Identification of Another B-Cell Epitope in the Type-Specific Region of Equine Herpesvirus 4 Glycoprotein G

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Recently, a novel 12-mer B-cell epitope, MKNPIYSEGSL, in the type-specific region of equine herpesvirus 1 (EHV-1) glycoprotein G (gG) was identified and used as an antigen for enzyme-linked immunosorbent assay (Maeda et al., J. Clin. Microbiol. 42:1095–1098, 2004). Although our prototype strain, TH20p, possesses two repeat sequences containing the B-cell epitope, the EHV-4 NS80567 strain has two repeat sequences that are not identical. One repeat sequence stretch contained the B-cell epitope, while the other contained the 11-mer, MKNPYVYSESL (underlining indicates a different amino acid). In this study, heterogeneity of the type-specific region was compared among Japanese EHV-4 isolates. The 11-mer peptide, MKNPYVYSESL, specifically reacted with sera from horses naturally infected with EHV-4 but not with sera from horses experimentally infected with EHV-4 TH20p. The 11-mer peptide may be another B-cell epitope in the type-specific region.

Equine herpesviruses 1 (EHV-1) and 4 (EHV-4) are causative agents of equine rhinopneumonitis. Because both viruses are closely related genetically and immunologically, distinguishing the two has been very difficult. Crabb et al. identified the type-specific region at the C-terminal region of glycoprotein G (gG) and established an enzyme-linked immunosorbent assay (ELISA) using these regions as antigens (1, 2). Our group also developed a type-specific ELISA using Japanese isolates (3) and used the ELISA for epizootiological and immunological studies in Japan (5, 9).

Because the ELISA antigens were expressed in Escherichia coli as fusion proteins, the purification was time-consuming. Therefore, the type-specific 12-mer B-cell epitope of EHV-4, MNKNPIYSEGSL, was identified and used as an ELISA antigen (4). Although the Japanese prototype strain, TH20p, has two repeat sequences containing the 12-mer B-cell epitope in the type-specific region, NS80567 has one stretch of repeat sequences containing the 12-mer B-cell epitope and another one containing similar, but not identical, amino acids, MKNPYVYSESL (underlining indicates a different amino acid) (7).

In this study, the heterogeneity of the type-specific region was compared among Japanese EHV-4 field isolates. In addition, ELISA using the 11-mer peptide was also established and compared with that using our recently identified 12-mer B-cell epitope.

MATERIALS AND METHODS

Viruses and cells. Twenty-one EHV-4 field isolates and two laboratory strains were used. The H45 strain was isolated from an aborted fetus in Japan and has been used as the Japanese prototype strain of EHV-4 (6). The TH20p strain was plaque purified from strain TH20, isolated from a colt suffering from respiratory disease (3). The other 21 strains were isolated between 1981 and 1991 and were propagated in fetal horse kidney (FHK) cells. Field isolates were used in the third passage and were never plaque purified. The 19 strains were isolated from foals with respiratory disease; 91c1 was from an aborted fetus, and 88c162 was from horses without any clinical signs. Two pairs of isolates, 88c160 and 88c162 as well as 88c180 and 88c186, were simultaneously isolated from different horses from the same farm and are therefore considered epidemiologically related.

DNA extraction. Twenty-one field isolates and the TH20 and H45 strains were used for DNA analyses. Viruses were propagated at low multiplicities of infection in FHK cells. Infected cells were treated with 1% sodium deoxycholate and protease K (0.1 mg/ml) in a solution containing 0.1 M Tris-HCl, 0.1 M NaCl, 5 mM EDTA (pH 9.0) at 37°C overnight. Following DNA extraction with phenol and chloroform-isooamyalkohol (24:1), ethanol-precipitated DNA was dissolved in water.

Amplification of the type-specific regions. The type-specific regions were amplified from extracted DNA of field isolates by PCR using two primers, gG4P-F and gG4P-R (8). Amplified samples were separated by electrophoresis on 1 to 2% agarose gels. Gels were stained with ethidium bromide and analyzed.

Nucleotide sequences. The amplified fragments were cloned into the pCR II vector (Invitrogen). The plasmids were analyzed by the ABI sequencer analyzer. Nucleotide sequences were obtained for three independently cloned plasmids.

Synthetic peptides. Two peptides, G1 (MKNPIYSEGSL) and G13 (MKNPYVYSESL) (underlining indicates a different amino acid) (7). In this study, the heterogeneity of the type-specific region was compared among Japanese EHV-4 field isolates. In addition, ELISA using the 11-mer peptide was also established and compared with that using our recently identified 12-mer B-cell epitope.

Comparison of the type-specific region by PCR. The type-specific regions of 21 EHV-4 field isolates and two laboratory
strains, TH20p and H45, were amplified by PCR using primers gG4P-F and gG4P-R. As expected, approximately 300-bp fragments were amplified from 12 isolates and TH20p. However, approximately 370- and 450-bp fragments were detected from the other nine field isolates and H45, respectively (Fig. 1A).

Comparison of amino acid sequences in the type-specific region. Nucleotide sequences of three representative EHV-4 strains were compared. The results showed that the size of the type-specific regions of TH20p, 87c34, and H45 were 295, 367, and 449 bp, respectively, and the number of repeat sequences was different (data not shown). Next, the amino acid sequences deduced from nucleotide sequences were compared (Fig. 1B). Interestingly, although TH20p had two repeat sequences that were identical, 87c34 and H45 had two kinds of repeat sequences. One contained the previously identified B-cell epitope MKNNPIYSE-G1, and another contained a similar but not identical amino acid sequence, MKNNPVYSE-SL (Fig. 1B).

ELISA using an 11-mer synthetic peptide, MKNNPVYSESL. Because the 11-mer amino acid (G13), MKNNPVYSESL, was very similar to the previously identified 12-mer B-cell epitope (G1), we expected that EHV-4-infected horse sera would also recognize the peptide. However, horse sera experimentally infected with TH20p did not react with the peptide, and the reaction to G13 was significantly less than that to G1 (P < 0.01) (Fig. 2). Paired sera from horses naturally infected with EHV-4 were examined by ELISA using G1 or G13 as antigen. The results revealed that G13 significantly reacted with EHV-4-infected horse sera (Fig. 3). However, the reaction to G13 was observed to be less than that to G1, and paired sera from one EHV-4-infected horse did not react with G13 at all (Fig. 3, open circle).

FIG. 1. PCR and amino acid sequences of the type-specific region of EHV-4 field isolates. (A) The type-specific regions were amplified from 23 EHV-4 isolates by PCR using primers gG4P-F and gG4P-R. (B) Amino acid sequences deduced from the nucleotide sequences of the type-specific regions of TH20p, 87c34, H45, and NS80567 (7) were compared. Bold letters and letters shown by underlines show repeat sequences containing G1 and G13, respectively.

FIG. 2. ELISA using two synthetic peptides against sera from horses experimentally infected with EHV-4. Two synthetic peptides (G1 and G13) were used as ELISA antigens. Sera collected from six foals that were experimentally infected with EHV-4 TH20p were diluted 1:1,000. Peptide reactivity is shown as the mean absorbance at 405 nm. Vertical error bars represent standard deviations of the means.
addition, it is interesting that both of the two peptides still possess immunogenicity to induce antibodies to horses in spite of their different specificities.

Although horses naturally infected with EHV-4 had antibodies to G13 as well as G1 (Fig. 3), TH20p-infected horses had antibodies to G1 only (Fig. 2). These results are understandable, as TH20p did not have a repeat sequence containing G13. Comparison of ELISA using G1 and G13 showed that most horses had antibodies to both epitopes, except for one horse that had antibodies only to G1 (Fig. 3, open circles). These results indicated that most EHV-4 probably have repeat sequences containing both G1 and G13, though some EHV-4, including TH20p, have repeat sequences containing only G1. To the best of our knowledge, all EHV-4 field isolates had the repeat sequence containing G1, indicating that ELISA using G1 may be sufficient for seroepizootiological studies and for the diagnosis of EHV-4. However, there is a possibility that some field isolates might have repeat sequences containing only G13 and not G1. Therefore, both G1 and G13 should be used as ELISA antigens for the diagnosis and epizootiological studies of EHV-4.

In this study, heterogeneity in the type-specific region was compared among field isolates, and another B-cell epitope, G13, was identified. Our study indicated that ELISA using both G1 and G13 as antigens should be conducted for seroepizootiological studies and diagnosis of EHV-4 infection.

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