Journal of Virological Methods 150 (2008) 34–40

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Characterization of polyclonal antibodies against the capsid proteins of Ljungan virus

Conny Tolf[a], Jens-Ola Ekström[a,1], Maria Gullberg[a], Gustav Arbrandt[b], Bo Niklasson[b,c], Gun Frisk[d], Jan-Åke Liljeqvist[e], Kjell Edman[a], A. Michael Lindberg[a,*]

[a] School of Pure and Applied Natural Sciences, University of Kalmar, SE-391 82 Kalmar, Sweden
[b] Apodemus AB, Grevgatan 38, SE-114 53 Stockholm, Sweden
[c] Department of Medical Cell Biology, Uppsala University, Box 571, SE-751 23 Uppsala, Sweden
[d] Department of Women's and Children's Health, University Hospital, Uppsala University, SE-751 85 Uppsala, Sweden
[e] Department of Clinical Virology, Göteborg University, Guldhedsgatan 10B, SE-413 46 Göteborg, Sweden

ARTICLE INFO

Article history:
Received 29 October 2007
Received in revised form 11 February 2008
Accepted 12 February 2008
Available online 9 April 2008

Keywords:
Parechovirus
Protein expression
Polyclonal antibodies
Virus detection
Immunodetection

ABSTRACT

Ljungan virus (LV) is a suspected human pathogen isolated from voles in Sweden and North America. To enable virus detection and studies of localization and activity of virion proteins, polyclonal antibodies were produced against bacterially expressed capsid proteins of the LV strain, *87-012G*. Specific detection of proteins corresponding to viral antigens in lysates of LV infected cells was demonstrated by immunoblotting using each one of the generated polyclonal antibodies. In addition, native viral antigens present in cell culture infected with LV strains *87-012G* or *145SLG* were detected in ELISA and by immunofluorescence using the antibodies against the VP0 and VP1 proteins. The anti-VP3 antibody did not react with native proteins of the LV virion, suggesting that the VP3 is less potent in evoking humoral response and may have a less exposed orientation in the virus capsid. No activity of the antibodies was observed against the closely related human parechovirus type 1. The polyclonal antibody against the VP1 protein was further used for detection of LV infected myocytes in a mouse model of LV-induced myocarditis. Thus, polyclonal antibodies against recombinant viral capsid proteins enabled detection of natural LV virions by several different immunological methods.

1. Introduction

Ljungan virus (LV) was discovered in Swedish bank voles (*Myodes glareolus*, previously referred to as *Clethrionomys glareolus*) during the search for an infectious agent causing an outbreak of lethal myocarditis in young athletes (Niklasson et al., 1998, 1999). LV infection in humans has been proposed to cause myocarditis, type 1 diabetes and intrauterine fetal deaths (Niklasson et al., 1998, 1999, 2003a). The connection between human disease and LV infections remains to be elucidated, but recent studies have shown a coincidence between LV infection and signs of diabetes in wild captured bank voles (Niklasson et al., 2003a,b). Furthermore, LV infections in a CD-1 mouse model have been shown to cause myocarditis, diabetes-like symptoms and reproductive problems including neonatal death (Niklasson et al., 2006a,b). After the discovery of two genotypes in Swedish bank voles, additional LV types have been identified among previously collected samples from North American voles, indicating a worldwide spread of LV (Donoso Mantke et al., 2007; Johansson et al., 2003; Johnson, 1965; Main et al., 1976; Whitney et al., 1970).

The LV prototype strain, *87-012*, was propagated initially in brains of suckling mice, but has subsequently been adapted to cytolytic growth in cell culture, resulting in a rapidly replicating variant, *87-012G* (Johansson et al., 2004). In addition, another Swedish strain, *145SL*, representing the second Swedish genotype has also been adapted to cytolytic replication in cell culture (data not shown). This strain variant is referred to as *145SLG*. These rapidly replicating cell culture adapted variants of the parental LV strains facilitate studies of virus–host cell interactions. Phylogenetic analyses have shown that LV belongs to *Picornaviridae* (Lindberg and Johansson, 2002), and form, together with human parechoviruses (HPEV), the *Parechovirus* genus (Stanway et al., 2005). Picornaviruses are small, icosahedral and nonenveloped viruses with a single-stranded RNA genome of positive polarity (Racaniello, 2001). The viral RNA is translated to a polyprotein, which is processed into capsid forming structural proteins and nonstructural proteins important for viral replication. Characterization of the LV genome has revealed several interesting properties

* Corresponding author. Tel.: +46 480 446240; fax: +46 480 446262.
E-mail address: michael.lindberg@hik.se (A.M. Lindberg).
[1] Present address: Umeå Center for Molecular Pathogenesis (UCMP), Byggnad 6L, Umeå University, SE-901 87 Umeå, Sweden.

0166-0934/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2008.02.012

including the coding of two different consecutive 2A protein motifs and a predicted extension at the C-terminus of the VP1 protein in comparison with members of the HPEV species (Johansson et al., 2002, 2003). As previously demonstrated for Aichi virus and for HPEV, and based on sequence analysis of the structural genes, the LV capsid was predicted to consist of only three structural proteins, VP0, VP1 and VP3 (Johansson et al., 2002; Stanway et al., 1994; Yamashita et al., 1998). This prediction was supported by initial analysis of the protein composition of the LV capsid (Johansson et al., 2004), although two of the proteins, VP0 and VP3, when subjected to SDS-PAGE, were impossible to distinguish due to similar molecular masses. The ability to assay the mechanisms a particular virus use to direct the cell to produce new virions by controlling the cell metabolism are important elements in the characterization of virus. The location and interaction of viral and cellular proteins are often studied by antibody-based identification methods. To detect cells and tissues infected with LV and to study immunogenic properties of the LV capsid, as well as characterize the interaction of LV with its target cells, polyclonal antisera against the individual structural proteins of the LV capsid were generated in rabbits. Immunoblotting, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence staining were used to evaluate the activity and specificity of polyclonal antibodies purified from antisera. The diagnostic potential of the antibody generated against the VP1 protein was further demonstrated in a mouse model of LV-induced myocarditis.

2. Materials and methods

2.1. Bioreagents

The gene sequences encoding predicted LV capsid proteins were cloned into the pGEX-4T-3 bacterial expression vector (GE Healthcare). The expression vector and recombinant plasmids were amplified in the *Escherichia coli* strain, DH5-alpha, and subsequently transferred to *E. coli* BL-21 (DE3) for protein expression.

Viruses were propagated in African green monkey kidney (GMK) cells. The Swedish strains, *87-012* and *145SL*, has been adapted previously to lytic cell culture propagation by serial passages in GMK cells, and were therefore designated *87-012G* and *145SLG*, respectively (Johansson et al., 2004). The *87-012G* virus was generated from an infectious cDNA clone (Ekström et al., 2007), while *145SLG* was first propagated in the laboratories of G. Frisk and J-Å. Liljeqvist. Confluent monolayers of GMK cells were infected with viruses for serological examination of natural viral antigen.

The type 1 HPEV strain *Harris* (HPEV1) and the enterovirus coxsackievirus B2 strain *Ohio-1* (CVB2) were used for cross reactivity analyses (Hyypiä et al., 1992; Polacek et al., 1999). Reference rabbit antisera against the VP1 capsid protein of HPEV1 was produced by the research group of T. Hyypiä, University of Turku, Turku, Finland (Alho et al., 2003), and the enterovirus-specific polyclonal rabbit antisera (KTL-482; Härkönen et al., 2002), was kindly provided by M. Roivainen, KTL, Helsinki, Finland.

2.2. Molecular cloning

The gene sequences encoding the predicted capsid protein VP0, VP1 and VP3 of the LV strain, *87-012G* (Johansson et al., 2003, 2004), were amplified using gene specific primers including restriction enzyme cleavage sites for *Bam*HI and *Not*I (underlined) and stop codons (boldface) for cloning and subsequent expression. The sense primer 5′-TTATTAGGATCCGCATCCAAAATGAATCCCGTTGGCAACCTG-3′ and the antisense primer 5′-TAATAAGCGGCCGC**TTA**TTGTGTGTAAATTACTGTGTCTGCACCAGGAGG-3′ were used to amplify the VP0 gene (770 bp), except that the first two codons in the N-terminus (codons in the VP0 and the VP1 genes were omitted to avoid any proteolytic processing of fusion proteins). The VP1 gene including the 2A1 motif (Johansson et al., 2002), except the two last C-terminal codons (948 bp) was amplified using the sense primer 5′-TTATTAGGATCGGTTTGTACTCATGGGGTTCAGAA-3′ and the antisense primer 5′-TAATAAGCGGCCGC**TTA**TGGGTTGGTTTCCACGTCACCACATTGGTTCAA-3′, while the VP3 gene (732 bp) was amplified using the sense primer 5′-TTATTAGGATCCGGGAAAAGAACTGTTCGAAAGACCAAGACATC-3′ and the antisense primer 5′-TAATAAGCGGCCGC**TTA**TTGCCAAACTAGATTAGATGTGGT-3′. Amplified PCR products were analyzed by agarose gel electrophoresis, and expected amplicons were extracted, then digested with *Bam*HI and *Not*I, and subsequently, cloned into restriction enzyme digested expression vectors. Single clones containing the capsid protein genes were isolated and verified by sequencing.

2.3. Expression and purification of viral capsid proteins

Recombinant plasmids or the expression vector without insert (negative control) were transformed into *Escherichia coli* BL21 (DE3) to express LV proteins as GST fusion proteins or as the GST tag protein alone. Transformed bacteria were grown in Luria Broth media supplemented with ampicillin (100 μg/ml, Sigma–Aldrich) at 37 °C to an optical density (OD) of 0.6 at 600 nm, before inducing protein expression with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After induction for 3–4 h at 22 °C, the cells were harvested and washed in ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and then stored at −20 °C, until protein purification was performed. Frozen bacteria pellets were suspended in ice-cold PBS supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma–Aldrich) and 1 mM ethylenedinitrilotetraacetic acid (EDTA, Merck). Suspended bacteria were disrupted by sonication (Sonics and Materials Inc.) and then incubated with gentle agitation during 1 h at 8 °C in 1% Triton X-100 (Sigma–Aldrich). Proteins soluble in the supernatant fractions was obtained by centrifugation at 20,000 × *g* for 1 h, and incubated overnight with a glutathione-agarose matrix (Sigma–Aldrich) at 4 °C. Proteins bound to the agarose were washed with adenosine-5′-triphosphate (ATP) and urea, as previously described (Chen et al., 2001; Thain et al., 1996), to purify LV proteins from a tightly associated bacterial GroEL chaperonin protein. Briefly, fusion proteins attached to the agarose beads were incubated with 5 mM ATP (Sigma–Aldrich) and 5 mM $MgCl_2$ in buffer-A (50 mM Tris–HCl, pH 8) and then with buffer-A containing 3 M urea. The urea was subsequently washed off, and proteins bound to the agarose were eluted with 20 mM reduced glutathione (Sigma–Aldrich) solved in buffer-A. Finally, the solvent of the eluted fractions was exchanged for PBS using centrifugal filters (Millipore). The GST protein was purified according to instructions provided with the expression vector (GE Healthcare).

2.4. Production of polyclonal antisera

Polyclonal antisera against the purified fusion proteins GST-VP0, GST-VP1, GST-VP3 and the GST protein alone were raised in rabbits by subcutaneous injections with 200 μg doses of the individual proteins in Freund's adjuvant followed by three booster doses, the first 20 days after initial immunization and the following at 30-day intervals. The antisera were collected 4 weeks after the last immunization, and the IgG fractions of generated antisera (anti-VP0, anti-VP1, anti-VP3 and anti-GST) were purified using Protein A-Sepharose (GE Healthcare). To remove antibodies reactive to the