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Molecular Linkage of Hantavirus Pulmonary Syndrome to the White-Footed Mouse, *Peromyscus leucopus*: Genetic Characterization of the M Genome of New York Virus

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The complete M segment sequences of hantaviruses amplified from tissues of a patient with hantavirus pulmonary syndrome in the northeastern United States and from white-footed mice, *Peromyscus leucopus*, from New York were 99% identical and differed from those of Four Corners virus by 23%. The serum of this patient failed to recognize a conserved, immunodominant epitope of the Four Corners virus G1 glycoprotein. Collectively, these findings indicate that *P. leucopus* harbors a genetically and antigenically distinct hantavirus that causes hantavirus pulmonary syndrome.

Hantaviruses are enveloped, spherical, minus-sense RNA viruses of the family *Bunyaviridae*. The hantavirus genome consists of three segments, known as large (L), medium (M), and small (S) segments. The L segment encodes an RNA-dependent RNA polymerase, and the S segment encodes the nucleocapsid protein. The envelope glycoprotein precursor, specified by a single open reading frame of the complementary strand of the M segment, is processed into mature G1 and G2 proteins during translation by a cellular protease (12).

Hantavirus pulmonary syndrome (HPS), an acute respiratory disease with a high mortality rate, was first recognized in 1993 after an investigation into a series of unexplained deaths that occurred in the Four Corners region of the southwestern United States (29), the only site in the United States where four states (Arizona, New Mexico, Utah, and Colorado) have a common border. The etiologic agent in the outbreak of HPS is known as the Four Corners virus (FC; also called Sin Nombre virus and Convict Creek virus) (11, 32).

All hantaviruses are harbored by specific rodent (family *Muridae*) or insectivore hosts. Hantaviruses are transmitted to humans through accidental inhalation of virus-contaminated rodent excreta. Previously, several hantaviruses have been recognized as etiologic agents of hemorrhagic fever with renal syndrome, which occurs in seasonal epidemics in Europe and Asia (43). These viruses include Hantaan virus, Dobrava/Belgrade virus, Seoul virus (SEO), and Puumala virus (PUU) (3, 13, 22, 23). The rodent hosts for these viruses are the striped field mouse (*Apodemus agrarius*), the yellow-necked field mouse (*A. flavicollis*), the Norway rat (*Rattus norvegicus*), and the bank vole (*Clethrionomys glareolus*), respectively. Prospect Hill virus (PH), the prototypic American hantavirus harbored by the meadow vole (*Microtus pennsylvanicus*), has not been associated with human disease (24, 25, 44).

FC is a new species of the genus *Hantavirus* (16, 28). It is carried by the deer mouse, *Peromyscus maniculatus*, which occurs throughout the western United States and Canada (8, 28). When sought, FC genetic material has been identified in the tissues or blood samples of all United States and Canadian patients with acute HPS from western states and provinces (4, 15, 18, 35). Patients with HPS in western states and provinces uniformly develop antibodies directed against the G1 glycoprotein and the nucleocapsid protein of FC (19). In several patients with a remote history of HPS, in whom viral genetic material can no longer be detected, antibodies to the FC G1 glycoprotein have been detected (15, 19, 38). This antibody reactivity appears to be specific for infection by FC.

Several hantavirus infections resulting in HPS have been contracted outside the habitation range of *P. maniculatus*. These cases have resulted in the recognition of an expanded clade of antigenically and genetically similar pathogenic hantaviruses. Cases in Louisiana and Florida have been shown to be caused by hantaviruses known as Bayou virus and Black Creek Canal virus, respectively (5, 27). The only case of HPS in the northeastern United States occurred in a patient (RI-1) whose complex travel history suggested possible exposures in Rhode Island and New York, including New York City, Long Island, and Shelter Island (2, 6, 17, 34). Unlike patients from the western United States, this patient lacked serum antibody against the FC G1 glycoprotein. We previously reported the complete S genome and nucleocapsid protein sequences of this hantavirus. Although its genetic sequence suggested that this patient was infected with a novel hantavirus, that conclusion could not be definitive in the absence of information about host associations and viral genetic data from candidate rodent hosts (14, 17). Furthermore, the strong cross-reactivities among the nucleocapsid proteins of related hantaviruses made it seem unlikely that we would be able to distinguish antigenic differences between the FC and RI-1 nucleocapsid proteins.

In a previous report, we demonstrated a high prevalence of hantavirus infection among white-footed mice, *P. leucopus*, captured on Shelter Island retrospectively and currently (26). We suspected that patient RI-1 might have contracted HPS during his stay on Shelter Island. We collected 16 rodents (each *P. leucopus*) from Shelter Island and determined that 4 were seropositive for hantavirus. Preliminary studies of the

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partial M and S genome sequences of one of these specimens suggested that the Shelter Island mice harbored a hantavirus with marked genetic similarity to the virus that infected patient RI-1 (26, 34). To verify that linkage, we analyzed partial M segment sequences from all four positive Shelter Island animals and complete M segment sequences from two specimens. Comparison with the complete sequence of the RI-1 M segment established that patient RI-1 was infected with a hantavirus virtually identical to that in circulation among white-footed mice on Shelter Island.

The results of serologic, clinical, and epidemiologic studies performed in connection with the fatal infection of patient RI-1 have been reported previously (2, 6, 17). To sample rodent populations at the Shelter Island site, 100 Sherman traps were set for four nights in October 1994 in 0.5 ha of broadleaf forest. Sixteen specimens were captured. After the administration of anesthesia, blood samples were obtained via the retroorbital sinus with a capillary tube. The rodents were then sacrificed, and tissues (lung, heart, kidney, liver, and spleen) were removed and frozen on dry ice. Blood samples were analyzed for reactivity against hantavirus (PH, SEO, and PUU)-infected cells by indirect immunofluorescence assay (IFA) as previously described (10). Since antibody titers against PUU were low, titration studies used only PH- and SEO-infected cells. Additional supplementary antibody testing was done by Western blot (immunoblot) analysis with the nucleocapsid antigen of FC expressed as a TrpE fusion protein in *Escherichia coli* as previously described (19, 42).

Reverse transcription PCR was conducted with nested and heminested strategies as previously described (16). Initial amplification reactions used PH and FC consensus primers; previously reported M segment consensus primers (35) did not amplify the RI-1 or New York virus (NY) M segment. The products of amplification with inner primers were ligated by TA cloning into the plasmid vector pCRII according to the manufacturer's (Invitrogen, San Diego, Calif.) instructions. The sequences of the inserts were determined with Sequenase (United States Biochemical, Cleveland, Ohio) according to the manufacturer's instructions. Additional primers were synthesized to allow double-stranded sequencing of at least two clones of each amplimer. Reverse transcription PCRs to obtain overlapping amplimers from contiguous portions of the M segment used primers designed from NY or RI-1 sequences in combination with FC and PH consensus primers. The sequences of primers used in this study are available upon request.

Maximum-parsimony analysis of G1 gene sequences was performed as described in the legend to Fig. 1 by using the MULPARS option of PAUP 3.1.1 (36). Prototype hantavirus sequences with the following GenBank accession numbers were used in this study: Hantaan virus 76-118 (33), M14627; SEO SR-11 (1), M34882; PUU P360 (41), L08755; PH-1 (30), X55129; THAI 749 (41), L08756; Bayou virus (27), L36930.

Four of 16 white-footed mice (designated NY-1, NY-2, NY-3, and NY-4) studied on Shelter Island were found to be seroreactive to PH and weakly reactive to PUU by IFA. Their antibody titers ranged from 1:256 to 1:512 against PH and from 1:128 to 1:256 against SEO. All four were strongly positive by Western blot for antibodies to FC nucleocapsid protein at a 1:1,000 dilution (Table 1). The high seroprevalence we noted among white-footed mice on Shelter Island is similar to the prevalence of FC among deer mice associated with case homes in the Four Corners region during the HPS outbreak of 1993 (8, 26).

A 220-nucleotide portion of the G2 gene (M segment coordinates 2640 to 2860) was amplified from all four seropositive mice and from lung RNA of patient RI-1. All of the rodent M segment amplimers were of identical sequence. They differed from the RI-1 sequence at a single residue, indicating that a very similar virus was present in all samples. To further characterize the similarity between the hantaviruses from the patient (RI-1) and the Shelter Island rodents (NY-1, NY-2, NY-3, and NY-4), additional nested and heminested PCRs were conducted to allow the amplification, cloning, and sequencing of the entire M segment of NY from two rodent specimens (NY-1 and NY-2) as well as that of the RI-1 virus. The M segments of the hantavirus from NY-1, NY-2, and RI-1 were found to be 3,668 nucleotides in length, 28 nucleotides shorter than that of FC (35). The difference reflects a trunca-

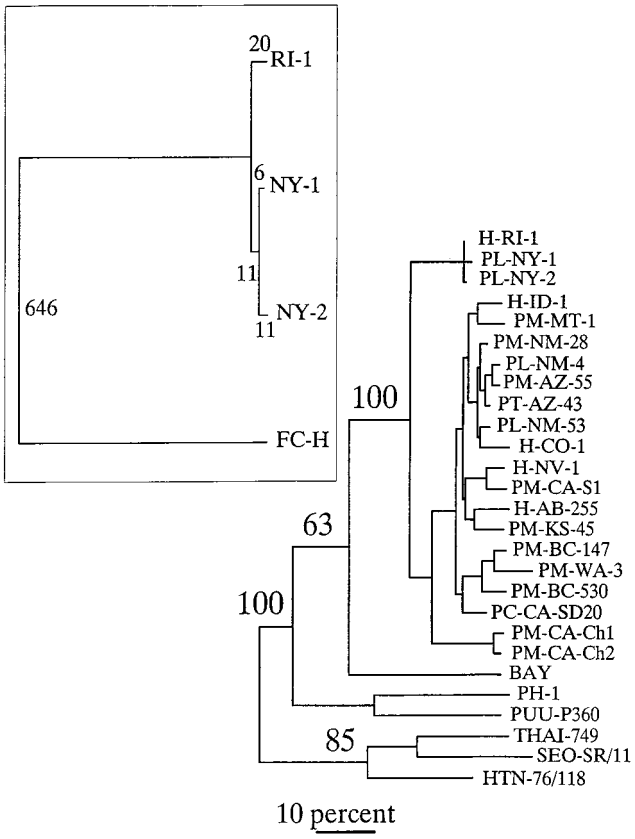

FIG. 1. Unweighted maximum-parsimony tree analysis of the G1 glycoprotein gene (residues 72 to 345) comparing geographically diverse viruses of *P. maniculatus* and human patients with HPS in the western United States and Canada with the homologous sequences of RI-1, NY-1, NY-2, and prototypical hantaviruses PH-1, PUU, SEO, Hantaan virus (HTN), THAI, and Bayou virus (BAY). The prefix for each taxon designation indicates the host of origin (H, human; PL, *P. leucopus*; PM, *P. maniculatus*; PC, *P. californicus*; PT, *P. truei*). A state or provincial location, a regional designation (California specimens only), and then a specimen number follow. RI, Rhode Island; NY, New York; ID, Idaho; MT, Montana; BC, British Columbia; WA, Washington; CA, California; NM, New Mexico; AZ, Arizona; CO, Colorado; AB, Alberta; KS, Kansas; NV, Nevada; SD, San Diego County; S, Siskiyou County; Ch, Channel Islands. Of the 274 characters in this set of sequences 224 were variable. The tree shown was generated by branch and bound analysis and is one of six equally parsimonious trees. The six trees differed only in the placement of taxa of very short branch lengths. Horizontal branch lengths are proportional to distance, but vertical distances are for clarity only. The scale bar indicates a 10% genetic distance. The percentage of bootstrap replicates (of 500) supporting each major node is indicated. The inset shows a maximum-parsimony tree based upon the entire 3,423-nucleotide genes for the glycoprotein precursors of RI-1 and NY-1 and NY-2 in comparison to that of FC (case H [35]). The number of nucleotide substitutions represented by each horizontal branch is indicated.

TABLE 1. Titers of antibody against SEO- and PH-infected cells in serum by IFA and sequenced M segment coordinates of four seropositive *P. leucopus* specimens from Shelter Island, N.Y.[a]

Specimen	Titer by IFA		M segment coordinates sequenced	
	PH	SEO	G1 gene	G2 gene
NY-1	1:256	1:256	Entire G1	Entire G2
NY-2	1:256	1:256	Entire G1	Entire G2
NY-3	1:512	1:128		2640–2860
NY-4	1:512	1:256		2640–2860

[a] Each specimen had a Western blot reactivity against FC nucleocapsid protein (at a 1:1,000 serum dilution) of 3+.

tion in the 3′ untranslated region of NY. The complementary strands of the NY and RI-1 M segments contained a single long open reading frame of 3,420 nucleotides, beginning with the initiating methionine codon at mRNA position 52. As with the corresponding FC segment, the reading frame specifies a predicted 1,140-amino-acid glycoprotein precursor. The calculated molecular mass of the NY/RI-1 protein is 125,500 Da. The G1 glycoprotein contains an 18-amino-acid signal sequence and is predicted to terminate after a consensus sequence, WAASA, at residue 652. The WAASA motif is believed to be a signal for cotranslational cleavage by a cellular protease, resulting in the production of G1 and G2 glycoproteins (12). The G1 and G2 proteins of NY/RI-1 are thus 652 and 488 amino acids in length, respectively. There were 47 amino acid substitutions between the G1 proteins of RI-1 and FC (7.2%) and 12 substitutions between the G2 proteins (2.5%). Thirteen substitutions were found in the first 120 amino acids of G1. Three conserved N-linked glycosylation sites were present in the G1 protein, and one was present in G2. The 57 conserved cysteine residues found in the glycoprotein precursors of other hantaviruses were conserved in NY and RI-1.

Patients, but not rodents, infected with FC consistently develop an immunoglobulin G response against the G1 glycoprotein (19). Most or all of the reactivity against G1 is localized to a single 31-residue linear epitope corresponding to amino acids 58 to 88. The primary sequence of the FC G1 epitope is conserved across a significant portion of the range of *P. maniculatus* (15). Patient RI-1 lacked a detectable immunoglobulin G response to the FC G1 epitope (17). Some insight into a possible basis for the aberrant pattern of serologic reactivity in patient RI-1 can be gained by considering that the RI-1 virus homolog of the FC G1 epitope, LKIESSCNFDLHVPSTSIQ KYNQVEWAKKSS, differs from that of the FC G1 protein, LKIESSCNFDLHVPATTTQKYNQVDWTKKSS, at five positions. Initial attempts to express the RI-1 virus G1 protein have been unsuccessful (data not shown), so it is not possible at present to demonstrate the presence of serum antibodies against the RI-1 virus G1 protein in patient RI-1. It is unknown at present whether antibodies to RI-1 virus neutralize FC or vice versa, but it is known that many monoclonal antibodies against G1 are neutralizing (9).

Hantaviruses are subject to considerable geographic genetic variation, but there is a high degree of conservation at the level of protein sequence within a given hantavirus species (14, 15, 31). Comparisons between the RI-1 S genome and that of FC revealed a 17% difference at the nucleotide level, with a 6.3% difference at the amino acid level (17). A similar comparison of the NY/RI-1 M segment and that of FC showed an overall nucleotide distance of 23%; the amino acid distances were 7% for G1 and 2.5% for G2. By contrast, the nucleotide and amino acid distances between NY-1 and RI-1 glycoprotein precursor genes were 1.1 and 1.2%, respectively. Of the 14 amino acid differences between NY and RI-1, 12 were located in the G1 protein and 2 were located in the G2 protein. Phylogenetic analyses supported the data from pairwise comparisons, with clear evidence for phylogenetic separation of NY and RI-1 from the FCs from the western United States (Fig. 1), and defined a new branch of hantaviruses associated with HPS.

We have shown that a pathogenic hantavirus, designated NY, occurs in white-footed mice (*P. leucopus*) in New York and that NY is virtually identical to RI-1, a virus implicated in a fatal case of HPS. Hantavirus antibodies have been detected previously in *P. leucopus* in the eastern United States (7, 20, 25, 26), albeit in a spotty distribution (37). Recently, a second case of HPS due to NY was identified within a few kilometers of Shelter Island. The occurrence of cases of HPS outside of the distribution of the deer mouse, *P. maniculatus*, highlights the importance of obtaining an understanding of hantavirus ecology and prevalence throughout the Americas.

There is a growing body of circumstantial evidence that hantaviruses evolve in a close relationship with their predominant rodent hosts (14, 41). As a result, there is a consistency to hantavirus-host evolutionary relationships that can lead to meaningful predictions about the properties of a hantavirus that might be found in association with a given rodent host or about a rodent that might be host to a given hantavirus. The predictability of these relationships is strongly reinforced by the present study, which shows that a hantavirus that is allopatric with but genetically related to that of *P. maniculatus* is present in a host of the same genus.

P. leucopus, the carrier of *Babesia microti* and *Borrelia burgdorferi*, is the predominant peromyscine rodent of the coastal northeastern United States. The range of *P. leucopus* extends as far west as Montana and Arizona (21), but in inland and western states, *P. maniculatus* assumes ecological dominance over other peromyscine rodents. Previous analysis of the S genome of RI-1 virus showed a relatively high phylogenetic affinity for FC and suggested that RI-1 virus could be associated with a peromyscine rodent such as the white-footed mouse. However, genetic studies of the hantaviruses of *Peromyscus* spp. captured in the western United States revealed that FC, not RI-1 virus, is in circulation among western *P. leucopus* animals (17). One explanation for this paradox is that FC has evolved with and become optimized for success in *P. maniculatus* and that other sympatric peromyscine rodents serve as secondary hosts for this ecologically dominant hantavirus. Outside of the range of FC and *P. maniculatus*, NY/RI-1 virus has achieved ecologic success in *P. leucopus.*

Several lines of evidence support our conclusion that NY/RI-1 virus is distinct from FC. Patient RI-1 lacked serologic reactivity against recombinant FC G1 glycoprotein, despite the universal presence of such antibodies in patients with HPS in the western United States. Analysis of the primary sequence of the 31-amino-acid immunodominant epitope of G1 provides a plausible explanation: NY/RI-1 virus has five amino acid substitutions relative to FC. The G1 glycoprotein of NY/RI-1 virus has more amino acid substitutions compared with that of FC than is observed among the most divergent *Peromyscus*-associated viruses in the West. For example, in a comparison of RI-1 and FC there are 10 amino acid substitutions in a 91-amino-acid region of G1 that has been studied extensively for genetic sequence variation. By comparison, even the highly divergent FCs of island-bound (Channel Islands, California) *P. maniculatus* showed a maximum of five substitutions relative to the FCs on the adjacent mainland (15).

Perhaps the most compelling evidence for the novelty of NY/RI-1, however, is based upon the distinctive phylogenetic position of NY/RI-1, as seen in Fig. 1. All western *Peromyscus*- and human-associated hantaviruses, including those of the Channel Islands, constitute an in-group with other FCs, in comparison to NY/RI-1. The distinctive phylogenetic position of NY/RI-1 is consistent with the phylogenetic position of the white-footed mouse, which is well-separated from *P. maniculatus* (39).

To date, the hantaviruses associated with HPS are known or suspected to be associated with rodent hosts of the subfamily Sigmodontinae, family Muridae. The propensity to induce severe pulmonary disease in humans may be a distinctive feature of hantaviruses associated with this rodent subfamily.

Nucleotide sequence accession numbers. The M segment sequences determined in this study have been deposited under GenBank accession numbers U36801 through U36821.

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