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RRH: HANTAVIRUS AND LIVER DYSFUNCTION

LRH: LLEDO AND OTHERS

Title:

PREVALENCE OF ANTI-HANTAVIRUS ANTIBODIES IN PATIENTS WITH
HYPERTRANSAMINEMIA IN MADRID (SPAIN)

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ABSTRACT

The hantaviruses are involved in a number of clinical syndromes of different severity and prognosis. Hantaviruses are widely distributed around the world, but the spectrum of illnesses they cause outside recognized endemic areas is unclear. A retrospective analysis was performed to detect anti-hantavirus antibodies in the serum of patients with hypertransaminemia of unknown etiology, and in that of healthy members of the general population of Madrid (Spain). Antibodies were detected by indirect immunofluorescence and enzyme immunoassay; positive results were confirmed by Western blotting. Of the 182 patients with hypertransaminemia, 11 (6%) were positive for anti-hantavirus IgG antibodies; Western blotting using recombinant Puumala virus N antigen showed one of these patients to have hantavirus-specific IgM antibodies. Among the 146 healthy subjects from the general population, three (2%) were positive for anti-hantavirus IgG antibodies. These results show that anti-hantavirus antibodies are more commonly detected in patients with hypertransaminemia than in healthy people.

KEY WORDS: Hantavirus, hypertransaminemia, liver dysfunction, prevalence, Spain.

INTRODUCTION

Hantaviruses belong to the genus Hantavirus (family *Bunyaviridae*). Some hantaviruses cause severe human disease, including hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), while others are non-pathogenic¹. HFRS is endemic in Euroasia and HPS in North and South America. An association exists between disease severity and the type of infecting virus. In areas that are non-endemic for these infections, information on the agents involved and the diseases they may cause is scarce. In such areas, the clinical manifestations of infection may be atypical².

Two hantaviruses, Puumala virus (PUUV) and Dobrava virus (DOBV), have been shown to cause thousands of cases of HFRS every year in Europe³. More recently, Saaremaa virus (SAAV) has been identified as another possible cause of this disease^{4,5}. There are also indications that still unknown hantaviruses are circulating in Europe⁶. In Spain, information on the infections caused by these viruses is limited to the results of epidemiological studies involving the general population and employment-associated risk groups^{7,8,9,10}. The clinical significance of hantavirus infection is also unclear. Two studies involving very small numbers of patients with symptoms compatible with hantavirus infection showed those with liver disease to most commonly possess anti-hantavirus antibodies^{7,9}. There have only ever been two reported cases of imported disease^{11,12}. Our knowledge of animal reservoirs for hantaviruses in Spain is also limited, although seven rodent species carrying specific antibodies have been identified (Gegúndez MI and others, unpublished data). The number of ecological/rodent surveys needs to be increased; this would help to provide a better picture of the epidemiology of hantavirus infection in Spain.

Although the infection rates detected in the above Spanish studies were low, the detection of antibodies indicates hantaviruses are present (perhaps a presently unknown serotype). It is therefore possible that sporadic infection may go undiagnosed, especially if the clinical manifestations are atypical².

The aim of this work was to improve our knowledge of hantavirus infection in Spain in patients with sentinel disease. The seroprevalence of anti-hantavirus antibodies in patients with hepatic dysfunction, identified by high transaminase levels, was measured and compared to that seen in healthy subjects.

PATIENTS AND METHODS

Serum specimens: Serum was collected from 182 patients (122 males, 60 females; age range 1--81 years, mean 40.7 years, standard deviation [SD] 17.14 years) with hypertransaminemia: serum aspartate aminotransferase (AST) >38 U/l and/or alanine aminotransferase (ALT) >35 U/l (see Table 1). Patients with known hepatic viral infections, who were alcoholic, or who were undergoing treatment with hepatotoxic drugs, were excluded. The selected patients were under clinical study for different reasons: 99 for known hypertransaminemia, three for acute hepatitis, six for fever, 13 for chronic liver disease, two for thrombocytopenia, and three for fatigue and weakness. The rest had come for a routine check-up of their health. Table 2 shows the analytical abnormalities found in this group of patients.

The serum of 146 people from the general population (70 males, 76 females; age range 2--89 years, mean 41.3 years, SD 18.88 years - population-based calculations were performed according to the National Institute of Statistics) with normal ALT and AST values was also examined (control group). These subjects came from the same geographical region as the patients (Fig. 1) (Health Area 3 of the Madrid Autonomous

Region, which is also home to the Príncipe de Asturias University Hospital). The occupation of the patients and controls, their area of residence, travel history, their history of contact with rodents, and medical history, were recorded.

All serum samples were maintained at -20°C until analysis. All subjects gave their informed consent to be included in the study, in compliance with the ethical standards of the Human Experimentation Committee of the University of Alcalá de Henares and the Helsinki Declaration of 1964 (as revised in 2004).

Methods

Indirect immunofluorescence assay (IFA). Sample sera were assayed as described by Lee HW and other¹³. Puumala virus (PUUV; strain Cg18/20) and Seoul virus (SEOV; strain 80/39) were used as antigens. These were propagated in VeroE6 cells (ATCC CRL 1586) and fixed on spot slides. The fluorescein-labeled conjugate used was a rabbit anti-human IgG serum (Sigma, St Louis, MO), diluted 1/128 in PBS containing Evan's blue. Sera showing a typical pattern of fluorescence at titers $\geq 1/32$ were considered positive. Spots of uninfected Vero E6 cells were used to provide negative control antigens.

IgG Enzyme immunoassay (EIA): Anti-hantavirus IgG antibodies were detected using the EIA IgG kit (Focus, Cypress; CA), following the manufacturer's instructions (serum samples diluted 1:400). The antigens used were the recombinant N protein of Seoul (SEOV) and Sin Nombre virus (SNV).

Western blot analysis: Serum samples with IgG titers of $\geq 1:32$ (as determined by IFA), and/or showing IgG reactivity in EIA, were analyzed by Western blotting (WB) to detect anti-hantavirus IgG and IgM antibodies. Blotting was performed exactly as described by Hjelle B and others¹⁴. The recombinant N proteins of PUUV, SNV, SEOV and Dobrava virus (DOBV) were expressed using the pET23b vector (Novagen, Madison, WI) in

Escherichia coli and purified in a metal chelation column using a C-terminal polyhistidine moiety, as described by Bharadwaj M and others¹⁵.

Positive and negative control sera (gifts from European Network for Diagnostics of Imported Viral diseases ENIVD) were also examined.

Statistical analyses. The differences in proportions in 2-way tables were analyzed using the χ^2 or Fisher exact test. Significance was set at < 0.05 .

RESULTS

Eleven patients with hypertransaminemia (6%; 2 females [seroprevalence 3.33%], and 9 males [seroprevalence 7.37%]) had anti-hantavirus IgG antibodies. Seropositive subjects were aged between 1 and 64 years (mean 37 years, SD 19 years). Table 3 shows the clinical laboratory results for all seropositive patients (four patients had come to the clinic due to known hypertransaminemia, one for thrombocytopenia, and six for a routine check-up). One year later, eight patients saw their transaminase levels normalize and one continued with high levels of ALT; two patients did not keep further appointments and were lost to analysis. Only one sample was confirmed positive for IgM antibodies against PUUV recombinant N protein (Fig 2).

One of the seropositive patients was an immigrant; the rest had no history of international travel. None referred to contact with rodents. The jobs of only two positive patients were known: one was a construction worker, the other worked in a glass factory. Only one patient had a medical background of interest: one year before the study he had suffered community-acquired pneumonia of unknown etiology.

IgG antibodies to hantavirus were found in three of the serum samples collected from the general population (seroprevalence 2%; 1 female [seroprevalence 1.3%] and 2 males [seroprevalence 2.8%]). The mean age of these subjects was 53.3 years (SD 16.4

years; range 39--78 years). These positive subjects had no history of contact with rodents, had no occupational risks, and had not traveled to high-risk areas.

The seropositivity of the patient and general population groups was significantly different ($p < 0.05$; $\chi^2 = 4.20$), with more positive results among the patients. No significant differences were found in seroprevalence with respect to sex or age in either group.

Table 4 shows the results of the serological tests performed on the seropositive subjects. As shown in Figures 3 and 4, the serum of seropositive members of the general population reacted only with SEOV; that of the seropositive patients reacted specifically with PUUV (63.6%).

DISCUSSION

Hantaviruses have a worldwide distribution, but our knowledge of their epidemiology and their impact on health differs according to the geographical area in question^{16,17}. The present results are important from the point of view of the epidemiology of infection, but not with respect to the diagnosis of acute infection (IgM antibodies were only sought in samples who were positive for IgG antibodies). The present work can be considered a pilot study in a country where it is still unknown whether hantavirus circulates or whether it causes disease. For this reason, two screening techniques were used involving a large number of antigens with high level cross reactivity among numerous serotypes; this reduces the chance of missing any positive result. However the problem of non-specific IFA or EIA reactions is well known, and large numbers of false positive results are unfortunately common. In the present work, discrepant results were obtained with the two screening tests used, although differences between EIA and IFI results have also been reported by other

authors^{18,19}. Positive results were therefore confirmed by the more specific WB procedure. Moreover, the numerous cross reactions among serotypes suggests that neutralization tests should be performed in order to identify the serotypes in positive cases and it is also important that the antibody responses may be directed at an as yet unidentified hantavirus, that it has been able to be detected using very sensitive assays as EIA and IFI.

The results show the patients with hypertransaminemia more commonly possessed antibodies to hantavirus than the members of the control group (who were from the same geographical region). The infection rate in the general population was low, as reported in other Spanish studies^{7,8,9}.

In both groups the epidemiological results with respect to sex and age were similar. However, two serological patterns were detected. The patients showed reactivity against PUUV, while the seropositive healthy controls showed reactivity against SEOV. In previous studies involving the general population of Madrid, the most common seroreactivities detected were against both these viruses⁸. Studies from other countries involving patients with hantavirus infection and hepatic dysfunction (including hepatitis) make no reference to the involvement of specific viruses²⁰. However, it is known that SEOV can cause more severe liver dysfunction than other hantaviruses²¹. Unexpectedly, one of the eleven seropositive patients detected was found to harbor SNV antibodies exclusively; other authors have reported the same phenomenon¹⁹.

HFRS is mainly characterized by fever and kidney dysfunction, sometimes with hemorrhaging. In typical HFRS, a significant number of patients suffer lung and liver problems^{22,23,24}, although the disease in non-endemic areas may be atypical: patients

with symptoms of hepatitis but minimal renal involvement have been recorded in such areas²⁵.

Some authors think that hantavirus may cause hepatitis since patients with acute hepatitis but no extrahepatic manifestations commonly have anti-hantavirus antibodies^{26,27}. It is speculated that if a hepatotropic lineage of hantavirus exists, it may result in liver dysfunction with clinical manifestations different to those of HFRS and HPS.

The hepatic manifestations of hantavirus infection have not been well described, although liver dysfunction has been reported, and it is not known whether chronic sequelae develop²⁸, e.g., chronic hepatitis. Ledina D and others²⁹ showed that hantavirus infection may be associated with chronic hypertension and chronic liver damage. Some authors believe the involvement of the liver to be an ominous prognostic sign in some patients^{30,31}, yet the experience of others suggests infection runs a benign clinical course³².

The spectrum of illness caused by hantavirus therefore remains unclear, especially outside endemic areas; indeed, the symptoms of infection in such areas may vary from those of classical HFRS and HPS. For this reason the present study involved patients with hantavirus-compatible disease. It has been shown that transaminases are often elevated in hantavirus infection; this has been recorded in 28%³⁰, 46%²³, and even 78%²¹ of those infected. In some cases the predominant symptom is a persistently marked elevation of serum transaminase levels². In the present work, 6% of the patients with hypertransaminemia had anti-hantavirus antibodies. This is lower than that found in patients with acute hepatitis in China (13.2%)²⁶, but higher than that reported in Japan²⁰ in patients with hepatic dysfunction (3%). However, this result is important

since it is the highest figure ever detected in all of the epidemiological studies performed in Spain.

It should be noted that, although the aim of the present work was not to search for acute infections, anomalies were commonly seen in the reactive patients that were very characteristic of hantavirus infection, e.g., thrombocytopenia and lymphocytosis²¹. Anti-hantavirus IgM antibodies were also detected by WB in one patient, although he had no recent history of illness compatible with classical hantavirus infection.

In conclusion, further epidemiological studies should involve more patients with disease compatible with hantavirus infection, as well as patients with less obvious symptoms since in non-endemic areas the clinical manifestations of infection may be atypical. The role of these viruses in hepatitis of unknown etiology also needs to be investigated. Further serological tests of patients with acute and chronic hepatitis should be performed to determine the relationship between hantavirus and hepatitis in the Madrid area.

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Table 1. Levels of ALT and AST: media values and ranges

	Patients n (%)	Media	Range
ALT >35 U/l	145 (79.6)	95.8	36-2150
AST >38 U/l	77 (42.7)	93.5	39-1480

72 patients (39.5%) had high levels of ALT and AST.

Table 2- Analytical abnormalities

Clinical laboratory findings	%
Elevation of GGT (>43 U/l)	56
Lymphocytosis	36
Lymphopenia	27.8
Elevated LDH (Lactate dehydrogenase)	22.5
Hyperbilirubinemia	16
Thrombocytopenia	10.5
Thrombocytosis	3.7
Elevated hematocrit	1.5
Reduction of LDH	1
Elevated iron	0.5

Table 3- Analytical abnormalities in seropositive patients

Clinical laboratory findings	%
Lymphocytosis	36
Hyperbilirubinemia	18
Elevated LDH (Lactate dehydrogenase)	18
Thrombocytopenia	18
Thrombocytosis	9

Table 4- Summary of serological data for hantavirus-reactive samples

Serum	IFA IgG		*EIA IgG	BLOT NATIVE N							
	<i>PUUV</i>	<i>SEOV</i>	<i>SEOV</i> and <i>SNV</i> N	<i>PUUV</i> IgG	<i>PUUV</i> IgM	<i>SEOV</i> IgG	<i>SEOV</i> IgM	<i>SNV</i> IgG	<i>SNV</i> IgM	<i>DOBV</i> IgG	<i>DOBV</i> IgM
P-1	+	-	+	+	-	-	-	+	-	-	-
P-2	-	+	-	-	-	-	-	-	-	+	-
P-3	-	+	+	-	-	-	-	-	-	+	-
P-4	+	-	+	+	-	-	-	-	-	-	-
P-5	-	-	+	-	-	-	-	+	-	+	-
P-6	+	-	-	+	-	-	-	-	-	-	-
P-7	+	+	+	+	-	-	-	+	-	-	-
P-8	+	-	+	+	-	-	-	+	-	-	-
P-9	+	-	-	+	-	-	-	-	-	-	-
P-10	+	-	-	+	+	-	-	-	-	-	-
P-11	-	-	+	-	-	-	-	+	-	-	-
GP-1	+	+	+	-	-	+	-	-	-	-	-
GP-2	+	+	+	-	-	+	-	-	-	-	-
GP-3	+	+	-	-	-	+	-	-	-	-	-

*Optical density (450 nm), cut-off value 0.100

P patient with hypertransaminemia

GP general population

FIGURE LEGENDS

Figure 1. The Madrid province - the geographical area of the study.

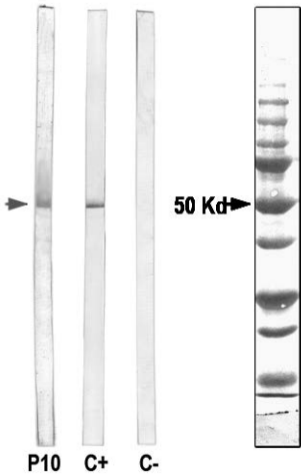
Figure 2. Positive sample (IgM) by WB from patients with hypertransaminemia

Figure 3.- Positive samples (IgG), as detected by Western blotting, from people of the general population.

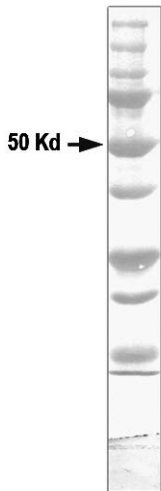
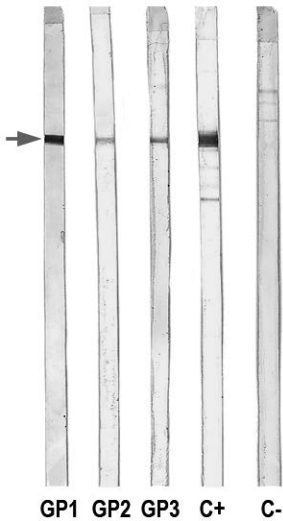
Figure 4.- Positive samples (IgG), as detected by Western blotting, from patients with hypertransaminemia.



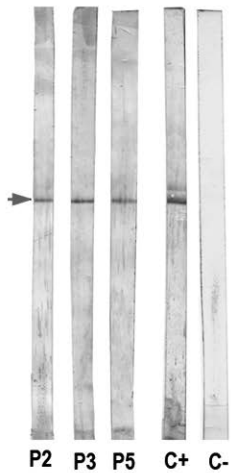
PUU N Prot



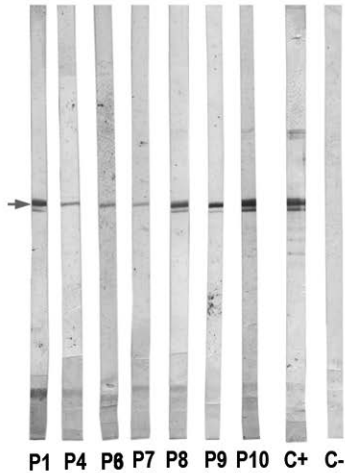
SEO N Prot



DOB N Prot



PUU N Prot



SN N Prot

