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Cells infected with vaccinia viruses expressing the equine infectious anaemia virus (EIAV) *gag* gene (VGag) or *gag* plus the 5′ *pol* encoding protease (VGag/PR) were evaluated with monoclonal antibody to a p26 capsid protein linear epitope (QEISKFLTD). Both recombinant viruses expressed Gag precursor protein (55K) whereas only VGag/PR expressed a detectable Gag–Pol fusion protein (82K) with a functional protease, shown by subviral particles containing processed p26. Horses inoculated with VGag/PR produced antibodies reactive with EIAV Gag proteins.

Horses infected with equine infectious anaemia virus (EIAV) have a life-long infection, characterized in the first few months by recurrent viraemia with fever, anaemia, and thrombocytopenia (Cheevers & McGuire, 1988; Clabough *et al.*, 1991). During viremic episodes, cell-free antigenic variants defined by *in vitro* neutralizing antibody assays can be isolated from plasma (Kono *et al.*, 1973). Mutations occurring in the *env* gene encoding gp90 and gp45 cause these antigenic variants (Montelaro *et al.*, 1984; Payne *et al.*, 1987). Despite variation of neutralization-sensitive epitopes and other mechanisms of viral escape, most EIAV-infected horses eventually become clinically quiescent carriers (Kono *et al.*, 1976). That lymphocyte responses are involved in EIAV control is demonstrated by the failure of foals with congenital severe combined immunodeficiency, which lack B and T lymphocytes, to terminate viraemia (Perryman *et al.*, 1988). Also, EIAV carrier horses treated with immunosuppressive drugs have viraemic episodes (Kono *et al.*, 1976).

EIAV could be controlled by several potentially protective immune responses including neutralizing antibody, cytotoxic T lymphocytes or antibody-dependent mechanisms of cytotoxicity. Studies of protective immune responses to lentiviruses have emphasized envelope glycoproteins. However, some have demonstrated cytotoxic T lymphocyte responses to human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus Gag proteins (Riviere *et al.*, 1989; Johnson *et al.*, 1991; Littaua *et al.*, 1991; Yasuhiro *et al.*, 1993). Immune responses to epitopes on the *gag*-encoded proteins may be important because the rate of divergence in *gag* is low compared to *env* in HIV-1 (Li *et al.*, 1988) and in EIAV (Perry *et al.*, 1992). The EIAV *gag* gene contains 1458 nucleotides (nt) encoding a 55K Gag precursor protein (Montelaro *et al.*, 1982; Stephens *et al.*, 1986; Hussain *et al.*, 1988) which is proteolytically cleaved into proteins including p15 matrix, p26 capsid, p11 nucleocapsid, and p9 of unknown function (Henderson *et al.*, 1987). A 10K protease encoded by the EIAV *pol* gene and expressed in *Escherichia coli* cleaves the Gag precursor to yield p26 (Rushlow *et al.*, 1992). To evaluate Gag protein synthesis and processing in eukaryotic cells and to aid the dissection of immune responses to Gag proteins, recombinant vaccinia viruses expressing EIAV Gag proteins and protease were evaluated.

Viral RNA for cDNA cloning was isolated (O'Rourke *et al.*, 1991) from EIAV-WSU5, a pathogenic cell culture-adapted strain isolated after three sequential passages of prototype EIAV in horses and then three limiting dilutions in cell cultures (O'Rourke *et al.*, 1988). First-strand synthesis was primed with an oligonucleotide corresponding to nt 2582 to 2599 of prototype EIAV provirus (Kawakami *et al.*, 1987) followed by second-strand synthesis (Gubler & Hoffman, 1983). Plasmids made by annealing dG-tailed cDNA to dC-tailed pUC19 were used to transform DH5α *E. coli*. Isolated plasmid pEIA5G contained a 2·2 kb EIAV cDNA insert and both strands were sequenced (GenBank accession no. L06609) using the dideoxynucleotide chain termination method. The sequence corresponded to nt 340 to 2578 of prototype provirus (Fig. 1) with the 1458 nt EIAV-WSU5 *gag* sequence being identical to that of prototype *gag* (Kawakami *et al.*, 1987). However, there were three

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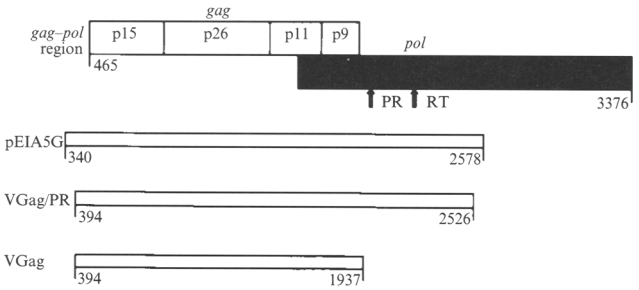

Fig. 1. Diagram of EIAV genes used to construct recombinant vaccinia viruses. EIAV proviral *gag* and 5′ *pol* (from Kawakami *et al.*, 1987; the complete *pol* extends to nt 5120) is at the top followed by a cDNA clone of the EIAV-WSU5 *gag* and 5′ *pol* in pEIA5G and the fragments of pEIA5G used to construct recombinant vaccinia viruses, VGag/PR and VGag. RT, Reverse transcriptase.

nucleotide differences in 5′ *pol*: an A at position 2058 changing arginine to lysine in the predicted protease sequence, a synonymous C at 2341, and an A at 2441 changing glycine to serine in the predicted reverse transcriptase. By comparison, the WSU5 *env* gene has 13 nt and six amino acid differences from the 2580 nt of prototype *env* (McGuire *et al.*, 1990). Like prototype EIAV, expression of Pol proteins from the WSU5 *gag–pol* transcript requires a ribosomal frameshift (Stephens *et al.*, 1986). The frameshift may occur at a heptanucleotide site associated with ribosomal frameshifting in other retroviruses (Hatfield *et al.*, 1992) and predicted for EIAV (Montelaro *et al.*, 1993); this is AAAAAAC located 40 nt into the *gag–pol* gene overlap.

To identify EIAV Gag proteins expressed by recombinant viruses, a monoclonal antibody (MAb) binding p26 was defined. The hybridoma producing MAb EIA6A1, an IgG1 isotype, was derived by fusing spleen cells from a BALB/c mouse immunized with EIAV prototype strain with X63.AG8.653 myeloma cells and cloned twice by limiting dilution (Davis *et al.*, 1983). This MAb reacted in immunoblots with 55K and 26K EIAV proteins and two other minor proteins (Fig. 2*a*, lane 7). The specificity of MAb EIA6A1 for p26 was defined by mapping the epitope using overlapping hexamers of the p26 amino acid sequence synthesized on plastic pegs (Geysen *et al.*, 1984; Cambridge Research Biochemicals). Significant MAb EIA6A1 binding occurred with hexamers encompassing the amino acid sequence QEISKFLTD (Table 1) corresponding to amino acids 179 to 187 of processed p26 (Stephens *et al.*, 1986). This epitope is also in the 83 amino acid C terminus of p26 previously identified as a highly immunogenic domain (Chong *et al.*, 1991).

To demonstrate that the EIAV 55K Gag precursor was processed by the *pol*-encoded protease rather than cellular proteases and to have vectors which correctly processed Gag proteins, two recombinant vaccinia viruses were made. VGag contained only the *gag* gene

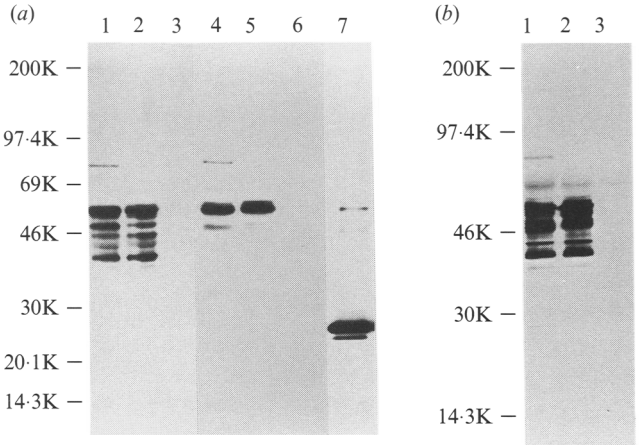

Fig. 2. Immunoblot of recombinant vaccinia virus-expressed *gag* proteins. (*a*) Lysates from HeLa cells (lanes 1 to 3) and equine kidney cells (lanes 4 to 6) infected with VGag/PR, VGag or VSC11, respectively, and 2 μg EIAV (lane 7) were reacted with MAb EIA6A1. (*b*) Lysates of TK$^-$ 143B cells (lanes 1 to 3) infected with VGag/PR, VGag or VSC11, were reacted with serum from horse A-1924 taken 60 days after EIAV infection. Neither an isotype control MAb nor preinfection serum from A-1924 reacted with duplicate immunoblots (data not shown). M_r standards are shown on the left.

Table 1. *Reactivity of MAb EIA6A1 with overlapping hexamers derived from the EIAV p26 amino acid sequence*

Peptide no.	Amino acid sequence	ELISA A_{405}
180	GHPQEI	0·017
181	HPQEIS	0·016
182	PQEISK	0·012
183	QEISKF	0·202*
184	EISKFL	0·306*
185	ISKFLT	0·298*
186	SKFLTD	0·122*
187	KFLTDT	0·027
188	FLTDTL	0·016
189	LTDTLT	0·015

* The A_{405} values for peptides 1 to 182 and 187 to 250 did not exceed 0·032.

(Fig. 1) and was made with an insertion plasmid derived by ligating the *Sma*I–*Xba*I fragment of EIAV cDNA from pEIA5G containing nt 394 to 1937 (Kawakami *et al.*, 1987) to *Sma*I-digested pSC11 (Chakrabarti *et al.*, 1985). Prior to ligation, the 5′ overhang resulting from the *Xba*I digest was filled using the Klenow fragment of DNA polymerase I. The *Sma*I cloning site in pSC11 is downstream of the P7·5 vaccinia virus promoter and the translation initiation codon for the cloned EIAV *gag* gene was the first ATG in the *Sma*I–*Xb*I fragment which was located 72 nt downstream from the *Sma*I site.

VGag/PR contained *gag* and 5′ *pol* genes encoding protease (Fig. 1). VGag/PR was made with an insertion plasmid derived by ligating the *Sma*I fragment from pEIA5G containing nt 394 to 2526 to *Sma*I-digested

pSC11. It was predicted to make the Gag precursor and also to have a ribosomal frameshift to produce a detectable Gag–Pol fusion protein (Stephens *et al.*, 1986). A control vaccinia virus, VSC11, was made with pSC11 without an EIAV cDNA insert. The insertion plasmids were characterized by restriction endonuclease digestion and nucleotide sequencing of all ligation sites. The insertion plasmid, pSC11, also contains the *β*-galactosidase gene driven by the P11 vaccinia virus promoter (Chakrabarti *et al.*, 1985) and following homologous recombination with vaccinia virus WR strain in infected cells, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside was added to the agarose overlay allowing visual detection of recombinant virus as a blue plaque. Selected recombinants were plaque-purified three times in BSC-1 cells.

The Gag proteins made by recombinant vaccinia viruses were identified by immunoblotting infected cell lysates. HeLa and neonatal equine kidney cells, in wells of a 24-well plate, were infected with 10 p.f.u./cell for 24 h. Infected cells were lysed with 0·5% NP40 in 50 mM-Tris–HCl pH 8, containing 5 mM-EDTA, 5 mM-iodoacetamide, 0·1 mM-TLCK and 1 mM-PMSF for 30 min on ice and centrifuged at 12000 ***g*** for 5 min. Proteins in the supernatant were separated by SDS–PAGE, transferred to nitrocellulose and probed with either MAb or serum from an Arabian horse 60 days after infection with EIAV-WSU5 (Perryman *et al.*, 1988). Bound primary antibodies were detected with either anti-mouse or anti-horse immunoglobulin antibodies conjugated to horseradish peroxidase and substrate for chemiluminescence (McGuire *et al.*, 1992). Cell cultures infected with VGag and VGag/PR produced a 55K protein, identified as the Gag precursor by its size and reactivity in immunoblots with MAb EIA6A1 (Fig. 2*a*, lanes 1, 2, 4 and 5). In addition, cell cultures infected with VGag/PR, but not VGag, expressed an 82K protein reactive with MAb EIA6A1 (Fig. 2*a*, lanes 1 and 4). This protein corresponded to the predicted truncated Gag–Pol fusion protein beginning with an initiation codon at nt 465, having a ribosomal frameshift in the overlap region, and continuing through nt 2526 to a termination codon 18 nt downstream in pSC11. If VGag encoded a Gag–Pol fusion protein involving the 244 nt overlap region, it was predicted to be approximately 55K and indistinguishable from the 55K Gag precursor protein by immunoblots using the antibodies described. Reactivity of MAb EIA6A1 with isolated EIAV resulted in a strong p26 band and a weak reaction with the 55K precursor protein (Fig. 2*a*, lane 7), presumably because of the low amount of this latter protein in EIAV. Serum antibodies from an EIAV-infected horse bound both the 55K and 82K proteins in cell lysates whereas serum taken before infection did not bind these proteins (Fig. 2*b*). p26 was not demonstrated in any of these immunoblots; however, MAb EIA6A1 bound 41K, 44K, 47K and 50K proteins in lysates from VGag- and VGag/PR-infected HeLa cells (Fig. 2*a*, lanes 1 and 2) and horse serum antibodies to EIAV bound similar proteins in VGag- and VGag/PR-infected thymidine kinase-negative (TK^-) 143B cells (Fig. 2*b*). Since these partially processed Gag proteins were present in lysates of cells infected with either construct, they did not result from the protease encoded by EIAV *pol*. It is likely these fragments were generated by cellular or vaccinia virus proteases. Cells infected with a recombinant vaccinia virus expressing HIV-1 *gag–pol* genes processed the 55K Gag precursor to p24 with three intermediates including p39, p41a and p41b (Gowda *et al.*, 1989*a*). Whether these HIV-1 Gag protein intermediates are similar to EIAV Gag proteins described above and attributed to cellular or vaccinia virus proteases is unknown.

Failure to detect p26 in recombinant virus-infected cell lysates prompted an examination of these cells for subviral particles which might contain processed p26 (Gowda *et al.*, 1989*b*; Karacostas *et al.*, 1989). For transmission electron microscopy, equine kidney cells in 25 cm^2 tissue culture flasks were inoculated with 10

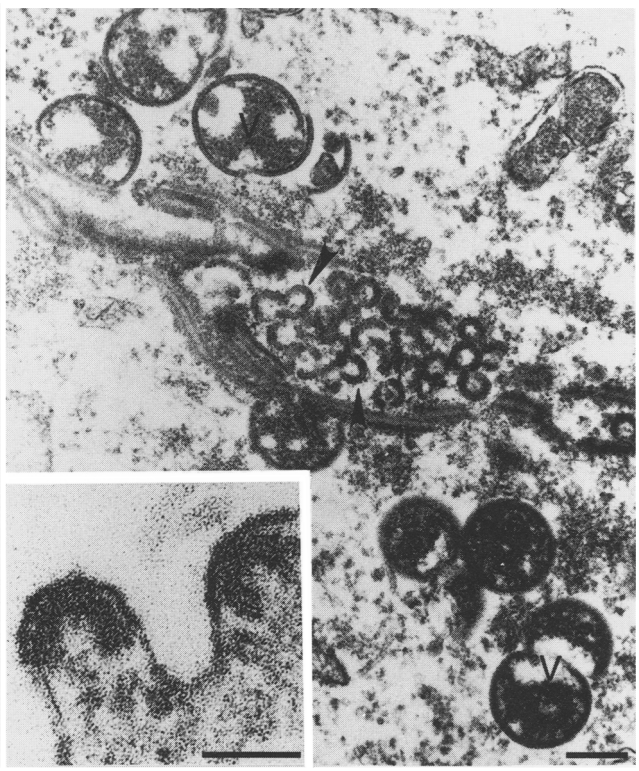

Fig. 3. Electron micrographs of retrovirus-like particles in thin sections of VGag/PR-infected equine kidney cells. Both vaccinia virus particles (V) and incomplete EIAV particles (arrowheads) are present in the cytoplasm. Bar represents 200 nm. Inset: retrovirus-like particles budding from the cell membrane; bar represents 100 nm.

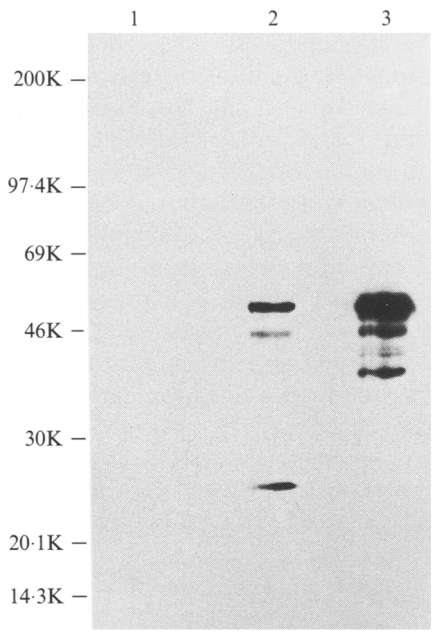

Fig. 4. Immunoblot of subviral particles isolated from recombinant vaccinia virus-infected cell cultures. Lanes 1 to 3 contained particles isolated from culture supernatants from equine kidney cells infected with VSC11, VGag/PR and VGag, respectively. All lanes were reacted with MAb EIA6A1. An isotype control MAb did not react with a duplicate immunoblot. M_r standards are shown on the left.

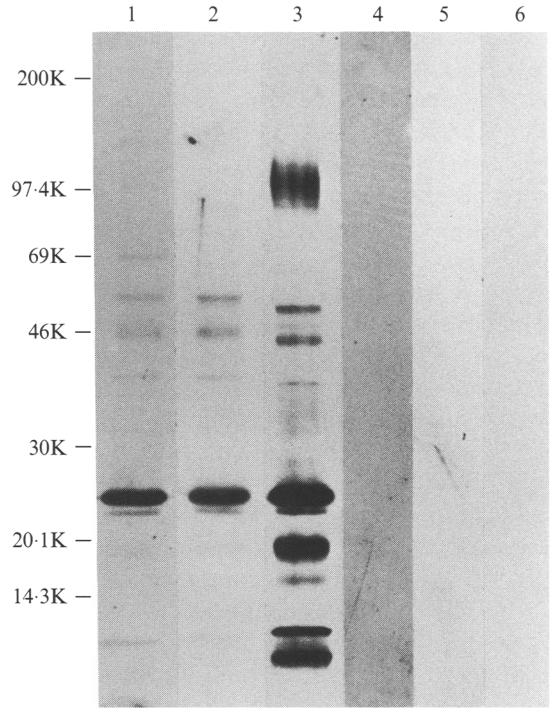

Fig. 5. Immunoblot of EIAV proteins with antibodies from horses immunized with VGag/PR. Every lane contained 0·5 μg EIAV and all antibodies were diluted 500-fold. Lanes 1 and 2 were reacted with sera from VGag/PR-immunized horses H-472 and H481, respectively. Lanes 3 and 6 were reacted with sera from horse A-1924 after and before EIAV infection, respectively. Lanes 4 and 5 were reacted with sera from VSC11-immunized horses H-479 and H-482, respectively. M_r standards are shown on the left.

p.f.u./cell of either VGag/PR, VGag or VSC11. After 24 h, cells were fixed with 4% paraformaldehyde/2% glutaraldehyde in 0·1 M-cacodylate buffer at pH 7·4. Fixed cells were removed by scraping, pelleted in agarose, post-fixed in 1% osmium tetroxide, and embedded in Epon. Ten ultrathin sections from each group were stained with uranyl acetate and lead citrate and examined. Cells infected with VGag/PR had both retrovirus-like particles and vaccinia virus particles that were distinguishable by size and morphology (Fig. 3). The retrovirus-like particles in VGag/PR-infected cells were not complete and were found free in the cytoplasm and budding from the cell membrane. Budding particles had a double-layered membrane with an electron-lucent centre and ranged from 90 to 110 nm in diameter (Fig. 3). Their morphology was similar to the particles described in cells infected with recombinant vaccinia virus or baculoviruses expressing the HIV-1 *gag* gene (Karacostas *et al.*, 1989; Gheysen *et al.*, 1989). Cells infected with VGag had subviral and vaccinia virus particles similar to those described for VGag/PR whereas cells infected with VSC11 had vaccinia virus, but no subviral retrovirus-like particles.

To examine subviral particles for p26, they were isolated and immunoblotted as described for cell lysates. Particles were removed from culture medium by centrifugation at 120000 *g* for 3 h, resuspended in PBS, layered onto a 15% sucrose cushion, and centrifuged at 120000 *g* for 90 min (Haffar *et al.*, 1991). Particles from VGag/PR-infected cells, and not from VGag-infected cells, contained processed p26 which reacted with MAb EIA6A1 (Fig. 4) demonstrating that processing of the p26 depended on the viral protease. Presence of capsid protein in EIAV subviral particles here and in HIV-1 particles (Gowda *et al.*, 1989*b*; Karacostas *et al.*, 1989) may be accounted for by protease cleavage of Gag precursor protein after particle assembly.

To determine whether VGag/PR would induce an immune response to EIAV Gag proteins, two horses (Shetland ponies H-472 and H-481) were immunized intradermally in each of four locations with 10^8 p.f.u. of VGag/PR and were boosted with the same dose at 4 and 12 months later. Two control horses (Shetland ponies H-479 and H-482) were similarly immunized with VSC11. Sera collected 2 weeks after the last boost were analysed for antibodies to EIAV by immunoblotting. The VGag/PR-immunized horses produced antibodies to EIAV Gag proteins, particularly p26 (Fig. 5, lanes 1 and 2). Longer development of these immunoblots demonstrated antibodies to p15 and p11 proteins (data not

shown). The VSC11-immunized horses did not produce antibodies to EIAV Gag proteins (Fig. 5, lanes 4 and 5).

Several conclusions can be drawn from the structural analysis of the EIAV *gag* and 5′ *pol* genes and the functional and immunological analysis of Gag proteins produced by recombinant vaccinia viruses. (i) Following sequential *in vivo* passages, the *gag* precursor sequence was more highly conserved in the clone we sequenced than either the 5′ *pol* or *env* sequence. (ii) Cells infected with VGag/PR expressing the *gag* and 5′ *pol* genes produced a predicted 82K Gag–Pol fusion protein demonstrating that the ribosomal frameshift necessary for translation of the protease gene from the *gag–pol* transcript occurred. (iii) The protease in the 82K Gag–Pol fusion protein was functional in equine cells (the only cell line examined for subviral particles) since the 55K Gag precursor was processed to p26 in subviral particles. This also indicated that the viral protease was unaffected by a naturally occurring mutation changing arginine to lysine. (iv) Expressed Gag proteins were bound by antibodies in serum from an EIAV-infected horse, and horses immunized with VGag/PR produced antibodies which bound EIAV Gag proteins. Based on these results, recombinant vaccinia virus VGag/PR produced functional Gag proteins and can be used further to examine T lymphocyte responses in EIAV carriers.

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