SEROLOGICAL CHARACTERIZATION OF A HANTAVIRUS FROM HUBEI, CHINA

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Received September 2, 2003; accepted January 22, 2004

Summary. – Hantavirus HV114, isolated from urine of a patient during epidemic of hemorrhagic fever with renal syndrome (HFRS) in China, was subjected to a detailed serological characterization using enzyme-linked immunosorbent assay (ELISA), neutralization test and indirect immunofluorescence antibody assay (IFA). It has been found that HV114 is antigenically similar to the hantavirus A9 strain isolated in China and to the Hantaan 76-118 virus (HTNV 76-118), but different from the hantaviruses isolated from *Apodemus agrarius* in the region endemic for HFRS.

Key words: hantavirus; HV114 virus; serology; ELISA; neutralization test; immunofluorescent assay

Introduction

Viruses of the genus *Hantavirus*, family *Bunyaviridae,* cause HFRS, also known as epidemic hemorrhagic fever (EHF) in China, Korean hemorrhagic fever (KHF) in Korea, nephropathia epidemica in northern Europe, and hantavirus pulmonary syndrome (HPS) in America. The genus *Hantavirus* contains several species: *Hantaan virus* (HTNV, type species contains 5 viruses: CUMC-B11, Hantaan 76-118 virus, Mojo virus, HV114 virus, and Lee virus), *Dobrava-Belgrade virus* (contains only a single virus, Dobrava-Belgrade virus (DOBV)), *Prospect Hill virus* (contains only a single virus, Prospect Hill virus (PHV)), *Puumala virus* (contains 10 viruses), *Seoul virus* (contains 9 viruses), and *Sin Nombre virus* (contains 6 viruses). R22 virus, K27 virus, and DOBV have been found to be associated with HFRS. Both HTNV 76-118 virus and R22 virus occur in Asia, while K27 virus is one of several viruses endemic in Europe and Asia; DOBV circulates mainly in eastern Europe. In China, HFRS is an endemic disease with annual incidence of 50,000–100,000 cases (Song, 1999). Furthermore, viruses antigenically related to HTNV 76-118 and R22 virus have been found co-circulating in many areas endemic for HFRS (Zhang *et al.*, 1996; Wang *et al.*, 2000). The Hubei Province is one of the areas in China with severe HFRS. Although hundreds of virus isolates of hantaviruses from the Hubei Province have been isolated, they have not been characterized yet. The pathogenesis of HFRS is still not completely understood (Ulrich *et al.*, 2002; Khan and Khan, 2003). In order to progress in this direction and to design more efficient disease control measures, it is necessary to explore in detail (i) the etiological agent of HFRS and (ii) its interactions with other viruses and host cells and (iii) with the host immune system.

Since the first isolation of the prototype virus of KHF (HTNV 76-118) in Korea in 1978 (Lee *et al.*, 1978), a number of isolates have been identified in China. We isolated a virus designated HV114 from the urine of a HFRS patient in acute phase of the disease, characteristic with a primary renal

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Abbreviations: DOBV = Dobrava-Belgrade virus; EHF = epidemic hemorrhagic fever; ELISA = enzyme-linked immunosorbent assay; ER = endoplasmic reticulum; HFRS = hemorrhagic fever with renal syndrome; HPS = hantavirus pulmonary syndrome; HTNV = Hantaan virus; IFA = indirect immunofluorescence antibody assay; KHF = Korean hemorrhagic fever; MAb(s) = monoclonal antibody(ies); PHV = Prospect-Hill virus

damage. Isolation of the virus from urine indicated that it infected kidneys, from which it was shed into urine.

The studies on the M genome segment of HV114 by Xiao *et al.* (1993) have revealed interesting facts inclusive of some differences between this and other hantaviruses.

Here we report new data on serological characterization of HV114, which will hopefully contribute to the understanding of pathogenesis of HFRS.

Materials and Methods

Cells and viruses. Vero E6 cells and the viruses HTNV 76-118, K27, R22, and PHV were provided by Prof. G. Song, Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, China. The Hubei-1 isolate was obtained from the blood of a HFRS patient in China (Liang *et al*., 1994).

Specimens. The first morning urine specimens from six HFRS patients were collected and kept at -20°C. Before inoculation, the specimens were treated with 4,000 U/ml penicillin and 4,000 µg/ml streptomycin for 24 hrs. Their pH was then adjusted to 7.2–7.5. Positive sera were collected from different endemic areas in the Hubei Province 4–6 weeks after the onset of the illness diagnosed clinically as HFRS and kept at -20°C

Monoclonal antibodies (MAbs) and immune sera. The following MAb clones were supplied by Dr. J.W. Huggins, the U.S. Army Medical Research Institute for Infectious Diseases (USAM RIID), Frederick, USA: HF-25-1 directed against the nucleocapsid protein of the A9 strain; ECO2, BDO1 and FDO3 directed against the nucleocapsid protein of HTNV 76-118; HCO2 directed against the G2a glycoprotein of HTNV 76-118; EBO6 directed against the G2b glycoprotein of HTNV 76-118; GDO5 directed against the G2f glycoprotein of HTNV 76-118, and EDO4 directed against the G2g glycoprotein of HTNV 76-118. To prepare antisera against the A9 strain, New Zealand white rabbits were inoculated intramuscularly with 1 ml of stock virus and were bled after 30 days.

ELISA was performed according to Yoshimatsu *et al.* (1996). Pooled MAbs against HTNV 76-118 and A9 (100 µl per well) were incubated in 96-well microtiter plates overnight at 4°C. After threefold washing with phosphate-buffered saline pH 7.4 (PBS), a lysate from cells infected with HV114 (100 µl per well) was added and the plates were incubated at 37°C for 2 hrs and washed three times with PBS. Then rabbit antisera were added and the plates were incubated at 37°C for 1 hr, washed three times with PBS and a biotinylated goat anti-rabbit antibody conjugated to horseradish peroxidase (1:250 dilution, 100 µl per well) was added. The plates were incubated at 37°C for 1 hr and washed seven times with PBS. Hundred µl of a substrate solution (33 ml of 0.1 mol/l citric acid, 66 ml of 0.1 mol/l Na_2HPO_4 and 40 mg of tetramethylbenzidine) and 1.5 ml of 30% hydrogen peroxide was added per well and the plates were incubated at 37°C for 15 mins in dark. The reaction was terminated with 2N H_2SO_4 and A_{450} was read

IFA was performed according to Yoshimatsu *et al.* (1993). After inactivation at 56°C for 30 mins 15 µl per well of selected sera diluted 1:20 with PBS was added to 10-well slides with different viral antigen smears. The slides were incubated at 37°C for 45 mins in wet and were washed twice with PBS. Then 15 µl of a goat anti-human FITC IgG (Chemicon, USA) (the dilution containing 8 U of FITC and Evans Blue diluted 1:60,000 with PBS) was added per well. The slides were incubated at 37°C for 30 mins in wet and were washed twice with PBS and one time with double distilled H_2O. Dried by blowing, the slides were sealed with glycerine-PBS pH 9.0 (9:1). Detected by a fluorescent microscope (Olympus BH-2, Japan), yellow-green grains were regarded as positive for antigen.

Neutralization test. A focus-reduction neutralization test was carried out as described by Tanishita *et al.* (1984). Briefly, rabbit A9 antisera diluted serially were mixed with A9 and HV114 (200 PFU/ml), incubated at 37°C for 1 hr and added to wells of six-well tissue culture plates containing confluent Vero E6 cell monolayers. The wells were overlaid with a mixture of agarose and tissue culture medium and incubated for 7–13 days. The overlay was removed, the cells were fixed and stained with Neutral Red and the foci were enumerated. An 80% reduction of the number of foci, as compared to the virus control, was considered the end point of virus neutralization titer of the antiserum tested.

Results

Virus propagation in Vero E6 cells

When HV114 was propagated in Vero E6 cells, the concentration of viral antigen increased with the propagation time. As demonstrated by ELISA, the A_{450} values were 0.79 ± 0.28, 1.21 ± 0.37 and 1.89 ± 0.42 at days 20, 40 and 60 p.i., respectively. When reacted with convalescent sera from eight HFRS patients in ELISA, the antibody titer against the HV114 antigen increased more than 4-fold compared to the titers of acute phase sera (titers from 200 to >12,800). No reaction was obtained with control sera.

The antigenicity of HV114 in comparison with selected hantaviruses, their strains or isolates was determined by IFA and neutralization test (Tables 1–3). The IFA showed (Table 1) that HV114, HTNV 76-118, K27 and R22 are closely related. They reacted with the sera Nos. 1, 2, 3, and 4 with similarly high titers (5,120–10,240) except the serum No. 2 with K27 (160), the serum No. 3 with HTNV 76-118 (80), and the serum No. 3 with HV114 (2560), which all gave low titers. On the other hand, the sera Nos. 5–10 gave low or medium titers with the abovementioned viruses except the serum No. 5 with HTNV 76-118. Based on these results, the sera could be divided into 2 groups, one consisting of sera Nos. 1–4 and another consisting of sera Nos. 5–10.

The antigenic relationship between HV114 and A9 was investigated by IFA and neutralization test (Table 2). As HV114 reacted with most antisera against A9 the two viruses were considered closely related.

The antigenic relationship between HV114, HTNV 776-118 and Hubei-1 was studied by IFA using several MAbs (Table 3). HTNV 76-118 reacted with all the MAbs. All the

Table 1. The reactivity of selected hantaviruses with convalescent sera from HFRS patients from Hubei, China in IFA

Serum No.	HV114	HTNV 76-118	K27	R22V	PHV
1	10,240	10,240	10,240	10,240	1,280
2	5,120	10,240	10,240	160	640
3	10,240	80	10,240	10,240	320
4	2,560	10,240	10,240	10,240	1,280
5	2,560	10,240	160	>40	>40
6	1,280	640	1,280	2,560	>40
7	640	80	2,560	>40	80
8	40	320	80	160	>40
9	>40	>40	1280	>40	>40
10	>40	>40	>40	>40	>40

Table 2. Antigenic relationship between HV114 and A9 as determined by IFA and neutralization test with rabbit antisera raised against A9

Antiserum No.	IFA		NT	
	A9	HV114	A9	HV114
	Titer of antibodies			
1	5,120	5,120	1,024	1,024
2	>5,120	>5,120	1,024	1,024
3	>5,120	>5,120	512	512
4	640	320	128	128
5	640	320	256	128

NT = neutralization test.

three viruses reacted with MAbs HF-25-1, BDO1 and ECO2, while HV114 and Hubei-1 did not react with MAbs EBO6, EDO4 and FDO3.

Comparing the reactivity of HV114, Hubei-1 and HTNV 76-118 we concluded that the antigenicities of nucleocapsid proteins of the three viruses and the antigenicities of G2 glycoproteins of HV114 and Hubei-1 or HTNV 76-118 were similar in certain properties but different in others.

Table 3. The reactivity of HV114, Hubei-1 and HTN 76-118 with selected MAbs in IFA

MAb	HV114	Hubei-1	HTNV 76-118
HCO2	+	–	+
EBO6	–	–	+
GDO5	+	–	+
EDO4	–	–	+
ECO2	+	+	+
BDO1	+	+	+
FDO3	–	–	+
HF-25-1	+	+	+

Discussion

The results of antigenic characterization of HV114 suggest that this virus belongs to hantaviruses and has a close relationship to HTNV 76-118, K27 and R22. In addition, it also indicates that there might be at least two different hantaviruses circulating in the Hubei Province, China. Whereas one induces a high titer antibody, the other induces a low titer antibody. HV114 may belong to the former. Since some hantaviruses have been isolated from both *Apodemus agrarius* and *Ratus novegicus* in Wuhan, the Hubei Province, these have close association with their rodent hosts (Schmaljohn and Hjelle, 1997). The patient that was the source of HV114 was probably infected with a wild virus that usually induces a high titer antibody. Furthermore, the fact that some patient sera from the Hubei Province reacted with different pathogenic hantaviruses like HTNV 76-118, R22 and K27 suggests that a complex variation of the hantaviral antigen may have taken place in the Hubei Province.

The comparison of HV114, A9 and HTNV 76-118 revealed that HV114 is antigenically similar to the two viruses. On the other hand, there were some differences between HV114, the virus isolated from the urine of an HFRS patient, Hubei-1, the isolate from the blood of an HFRS patient, and HTNV76-118, the virus isolated from a rodent. Whether the origin of HV114 and its transmission from rodents to humans was associated with changes in antigenicity and pathogenesis needs further study.

Acknowledgment. We thank Prof. Dr. R.M. Shah, Faculty of Dentistry, University of British Columbia, and Prof. Dr. W.R. Bell, Division of Hematology, The John Hopkins University School of Medicine for their help in preparation of the manuscript. This study was supported by the National Natural Science Foundation of China (No. 39870659).

References

Khan A, Khan AS (2003): Hantaviruses: a tale of two hemispheres. *Panminerva Med.* **45**, 43–51.

Lee HW, Lee PW, Johnson KM (1978): Isolation of the etiologic agent of Korean Hemorrhagic fever. *J. Infect. Dis.* **137**, 298–308.

Liang M, Li D, Xiao SY, Hang C, Rossi CA, Schmaljohn CS (1994): Antigenic and molecular characterization of hantaviruses isolates from China. *Virus Res.* **31**, 219–233.

Schmaljohn C, Hjelle B (1997): Hantaviruses – a global disease problem. *Emerg. Infect. Dis.* **3**, 95–104.

Song G (1999): Epidemiological progresses of hemorrhagic fever with renal syndrome in China. *Chin. Med. J.* **112**, 472–477.

Tanishita O, Takahashi Y, Yamanishi K, Takahashi M (1984): Evaluation of focus reduction neutralization test with peroxidase-antiperoxidase staining technique for hemorrhagic fever with renal syndrome virus. *J. Clin. Microbiol.* **20**, 1213–1215.

Ulrich R, Hjelle B, Pitra C, Kruger DH (2002): Emerging viruses: the case 'hantavirus'. *Intervirology* **45**, 318–327.

Wang H, Yoshimatsu K, Ebihara H, Ogino M, Araki K, Kariwa H, Wang ZX, Luo ZZ, Li DX, Hang CS, Arikawa J (2000): Genetic diversity of hantaviruses isolated in China and characterization of novel hantaviruses isolated from *Niviventer confucianus* and *Rattus rattus. Virology* **278**, 332–345.

Xiao SY, Liang MF, Schmaljohn CS (1993): Molecular and antigenic characterization of HV114, a hantavirus isolated from a patient with haemorrhagic fever with renal syndrome in China. *J. Gen. Virol.* **74**, 1657–1659.

Yoshimatsu K, Arikawa J, Kariwa H (1993): Application of a recombinant baculovirus expressing hantavirus nucleocapsid protein as a diagnostic antigen in IFA test: cross reactivities among 3 serotypes of hantavirus which cause hemorrhagic fever with renal syndrome (HFRS). *J. Vet. Med. Sci.* **55**, 1047–1050.

Yoshimatsu K, Arikawa J, Tamura M (1996): Yoshida R, Lundkvist A, Niklasson B, Kariwa H, Azuma I, Characterization of the nucleocapsid protein of Hantaan virus strain 76-118 using monoclonal antibodies. *J. Gen. Virol.* **77**, 695–704.

Zhang ZR, Zhao HR, Li Q (1996): Study on the geographic distribution and serological typing of HFRS in Hebei Province. *Zhonghua Liu Xing Bing Xue Za Zhi* **17**, 87–90.