access to the cytosol. As shown in Fig. 7C, the addition of α-sarcin to the incubation with wild-type recoated particles led to prompt loss of 35S incorporation; the use of VP7 S-S recoated particles or non-infectious DLPs yielded little to no reduction in label accumulation. Thus, introduction of the disulfide cross-link prevents the particle from completing the sequence of conformational events that lead to membrane disruption and penetration. The same approach has shown that antibodies directed against VP7 act at a similar stage (7, 39).

**DISCUSSION**

Examining the high-resolution structure of VP7 led us to group the various epitopes, identified by monoclonal antibody escape mutation and other classical approaches, into two re-
regions, designated 7-1 and 7-2, as shown in Fig. 1 (1). We also found that Fab 4F8, seen in the crystal structure to contact residues on either side of the subunit boundary within a trimer, neutralized infectivity, presumably by stabilizing the trimer and preventing uncoating (1). Neutralization by the Fab was less potent than neutralization by the intact antibody, as expected from the contribution of divalency to overall IgG avidity. Our suggestion that noncovalent cross-linking of VP7 is a general mechanism for neutralization by antibodies that bind region 7-1 appeared to be at odds with data in the literature, which reported failure of neutralization by Fabs from certain anti-

Antibody valency also affects its serotype recognition breadth. Antibodies 159 and 4C3, both IgGs, neutralize a relatively narrow range of serotypes; both have escape mutations at residue 94. Viruses with mutations at this position in region 7-1 appeared to be at odds with data in the literature, which reported failure of neutralization by Fab (46). We have reinvestigated this point for MAbs 159, 4C3, and 4H3 and shown that, at suitable concentrations, their Fab fragments indeed block infection. The concentrations required to neutralize are between 3- and 70-fold higher than for the intact antibody, fully consistent with probable differences in avidity.

Region 7-2 lies at a domain boundary within one VP7 subunit. In principle, monovalent binding might be sufficient for neutralization, if the mechanism were to involve rigidification of the VP7 subunit to an extent requiring expulsion of the antibody for VP7 to dissociate from the DLP. Fab RV3:4, from a region 7-2-directed MAAb, did not, however, block infection. The slight (43.5%) reduction observed at the highest concentration could be due to a trace of remaining intact IgG, nonspecific interference with viral uptake when particles are fully decorated with Fab, or steric interference with VP4 conformational changes necessary for viral entry (14, 57, 61). Bridging two RV3:4 Fabs with a Fab-directed secondary antibody restored neutralization. We conclude that cross-linking of sites on two subunits, either within a VP7 trimer or between two of them on the TLP surface, is the basis for neutralization by antibodies that recognize 7-2 epitopes. Both intra- and intertrimer cross-linking are possible, given the distances modeled on the cryoelectron microscopy reconstruction of the VP7 recoated particle (3). Reconstructions of intact, divalent antibodies bound to virus particles indicate that flexibility of the Fc-Fab hinge allows a generous distance range (60 Å to 140 Å) between Fab footprints (30, 54). Intertramer binding would be across a local dyad, matching the rough symmetry of the IgG.

Cross-linking of surface proteins is a mechanism by which neutralizing antibodies block cell entry of a number of other viruses. The classic example is poliovirus: a cross-linking mechanism accounts for the relatively small number of antibody molecules per virion needed to block infection, because the expansion transition needed to initiate entry is highly cooperative (16, 17, 31). West Nile virus antibodies cross-link adjacent subunit domains to prevent the dimer-to-trimer transition associated with entry (34, 44). In other cases, Fabs neutralize without a second, bridging antibody. The mechanism appears to involve interference with a conformational change in the target protein by stabilization of its pretransition conformer. Fabs targeting the base of the influenza hemagglutinin increase the threshold necessary for pH-mediated conformational changes that permit entry from endosomes (15, 55).

To generate an efficient neutralizing antibody response that targets VP7, the immunogen must contain VP7 in the form of

FIG. 7. Properties of virus DLPs recoated with VP4 and wild-type or disulfide cross-linked VP7. (A) Immunoblot showing trypsin cleavage of VP4 to VP5* on wild-type (wt) and disulfide-linked (VP7 S-S) recoated particles, before (left, +T) or after (right, +E/T) the addition of EDTA to remove VP7. –, untreated samples. The VP4 antibody (46) used for detection recognizes an epitope protected from protease digestion when VP4 is properly assembled on particles. (B) Infectivity of wild-type and disulfide-linked recoated particles. (C) Coentry of α-sarcin, measured by loss of 35S incorporation, following exposure of cells to medium only, to DLPs recoated with wild-type or disulfide-linked VP7, or to uncoated DLPs.
assembled oligomers, e.g., on authentic virions, inactivated virions, virus-like particles, or the surface of cells expressing an engineered, membrane-anchored VP7 (6, 9, 13, 28, 32, 33, 59). Antiserum generated from VP7 peptides, recombiant wild-type VP7, or VP7 S-S have only modest effects on viral infectivity (22, 29; G. W. Both, personal communication, H. B. Greenberg, unpublished data, and N. Feng, S. T. Aoki, P. R. Dormitzer, and H. B. Greenberg, unpublished data). A high degree of polyvalency appears to be a useful attribute of many effective immunogens. It is also possible that obscuring non-neutralizing, immunodominant epitopes on VP7, by VP6 interactions or membrane tethering, enhances the selectivity of B-cell stimulation.

The results reported here also provide some additional information about VP7 uncoating during viral entry. Although the disulfide does not prevent the release of VP7 when a chelator is added in excess of calcium to virions in solution, it is likely to stabilize its attachment to DLPs when the virion is in the confines of an endocytic vesicle with a low (but nonzero) concentration of calcium. Previous work (35) has shown that VP4 hydrophobic loops, at the tip of the VP5*-sarcin entry suggests that this conformational change in recombinant herpes simplex viruses-1-expressed rotavirus VP7, Virology 189:782–832.


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