Contact Residues and Predicted Structure of the Reovirus Type 3-Receptor Interaction*

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Sequence similarity between the reovirus type 3 hemagglutinin (HA3) and an anti-idiotypic monoclonal antibody (87.92.6) has been shown to define the site of interaction with a neutralizing (idiotypic) monoclonal antibody (9B.G5) and the cellular receptor for the virus. A synthetic peptide (Vpeptide) derived from the anti-idiotypic sequence inhibits viral binding to the receptor. In this study, variants of the Vpeptide were utilized to probe specific amino acid residues involved in binding the neutralizing antibody and the receptor. These studies indicate that the —OH groups of several residues are involved in contacting the reovirus type 3 receptor, including Tyr49, Ser52, Ser54, and Thr57 in the anti-idiotypic sequence, corresponding to Tyr28, Ser27, Ser29, and Ser30 in HA3, respectively. In contrast, only Ser54 of the anti-idiotypic sequence, corresponding to Ser327 of HA3, significantly altered neutralizing antibody binding.

Additional studies implicate sialic acid as a potential receptor type 3 receptor on some cells. This includes inhibition of binding of reovirus type 3 and 87.92.6 to L cells by heavily sialylated glycoproteins. Sialic acid was therefore utilized as a candidate receptor to analyze potential interaction schemes with HA3/87.92.6.

Sequence similarity to other immunoglobulin structures with similar sequences allowed modeling of the three-dimensional structure of these epitopes. These models reveal that similar amino acid residues and side-chain geometries may be utilized by the reovirus type 3 and influenza hemagglutinins in their interactions with cell-surface receptors.

The attachment of reovirus to both red blood cells (hemagglutination) and other cells (i.e. neuronal cells) is the primary step in the infectious process. The reovirus type 3 cell-attachment site is associated with the e1 polypeptide, which is also the reovirus type 3 hemagglutinin (HA3). This HA3 epitope for cell attachment is defined by neutralizing monoclonal antibody 9B.G5 (1, 2). A murine anti-idiotypic monoclonal antibody developed against 9B.G5, termed 87.92.6, competes with reovirus type 3 for binding to its specific cellular receptors (reovirus type 3 receptors (Reo3R)) (3, 4). The deduced amino acid sequence of HA3 was found to share sequence similarity with a combined determinant comprised of the 87.92.6 heavy and light chain variable region second complementarity-determining regions (CDR II) (5). Synthetic peptides have been utilized to determine whether the sequence similarity 87.92.6 and HA3 defines amino acids essential for epitope recognition. Prior studies (6) indicate that a peptide corresponding to the 87.92.6 light chain CDR II (Vpeptide) inhibits the interactions between 9B.G5 and HA3, 9B.G5 and 87.92.6, 87.92.6 and the Reo3R, and HA3 and Reo3R. A corresponding peptide derived from the HA3 sequence (receptor peptide) is capable of eliciting a neutralizing immune response specific for reovirus type 3 (7). These studies strongly suggest that this epitope is directly involved in reovirus binding to the Reo3R.

The epitope defined by the two peptides appears to have additional biological functions. Cross-linking of the Reo3R by either virus or anti-receptor antibodies results in inhibition of cellular growth and down-modulation of the Reo3R (8). These effects were reproduced by dimeric forms of the Vpeptide (9). Thus, the interaction between Vpeptide/87.92.6/HA3 and the Reo3R is also capable of transducing transmembrane signals in Reo3R-bearing cells. Three-dimensional models of the corresponding epitopes on HA3 and in 87.92.6 have been developed (10). Studies characterizing the Reo3R on murine L cells implicate sialic acid as a potential site for HA3 binding (10, 11). Data consistent with sialic acid participation in reovirus type 3 binding include decreased binding of reovirus type 3 following neuraminidase treatment of L cells as well as inhibition of reovirus type 3 binding by sialylated glycoproteins and sialic acid-containing carbohydrates (10, 11). Identification of HA3 as the viral polypeptide for sialic acid binding was accomplished by analysis of reovirus reassortment clones derived from reovirus type 1 and 3 genetic crosses, suggesting a direct interaction between HA3 and sialic acid.

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† The abbreviations used are: HA3, type 3 hemagglutinin; Reo3R, reovirus type 3 receptor; CDRs II, second complementarity-determining regions; BSM, bovine submaxillary mucin; BSA, bovine serum albumin; RIA, radioimmunoassay; PBS, phosphate-buffered saline; IHA, influenza hemagglutinin; anti-Id3, type 3 anti-idiotypic serum; SLC, sialic acid binding conformation.
In this study, we utilize variants of the V₃ peptide to determine specific amino acid side chains potentially involved in contacting the Reo3R on L cells. We also utilize sialylated glycoproteins to explore potential interactions between sialic acid and the HA3/87.92.6 binding epitope. By combining these observations, we have developed preliminary models of the interactions between sialic acid and HA3 and between sialic acid and 87.92.6.

**MATERIALS AND METHODS**

**Peptides and Proteins**—Bovine submaxillary mucin (BSM) and bovine serum albumin (BSA) were purchased from Sigma. All peptides were synthesized by solid-phase methods, deprotected, and released from the resin utilizing anhydrous HF. Peptides were lyophilized and further purified by passage over a Sephadex G-25 superfine column and lyophilized. Purity was assessed by high-performance liquid chromatography utilizing a C₁₈ column and a 0–70% acetonitrile gradient. All peptides purified were >90% homogeneous.

**Monoclonal Antibodies**—Neutralizing anti-reovirus type 3 monoclonal antibody 9B.G5 (murine IgG₂a,k) or isotype-matched monoclonal antibody 9H.11 was isolated from the culture supernatant by 50% ammonium sulfate precipitation; dialyzed against phosphate-buffered saline (PBS); bound to a staphylococcal protein A column followed by elution with 0.1 M citric acid, pH 3.5; neutralized with 0.5 M Tris-HCl, pH 8.5; dialyzed against PBS; and concentrated on an Amicon protein concentrator. Purified antibody was radiiodinated (5). Purified antibody 9B.G5 was adsorbed to the wells by incubation of 50 μl of a 10 μg/ml solution in 1% BSA, PBS, 0.1% NaN₃; and purified 9B.G5 was adsorbed to the wells by incubation of 50 μl/well of a 5 μg/ml solution of 87.92.6 and HO13.4 (both murine IgM,k) were grown as ascites from hybridoma cells. For 87.92.6, the ascites was filtered and stored at −70°C prior to use. This was necessary due to the observed instability of this antibody when stored in purified form. For HO13.4, the antibody was immunoaffinity-purified as previously described (6) and utilized at a concentration of 10 μg/ml in PBS, which gave similar binding on fluorescence-activated cell sorting analysis as the similar volume of 87.92.6 ascites.

**Virus**—Purified reovirus type 3 was prepared as previously described (6). Purified virus was radiiodinated by the chloramine-T method (6).

**Radioimmunoassay (RIA)**—RIA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with peptides by evaporation of 87.92.6 ascites. For 87.92.6, the ascites was filtered and stored at −70°C.

**Competitive RIA**—RIA plates were coated with staphylococcal protein A (Sigma) by incubation of 50 μl/well of a 5 μg/ml solution overnight at 4°C. The wells were washed with PBS, blocked with 2% BSA in PBS with 0.1% NaN₃, and washed with PBS. 125I-Labeled 9B.G5 was added at 50,000–100,000 cpm/well in 1% BSA in PBS for 1–2 h at 37°C. The wells were washed; and competitors were added at various concentrations in 100 μl of 0.5% BSA, 0.45% NaCl, phosphate buffer for 1 h at 37°C. 125I-Labeled reovirus type 3 was added for an additional 45 min at 5–10 × 10⁶ cpm/well. The wells were washed extensively, and the counts/minute bound was determined. Specific counts/minute bound was determined by subtracting counts/min bound to uncoated wells from counts/min bound to peptide-coated wells.

**Flow Cytometry Analysis**—L cells were centrifuged, washed twice in ice-cold PBS, and specific counts/minute bound was determined as noted above. Percent inhibition of binding was calculated by the formula noted above.

**Flow Cytometry Analysis**—The ability of sialylated glycoproteins to inhibit antibody binding to cells was determined by preincubation of the antibody with varying amounts of inhibitor in (in PBS) for 30 min to 1 h at 23°C. Cells (either L cells grown as described above or R1.1 cells grown in RPMI 1640 medium with 10% fetal calf serum and added antibiotics and L-glutamine from GIBCO) was washed in 1% BSA, PBS, 0.1% NaN₃ and resuspended at 10⁶/ml. Cells (100 μl) were then added in 1% BSA, PBS, 0.1% NaN₃, and the incubation was continued for 20–30 min. Ice-cold 1% BSA, PBS, 0.1% NaN₃ was added, and the cells were centrifuged. washed prior to counterstaining with a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Fisher) in 1% BSA, PBS, 0.1% NaN₃. The cells were washed twice, and fluorescence intensity was determined as previously described (6). Inhibition of binding was calculated as noted above with Δ mean channel number utilized in place of counts/minute.

**Neuraminidase Treatment**—L cells were centrifuged, washed twice in PBS, and resuspended at 4 × 10⁵/ml in PBS. Type VIII neuraminidase from Clostridium perfringens (Sigma) was dissolved in PBS at 5 units/ml and added to a final concentration of 20–25 millunits/ml in tissue culture flasks. Following a 1-h incubation, the cells were centrifuged, washed twice in ice-cold PBS, and processed for flow cytometry as described above. Control cells were treated identically without the addition of neuraminidase.

**Modeling of HA3 and V₃ Peptides Interaction with Sialic Acid**—The strategy for the modeling studies was comparative model building based on a nonimmunoglobulin-like domain of the human collagenous hypervariable loops. By combining molecular mechanics and energy optimization, the development of starting geometries followed two knowledge base approaches: (i) consideration of sequence similarity between fragments and (ii) consideration of loop size and side-chain orientation of hypervariable loops. Starting geometries for the V₃ and HA3 epitopes were derived as previously described (6). The starting geometry for sialic acid was derived from that of Weiss et al. (13). Energy parameters were those of Kollman and co-workers (14). The program DISCOVER (BIOSYM Technologies) was used for the calculations. A sliding scale dielectric was used in the calculations. Only nonbonded interactions for atom pairs closer than 7.5 Å were calculated, with typical convergence criteria assumed in optimization. Model building was performed using the program INSIGHT (BIOSYM Technologies).

**RESULTS**

**Interaction of 9B.G5 with V₃ Peptide and Variants**—To determine the relative affinity of binding of neutralizing anti-HA3 monoclonal antibody 9B.G5 to the variants of the V₃ peptide, a simple solid-phase RIA was performed utilizing peptides dried onto microtiter wells. Binding of directly radiiodinated 9B.G5 to increasing amounts of peptides was quantitated (Fig. 1). First, binding to peptides of increasing length was determined (Fig. 1, upper). This revealed significant binding of 9B.G5 to a 10-residue peptide (V₁₋₁₀ peptide) corresponding to the carboxyl-terminal decamer of the V₃ peptide, without significant binding to similar nonamer, octamer, and heptamer peptides (V₇, V₈, and V₉ peptides). This indicates that the 10 amino acids in the V₁₋₁₀ peptide are the minimal length that elicits significant 9B.G5 binding in this assay.

To examine the contributions of individual amino acid side chains in this interaction, binding to the V₃ peptide variants noted in Table I was examined (Fig. 1, lower). This revealed that a Ser → Ala substitution at position 12 (V₁₋₁₂) significantly diminished binding of 9B.G5 as determined by RIA wells. It is seen that neither L cell line treated identically without the addition of neuraminidase.

None of the other substitutions diminished the binding of 9B.G5 below that of the unaltered V₃ peptide. Together, these studies imply that deletion of the —OH group from position 12 (corresponding to Ser⁹ in the CDR II and Ser²⁷ in HA3) significantly diminished the binding of 9B.G5. 9B.G5 bound
FIG. 1. Binding of 9B.G5 to V, peptide variants. Binding of 125I-labeled 9B.G5 to V, peptide variants on solid-phase RIA was determined as described under "Materials and Methods." Specific counts/minute bound is shown as a function of increasing amounts of peptide/well. The mean ± S.E. is shown for duplicate determinations on replicate wells. Upper, results for peptides of increasing length; B, results for peptides with specific substitutions (see Table I). The V, peptide sequence is CQGLEWIGRIDPANG.

Table I

Amino acid sequences of synthetic peptides used and correspondence to the HA3 and 87.92.6 VI, CDR II sequences

| Peptide sequences | HA3 (317)QSM-WIG1VYS5GSGLNL(332) | VI, CDR II (39)KPGKTNKLIYSGSTLQ(55) |
|-------------------|---------------------------------|-------------------------------------|
| V,                | KPGKTNKL1IYSGSTLQ               | ++ ++++++ ++                        |
| V,SH              | CKPGKTNKL1IYSGSTLQ              |                                     |
| V,A6              | KPGKANKKL1IYSGSTLQ              |                                     |
| V,F11             | KPGKTNKL1EIGSTLQ                |                                     |
| V,A12             | KPGKTNKL1YSGSTLQ                |                                     |
| V,A13             | KPGKTNKL1YSGSTLQ                |                                     |
| V,A14             | KPGKTNKL1YSGSTLQ                |                                     |
| V,A15             | KPGKTNKL1YSGSTLQ                |                                     |
| V,7               | YSGSTLQ                         |                                     |
| V,8               | IYSGSTLQ                        |                                     |
| V,9               | LIYSGSTLQ                       |                                     |
| V,10              | LLIYSGSTLQ                      |                                     |

It was found that some of the substitutions appeared to enhance binding in the solid-phase assay. This may not have been due to a direct interaction with 9B.G5, but instead may have been due to enhanced binding of the particular peptides to the RIA plates. To eliminate this variable, aqueous-phase competition assays were utilized to determine the ability of these peptides to compete with 125I-labeled reovirus type 3 for binding to 9B.G5. Peptides were added to 9B,G5-coated microtiter wells, and inhibition of subsequent 125I-labeled reovirus type 3 binding was determined (Fig. 2). The V,10 decamer was an effective inhibitor in this assay with the shorter heptamer, octamer, and nonamer peptides being ineffective (Fig. 2, upper). This confirms the ability of V,10 to bind to 9B.G5 and infers binding at the site of interaction with HA3.

When the substituted V, peptide analogs were tested, the V,A12 variant demonstrated diminished ability to inhibit the 9B.G5-HA3 interaction (Fig. 2, center and lower). None of the other substitutions demonstrated diminished interaction with the binding site of 9B.G5 by this assay. The Ser → Ala substitution at position 5 (V,A5), which is outside the region of sequence similarity, while enhancing binding on solid-phase RIA (Fig. 1, lower), had no effect on the aqueous-phase competition assay. Similarly, the Tyr → Phe substitution at position 11 (V,F11) enhanced 9B.G5 binding in the solid phase (Fig. 1, lower) without affecting competition in the aqueous phase (Fig. 2, lower). Of interest was the increased ability of the Gly → Ala-substituted V,A13 and dimeric V,SH peptides to compete for 9B.G5 binding in the aqueous-phase assay. The dimeric V,SH peptide is likely to have increased avidity for the cell-bound Reo3R, whereas altered conformational properties of the V,A13 peptide may affect its apparent increased affinity for 9B.G5 (see below).

Together, these results suggest that deletion of the —OH group from position 12 (corresponding to Ser527 in the CDR II and Ser527 in HA3) diminishes the ability of the peptide to inhibit HA3 binding by 9B.G5. Dimerization of the V, peptide (V,SH peptide) or addition of a —CH3 group to position 13 (V,A13 corresponding to Gly530 in the CDR II and Gly530 in HA3) enhances the ability of the peptide to inhibit HA3

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FIG. 2. Inhibition of 9B.G5 binding to reovirus type 3 by peptides. Inhibition of 125I-labeled reovirus type 3 binding to 9B.G5-coated wells was determined as described under "Materials and Methods." Percent inhibition of duplicate determinations on triplicate wells is shown versus increasing amounts of peptide inhibitors. Upper, results for peptides of increasing length; center and lower, results for peptides with specific substitutions (see Table I).

binding by 9B.G5. A decamer (V_{10}, corresponding to amino acids 323–332 of HA3 and amino acids 46–55 of the 87.92.6 V_{I}, CDR II) is sufficient to inhibit HA3 binding by 9B.G5.

Interaction of Reovirus Type 3 Receptor with V_{I} Peptide and Variants—The capacity of these peptides to interact with the Reo3R was next determined. This was quantitated by their ability to inhibit binding of 125I-labeled reovirus type 3 to murine L cells in aqueous-phase binding assays. The short hepta to decapeptides tested were ineffective in inhibiting reovirus type 3 binding to L cells (data not shown). When the variant peptides were evaluated, the Ser → Ala substitution at position 5 (V_{I},A5) had no effect on binding inhibition compared with the unsubstituted V_{I} peptide (Fig. 3, upper). The dimeric form of the V_{I} peptide (V_{I},SH) was more effective on a molar basis than the V_{I} peptide. Thus, these substitutions had similar effects on interactions with the Reo3R and 9B.G5.

In contrast, loss of —OH groups from position 11, 12, 14, or 15 (V_{I},F11, V_{I},A12, V_{I},A14, and V_{I},A15) resulted in diminished ability of the peptides to inhibit 125I-labeled reovirus type 3 binding (Fig. 3, lower). The loss of —OH groups at positions 14 and 15 had a more pronounced effect than that at positions 11 and 12. This is in contrast to binding to 9B.G5, where position 12 seemed to make the major contribution (Figs. 1 and 2). Also at variance with the 9B.G5 results was the diminished ability of the V_{I},A13 peptide to inhibit the reovirus type 3-Reo3R interaction, where this peptide had a greater apparent affinity for 9B.G5 in a similar assay (compare Figs. 2 (center) and 3 (upper)).

These results suggest that deletion of —OH groups from position 11, 12, 14, or 15 (corresponding to Tyr^{39}, Ser^{50}, Ser^{55}, and Thr^{251} in the CDR II and to Tyr^{290}, Ser^{297}, Ser^{298}, and Ser^{235} in HA3, respectively) reduces the ability of the peptides to inhibit HA3 binding. Deletion of the —OH group at position 5 has no effect, whereas addition a of —CH_{3} group at position 13 reduces the ability of the peptide to inhibit HA3 binding. Dimerization of the V_{I} peptide (V_{I},SH) enhances its ability to inhibit HA3 binding to L cells. Thus, some of the peptide substitutions affect 9B.G5 and Reo3R binding differently (dimerization and the Ser → Ala substitution at position 5), whereas others have different effects (substitutions at positions 11–15).

Sialylated Glycoproteins Interact with Reovirus Type 3 and 87.92.6—Prior studies (10, 11) implicate sialic acid as an important carbohydrate component of the L cell receptor for reovirus type 3. Studies utilizing heavily sialylated glycoprotein BSM demonstrate that BSM binds reovirus type 3 and inhibits binding of reovirus type 3 to L cells (10, 11). This inhibition was dependent on the presence of sialic acid moieties in BSM as treatment with agents that removed sialic acid (such as sodium borohydride) diminished the ability of BSM to bind reovirus type 3. In addition, the binding of BSM was dependent on the HA3 molecule as a reovirus type 3 genetic reassortant containing the hemagglutinin of reovirus type 1 (reovirus type 3.HA1) did not bind BSM. Thus, a specific interaction between the sialic acid moieties of BSM and HA3 was inferred.

To confirm the prior studies, and to determine the capacity of sialic acid to bind to the reovirus type 3 analog 87.92.6, the ability of BSM to inhibit binding of reovirus type 3 and 87.92.6 to L cells was assessed. In Fig. 4, inhibition of 125I-labeled reovirus type 3 binding to L cells by increasing amounts of BSM is compared to inhibition by BSA, which
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FIG. 3. Inhibition of reovirus type 3 binding to L cells by V1 peptide variants. Inhibition of 125I-labeled reovirus type 3 binding was performed as described under "Materials and Methods." The mean percent inhibition ± S.E. of binding versus increasing peptide concentration from two experiments is shown.

FIG. 4. Inhibition of reovirus type 3 binding to L cells by sialylated glycoprotein. The ability of BSM versus BSA to inhibit 125I-labeled reovirus type 3 binding to L cells was performed as described under "Materials and Methods." Percent inhibition of 125I-labeled reovirus type 3 binding is shown versus increasing amounts of BSA/BSM. The mean ± S.E. of replicate determinations on duplicate wells is shown.

Percent inhibition of binding is shown for BSM versus BSA. Since an isotype-matched IgM monoclonal antibody binding a distinct surface molecule on L cells was not available for comparison, the effect of BSM on the binding of HO13.4 to murine R1.1 cells was utilized to control for nonspecific effects. Fig. 5 demonstrates that BSM has no effect on binding of HO13.4 to Thy1 molecules present on R1.1 cells. In contrast, BSM markedly inhibited 87.92.6 binding to L cells. As an additional control, the ability of BSM to inhibit increasing amounts of antibody binding was also assessed (Fig. 6). Preincubation of increasing amounts of antibodies with a fixed amount of BSM was compared to preincubation with BSA to determine inhibition of binding. Again, specific inhibition was obtained with BSM, and this effect could be overcome by addition of increasing amounts of 87.92.6. This implies an interaction between BSM and 87.92.6 that inhibits 87.92.6 binding to L cells. The ability of increasing amounts of 87.92.6 to overcome the inhibition by BSM suggests that BSM is interacting with 87.92.6 and not with the Reo3R, as the number of L cells was held constant. These studies indicated that whereas BSM specifically inhibited 87.92.6 binding to L cells, it did not inhibit HO13.4 binding to R1.1 cells. Thus, a specific interaction of BSM with the binding site of 87.92.6 is implied.

The effects of neuraminidase treatment on L cell binding of 87.92.6 was evaluated by flow cytometry (Fig. 7). A marked decrease in fluorescence intensity followed neuraminidase treatment of cells. This implies a direct interaction between a neuraminidase-sensitive substance on the surface of L cells and the 87.92.6 antibody. Sialic acid is a likely target for the effect of neuraminidase in this experiment.

Molecular Modeling of HA3/87.92.6-Sialic Acid Interaction—In light of the above results, a direct interaction between the V1 peptide-defined epitope and sialic acid seems unlikely. The data from variants of the V1 peptide (Fig. 3) implicate the hydroxyl groups of residues 11, 12, 14, and 15 of the V1 peptide in the binding interaction of the V1 peptide to sialic acid. Therefore, models can be developed utilizing
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**Fig. 5.** Inhibition of 87.92.6 binding to L cells by BSM. Competitive binding of 87.92.6 to L cells and HO13.4 to R1.1 cells was performed as described under "Materials and Methods" for flow cytometry. BSA or BSM was added at 500 μg/ml. In this study, antibodies were utilized at 5 μl/sample. The extent of binding is measured by Δ mean channel number on flow cytometry (A). Percent inhibition of binding is as shown in B.

**Fig. 6.** Inhibition of 87.92.6 binding to L cells by BSM. Competitive binding analysis was performed as described for Fig. 5. BSM and additional BSA were utilized at a final concentration of 720 μg/ml. Percent inhibition of binding is shown versus increasing volume of antibodies added.

**Fig. 7.** Effect of neuraminidase treatment on antibody binding to cells. L or R1.1 cells were subjected to neuraminidase treatment as described under "Materials and Methods" and subsequently stained with antibodies for flow cytometry analysis. The Δ mean channel number (mean channel number with primary antibody − mean channel number without primary antibody) was compared for treated and untreated cells to derive percent decrease. The mean ± S.D. for two experiments is shown.

Structural analysis of potential intermolecular interactions and the peptide with sialic acid that will allow a molecular understanding of the binding sites on 87.92.6 and HA3.

Our previously published structures for reovirus HA3 (residues 323–332) and the 87.92.6 Vt CDR II were utilized as starting geometries (Fig. 8). These structures are characterized by differences in primary structure and loop conformation. Amino acids 326–329 of HA3 are identical to amino acids 49–52 of the 87.92.6 Vt CDR II (Tyr-Ser-Gly-Ser). These correspond to positions 11–14 of the Vt peptide. In the model structures, Tyr35 of the 87.92.6 Vt CDR II occupies the first position in a distorted 3:5 β hairpin loop; and this corresponds to Tyr38 in the HA3 structure, which occupies the second position in a type 1 β turn with a G1 β bulge. Comparison of the HA3 and Vt models reveals that when the amino acids with maximal sequence similarity are aligned (Tyr-Ser-Gly-Ser), the first position in the β turn of HA3 (Ser32) and the fifth position of the β turn of the Vt peptide (Thr35) occupy equivalent positions. For the hydroxyl groups of amino acids 49–52 of the 87.92.6 Vt CDR II to occupy similar positions in space to those of HA3 amino acids 326–329, the structures are rotated 90° with respect to each other (Fig. 9C) (6). When this is performed, it is found that the hydroxyl group from Ser35 in HA3 (the first position of the reverse turn) overlaps that of the hydroxyl group from Thr35 in the 87.92.6 Vt CDR II (the fifth position in the reverse turn), and all the other residues in the Tyr-Ser-Gly-Ser- sequence occupy similar positions in space. Thus, potential interactions with all of the hydroxyl groups of 87.92.6 Vt CDR II positions 49–53 (represented by Vt peptide positions 11–
FIG. 8. Modeling of HA3 (left) and 87.92.6 V\(_{\text{L}}\) CDR II (right) epitopes. Left, the hydrogen bonding scheme of the 3:5 hairpin loop (a type 1 \(\beta\) turn and a G1 \(\beta\) bulge) observed in the CDR 2H of the crystal structure of human Fab NEW. The sequence shown is of the HA3 epitope mutated at positions dictated by the sequence homology between NEW and the HA3 epitope (6). The five positions defining the turn are occupied by Ser in position 1, Tyr* in position 2, Ser in position 3, Gly in position 4, and Ser in position 5. Right, the hydrogen bonding scheme of the distorted 3:5 \(\beta\) hairpin loop observed in the CDR 2L of the crystal structure of the light chain dimer of REI. The sequence shown is of the V\(_{\text{L}}\) peptide mutated at positions dictated by sequence similarity between the REI sequence and the V\(_{\text{L}}\) peptide sequence. The five positions defining the turn are occupied by Tyr* in position 1, Ser in position 2, Gly in position 3, Ser in position 4, and Thr in position 5. Please note that Tyr* in Fig. 5 occupies a different position in the 3:5 \(\beta\) turn.

15) have corresponding hydroxyl groups in HA3 positions 325–329.

An interaction scheme for sialic acid and the influenza hemagglutinin (IHA) has been published with data derived from x-ray crystallographic analysis (13). The starting geometry of sialic acid bound to IHA was utilized to develop our preliminary structures for the epitope binding conformations. The orientation of sialic acid with regard to the HA3/87.92.6 V\(_{\text{L}}\) CDR II epitope in the preliminary structure was based on the relative positions of potential hydrogen bond partners on sialic acid for the implicated hydroxyl groups on the HA3/87.92.6 V\(_{\text{L}}\) CDR II epitope. Utilizing our data, several orientations of sialic acid with respect to the HA3/87.92.6 V\(_{\text{L}}\) CDR II epitope were possible. With the caveat that position 2 (involved in linkage of sialic acid to glycoproteins) was not available, groups that represented the most likely potential hydrogen bond partners on sialic acid included the carboxylic acid group, the acetamido group, and glycerol hydroxyl groups, all of which are involved in hydrogen bonding to the influenza hemagglutinin (Table II). These groups were utilized to orient the sialic acid with respect to the HA3/87.92.6 V\(_{\text{L}}\) CDR II epitope in a manner that would allow hydrogen bonding to all four hydroxyl groups implicated on the HA3/87.92.6 V\(_{\text{L}}\) CDR II epitope.

For the V\(_{\text{L}}\) structure, the principal contacts involve the hydroxyl groups from Tyr\(^{69}\), Ser\(^{69}\), Ser\(^{72}\), and Thr\(^{75}\). The residue shifting in the conserved region of HA3 based on the NEW geometry allows for a set of equivalent interactions between HA3 and sialic acid involving Tyr\(^{206}\) (Tyr\(^{69}\)), Ser\(^{207}\) (Ser\(^{69}\)), Ser\(^{209}\) (Ser\(^{72}\)), and Ser\(^{215}\) (Thr\(^{75}\)). These models reveal the predominant planar nature of the epitopes in both cases, with intermolecular hydrogen bonds all directed from the same face of the reverse-turn structures. These hydrogen bonds pair with sialic acid side chains as noted in Table II. In Table II, the resulting heavy atom distances representative of hydrogen bond pairs are shown in comparison with the known pairs involved in the IHA-sialic acid interaction. The model structures for the HA3 and 87.92.6 V\(_{\text{L}}\) CDR II epitopes...
parameters estimated for the sialic acid moiety. The structure of the immunoglobulin NEW heavy chain CDR I1 was examined to determine the spatial orientations of the side chains that define the geometry of the sialic acid binding conformations (modes) are similar. The conformational energy for this binding mode was evaluated utilizing both the HA3 and 87.92.6 VI CDR II epitopes (Table III). This was approached in a sequential fashion. In constructing the model for the HA3 structure, the reverse turn of the immunoglobulin NEW heavy chain CDR II was utilized as a template, whereas for the 87.92.6 VI CDR II epitope, the REI light chain CDR II was utilized (6, 12). To examine the sequence-independent properties of the starting conformations, polyalanine forms of these starting geometries were developed, and their stabilization energies were determined (Table III, Column 1). The sequence-independent effect of the conformation change in assuming the modes of these epitopes was then calculated for these polyalanine forms (Column 2). The root mean square deviations for the transitions of the starting geometry to the respective binding mode are presented by the relatively low root mean square deviations of crystallographically determined CDR II regions of light chains (data not shown). In Column 5, the corresponding conformational energies for the HA3 and VI CDR II epitopes with sialic acid are shown utilizing each other's starting geometries. In this column, the HA3 epitope is in the V I/SLC conformation, whereas the VI CDR II epitope is in the HA3/SLC conformation. Comparison of HA3 (V I/SLC) with HA3 (SLC) (Columns 5 and 4) indicates that the two conformations are close in energy. Thus, whereas it is impossible to distinguish the true binding mode, several potential geometries are suggested by these analyses.

The interaction models between the HA3/87.92.6 VI CDR II epitopes and sialic acid are shown in Figure 9 (A and B). The superposition of the contact residues with the two modeled epitopes rotated ~90° with respect to one another (similar to Ref. 6) is shown in Figure 9C. Dynamics run of these structures are predicted to form hydrogen bonds with identical partners on sialic acid. These differ from the IHA structure in two respects. The carbohydrate at position 4 of sialic acid is utilized in these model structures, but not in the IHA structure. Conversely, the glycerol hydroxyl group at position 9 of sialic acid interacts with IHA, but not with the current model structure. Otherwise, the intermolecular distances of the current models are very similar to those in the IHA-sialic acid pair (Table II).

In the absence of crystallographic or NMR information, the placement of hydrogens in the donor-acceptor scheme in any model is tentative at best. In our models of the possible binding mode of the epitopes, the models reflect subtle differences in the donor-acceptor pairings. For example, the Tyr is a donor in the VI CDR peptide-sialic acid model, whereas it is an acceptor in the HA3-sialic acid model. The differences are indicative of the model geometries as well as the partial energy parameters estimated for the sialic acid moiety. The structures reflect in vacuo explorations of conformational space to generate models that are useful and stereochemically valid. The molecular models indicate that the HA3 and VI conformers have different backbone conformations. However, the spatial orientations of the side chains that define the geometry of the sialic acid binding conformations (modes) are similar. The conformational energy for this binding mode was evaluated utilizing both the HA3 and 87.92.6 VI CDR II epitopes (Table III). This was approached in a sequential fashion. In constructing the model for the HA3 structure, the reverse turn of the immunoglobulin NEW heavy chain CDR II was utilized as a template, whereas for the 87.92.6 VI CDR II epitope, the REI light chain CDR II was utilized (6, 12). To examine the sequence-independent properties of the starting conformations, polyalanine forms of these starting geometries were developed, and their stabilization energies were determined (Table III, Column 1). The sequence-independent effect of the conformation change in assuming the modes of these epitopes was then calculated for these polyalanine forms (Column 2). The root mean square deviations for the transitions of the starting geometry to the respective binding mode is ~0.68. However, the poly(Ala) forms of the complex conformation are ~4 kcal/mol closer in energy than the starting conformations. The root mean square value for the backbone conformation of the structures in Column 2 approaches 3.5. The poly(Ala) forms of the complex conformation are 4 kcal/mol closer in energy than the starting conformation. The conformational energies of the HA3 epitope before and after binding sialic acid are shown in Column 3, whereas the corresponding energies for the VI CDR II epitope are shown in Column 4. In both cases, the modeled sialic acid binding mode is the preferred conformation for the derived structures by ~16 kcal/mol, with a 0.68 root mean square for their backbones. This energy difference appears to be due to the side chains as the poly(Ala) forms for all of the conformations are similar (Columns 1 and 2). This implies that there is an energy cost for forcing the side chains into the REI conformation (for the VI peptide) or the NEW conformation (for HA3). The degree of backbone structural variability represented by the relatively low root mean square is in keeping with the comparisons of root mean square values of crystallographically determined CDR II regions of light chains (data not shown). In Column 5, the corresponding conformational energies for the HA3 and VI CDR II epitopes with sialic acid are shown utilizing each other's starting geometries. In this column, the HA3 epitope is in the VI/SLC conformation, whereas the VI CDR II epitope is in the HA3/SLC conformation. Comparison of HA3 (VI/SLC) with HA3 (SLC) (Columns 5 and 3, respectively) indicates that the two conformations are close in energy. The same is true for VI (HA3/SLC) and VI (SLC) (Columns 5 and 4, respectively). This indicates that although the low energy forms of the HA3 and VI epitopes modeled with different starting geometries give vastly different backbone structures (root mean square difference of 3.5), the energies of these conformations complexed with sialic acid differ by 1.5 (for HA3) to 2.5 (for the VI peptide) kcal/mol. Whereas the REI-type conformation may be more preferred for both HA3 and VI structures, the closeness in energy of the two structures may indicate that either conformation may be observed 50% of the time. Thus, whereas it is impossible to distinguish the true binding mode, several potential geometries are suggested by these analyses.
before sialic acid binding indicate that intramolecular hydrogen bonds involved in stabilizing the reverse-turn structures involve most of the residues involved in binding sialic acid, with the possible exception of Tyr. This may result in a new lower stabilization energy than would otherwise be expected. The pairing of the hydroxy group corresponding to Thr° of the Vc peptide with the charged carboxylic acid group of sialic acid may account for its relatively large contribution to the binding interaction (Fig. 3). Similarly, the pairing of the hydroxy group corresponding to Ser° of the Vc peptide with the carbohydrate hydroxy group at position 4 of sialic acid would be expected to form a relatively strong hydrogen bond. In contrast, the Tyr°-glycerol—OH pair at position 8 results in entropic loss from the glycerol side chain losing conformational flexibility, whereas the Ser°-acetamido—NH utilizes a less polar—NH group. This could result in weaker interaction energies for these pairs, accounting for the lesser contribution of the Vc peptide hydroxyl groups at positions 11 and 12 in binding.

**DISCUSSION**

The neutralizing anti-reovirus type 3 monoclonal antibody 9B,G5 is a Reo3R analog by several criteria. 9B,G5 competitively inhibits binding of reovirus type 3 to the Reo3R (15, 16). Antibodies that bind 9B,G5 also recognize the Reo3R (3, 4, 15, 16). One antibody developed against 9B,G5 (87.92.6) binds both 9B,G5 and the Reo3R in a competitive manner (3, 4, 87.92.6 also exhibits sequence similarity to HA3 (5), indicating a primary structural basis for both 9B,G5 and Reo3R recognition. Peptides derived from this region of sequence similarity bind both 9B,G5 and the Reo3R with similar affinity (6). Thus, 9B,G5 and the Reo3R display many similar binding interactions.

However, binding of synthetic peptides to 9B,G5 and the Reo3R reveals differences in binding strategies to these moieties. A 10-aminoc acid peptide corresponding to the region of sequence similarity between HA3 and the 9B,G5 CDR II inhibits binding of HA3 with 9B,G5 (Fig. 2), but does not inhibit HA3 binding to the Reo3R on L cells. Longer versions of these peptides are currently under development to evaluate the minimum-sized peptide necessary to inhibit binding of reovirus type 3 to L cells. Within this region, the —OH from Ser° of the CDR II (corresponding to Ser° in HA3) is likely to directly interact with 9B,G5, as indicated by the diminished ability of the Vc12 peptide to interact with 9B,G5 (Figs. 1 and 2). In contrast, —OH groups from Tyr°, Ser°, Ser°, and Thr° of the CDR II (corresponding to Tyr°, Ser°, Ser°, and Ser° in HA3, respectively) are all likely to directly interact with the receptor structure as VcF11, VcA12, VcA14, and VcA15 peptides all have diminished ability to interact with the Reo3R. Thus, whereas general epitope bound by 9B,G5 and the Reo3R represented by the Vc peptide and its analogs seems to encompass the same stretch of amino acids, specific intermolecular interactions involved in binding 9B,G5 may differ from those involved in binding the Reo3R.

Since small peptides in solution are thought to exist in a multiplicity of transient conformational states in dynamic equilibrium, it is of interest to examine the conformational possibilities of structural analogs of Vc and Vc reverse-turn loops that have shown biological activity in an effort to establish design criteria in the development of biologically active peptides derived from antibody templates. Unlike previous studies of the conformational properties of CDR loops that were interested in defining the accuracy of prospective predictions to actual crystallographic structures (17, 18), this approach is more concerned with an analysis of the local energy minimum of analogs of well-defined Ig crystallographic templates. In this regard, the differences in binding the Vc,A13 peptide demonstrated in Figs. 2 and 3 are also of interest. The Gly→Ala substitution of this residue results in increased binding to 9B,G5 (Figs. 1 and 2), but diminished binding to the Reo3R (Fig. 3).

In an effort to explain these observations in terms of molecular structure, molecular dynamics calculations (employing starting geometries suggested by the molecular modeling studies) were performed for the Vc and Vc,A13 peptides. These preliminary calculations (data not shown) indicate that the introduction of Ala at position 13 restricts the in vacuo conformational flexibility of the Vc,A13 peptide. One structural interpretation of these calculations is that whereas both the Vc and Vc,A13 peptides can populate conformations that represent the 9B,G5 binding mode, the energy cost for conformational adjustment in adopting the 9B,G5 binding mode conformation from the respective low energy “solution” structures may be greater for the Vc peptide than for the Vc,A13 peptide. In other words, whereas the Vc,A13 peptide is less conformationally flexible than the Vc peptide, its low energy solution conformation may be close to the 9B,G5 binding conformation, and relatively less conformational adjustment is needed to assume this conformation in comparison with the Vc peptide. Conversely, the Vc,A13 peptide may lack the conformational flexibility needed to easily assume the Reo3R binding mode. This implies that somewhat different conformations of the Vc peptides may be optimally bound by 9B,G5 versus the Reo3R.

**Analysis of Reo3R on Different Cells**—Previous data from this laboratory biochemically characterized the Reo3R on some cells (19-21). The receptor of murine R1.1 (thymoma) cells has been characterized utilizing anti-idiotypic anti-reovirus type 3 polyclonal serum (anti-Id3), which resembles 87.92.6 in its binding interactions. Anti-Id3 identified a ~65-kDa glycoprotein with a pI of 5.8~6.0 on the surface of R1.1 cells. This protein was shown to possess binding characteristics similar to those of the β-adrenergic receptor (20, 21). Treatment of the R1.1 Reo3R with inhibitors of glycosylation (including neuraminidase) did not inhibit the ability of anti-Id3 to immunoprecipitate the receptor (19, 20). Interestingly, whereas R1.1 cells bind anti-Id3, 87.92.6, and reovirus type 3 in a specific/saturable manner, these cells are not infected by reovirus type 3.

The Reo3R on murine L cells is sensitive to neuraminidase treatment. These cells are easily infected by reovirus type 3, and they possess little if any β-adrenergic receptor-like binding activity (22). Thus, by several criteria, the Reo3R on L cells may be distinct from that on R1.1 cells. It is noteworthy that BSM and neuraminidase treatment do not appreciably inhibit 87.92.6 binding to R1.1 cells. Thus, these cells may possess a distinct receptor for reovirus type 3. This receptor may divert virus from sialic acid to a distinct binding site, which may not allow virus internalization, or may divert the virus to an intracellular compartment nonpermissive for subsequent steps in the infectious cycle. This mechanism could play a role in influencing the tissue tropism of reovirus type 3 or other viruses.

**Implications of Structural Studies**—Sialic acid is a common carbohydrate on the surface of many mammalian cells. Viruses have been shown to utilize sialic acid as both a receptor and substrate. Prior structural analysis of the IHA-sialic acid interaction (13) indicates hydrogen binding to four groups on sialic acid. Our study implicates three of these four groups as potential hydrogen bond partners with reovirus HA3. This

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may indicate a ready geometric availability of these groups for developing intermolecular interactions with biologically important molecular structures. Whereas this study derives the hydrogen bond partners from model structures, similar amino acid residues are implicated as contact residues in this model, as were found in the crystallographically determined IHA-sialic acid structure (Table III). This may indicate a propensity for certain amino acid residues to form hydrogen bond partners with the sialic acid groups, suggesting a chemical basis for viral/antibody binding. However, the specificity of binding for viruses to sialic acid may also relate to other aspects of the sialylated glycoproteins as well as additional interaction sites on the virus.

The utility of molecular modeling coupled with examination of peptide analogs of binding sites allows development and verification of specific structural data of biological importance. This approach allows development of testable hypotheses regarding binding interactions. These can be probed by additional experimentation, and more refined models can be developed. By utilizing these complementary techniques, substances with specific binding activity can be developed. This should allow rational development of biologically active compounds.

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