A 29K envelope glycoprotein of equine arteritis virus expresses neutralization determinants recognized by murine monoclonal antibodies

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A panel of six neutralizing murine monoclonal antibodies (MAbs) to equine arteritis virus (EAV) was produced. The MAbs were characterized by Western immunoblotting assay and competitive ELISA. The six MAbs identify a single neutralization site on a 29K envelope glycoprotein. Deglycosylation of viral proteins prior to immunoblotting showed that the 29K protein is the glycosylated form of a 20K protein. Equine anti-EAV serum also strongly bound the 29K glycoprotein, as well as an unglycosylated protein of 17K. The equine antisera to EAV blocked the binding of a selected MAb to EAV, whereas normal equine serum did not. Two neutralization-resistant escape mutant (EM) variants of the EAV prototype were produced using MAb 6D10. The phenotypic properties of the EM viruses were characterized by neutralization and immunoblotting assays with two MAbs (6D10 and 5G11). The two MAbs failed to neutralize either EM virus, and they did not react in an immunoblot assay with any proteins of the EM viruses. In contrast, binding of the equine antiserum to viral proteins was equivalent with prototype and EM virus strains. These data clearly indicate that a 29K envelope glycoprotein expresses at least one neutralization determinant of EAV.

Equine arteritis virus (EAV) was first isolated from the lung of an aborted fetus at Bucyrus, Ohio, U.S.A. (Doll et al., 1957). EAV causes epizootic episodes of respiratory disease in susceptible horses and, of greater concern to the equine breeding industry, both abortion in pregnant mares and persistent infection in stallions (Timoney et al., 1987). Serological surveys indicate that EAV is widely distributed throughout the world (Timoney & McCollum, 1988). EAV is an enveloped, positive-stranded RNA virus and until recently it was classified as the sole member of the genus Arterivirus in the family Togaviridae (Westaway et al., 1985). This classification was based on the morphological, physical and chemical properties of EAV. However, recent studies have shown that the genomic organization and replication strategy of EAV are very similar to those of the Corona- and Toroviridae (den Boon et al., 1991). It was proposed, therefore, that EAV should be classified as being in a coronavirus-like superfamily. The EAV genome (12.7 kb) contains eight open reading frames (ORFs) and during replication a nested set of six 3'-coterminal viral subgenomic mRNAs is produced (den Boon et al., 1991). de Vries et al. (1992) identified four structural proteins of EAV, and their respective ORFs, by expression of individual ORFs and immune precipitation of the encoded proteins with appropriate monoclonal and antivirion sera. Their results indicate that the structural proteins of EAV include a 14K nucleocapsid protein and three membrane proteins of 16K, 25K and 30K to 42K. These proteins are identified as N, M, Gs and Gl, respectively. The M protein is unglycosylated whereas the Gs and Gl proteins are N-glycosylated and the variable size of the Gl protein is the result of heterogeneous N-glycosylation. The results obtained by de Vries et al. (1992) differ from some of those previously described by others (Hyllseth, 1973; Zeegers et al., 1976; van Berlo et al., 1982, 1986; Iwashita & Harasawa, 1987a, b; Chirinside, 1992). For instance, Zeegers et al. (1976) and Iwashita & Harasawa (1987a, b) described an additional 21K glycosylated structural protein.

Horses naturally infected with EAV or vaccinated against the virus develop long-lasting, probably life-long immunity (McCollum, 1976). However, the viral protein or proteins that express the neutralization determinants of EAV have not been clearly identified. In this study we describe a panel of neutralizing murine monoclonal antibodies (MAbs) to EAV, and their characterization by Western immunoblotting assay and competitive ELISA using prototype and neutralization-resistant strains of EAV.

The Bucyrus strain of EAV and rabbit kidney 13 (RK-13) cell line were generously provided by Dr A. Castro (California Veterinary Diagnostic Laboratory, Davis, Calif., U.S.A.). The virus was three times plaque-picked from agar-overlaid Vero cells. Virus stocks were prepared
by passage in RK-13 cells and stored at −70 °C. MAbs to EAV were produced according to standard protocols similar to those previously described (Heidner et al., 1988; Whetter et al., 1989). Mice were initially immunized by intravenous (i.v.) inoculation of unpurified supernatant from EAV-infected RK-13 cells, and subsequently boosted three times (2 weeks apart) by intraperitoneal inoculation of the same antigen (1:2 emulsion in Freund’s complete or incomplete adjuvant). Finally, the mice were inoculated i.v. with EAV at 3 days prior to fusion. Fusion of splenic lymphocytes with cells from the P3X63Ag8.653 murine myeloma cell line (Kearney et al., 1979) was done in the presence of polyethylene glycol in RPMI medium. Supernatant fluids from hybridoma cultures were screened by microneutralization assay (Richards et al., 1988) using RK-13 cells. Twenty-five different hybridomas that produced EAV-specific antibodies were identified and 13 of these were cloned (three times) by limiting dilution in 96-well plates (Lietzke & Unsicker, 1985). Six hybridomas (designated 6D10, 10F11, 10H4, 5G11, 7E5 and 9F2) which gave the highest neutralization titres were used for production of ascitic fluid. The neutralization titres of ascitic fluid from each hybridoma ranged from 800 (MAb 9F2) to greater than 6400 (MAbs 6D10, 10F11 and 10H4). Immunoglobulin isotyping was done by agar gel immunoprecipitation (Ouchterlony & Nilsson, 1986). Anti-EAV equine serum was kindly provided by Drs P. J. Timoney and W. H. McCollum (University of Kentucky, Gluck Equine Research Center, Lexington, Ky., U.S.A.). Purified IgG from each MAb and the polyclonal equine antiserum were prepared with ABX 40 μm ion exchange resin (J. T. Baker). Each MAb was biotinylated according to a method previously described (Goding, 1986; Liddell & Cryer, 1991).

All MAbs were characterized by both Western immunoblot assay and competitive ELISA. For these assays EAV was purified on a continuous CsCl gradient following Freon 113 (1,1,2-trichloro-1,2,2-,trifluoro-ethane, Sigma) extraction. Briefly, confluent monolayers of RK-13 cells were infected at an m.o.i. of 10 and incubated for 48 h at which time c.p.e. was complete. The supernatant with cellular debris was extracted once with cold Freon 113 and 25% Sphadex G200 (Sigma). The aqueous phase was extracted a further three times in a half volume of Freon 113, and the fluorocarbon phases from each extraction were pooled. NET buffer (150 mM-NaCl, 5 mM-EDTA and 50 mM-Tris–HCl pH 7.5) was added to the pooled fluorocarbon phases, and the aqueous phase was extracted a final time and pooled with the previous extracts. The aqueous phase pool was centrifuged through an 11% CsCl button (120000 g for 4 h) at 4 °C. The virus pellet was resuspended in NET buffer and sonicated for 3 min, then overlaid onto an 11 to 33% continuous CsCl gradient and ultracentrifuged overnight at 103600 g at 4 °C. The virus band was collected and repelleted by ultracentrifugation. Purified virus was resuspended in different buffers depending on its intended use. A portion of each virus pellet was resuspended in NET buffer and the virus titre determined by microtitration using RK-13 cells. Monolayers were stained with 1% crystal violet and titres determined by the method of Reed & Muench (1938). Control antigen was prepared from uninfected confluent monolayers of RK-13 cells. Cell lystate with supernatant was subjected to the same purification procedure as was the virus, and this antigen was used as a negative control in the Western immunoblot assay and competitive ELISA.

The Western immunoblot assay was done essentially as described previously (Harlow & Lane, 1988). Freon 113-extracted, CsCl gradient-purified EAV was resuspended in 1 × Laemmli sample buffer and subjected to SDS–PAGE (Laemmli, 1970) using a 12% acrylamide resolving gel and 5% stacking gel. Proteins were electrophoreted onto a membrane (Immobilon-P, 0.45 μm, Millipore) and purified IgG from individual MAbs (1 μg/ml) or equine anti-EAV serum (10 μg/ml) was added. Membranes were washed and incubated with affinity-purified biotinylated anti-mouse IgG (Zymed) or anti-equine IgG (Vector). Bound IgGs were detected with horseradish peroxidase (HRP) conjugated to avidin (Zymed). Finally, the blots were exposed to enhanced chemiluminescence Western blotting detection reagents (Amersham) for 1 min, wrapped in Saran Wrap, and exposed to Kodak XAR 5 film. All six MAbs strongly reacted by Western immunoblot assay with a 29K protein (representative example Fig. 1, lane 2). Prolonged exposure of blots with lower affinity MAbs (5G11, 7E5
Fig. 2. Immunoblotting of prototype and neutralization-resistant EM strains of EAV. Lanes 1 and 4 contain proteins of prototype EAV, lanes 2, 3, 5 and 6 contain proteins from EM virus variants 1 and 2. Lanes 1 to 3, anti-EAV equine IgG; lanes 4 to 6, MAb 5G11. The Mr of significant protein bands are indicated on the left. The bands of Mr greater than 60K which are present in all lanes reflect non-specific binding of the HRP-avidin conjugate.

Fig. 3. Competitive binding assay with unbiotinylated and biotinylated MAbs. The percentage displacement of biotinylated MAb 6D10 by various concentrations of each unbiotinylated MAb is depicted (■, 5G11; ○, 7E5; □, 9F2; △, 6D10; ▲, 10F11; Δ, 10H4).

and 9F2) revealed bands of higher Mr (44K and 60K), presumably as a result of contamination of these proteins with the 29K protein (representative example Fig. 2, lane 4). The higher affinity MAbs 6D10, 10F11 and 10H4 (Fig. 3) consistently identified the two higher Mr proteins contaminated with the 29K protein. The equine anti-EAV serum also strongly recognized the 29K protein, as well as another protein of 17K (Fig. 2, lane 1). Equine serum also produced a weaker band of 44K. It is concluded that the 29K protein expresses the neutralization determinants of EAV, since this was the only protein which was consistently identified by the MAbs.

To confirm the specificity of the immunoblotting procedure, two neutralization-resistant escape mutant (EM) viruses were produced. EM viruses were produced by propagation of prototype EAV in the presence of MAb 6D10, and selection of neutralization-resistant variants. Both EM viruses were resistant to neutralization by MAbs 6D10 and 5G11. These two EM viruses were cloned, then purified on a continuous CsCl gradient following Freon 113 extraction and subjected to Western immunoblot assay with MAbs 6D10 and 5G11. The MAbs did not identify any viral protein in the EM viruses even after prolonged exposure (representative example Fig. 2, lanes 5 and 6), whereas binding of the equine antiserum was unaffected (Fig. 2, lanes 2 and 3). The data confirm the specificity of the immunoblot assay. The data also are consistent with the hypothesis that bands other than that of 29K in the blots of the prototype strain of EAV with MAbs (Fig. 2, lane 4) reflect contamination of higher Mr proteins with the 29K protein.

Gradient-purified EAV was treated with endoglycosidase F (glyco-F; Boehringer Mannheim) to determine whether the 29K protein is glycosylated. Deglycosylation of viral proteins was done as described (de Vries et al., 1992). Gradient-purified EAV was resuspended in glyco-F incubation buffer (50 mM-sodium phosphate buffer, 20 mM-EDTA, 0.15% SDS, 1% Nonidet P40, 1% 2-mercaptoethanol) containing aprotinin, leupeptin and pepstatin A (1 µg/ml), and subjected to glyco-F treatment (100 to 200 milliunits/reaction tube). The reaction was carried out overnight at 30 °C in a shaker incubator. Control reaction tubes were treated in the same manner, except an equivalent volume of sterile PBS was added instead of enzyme. Samples were supplemented with an equal volume of 2 × Laemmli sample buffer and solubilized at 100 °C for 20 s before SDS–PAGE and transfer to a membrane for Western immunoblotting analysis with MAbs or polyclonal equine antisera to EAV. All six MAbs strongly reacted with the 20K protein in glyco-F-treated EAV, and the band of 29K dramatically decreased in intensity, which clearly indicates that the 20K protein is the deglycosylated product of the 29K glycoprotein of untreated EAV (representative example Fig. 1, lane 3). The equine serum also identified a band of 20K in the glyco-F-treated EAV (data not shown). The 17K band recognized by equine antisera was unaffected by glyco-F treatment (data not shown), indicating that this protein is not glycosylated. Results of deglycosylation studies indicate that the 29K protein is a glycosylated product of a precursor protein of 20K.
The MAbs were further characterized by competitive binding assay to define the number of neutralizing epitopes recognized by the panel. A competitive ELISA modified from that previously described for bluetongue virus (Reddington et al., 1991) was used. ELISA plates were coated with Freon 113-extracted, CsCl gradient-purified virus in coating buffer (150 mM-NaCl, 70 mM-sodium phosphate buffer, 1% lactose, pH 8.0). Tenfold dilutions (50 ng/ml to 50000 ng/ml) of unbiotinylated inhibitory MAb and biotinylated test MAb (2000 ng/ml) were reacted to determine the ability of unbiotinylated MAb to block the binding of each biotinylated MAb. Bound biotinylated MAbs were detected with HRP conjugated to avidin and o-phenylenediamine dihydrochloride (OPD) enzyme-substrate system. Inhibition of binding of the biotinylated MAb by each unbiotinylated MAb was then calculated and expressed as a percentage. Competitive binding studies with biotinylated and unbiotinylated MAbs produced two distinct patterns of inhibition (representative example Fig. 3). MAbs 6D10, 10F11 and 10H4 consistently had a higher percentage of inhibition than did MAbs 5G11, 7E5 and 9F2 at each concentration of inhibitory antibody. This pattern was consistent regardless of the biotinylated MAb used, indicating that the two patterns of inhibition reflect only differences in binding affinity of the MAbs. The three MAbs with highest binding affinity (6D10, 10F11 and 10H4) also consistently had higher neutralization titres (> 6400) than did the low affinity MAbs (5G11, 7E5 and 9F2; titres 800 to 6400). These six MAbs either recognize the same epitope or distinct but interacting epitopes. Competitive binding studies were also done with the equine anti-EAV serum and biotinylated MAb 6D10.

The equine anti-EAV serum inhibited the binding of biotinylated MAb 6D10, whereas the control equine serum did not (Fig. 4). Clearly, antibodies in the equine antiserum either recognize the same epitope as does MAb 6D10 or they sterically interfere with the binding of MAb 6D10.

The goals of this study were to develop a panel of neutralizing MAbs to EAV, to identify the viral protein that expresses the neutralization determinants of the virus, and to determine the number of neutralizing determinants in that protein. All six neutralizing MAbs clearly bind the 29K protein, indicating that it expresses at least one epitope responsible for the neutralization of EAV. Studies with neutralization-resistant EM variants of EAV confirm the specificity of the immunoblot assay used. Results of deglycosylation studies indicate that the 29K protein is a glycosylated product of a precursor protein of 20K. It is concluded that a 29K envelope glycoprotein of EAV expresses determinants recognized by all six neutralizing murine MAbs, and that the MAbs either recognize the same epitope or interacting epitopes in a single neutralization site. Competitive binding studies suggest that this site also is important in the horse humoral immune response to EAV. Further studies are needed to identify the ORF that encodes the 29K envelope glycoprotein. At least four EAV ORFs (numbers 2 to 5) encode proteins of similar size, especially when putative N-glycosylation sites are considered. Of these, ORFs 2 and 5 encode membrane proteins identified as Gα and Gβ by de Vries et al. (1992), whereas the ORF 3 and 4 products are unidentified. The characterization of proteins expressed from these four ORFs with neutralizing MAbs will establish which ORF encodes the 29K envelope glycoprotein of EAV.

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