EPIZOOTIOLOGY OF *HANTAVIRUS* INFECTIONS IN BALTIMORE: ISOLATION OF A VIRUS FROM NORWAY RATS, AND CHARACTERISTICS OF INFECTED RAT POPULATIONS

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Rats trapped from 14 locations in Baltimore, Maryland, were shown to have antibody to *Hantavirus*. Antibody prevalence rates were higher in residential locations than in parks. Infected rats were obtained continuously over a six-year period from 1980–1985 at five locations, indicating the enzootic nature of this infection in urban rats in the United States. Prevalence of antibody and geometric mean titers increased with rat mass and sexual maturity, suggesting that infections in rats are acquired through age-related mechanisms. Three isolates of *Hantavirus* were obtained from rats. One of these isolates was shown by serologic tests and monoclonal antibody reactivity patterns to be similar, if not identical, to rat virus previously obtained from Philadelphia. The presence of rats in many urban settings and the high prevalence of *Hantavirus* infection in this species suggest that human exposure to this virus is occurring in the United States, although human disease from this exposure has not been recognized.

disease reservoirs; hemorrhagic fever, epidemic; *Rattus norvegicus*

Hantaan virus, the causative agent of Korean hemorrhagic fever in humans, was originally isolated in Korea from the striped field mouse, *Apodemus agrarius coreae* (1). Adaptation of the virus to cell culture (2), and development of serologic assays, allowed demonstration that antigenically related viruses are the cause of diseases in humans, referred to as hemorrhagic fever with renal syndrome, throughout Asia (3), Japan (4), the Soviet Union (5), and Northern Europe (6, 7). Within the past few years the geographic boundaries delimiting areas of known disease in humans have expanded to include Greece (8), Belgium (9), and France (10). Serosurveys have documented human antibody to Hantaan or related virus(es) in the United States (11–13), Canada (14), and Africa (15), although to our knowledge no evidence of human disease has yet been reported.

Hantaan virus has been characterized by biochemical and molecular analyses and is believed to constitute a new genus, *Hantavirus*, in the family Bunyaviridae (16, 17).

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Comparisons of Hantaan and related agents causing hemorrhagic fever with renal syndrome have revealed significant cross-reactive relations that appear to be associated with disease patterns and the different rodent host species (18–20).

Prototype Hantaan virus is maintained in nature by sylvatic rodents primarily of the genus *Apodemus* (1, 21), and is associated with the classic severe form of hemorrhagic fever with renal syndrome called Korean hemorrhagic fever or epidemic hemorrhagic fever (1, 22). Puumala virus is associated primarily with *Clethrionomys glareolus* (23), and is the cause of a milder form of this hemorrhagic fever, called nephropathia epidemica, in Scandinavia, Western Soviet Union, and Europe (24). Prospect Hill virus has been isolated from *Microtus pennsylvanicus* in the United States (25), and although antibodies to this virus have been detected in persons, no human disease has been described (26). A Hantaan-related virus associated with wild *Rattus norvegicus*, and to a lesser extent with *R. rattus*, has been incriminated in urban outbreaks of hemorrhagic fever with renal syndrome in Korea and China (22, 27). Rat-associated viruses have also been implicated in laboratory outbreaks of this hemorrhagic fever in Asia (28, 29), Belgium (30), and England (31).

Experimental *Hantavirus* infections are typically asymptomatic in natural rodent reservoirs such as *Apodemus* (21), *Clethrionomys* (32), and *Rattus* (33). Infected animals show long-term persistence of viral antigen in tissues, accompanied by shedding of virus in bodily secretions and excretions (21, 34). Transmission is believed to occur via fomites contaminated with infectious urine, feces, or saliva (34).

In 1982, *R. norvegicus* from cities in the United States were shown to be infected with a Hantaan-related virus (35, 36), and recently two closely related hantaviruses have been isolated from indigenous rats (13, 37). The prevalence of infection in these rodents varies but can be greater than 50 per cent in some locations (13, 37, 38).

Although the geographic distribution of *Hantavirus* infections in rats has been mapped by various serosurveys (13, 37, 38, 40), few studies have examined the ecology, virology, and epizootiology of infection in a given locale. This report describes the isolation of a Hantaan-related virus from rats in Baltimore, Maryland, and the pertinent characteristics of the rat population in Baltimore as they relate to infection with this agent.

MATERIALS AND METHODS

Collection of wild rats

R. norvegicus were trapped from 14 locations within Baltimore (figure 1), in 1980–1985, using live traps (Tomahawk Trap Co., Tomahawk, WI). Animals were only sporadically collected during 1980 for
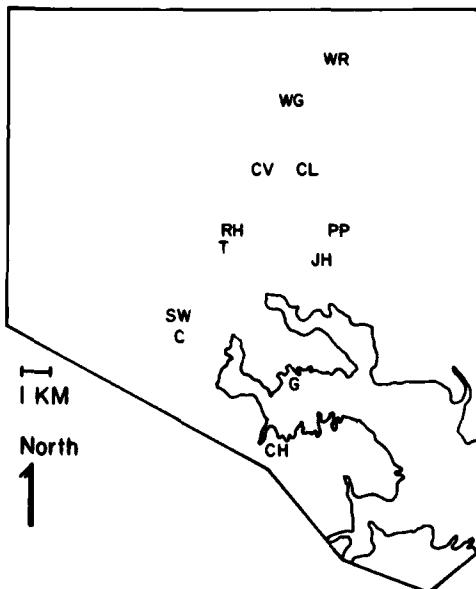
FIGURE 1. Outline map of Baltimore, MD, showing trapping locations used for collecting rats in 1980–1985. The irregular outline indicates the harbor, and granary storage facilities are marked G. Trapping locations were: CV, Charles Village; CH, Cherry Hill; CL, Chilton St.; C, Christian St.; JH, Johns Hopkins; PP, Patterson Park; RH, Reservoir Hill; SW, Smallwood St.; T, Tiffany St.; WR, Western Run; WG, Winston-Govane. The land-use classifications of each site are shown in table 1.

an unrelated study, and intensive collecting of rats did not commence until 1984. Twelve study sites were in multiunit residential areas and two were in urban parklands. At residential locations rats were trapped in alleys located behind dwellings, and in parkland areas rats were trapped along a streambed (Western Run), or on a shrub-covered hillside (Cherry Hill) formerly used as a landfill site. Trap sites were scattered along a north-south axis within the city limits, ranging from harborside locations to areas 10 km from port facilities (figure 1).

Traps were placed in locations approximately one hour before sundown, and were left either 3-4 hours or overnight. Beginning in 1984, locations were sampled with 30 traps placed for three consecutive nights per month, and an effort was made to consistently place traps in the same locations in the alleys. Animals were anesthetized with ketamine HCl and xylazine, sexed, weighed and bled via cardiac puncture. Rats were examined for evidence of sexual maturity (scrotal testes in males; perforate vaginal orifices and/or pregnancy in females), and body mass was used as an indicator of chronologic age (38, 39).

Lungs, kidneys, and spleen were removed aseptically, placed in plastic bags and either stored at −70 C or placed directly in maintenance media (see below) for mechanical dissociation and viral isolation attempts. Adult rats from three sites weighing >300 g were chosen for isolation attempts since animals of this mass were frequently seropositive (38), and previous experience demonstrated that virus could be obtained from seropositive rats (37).

Virus isolation attempts

Details of the procedure used to isolate Hantaan-like viruses have been published previously (37). In the present isolation we used frozen and thawed (n = 2) or fresh lungs (n = 3) from five wild rats dissociated by mechanical blender (Stomacher Blender Model 80, Tekmar Co., Cincinnati, OH) in a 10 per cent (weight/volume) suspension

of maintenance media (Eagle's minimum essential medium containing 10 per cent inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.5 μg/ml fungizone). Dissociated suspensions were allowed to settle without centrifugation, and supernatant fluids were either intramuscularly inoculated into five seronegative Wistar rats (6-8 week-old inbred females; 1.0 ml/rat), or placed on nearly confluent monolayers of Vero E-6 cells at 0.5, 1.0, and 2.0 ml/25 cm^2 flask. Inoculated rats were housed under a laminar flow cage cover with a cage of sham-inoculated control animals. Rats were initially bled at day 30 postinoculation, and then at approximately two-week intervals for evidence of seroconversion. Selected seropositive rats were sacrificed and lung tissues were processed as with wild rat tissues for co-cultivation with Vero E-6 cells.

Tissue-inoculated cell culture flasks were incubated at 37 C for 14 days, decanted, and culture fluids stored at −70 C pending assay. Cell suspensions, obtained from trypsinized monolayers, were 1) passed to cell-free 25 cm^2 flasks; 2) inoculated onto fresh monolayers of 50-70 per cent confluent Vero E-6 cells; 3) placed on 10-well spot slides, incubated overnight at 37 C, then fixed in cold acetone (8 minutes at −20 C). Spot slides were examined for characteristic cytoplasmic *Hantavirus* antigen by indirect immunofluorescent antibody assay, using sera known to contain antibody to Hantaan virus. Cells were similarly passed and examined at weekly intervals until day 45, at which time Hantaan antigen had either been detected or cell adaptation of virus was considered unsuccessful.

Serologic techniques

All wild and laboratory rat sera were screened by indirect immunofluorescent antibody tests using spot slides of Vero E-6 cells, 50-70 per cent infected with prototype 76-118 strain Hantaan virus (prepared by Dr. G. French, Salk Institute, Swiftwater, PA), as described previously (37, 38). Sera diluted at 1:8 and 1:32 were incubated

on spot slides for 30 minutes at 37 C, washed twice in phosphate buffered saline, then incubated for 30 minutes after the addition of fluorescein conjugated anti-rat immunoglobulin G conjugate (Cappel Laboratories, Westchester, PA). Spot slides were washed, dried, and covered after mounting in 10 per cent glycerol in phosphate buffered saline. Slides were examined for characteristic fluorescence, and sera positive at 1:32 were reexamined by titration in fourfold steps to 1:2,048. Previously, we have shown an indirect immunofluorescent antibody titer of 1:128 to be a better criterion for indicating the presence of neutralizing antibody in rat sera (38), but a titer of 1:32 was considered positive here to maintain continuity with recently published reports on rats from other locations (40, 41). Titer was recorded as the highest serum dilution giving characteristic fluorescence, and titers clearly greater than 1:2,048 were considered equal to 1:4,096 for the purpose of determining geometric mean titer. This procedure underestimated the overall geometric mean titer since titers up to 1:81,920, which have been found in wild rats in Baltimore, were not represented. Geometric mean titer was computed for various groups of rats on the basis of seropositive (≥1:32) animals only.

Plaque reduction neutralization tests were conducted using prototype Hantaan virus (strain 76-118), Girard Point virus, from a rat captured in Philadelphia (37), and a Baltimore isolate of virus, following procedures detailed previously (37). Duplicate 25 cm² flasks of Vero E-6 cells were inoculated with a fixed amount of virus (approximately 100 plaque-forming units) mixed with fourfold dilutions of serum, after prior incubation of the virus-serum mixture at 4 C overnight. Virus-serum inocula were allowed to absorb to cell sheets for one hour, after which flasks were overlayed with a 1 per cent nutrient agarose containing 10 per cent fetal bovine serum, 4 per cent L-glutamine, and 1 per cent nonessential amino acids in addition to Eagle's minimum essential medium mixed with antibiotics. Flasks were incubated for 7-9 days, depending on virus strains, then overlayed and stained with a second agarose sheet containing a final dilution of 0.167 mg/ml neutral red. Plaques were counted at 24-48 hours post-staining, and 80 per cent and 50 per cent titers were computed on the basis of reductions in plaque dose. Negative and positive control sera were included in all indirect immunofluorescent antibody and plaque-reduction neutralization tests, and neutralization tests were replicated at least twice for each virus-serum combination.

Antigenic characterization of isolates

Spot slides of Girard Point virus and a Baltimore isolate obtained from tissue culture were prepared by mixing cells from cultures exhibiting nearly 100 per cent characteristic Hantaan antigen fluorescence, with equal volumes of uninfected Vero E-6 cells. Mixed cell suspensions were placed on 10-well spot slides, incubated overnight at 37 C, fixed in cold acetone (−20 C, 8 minutes) and stored at −70 C prior to use. Parallel indirect immunofluorescent antibody and plaque-reduction neutralization assays were run using the 76-118 strain of Hantaan virus, Girard Point virus, and Baltimore isolate. Each virus was tested against 1) Hantaan monoclonal antibody or mouse hyperimmune ascites fluid (hybridoma prepared by Dr. J. B. McCormick, Centers for Disease Control, Atlanta, GA); 2) convalescent-phase sera from patients with Korean hemorrhagic fever; 3) positive and negative wild rat sera; 4) convalescent-phase sera from patients with nephropathia epidemica; and 5) polyvalent reovirus antiserum.

Data analysis

Statistical analyses of data were performed using 1984 SAS programs (42) resident on an IBM 370 system located at The Johns Hopkins University School of Hygiene and Public Health. Specific tests and significance levels are reported in the text.

RESULTS

Geographic distribution and prevalence of antibody in Norway rats

Overall, 525 Norway rats were trapped from 1980–1985 and 253 (48.2 per cent) had indirect immunofluorescent antibody titers of ≥32 to Hantaan virus. Seropositive rats were found at all sites (table 1). Crude prevalence rates, from areas where at least 20 rats were captured, varied considerably, ranging from 69.5 per cent at Port Street to 20.0 per cent at Western Run. Rats trapped from residential sites had higher prevalences compared with animals from parkland locations. Rats from parkland locations were also significantly smaller than those originating from residential sites, which probably influenced the infection rate (see below). Conclusions concerning trends in prevalence among these study sites were limited by variations in sample

sizes and population dynamics over the six years of study. However, the occurrence of positive animals at each sampled site indicates the widespread dissemination of this virus in rats in Baltimore.

At five locations rats were trapped at intervals spanning 5–6 years (table 2). Although sample sizes were sometimes small, the data indicate that infected rats have been present at each site continuously over the entire sampling period, and that a Hantaan-related virus has been enzootic in rat populations in Baltimore for at least six years.

Association between antibody prevalence and titer with body mass

The relations between body mass and antibody prevalence (figure 2) and geometric mean titer (figure 3) were determined for the entire sample of 525 rats. Antibody prevalence rates increased steadily with

TABLE 1

Summary of serosurvey results for Norway rats in Baltimore, conducted in 1980–1985, for antibody to Hantaan virus as measured by indirect immunofluorescent antibody tests

Trap location	Habitat	n	% positive*
Patterson Park	MUR†		
Port St.		96	69.8
Chase St.		62	43.5
Bradford St.		38	63.2
Reservoir Hill	MUR	74	48.6
Winston-Govane	MUR	66	50.0
Charles Village	MUR	26	38.5
Johns Hopkins	MUR		
Castle St.		15	40.0
Washington St.		33	36.4
Smallwood St.	MUR	6	66.7
Christian St.	MUR	5	40.0
Tiffany St.	MUR	2	100.0
Chilton St.	MUR	2	100.0
Unspecified§	MUR	6	33.3
Cherry Hill	UP‡	74	29.7
Western Run	UP	20	20.0
Total		525	48.2

* Indirect immunofluorescent antibody titer ≥1:32.
† MUR, multiunit residential area of high human population density.
‡ UP, urban parkland area.
§ Obtained from Bureau of Rat Control, Baltimore City.

TABLE 2

Long-term trapping results for Norway rats collected in 1980–1985 from five sites in Baltimore, showing evidence of enzooticity of Hantaan-related infections in rats

Location and year	n	% positive*	GMT†
Port St.			
1980	2	100.0	12.0
1983	13	61.5	11.6
1984	65	73.8	10.3
1985	16	56.3	11.0
Chase St.			
1980	21	33.3	10.6
1983	4	25.0	12.0
1984	13	69.2	10.7
1985	24	41.7	9.0
Bradford St.			
1980	10	80.0	10.7
1982	9	66.7	9.4
1985	19	52.6	10.7
Charles Village			
1980	19	42.1	11.0
1984	7	28.6	7.0
Reservoir Hill			
1980	51	56.9	10.3
1981	8	25.0	12.0
1983	5	40.0	11.5
1985	10	30.0	9.3

* Indirect immunofluorescent antibody titer ≥1:32.
† Log₂ geometric mean titer of seropositive rats.
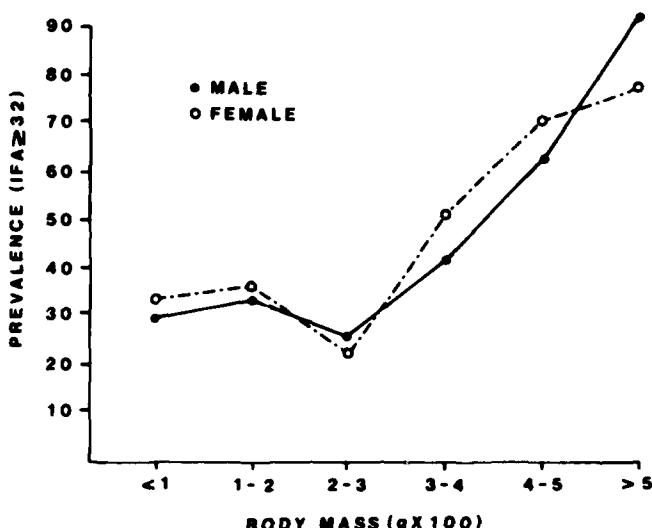
FIGURE 2. The relation between antibody prevalence (indirect immunofluorescent antibody titer ≥1:32 considered positive) and body mass for 525 Norway rats captured in Baltimore in 1980–1985. Rats were separated by sex then grouped into 100 g mass classes.
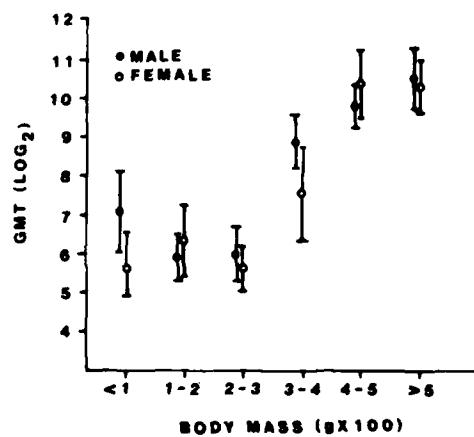

FIGURE 3. The relation between $\log_2$ geometric mean titer ($\log_2$ GMT) and body mass for 253 seropositive Norway rats captured in Baltimore in 1980–1985. Rats were separated by sex then grouped into 100 g mass classes. Bars indicate 95 per cent confidence intervals.

mass above 300 g. Rats under 300 g, when compared with larger animals, were less frequently seropositive and had lower antibody titers when positive. Using a G test, two nonoverlapping sets of prevalence classes could be distinguished based on mass; rats ≤400 g formed a low prevalence

group with overall (both sexes combined) prevalences ranging from 24.4 per cent to 45.2 per cent, and rats >400 g formed a high prevalence group with prevalences ranging from 65.6 per cent to 85.0 per cent ($G = 84.11; p < 0.001$, 5 degrees of freedom).

In comparison, analysis of variance of the geometric mean titers, based on body mass, distinguished three separate groups, although the overall trend was similar to that found for prevalence. The three groups were comprised of rats in mass class <300 g, with a $\log_2$ geometric mean titer ranging between 5.7 and 6.3; rats in the mass class 300–399 g with a geometric mean titer of 8.37; and rats ≥400 g with geometric mean titers ranging from 10.1 to 10.5 ($F = 31.34$; $p < 0.0001$; GT2 test). On the basis of the relations between prevalence, geometric mean titer, and mass, we have divided our rats into two groups for future analyses, using the body mass of 300 g as the dividing criterion.

Variation of antibody prevalence with rat sex and location

No stratification of rats on basis of sex was necessary, because overall antibody

prevalence rates did not differ between males and females (49.1 per cent males positive, $n = 286$; 47.0 per cent females positive, $n = 239$: $\chi^2 = 1.97$; $p = 0.66$), and no difference in prevalence was found between the sexes in any mass class. Similarly, males and females showed no significant difference in geometric mean titers (males, 8.93 ± 2.16; females, 8.63 ± 2.10; $F = 0.48$; $p = 0.49$).

Rat mass varied with trapping locations and sex. Two-way analysis of variance showed that male rats were significantly heavier than females (mean body mass, males = 335.1 ± 138.4; females = 307.7 ± 148.8; $F = 5.48$; $p = 0.02$), and residential rats were significantly heavier than parkland rats (mean body mass, residential rats = 346.3 ± 143.1; parkland rats = 217.5 ± 89.9; $F = 73.4$; $p < 0.0001$). The interaction of sex and habitat was insignificant, so that male rats tended to be heavier than female rats regardless of habitat, and residential rats tended to be heavier than parkland rats regardless of sex. Partitioning for these covariates showed there were no significant differences between residential rats and parkland rats regarding mass associations with antibody prevalence or with geometric mean titer.

Indices of sexual maturation

Sexual maturation was correlated with rat mass (table 3). The proportions of male rats with scrotal testes and female rats with perforate vaginal orifices increased through mass classes up to 200 g, at which time nearly 100 per cent of the animals displayed sexual signs of adulthood. Pregnancy in females lagged slightly behind the presence of perforate vaginal orifices. The per cent of pregnant females was relatively constant at 32.6–40.9 per cent in the mass classes between 200 and 499 g, while an increase was seen in the largest mass class (>500 g). This difference in pregnancy rates in the four mass classes above 200 g was significant ($\chi^2 = 10.06$; $p = 0.02$), although there was no significant difference in mass classes between 200 and 499 g ($\chi^2 = 0.49$; $p = 0.78$).

Seasonal patterns in antibody prevalence

City-wide, the antibody prevalence rates for rats showed significant variation by season. The lowest rate occurred in the spring (March–May), and prevalence increased linearly through winter (December–January). During this interval, crude prevalence almost doubled from 36.8 per cent ($n = 114$) to 68.5 per cent ($n = 54$) ($\chi^2 = 20.38$; $p < 0.0001$). This variation was primarily due to changes in antibody prevalence among rats <300 g, which increased from 17.2 per cent in spring ($n = 58$) to 50.0 per cent ($n = 16$) in winter ($\chi^2 = 8.12$; $p = 0.044$). Rats with body mass >300 g showed no significant seasonal variation in prevalence ($\chi^2 = 6.38$; $p = 0.10$). The significant trend in antibody prevalence by season in smaller animals may have been due to the tendency for larger rats to lose mass during the winter and therefore appear in lower mass classes. In addition, trapping effort was not equally distributed across all city locations over all seasons, so that a trap location bias may be present.

When we examined seasonal antibody

TABLE 3

Measures of sexual maturation in Norway rats in Baltimore, 1980–1985, based on 100 g mass classes

	Mass class (g)					
	0–99	100–199	200–299	300–399	400–499	≥500
Scrotal males*	8/18†	21/33	40/40	76/77	65/66	29/29
Perforate females‡	3/23	33/47	36/39	41/41	48/48	29/29
Pregnant females	0/22	0/27	9/22	12/32	15/46	17/24

* Male rats with descended testes indicating sexual maturity.
† No. of rats with trait per mass class/no. of rats examined per mass class.
‡ Female rats with perforate vaginal orifices indicating sexual maturity.
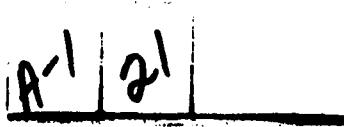
prevalence from a single alley, from which a sufficient number ($n > 10$) of rats were trapped during all seasons, there appears to be no consistent trend. A detailed analysis, from Port Street, of antibody prevalence rates and indirect immunofluorescent antibody titers as a function of rat mass and season is shown in figure 4. Crude prevalence rates varied from a high of 87.5 per cent in winter (December 1983–February 1984) to a low of 61.5 per cent in fall (August–October 1983). Large rats (>300 g) have consistently higher prevalence rates (81.5 per cent positive), and higher geometric mean titers (10.3 ± 1.5) than small (<300 g) rats (38.9 per cent positive; geometric mean titers = 6.5 ± 1.1), but overall seasonal variations were slight. The large number of small rats caught during the summer and fall months tended to decrease crude prevalence rates.

Isolation of virus

Wistar rats inoculated with wild rat lung tissue first showed evidence of seroconversion to Hantaan virus at day 26–3P (table 4), with the majority seroconverting by day 40–44 (56 per cent). Only one group of rats (receiving tissue from wild rat 30) failed to seroconvert to high titers (maximum indirect immunofluorescent antibody titer = 1:32), and no virus was isolated from these animals. Rats in other inoculated groups, with the exception of groups receiving tissues from rat 58, seroconverted with most (14 of 15) indirect immunofluorescent antibody titers becoming >2,048. The five rats receiving tissues from rat 58 achieved only moderate titers (range 1:32–1:512), but virus was successfully isolated in cell culture directly from the wild rat tissue. The inoculated rats maintained high indirect immunofluorescent antibody titers to the Baltimore virus for more than one year, at which time all rats were sacrificed (table 4).

Three virus isolates were obtained from either direct co-cultivation of dissociated wild rat lung tissue with Vero E-6 cells (rat 58), or from subsequent co-cultivation of lung tissue obtained from seropositive Wistar rats (rats 59 and 60). Isolation of virus from rat 51 was considered unnecessary. Distinctive cytoplasmic immunofluorescence was first seen on spot slides prepared from cell suspensions 21 days following di-
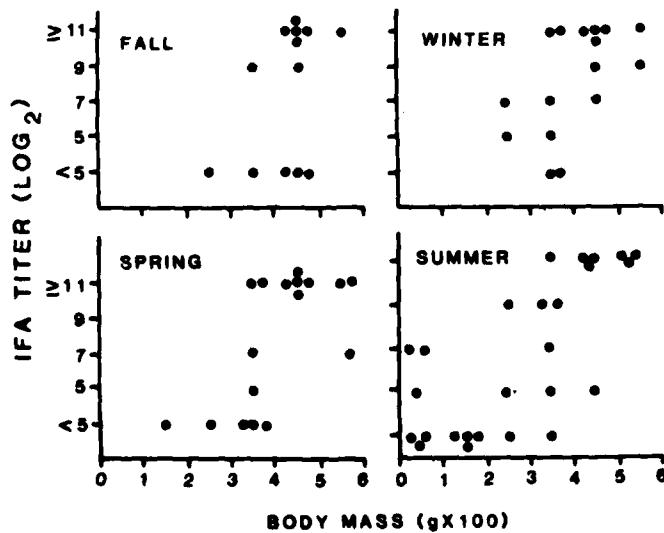

FIGURE 4. Seasonal variation in indirect immunofluorescent antibody titers and body mass for 72 Norway rats trapped from a single alley in Baltimore in 1983–1984.

TABLE 4

Seroconversion of groups of five Wistar rats to a Hantaan-related virus following inoculation with wild caught Baltimore rat lung tissues

Wild rat no.	IFA titer	Day postinoculation (IFA* titers >1:128)					
		0	26–30	40–44	60–64	90–94	360–364
30	>1:2,048	0/5†	0/5	0/5	0/5	0/5 (5)‡	NA§
51	>1:2,048	0/5	0/5	3/5	3/5 (2)	3/3 (3)	NA
58	1:2,048	0/5	0/5	1/5	3/5 (1)	3/4 (4)	NA
59	>1:2,048	0/5	3/5	5/5 (1)	4/4	4/4	4/4
60	1:128	0/5	4/5	5/5 (1)	4/4	4/4	4/4
Control	<1:8	0/5	NA	0/5	0/5	0/5 (5)	NA

* Indirect immunofluorescent antibody (IFA) test.
† No. of rats with antibody >1:128/no. of inoculated rats.
‡ Number of rats sacrificed is shown in parentheses.
§ NA, not available, either all rats sacrificed or not tested.

rect inoculation of tissues from rat 58 onto flasks containing Vero E-6 monolayers. Approximately 10 per cent of the cells examined on spot slides contained antigen on day 21 postinoculation, 75 per cent on day 28, and 100 per cent on day 35. Aliquots of day 35 supernatant fluid were frozen, and infectivity was demonstrated by plaque assay. Virus seed stock was made by inoculation of 1 ml of this fluid onto each of five 150 cm² culture flasks containing Vero E-6 cells and harvesting the supernatant fluids on day 10. The plaque-forming titer of this material was determined to be approximately 1.1 × 10⁶ plaque-forming units/ml, and this virus seed stock was used in all subsequent analyses.

Co-cultivation of seropositive Wistar lung tissue with Vero E-6 cells yielded similar results, although cytoplasmic immunofluorescence was slower to appear. Approximately 5 per cent of cells examined were infected on day 28, and 100 per cent fluorescence did not occur until day 42. Reference virus seed stocks of these isolates were made in the manner described previously.

Antigenic characterization of isolates

Spot slides of cells infected with the 76-118 and Girard Point strain of *Hantavirus* and the Baltimore isolate of virus were tested against serial twofold dilutions of nine immune reagents by indirect immu-

nofluorescent antibody (table 5). Comparable endpoint antibody titers were seen against all three antigens, with the largest difference being a twofold lower reaction of convalescent-phase serum from a Korean hemorrhagic fever patient against the two United States rat isolates. No reactions were detected with reference reovirus antiserum or negative control rat serum.

When tested by plaque-reduction neutralization tests, homologous reactions of convalescent-phase Korean hemorrhagic fever patient serum with Hantaan virus were clearly distinct from heterologous reactions with Girard Point or the Baltimore isolate (table 6). A monoclonal antibody, directed against one, or both, of the glycoproteins of Hantaan virus (C. S. Schmaljohn, personal communication, 1985) neutralized all strains of virus, although a fourfold greater 50 per cent plaque-reduction neutralization titer was seen against Hantaan. Baltimore and Girard Point reacted similarly to all antisera with the greatest difference in endpoint titer being twofold. None of the isolates was neutralized by nephropathia epidemica patient serum, negative rat serum, or reference reovirus antiserum. These results suggest that the Baltimore isolate and Girard Point virus are closely related, and both are distinct from Hantaan virus.

The Baltimore isolate was further compared with Hantaan and Girard Point vi-

TABLE 5

Comparison of Hantaan, Girard Point, and a Baltimore isolate of Hantavirus by indirect immunofluorescent antibody assay

Antibody	Virus		
	Hantaan	Girard Point	Baltimore
Hantaan MHAF*	2,048†	2,048	2,048
Korean hemorrhagic fever patient serum‡	2,048	1,024	1,024
Girard Point lab rat§	16,384	16,384	16,384
Baltimore lab rat§	32,768	32,768	32,768
Baltimore wild rat§	4,096	4,096	4,096
Nephropathia epidemica patient serum‖	16,384	16,384	16,384
Negative rat	<8	<8	<8
Polyvalent reovirus	<8	<8	<8

* Mouse hyperimmune ascitic fluid against Hantaan virus, strain 76-118.

† Reciprocal of highest serum titer showing characteristic fluorescence; italicized numbers indicate homologous relation.

‡ Convalescent-phase serum.

§ Rat immune sera from laboratory animals or wild caught animals infected with Girard Point or Baltimore isolates of *Hantavirus*.

‖ Convalescent-phase serum.

TABLE 6

Comparison of Hantaan, Girard Point, and Baltimore isolates of Hantavirus by plaque reduction neutralization tests

Antibody*	Virus					
	Hantaan		Girard Point		Baltimore	
	80†	50	80	50	80	50
HC02-BH11‡	64	512§	64	128	64	128
Korean hemorrhagic fever patient serum	64	128	<8	16	<8	8
Girard Point lab rat	16	64	128	256	64	128
Baltimore wild rat	<8	8	>2,048	>2,048	>2,048	>2,048
Nephropathia epidemica patient serum	<8	<8	<8	<8	<8	<8
Negative rat	<8	<8	<8	<8	<8	<8
Polyvalent reovirus	<8	<8	<8	<8	<8	<8

* As designated in table 5, except as noted.

† Per cent reduction in plaque dose.

‡ Mouse monoclonal antibody produced against Hantaan virus, strain 76-118.

§ Reciprocal of highest titer neutralizing plaque dose; italicized numbers indicate homologous relation.

ruses by three different groups of monoclonal antibodies raised against prototype Hantaan virus, strain 76-118 (table 7). Monoclonal antibodies in the groups EC02 and HC02 were broadly crossreactive against all three viruses. Monoclonal antibody HC02-BH11 reacted at lower serum dilutions against Baltimore, compared with Hantaan and Girard Point, although this antibody neutralized Baltimore and Girard Point viruses to the same extent. Monoclonal antibodies in the group BD01 reacted with Hantaan virus but not with either Baltimore or Girard Point, again indicating the similarities between the two domestic isolates and their distinctiveness from Hantaan.

DISCUSSION

The serosurvey of Norway rats in Baltimore indicates that *Hantavirus* infections are geographically widespread, and have been enzootic in this city for at least six years. Seropositive rats were found at all

TABLE 7

Comparison of Hantaan virus, Girard Point virus, and a Baltimore isolate of Hantavirus by immunofluorescent antibody assay using monoclonal antibodies produced against Hantaan virus, strain 76-118

Monoclone*	Virus		
	Hantaan	Girard Point	Baltimore
EC02-BG01	>2,048†	>2,048	>2,048
EC02-BG05	>2,048	>2,048	>2,048
EC02-BA01	>2,048	>2,048	>2,048
HC02-BH11	>2,048	512	>2,048
HC02-BC10	>2,048	>2,048	>2,048
HC02-BE11	2,048	2,048	2,048
BD01-BD12	>2,048	<8	<8
BD01-BB08	>2,048	<8	<8
BD01-BG01	>2,048	<8	<8

* Hybridoma produced by Dr. J. B. McCormick, Centers for Disease Control, Atlanta, GA; ascitic fluids produced in volume by Dr. G. French, Salk Institute, Swiftwater, PA.

† Reciprocal of highest dilution tested giving characteristic fluorescence.

14 sampled locations, during all sampled time intervals, although antibody prevalence rates and geometric mean titers showed considerable variation. This variation is expected when considering only crude prevalence rates, since our data clearly establish that the presence of indirect immunofluorescent antibody and endpoint titers are strongly associated with rat mass.

As we have commented previously (38), associations between increasing prevalence of anti-Hantavirus antibody and geometric mean titers with increasing rat mass are of particular interest since they may indicate an age or maturational stage when viral transmission is occurring. Mass is a good indicator of age in wild-caught, pen-reared Norway rats (43), and has been used to group wild-trapped Norway rats in Baltimore into maturational stages (39). Our data indicate that the median mass at sexual maturity for both sexes is between 100–200 g, although no females <200 g were pregnant. Based on comparison with pen-

reared populations, this indicates an approximate median age at sexual maturity of 100 days. Rates of growth vary between the sexes and between seasons of the year; however, we believe that mass is a good indicator of chronologic and functional age and is useful in determining natural patterns of Hantavirus transmission in wild rodent populations.

The data show that low antibody prevalence rates and titers are associated with juvenile or young adult rats. High prevalence rates and high geometric mean titers are associated with large, sexually mature animals. This suggests that rats are becoming infected with this Hantavirus in some age-dependent manner that may relate to time of exposure or susceptibility. Exposure to this virus may be relatively constant since prevalence rates continue to increase as rats gain mass (figure 2), although the geometric mean titer levels off after the 400 g mass class (figure 3). Exposure to this virus probably begins at an early age, assuming some lag time between infection and production of antibody.

Recently, Arikawa et al. (41) reported that a higher proportion of Norway rats over six months of age were seropositive (64 per cent), compared with younger animals (9 per cent), in Japan. In this study, eye lens weight was used as an aging technique and the authors hypothesized that close contact in contaminated nests over the winter may be important for effective Hantavirus transmission (41). Prior to this publication, which supports our original observations on age-dependent transmission in urban rats (38), Arikawa et al. (44) reported higher antibody prevalence rates, but lower antibody titers, in very small rats (mass <50 g) relative to larger individuals (44). The apparent discrepancies between these two reports have not been resolved and it is impossible to state that age-dependent transmission of hantaviruses is a consistent feature of rat infection.

Laboratory experiments in which Hantaan virus, or Hantavirus containing tissue, has been inoculated into rats demonstrate

that peak immunofluorescent antibody titers are obtained by day 40 postexposure (21, 33). Our inoculated rats showed a similar response, and maintained high immunofluorescent antibody titers for one year (table 4). Although direct tissue inoculation of virus is probably not a major route of infection, except in instances of intraspecific wounding, these data demonstrate that rats can show a detectable antibody response within weeks after infection. Our data suggest that most seroconversions are occurring after rats reach masses of 300 g, or about 100–150 days of age. Weaning is completed at approximately 40 days of age (43), so it appears rats are beginning to become infected soon after this time.

Weaning may be a critical time for exposure to enzootic pathogens in rats. Rats receive maternal antibody across the placenta, but the majority of passively acquired antibody is obtained by nursing during the first 20 days following birth (45). It is possible that young rats from seropositive mothers are protected from *Hantavirus* infection for extended periods of time after birth. Most reproductively active rats in Baltimore are already seropositive (figure 2; table 3). Our smallest rats, which are all post-weaning, have only low virus-specific immunoglobulin G titers that may represent the waning of maternally derived antibody. However, these data do not preclude the possibility of earlier, or even congenital, infection with a delayed onset of antibody production. We plan to test these hypotheses by looking at immunoglobulin M levels and the presence of viral antigen in tissues obtained from different mass rats, and by breeding experimentally infected laboratory females and examining antibody levels in the young. We hope to eventually challenge young rats of different ages, with and without maternal antibody, to determine if this antibody is protective.

The demonstration that seropositive rats were present in Baltimore, at least as early as 1980, strengthens the hypothesis that *Hantavirus* infection in commensal rats represents a stable, long-term association between virus and rat, and not a recently introduced infection originating from port cities of Asia (35, 36). Transmission may require close animal-to-animal contact (41) and Norway rat movements in urban settings cover short distances (<100 m) (46). These factors would suggest that *Hantavirus* spread would be slow, and would require a significant period of time to spread throughout the city.

Seropositive rats have now been found worldwide (40), and viral isolates have been made from rats in the United States (13, 37), South America (47), Korea (27), China (48), and Japan (18). It is apparent from the results of our cross-neutralization tests and monoclonal antibody reactivity that the Baltimore rat isolate should be considered a member of this complex of viruses, since it is virtually indistinguishable from Girard Point virus. All of the rat virus isolates examined by serologic methods have been found to be closely related (20). It now appears that hantaviruses may be antigenically grouped, using immunologic methods, more accurately by their rodent host than by geographic region of isolation.

The current lack of recognition of human disease associated with hantaviruses from wild rats outside of Asia may be due to many factors, including low frequency of occurrence, misdiagnosis and geographic strain variation in virus(es). Rat-associated hantaviruses have been implicated in human cases of hemorrhagic fever with renal syndrome in laboratory settings outside of Asia (30, 31). Antibody to various hantaviruses has been shown in a number of persons from a variety of locations in North America (11–14, 26), although neutralizing antibody has been less frequently found than indirect immunofluorescent antibody. In part, some of the difficulty in demonstrating specific neutralizing antibody may be the lack of appropriate virus strains to use as screening agents. The results of cross indirect immunofluorescent antibody tests show the utility of this test as a screening tool (table 5), but the cross-neutralization tests unequivocally demonstrate the need

to match homologous serum and virus to differentiate strains of *Hantavirus* (table 6). Future serosurveys in the United States need to include a panel of hantaviruses, such as a rat virus, Hantaan virus, and a vole isolate such as Prospect Hill virus (26), as test agents in plaque-reduction neutralization assays to help distinguish the virus responsible for human antibody.

The common occurrence of rats in urban centers, such as Baltimore, coupled with the persistent nature of this infection in these animals, suggests that the risk of human exposure to this agent may be high. Epidemiologic studies are needed to examine the association between antibody-positive humans and the presence of specific risk factors, such as exposure to rodents at home or workplace. The possibility of clinical disease due to rat-borne hantaviruses in the United States is real, and the medical community should be aware of these viruses as potential pathogens when making differential diagnoses of patients with acute onset of febrile illnesses with accompanying renal involvement.

REFERENCES

1. Lee HW, Lee PW, Johnson KM. Isolation of the etiological agent of Korean hemorrhagic fever. J Infect Dis 1978;137:298–308.
2. French GR, Foulke RS, Brand OA, et al. Korean hemorrhagic fever: propagation of the etiologic agent in a cell line of human origin. Science 1981;211:1046–8.
3. Lee PW, Gajdusek DC, Gibbs CJ Jr, et al. Aetiological relation between Korean hemorrhagic fever with renal syndrome in People's Republic of China. Lancet 1980;1:819–20.
4. Lee HW, Lee PW, Tamura M, et al. Etiological relation between Korean hemorrhagic fever and epidemic hemorrhagic fever in Japan. Biken J 1979;22:41–5.
5. Chumakov MP, Gavrilovskaya VA, Boiko MA, et al. Detection of hemorrhagic fever with renal syndrome (HFRS) virus in the lungs of bank voles *Clethrionomys glareolus* and red backed voles *Clethrionomys rutilus* trapped in HFRS foci in the European part of U.S.S.R., and serodiagnosis of this infection in man. Arch Virol 1981;69:295–300.
6. Svedmyr A, Lee HW, Berglund A, et al. Epidemic nephropathy in Scandinavia is related to Korean hemorrhagic fever. (Letter.) Lancet 1979;1:100.
7. Lee HW, Lee PW, Lähdevirta J, et al. Aetiological relation between Korean hemorrhagic fever and nephropathia epidemica. Lancet 1979;1:186–7.
8. Lee HW, Antoniadis A. Serological evidence for

Korean haemorrhagic fever in Greece. (Letter.) Lancet 1981;1:832.
9. Van Ypersele de Strihou C, Vandenbroucke JM, Levy M, et al. Diagnosis of epidemic and sporadic interstitial nephritis due to Hantaan-like virus in Belgium. (Letter.) Lancet 1983;2:1493.
10. Chanard J, Melin J-P, Milcent T, et al. Focus of Muroid virus nephropathy in France. (Letter.) Lancet 1984;1:98.
11. Gibbs CJ Jr, Takenaka A, Franko M, et al. Seroepidemiology of Hantaan virus. Lancet 1982;2:1406–7.
12. Yanagihara R, Chin C-T, Weiss MB, et al. Serological evidence of Hantaan infection in the United States. Am J Trop Med Hyg 1985;34:396–9.
13. Tsai TF, Bauer SP, Sasso DR, et al. Serological and virological evidence of a Hantaan virus-related enzootic in the United States. J Infect Dis 1985;152:126–36.
14. Lee HW, Seong IW, Baek LJ, et al. Positive serological evidence that Hantaan virus, the etiologic agent of hemorrhagic fever with renal syndrome, is endemic in Canada. Can J Microbiol 1984;30:1137–40.
15. Gonzales JP, McCormick JB, Baudon D, et al. Serological evidence for Hantaan-related virus in Africa. Lancet 1984;2:1036–7.
16. Schmaljohn CS, Hasty SE, Harrison SA, et al. Characterization of Hantaan virions, the prototype virus of hemorrhagic fever with renal syndrome. J Infect Dis 1983;48:1005–12.
17. Schmaljohn CS, Dalrymple JM. Analysis of Hantaan virus RNA; evidence for a new genus of Bunyaviridae. Virology 1983;131:482–91.
18. Yamanishi K, Dantas JR Jr, Takahashi M, et al. Antigenic differences between two viruses, isolated in Japan and Korea, that cause hemorrhagic fever with renal syndrome. J Virol 1984;52:231–7.
19. Song G, Hang C-J, Liao H-X, et al. Antigenic difference between viral strains causing classical and mild types of epidemic hemorrhagic fever with renal syndrome in China. J Infect Dis 1984;150:889–94.
20. Schmaljohn CS, Hasty SE, Dalrymple JM, et al. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science 1985;227:1041–4.
21. Lee HW, French GR, Lee PW, et al. Observations on natural and laboratory infection of rodents with the etiologic agent of Korean hemorrhagic fever. Am J Trop Med Hyg 1981;30:477–82.
22. Song G, Hang C-S, Qui X-Z, et al. Etiologic studies of epidemic hemorrhagic fever (hemorrhagic fever with renal syndrome). J Infect Dis 1983;147:654–9.
23. Brummer-Korvenkontio M, Vaheri A, Hovi T, et al. Nephropathia epidemica: detection of antigen in bank voles and serologic diagnosis of human infection. J Infect Dis 1980;141:131–4.
24. Lahdevirta J. Nephropathia epidemica in Finland. Ann Clin Res 1971;3(suppl 8):1–154.
25. Lee PW, Amyx HL, Gajdusek DC, et al. New haemorrhagic fever with renal syndrome-related virus in indigenous wild rodents of the United States. (Letter.) Lancet 1982;2:1405.

26. Yanagihara R, Gajdusek, GC, Gibbs CJ Jr, et al. Prospect Hill virus: serologic evidence for infection in mammalogists. N Engl J Med 1984;310:1325-6.

27. Lee HW, Baek LJ, Johnson KM. Isolation of Hantaan virus, the etiologic agent for Korean hemorrhagic fever, from wild urban rats. J Infect Dis 1982;146:638-44.

28. Lee HW, Johnson KM. Laboratory acquired infections with Hantaan virus, the etiologic agent of Korean hemorrhagic fever. J Infect Dis 1982;146:645-51.

29. Umenai T, Lee HW, Lee PW, et al. Korean haemorrhagic fever in staff in an animal laboratory. Lancet 1979;1:1314-6.

30. Desmyter J, LeDuc JW, Johnson KM, et al. Laboratory rat associated outbreak of haemorrhagic fever with renal syndrome due to Hantaan-like virus in Belgium. Lancet 1983;2:1445-8.

31. Lloyd G, Bowen ETW, Jones N, et al. HFRS outbreak associated with laboratory rats in UK. Lancet 1984;1:1175-6.

32. Yanagihara R, Svedmyr A, Amyx H, et al. Isolation and propagation of nephropathia epidemica virus in bank voles. Scand J Infect Dis 1984;16:225-8.

33. Lee PW, Amyx HL, Gibbs CJ Jr, et al. Propagation of Korean hemorrhagic fever virus in laboratory rats. Infect Immun 1981;31:334-8.

34. Lee HW, Lee PW, Baek LJ, et al. Intraspecific transmission of Hantaan virus, etiologic agent of Korean hemorrhagic fever, in the rodent Apodemus agrarius. Am J Trop Med Hyg 1981;30:1106-12.

35. Tsai TF, Bauer SP, Sasso DR, et al. Preliminary evidence that Hantaan or a closely related virus is enzootic in domestic rodents. N Engl J Med 1982;307:623-4.

36. LeDuc JW, Smith GA, Bagley LR, et al. Preliminary evidence that Hantaan or a closely related virus is enzootic in domestic rodents. (Letter.) N Engl J Med 1982;307:624.

37. LeDuc JW, Smith GA, Johnson KM. Hantaan-like viruses from domestic rats captured in the United States. Am J Trop Med Hyg 1984;33: 992-8.

38. Childs JE, Korch GW, Smith GA, et al. Geographical distribution and age related prevalence of antibody to Hantaan-like virus in rat populations of Baltimore, Maryland, USA. Am J Trop Med Hyg 1985;34::385-7.

39. Farhang-Azad A, Southwick CH. Population ecology of Norway rats in the Baltimore Zoo and Druid Hill Park, Baltimore, Maryland. Ann Zool 1979;15:1-42.

40. LeDuc JW, Smith GA, Childs JE, et al. Global survey of antibody to Hantaan-related viruses among peridomestic rodents. Bull WHO 1986;64:139-44.

41. Arikawa J, Takashima I, Hashimoto N, et al. Epidemiological studies of hemorrhagic fever with renal syndrome (HFRS) related virus infection among urban rats in Hokkaido, Japan. Arch Virol 1986;88:231-40.

42. SAS user's guide: statistics. Version 5 ed. Cary, NC: SAS Institute, Inc., 1985.

43. Calhoun JB. The ecology and sociology of the Norway rat. Washington DC: US GPO, 1983.

44. Arikawa J, Takashima I, Hashimoto N, et al. Epidemiological study of hemorrhagic fever with renal syndrome related virus infection among urban rats in two islands in Tokyo Bay, Japan. Acta Virol 1985;29:66-72.

45. Halliday R. Prenatal and postnatal transmission of passive immunity to young rats. Proc R Soc Lond 1955;144:427-30.

46. Davis DE, Emlen JT, Stokes AW. Studies on home range in the brown rat. J Mammology 1948;29:207-25.

47. LeDuc JW, Smith GA, Pinheiro P, et al. Isolation of a Hantaan-related virus from Brazilian rats and serologic evidence of its widespread distribution in South America. Am J Trop Med Hyg 1985;34:810-5.

48. Song G, Hang C, Liao H, et al. Isolation of EHF-related agent from Rattus norvegicus captured from patients' home in an endemic area of the mild type of hemorrhagic fever. Acta Microbiol Sin 1982:22;273-7.