MAJOR ARTICLE

Plasma Exchange–Associated Immunoglobulin M–Negative Hantavirus Disease after a Camping Holiday in Southern France

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A 37-year-old Belgian patient presented with acute nephropathia epidemica (NE) shortly after a camping holiday in southern France. Unusual symptoms were initial noncardiogenic lung involvement, followed by severe acute renal failure, acute acalculous cholecystitis, presence of immunoblasts in the bone marrow, and hemolytic anemia, presenting as hemolytic uremic syndrome. Positive immunoglobulin (Ig) A and rising IgG titers against Puumala hantavirus (PUUV) were detected, but IgM remained negative on days 8 and 20. The results of reverse-transcriptase–polymerase chain reaction performed on day 8 were positive for PUUV. This is the first report of an iatrogenically IgM-negative hantavirus case due to the selective removal of heavyweight molecules during plasma exchange via the centrifugation technique. This is also the first report of proven NE from the Mediterranean part of France.

Hantaviruses are members of the *Bunyaviridae* family, genus *Hantavirus.* Chronically infected but asymptomatic wild rodents are the most important reservoirs; they transmit infection to humans via aerosolized excreta. Hemorrhagic fever with renal syndrome (HFRS) is the common denomination of Old World human hantavirus infections and can be caused by any of several pathogenic virus strains, including Hantaan (HTNV), Seoul, Puumala (PUUV), Dobrava, and Amur [1, 2]. The clinical presentation, particularly of the PUUV-induced HFRS cases, also called nephropathia epidemica (NE), is often mild or atypical, necessitating laboratory confirmation. According to criteria issued by the World Health Organization and the Centers for Disease Control and Prevention, this should preferably occur via a hantavirus-specific IgM diagnostic assay. Indirect immunofluorescence assay (IFA) and particularly ELISA can readily be used for IgM seroconfirmation. We report here what is, to our knowledge, the first (iatrogenic) exception to this rule.

CASE REPORT

A 37-year-old white man, a Belgian industrial meat worker, had been camping and sleeping in a 1-person tent in the French Pyrenean mountains (region of Perpignan) during the last 2 weeks of August 1999. Three days after his return to Belgium (3 September 1999; day 1), he developed a flulike syndrome, consisting of a dry cough with white sputa, dyspnea, shivers, headache, anorexia, nausea and vomiting, and diffuse myalgias, for which he presented at the hospital on day 3. At admission, the temperature was 38.5°C, blood pressure was 112/49 mm Hg, and respiratory rate was 22 breaths/min. Abdominal palpation revealed nothing ab-

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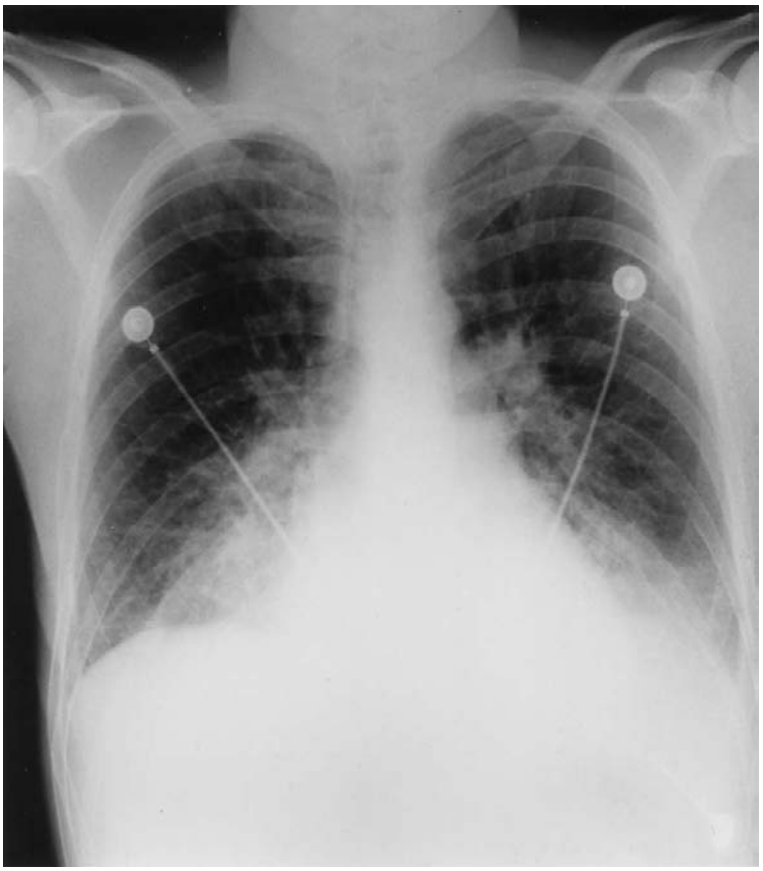

Figure 1. Thorax radiograph on day 7 of acute nephropathia epidemica, showing vascular congestion in both lungs, with presence of bibasal infiltrates and left pleuritis, but no cardiac enlargement.

normal, but right renal percussion was painful. The hemoglobin (Hb) level was 15.9 g/dL, the WBC count was 11.7×10^9 cells/L, the platelet count was 132×10^9 platelets/L, the C-reactive protein level was 38 mg/L, and the lactate dehydrogenase (LDH) level was 482 U/L, but the serum creatinine was still normal on day 3, at 1.20 mg/dL. Blood gas analysis breathing ambient air showed a pH of 7.57, partial pressure of oxygen of 82 mm Hg, partial pressure of carbon dioxide of 20 mm Hg, and a saturation of 98%. The chest radiograph revealed a normal heart and mediastinum, but pulmonary vascular congestion and Kerley B-lines were present at the right lung basis. The next day (day 4), the patient insisted on leaving the hospital, despite still having a fever (temperature, 38.6°C), an increase in the Hb level to 17.1 g/L, and a further decrease in the platelet count to 52×10^9 platelets/L.

Two days later (day 6), the patient was readmitted with malaise, increased dyspnea, diffuse myalgias, and vomiting, this time complicated by minor hematemesis and progressive oliguria. A positive Murphy's sign was found in the upper right quadrant. A peripheral blood smear revealed a net increase in the WBC count (38×10^9 cells/L) with left shift and with the presence of numerous atypical lymphocytes, together with immature WBC forms, including 1 myeloblast, 1 myelocyte, and 1 metamyelocyte. The presence of 2 schistocytes per 1000 RBCs was noted. The platelet count decreased further to 44×10^9 platelets/L, and then (day 7) to 24×10^9 platelets/L, and the Hb level decreased to 7.9 g/dL. Moreover, there was a sharp increase in the C-reactive protein level (121 mg/L), the LDH level (1854 U/L), and the creatinine level (peak value, 9.09 mg/dL). The lipid profile showed a triglyceride level of 284 mg/dL, contrasting with a total cholesterol level of only 87 mg/dL. Urine examination revealed 20 RBCs per high-power field and a marked proteinuria (protein level, 7.8 g/L). On day 7, diuresis was only 175 mL/24 h, and proteinuria 4.35 g/24 h. A new chest radiograph (day 7) showed marked progression of the vascular congestion of both lungs (figure 1). Results of several hemocultures remained negative, and, consequently, the patient was not provided any antibiotic therapy. Abdominal ultrasonography showed ascites formation, hydrops (10.5×4.5 cm) of the gallbladder with bile sludge, a thickening of the gallbladder wall (4.5 mm), and swollen kidneys (transaxial diameter, >13 cm) without signs of urinary obstruction. Findings of the analysis of a bone marrow biopsy sample were normal, except for the presence of 1 or 2 immunoblasts per high-power

Table 1. Serological and renal function in a Belgian case of acute nephropathia epidemica.

Test and virus strain or serological value	Time after onset			
	Day 8[a]	Day 20	Month 4	Month 33
ELISA PUUV				
IgG[b]	2.068 (+)	ND	2.964 (+)	1.910 (+)
IgM[b]	0.690 (−)	ND	2.790 (+)	0.410 (−)
IgA[c]	2.299 (+)	ND	0.120 (−)	ND
ELISA PUUV-rNp-118				
IgG[d]	1.920 (+)	ND	3.370 (+)	ND
IgM[d]	0.190 (−)	ND	1.473 (+)	ND
IgA[d]	2.193 (+)	ND	0.430 (−)	ND
ELISA HTNV				
IgG[b]	0.026 (−)	ND	1.861 (+)	ND
IgM[b]	0.081 (−)	ND	0.046 (−)	ND
IFA HTNV				
IgG[e]	512 (+)	2048 (+)	ND	ND
IgM[e]	−	−	ND	ND
Serum creatinine level, mg/dL	8.08	3.20	1.60	1.15

NOTE. HTNV, Hantaan; ND, not done; OD, optical density; PUUV, Puumala; +, positive; −, negative.

[a] Acute sampling on day 8 performed after the first plasma exchange session.

[b] Recombinant whole nucleocapsid protein PUUV and HTNV as antigen. Starting dilution 1/201. Results given as OD patient serum divided by OD of a reference positive control serum. Results considered positive if >1.5 for IgG and if >2 for IgM.

[c] Recombinant whole nucleocapsid protein of PUUV, starting dilution 1/201. Results were considered positive if higher than the mean OD of 10 negative samples plus 3 SD (cutoff: 0.827).

[d] Homemade ELISA based on a recombinant, truncated PUUV nucleocapsid protein of 118 amino-terminal amino acid (cutoff IgG, 0.396; IgM, 0.592; IgA, 0.526).

[e] Vero E6 cells infected with prototype strain HTN 76–118. Starting dilution 1/16. Results given as the reciprocal of highest dilution still IFA positive.

field. Gastroscopy showed hemorrhagic gastritis and peptic esophagitis grade 3.

Given the picture of acute renal failure, severe thrombocytopenia, and rapid-onset "microangiopathic" anemia with schistocytes and an increased LDH level, a diagnosis of hemolytic uremic syndrome (HUS) was initially forwarded, and plasma exchange (PEX) via centrifugation was performed on days 8 and 9, together with peritoneal dialysis. Because of the extreme thrombocytopenia, a pulmonary artery catheter was not inserted, but hemodynamic data remained stable during and after PEX, and cardiac signs of fluid overload were never recorded. Symptoms improved from day 9 onward, the body weight decreased from 62.5 kg (day 14) to 54 kg (day 21), and the patient was discharged on day 27 with a serum creatinine level of 2.15 mg/dL and a normal complete blood cell count. The patient was seen on follow-up visits after 4 and 33 months and appeared completely healed, with renal function restored to normal (serum creatinine level, 1.6 and 1.15 mg/dL, respectively; table 1), with normal blood pressure and absence of proteinuria.

The differential diagnosis of severe NE instead of HUS was considered only at the end of day 8, and samples were obtained for serological tests after the first PEX. Routine IFA serological tests with prototype HTNV were performed at the Institute of Tropical Medicine (Antwerp, Belgium), and more specific assays on conserved serum samples were carried out retrospectively at the Hantavirus Reference Laboratory at the University of Leuven, Belgium. Testing for leptospirosis was negative, and both hantavirus IFA and ELISA assays gave increased IgG titers for PUUV or for (cross-reacting) HTNV, but the results remained negative for IgM during the acute phase (days 8 and 20) (table 1). However, the results of 2 homemade tests for PUUV IgA were clearly positive. Knowing that PUUV is the only pathogenic serotype present in both Belgium and France [3], and despite the then-unexplained IgM seronegativity, it was decided to perform RT-PCR on the acute-phase serum sample obtained after the first PEX on day 8, which was still available and which had been stored at −20°C. PUUV-specific primers (forward: 5′-GAC TCC TTG AAA AGC TAC TAC G-3′; reverse: 5′-ATT CAC ATC AAG GAC ATT TCC-3′) were used for the amplification of a 346-bp fragment in the conserved region of the S-segment, common to all known PUUV strains. A positive PCR control was performed on supernatant of PUUV (Cg 18–20)–infected Vero E6 cells. Nucleotide sequencing of the amplicon was performed, and a GenBank search revealed that the obtained sequence was indicative of a novel PUUV strain. This strain was called "France/Perpignan1999," and the partial sequence of the S-segment was deposited in GenBank (accession number AY101391; figure 2).

DISCUSSION

To our knowledge, this is the first RT-PCR–proven case of human PUUV infection in France or in Belgium. This Belgian patient was probably infected during his camping holiday in France, right before he became ill. Camping—and, particularly, sleeping on the ground in forested areas—greatly increases the risk of inhaling aerosolized excreta of wild rodents [7, 8]. The incubation time for a PUUV infection is generally estimated to be 2–4 weeks, and in the weeks and months before his holiday in France, the patient had not traveled, had not camped in Belgium, and had not visited the Ardennes, a region in Belgium where NE is highly endemic [8, 9]. His profession as a meat cutter in refrigerated rooms in Antwerp (northern Belgium) did not expose him to rodents or their excreta, and he denied any possible other rodent contact. The most likely hypothesis is that the patient contracted hantavirus disease during his sojourn in the region of Perpignan in southern France. This would be the first report of proven NE from the Mediterranean

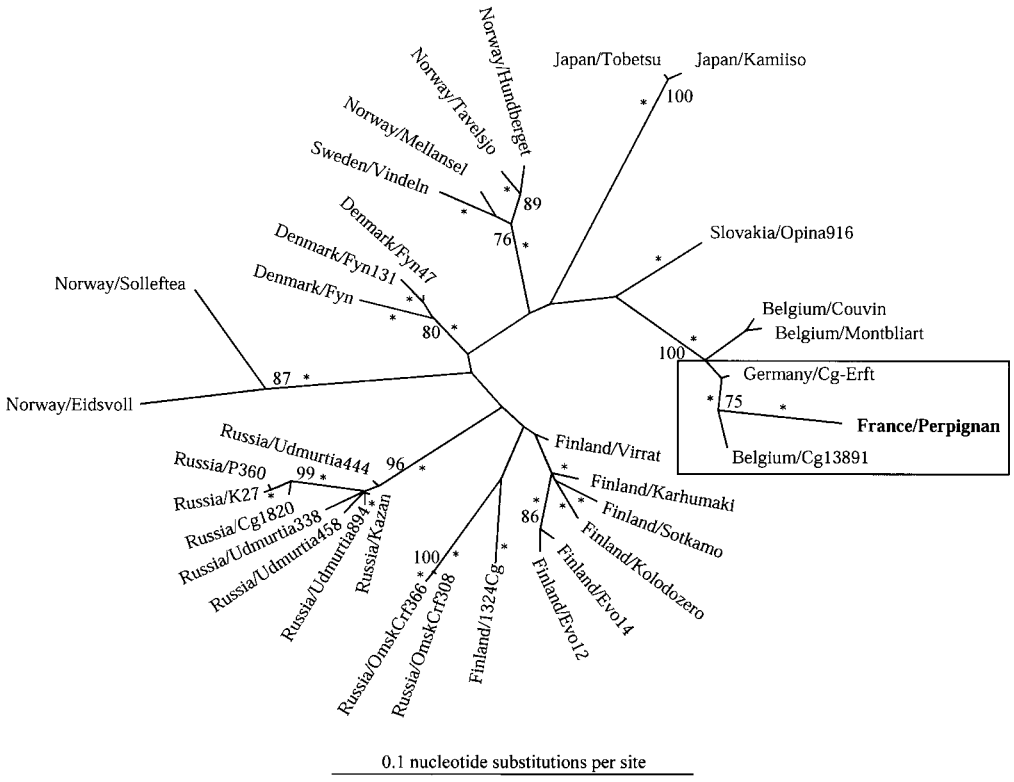

Figure 2. Phylogenetic relationships of France/Perpignan1999 (GenBank accession no. AY101391) and other hantaviruses. An alignment of 251 nucleotides was made using ClustalX, version 1.8 [4]. The correct model of evolution was tested with the program Modeltest [5]. Phylogenetic trees were reconstructed by algorithms implemented in PAUP*, version 4.0b8 [6]. A neighbor-joining tree (NJ) was inferred under the appropriate model of evolution. This tree was used as input for a heuristic maximum likelihood (ML) search according to the tree bisection reconnection algorithm. A heuristic parsimony search resulted in 3 most parsimonious trees for which a majority-rule consensus tree was obtained. Finally, a majority-rule consensus tree was reconstructed for the NJ, ML, and parsimony consensus tree. The values next to the branches indicate the bootstrapping confidence limits (in percentages) from 1000 replicates. Branches with asterisks were supported by significant P values ($P < .01$) in likelihood ratio tests for zero branch lengths. Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 nucleotide replacements per site. The following Puumala virus S segment sequences were used: Russia/Udmurtia894 (GenBank accession no. Z21497), Russia/Udmurtia458 (Z30707), Russia/Udmurtia338 (Z30708), Russia/Udmurtia 444 (Z30706), Russia/Kazan (Z84204), Russia/OmskCrf308 (AF367070), Russia/OmskCrf366 (AF367071), Russia/Cg1820 (M32750), Russia/P360 (L11347), Russia/K27 (L08804), Slovakia/Opina916 (AF294652), Belgium/Montbliart (AJ277031), Belgium/Couvin (AJ277034), Belgium/Cg13891 (U22423), Germany/Cg-Erft (AJ238779), Japan/Tobetsu (AB010731), Japan/Kamiiso (AB010730), Sweden/Vindeln (Z48586), Norway/Mellansel (AJ223375), Norway/Tavelsjö (AJ223380), Norway/Hundberget (AJ223371), Norway/Eidsvoll (AJ223369), Norway/Sollefteå (AJ223376), Denmark/Fyn (AJ238791), Denmark/Fyn47 (AJ278092), Denmark/Fyn131 (AJ278093), Finland/Virrat (Z69985), Finland/Karhumaki (AJ238788), Finland/Sotkamo (X61035), Finland/Kolodozero (AJ238789), Finland/Evo14 (Z30704), Finland/Evo12 (Z30702), and Finland/1324Cg (Z46942).

part of France. The main, if not the only, rodent reservoir for PUUV in Europe, the bank vole *(Clethrionomys glareolus)*, is absent in the biotope of so-called "Mediterranean shrub" occupying most of southern European coastal regions, but it is present, albeit in sparser population densities, in the forested Pyrenean mountains [10].

The sequence of our France/Perpignan1999 PUUV could not be compared with other human or rodent French PUUV lineages. The previously described French strain PUU90-13, which is thought to be isolated from a human case in 1990 in France [11], is, in fact, an isolate from a bank vole captured in Turnhout in Belgium and called Cg13891 [12], as clarified later by the original authors [13–15]. The same authors propose that in all former literature, and from now on, each mentioning of the so-called "French isolate PUU90-13" should be replaced by "the Belgian strain Cg13891" [13–15]. Although the amplified 346-bp fragment of the S-segment is fairly small, analysis of the phylogenetic tree nevertheless showed that France/Perpignan1999 forms a separate clade with Cg13891, both situated at a short evolutionary distance from a German strain Cg-Erft (figure 2). Two other previously described Belgian rodent strains, however, are at a slightly longer distance. Such a distinct western European PUUV clade was previously described by others [11, 14, 16].

This is also the first report of a RT-PCR–proven case of typical acute hantavirus disease that remained IFA and ELISA

IgM negative throughout an acute phase of ⩾20 days. PEX, particularly when the centrifugation technique is used, can selectively discard from the blood compartment heavy-weight molecules, such as pentamer IgM, while sparing lighter molecules, such as IgA and IgG [17]. This effect can persist for several weeks, depending on the half-life of the studied molecules and the number of PEX sessions applied. The first PEX performed on day 8 can thus explain the IgM negativity (despite IgA, IgG, and PCR positivity on the same serum sample), because this aliquot of day 8 was obtained only after the epuration session. Although late IgM seroconversion can occur in NE, negative PUUV IgM antibodies >7 days after the onset of symptoms have not been documented to date [18]. Thus, the consecutive IFA IgM negativity on day 20 despite increasing IgG titers can also be explained as a lasting iatrogenic effect of an additional second PEX, performed on day 9. However, detection with 2 different ELISA formats of IgM antibodies in month 4—that is, after full convalescence (table 1)—provides proof of a recent PUUV infection, after which a gradual disappearance of IgM antibodies up to 9 months after infection can be expected [19, 20]. In contrast to IgM, specific PUUV IgA were detected on day 8, but were already negative at month 4 (table 1). This is in accordance with our previous findings, whereby ELISA IgA seropositivity was demonstrated in all acute-phase NE samples up to day 25, after which IgA titers rapidly decreased [20].

Although it is admittedly an exceptional intensive care therapeutic measure for acute viral infections, PEX should be considered for the interpretation of unexpected results in classic serology, especially if the serosampling was performed only after the start of the PEX sessions. Other possible indications for PEX in viral infections may be Guillain-Barré syndrome or disseminated intravascular coagulation, both of which have been described as rare complications of hantavirus infections.

Several other interesting clinical and diagnostic lessons may be learned from this NE case. First, minor lung involvement clearly preceded the renal involvement, before any fluid overload could occur. At the first admission, the patient was rather dehydrated, the result of his frequent vomiting and because his diuresis and renal function were still normal. Although never so severe as to induce a full-blown hantavirus pulmonary syndrome (HPS), typical for the New World Sin Nombre virus (SNV) or SNV-like infections, noncardiogenic acute lung edema with typical thoracic radiography findings, does occur in some NE cases [15, 21, 22]. All signs described as discriminatory for presumptive HPS [23]—that is, myalgias, myelocytes, metamyelocytes, thrombocytopenia, hemoconcentration, and hypocapnia—were also present in our patient with NE.

Second, a substantial decrease in the platelet count often heralds clinical complications, particularly if it is accompanied by a sudden increase in Hb or hematocrit. Thrombocytopenia combined with hemoconcentration is probably an expression of an endothelial dysfunction, resulting in plasma leakage, generally accepted as the main pathophysiological mechanism found in all hantavirus infections [3, 23]. This leads to edematous infiltration and swelling of various abdominal organs (the kidneys in particular). In severe cases, like the one presented here, fluid accumulation in abdominal and pleural cavities together with laboratory signs of hemoconcentration can also be documented.

Third, acute acalculous cholecystitis as an acute complication of hantavirus infection has been described to date only in a Korean HFRS series [24], whereby gallbladder-wall thickening of ⩾4 mm was correlated with a more severe degree of thrombocytopenia, acute renal failure, and abdominal and pleural fluid effusions, as in our patient. Bile sludge and even hydrops of the gall bladder in an intensive care unit patient could be ascribed to prolonged fasting and/or nasogastric feeding. However, both these intensive care unit conditions were absent in our patient, and they cannot account for the observed sonographic gallbladder-wall thickening of 4.5 mm, which was even and diffuse. We believe that gallbladder-wall thickening is just another expression of plasma leakage with transient edematous interstitial infiltration, as showed in other full organs (e.g., the

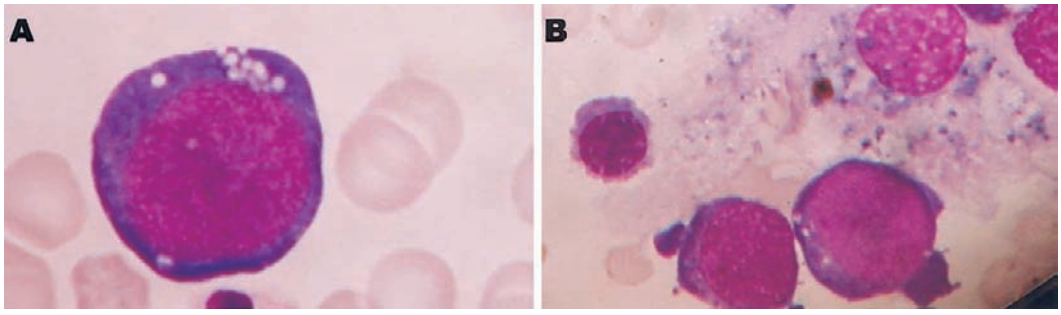

Figure 3. Two sections of the bone marrow biopsy sample obtained on day 7 of acute nephropathia epidemica. *A,* Typical immunoblast with high nuclear-cytoplasmic ratio, intense basophilic cytoplasm, small intracytoplasmatic vacuoles, and small nucleoli in the immunoblastic nucleus. *B,* Presence of 2 immunoblasts adjacent to each other near the lower border of the high-power field.

kidney). Acute acalculous cholecystitis may mimic an acute abdomen, particularly if accompanied by positive Murphy's sign and pronounced leukocytosis, as in this case. This can lead to unnecessary surgical interventions.

Fourth, as noted before, paradoxical lipid perturbations (i.e., low fasting serum cholesterol level in contrast with elevated triglyceride levels) are characteristic for NE [25] and for HFRS [26] and are indicative of its severity.

Fifth, hemolytic anemia can be so pronounced in NE as to suggest HUS, particularly when accompanied by severe acute renal failure and profound thrombocytopenia. Except for raised LDH levels, this rare hantavirus complication has not been mentioned so far in European or in extensive Asian series [3, 25, 26]. It should be distinguished, however, from real HUS, because PEX and/or substitution of coagulation factors is not warranted in NE, for which these intensive care unit treatments are probably deleterious.

Sixth, in a recent Finnish study, it was suggested that genetic susceptibility to development of severe NE may be linked to the presence of HLA B8 and DR3 alleles [27]. In our patient, HLA genotyping by PCR followed by reverse dot blot hybridization with specific oligonucleotide probes gave following results: A*03, A*74, B*3508, B*44, DRB1*07, and DRB1*11. The absence of both HLA B8 and DR3 alleles in our patient shows that at least in some individual cases, severe NE can occur without the presence of this haplotype, which is commonly found in the white population of Europe, even outside of Finland.

And finally, to date, the diagnostic triad of leukocytosis with left shift, low platelet counts, and immunoblasts was described only in American SNV(-like)–induced forms of HPS [23]. In our patient, the abundant "atypical lymphocytes," viewed from day 6 on, together with several forms of immature WBCs on routine (i.e., nonfixed) peripheral blood smears, were most probably immunoblasts, the presence of which was retrospectively confirmed on fixed sections of the bone marrow performed on day 7 (figure 3). To our knowledge, this is the first report of an Old World hantavirus infection documenting such a triad. In HTNV-induced HFRS, "atypical lymphocytes" are often present between days 4 and 14 of illness, together with immature WBCs [26]. To what extent some of these "atypical lymphocytes" are in fact immunoblasts has yet to be established. Careful inspection of the peripheral smear for the presence of immunoblasts [23] is indicated in all severe hantavirus infections worldwide. If confirmed in other NE cases, this could point again to similarities between NE and HPS and to the possibility of common pathophysiological mechanisms, thus explaining the growing list of similar points in clinical presentation [15, 21, 22].

In conclusion, in the rare cases of PEX treatment for a viral condition, diagnostic serosampling should be planned before the first epuration session to avoid iatrogenic IgM negativity, as we encountered in the patient under discussion.

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