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A novel method for cloning of non-cytolytic viruses

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Abstract

Hantaviruses are rodent-borne pathogens with a segmented single-stranded RNA genome of negative polarity. Spontaneous occurrence of variants with genetic heterogeneity have been observed both in vivo and in vitro. The objective of this study was to establish a method for the cloning of genetically homogenous hantaviruses which can be used for subsequent functional studies. Infected VeroE6 cells were incubated with an agarose/medium overlay to prevent uncontrolled distribution of de novo synthesized virus. Thereafter, the overlay was removed and stored for isolation of the diffused virus. The cell layer was fixed and viral antigen-containing foci were detected by immunochemistry. The relative location of the foci on the culture dish was used to trap individual virus clones in the corresponding overlay. The clones were picked and used for re-infection. According to this novel protocol three different hantaviruses, i.e. Hantaan, Puumala, and Tula virus, were purified. In the course of purification the titers of the resulting virus stocks were increased by 10–1000-fold. In addition, this method was used to purify a minor Puumala virus variant from a parental stock containing a mixture of two variants. Taken together, the method presented is well suited to isolate genetically homogenous hantaviruses and might also be applicable for other non-cytolytic viruses.

Keywords: Puumala virus; Genetic heterogeneity; Variants; NSs; Non-cytolytic virus; Virus cloning method

1. Introduction

Hantavirus represents a distinct genus of the *Bunyaviridae* family. Human hantavirus infections can lead to hantavirus cardiopulmonary syndrome (HCPS) or hemorrhagic fever with renal syndrome (HFRS) (Schmaljohn and Hjelle, 1997; Krüger et al., 2001; Ulrich et al., 2002). Whereas members of the other *Bunyaviridae* genera are transmitted by arthropods, the natural hosts of hantaviruses are rodents infected persistently within the family Muridae, subfamilies Arvicolinae (voles), Murinae (Old World mice and rats), and Sigmodontinae (New World mice and rats). The prototype member of the genus *Hantavirus* is the Hantaan virus (HTNV) carried by the striped field mouse *Apodemus agrarius* (subfamily Murinae) causing HFRS with high case fatality ratios (Lee and van der Groen, 1998). In Europe most cases of HFRS are due to infections with Puumala virus (PUUV) transmitted by the bank vole *Clethrionomys glareolus* (subfamily Arvicolinae) or with members of Dobrava Belgrade virus transmitted by the yellow-necked mouse *Apodemus flavicollis* or the striped field mouse *Apodemus agrarius* (for reviews, see Krüger et al., 2001). Tula virus (TULV) represents another Arvicolinae-associated hantavirus species harboured by common and field voles (*Microtus arvalis* and *M. agrestis*; subfamily Arvicolinae). It causes only rarely specific illness in humans (Klempa et al., 2003).

Hantaviruses contain a tripartite single-stranded RNA genome of negative polarity. According to their size the three segments are designated as S- (small), M- (medium), and L- (large) RNA and code for the nucleocapsid protein (N protein), the envelope glycoproteins, and the RNA-dependent RNA polymerase, respectively (Elliott et al., 1991; Schmaljohn et al., 1985). In addition to the N protein, the S segment of Bunyamwera virus, genus *Orthobunyavirus*, encodes a non-structural protein (NSs) that was demonstrated to thwart antiviral innate immune responses by blocking the interferon response (Kohl et al., 2003; Thomas et al., 2004). In almost all Sigmodontinae- and Arvicolinae-associated hantaviruses a second open reading frame (ORF2) is conserved within the genomic S segment encoding for a putative NSs protein (Plyusnin and Morzunov, 2001). In PUUV this ORF2 ranges from nucleotide positions

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83–352 of the genomic S segment encoding for a potential protein with 90 amino acids. However, the functional relevance of this ORF2 and its putative gene product are unknown.

In general, RNA virus polymerases lack proof-reading activities resulting in high mutation rates (Sironen et al., 2001). Thus, long term propagation of RNA viruses in vivo and in vitro lead to creation of genetic variants and heterogeneous virus populations. One important prerequisite for a detailed functional analysis of the different hantaviruses (e.g. virus-mediated pathogenesis) is the use of genetically homogenous and well characterized virus stocks. Since hantaviruses are non-cytolytic in vitro, purification of genetically homogenous strains by the classical plaque purification is not possible. Therefore, a novel purification procedure was established for cloning of non-cytolytic hantaviruses by adopting the classical plaque purification procedure in combination with immunodetection methods. This novel procedure was successfully applied for purification of three different hantavirus strains and to isolate a genetically homogenous ORF2-deficient PUUV variant from a mixed virus population.

2. Materials and methods

2.1. Viruses and cell culture

VeroE6 cells used for virus propagation and titration were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 IU penicillin, and 100 μg/ml streptomycin. For production of virus stocks about 1×10^7 VeroE6 cells were infected with HTNV, strain 76–118 (Lee et al., 1978), TULV, strain Moravia (Vapalahti et al., 1996), or PUUV, strain Sotkamo (Brummer-Korvenkontio et al., 1982). After incubation for 7–10 days the cell culture medium was collected, centrifuged at 2000 g, aliquoted, and stored at −80 °C. Virus stocks were shown to be free of mycoplasma contamination by using the VenorGeM mycoplasma detection kit (Minerva Biolabs, Berlin, Germany).

2.2. Determination of virus titers

Virus titers were quantified as described previously (Heider et al., 2001). Briefly, VeroE6 cells grown in 6-well plates were infected by different dilutions of HTNV, TULV, or PUUV for 1 h at 37 °C, fed with medium containing 0.5% agarose, and incubated for 7–10 days at 37 °C. Thereafter, the medium/agarose overlay was removed, and cells were washed with PBS and fixed with methanol for 10 min. TULV- and PUUV- or HTNV-infected cell foci were detected using polyclonal sera from rabbits immunized with recombinant N protein from TULV strain Malacky (Sibold et al., 1999) or HTNV strain Fojnica (Ražanskienė et al., 2004), respectively. The formed antigen–antibody complexes were visualized using Chemiluminescence super signal west dura according to the protocol supplied by the manufacturer (Pierce, Perbio, Bonn, Germany). The number of antigen-positive foci were counted to determine the corresponding virus titers which are expressed as focus forming units per milliliter (FFU).

2.3. Focus purification

The procedure of the focus purification is depicted in Fig. 1. For cloning virus-specific foci were detected as described in Sec-

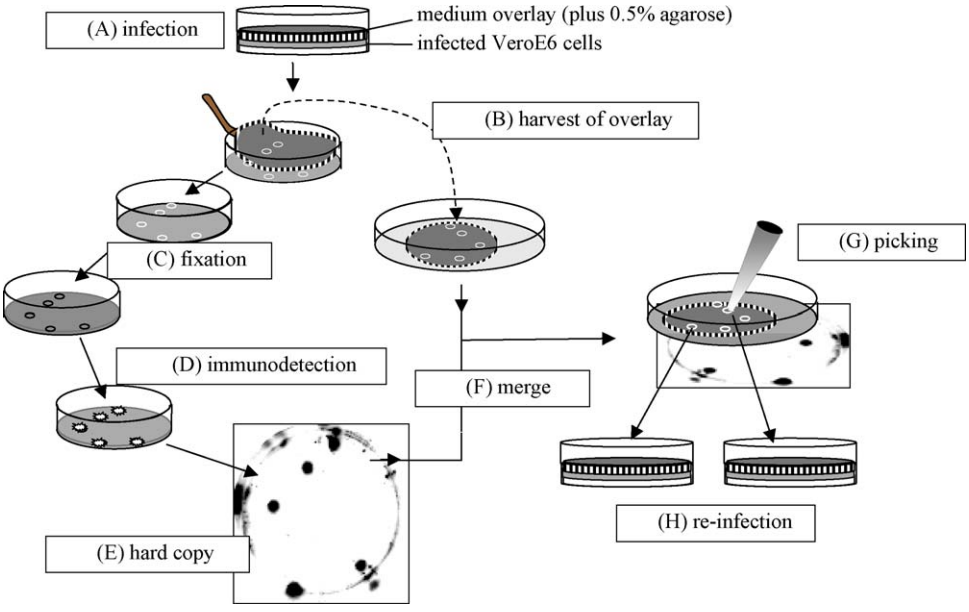

Fig. 1. Scheme of the procedure for focus purification of hantaviruses. (A) VeroE6 cells infected by different dilutions of hantaviruses were overlaid with an agarose/medium mix and incubated for 7–10 days. (B) The overlay was removed and stored for isolation of the virus diffused into it. (C) Cells were fixed with methanol. (D) Viral antigen-containing foci were detected via enhanced chemiluminescence (Pierce) with virus-specific antisera raised against recombinant HTNV or TULV nucleocapsid proteins. (E and F) The relative location of the detected foci was used as map to trap individual virus clones diffused into the corresponding region of the overlay. (G and H) Several clones were picked and each was used for re-infection on a separate plate. Three cycles of this procedure were performed for production of genetically homogenous virus stocks.