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Virus Research 53 (1998) 107–120

Virus Research

Immune response to recombinant visna virus Gag and Env precursor proteins synthesized in insect cells

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Received 1 April 1997; received in revised form 3 November 1997; accepted 3 November 1997

Abstract

Two different recombinant visna virus (VV) *gag*-baculoviruses were constructed for the expression of precursor VV Gag in insect cells. Both recombinant Gag viruses expressed proteins migrating on SDS–PAGE at the predicted rate for VV Gag precursor, $Pr50^{gag}$. However, differences were seen in the morphology of the virus-like particles produced. Monoclonal antibody directed against the VV Gag capsid protein (p25) and sera from sheep infected with ovine lentiviruses reacted to both 50-kDa proteins. A recombinant VV *env*-baculovirus was constructed, substituting sequences encoding the signal peptide of VV Env with the murine IFN-γ analogue. Sera from ovine lentivirus infected sheep reacted in immunoblots with two proteins of approximately 100 and 200 kDa found in the plasma membrane of insect cells infected with *env*-recombinant virus. Sheep immunized with either the recombinant Gag or the Env proteins developed high antibody titers to VV in ELISA. The serum of sheep and ascitic fluid of mice immunized with the recombinant Gag reacted with native $Pr50^{gag}$ and the processed Gag proteins in immunoblots, whereas serum of the recombinant Env immunized sheep reacted with VV gp135 and a putative oligomer of gp135. The immunized sheep responded specifically to visna virus by lymphocyte proliferation in vitro.

Keywords: Visna virus; Visna virus-*gag*-recombinant; Visna virus-*env*-recombinant; Baculovirus expression system; Visna virus recombinant protein immunization

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0168-1702/98/$19.00
PII S0168-1702(97)00141-X

1. Introduction

Maedi-visna virus (MVV) is prototypic of the lentivirinae genus of the retroviridae family and causes a systemic infection in sheep (Pétursson et al., 1976). Predominant pathological manifestations include interstitial pneumonia (maedi) and encephalomyelitis (visna) (Georgsson, 1990). The causative agent was first isolated from the brain of an infected sheep and called visna virus (VV) (Sigurdsson et al., 1960). A second virus was isolated from the lungs of an infected sheep and named maedi (Sigurdardóttir and Thormar, 1964). Later it was shown that the two isolates represented different strains of the same virus, frequently referred to as MVV.

Like other lentiviruses the VV genome contains three open reading frames (ORFs) *gag*, *pol* and *env*, which encode the internal proteins (Gag), catalytic (protease, reverse transcriptase, integrase and dUTPase) and envelope proteins respectively, as well as a number of small ORFs, which encode regulatory proteins. The largest of the *gag*-encoded proteins is a non-glycosylated precursor molecule of 50 kDa ($Pr50^{gag}$) (Vigne et al., 1982). $Pr50^{gag}$ is processed by the viral protease through intermediate proteins including p35 to form the mature CA, MA and NC proteins with apparent molecular masses of 25 (p25), 16 (p16) and 14 (p14) kDa respectively. The precursor of the mature envelope protein is a glycosylated polyprotein of approximately 150 kDa, which is processed to an apparent mass of 135 kDa. The transmembrane part of the glycoprotein is referred to as gp44 (Vigne et al., 1982; Sonigo et al., 1985).

Although considerable information on the virus and function of various parts of the viral genome, as well as processing of structural proteins, has been obtained in in vitro systems several problems in host-virus interactions have not been solved. The identification of structural proteins important to the induction of potentially protective immune responses is of primary interest. To study these problems it was necessary to produce a considerable quantity of purified antigenic viral proteins.

Several lentivirus Gag precursors have been produced in insect cells infected by recombinant baculoviruses (reviewed in Tobin et al., 1995). The Gag precursors assemble at the plasma membrane and bud into the extracellular space as virus-like particles (VLPs) which resemble immature virions. The VLPs are produced in great abundance and can be purified by standard virological techniques. The baculovirus-insect cell system is also capable of post-translational glycosylation and was therefore chosen for expression of the Env precursor (Rasmussen et al., 1992; O'Reilly et al., 1994; Davis and Wood, 1995). Here we report the construction of recombinant baculoviruses for the expression of VV Gag and Env precursor proteins and the immune responses to these products in infected and immunized sheep.

2. Materials and methods

2.1. Cells and culture medium

Wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant viruses were propagated in *Spodoptera frugipeda* (Sf-9) insect cells. Sf-9 cells were maintained at room temperature (RT) as suspension cultures in Grace's insect medium (Gibco), supplemented with 8–10% fetal calf serum (FCS) (Gibco) and gentamicin sulfate, 50 μg/ml. Sheep choroid plexus (SCP) cells (Pétursson et al., 1976) were cultured at 37°C in a humidified atmosphere containing 5% CO_2 using Dulbecco's MEM (DMEM), (Gibco) supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin and 1–10% normal lamb serum (LS). During antigen production SCP cells were cultured in TC 199 medium (Gibco) supplemented with 2 mM glutamine, antibiotics, 1% LS or 0.5% FCS. Lymphocyte blastogenesis assays were performed in AIM-V serum-free medium (Gibco) supplemented with 2 mM glutamine and 5×10^{-5} M 2-mercaptoethanol.

2.2. Construction of AcNPV-transfer vectors containing visna gag and env genes

All plasmid DNA manipulations were carried out using standard molecular biological techniques (Sambrook et al., 1989). The kv72/67r molecular clone of KV1772 that has been shown to be infectious in vitro and pathogenic in vivo

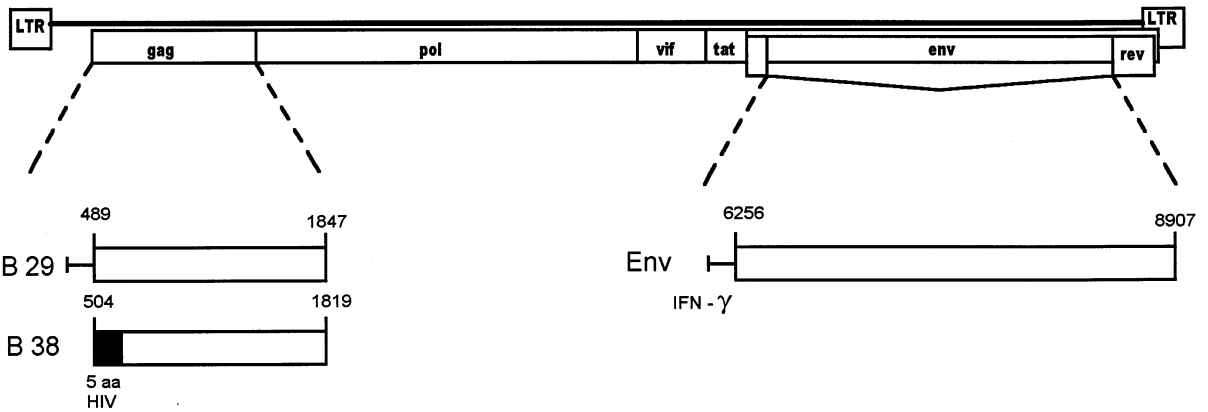

Fig. 1. Visna virus genome structure and structural gene fragments used to make recombinant baculoviruses. Diagram of visna virus genome structure showing relative positions of ORFs. Diagrams of constructs, *gag*-B29, *gag*-B38 and *env*, showing pkv72/67r nucleotides used for expression and the location of the sequences encoding the five HIV-1 aminoacids and the signal peptide of mouse IFN-γ.

was used as a source of VV DNA (Andrésson et al., 1993). All plasmid constructs were confirmed by DNA sequence analysis. AcNPV DNA for co-transfections was purified according to procedures previously described (Summers and Smith, 1987).

2.2.1. Gag gene

Visna *gag* sequences were amplified by polymerase chain reaction (PCR) from the molecular clone KV1772-kv72/67r, using synthetic primers. The product was cloned first into pCRII (Invitrogen) and then transferred directionally into the transfer vector pBacPac 1 (Invitrogen). Plasmids were co-transfected with AcMPV as described (Summers and Smith, 1987). Two *gag*-baculovirus transfer vectors were made (Fig. 1). The first (Gag-B29) used oligonucleotide primers: 5′-CATGCGACAGATCTGGTAAGGAAGCCGCCGTGGTGA-3′ and 5′-CATGCGACAGGCCTCTTTTATTTCTATTTTGGGTGG-3′ to amplify the entire *gag* gene (nt 453–1847). The forward and the reverse primers contained BglII and StuI sites respectively to facilitate directional cloning. The second (Gag-B38) utilized primers 5′-ATGGGTGCGAGAGCGTCAAAGGAGAAAAAGGGATACCCCG-3′ and 5′-CTATGTTACAACATAGGGGGCGCGGAC-3′. To determine the effect of N-terminal myristylation signals on the budding of VV Gag VLPs, the forward primer replaced the first five amino acids of the VV Gag precursor (MAKQG) with the first five residues of the HIV-1 (H × B2) Gag precursor (MGARA).

2.2.2. Env gene

Initially, a baculovirus transfer vector was generated to express the entire VV Env glycoprotein using standard PCR cloning techniques (data not shown). The resultant recombinant baculovirus produces very low yields of the recombinant Env glycoprotein. In an attempt to generate reagent quantities of Env, the native Env signal peptide was replaced with that of mouse γ-interferon (IFN). A linker, encoding the signal peptide for mouse γ-IFN (Gray and Goeddel, 1983; Dijkmans et al., 1985) and containing SacI and XbaI compatible ends, was cloned into the homologous sites in pBacPacI-IFN. The linker was composed of the following oligonucleotides: 5′-CATGAACGCTACACACTGCATCTTGGCTTTGCAGCTCTTCCTCATGGCTGTTTC TGGCT-3′ (upper strand) and 5′-CTAGAGCCAGAAACAGCCATGAGGAAGAGCTGCAAAGCCAAGATGCAGTGAGTAGCGTTCATGAGCT-3′ (lower strand).

Visna *env* sequences (nt 6256–8907) were amplified from the molecular clone pkv72/67r, using

the primers 5′-TATGCGACGTACTAGTGAAG-TGAGAAAAGGAAACCAG-3′ (forward) and 5′-ATATCGCATGCGGCCGCTACTATAACTC-TACATAGTCATTTTCCAG-3′ (reverse) which contained Spe1 and EagI restriction sites respectively and duplication of the stop codon. The DNA was cloned directionally into the pBacPac1-IFN to generate pBacPac1-IFN-visna-Env (Fig. 1).

Predicted molecular masses of recombinant and native VV proteins were calculated, using PEPTIDESORT from the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin, Madison, WI, USA.

2.3. Isolation of recombinant baculoviruses

Recombinant baculoviruses were produced as previously described (Summers and Smith, 1987). Briefly, AcNPV genomic DNA was co-transfected into Sf-9 cells with transfer vector plasmid DNA, using the calcium phosphate transfection method. Recombinant virus was initially selected, using terminal dilutions of virus in 96-well plates, containing Sf-9 cells and screened by dot-blot hybridization with ^{32}P-labelled VV specific gag or env DNA probes. Supernatants, containing recombinant viruses, were then purified by three successive plaque isolations.

2.4. Electron microscopy

Sf-9 cells, which were infected with recombinant baculoviruses, were pelleted gently and fixed in 1.25% buffered glutaraldehyde, then in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in epoxy resins. Thin sections were cut and stained with uranyl acetate and lead citrate as previously described (Gonda et al., 1985).

2.5. Expression and preparation of antigens

2.5.1. Gag B29 and B38 proteins

Sf-9 cells were infected with recombinant virus at multiplicity of infection (MOI) of 10 p.f.u./cell. After 3 days of infection culture supernatants were harvested and VLPs purified as previously described (Rasmussen et al., 1990). Briefly, the proteins were PEG precipitated, the pellet resuspended in TNE buffer, layered either onto a 20–60% linear sucrose gradient or a 20–45% discontinuous gradient and centrifuged at $100\,000 \times g$ for 90 min. The portion of the gradient containing the VLPs (1.16 g/ml) was collected and high salt purified as previously described (Torsteinsdóttir et al., 1992). The final sample was resuspended in PBS or TNE buffer and stored at 4°C.

2.5.2. Recombinant Env cell membrane antigen

Recombinant Env protein was expressed in the cell membrane of recombinant virus infected cells. Sf-9 cells were infected with recombinant virus at MOI of 10 p.f.u./cell. After 2 days the cells were pelleted, resuspended in HEPES disruption buffer (10 mM HEPES, pH 7.3; 1 mM EGTA; 2 mM $MgCl_2$; 1 mM benzamidine; 1 μM 1,10-phenanthroline) and lysed in a Dounce homogenizer. The sample was layered onto a 15–60% sucrose step gradient in HBS buffer (10 mM HEPES, pH 7.4; 150 mM NaCl), centrifuged $100\,000 \times g$ for 4 h and removed from the interface between 15 and 60% sucrose. Gradient purified antigen was either used directly or diluted 1:3 in HBS buffer, pelleted at $150\,000 \times g$ for 2 h and resuspended in HBS buffer.

2.5.3. Visna virus antigen

The VV antigens for ELISA and immunoblots were prepared as follows: Approximately 1 l of supernatant from visna infected SCP cells, cultured in TC199 medium supplemented with 0.5% FCS for ELISA or 1% LS for immunoblots, was clarified by centrifugation at $13\,200 \times g$ for 15 min. The supernatant was collected and centrifuged overnight (16 h) at $14\,600 \times g$ or $100\,000 \times g$ for 90 min. The pellet was resuspended in 1 ml TNE buffer and high salt purified as previously described (Torsteinsdóttir et al., 1992). Antigen for the lymphocyte stimulation test was produced from VV infected SCP cells and negative control antigen from a mock-infected SCP culture, as previously described (Torsteinsdóttir et al., 1992).

2.6. Immunizations

2.6.1. Recombinant visna Gag-B29 antigen

High salt purified Gag-B29 0.2 ml was mixed with 0.8 ml complete Freund's adjuvant (Sigma) for the first inoculation and incomplete Freund's adjuvant (Sigma) for two subsequent inoculations, which were given at 2-week intervals. Administration was intraperitoneal to three pristane primed Balb/c mice (Overkamp et al., 1988) or subcutaneous to outbred Icelandic sheep.

2.6.2. Recombinant Env and baculovirus antigen

Two days following infection with either recombinant VV Env or non-recombinant baculovirus, 8×10^6 Sf-9 cells were pelleted, washed once in PBS and resuspended in 0.2 ml PBS. The antigens were mixed with 0.8 ml complete Freund's adjuvant and injected subcutaneously into two sheep: one for recombinant Env and the other for non-recombinant baculovirus control. For the subsequent two immunizations, spaced at 2-week intervals, the cells were dissolved in 1.0 ml PBS and administered without adjuvant.

2.7. Serological tests

2.7.1. Immunoblots

Recombinant and native viral antigens were resolved by denaturing polyacrylamide electrophoresis and transferred to nitrocellulose membrane, using standard immunoblotting techniques (Coligan et al., 1994). Briefly, antigen containing membranes were blocked for 1 h at RT in TBS-T (0.1 M Tris–HCl buffered saline pH 7.8, 150 mM NaCl, 0.5% Tween 20) and cut into strips where indicated. Membranes were incubated overnight at 4°C on a rotator in either primary sera or ascites fluids, (diluted in TBS-T), washed with several changes of TBS-T and incubated with either rabbit anti-goat IgG or goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma A4174 and A2554). Membranes were rewashed in TBS-T and bound antibodies were detected by chemiluminescence and exposure to film (Amersham).

2.7.2. Glycoprotein detection

ECL glycoprotein detection system, which detects total carbohydrate (Amersham), was used according to manufacturer's instructions.

2.7.3. ELISA

ELISA was preformed as previously described (Torsteinsdóttir et al., 1992). Briefly, serum samples were diluted in PBS and applied to visna antigen coated plates for 1 h at room temperature, using three-fold dilutions, starting at 1:200. Rabbit anti-goat IgG conjugated to horseradish peroxidase (Sigma A4174) was used and bound antibody detected with H_2O_2/*o*-phenylenediamine dihydrochloride (OPD) (Dako) in phosphate-citrate buffer at pH 5.0.

2.7.4. Neutralization test

100 $TCID_{50}$ of VV was mixed with serial two-fold dilutions of serum in DMEM medium supplemented with 1% LS, starting with dilution 1:4. The samples were incubated at room temperature for 24 h and then inoculated in quadruplicate onto monolayers of SCP cells in 96-well tissue culture plates. Cytopathic effect was monitored by microscopic examination 7, 14 and 21 days after inoculation.

2.7.5. Lymphocyte stimulation test

Serial two-fold dilutions of virus and control antigen in AIM-V medium were dispensed in triplicate into round bottom 96-well plates, using 100 μl/well. Six wells containing only AIM-V medium and six wells with 0.5 μg/ml phytohemagglutinin (PHA) served as negative and positive controls, respectively. Peripheral blood lymphocytes (PBL) were obtained by sedimentation of anticoagulated sheep blood on Histopaque-1072 (Sigma), washed several times in PBS and resuspended at 2×10^6 cells/ml in AIM-V (Gibco). A suspension of 2×10^5 cells was added to each well in 100 μl and the plates were incubated in humidified atmosphere containing 5% CO_2 at 37°C for 6 days. During the last 8 h 1 μCi [^{3}H]thymidine (Amersham) was added to each well. Cells were harvested on glass fiber filters and radioactivity was quantitated by liquid scintillation. The mean c.p.m. of triplicate cultures was used to calculate a relative index

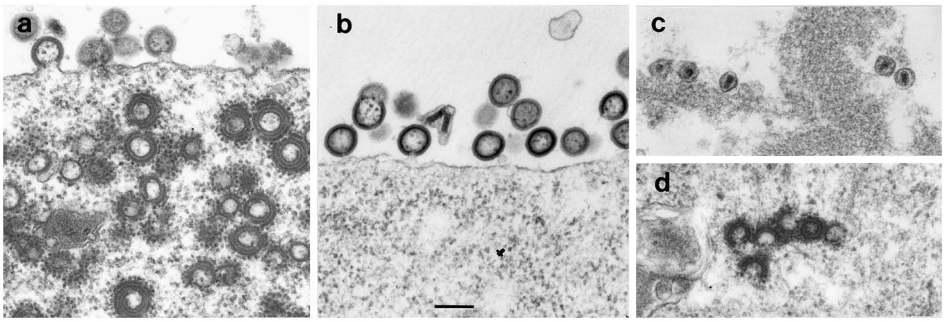

Fig. 2. Electron micrographs of insect or mammalian cells producing visna virus-like particles (VLPs) or visna KV1514 virus respectively. (a) Sf-9 insect cell infected with gag-B29 baculovirus showing VLPs composed of multiple bilayer membranes. (b) Sf-9 cell infected with gag-B38 baculovirus showing VLPs with single bilayer membranes. (c) SCP mammalian cell infected with visna virus KV1514 showing extracellular virus particles with condensed core structures and single bilayer membranes. (d) SCP cell infected with KV1514 showing intracellular virus particles with multilayered membranes. The length of the size bar is 0.2 μm.

(RI). Values were used only if the antigen dilutions showed a titration effect.

The relative index (RI) was calculated as:

$$\mathrm{RI} = \frac{\text{c.p.m. of cells with antigen} - \text{c.p.m. of cells with control antigen}}{\text{c.p.m. of cells in medium alone}}$$

The stimulation of cells in medium alone ranged from 200 to 1000 c.p.m. and in PHA 50 000 to 200 000 c.p.m.

3. Results

3.1. Electron microscopy

Electron microscopic examination of Sf-9 cells, infected with either Gag-B29 or B38 recombinant baculoviruses, demonstrated that large quantities of VLP were present both intracellularly and budding from the cell membrane. Differences in VLP morphology could be seen. Cells infected with Gag-B38 produced VLP structures with single layer cores, which were similar in ultrastructure to those produced from the expression of HIV-1 and BIV Gag precursors in insect cells (Fig. 2b) (Gheysen et al., 1989; Rasmussen et al., 1990). In contrast Gag-B29 infected insect cells had structures with both single and multilayered cores (Fig. 2a), that resembled particles seen in visna virus infected cells (Fig. 2c,d). Electron microscopy was not performed on the recombinant baculovirus infected cells expressing VV Env.

3.2. Detection of recombinant proteins in immunoblots

Purified VLPs were electrophoresed on acrylamide gels, immunoblotted onto nitrocellulose and tested with visna specific antibodies. Polyclonal mouse ascites produced against whole visna virus (Fig. 3a) and a mAb specific to MVV p25 (capsid protein) (Fig. 3b) reacted strongly to both Gag-B29 and Gag-B38 expressed recombinant VV proteins at the predicted size of 50 kDa in immunoblots. Post-infection sera from sheep experimentally infected with the visna virus strains KV1514 (Pétursson et al., 1976), KV1772-kv72/67r (Andrésson et al., 1993) and a field maedi strain KM1071 also reacted strongly with the recombinant $Pr50^{Gag}$. Pre-infection sera did not react with these proteins (Fig. 4a,b). The use of these sera demonstrated a difference between the two recombinant $Pr50^{Gag}$ antigens: Gag-B29 contained two reactive protein bands in the range of 50 kDa (Fig. 4a), whereas Gag-B38 contained only one band, which appeared to be intermediate in size (Fig. 4b). In addition both constructs contained a Gag-specific protein, which migrated at the same rate as was described for native Gag intermediate of 35 kDa (Vigne et al., 1982).

Since the transmembrane part of the Env protein was included in the construct, the recombinant Env protein was retained in the cell membrane of recombinant virus infected cells (Rasmussen et al., 1992; Clements and Zink, 1996). The protein was partially purified as a cell membrane extract and detected in immunoblot with sera from MVV experimentally infected sheep. Antibodies in post-infection sera recognized two species of recombinant Env proteins, one was approximately 100 kDa, which was close to the calculated mass of the native pre-glycosylated gp135 protein and the other was approximately 200 kDa (Fig. 4c). Pre-infection sera from the same sheep showed no reaction to those proteins. Because lentivirus Env proteins have been reported to be at least partially glycosylated in insect cells (Rusche et al., 1987; Rasmussen et al., 1992), the ECL glycoproteins detection system (Amersham) was used to assess carbohydrate addition to the Env protein. No glycosylation was detected (result not shown).

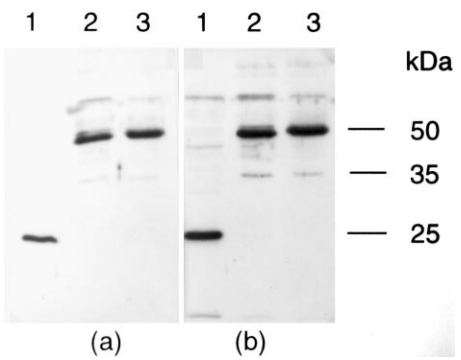

Fig. 3. Immunoblot of recombinant Gag-B29 and Gag-B38 with mouse antibodies. Immunoblot of whole visna virus (lane 1), recombinant Gag-B29 (lane 2), and Gag-B38 (lane 3) electrophoresed in denaturing 14% polyacrylamid gel. The nitrocellulose membranes were reacted with: (a) monoclonal mouse ascites fluid (1:2000 dilution) raised against p25 (capsid), (b) polyclonal ascites fluid (1:4000 dilution) from mice immunized with whole visna virus antigen. The position of the major 50-kDa Gag precursors, p35 (intermediate) and p25 are indicated.

3.3. Immunizations with the recombinant proteins

Mice immunized with Gag-B29 developed a strong antibody response to the Gag precursor $Pr50^{gag}$, the intermediate protein p35, and mature proteins p25, p16 and p14 of native visna virus antigen (Fig. 5). Immunization of mice with the recombinant Env did not generate immune responses specific to any native viral proteins (data not shown).

One sheep was immunized subcutaneously with Gag-B29, another with Sf-9 cells infected with *env*-baculovirus and a control sheep with Sf-9 cells infected with non-recombinant baculovirus. Endpoint ELISA values indicated that the sheep that had been immunized with recombinant VV proteins developed humoral responses, that recognized native virus antigen. The animal immunized with cells infected with non-recombinant baculovirus did not (Fig. 6). The ELISA titers of the immunized sheep were comparable to or higher than those from sheep that had been experimentally infected with VV KV1772-kv72/67r. Serum from sheep immunized with Gag-B29, demonstrated a strong reaction to mature Gag antigens p25, p16 and p14 and a somewhat weaker reaction to Gag precursors Pr50 and p35 (Fig. 7a). Serum from the sheep inoculated with the recombinant Env preparations reacted strongly to gp135 and a protein larger than 200 kDa but not to the processed transmembrane glycoprotein gp44. In contrast serum from VV experimentally infected sheep reacted to the gp135 and gp44 but not to the large protein (Fig. 7b). The serum from the sheep immunized with cells infected with non-recombinant baculovirus showed no response to VV antigens (Fig. 7a,b).

Neutralizing antibodies could not be demonstrated in the sera from the immunized sheep.

3.4. Lymphoproliferation of sheep immunized with $Pr50^{gag}$ B29 or recombinant Env protein

PBL from the three immunized sheep were tested for stimulation in response to visna virus antigen (Fig. 8). Blastogenic responses of the PBL from the sheep immunized with either recombinant Gag-B29 or Env were similar to those generated from PBL

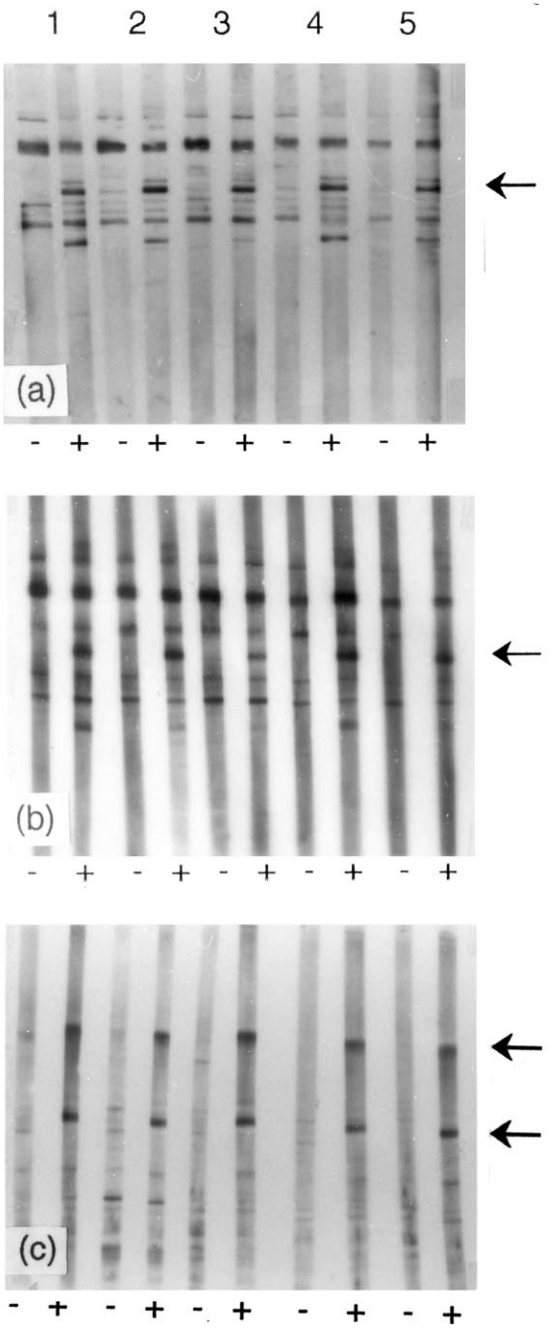

Fig. 4. Immunoblot of recombinant Gag-B29, Gag-B38 and Env with sera from sheep experimentally infected with MVV. Purified VLPs of Gag-B29, panel (a), and Gag-B38, panel (b), were electrophoresed in denaturing 14% polyacrylamid gels, recombinant Env protein, panel (c) in denaturing 10% polyacrylamide gels. The nitrocellulose membranes were cut into strips and reacted with pre-infection (−) and post-infection (+) sera (1:200 dilution) from sheep experimentally infected with visna viruses KV1514 (pair 1), KM1071 (pair 2) and KV1772-kv72/67r (pairs 3, 4, and 5). The position of the 50-kDa gag precursor bands and the two Env protein bands are indicated by arrows.

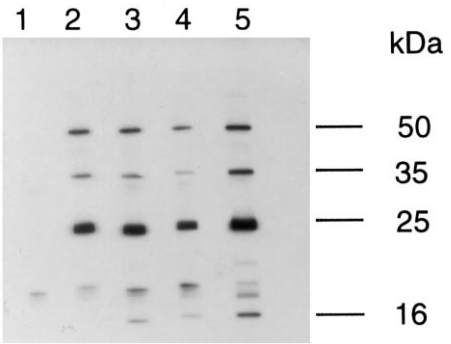

Fig. 5. Antibody response of mice immunized with Gag-B29 protein. Mice were immunized with either purified Gag-B29, KV1514 or hybridomas raised against AsaP1 (*Aeromonas salmonicida* subsp. *achromogenis*) as negative control and ascitic fluid containing antibodies were generated. A previously characterized mAb raised to p25 (capsid) was used as a control. Whole visna virus antigen was electrophoresed in denaturing 14% polyacrylamid gels, and transferred to nitrocellulose. The membrane was cut into strips and reacted with ascitic fluid from mice inoculated with hybridomas raised against AsaP1 (1:2000 dilution, lane 1), an anti-p25 hybridoma (1:2000 dilution, lane 2), purified Gag-B29 (1:2000 dilution, lane 3; 1:4000 lane 4) or whole KV1514 virus (1:5000 dilution, lane 5). The position of the main Gag protein bands are indicated.

of sheep experimentally infected with VV KV1772-kv72/67r. PBL from the sheep immunized with non-recombinant baculovirus showed no response to VV antigens (Fig. 8).

4. Discussion

The mature MVV Gag proteins p25 and p16 have been produced in pGex vectors as bacterial fusion proteins (Kwang and Cutlip, 1992) and the p25 also in yeast (Reyburn et al., 1992). The MVV transmembrane Env protein gp44 and the external protein gp70 have been produced in pGex vectors as bacterial fusion proteins (Kwang

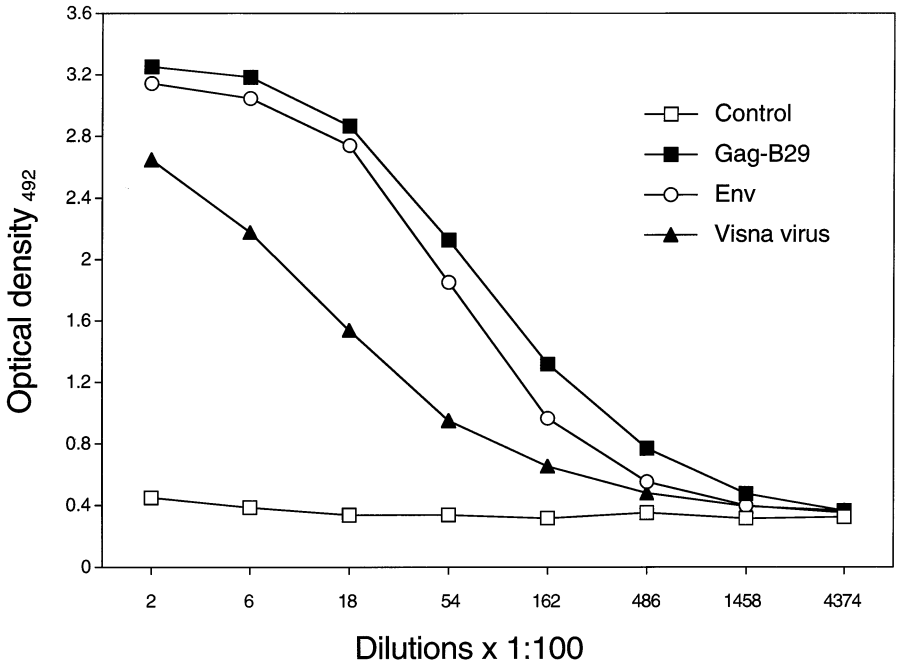

Fig. 6. Whole virus ELISA values of sera from immunized sheep. Diluted sera from sheep injected with purified Gag-B29 antigen (—■—), lysate from insect cells expressing recombinant Env (—○—), lysate from non-recombinant baculovirus infected cells (—□—) or infectious KV1772-kv72/67r virus (—▲–) were assayed for reactivity to immobilized whole visna virus by ELISA. Optical densities (OD) (492 nm) were plotted against dilutions, starting with 1:200 dilution. OD values below 0.4 were considered negative.

and Cutlip, 1992) and gp70 has also been produced in the baculovirus system (Kwang et al., 1995). In caprine arthritis-encephalitis virus (CAEV), the closest known relative of MVV, the Env gp135 has been cloned and expressed in a vaccinia vector (Lichtensteiger et al., 1991). The smaller CAEV proteins of Gag and Env have been cloned and expressed, using λgt11 or pGex vectors (Bertoni et al., 1994; Rimstad et al., 1994; Clavijo and Thorsen, 1995; Rosati et al., 1995). However, this is the first report of the expression of recombinant visna virus Gag and Env precursor proteins in insect cells from recombinant baculoviruses and the immunological characterization of these proteins after inoculation into sheep.

HIV and SIV Gag precursors appear to require myristylation of the penultimate N-terminal glycine residue for the production of extracellular particles in both mammalian and insect cell systems (Göttlinger et al., 1989; Bryant and Ratner, 1990). Substitution of alanine for glycine-2 in the SIV Gag precursor permits the formation of intracellular particles but prevents budding (Delchambre et al., 1989). In contrast to the primate lentiviruses, the VV Gag precursor contains an alanine-2 residue, which is not a site for myristylation. Although the N-termini of the majority of retrovirus Gag precursor or MA proteins have been shown to undergo post-translational modification, the BIV Gag precursor lacks any fatty acid and is consequently not blocked to Edmund degradation (Tobin et al., 1994). It is not clear whether the VV Gag precursor requires alternative fatty acid modification (e.g., acetylation) or remains unmodified. The effect of myristylation was examined in this present study by the expression of VV Gag precursors, that contain either the homologous N-terminus (Gag-B29) or the five

N-terminal amino acids encoded by the HIV-1 *gag* gene (Gag-B38). Although both recombinant baculoviruses expressed Gag proteins, that formed extracellular particles, different ultrastructural morphologies were observed. Gag-B29 VLPs accumulated in the cytoplasm and budded from the membrane as multilayered structures similar to those occasionally seen in mammalian cells productively infected with VV. In contrast, intracellular and extracellular Gag-B38 VLPs have single bilayer cores, are highly uniform in appearance and are more similar to the VLPs of HIV-1, SIV and BIV (Delchambre et al., 1989; Gheysen et al., 1989; Rasmussen et al., 1990). Our data indicate that the differences in membrane morphology map to the N-terminus of the Gag precursor.

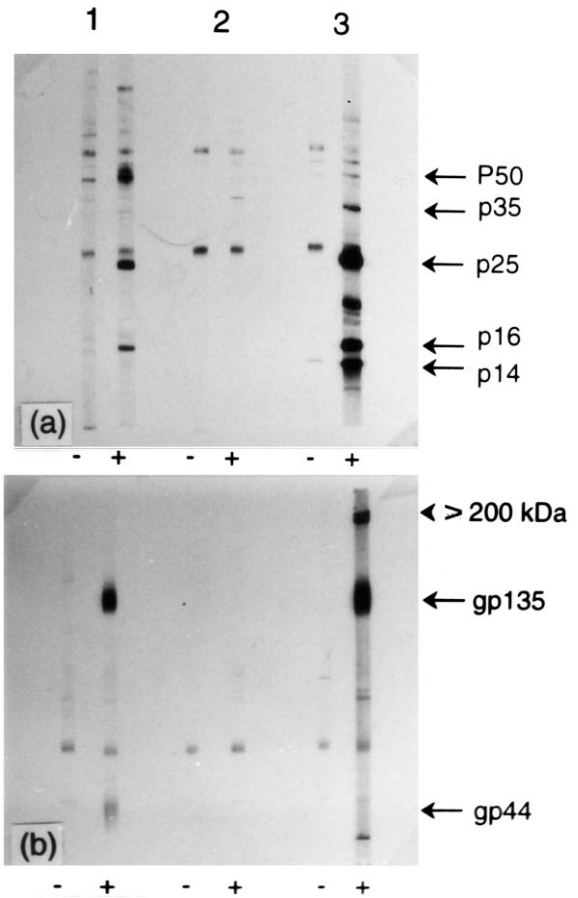

Fig. 7. Immunoblot of whole visna virus antigen reacted with sera from immunized sheep. Visna virus antigen was electrophoresed in denaturing polyacrylamide gels 14% panel (a) or 10% panel (b), transferred to nitrocellulose and cut into strips. The strips were reacted with pre-immune (−) or post-immune (+) sera from sheep experimentally infected with visna virus (KV1772-kv72/67r), dilution 1:1000, pair 1 (a) and (b), immunized with lysate from non-recombinant baculovirus infected cells, dilution 1:5000, pair 2 (a) and (b), with purified Gag-B29, dilution 1:10000, pair 3 (a) and with lysate from insect cells expressing recombinant Env, dilution 1:5000, pair 3 (b). Position of the main Gag and Env proteins are indicated with arrows.

In our initial efforts to express Env, using the homologous signal peptide, no significant quantities of Env were produced. Similar efforts to express HIV-1 and BIV Env precursors also resulted in low yields (data not shown; Rasmussen et al., 1992). We hypothesized that the signal sequence of lentivirus Env glycoproteins may be either toxic or inefficient in insect cells. Because IFN-γ is normally secreted, we engineered baculovirus to express VV Env precursor, using the signal peptide of mouse IFN-γ. The chimeric IFN-Env-baculovirus directed expression of two species of Env proteins one close to 100 kDa and the other approximately 200 kDa. Based upon the predicted amino acid sequence the unmodified translation product of the Env construct with the IFN-γ signal sequence attached is 104 kDa; if the signal sequence was removed during post-translational processing, the recombinant Env protein would be 102 kDa. Either size is consistent with the appearance of the 100 kDa species. The appearance of a higher molecular weight species of the recombinant Env suggests that a significant fraction of the protein is present as a dimer. Dimers and multimers of other recombinant lentiviruses have been reported (Schawaller et al., 1989; Earl et al., 1990; Rasmussen et al., 1992). The oligomers of lentivirus Env appear to be either highly resistant to the denaturants employed prior to electrophoresis or are able to reform after the material has entered the gel. Attempts to detect additions of carbohydrates to the recombinant Env were negative, suggesting that the recombinant Env is not glycosylated. Because native IFN-γ is glycosylated and the signal peptide influences post-translational modifications, lack of glycosylation could be due to incompatibility between the murine signal peptide and the insect cell system.

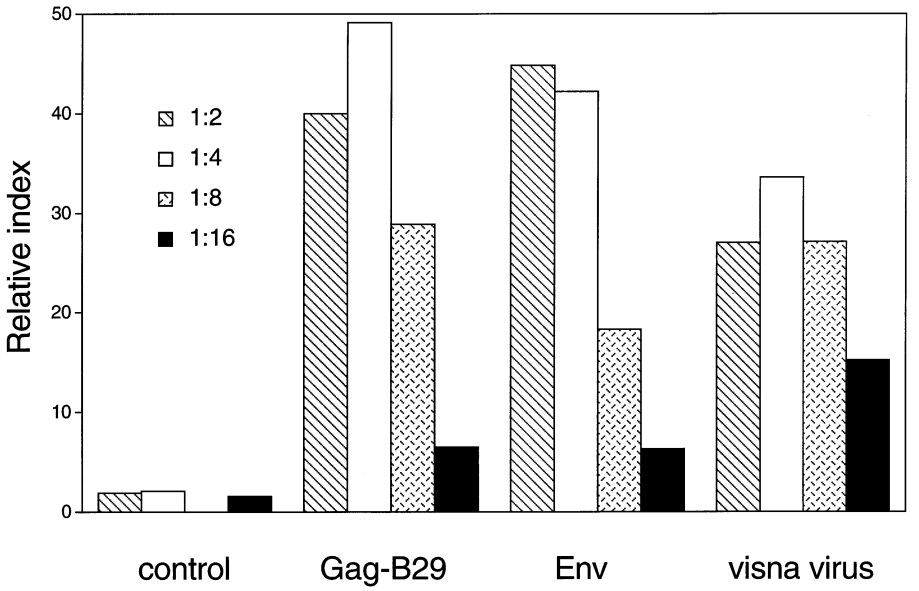

Fig. 8. Blastogenic responses of PBLs from visna infected and immunized sheep. PBLs were prepared from sheep inoculated with either lysate from non-recombinant baculovirus infected cells (control), purified Gag-B29, lysate from insect cells expressing recombinant Env, or infected with KV1772-kv72/67r virus. Results from [^{3}H]thymidine incorporation are expressed as a relative index of two-fold virus dilutions, one representative experiment out of three.

The sheep immunized with the visna virus recombinant Gag and Env proteins rapidly developed antibody responses to these viral proteins. The concentration of the virus-specific antibodies in the immunized animals was greater than that found in experimentally infected sheep. The sheep immunized with Gag-B29 responded strongly to both precursor and processed VV Gag proteins, whereas the sheep immunized with recombinant Env reacted to visna gp135 and a larger protein but not to the processed transmembrane gp44. In contrast, sera from experimentally infected sheep produced antibody reaction with gp135 and gp44 but not with the large protein in the visna antigen. The lack of response to gp44 by immunized sheep may indicate that the gp44 epitopes of the recombinant Env precursor were not folded into conformations similar to those of its native counterpart. Therefore, serum from sheep immunized with the recombinant Env recognizes a multimer in the visna virus antigen. Additional immunological reagents, including recombinant fragments of gp135 and corresponding antibodies, will be required to fully characterize the recombinant Env protein.

Neutralizing antibodies were not detected in the sheep immunized with the recombinant VV Env despite a strong antibody response. It is possible that additional inoculations of recombinant VV Env may result in the development of neutralizing antibodies. Similarly, goats immunized with CAEV gp135 expressed by recombinant vaccina virus also failed to develop neutralizing antibodies; in addition these goats were not protected when challenged with virus (Cheevers et al., 1994). However, low titers of neutralizing antibodies have been detected in sheep immunized with recombinant MVV Env gp70 (Kwang et al., 1995).

The immunized sheep showed a similar VV specific lymphocyte proliferation response in vitro as sheep infected experimentally with VV. Although the cellular immune response in MVV infection has not been characterized as extensively as the humoral response, specific lymphocyte proliferation (Griffin et al., 1978; Sihvonen, 1981; Larsen et al., 1982; Torsteinsdóttir et al., 1992) and MVV specific cytotoxic T-cells have been reported in experimental MVV infection (Bird et al., 1993).

Mice immunized with the Gag-B29 developed strong antibody response to all major Gag proteins. Previous studies have shown that virus-like particles of HIV-1 and other lentiviruses produce strong immune responses in mice and other species (Gheysen et al., 1989; Tobin et al., 1994). In contrast mice inoculated with recombinant Env protein did not generate a measurable immune response. The lack of humoral response to recombinant Env is consistent with results reported in previous studies that showed that BALB/c mice responded very poorly or not at all to visna virus Env (Stanley et al., 1987; Houwers, 1988).

Despite active immune responses, lentiviruses persist in the infected animal. In natural and experimental MVV infections, antibodies to Gag and Env proteins are detected early and neutralizing antibodies to gp135 later (Pétursson et al., 1976; Kajikawa et al., 1990; Torfason et al., 1992). Although an early rise in neutralizing antibodies to VV has been found to be somewhat protective (Georgsson et al., 1993) there are several indications that lesions in the central nervous system (CNS) in visna are immune-mediated (Nathanson et al., 1976, 1981). We previously demonstrated that a strong cellular immune response and CD8 positive cells, rather than the antibody response, might be the important effectors in the induction of CNS lesions (Torsteinsdóttir et al., 1992). In CAEV, antibodies to gp135 can have a deleterious immunopathological effect (McGuire et al., 1986; Knowles et al., 1990). This phenomenon of enhancing antibodies and exacerbation of disease in immunized animals is also known from other lentiviruses such as HIV-1 (Robinson et al., 1988), SIV (Montefiori et al., 1990) and EIAV (Wang et al., 1994).

It is therefore of great importance to dissect out the antigens and the types of immune response that are beneficial rather than deleterious to the animal. The recombinant visna Gag and Env proteins will be useful tools in future experiments designed to approach this problem.

Acknowledgements

We are indebted to Lyn Rasmussen, Svava Högnadóttir, Sigríður Matthíasdóttir, Karl Skírnisson, Sigurður Torfi Guðmundsson and Guðmundur Einarsson for expert technical help. This work was supported by The Icelandic Research Council, grants no 91-L-014, 94-L-050, and 95-L-045

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