Antibodies to the Buried N Terminus of Rhinovirus VP4 Exhibit Cross-Serotypic Neutralization

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Development of a vaccine for the common cold has been thwarted by the fact that there are more than 100 serotypes of human rhinovirus (HRV). We previously demonstrated that the HRV14 capsid is dynamic and transiently displays the buried N termini of viral protein 1 (VP1) and VP4. Here, further evidence for this “breathing” phenomenon is presented, using antibodies to several peptides representing the N terminus of VP4. The antibodies form stable complexes with intact HRV14 virions and neutralize infectivity. Since this region of VP4 is highly conserved among all of the rhinoviruses, antiviral activity by these anti-VP4 antibodies is cross-serotypic. The antibodies inhibit HRV16 infectivity in a temperature- and time-dependent manner consistent with the breathing behavior. Monoclonal and polyclonal antibodies raised against the 30-residue peptide do not react with peptides shorter than 24 residues, suggesting that these peptides are adopting three-dimensional conformations that are highly dependent upon the length of the peptide. Furthermore, there is evidence that the N termini of VP4 are interacting with each other upon extrusion from the capsid. A Ser5Cys mutation in VP4 yields an infectious virus that forms cysteine cross-links in VP4 when the virus is incubated at room temperature but not at 4°C. The fact that all of the VP4s are involved in this cross-linking process strongly suggests that VP4 forms specific oligomers upon extrusion. Together these results suggest that it may be possible to develop a pan-serotypic peptide vaccine to HRV, but its design will likely require details about the oligomeric structure of the exposed termini.

Rhinoviruses are the major causative agents of the common cold and cost the United States economy approximately $40 billion per year (6). Therefore, it is of great interest to prevent or ameliorate the symptoms of the common cold. The rhinovirus genus is a member of the picornavirus family and is characterized by nonenveloped capsid with a diameter of ~300 Å containing a single-stranded, plus-sense RNA genome (19). Other members of the picornavirus family include foot-and-mouth disease virus, poliovirus, encephalomyocarditis virus, and hepatitis A virus. The capsids exhibit pseudo T₄ containing a single-stranded, plus-sense RNA genome (19). Other members of the picornavirus family include foot-and-mouth disease virus, poliovirus, encephalomyocarditis virus, and hepatitis A virus. The capsids exhibit pseudo T₄ containing a single-stranded, plus-sense RNA genome (19).
capsid at the capsid-RNA interface (8). These results suggested that the picornavirus capsid was more dynamic than indicated by the crystal structure and that these termini are presented to the exterior of the virion in a temperature-dependent and reversible manner. While the role of capsid dynamics in the viral life cycle was not clear, it was suggested that the N termini of VP1 and VP4 might facilitate cell membrane attachment and subsequent entry of the virus into the host cell (3, 4).

More recently, evidence for capsid dynamics has been found in other viruses as well. In the cases of swine vesicular disease virus (10) and coxsackievirus A9 (18), antibodies were raised against the whole virus in pigs and rabbits, respectively. These polyclonal antibodies demonstrated a strong reaction to the peptides corresponding to the N termini of VP1 and VP3 of swine vesicular disease virus and coxsackievirus A9, respectively. In a similar study, antibodies from the plasma of patients suffering from type I diabetes were found to target VP4 protein of coxsackievirus B3, again suggesting the exposure of VP4 peptide during coxsackievirus infection (23). These results imply that capsid “breathing” may be a phenomenon common to many proteinaceous capsids.

Using a very different approach, the dynamic nature of HRV14 was analyzed using limited proteolysis and mass spectrometry (matrix-assisted laser desorption ionization [MALDI]) analyses (14). In these experiments, the virus was treated with both matrix-bound and soluble forms of trypsin for various periods of time, and the resulting proteolytic fragments were identified by MALDI. Surprisingly, the N termini of VP4 and VP1 were found to be the most proteolytically sensitive portions of the capsid in spite of being buried inside the viral capsid. As an additional control, the antiviral “WIN” compounds, which had been previously shown to stabilize the virions against thermal and acid denaturation, were added during digestion. While these WIN compounds did not affect the intrinsic proteolytic activity of trypsin, they nearly completely protected the VP1 and VP4 termini from proteolysis for an extended period. Together, these results suggested that HRV14 is transiently exposing these termini in a “breathing” process and that the empty hydrophobic drug-binding region apparently plays an important role in facilitating these dynamics.

In this study we further examined HRV14 capsid dynamics by raising polyclonal antibodies against several peptides representing the N termini of VP1 and VP4. In these experiments, only the antibodies against the VP4 N terminus were found to successfully neutralize viral infectivity in vitro. Further, we demonstrate that the HRV14 VP4 antisera cross-reacts with other serotypes of rhinovirus (HRV16, and HRV29), which is likely due to the high degree of conservation of VP4. Antibody neutralization closely parallels the MALDI analysis in that antibody neutralization and proteolysis are enhanced at 37°C in the case of HRV16 whereas the elevated temperatures are not required for either phenomenon in the cases of HRV14 and HRV29. Epitope mapping of the N-terminal 30 residues of VP4 suggests that it adopts a nonlinear conformation, and this is further substantiated by results showing that all of the copies of VP4 in the Ser5Cys HRV14 mutant at room temperature form cysteine cross-linked dimers. This cysteine cross-link does not form at 4°C, suggesting that capsid breathing is essential for VP4 exposure and interactions. Since VP4 dimerization does not affect viral infectivity, it seems likely that VP4 exposure is a normal part of the cell attachment and entry process of rhinovirus. Together, these results suggest that VP4 might be useful as a pan-serotypic rhinovirus vaccine, but it seems likely that better understanding of the VP4 oligomeric structure will be necessary for further optimization.

MATERIALS AND METHODS

Production of antibodies against the N termini of HRV14 VP4 and VP1. Antibodies were raised against peptides corresponding to the N-terminal amino acids of VP4 and VP1. In the case of VP4, two different peptides (Fig. 1) were used for immunization, a peptide corresponding to HRV14 VP4 without the N-terminal myristoyl moiety and one that represented a consensus sequence of all the rhinovirus serotypes with the N terminus myristoylated (peptide VP4.2). The polyclonal antibody preparations were produced by Syngene Corporation in the case of the HRV14 peptide and by Sigma Genosys in the case of peptide VP4.2. Briefly, unmyristoylated peptide synthesis was performed using the standard t-butyloxycarbonyl chemistry. The peptide was conjugated with keyhole limpet hemocyanin using 1-ethyl-3-(3-dimethylamino)propyl)carbodiimide hydrochloride as a cross-linker. The peptides were high-pressure liquid chromatography purified to >70% prior to injection. Polyclonal antibodies were produced in New Zealand White rabbits by injecting 200 μg of antigen in complete Freund’s adjuvant initially, and subsequent booster injections were given every 2 weeks for 2 months and contained 100 μg of the peptide in incomplete Freund’s adjuvant. Serum samples were collected every 2 weeks beginning 49 days after the first injection.

Virus purification. HRV was produced using previously described protocols (5). In brief, HeLa cells were infected with HRV14 at a multiplicity of infection of ~10. After incubation of the infected cells at 35°C for 9 to 10 h, the virus was purified from lysed cells treated with N-laurylsarcosine to solubilize cellular debris. However, unlike in the previously described protocol, the lysed cellular material was not treated with trypsin, since even this brief treatment resulted in cleavage of VP1 and VP4 (14). Virus particles were pelleted by ultracentrifugation at 280,000 × g for 2 h. The virus was then resuspended in 20 mM Tris buffer, pH 7.6, and further purified using 7.5 to 45% sucrose gradients centrifuged at 2 × 10^14 × g for 1.5 h. The virus bands were collected, pooled, and dialyzed overnight at 4°C against 20 mM Tris buffer, pH 7.6. The HRV14 concentration was determined spectrophotometrically using an extinction coefficient of 7.7 ml/mg · cm at 260 nm and stored at 4°C.

Cells, media, and virus stocks. The Wisconsin-HeLa (WI-HeLa) cell line was passaged in suspension culture in medium B supplemented with 10% bovine serum (Invitrogen, Carlsbad, CA). Virus stocks for HRV14 have been described elsewhere (7). Purified HRV16 used for MALDI analysis was a gift from Wai-Ming Lee (University of Wisconsin-Madison). HRV16 stocks were prepared from HRV16 cDNA provided by Wai-Ming Lee (13). HRV29 was obtained from the ATCC and was further amplified using HeLa cell monolayers, as instructed in the ATCC product information sheet.

Plaque assays. Plaque assays were performed as previously described (24). Briefly, 1.4 × 10^6 HeLa cells in 5 ml of AH medium (supplemented with 10% bovine serum), were layered on 0.6-mm cell culture plates and incubated at 35°C for 8 to 10 h until monolayers formed. The monolayers were washed with phosphate-buffered saline (PBS) prior to infection. Virus samples were added to the monolayer and allowed to attach for 1 h at room temperature. The monolayers were then washed with PBS, and overlaid with 2.5 ml of P6 medium mixed with 0.8% agar, and then topped with 2.5 ml of P6 medium. The plates were incubated at 35°C under 5% CO2 for 48 h and then stained with crystal violet for PFU determination. PBS was used for all virus and serum dilutions.

Succrose gradient fractionation of antibody/virus complexes. Purified HRV14 was mixed with various amounts of the anti-VP4 serum and incubated at room
temperature for 8 to 10 h. The virus-serum mixture was then layered on continuous sucrose gradients of 7.5 to 45% and centrifuged at 2 x 10^14 × g for 1.5 h. The sucrose gradients were then fractionated in 1.5-ml fractions using an ISCO gradient fractionator (Teledyne Technologies Inc., Thousand Oaks, CA). The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

ELISA. Standard enzyme-linked immunosorbent assay (ELISA) protocols were used for all the epitope mapping experiments. ELISA plates were coated with 100 μl of 1- to 5-μg/ml concentrations of peptides using carbonate-bicarbonate buffer (pH 9.6) and left overnight at 4°C. The coated plates were blocked with 200 μl of 3% bovine serum albumin (BSA) or 5% dry milk prepared in coating buffer and incubated at room temperature for 1 to 1.5 h. The blocking solution was removed, and the plates were rinsed once with wash buffer (150 mM NaCl and 0.05% Tween 20) before addition of 100 μl culture supernatants from hybridoma cultures. The plates with hybridoma cultures were incubated at room temperature for 2 h before the plates were washed four times with wash buffer. Secondary antibodies (anti-mouse and anti-rabbit) conjugated with horseradish peroxidase in appropriate dilutions as suggested by the manufacturer (Sigma-Aldrich, St. Louis, MO) were added to the plates and incubated for 1 to 1.5 h. The dilutions of the secondary antibody were made in buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, and 0.05% Tween 20. The pH was adjusted to 7.4 before addition of 0.1% BSA. The secondary antibody was removed from the plates before they were washed four times with wash buffer. HRP was detected using 3,3′,5′,5′-tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO) as per the manufacturer’s instructions. The reaction was stopped within 30 min by the addition of 100 μl of 1 N H2SO4, and absorbance measured at 450 nm using an ELISA plate reader.

Mutagenesis of VP4. Site-directed mutagenesis of HRV14 cDNA was performed as described earlier (11). HRV14 cDNA that produces infectious RNA upon in vitro transcription was used as the template for mutagenesis by the PCR overlap method. In the infectious clone cDNA, Ser5 is bounded by two unique restriction sites, KasI and AflII. To mutate Ser5 to Cys, two oligonucleotides were synthesized: one in the 5′-3′ orientation including the KasI site and one in the 3′-5′ orientation encompassing the AflII site. Fragments of the cDNA encoding VP4 Ser5 were amplified by PCR using the primers with the mutation. The fragments were then used as primers for subsequent PCRs to make full-length KasI/AflII fragment. The fragments containing the VP4 Ser5Cys mutation were inserted into the HRV14 cDNA using the KasI and AflII restriction sites. The assembled mutand cDNA was sequenced for verification.

Transfection and amplification of the HRV14 VP4 Ser5Cys mutant. RNA transcripts from full-length wild-type and mutated HRV14 cDNAs were made using an in vitro transcription reaction. The RNA transcripts were diluted in HEPES-buffered saline containing 200 μg/ml DEAE-dextran/ml. The dilutions were added to HeLa cell monolayers (1.41 × 10^6) and incubated at room temperature for 60 min. The cells were washed to remove DEAE-dextran and supplemented with 4 ml of AH medium as a liquid overlay. The plates were incubated for 48 h before harvesting the cells, and virus was released by repeated freeze-thawing. The presence of virus particles was determined by plaque assays. Virus titer amplification was accomplished using monolayers of HeLa cells in T75 flasks and later by HeLa cell infections in suspension culture until the titer was close to 10^10 PFU/ml. After several passages, the RNA from the VP4 Ser5Cys mutant was sequenced to check for reversion and was found to be genetically stable for several passages, but the mutant eventually reverted after 10 to 15 passages.

Development of MAbs. Mouse monoclonal antibodies (MAbs) were developed by the Washington University Hybridoma Center using the 30-residue N-terminal peptide of HRV14 VP4 (top peptide in Fig. 1). The peptide conjugated with keyhole limpet hemocyanin was used as the immunogen. Three rounds of screening were performed to select for hybridoma clones producing antibodies binding to the peptide described above. However, for screening purposes, peptide conjugated with BSA was used in order to select for clones that recognize the peptide and not the conjugate.

RESULTS

Immunoprecipitation of HRV14 with VP4 antiserum. From the previous MALDI studies (14), it is clear that the N termini of VP1 and VP4 are transiently extruded during capsid “breathing.” The next question was whether it was possible to take advantage of this dynamic process to create novel neutralizing antibodies. To this end, polyclonal antiserum was first raised against a polypeptide corresponding to the first 30 residues of HRV14 VP4. To demonstrate interaction between these antibodies and intact HRV14, purified HRV14 was mixed with the undiluted antiserum, incubated at room temperature for 8 to 10 h, and then examined using sucrose gradients. The various antibody/virus species were separated using a continuous sucrose gradient of 7.5 to 45% and fractionated with an ISCO (Teledyne Technologies Inc) device. The fractions were collected as 1.5-ml aliquots starting at the top of the tube and analyzed using silver-stained SDS-PAGE (Fig. 2). Samples of antiserum alone and virus alone were used as controls. As expected, protein (mostly antibody) is not observed beyond the third fraction from the top in the sample with antiserum alone. In the virus-only sample, a narrow band formed in the middle of the gradient corresponding to intact, unaggregated virions. In contrast, the virus/antiserum complex exhibited a distribution pattern typical of aggregating antibodies. In the presence of the antibodies, the middle band corresponding to the virus is broader than that for the virus-only sample and antibody is now observed throughout the gradient. This strongly suggests that the antibody binds to and aggregates the virus particles. The strongest indication of antibody-mediated aggregation is the fact that there was immunocomplex pelleted at the bottom of the gradient, while this was not the case for antibody and virus alone (note fraction 7 of the complex in the gel in Fig. 2).
HRV14 neutralization by polyclonal antibodies to VP1 and VP4 peptides. The sucrose gradient studies clearly demonstrated that these antibodies, raised against a synthetic peptide corresponding to the N terminus of VP4, did indeed bind to the intact virions. The next question was whether these antibodies could neutralize infectivity. Using plaque assays, the dose and temperature dependencies of HRV neutralization by VP4 and VP1 antisera were measured. Four independent experiments were performed in which each of the virus-antiserum dilutions were incubated at room temperature or 37°C for 2 hours or for 8 hours. The mixtures were then added to the HeLa cell monolayers for plaque assays, and the results are shown in Fig. 3. There is a clear neutralization of the virus infectivity by the VP4 antiserum in a dose-dependent manner, with an 50% effective dose of approximately 1/5 dilution when incubated with the virus for 2 hours.

However, there was no apparent neutralization of infectivity when the VP1 antiserum was used (data not shown). In the case of poliovirus, antiserum raised against residues 24 to 40 from the N terminus of VP1 was able to neutralize infectivity (15). It is possible that the conformation of the synthetic peptide corresponding to the first 30 residues of HRV14 VP1 differs significantly from that region, as it is exposed during the breathing process.

As shown in Fig. 3, neutralization efficacy is equivalent when the virus is incubated with the antiserum at room temperature or at 35°C. This is in contrast to the case for poliovirus, where neutralization of the virus by the antiserum raised against the internal residues required incubation at 37°C (15). However, these results are consistent with our previous capsid “breathing” experiments with HRV14 (14) demonstrating that the N termini of VP1 and VP4 are displayed at room temperature.

From these results it is also apparent that neutralization proceeds in a time-dependent manner. As shown in Fig. 3, when the virus-polyclonal antiserum mixture was incubated for 8 hours, neutralization increased by about 25%. This time-dependent process was also observed in the case of poliovirus (15). Since these experiments were performed by allowing the virus-antiserum mixture to attach to the HeLa cells for 1 hour and then washing with PBS, these antibodies apparently affect the early stages of viral attachment.

Cross-reactivity of HRV14 VP4 antiserum with other rhinovirus serotypes. To ascertain the specificity of these anti-HRV14 VP4 antibodies, neutralization assays were performed using another rhinovirus serotype, HRV16. Somewhat unexpectedly, antibodies to VP4 of HRV14 also neutralized HRV16 (Fig. 3). Similar to what was observed in the case of HRV14, there is a clear time dependency in this neutralization process. However, unlike that of HRV14, the neutralization of HRV16 was greatly enhanced when the virus-antiserum mix was incubated at 37°C prior to attachment to monolayers of HeLa cells. Indeed, the longer incubation at higher temperatures enhanced neutralization to more than 98%. The enhanced neutralization at higher temperatures is similar to what was previously observed in the case of poliovirus (15). As an additional control, the well-characterized antibody MAb 17-1A (27) was used in HRV16 neutralization assays and, as expected, did not abrogate infectivity (data not shown). Therefore, this cross-serotypic activity is specific to the anti-VP4 antibodies. These results demonstrate a direct correlation between the “breathing” observed using limited MALDI analysis and the neutralization by anti-VP4 antibodies. As demonstrated in our earlier studies (11), HRV16 capsid is fairly resistant to proteolysis at room temperature but not at the elevated temperature of 37°C. The mechanism of in vitro neutralization cannot be discerned from these studies, but previous studies with MAbs that recognize epitopes on the viral surface suggest that abrogation of cellular attachment is a likely possibility (26).

Cross-serotypic neutralization by anti-VP4 antiserum. The antiserum to HRV14 VP4 clearly neutralizes HRV16, but it remained to be seen whether it could neutralize other serotypes as well. To that end, neutralization assays were performed on HRV29 (Fig. 3). When the antiserum against HRV14 VP4 was mixed with HRV29 and incubated for 8 hours at room temperature prior to attachment, HRV29 infectivity was inhibited by more than 90%. This strongly sug-
suggests that the N terminus of HRV14 VP4 does indeed elicit cross-serotypic neutralizing antibodies.

To ascertain whether there is unique homology in VP4 among all of the 100 serotypes, the known sequences of HRV VP1 and VP0 capsid proteins were aligned (Fig. 4). As previously noted (12), the serotypes of HRV fall into two main groups of 72 and 25 serotypes. This clustering is not related to which cell receptor is utilized by that particular serotype. It could be argued that the sequence of VP4 is highly conserved, and hence can elicit pan-serotypic antibodies, since it is entirely buried at the capsid/RNA interface. To examine this more closely, the aligned sequences of portions of VP0 (VP4/VP2) and the entirety of VP1 from the large cluster of 72 serotypes (Fig. 4) were aligned and the percent identity was plotted versus residue number and compared to external solvent accessibility. From this analysis, it is clear that the external portions of the capsid have a higher content of nonidentical residues. However, there is still marked heterogeneity in even the buried portions of the capsid. What is particularly interesting is that the buried N terminus of VP1 is not nearly as conserved as VP4. Together, these findings suggest that conservation is not entirely proportional to the extent of being externally displayed and that there is some additional pressure on VP4 that enforces a higher degree of conservation.

HRV14 falls into the smaller group of 25 serotypes, while HRV16 and HRV29 belong to the 72-member group. Since antiserum raised against HRV14 VP4 neutralizes members of the larger group of serotypes, it was then necessary to determine whether antiserum against the peptide corresponding to the VP4 sequence from the larger serotype group could neutralize HRV14. For the subsequent studies, three different peptides were used: a 30-mer peptide corresponding to the N terminus of HRV14 VP4 (top of Fig. 1; described in Materials and Methods); a 24-mer peptide, VP4.1, corresponding to the first 24 residues of VP4; and a 24-mer, VP4.2, representing a consensus sequence of the first 24 residues in HRV VP4 (peptide at the bottom of Fig. 1). When polyclonal antibodies were raised against VP4.2, the resulting antiserum was able to neutralize both HRV14 and HRV16, but with less efficacy than the anti-HRV14 VP4 antiserum (Fig. 5). Therefore, it is apparent that the N termini of the various HRV serotypes are immunologically similar but that there are some important differences.

Epitope mapping experiments. To improve the cross-serotypic efficacy of this peptide, it is necessary to find the most immunologically important segment. To this end, epitope mapping experiments were performed using polyclonal antibodies and MAbs developed against the 30-mer and 24-mer HRV14 VP4 peptides. The polyclonal antibodies and the MAbs raised against the 30-mer HRV14 VP4 peptide reacted weakly with...
the HRV14 24-mer but not at all with the consensus sequence 24-mer (Fig. 6). Importantly, the MAbs reacted strongly with the full-length VP4 in Western blots (data not shown), and purified antibodies neutralized HRV14 with similar efficacy as the polyclonal antibodies (Table 1 and Fig. 7). This neutralization of HRV14 by the purified MAbs is a strong affirmation that neutralization is due to specific interactions between the antibodies and VP4.

It seems highly unlikely that MAbs and polyclonal antibodies raised against the 30-mer peptide reacted weakly to the two 24-mer peptides (VP4.1 and VP4.2) because all of the epitopes of all of the antibodies resided in the last six residues of the 30-mer. It seemed much more likely that the folded structures of the two peptides differed significantly because of the six residues at the carboxyl end. To try to find a core sequence of VP4 that was less sensitive to possible folding problems and to truncation of either end of the inoculation peptide, the polyclonal antibodies were no longer able to bind. One interpretation of these results is that there is that there is an essential “core” of the peptide that, for folding reasons, does not tolerate truncation. However, it seems unlikely that all of the antibody species within the polyclonal response recognize those eight residues in the middle of a putative random coil. It is interesting to note, however, that the antibodies raised against VP4.1 (the HRV14 sequence) did indeed cross-react with the consensus sequence VP4.2. This suggests that these peptides can elicit a pan-serotypic immune response once the right segment of VP4 is identified. Since the MAbs raised against the HRV14 VP4 30-mer peptide recognize unique epitopes on VP4, they were also tested against the panel of synthetic peptides. As shown in Fig. 9, none of the MAbs reacted with any of the smaller VP4 fragments or even with the 24-mers, VP4.1 and VP4.2. While antibody 6E1.12 appeared to have a weak reactivity with the fragments, this ELISA signal dropped to background when other ELISA blocking agents were used. Interestingly, all four MAbs react with native VP4 peptide in a Western blot assay (data not shown). This is consistent with the idea that the longer VP4 peptides are folding in a manner distinct from any of the peptides that are 24 residues in length or smaller.

**Dimerization of VP4 in the HRV14 Ser5Cys mutants.** To better understand how and where the N termini of VP4 are being extruded from the capsid, Ser5 in VP4 was mutated to a cysteine to introduce a potential modification site for monomaleimido nanogold (MMNG) that could potentially trap VP4 in the extruded state and also could be used in cryo-TEM analysis. This mutation would introduce a highly specific modification site, since VP4 does not contain any cysteine residues nor are there any exposed cysteines on the HRV14 capsid surface. The VP4 Ser5Cys mutation did not have any apparent affect on infectivity (e.g., plaque size and particle/PFU ratio); however, it must not be quite as viable as wild-type virus since it slowly reverted back to wild type after ~10 to 15 passages.

When attempts were made to modify the mutant virus with MMNG, it became apparent that the extruded termini were forming strong interactions. As per the manufacturer’s instructions, the virus-MMNG mixture was incubated at room temperature for 2 h, followed by overnight incubation at 4°C. The sample was then analyzed using SDS-PAGE and silver stain analysis under reducing and nonreducing conditions (Fig. 10). Since the VP4 band shifted to a molecular mass of ~22 kDa under nonreducing conditions, it was initially thought that MMNG was indeed reacting to the Ser5Cys mutant form of VP4. In the absence of MMNG, the molecular weight of VP4 in the Ser5Cys mutant did not shift when this control sample was kept at 4°C. However, further investigation demonstrated

**FIG. 6.** Cross-reactivity of antiserum from inoculation with the HRV14 VP4 30 residue peptide with the other forms of the VP4 peptides. Shown are the raw ELISA results when antibodies to the 30-residue HRV14 VP4 peptide were added to the 24-residue peptides, VP4.1 (HRV14 VP4) and VP4.2 (consensus sequence [Fig. 1]), compared to preimmune serum. OD450 optical density.

**FIG. 7.** Example of neutralization of HRV14 infectivity by MAbs raised against the 30-mer of HRV14 VP4. The approximate neutralization efficacies for the MAbs are summarized in Table 1.

**TABLE 1.** Neutralization of HRV14 by purified MAbs to the 30-mer peptides corresponding to the N terminus of HRV14 VP4

| MAb   | Concentration (μg/ml) | Mean (SE) % neutralization
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>3H4.1.2</td>
<td>180</td>
<td>54 (7)</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>41 (1)</td>
</tr>
<tr>
<td>3H4.1.3</td>
<td>250</td>
<td>49 (2)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>33 (1)</td>
</tr>
<tr>
<td>3G8.1.1</td>
<td>200</td>
<td>50 (7)</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>43 (9)</td>
</tr>
<tr>
<td>5G10.2.1</td>
<td>225</td>
<td>48 (3)</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>52 (13)</td>
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*a The concentrations of antibody used are only an approximation since they aggregated upon purification. Assays were performed using the same samples of MAbs but at two different concentrations.

*b Neutralization by control serum was 0%.
that this shift in the apparent molecular weight of VP4 in the Ser5Cys mutant occurs in the absence of MMNG at room temperature, but not at 4°C, in neutral pH buffers (pH 6.5 to 7.5). This is entirely consistent with MALDI analysis that demonstrated transient externalization of VP4 N termini at room temperature and not at 4°C (14). Negative-stain TEM analysis of the VP4 cross-linked sample of the Ser5Cys mutant did not show any appreciable aggregation of the virus particles, demonstrating that the observed cross-links are intraparticle in nature. Perhaps most interesting is that the SDS-PAGE analysis (Fig. 10) shows that essentially all of the VP4 is forming cysteine dimers. The complete nature of these cross-links strongly suggests that there is specific rather than stochastic organization of the extruded VP4 that is perhaps dimeric in nature. The particle-to-PFU ratios for the cross-linked and un-cross-linked forms of the Ser5Cys mutant were the essentially the same as those for the wild-type virus (data not shown), suggesting that locking VP4 into this extruded, dimerized form does not adversely affect infectivity.

DISCUSSION

In our previous studies we described the dynamic nature of the HRV14 capsid and demonstrated the transient and reversible exposure of the N termini of VP1 and VP4 to the outside of the capsid (14). Further studies (11, 20) clearly showed that capsid dynamics are indispensable for viral uncoating and are controlled by the flexibility in the canyon and the adjoining hydrophobic pocket. The results of the ultracentrifugation experiments presented here clearly demonstrate that the anti-VP4 antibodies aggregate the virions and can neutralize viral infectivity. This is further strong evidence that intact particles extrude the N terminus of VP4. While the overall efficacy of viral neutralization is not nearly as high as observed with some of the MAbs raised against the intact HRV14 capsid (e.g., MAb 17-IA), it is notable that it is similar to that of some of the aggregating, weakly neutralizing antibodies (2).

Of important significance with regard to possible applications for this peptide vaccine is that the polyclonal antibodies raised against the HRV14 30-mer are able to neutralize several HRV serotypes. Similar cross-reactivity with serum raised against the N terminus of VP1 was observed in the case of enteroviruses (9). Antibodies elicited against residues 37 to 53 of VP1 from the poliovirus type 3 Sabin strain showed cross-reactivity with other poliovirus and coxsackievirus serotypes. In the same study, the authors (9) reported similar cross-reactivity with 37 to 53 residues belonging to VP1 of rhinoviruses. There is greater sequence conservation in VP4, not only across the 97 serotypes of HRV but also across the picornavirus family, than in VP1, VP2, and VP3. The two distinct groups of HRV serotypes, A and B, based on the VP4 sequence conservation, cross-react with serum raised against the VP4 peptide belonging to the opposite group. Antibodies raised against the myristoylated N-terminal 24 residues of the group A VP4 peptide neutralized HRV14, and the efficacy was not markedly different from that of the antibodies against the HRV14 VP4 peptide. This cross-reactivity was further confirmed by the neutralization results for HRV29, which belongs to the group A class of serotypes. This makes a strong case for the possibility of developing a pan-serotypic vaccine. Interestingly, antibodies raised against the 30 N-terminal residues of VP1 did not affect virus infectivity, and it may simply have been the wrong portion of VP1 to elicit antibodies capable of recognizing the extruded portion of VP1.

The neutralization of HRV16 by HRV14 VP4 antiserum was enhanced at 37°C compared to room temperature. This is consistent with the capsid breathing studies of HRV16 using MALDI analysis, which demonstrated increased exposure of VP4 at 37°C compared to at room temperature (11). What is particularly notable is that the anti-VP4 polyclonal antibodies...
were more efficacious against HRV16 than against HRV14. It is not clear whether HRV16 is presenting a more accessible epitope than HRV14, if there is a difference between the two serotypes with regard to the effects of antibody binding to VP4, or if the differences in capsid dynamics lead to a more efficient neutralization. Nevertheless, it is surprising that antibodies raised against HRV14 are actually more effective against HRV16, and this certainly lends support to the idea of creating a pan-serotypic peptide vaccine against HRV.

In the crystal structures of rhinoviruses (HRV14, HRV3, HRV16, and HRV2), VP4 is ordered to various degrees. In HRV14 (22) and HRV3 (32), residues from H1 to 68 were observed, and other than a short helix, the structure has mainly a random-coil conformation. In HRV16 (16), residues 1 to 7 and 23 to 44 of VP4 were observed, with residues 1 to 5 and 26 to 29 forming a small β-sheet. In HRV2 (31), residues 2 to 7 and 25 to 43 were ordered and had β-sheet interactions at the N terminus that were similar to those for HRV16. There is an interesting correlation between the observed β-sheet and the epitope mapping experiments. The polyclonal serum against the 24-residue HRV14 VP4 peptide does not recognize

FIG. 9. Reactivities of the MAbs raised against the HRV14 VP4 30-residue peptide (Fig. 1) with various fragments of the HRV VP4 24-mer. Shown are the ELISA results for four MAbs against the N- and C-terminal truncations of the 24-mer HRV14 peptide. In addition, reactivities against the HRV14 30-mer and the two 24 residue peptides (VP4.1 and VP4.2) are shown. OD450, optical density at 450 nm. Error bars indicate standard deviations.

FIG. 10. Evidence of VP4 cysteine cross-linking upon extrusion from the HRV14 capsid in the HRV14 VP4 mutant, Ser5Cys. VP4 dimers form only at room temperature and not when incubated at 4°C, while dimers do not form with wild-type (WT) samples at either incubation temperature. The last two lanes correspond to independent replicates of the first two lanes except at higher virus concentrations.

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FIG. 10. Evidence of VP4 cysteine cross-linking upon extrusion from the HRV14 capsid in the HRV14 VP4 mutant, Ser5Cys. VP4 dimers form only at room temperature and not when incubated at 4°C, while dimers do not form with wild-type (WT) samples at either incubation temperature. The last two lanes correspond to independent replicates of the first two lanes except at higher virus concentrations.
smaller peptides where six to eight residues from either the C or N terminus are removed, suggesting that the middle core of the 24-residue peptide is essential for antibody binding. Further, the MAbs and the polyclonal sera against the 30-residue peptide do not recognize any of the truncated forms of the 24-mer peptide. This strongly suggests that these peptides are forming a number of different structures, the number of which is highly dependent upon the length. This may be related to the β-sheet observed most clearly in the HRV16 structure; removal of residues 25 to 30 may disrupt the sheet formation. This is assuming that the extruded conformation is related to that observed in the capsid. Future optimization of the peptide vaccine will undoubtedly require a systematic analysis of which peptide fragment works best to elicit a response to extruded VP4.

The results with the VP4 Ser5Cys HRV14 mutant suggest that peptide design will also have to include information as to the oligomers formed by VP4 upon extrusion from the capsid. VP4 with the Ser5Cys mutant forms cysteine dimers only when incubated at room temperature. Indeed, the mutant can be stored at 4°C without any VP4 dimer formation. The facts that VP4 with the Ser5Cys mutant forms cysteine dimers only when forming stochastically, then it seems more than likely that only a fraction of the VP4 peptides would from dimers. Further, it is tempting to speculate that these VP4 dimers form at or near the icosahedral twofold axes, since that general location would allow for complete dimerization of the mutated VP4.

It is also notable that virus infectivity is unaffected by the removal of residues 25 to 30 may disrupt the sheet formation. /H9252

REFERENCES

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