Identification of gag Precursor of Equine Infectious Anaemia Virus with Monoclonal Antibodies to the Major Viral Core Protein, p26

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SUMMARY

Monoclonal antibodies (MAbs) against the major core protein p26 of equine infectious anaemia virus (EIAV) were produced and characterized. Sensitive enzyme-linked immunosorbent assay and Western blot immunoassay were employed to confirm the specificity of these MAbs. Western blot analysis also indicated that MAbs to p26 reacted with another EIAV protein of 55 000 apparent M, (designated here as Pr55<sub>gag</sub>) present in density gradient-purified virus preparations. Rabbit antiserum prepared against p26 as well as MAbs to p26 detected Pr55<sub>gag</sub> and several other intermediate cleavage products in detergent-soluble lysates of virus-infected cells in Western blot and immunoprecipitation assays. The results suggest that Pr55<sub>gag</sub> is the gag polyprotein of EIAV.

Equine infectious anaemia virus (EIAV), a member of the subfamily Lentivirinae in the family Retroviridae, causes a naturally occurring disease in all members of the horse family (Issel & Coggins, 1979). The disease has a world-wide distribution and is of great economic importance to the horse industry. The virus shares many characteristics with the human immunodeficiency virus, the causative agent of acquired immunodeficiency syndrome. These non-oncogenic, chronic disease-causing lentiviruses have identical morphology, share genetic and antigenic relatedness, and genetic and/or antigenic variants of each of these viruses appear during persistent infection (Hahn et al., 1986; Issel et al., 1986; Montelaro et al., 1986; Salinovich et al., 1986; Hussain et al., 1987; Payne et al., 1987). EIAV contains two main surface glycoproteins (gp90 and gp45) and four major non-glycosylated internal proteins designated p26, p15, p11 and p9 (Parekh et al., 1980; Montelaro et al., 1982). The nucleotide sequence of the gag gene of EIAV provirus DNA and the N- and C-terminal amino acid sequence analysis of each virus core gag protein are in complete agreement (Stephens et al., 1986; Henderson et al., 1987; Ball et al., 1988). The major EIAV core antigen is the group-specific antigen, p26. This protein constitutes about 40% of the total virion protein mass and is the predominant viral protein employed in commercial diagnostic tests for the detection of EIAV antibodies in animals (Coggins & Norcross, 1970; Issel & Coggins, 1979; Parekh et al., 1980).

The present paper reports the generation of monoclonal antibodies (MAbs) against p26 and the identification of the gag-encoded polyprotein, from which the four major internal proteins of EIAV (p26, p15, p11 and p9) are derived.

Procedures used for the production and characterization of MAbs against p26 were essentially as described by Hussain et al. (1987). Briefly, BALB/c mice were immunized with density gradient-purified EIAV emulsified in Freund's complete adjuvant (200 μg of EIAV intraperitoneally and subcutaneously), followed by two bi-weekly booster injections in Freund's...
incomplete adjuvant. A similar dose diluted in phosphate buffer was given intravenously 3 days before fusion. Three days after the last immunization, serum samples from each mouse were assayed for antibodies against EIAV. Four days after the last injection, mice with high antibody titres were used as a source of spleen cells for fusion with Sp2/O-Ag-14 myeloma cells. Hybridomas were selectively isolated by growth in HAT medium and screened for antibody activity against EIAV proteins by ELISA using immune mouse serum and increasing concentrations of antigen; an optimal concentration of 1 μg/well of dissociated virus was chosen to increase the possibility of detecting hybridomas specific to EIAV antigens (Hussain et al., 1987). Cells from selected antibody-positive wells were cloned three times by the limiting dilution method and tested for continued antibody production at each step until they were phenotypically stable. Specificity of MAbs to EIAV p26 was determined in a Western blot immunoassay.

SDS–PAGE of EIAV proteins was performed as described previously (Montelaro et al., 1982). To identify immunoreactive components of EIAV, SDS–PAGE-resolved proteins were transferred to nitrocellulose membranes and immunoblotted with polyclonal serum or MAbs as described previously (Salinovich et al., 1986; Hussain et al., 1987).

The serological specificity of these hybridomas as monitored by Western blot immunoassay indicated that MAbs 141-4D5, 140-1C3, 149-4E3, 135-4E5 and 131-2D6 were specific for p26 (Fig. 1). Immunoglobulins produced by hybrids 149-4E3, 141-4D5, 140-1C3 and 131-2D6 were of the IgG1 subclass, whereas immunoglobulin produced by hybrid 135-4E5 was found to be IgG2b by the double immunodiffusion test using isotype-specific antisera. The p26-specific MAbs showed reactivity in Western blot immunoassay with another protein of apparent Mr 55 000 in density gradient-purified virus preparations (Fig. 1). The presence of multiple bands on the Western blot reflects antigenic relatedness and indicates that these proteins share antigenic determinants with the core protein, p26. Our reference EIAV-positive horse serum also recognized the 55K protein (data not shown). The fact that our hybridomas were cloned at least three times ensured that they were monoclonal and argues against the existence of multiple clones of hybridoma in our preparations.

To confirm further that the Pr55\textsuperscript{gag} recognized by the p26-specific MAbs is not due to impurities in our MAbs, the viral core protein (p26) of EIAV was purified by reverse-phase (RP) HPLC from a stock of density gradient-purified virus preparations (data not shown) and analysed by SDS–PAGE (Fig. 2a). One rabbit was injected with 800 μg of RP-HPLC-purified p26 emulsified in Freund’s complete adjuvant, followed by a booster dose of 400 μg of purified protein after 2 weeks. Ten days after the final immunization, the serum was assayed for its reactivity with p26 antigen. Anti-p26 activity was detected in agar gel immunodiffusion, dot blot and Western blot assays. This immune rabbit serum was used to examine the virus and virus-infected cultures for EIAV-related proteins. In addition to its reactivity with the p26 antigen in Western blot, this antiserum reacted with another EIAV protein of apparent Mr 55K in density gradient-purified virus preparations (Fig. 1). The results with polyclonal and monoclonal antibodies indicate that EIAV contains a minor protein of 55K (referred to here as Pr55\textsuperscript{gag}) which shares antigenic determinants with p26. However, the epitopes recognized by these MAbs must be located on the group-specific antigen (p26) since the MAbs showed reactivity with both the 55K precursor protein and one of its cleavage products, p26. The results also indicate that mature EIAV virions contain relatively low quantities of uncleaved precursor, suggesting that the cleavage and processing events of the precursor polyprotein take place rather late in the maturation pathway. Detectable levels of gag precursors have been observed in viral particles of other mammalian retroviruses (Jamjoom et al., 1975).

To obtain additional evidence for the gag-encoded polyprotein, immunoprecipitation and Western blot analyses were performed on lysates of persistently infected cell cultures. The presence of EIAV p26 in persistently infected foetal equine kidney (FEK) cells was monitored by immunofluorescence assay using MAb 141-4D5. Specific, well defined, fluorescent intracytoplasmic spots were observed in EIAV-infected cultures (Fig. 3). To prepare a detergent-soluble cell extract, FEK cells persistently infected with EIAV were grown in flasks, rinsed three times with phosphate-buffered saline, lysed with lysing buffer (0.15 M-NaCl, 1%
Fig. 1. Western blot analysis of p26-specific MAb. Proteins of density gradient-purified EIAV preparations were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The strips were incubated with MAb 131-2D6, 135-4E5, 140-1C3, 141-4D5 and 149-4E3 in lanes 2 to 6, respectively. A blot of Mr markers stained with amido black (lane 1) is included for reference. The arrowhead shows the location of the 55K protein.

deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM-Tris-HCl pH 7.8 and 2 mM-phenylmethylsulphonyl fluoride) and centrifuged at 10000 g for 30 min. For immunoprecipitation, the supernatant was incubated for 1 h at 4 °C with 80 μl of either p26-specific MAb or rabbit anti-p26 serum. Immune complexes were precipitated by 50 μl of 20% (w/v) suspension of Protein A–Sepharose (Sigma) for 30 min at 4 °C. Immune complexes were collected by centrifugation, the pellet was washed three times with wash buffer and boiled for 4 min in 80 μl of SDS-PAGE sample buffer. Eluted proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with either rabbit anti-p26 serum or p26-specific MAbs.
Fig. 2. SDS-PAGE, immunoprecipitation and Western blot analysis of the major core protein, p26, and EIAV gag-encoded polyproteins. (a) Lane 1, RP-HPLC-purified p26 stained with Coomassie Brilliant Blue; lane 2, EIAV virion proteins; lanes 3 and 4, low and high Mr markers, respectively. (b) Lanes 1 and 2, Western blots of EIAV reacted with MAb 140-1C3 and with rabbit antiserum, respectively; lane 3, Western blot of proteins in EIAV-infected cell lysates immunoprecipitated with rabbit anti-p26 serum.

Western blots of proteins immunoprecipitated from EIAV-infected FEK cells with anti-p26 serum or p26-specific MAb revealed a number of gag-related proteins of lower Mr, than the major precursor (Pr55), which are labelled here as Pr49, Pr43, Pr40 and Pr35 (Fig. 2). All the intermediate products contain p26 and appear to represent combinations of other viral proteins, i.e., p15, p11 and p9. The Mr of the 55K precursor protein, which shares antigenic determinants with p26, is in good agreement with the sum of the Mr of the four major internal proteins (p26, p15, p11 and p9), which were calculated previously using electrophoresis and guanidine
Fig. 3. Indirect immunofluorescence staining of EIAV-infected FEK cell cultures. A well defined cytoplasmic fluorescence was observed in acetone-fixed cells stained with p26-specific MAb 141-4D5. Bar marker represents 20 μm.

hydrochloride gel permeation chromatography (Parekh et al., 1980; Montelaro et al., 1981). The Pr49 protein shares antigenic determinants with p26, and its apparent $M_r$ is in good agreement with the sum of the $M_r$ for p15, p26 and p11. The Pr43 protein shares antigenic determinants with p26, and its $M_r$ is in agreement with those of p26, p11 and p9. This particular fragment has not been previously detected and we suggest that it could be a short-lived proteolytic product that may be present at different levels of processing in persistently infected equine cell cultures. In addition, our studies show that infected cells appear to contain a low concentration of this product compared to the rest of the cleavage products. The Pr40 protein shares antigenic determinants with p26, and its $M_r$ is in close agreement with those of p15 and p26. The Pr35 protein shares antigenic determinants with p26, and its $M_r$ is in close agreement with those of p26 and p11. These data seem to support the report of Henderson et al. (1987). Therefore, our data confirm that Pr55sag is proteolytically cleaved via several intermediate cleavage products in virus-infected cells to generate the low $M_r$ gag proteins found in viral particles. These data support the view that Pr55 is the putative gag precursor polyprotein of EIAV from which p26, p15, p11 and p9 would be derived by proteolytic cleavage. Previous studies using peptide mapping and serology demonstrated that the four major non-glycosylated internal proteins of EIAV (p26, p15, p11 and p9) are not related (Montelaro et al., 1982). Therefore, these proteins appear to be the cleavage products of the Pr55sag protein and it is suggested that the gag precursor is processed by proteases that cleave the molecule to yield the characteristic internal virion proteins.

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