Expression of the Nucleoprotein of the Puumala Virus from the Recombinant Semliki Forest Virus Replicon: Characterization and Use as a Potential Diagnostic Tool

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Puumala virus (Bunyaviridae family, Hantavirus genus) causes a mild form of hemorrhagic fever with renal syndrome (HFRS) called nephropathia epidemica in northern and central Europe. Serological tests are used for diagnosis, but antigen production is difficult because the virus grows poorly in tissue culture. We expressed the N protein (nucleoprotein) of Puumala virus via the Semliki Forest virus (SFV) replicon in mammalian cells and compared its antigenic properties with those of the native antigen derived from Puumala virus-infected cells. Detection of immunoglobulin G or immunoglobulin M by enzyme-linked immunosorbent assay (ELISA), μ-capture ELISA, and indirect immunofluorescence assay was (at least) as effective with the recombinant antigen as with the native antigen when HFRS patient sera or organ washes from wild rodents were tested. No nonspecific reaction was observed. Thus, the SFV-expressed N protein of Puumala virus appears as a valid antigen, specific and sensitive for serological investigations.

Hantaviruses are members of the Bunyaviridae family and possess three single-stranded negative-sense RNA genome segments called L, M, and S (segments named for their size, i.e., large, medium, and small, respectively) (19, 25, 27). Hantavirus infections in humans are associated with severe and often fatal diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome. Hantaan, Seoul, Dobrava, and Puumala viruses are known to cause HFRS in Russia, Asia, and Europe (22), whereas Sin Nombre and related viruses cause hantavirus pulmonary syndrome in the Americas (for reviews, see references 25 and 28). Each group of hantaviruses has a different rodent reservoir: Hantaan, Dobrava, and Seoul viruses are transmitted by rodents of the Murinae subfamily, Sin Nombre is transmitted by Sigmodontinae, and Puumala, Tula, Topografov, and Khabarovsk viruses are transmitted by Arvicolinae. Puumala virus, carried by the bank vole Clethrionomys glareolus, causes a relatively mild form of nephropathia epidemica in northern and central Europe, particularly in Scandinavia and in the western parts of Russia (22, 23). Numerous cases have been reported in Belgium, Germany, Austria, and in the Champagne-Ardenne region of France (4, 7, 11, 12, 20; B. Le Guenno, M. A. Camprasse, J. C. Guilbaut, P. Lanoux, and B. Hoen, Letter, Lancet 343:114-115, 1994). Clinical manifestations are fever, conjunctival infections, thrombocytopenia, and transient renal failure.

Detection of the viral genome by reverse transcription-PCR (RT-PCR) in blood or urine samples has been done largely (1, 10, 13, 31) because isolation of the virus in tissue culture is rarely successful. A rapid test by real-time RT-PCR was recently developed for Puumala virus (8). A sensitive immunoblot assay can also be used for the detection of viral antigens in human specimens (17). Although these techniques are useful to assess viremia, serological tests based on the detection of specific antibodies are widely used for routine diagnosis. During the acute phase of infection, the immunoglobulin M (IgM) level rises, followed by the production of IgG; the early antibody response is induced by nucleoprotein N, the major antigen (6, 18, 34, 40). Serological assays are based on viral antigens expressed in infected cells. However, massive production of viral proteins is rarely observed because the virus grows poorly in tissue culture. In addition, some hantaviruses must be manipulated in a high-security containment facility. Therefore, several laboratories have expressed the N protein as a recombinant protein in Escherichia coli (6, 9, 24, 37) or in insect cells (5, 29, 30, 33, 36).

In this study, we expressed the N protein of Puumala virus in mammalian cells via the Semliki Forest virus (SFV) replicon and compared its antigenic properties with those of the native antigen extracted from Puumala virus-infected cells. The recombinant antigen worked as well, or even better, than the native antigen, in the detection of IgM and IgG in patient sera by indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA). It was also used to analyze sera or lung and kidney washes from wild bank voles and found very efficient for all these serological investigations.

MATERIALS AND METHODS

Cells. BHK-21 cells were grown in Glasgow minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS), 10% tryptose phosphate, and 10 mM HEPES. BSR cells (a clone of BHK-21) were cultured in Glasgow MEM.
supplemented with 10% FCS, and Vero E6 cells were grown in Dulbecco modified Eagle medium supplemented with 5% FCS. Penicillin (5 U/ml) and streptomycin (5 μg/ml) were added. The cells were incubated at 37°C in a 5% CO₂ atmosphere.

**Virus and native antigen for ELISA.** Stocks of Puumala virus (strain Cg 13891) were produced by infecting semiconfluent Vero E6 cells at a low multiplicity of infection (MOI) of 10⁻² to 10⁻¹. To produce Puuma virus antigen for ELISA, Vero E6 cells were infected and incubated for approximately 2 weeks. The infected Vero E6 cells were trypsinized and mixed with an equal number of fresh cells, which were then used to seed new flasks. When 80% of the cells gave positive results in an IFA, which usually required three passages, the cells were collected, washed with borate buffer (50 mM sodium borate [pH 9.0]), 120 mM NaCl, and centrifuged at a low speed. The cell pellet was suspended in 10 volumes of borate buffer containing 1% Triton X-100. Cell lysis was achieved by sonication (four 30-s pulses) at maximal power (300 W). Cell debris was centrifuged at 6,000 × g, and the supernatant was frozen at −80°C.

**Serum panels.** An anti-Puumala virus hyperimmune serum was prepared by injecting hamsters intramuscularly with virus inactivated by β-propiolactone. The animals received one or two booster injections at 2-week intervals. Serum was collected 4 to 6 weeks postinfection by cardiac puncture.

Human sera from HFRS patients (mostly from the Champagne-Ardennes and Franche-Comté areas of France) were from the serum collection of the Centre National de Référence des Hantavirus (Centre National de Référence des Hantavirus, Arbovirus and Lymphocytic Choriomeningitis Virus Research, INRA, Montpellier, France) and kindly provided by L. Kaâraïnem. Serum panels were produced by infecting semiconfluent Vero E6 cells at a low multiplicity of infection (MOI) of 10⁻² to 10⁻¹. To produce Puuma virus antigen for ELISA, Vero E6 cells were infected and incubated for approximately 2 weeks. The infected Vero E6 cells were trypsinized and mixed with an equal number of fresh cells, which were then used to seed new flasks. When 80% of the cells gave positive results in an IFA, which usually required three passages, the cells were collected, washed with borate buffer (50 mM sodium borate [pH 9.0]), 120 mM NaCl, and centrifuged at a low speed. The cell pellet was suspended in 10 volumes of borate buffer containing 1% Triton X-100. Cell lysis was achieved by sonication (four 30-s pulses) at maximal power (300 W). Cell debris was centrifuged at 6,000 × g, and the supernatant was frozen at −80°C.

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**Serum panels.** An anti-Puumala virus hyperimmune serum was prepared by injecting hamsters intramuscularly with virus inactivated by β-propiolactone. The animals received one or two booster injections at 2-week intervals. Serum was collected 4 to 6 weeks postinfection by cardiac puncture.

**RESULTS**

Production of SFV-N virus and characterization of the recombinant N protein. Plasmid pSFV-Npuu was constructed by inserting the entire open reading frame of the Puuma virus N protein into pSFV1 at the unique BamHI site. Four positive clones were analyzed, and the orientation of the insert was determined by sequencing. We noted three nucleotide changes (A→C at position 59, A→G at position 140, and C→T at position 758) with regard to the published sequence (7) (GenBank accession number U22423). Three of these nucleotide changes modify the amino acids as follows: Asp→Ala, Asp→Gly, and Ser→Phe, respectively. The changes at positions 140 and 758 were found in all four clones sequenced, suggesting that they were not due to a PCR error. The difference observed at position 59 might represent a heterogeneous viral population (21). Plasmid pSFV-Npuu containing the insert in the antisense orientation was selected and used as a negative control. RNA synthesized in vitro from the DNA plasmid template was electroporated into BHK-21 cells together with the helper RNA derived from pSFV-H2, enabling production of suicide virus (14). These infectious particles enter the cell and replicate but cannot generate new virions due to the absence of genes coding for the structural proteins in the replicon. The expression of N protein was characterized in BSR cells by Western blotting and IFA. The N protein was revealed by using hamster serum raised against Puuma virus. Extracts of Vero E6 cells infected with Puuma or Hantaan virus were loaded in the gel as controls. The recombinant protein migrated as a single band to the same position as the viral nucleoprotein of Puuma virus, and Hantaan virus nucleoprotein migrated slightly faster (Fig. 1). As expected, no band was visible in noninfected (mock-infected) control cells (Fig. 1), in cells infected with the control replicon (lane SFV-1), or in the cells infected with SFV-Npuu anti virus (not shown).

Expression of the recombinant nucleoprotein was also revealed by IFA, using the hamster serum raised against Puuma virus (Fig. 2). The staining was found only in the
cytoplasm and exhibited an organized pattern like the one observed in Puumala virus infection. No reaction was detected in cells infected with the SFV-Npuu anti control virus or SFV-1.

Detection of antibodies against Puumala virus nucleoprotein in human sera. To test the reactivity of the N protein for diagnosis or serological surveys, a panel of 88 positive and 57 negative human sera was analyzed using the recombinant N in an ELISA. Of these sera, 47 positive and 11 negative sera were tested by IFA. All of the 47 sera from HFRS patients gave positive results with the native and recombinant antigens at the usual 1:64 dilution (Table 1). The 11 sera from HFRS-negative patients showed no reaction with either native or recombinant antigen, as expected. Six HFRS-positive sera were titrated. The titer, expressed as the highest reciprocal dilution giving a positive response, was similar with both antigens for all of the sera, except one for which the titer with the recombinant antigen was lower than that with the native antigen (256 with recombinant nucleoprotein compared to 1,024 with native antigen). This difference might be due to the contribution of the antibodies to the glycoproteins, which could be titrated only with the native antigen.

Detection of IgM by ELISA. Detection of anti-Puumala virus IgM was performed by an immunocapture test specific for the μ chains. All sera were analyzed at a dilution of 1:100. Eighty-six serum samples were positive for IgM with the two antigens (Table 2), and for most of these samples, the ODs were higher with the recombinant N than with the native antigen. Among these samples, one contained only IgM and no IgG, indicating an early stage of infection. In addition, 57 sera negative with the native antigen were also negative with the recombinant antigen. The IgM titer was determined for 4 sera, but only one titration curve is shown (Fig. 3) because the curves were similar. The titers (defined as the last reciprocal dilution giving an OD higher than the background) were consistently found to be at least twofold higher with the recombinant N than with the native antigen. In this expression system, the SFV non-structural proteins are present in the cell extracts, all the sera were tested simultaneously against extracts from cells infected by the control virus, SFV-Npuu anti containing the insert in the antisense orientation. As expected, antibodies directed against these proteins were not observed.

Detection of IgG by ELISA. The sera were analyzed at 1:100 dilution. The panel analyzed comprised 87 sera, 2 of which contained only IgG and no IgM (Table 2). All these sera were found positive with the recombinant nucleoprotein, and in most cases, the intensity of the reaction was higher with the recombinant nucleoprotein than with the native antigen. The
IgG titers determined for five positive sera were found 2 to 4 dilutions higher with the recombinant N than with the native antigen (Fig. 3). The 57 sera of patients negative for specific IgG (and IgM) were likewise negative with the recombinant nucleoprotein. Immunoglobulin G antibodies against SFV nonstructural proteins were not detected in any of the sera tested.

Detection of anti-Puumala virus antibodies in wild rodent samples. Since the bank vole Clethrionomys glareolus is the recognized reservoir of Puumala virus, circulation of the virus can be investigated by capture of these rodents and detection of specific IgG antibodies. Therefore, 47 samples (serum samples or lung or kidney washes) obtained from bank voles trapped during field studies were analyzed by IFA and ELISA for IgG, using both the native and recombinant antigens.

All the samples gave positive results (had anti-Puumula virus antibodies) when native antigen was used in an IFA, but the recombinant antigen failed to detect three samples that had anti-Puumula virus antibodies (Table 3). However, in an ELISA, these three samples were positive with both antigens and two other samples were negative with the native antigen but positive with the recombinant nucleoprotein (Table 3). Again, the ODs obtained with the recombinant antigen were identical or higher than those obtained with the native antigen. This was clearly shown when a pool of samples with anti-Puumula virus antibodies was titrated, leading to a higher titer when the recombinant antigen was used (Fig. 4). None of the 17 samples lacking anti-Puumula virus antibodies reacted with the recombinant N in ELISA and IFA.

**TABLE 1.** Comparison of the abilities of Puumala virus recombinant and native nucleoprotein antigens to detect HFRS antibodies in an IFA

| Patients      | No. of samplesa | rN Positiveb | rN Negative | nAg Positiveb | nAg Negative |
|---------------|-----------------|--------------|-------------|---------------|--------------|
| HFSR positive | 47              | 0            | 47          | 0             |
| HFSR negative | 0               | 11           | 0           | 11            |

a Number of samples that gave positive or negative results with recombinant nucleoprotein (rN) or native antigen (nAg). The same samples were used to test the recombinant and native antigens.

b Positive at the 1:64 dilution.

**TABLE 2.** Comparison of the abilities of Puumala virus recombinant and native nucleoprotein antigens to detect IgM and IgG antibodies in human sera in an ELISA

| Patients | IgM IgG | No. of samplesa | rN Positiveb | rN Negative | nAg Positiveb | nAg Negative |
|----------|---------|-----------------|--------------|-------------|---------------|--------------|
| HFSR positive | + − | 1 0 | 1 0 |
|           | + + | 85 0 | 85 0 |
|           | − + | 2 0 | 2 0 |
| HFSR negative | − − | 0 57 | 0 57 |

a Number of samples that gave positive or negative results with recombinant nucleoprotein (rN) or native antigen (nAg). The same samples were used to test the recombinant and native antigens.

b Positive at the 1:100 dilution.

DISCUSSION

Puumala virus circulates in northern Europe, where it causes thousands of cases of a mild form of HFRS each year. We expressed the nucleoprotein of Puumala virus via the SFV replicon in mammalian cells and compared it to the native antigen in IFA and ELISA for the ability to detect IgG and IgM antibodies in serum samples from patients affected by HFRS or in samples from bank voles. The recombinant antigen appeared to be very sensitive and specific and did not yield any false-positive results. The response to recombinant antigen was often higher than the response to the native antigen. In addition, the two methods, IFA and ELISA, used with the recombinant nucleoprotein appeared to be very efficient; IFA was possibly slightly less sensitive than ELISA, considering that 3 of 47 positive samples from wild rodents were not detected. ELISA using the recombinant protein is highly sensitive: the recombinant (but not the native) protein revealed the presence of HFRS-specific IgG antibodies in two rodents positive by
IIFA with both antigens. The high sensitivity of the recombinant antigen is probably due to the large amounts of proteins expressed by the SFV vector and to the fact that in contrast to the native antigen, it was prepared without detergent. Initially, the recombinant antigen was prepared like the native one, i.e., in a buffer containing Triton X-100, a method used to solubilize membranes and inactivate the virus (16, 18). The responses obtained with the detergent-treated recombinant nucleoprotein were lower, and some samples that were weakly positive with the native antigen were found negative. Therefore, the method used to prepare the antigens appeared to be of special importance.

In Europe, Puumala virus is a frequent source of infection, but Dobrava and Seoul viruses are also possible pathogens (39; Ahn, C., J. T. Cho, J. G. Lee, C. S. Lim, Y. Y. Kim, J. S. Han, S. Kim, and J. S. Lee. 1997). It would be interesting to assess the cross-reactivity of the recombinant Puumala N protein with sera from patients infected by other serotypes. Because of the relatively high degree of cross-reactivity between Puumala, Tula, and Topogravov viruses (32, 35), it seems likely that the recombinant Puumala virus antigen could be useful for the diagnosis or seroepidemiology of these viruses.

Hantavirus recombinant nucleoproteins reported so far were produced in E. coli (6, 9, 24, 37), lepidopteran cells, or Drosophila cells (5, 29, 30, 33, 36). The establishment of mammalian cell lines stably expressing the hantavirus nucleoprotein was reported recently (38), but the cell lines were not used in serological tests. It would be of great interest to compare the results obtained with these different antigens, including the nucleoprotein expressed via the SFV replicon, but unfortunately, these antigens were not available in our laboratory. Data published by others revealed that antigens expressed in bacteria suffer from low sensitivities and specificities (6, 36). By developing the SFV-based recombinant antigen, we chose a system enabling expression in mammalian cells rather than in insect cells because of the lower background of most of the sera when we used mammalian cell extracts rather than SF9 cell extracts as a negative control in ELISA (26). Also, in terms of biohazard, the SFV replicon is of special interest, since it is a noninfectious system which produces a suicide virus incapable of propagating; therefore, manipulation requires a classical (non-high-security) laboratory. In addition, it produced large amounts of the recombinant nucleoprotein: for instance, coating the wells with the recombinant antigen required two- to eightfold-smaller quantities of cell extracts than with the native antigen. Production of the recombinant antigen is more rapid than the native antigen (24 h versus approximately 3 weeks). Moreover, purification of the protein is not required, since cells infected with the SFV without the insert serve as a negative control. Reaction with the control antigen would also indicate the possible presence of antibodies against the non-structural proteins if the individual had been infected with SFV, a situation that we did not encounter with European samples but which could occur with African ones.

Altogether, our results indicate that the Puumala virus nucleoprotein expressed in the SFV replicon is a good antigen and a valid diagnostic tool for IFA and ELISA for the detection of IgM and IgG in sera from humans or rodents infected by Puumala virus.

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