Sequence Conservation and Antigenic Variation of the Structural Proteins of Equine Rhinitis A Virus

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The nucleotide and deduced amino acid sequences of the P1 region of the genomes of 10 independent equine rhinitis A virus (ERAV) isolates were determined and found to be very closely related. A panel of seven monoclonal antibodies to the prototype virus ERAV.393/76 that bound to nonneutralization epitopes conserved among all 10 isolates was raised. In serum neutralization assays, rabbit polyclonal sera and sera from naturally and experimentally infected horses reacted in a consistent and discriminating manner with the 10 isolates, which indicated the existence of variation in the neutralization epitopes of these viruses.

Equine rhinitis A virus (ERAV; formerly equine rhinovirus 1 (22, 23, 48]) was recently renamed and reclassified in the genus Aphthovirus of the family Picornaviridae (17) and is among many viruses that cause respiratory disease in horses worldwide. ERAV is emerging as a serious pathogen of horses (4, 23), and has also been shown previously to infect humans and other species (3, 32, 42). The isolation and characterization of ERAV were first reported in the United Kingdom in 1962 (33). Subsequently, ERAV has been isolated from horses worldwide (5, 12, 15; M. J. Studdert and L. J. Gleeson, Letter, Aust. Vet. J. 53:452, 1977). Most isolations of equine rhinitis viruses have come from the nasopharynx of horses with acute febrile respiratory disease. However, horses may carry and shed virus in their urine and feces for up to 4 weeks postinfection (28, 33). Seroepidemiologic studies indicate that morbidity rates may reach 50%, particularly where older horses are the majority of the population tested (3, 42).

Amino acid substitutions in picornavirus capsid proteins, particularly in the surface loops of the capsid protein VP1, are responsible for the antigenic variation seen between serotypes and strains (9, 20, 30). Antigenic variation has important implications in the design of effective vaccines to control infection and reinfecion and also in the design of diagnostic tests able to detect all serotypes and strains of these viruses. To understand the antigenic relationships between different ERAV isolates, we have determined the nucleotide sequences of the P1 region of 10 ERAV isolates from Australia, the United Kingdom, Switzerland, and the United States (Table 1) and developed a panel of monoclonal and polyclonal antibodies to probe the antigenic relationships between these viruses.

The nucleotide sequence of the complete viral capsid protein coding region (P1 region) was determined for 10 ERAV isolates, of which eight sequences were previously unknown (967/90, 360007, 544/82, 4066/79, V1722/70, P200/75, P346/75, and P1316/92). The nucleotide sequence of ERAV.PERV/62 contained five nucleotides that differed from the published sequence (48), and there were 14 nucleotide differences between the ERAV.393/76 sequence obtained for this study and the original published sequence (22). Considerable variability was found at the nucleotide level, with identities ranging between 79.6 and 96.6% among the 10 isolates (data not shown), where many of the sequence differences are in the third codon position and as such do not result in many amino acid changes (Fig. 1). The amino acid identity and similarity ranged from 96.8 to 99.3% and 98.4 to 99.7%, respectively, among the 10 isolates. Within sites that were variable, most of the differences represent conservative amino acid substitutions, which is analogous to accumulation of amino acid substitutions in foot-and-mouth disease virus (FMDV) (25, 26). ERAV.393/76, however, differs in several amino acids that are completely conserved in all other isolates. This might reflect a higher degree of adaptation to cell culture than is the case for all of the other, less passaged isolates (7, 14, 41). Phylogenetic trees for the 10 ERAV isolates representing the entire P1 region showed these isolates to be clustered closely into six main branches. Dendrograms were similar for both P1 (Fig. 2A) and VP1 (data not shown). The ERAV isolates formed a closely related cluster as a branch of the Aphthovirus genus as previously described (Fig. 2B) (22, 48). The branch lengths between the ERAV isolates are shorter than those between the FMDV serotypes, which is consistent with the view that these ERAV isolates represent a single serotype.

In order to understand the relationship of sequence changes to the antigenic sites of ERAV, a panel of monoclonal antibodies (MAbs) was prepared against the prototype isolate ERAV.393/76. Lymphocytes were taken either from the ingui-
nal and poliptite lymph nodes of mice that were immunized with purified UV-inactivated ERAV.393/76 (11) emulsified in complete Freund’s adjuvant or from the spleens of mice that had received two further boost of purified virus. Lymphocytes from the spleen and the lymph nodes were used separately in hybridoma fusions with Sp2/O-Ag14 myeloma cells as described previously (19). Hybridomas were screened in an enzyme-linked immunosorbent assay (ELISA) comprising plates coated with purified ERAV.393/76 and reactive hybridomas cloned twice by limiting dilution. The specificity of these MAbs were determined by Western blotting against ERAV.393/76 (data not shown), and the properties of these MAbs are summarized in Table 2. Of the seven MAbs, four recognized the VP2 protein, one recognized VP1, and one recognized VP3. The seventh MAb (L2G3) did not bind in Western blotting but was able to immunoprecipitate [35S]methionine-labeled viral capsid proteins and may therefore recognize a conformation-dependent epitope. None of the MAbs was able to neutralize virus infectivity, while most of the MAbs bound in immunofluorescence assays to six different ERAV isolates, suggesting conservation of the nonneutralization epitopes between these viruses. The epitopes of these MAbs were more precisely mapped using an ERAV.393/76 P1 gene-specific phage display library (40) and were further defined with a panel of glutathione S-transferase fusion proteins displaying parts of the isolated phagotopes. All the MAb epitopes identified represent segments of sequence compatible with their reactivity against viral proteins in Western blotting (Table 2 and data not shown). Furthermore, these epitopes were conserved among all 10 isolates (Fig. 1), and this explains their ability to bind to each of the representative isolates by immunofluorescence. The relationships among isolates were also examined using serum neutralization assays with ERAV polyclonal sera raised either in rabbits or experimentally and naturally in horses (11). Consistent results were obtained in two independent experiments, and the results from one of these experiments are shown in Fig. 3. The experimentally infected horse and rabbit sera showed the highest neutralizing antibody titers to the prototype ERAV.393/76 isolate with which they were infected or inoculated, and these antisera neutralized each of the other isolates to various degrees. In particular, each serum had a consistent and significantly (≥4-fold) lower neutralization titer than did isolate 393/76 with the polyclonal horse sera but not with the polyclonal rabbit serum. Isolate P1316/92 showed similar neutralizing antibody titers to 395/76 against all sera except SM, which was obtained from a horse naturally infected with ERAV (23). Isolates 360007, 4066/79, 967/90, V1722/75, and P200/75 were neutralized to similar levels as ERAV.393/76 by each of the sera tested. Therefore, despite the high levels of sequence conservation, some antigenic variation in neutralization epitopes of the viruses could be demonstrated.

The three-dimensional crystal structure of many picornaviruses has shown that major differences between picornavirus serotypes are concentrated within the surface-exposed loops of the virus capsid proteins (9, 16, 20, 21, 30). Amino acid variation within these loops alters the antigenic properties of the viruses and is responsible for the diversity of virus serotypes (27). Picornavirus capsid proteins share a great deal of structural homology, where VP1, VP2, and VP3 are each composed of eight-stranded β-barrels and differ in the size and conformation of the connecting loops between the strands and the extensions of their amino and carboxyl termini (37). Alignment of the predicted secondary structural elements of the ERAV capsid proteins with those of FMDV and other picornaviruses of known three-dimensional structure (2, 24, 36) suggests the location of loop and beta-sheet regions for the ERAV capsid (Fig. 1) (48). These alignments predict that ERAV VP1 contains longer connecting loops between β-barrel structures than does FMDV, with the exception of the βG-βH loop. The long βG-βH loop of FMDV contains the integrin-binding motif RGD and is the major epitope to which neutralizing antibodies are directed (6, 10, 27, 45–47). Four other antigenic sites of FMDV serotype O are also located across the P1 region (18). The βG-βH loop of ERAV is much smaller than that of FMDV and has no identifiable integrin-binding motif. Among the 10 ERAV isolates, amino acid variation appears to occur mostly in proposed loop regions of the capsid proteins, particularly in the βA2-βB loop of VP2 between amino acids 110 and 134 and the βE-βF loop in VP1 corresponding to amino acids 645 to 676 (Fig. 1).

The epitopes of three MAbs localized to proposed loop or C-terminal regions of the capsid proteins. The MAbs detected mostly linear epitopes across the P1 region (L5G12 and L4E4 to VP2, L4G4 to VP3, and L7E8 to VP1), although no MAbs were generated to the smallest (4-kDa) capsid protein, VP4. One MAb, L2G3, appeared to bind to a conformation-dependent epitope, since this MAb bound well to whole virus in the
ELISA and immunoprecipitation assay (data not shown) but did not bind to virus that had been fixed for immunofluorescence or reduced and denatured for Western blotting. The MAb epitopes identified by phage display mapped to regions of the VP2, VP3, and VP1 capsid proteins that were conserved across all 10 isolates. Conservation of the P1 amino acid sequences would predict that these viruses have a very close antigenic relationship, since the amino acid substitutions observed are mostly conservative and are dispersed randomly across the P1 region. The inability of these MAbs to neutralize virus infectivity and the conserved nature of these epitopes across the 10 ERAV isolates suggest that these regions are not subject to antibody-mediated selective pressures. Neutralizing MAbs were not obtained in successive fusions despite the presence of neutralizing antibodies in the serum obtained from these mice. This may be a reflection of bias introduced by selecting MAbs able to bind in ELISA, since virus bound to ELISA wells may have neutralization epitopes altered or unavailable for binding.

Antigenic sites of picornaviruses FMDV, poliovirus (PV), and others have been studied extensively (48). FIG. 1. Amino acid alignment of the P1 region of the 10 ERAV isolates. The amino acid sequence of ERAV 393/76 is given on the top line and is numbered from the first amino acid of P1. Identical amino acids are represented by dashes, nonidentical amino acids are indicated by single letters, and conservation of an amino acid among all 10 isolates is indicated by a star. An arrow indicates the location of the first amino acid for each of the four structural proteins. Thick lines indicate regions of proposed secondary structure (α-helices and β-barrels) as determined by homology with picornaviruses of known three-dimensional structures (48).

ELISA and immunoprecipitation assay (data not shown) but did not bind to virus that had been fixed for immunofluorescence or reduced and denatured for Western blotting. The MAb epitopes identified by phage display mapped to regions of the VP2, VP3, and VP1 capsid proteins that were conserved across all 10 isolates. Conservation of the P1 amino acid sequences would predict that these viruses have a very close antigenic relationship, since the amino acid substitutions observed are mostly conservative and are dispersed randomly across the P1 region. The inability of these MAbs to neutralize virus infectivity and the conserved nature of these epitopes across the 10 ERAV isolates suggest that these regions are not subject to antibody-mediated selective pressures. Neutralizing MAbs were not obtained in successive fusions despite the presence of neutralizing antibodies in the serum obtained from these mice. This may be a reflection of bias introduced by selecting MAbs able to bind in ELISA, since virus bound to ELISA wells may have neutralization epitopes altered or unavailable for binding.

Antigenic sites of picornaviruses FMDV, poliovirus (PV),
and human rhinovirus have been well characterized. Although FMDV differs markedly in the surface design from PV and human rhinovirus 14 (HRV14) (2), the antigenic sites of all three appear to localize to regions in and around their receptor-binding sites (44). FMDV serotype O has five recognized neutralization epitopes—antigenic sites across the P1 region (18). Site A was the first major epitope identified, located within the βG-βH loop of VP1 and encompassing the RGD integrin-binding motif (1, 21). PV and HRV14 each contain three major antigenic sites (13, 29, 31, 38, 39), which are highly variable and consist of surface loops surrounding the receptor-binding canyon on the surface of the virion (35). Despite the highly conserved amino acid sequence among the 10 ERAV isolates, polyclonal serum neutralization data suggest that some variation does exist between neutralization epitopes of ERAVs. PERV/62 was significantly different from the 393/76-like viruses based on the neutralizing antibody titers of polyclonal sera. PERV/62 is the earliest ERAV isolate used in this study. The amino acid differences of viruses isolated at later times may represent evidence of selective immunologic pressure or replication biases that have altered the antigenic structure of viruses over time, in order to escape neutralization by circulating antibodies to earlier isolates. The ERAVs isolated subsequent to PERV/62 show higher cross-neutralizing antibody titers to the polyclonal sera. The location of individual amino acid substitutions between ERAV isolates that may

![FIG. 1—Continued.](http://jvi.asm.org/)}
explain the variable neutralization results between ERAV isolates is not obvious. As with FMDV and other picornaviruses, amino acid substitutions across a number of regions may alter the capacity of antibodies to bind and neutralize ERAV. Elucidation of the neutralization epitopes of ERAV by selection of escape mutants resistant to neutralization or by mapping epitopes of neutralizing MAbs is necessary in order to more precisely define the critical amino acids involved for ERAVs. Although ERAVs are not as divergent as the FMDV serotypes, variation in the neutralization epitopes of ERAV isolates raises the possibility that a horse could be susceptible to multiple ERAV infections over a lifetime, since infection with a PERV/62-like isolate may not afford protection from a 393/76-like virus. Furthermore, the variation also has important implications for the design of effective vaccines that are able to protect against infection with all possible ERAV isolates.

FIG. 2. Unrooted phylogenetic trees inferred using the maximum likelihood method for the nucleotide sequences of the P1 region of the 10 ERAV isolates (A) and the P1 region of viruses representing the nine genera of the picornavirus family and the six representative ERAV isolates (B): FMDV serotypes FMDV.01K (accession no. X00871) and FMDV.SAT2 (accession no. AJ251473); erbovirus (ERBV; accession no. X96871); avian encephalomyocarditis-like virus (AEV; accession no. AJ225173) and human hepatitis A virus (HAV; accession no. M14707); Aichi virus (accession no. AB010145); coxsackievirus A9 (CV-A9; accession no. D00627), PV-2 (accession no. X00595), and human enterovirus 71 (HEV-71; accession no. U22521); HRV14 (accession no. X01087); echovirus 23 (EV-23; accession no. A005695); porcine enterovirus type 1 (PEV-1; accession no. P001380); and Theliers murine encephalomyelitis virus (TMEV; accession no. M16020) and encephalomyocarditis virus (EMCV; accession no. X74312). Each branch of these trees was highly significant (P < 0.01). Trees were inferred using the maximum likelihood method with the Ednaml program of the phylogeny inference package PHYLIP version 3.572c (8) and bootstrapped using the Eseqboot package by the same author.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>Specificity in Western blotting against 393/76</th>
<th>ELISA titer (μg/ml)b</th>
<th>No. of isolates bound in IFAc</th>
<th>Peptides displayed by MAb-enriched phagesd (minimum epitope identified)</th>
</tr>
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<tbody>
<tr>
<td>L7E8</td>
<td>IgG2a</td>
<td>VP1</td>
<td>2</td>
<td>6/6</td>
<td>758RPIPPAFTRA758 (758RPIPPAFTRA758) 303NGPPLPNAPVR338 303NGPPLPNPE310</td>
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<tr>
<td>L4E4</td>
<td>IgG2a</td>
<td>VP2</td>
<td>2</td>
<td>6/6</td>
<td>303NGPPLPNAPVR338 303NGPPLPNPE310 303NGPPLPNAPVR338 303NGPPLPNPE310</td>
</tr>
<tr>
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<td>VP2</td>
<td>0.07</td>
<td>6/6</td>
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<tr>
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<td>VP2</td>
<td>2</td>
<td>6/6</td>
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</tr>
<tr>
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<td>VP2</td>
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<td>1/6f</td>
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<tr>
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<td>IgG3</td>
<td>VP3</td>
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<td>6/6</td>
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</tr>
<tr>
<td>L2G3</td>
<td>IgG2b</td>
<td>Did not bind</td>
<td>0.01</td>
<td>0/6</td>
<td>ND</td>
</tr>
</tbody>
</table>

a ELISA titer is the concentration of MAb resulting in absorbance at 450 nm of 0.5 on wells coated with purified ERAV.393/76.
b Binding of MAb to six representative ERAV isolates (393/76, 967/90, PERV/62, 360007, P346/75, and P1316/92) in immunofluorescence assay (IFA).
c Amino acid numbering is from the first amino acid of P1 (Fig. 1).
d Weak binding to ERAV.393/76 only.
e ND, not determined.
f IgG2a, immunoglobulin G2a.
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REFERENCES


