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EPIDEMIOLOGY OF HANTAVIRUS INFECTIONS IN BALTIMORE

ANNUAL REPORT

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).



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INTRODUCTION

Hantaviruses (Family Bunyaviridae) are the etiological agents responsible for a spectrum of illnesses referred to collectively as hemorrhagic fever with renal syndrome (HFRS; Scmaljohn et al., 1985). The viruses are distributed worldwide and rodents are the primary reservoirs (Yanagihara and Gajdusek, 1987; Leduc et al., 1987), although carnivores and insectivores may also be infected (Tang et al., 1985; Zhao-zhuang et al., 1985). HFRS varies in severity depending on the particular virus involved. The primary clinical differences occur in the degree of hemorrhaging, hypotension, oliguria, and probability of mortality. Despite the widespread geographic range of hantaviruses, and the severity of HFRS, only the recent development of serodiagnostic tests has permitted evaluation of the importance of HFRS worldwide (French et al., 1981). Broad serosurveys of potential reservoir species (see LeDuc et al., 1987, for summary) show that infected animals exist far outside the recognized areas of endemic human disease, and hantaviruses are being recognized as causing fevers of unknown origin in an increasing number of countries where they were not thought to exist.

In 1982 several workers detected evidence of hantaviral infections among Norway rats (Rattus norvegicus) in cities of Korea and the United States (LeDuc et al., 1982; Lee H-W et al., 1982; Tsai et al., 1982), and subsequently two isolates were made in the USA (LeDuc, et al 1984 and Tsai et al, 1985). This was followed, in 1985 by the characterization of another Hantavirus (Prospect Hill virus; Lee P-W et al., 1985) in meadow voles (Microtus pennsylvanicus) first detected in Maryland (Lee P-W et al., 1982). Most recently, a third virus of the genus was described (Leakey virus) from house mice (Mus musculus), in Texas (Yanagihara et al., 1987).

Following several years of study, Childs et al. (1987a) characterized a Hantavirus and provided a detailed examination of the epizootiology of the virus in R. norvegicus of Baltimore, Maryland (Childs et al., 1985; Childs et al., 1987b). At that time they concluded that; the hantaviral infection in rats was widely disseminated throughout the city, representing a long time enzootic condition; the virus was closely related to Seoul virus (responsible for mild-type epidemic hemorrhagic fever in Asia); and based on ecological studies, there was potential for transmission to humans wherever the species came into close contact. As all three reservoir rodent species (Norway rats, house mice and meadow voles) known to carry hantaviruses in the United States exist within the city, it was also speculated that interspecific transmission could lead to coinfection with the remote possibility of genetic recombination of the viruses.

This was the rationale for submission of the proposal that forms the basis of contract DAMD17-87-C-7101, "The Epidemiology of Hantavirus Infections in Baltimore". Six major areas of research were proposed: 1) to isolate a library of viruses from

mammals within Baltimore, 2) to immunologically characterize the viruses responsible for infections of rodents and humans, 3) to examine the specificity of serological methods currently available, and develop protein-blotting methods (Western blots) to evaluate these methods, 4) to determine the prevalence of antibodies to hantaviruses within residents of Baltimore and determine to source (reservoir species) of the infections, 5) to determine the risk factors associated with human infection, and 6) to determine if human infection was associated with any clinical disease. Subsequently, it was proposed to deemphasize the first area of proposed research in favor of evaluation of the use of RNA probes obtained from hantaviruses for in situ hybridization studies of viral distribution and pathogenesis of infection among experimental animals.

METHODS

Comparative Serology

Recently, a number of studies have indicated the occurrence of hantaviral infections in humans outside of recognized endemic areas (Childs et al., 1988; Lee H-W et al., 1984; Yanagihara et al. 1985, Forthal et al. 1987,). In many cases only IFA data has been published with no confirmation of results by more specific assays such as PRN. We embarked on a major comparative serological examination of sera from Baltimore to assess the reliability and validity of serological results collected by different assays.

The indirect immunofluorescent antibody (IFA) test has been described in detail by LeDuc et al. (1984), and is considered the standard assay currently used in Hantavirus serosurveys. Briefly, test sera were diluted 1:8 and 1:32 in phosphate buffered saline (PBS). Dilutions were applied in duplicate to spot slides of Vero E-6 cells, 50-70% infected with prototype Hantaan virus (76-118), prepared by Dr. G. French. Slides were incubated for 30 minutes at 37 C, washed twice in PBS then incubated for 30 minutes with FITC conjugated goat anti-human heavy and light chain specific IgG. After washing and drying, spot slides were mounted with 10% glycerol in PBS and covered. Slides were then examined for characteristic cytoplasmic fluorescence. Seropositive samples were titered to an endpoint dilution in four-fold steps.

The indirect enzyme linked immunosorbent assay (ELISA) as developed at USAMRIID, has been briefly described by Childs et al. (1988). Monoclonal antibodies from mouse hyperimmune ascites fluid was coated onto a 96 well microtiter plate at a dilution of 1:5000 in PBS and incubated overnight at 4 C. Plates were washed twice in PBS and wells were coated with either supernatant fluid from Vero E-6 cell cultures infected with 76-118, or, as a control, uninfected cell supernatant fluid. Plates were incubated one hour at 37 C, washed, and 100 ul serum samples, diluted 1:100 in PBS were added, in duplicate, to wells with 76-118 and control antigens. The plates were incubated one hour at 37 C, washed twice in PBS and 100 ul of goat anti-human alkaline phosphatase conjugated IgG added. The plates were incubated for an hour at 37

C, washed twice in PBS and 100 μ l of P-Nitrophenyl Phosphate (PNP) in Diethanolamine (DEA) at 5 mg/ml added. Plates were read in a spectrophotometer at 406 nm, at 30, 45, and 60 minutes. Seropositive samples were tentatively identified as those with differences in the optical densities between 76-118 and control wells of at least two standard deviations above the mean differences for three negative human sera included on each plate. Sera were later titrated using a standard curve for a series of dilutions from a known positive.

Plaque reduction neutralization tests were conducted using 76-118, Baltimore rat virus and Prospect Hill virus according to the protocol described in Childs et al (1987a). Approximately 100 plaque forming units (PFUs) of each virus was mixed with four fold dilutions of test sera and incubated overnight at 4 C. Duplicate flasks (25 cm) of Vero E-6 cells were inoculated with the virus-serum mixtures, allowed to absorb them for one hour at 37 C, and then overlaid with a 1% nutrient agarose containing 10% fetal bovine serum, 4% L-glutamine, 1% nonessential amino acids, and Eagle's minimum essential medium mixed with antibiotics. Flasks were incubated for 7-9 days, then overlaid with a second agarose sheet containing neutral red. Plaques were counted 24-48 hours later, and titers determined as the highest serum dilution giving at least an 80% reduction in the number of plaques compared to control flasks. Sera neutralizing one virus with at least four fold higher titer compared to other viruses was considered to be specific to that virus.

Western blots were developed using Hantaan virus 76-118 and Baltimore rat virus as antigen sources. Antigen was obtained from virus seed stock maintained at USAMRIID, grown in Vero E-6 cell culture, and inactivated by BPL. Virus preparations were separated by SDS-PAGE by mixing equal volumes of cell culture supernatant fluid with SDS sample buffer and heating for 5 minutes at 100 C. Proteins were separated on a 5-17.5% gradient slab gels with 150 constant volts for 3.5 hours, using a 25 mM Tris/20 mM glycine buffer system (pH 6.8). Proteins were transferred to nitrocellulose sheets with 300 mAmps for 3 hours at 4 C. The sheets were dried and stored until needed at 4 C. Nitrocellulose sheets were wetted with PBS-Tween 20 containing a 1/50 dilution of test sera and allowed to incubate for 2 hours at 37 C. Sheets were then washed in PBS-T20, horseradish peroxidase conjugated goat anti-human IgG was added, incubated for one hour at 37 C, and washed in PBS-T20. Bands were visualized by adding 4-chloro-1-naphthol.

Immunologic Characterizations of Infections in Mammals

A variety of small mammals were examined by IFA and cross PRN tests to document the prevalence and occurrence of interspecific infections with different hantaviruses. Animals were examined for spatial and temporal patterns in the presence of antibody to Baltimore Rat virus and Prospect Hill virus. Details of this work are reported in the manuscript by Korch et al (Am. J. Trop. Med. Hyg. in press).

Small mammals (rats, voles, deer mice, house mice, and shrews) were trapped using live traps at 10 locations throughout Baltimore (see Childs et al. (1987) for sites). Animals were transported to the laboratory and a blood sample collected by either cardiac puncture or capillary tube puncture of the infraorbital sinus. Sera were collected by centrifugation and stored at -20 C until tested. Samples were screened by IFA (see above). Selected seropositive and negative samples were then tested by PRN (see above) to identify the viruses responsible for infections. Samples were screened from all areas, especially sites where more than one species was infected to test for interspecific transmission.

Viral isolation attempts were made by aseptically removing kidneys, spleens, lungs, and livers. Organs were dissociated by mechanical blender in a 10% suspension of maintenance medium (see PRN methods for composition) and allowed to settle. Supernatant fluids were placed on Vero E-6 cells in 25 cm flasks at 2 ml per flask. Flasks were incubated for 14 days at 37 C, decanted and culture fluids stored at -70 C for later assay. Monolayers were trypsinized and passed into cell-free flasks, inoculated into flasks of fresh Vero E-6 cells, or placed on spot slides for testing. Spot slides were fixed in cold acetone and examined for cytoplasmic fluorescence according to the IFA protocol. Cells were passaged at weekly intervals for up to 45 days.

Human Epidemiology

Epidemiological studies of Baltimore metropolitan residents were undertaken to determine: the prevalence of antibodies to hantaviruses; the patterns of antibody responses to various antigens; the risk factors associated with human infection; and to identify any clinical disease associated with hantaviral infection. Four populations were sampled: a predominantly young, inner city group using Baltimore City's Sexually Transmitted Disease (STD) Clinic, a predominantly middle aged population from Johns Hopkins Hospital (JHH), that were flagged for proteinuria, a population of individuals using chronic renal dialysis units within Baltimore City, and, finally, a group of individuals with cardiomyopathies taking part in a study at JHH.

Sera from all individuals were stored at -20 C until tested. Sera were initially screened by ELISA at dilutions of 1:100 (see above). All sera with OD readings at least 2 standard deviations above negative control sera were titered against a standard curve from a known positive sample. All positive, and selected negative sera, were tested by Western blot and PRN assays. Sera were identified as positive by these tests according to the criteria discussed above in the Comparative Serology section.

All patients at the STD clinic, inpatients at JHH, and some chronic renal dialysis patients were approached and asked to participate in the epidemiological survey. The survey (Appendix 1) examined the age, sex, race, birthplace, residence and workplace histories, exposure to domestic and commensal animals, and clinical symptoms consistent with HFRS. Charts of

seropositive patients at JHH who could not be interviewed were reviewed for historical information, clinical data on current complaints, and chronic illnesses. Charts of three randomly selected seronegative patients matched for age, sex, and ward were also examined without prior knowledge of the patient's serological status by the examiner. All interview forms were coded and stored on computer for analysis using SAS (1984) software.

Antigen and RNA Detection

1-3 day old Harlan mice were inoculated intracerebrally with .02 ml of culture supernatant fluid containing 10^5 PFUs of Hantaan or Baltimore Rat virus. Mice were housed 7 to a cage (1 adult female, 6 young), and were sacrificed on day 17-21 or as they showed signs of illness. Body cavities and brain cases were opened aseptically, and fixed in 10% buffered formalin for two weeks. Tissues, including brain, liver, lung, kidney, spleen, salivary glands, and lymph nodes were imbedded in paraffin, sectioned at 6 microns, and fixed to organosilane treated glass slides (2% organosilane in acetone). Slides were stored at room temperature until ready for use.

RNA probes to the S-segment of Hantaan virus were prepared from cDNA of the entire S segment cloned into pGEM-1 by Dr. C. Schmaljohn, of USAMRIID. The cDNA was cloned into the Pst I site in the Multiple Cloning Region (Schmaljohn et al., 1987). Viral sense probe (SB) was made from the preparation by Sal I digestion. Following digestion, DNA was precipitated in 3 M Sodium Acetate (1/10 volume) and 100% Ethanol (2.5 volumes) overnight at -20 C. The preparation was centrifuged at 10 K for 30 minutes at 4 C, the supernatant was decanted and the pellet dried in a vacuum for 10 minutes. The pellet was resuspended in 70% Ethanol (-70 C), centrifuged for 10 minutes, dried, and resuspended in TE.

Radioactively labeled probe was made by combining 1 ug of DNA template with 5X buffer, RNasin, and 10mM stocks of ATP, CTP, GTP, 35-S labeled UTP, and polymerase. The cocktail was mixed at room temperature, briefly spun in a microfuge, then incubated at 40 C for two hours. A 1:50 dilution of the cocktail was made in DEPC water to determine the amount of RNA incorporated and the specific activity of the probe. Cocktails with specific activities on the order of 10^9 were then treated with DNase, incubated at 37 C for 10 minutes followed by the addition of 0.1 M EDTA in DEPC water. The probes were then frozen at -80 C, overnight.

The next day, the probes were thawed on ice and a Sephadex column prepared. The probe cocktail was added to the column and radioactively labeled probe collected as the first peak of radioactive material passing through the column. Total and precipitable counts were determined, to assess the purity, and amount of RNA incorporated. Probes were frozen at -80 C until ready for hybridization studies.

For in situ hybridizations, probes were thawed at room temperature and the sample evaporated in a speed vacuum to a final concentration of 2 ng/ul was obtained. Tissue samples, previously mounted on organosilane treated slides, were deparaffinized in xylenes, and rehydrated in successive steps in 100%, 95%, 70% ethanols, and 2X SSC. Slides were treated with 25ug/ml Proteinase K in a buffer of 10mM Tris and 2mM CaCl₂, for 30 minutes at 37 C. Slides were acetylated with 0.1 M TEA-HCL with 0.25% Acetate Anhydride for 10 minutes, then placed in 2X SSC, 70%, 95% ethanols, and air dried. A cocktail of Formamide dextran sulfate and probe were made so that the final concentration of the probe was 0.5 ng/ul, and 20 ul of cocktail was added to each slide. Cover slips were placed over each tissue section and sealed with rubber cement. The slides were then placed on a sponge soaked with 1M NaCl, in an airtight container, and incubated at 46 C overnight.

The following day, the cover slips were removed and the slides washed in 0.5 M SSC containing 10mM DTT. The slides were washed twice in 0.5 M SSC and then in 0.5 M NaCl in 10 mM Tris. The slides were then transferred to a bath of 0.5 M NaCl in 10 mM Tris containing 40 ug/ml of RNase A, at 37 C for 40 minutes. The slides were then washed four times in 0.1 M SSC containing 10 mM DTT, at 60 C, for 10 minutes per wash, then dehydrated in 70% and 95% ethanols containing 0.3 M Ammonium Acetate. The slides were dipped in photographic emulsion, placed in a holding box, at 4 C until they were ready to be developed (3-21 days). Slides were developed using standard procedures, counter-stained in hematoxylin and eosin, and mounted with permount. Tissues were examined under high power light microscopy for the presence of characteristic silver grains associated with labeled RNA probe.

Antigen distribution in experimentally infected animals was examined by Avidin-Biotin Complex (ABC) method (modified from procedure developed by Dr. W. Hall, USAMRIID). As with in situ hybridizations, tissues mounted on slides were deparaffinized and placed in a protease solution containing Protease VIII (0.2 mg/ml) and sodium phosphate monobasic-sodium phosphate dibasic (pH 7.8) at 37 C for 30 minutes. Slides were washed in distilled water, rinsed in 95% ethanol, and placed in a 1:100 solution of 30% hydrogen peroxide and 100% methanol for 30 minutes. The slides were washed in PBS, and the excess removed. They were then placed on a water soaked sponges, in a tray, and normal goat serum applied for 20 minutes. The serum block was removed, and primary antibodies (either rabbit anti-Hantaan or rabbit anti-Seoul viruses) were added at 1:100 in PBS containing 0.1% bovine serum albumin. Slides were incubated for one hour at room temperature, washed in PBS, the excess moisture removed, the biotinylated secondary antibody (goat anti-rabbit) added, and incubated at room temperature for one hour. The secondary antibody was removed, the slides washed in PBS, and the ABC reagent added. Slides were incubated for one hour, at room temperature. BAD substrate (0.25 mg/ml) in water, 50 mM Tris and hydrogen peroxide was then applied to the slides for three

minutes. The slides were then transferred to water for five minutes, stained with hematoxylin, mounted in permount, and viewed under light microscope.

RESULTS and DISCUSSION

Mammal Survey

A total of 2801 mammals representing 13 species were sampled throughout Baltimore. They included six species of rodents, four carnivores, one lagomorph, one insectivore, and one marsupial (Table 1). IFA screening showed that there was at least one seropositive individual in each species, except among gray tree squirrels, Sciurus carolinensis, opossums, Didelphis virginianus, and eastern cottontail rabbits, Sylvilagus floridanus, although the latter was represented by only two individuals. Seroprevalences in most species were low, and titers rarely exceeded 1:128. In addition, the geographic distribution of seropositive animals was spotty, with no seropositive individuals sampled in most locations. In contrast, Norway rats and meadow voles were seropositive with high seroprevalences, ranging from 21 to 80%, had high IFA titers (often exceeding 1:2048), and seropositive animals were observed at every site where these species were captured. These two species also differed from the remaining group in that seroprevalence increased with body size, indicating an age related acquisition of exposure, while seroprevalence occurred nearly uniformly (at low rates) throughout the other species.

Table 1. Number of Animals Tested for Antibodies to Hantaan Virus, Strain 76-118, from Baltimore, Maryland, from 1980-1986.

Order	Species	Number tested	%Positive
Rodentia	<u>Rattus norvegicus</u>	853	42.0
	<u>Mus musculus</u>	603	3.6
	<u>Peromyscus leucopus</u>	185	1.1
	<u>Microtus pennsylvanicus</u>	184	16.8
	<u>Sciurus carolinensis</u>	33	0.0
	<u>Tamias striatus</u>	7	14.3
Carnivora	<u>Felis catus</u>	649	6.5
	<u>Canis familiaris</u>	164	1.2
	<u>Procyon lotor</u>	82	2.4
	<u>Mustela frenata</u>	12	8.3
Lagomorpha	<u>Sylvilagus floridanus</u>	2	0.0
Insectivora	<u>Blarina brevicauda</u>	19	5.1
Marsupialia	<u>Didelphis virginianus</u>	8	0.0

As many of these species overlapped both spatially and temporally at the sampling sites, the possibility of interspecific transmission was examined by PRN tests (Tables 2 and 3). Neutralization patterns indicate that at least two distinct hantaviruses are circulating concurrently within the

mammalian fauna of Baltimore. Norway rats were found to have neutralizing antibodies to Baltimore rat virus at every site where they were captured, while meadow voles had neutralizing titers to Prospect Hill virus everywhere they were captured. There was no evidence of cross-infection between these host species, even at the Cherry Hill site where both species were captured together. Only one of the other species examined had neutralizing titers against any of the other hantaviruses tested. Two raccoons (Procyon lotor) had a neutralizing titers of 1:10 against prototype Hantaan virus (76-118) but did not neutralize either Baltimore rat or Prospect Hill viruses.

Table 2. Comparison of Reciprocal IFA Titers to Hantaan Virus Antigen (HTNV) and PRN titers to Baltimore Rat Virus and Prospect Hill Virus for Norway Rats.

Location	IFA	PRN	
	HTNV	BRV	PHV
Patterson Park	32	160	40
	128	0	0
	4096	640	0
	4096	640	10
	8192	640	10
Winston Govane	32	0	0
	2048	40	0
	4096	>640	640
Cherry Hill	0 (5)	0	0
	32 (3)	0	0
	128 (3)	0	0
	128 (2)	80	10
	256	80	0
	512	0	0
	512	80	0
	2048	160	0
	4096	80	40

Note- numbers in () indicate sample size if >1.

Table 3. Comparison of Reciprocal IFA Titers to Hantaan Virus Antigen (HTNV) and PRN titers to Baltimore Rat Virus and Prospect Hill Virus for Meadow Voles.

Location	IFA	PRN	
	HTNV	BRV	PHV
Leakin Park	32	10	10
	512	10	320
	512	0	0
Herring Run	32	10	160
	128	0	320
	128	10	160
Cherry Hill	32 (2)	0	640
	32	0	40
	32	0	160
	64	0	10
	128	0	320
Hillen Park	64	0	320
	128	0	160
	128	0	320
	256	0	10

Note- number in () indicates sample size if >1.

Thus, the pattern of infections indicate that two major reservoir species exist in Baltimore; Norway rats and meadow voles. The two viruses, a Baltimore rat strain and a Prospect Hill strain, circulate independently in these hosts even within the same habitats, and there is no evidence of cross infection.

Virus isolations were attempted from two seropositive *M. musculus* and one *M. pennsylvanicus*. In both isolation attempts from IFA positive mice cells in culture were killed by day 10. Attempts were made to salvage any surviving cells by passaging to new and semi-confluent flasks. Cell sheets continued to be destroyed although there were no signs of gross contamination. After five passages supernatants were decanted, and frozen. Attempts to make spot slides from these cultures were typically unsuccessful as few cells were available to fix to slides. The isolation attempt from the vole was continued for three blind passages but was unsuccessful; no positive spot slides were found.

The low prevalences and titers in other species, and the geographically sporadic distributions may represent false positives by IFA. This hypothesis supported by the fact that (with the exception of two raccoons) none of the sera have neutralizing titers to any of the hantaviruses tested.

Alternatively, the remaining mammalian species may be infected with other hantaviruses. Neutralizing titers in two raccoons to prototype Hantaan virus would support this possibility. However, seroprevalence patterns would appear to argue against this as seropositive rates are low. Even house

mice, which may serve as reservoirs for Leakey virus in Texas, are rarely infected in Baltimore. Ultimate resolution of this question will depend on applying Western blotting to animal sera positive by IFA. Western blots, in humans, appear to respond to a variety of heterologous hantaviruses making detection of new hantaviruses possible without the difficulties of false positives. We have recently modified our Western blots for use with rats with good success. We will apply it in the future to sera from other small mammals to resolve these questions.

Human Epidemiology

As of March 1, 1988, we have collected sera from 2994 persons and obtained questionnaires from 539 of these individuals. Of this total, 962 sera have come from JHH (20 questionnaires), 1517 have come from the STD clinic (440 questionnaires), 405 have come from dialysis patients (79 questionnaires) and 110 have come from patients with cardiomyopathies (0 questionnaires).

The major characteristics of the persons sampled are shown in Tables 4 and 5. The figures for hospital groups (JHH and Dialysis) are combined for these analyses because of small sample sizes. The population seen at the STD clinic is composed predominantly of young, black, males, while the hospital population is older, comprised equally of males and females, and is also predominantly black. The 1980 census reported that 55% of Baltimore population of 786,775 was black (Goodman and Talalay 1981).

Table 4. Demographic Characteristics of Individuals from Different Sources from Whom Questionnaires Were Obtained.

Source	N	X Age	% Male	% Black
STD Clinic	440	24.6	67.7	92.5
Hospitals*	99	46.7	47.0	65.0

* JHH and Good Samaritan.

The majority of both populations were born in Baltimore, although more of the STD patients were born in the city (78.5%) than the hospital patients (50.5%). The majority of the individuals interviewed disclaimed any foreign travel. The countries most frequently visited were Canada, Mexico and Islands in the West Indies. A few individuals had histories of travel to HFRS endemic areas of Korea, (N=10) Japan (N=7), or to potentially NE endemic locations in Europe.

Table 5. History of Residence and Travel for Individuals from Different Sources from Whom Questionnaires Were Obtained.

Source	% Baltimore Born	X Yrs at Residence	% Foreign Travel
STD Clinic	78.5	7.3	14.3
Hospital*	50.5	14.4	29.5

* JHH and Good Samaritan

Risk factors for exposure to Hantavirus via contact with rodents have been established for 539 persons. Figure 1 shows the percentage of individuals indicating presence of rats and mice in their homes, the alleys and streets around their homes, or at their work places. Responses were graded as none, few or many sightings. Approximately 10% of all persons indicate having been exposed to Norway rats in their current homes at some time compared to 60% with exposure to mice. In contrast, approximately 65% of persons indicated rats were present in alleys around their homes compared to only 25% for house mice. The degree of contact with rats was approximately 20% at work compared to approximately 30% for mice.

Actual reported contacts with rats and mice via feces, trapping, handling or being bitten by the animal are shown in Figure 2. Approximately 13-20% of Baltimore residents report contact with rat feces (usually sweeping them up), 15-30% contact via trapping rats, 4-12% contact through handling rats (usually after trapping them) and 2% having been bitten by a rat (usually as a child). All these rates are approximately doubled for contact with house mice.

These data indicate a significant degree of contact with hantaviral reservoirs in Baltimore. At the present time we have too few positive sera to accurately attempt a statistical analysis of risk, but we anticipate such an analysis within the next year.

Prevalence of Antibodies to a Hantavirus

The prevalence of antibodies to a Hantavirus by IFA and ELISA assays are shown in Tables 6 and 7, respectively. Not all specimens were run by both assays so the numbers are not equivalent.

The ELISA results are most representative of the various groups studied and reveal an overall human prevalence of 1.30% with titers >100 and .67% with titers >200. Using the more conservative criteria for positive, which is 98.4% specific when compared against neutralization tests (see below) results in the prevalence rates of .56% for STD patients, .84% for JHH patients, .74% for dialysis patients and 0% for cardiac patients. This is a conservative prevalence estimate as 4/14 of the sera with ELISA titers between 100 and 199 were Western blot positive. In future

consideration of seropositivity we propose to use a ELISA titer of >200, or >100 with either confirmatory Western or neutralization as criterion of positive.

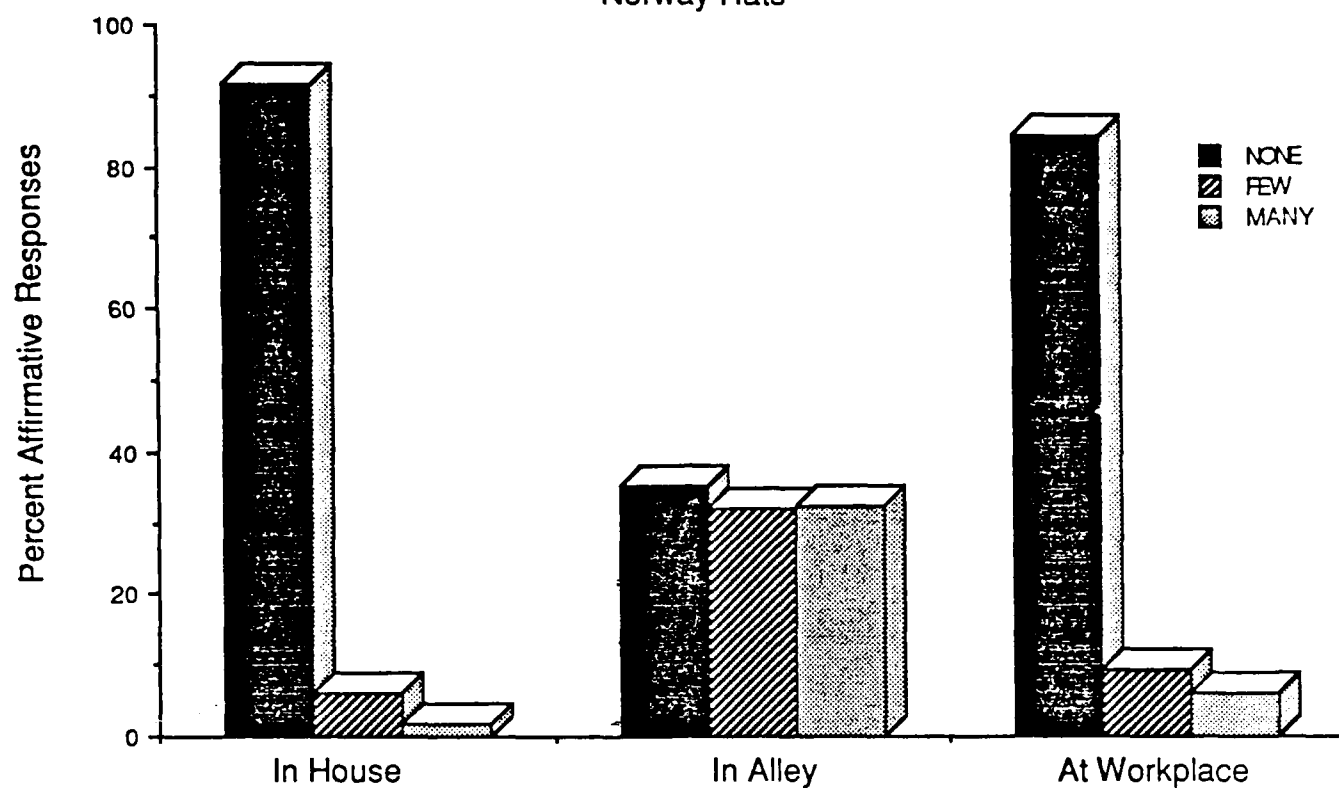
Table 6. Prevalence of ELISA Antibodies to a Hantavirus in Persons from Different Sources in Baltimore.

Titer	Source				Total
	JHH	STD	Dialysis	Cardiac	
< 100	945	530	391	110	1976
100 - 199	2	1	11	0	14
200 - 399	5 (2)	1	1	0	7 (4)
400 - 999	2	0	0	0	2
1000 - 1999	2	0	2	0	4
≥ 2000	6 (2)	2	0	0	8 (4)

Note-numbers in () indicate number of different persons.

The IFA results shown in Table 7 indicate an overall human antibody prevalence of 4.5% with titers >32 and 1.5% with titers > 128. Using the criterion of 128 as positive, which is 83.0% specific when compared against neutralization tests (see below) results in prevalence rates of 1.22 for STD patients, 1.33 for JHH patients, and 8.7% for a small sample of dialysis patients. Based on specificity analyses shown we consider the ELISA assay with a second confirmatory test to be best assay. This is a consciously conservative stance as we believe further claims of hantaviral infection based solely on IFA results do not provide rigorous proof of infection.

15
Norway Rats



House Mice

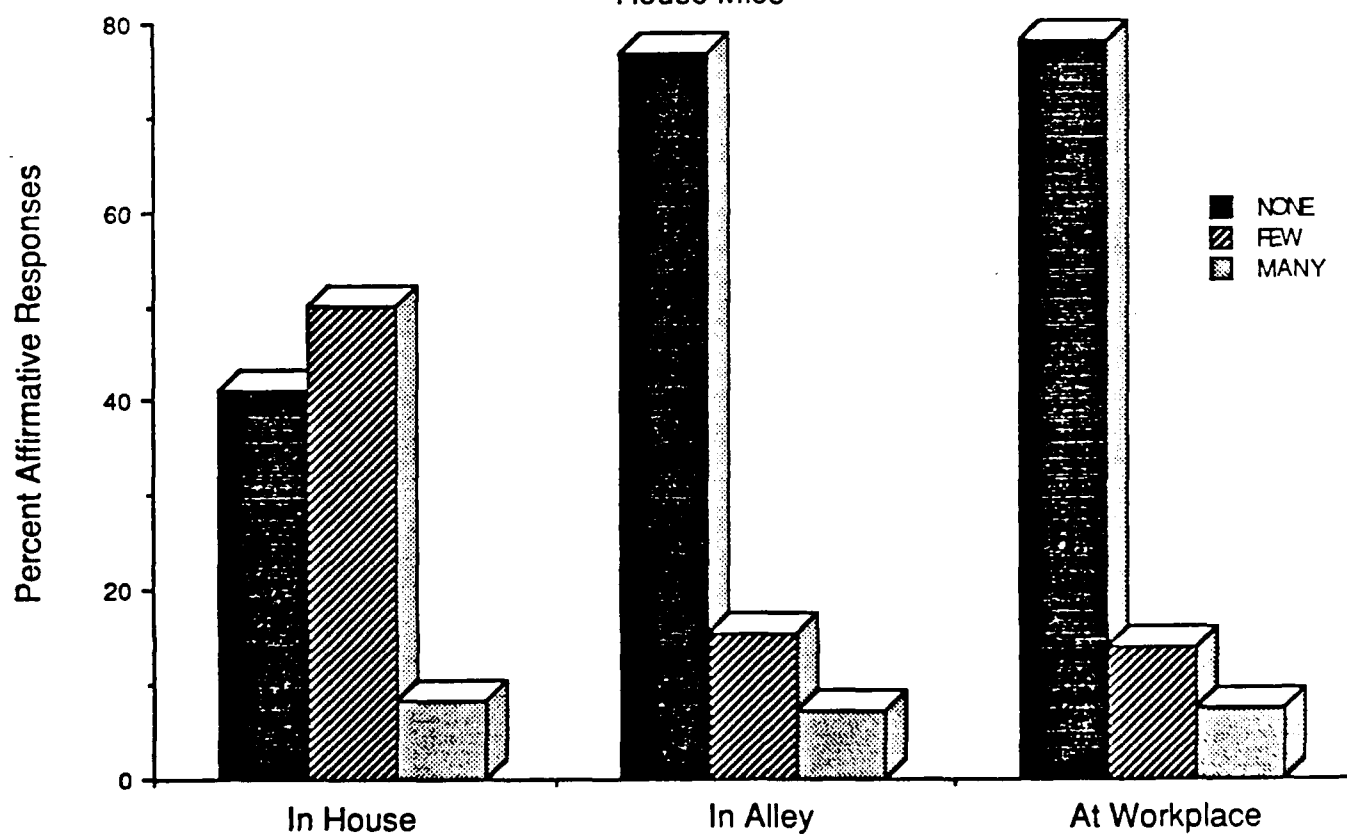


Figure 1. Responses from Baltimore residents to questions concerning sightings of Norway rats and house mice.

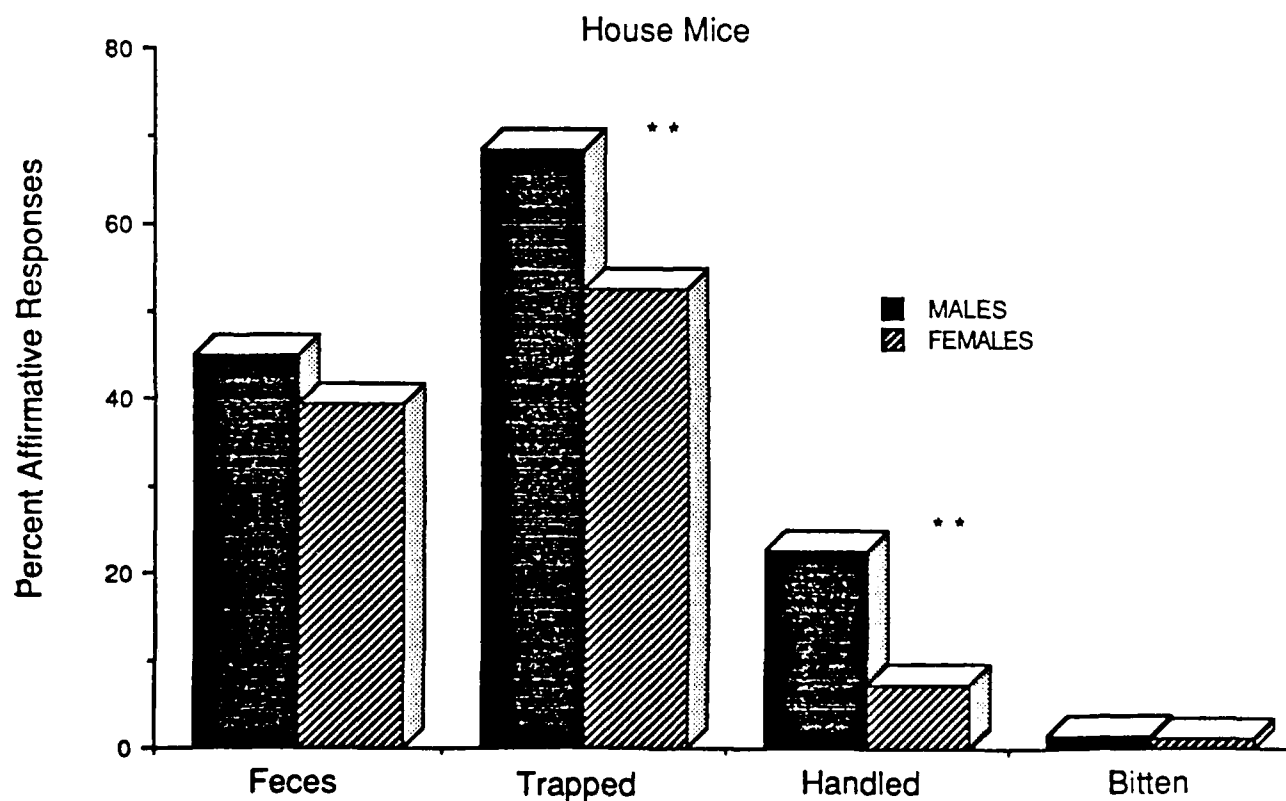
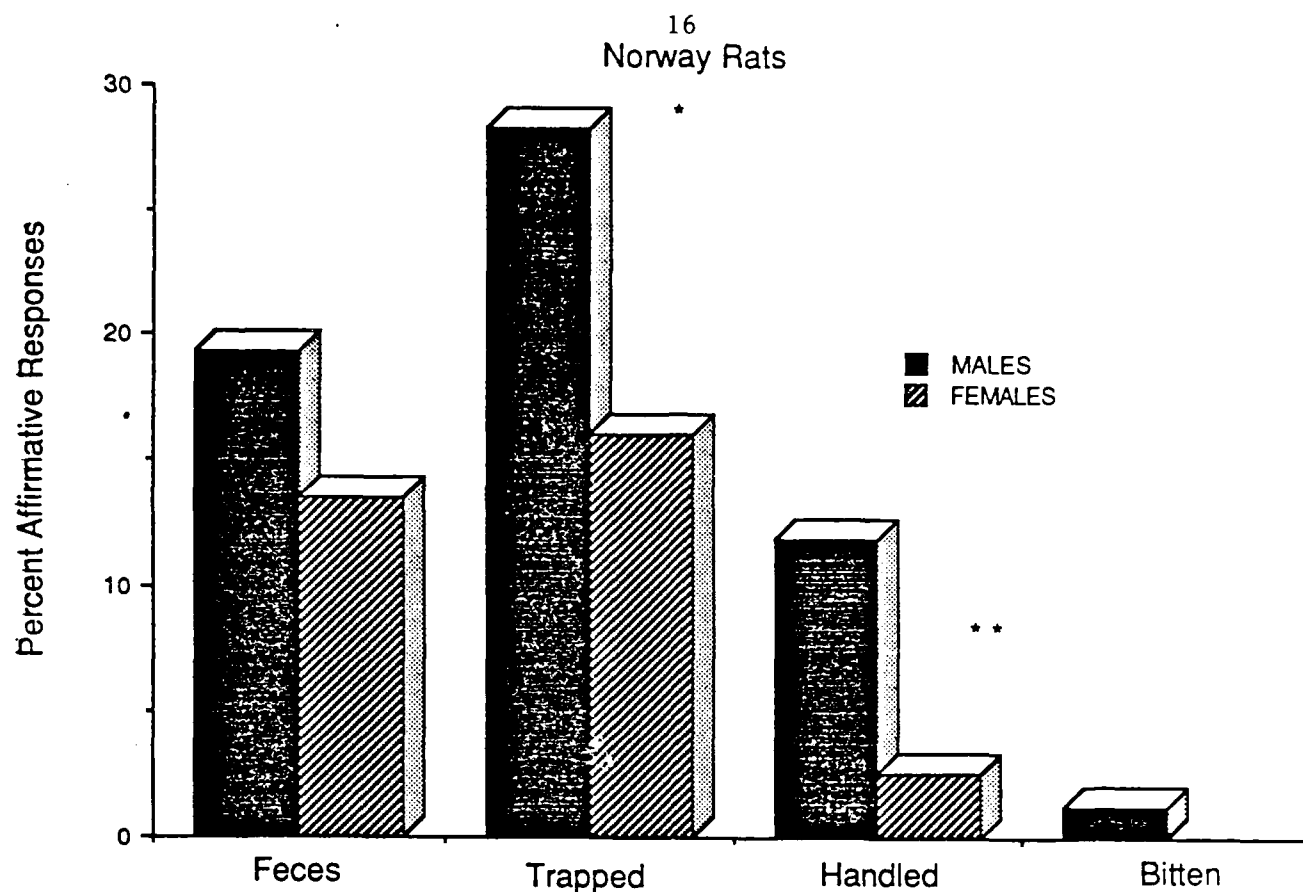


Figure 2. Responses from Baltimore residents to questions concerning contact with Norway rats and house mice. Asterisks indicate significant differences between the sexes at the .05 (*) or .01 (**) level.

Table 7. Prevalence of IFA Antibodies to a Hantavirus in Persons from Different Sources in Baltimore.

Titer	Source			Total
	JHH	STD	Dialysis	
0 - 31	861	1421	78	2360
32 - 63	24	20	6	50
64 - 127	9	18	0	27
128 - 511	14	13	7	34
≥ 512	3	5	1	9

Comparative Serology

Sera were compared by IFA, ELISA, PRN and by Western blotting. For purposes of determining sensitivity and specificity the Baltimore rat PRN results were used as the criterion for "true" positivity. This may result in conservative interpretation of results, especially if other hantaviruses are circulating in Baltimore humans. Sensitivity is over estimated and specificity underestimated as the entire sample of sera were not tested by PRN, but was biased toward specimens either IFA or ELISA positive on initial screening. However, the results do accurately indicate the relationship between serological tests for a wide range of samples, as tested in laboratories at JHU and USAMRIID.

The results of comparative assays are shown in Table 8. As expected, the IFA consistently gave positive results that could not be confirmed by additional assays. However, the sensitivity of this test as a screening tool was great. The specificity of the ELISA assay was excellent, especially at titers >200. The Western blot appears to give results comparable to PRN tests and could be considered a possible replacement assay in situations where PRN tests are not required to differentiate between different hantaviruses, but are used only as a confirmatory test for the less specific IFA.

Table 8. Sensitivity and Specificity of Various Serological Assays for Hantaviruses as Compared to the Plaque Reduction Neutralization Assay. Sera Collected from Baltimore Residents Only.

Test	Positive Criterion	Sensitivity %*	Specificity %
IFA	1:32	100	72.1
	1:128	100	83.0
ELISA	1:100	100	89.6
	1:200	100	98.4
WESTERN	Nucleocapsid Band	100	96.6

* Figures do not reflect PRN testing of entire collection of samples.

Only a single band of approximately 50,000 daltons, presumably nucleocapsid, was visualized in Western assays. Antisera against synthetic peptide fragments of G1 and G2 (kindly provided by Dr. C. Schmaljohn, USAMRIID) showed the presence of these glycoproteins on the nitrocellulose, so the destruction of conformational epitopes by denaturing conditions was inferred to account for the loss of bands. The inability to visualize glycoproteins may limit the ultimate usefulness of the Western assay in differentiating hantaviruses, but it is a useful confirmatory assay for IFA or ELISA that does not require high containment.

IFA was also compared to ELISA in a large number of additional samples, and the results are shown in Figure 3, along with graphical presentations of the correlations of IFA and ELISA data with PRN tests using the Baltimore rat isolate.

Evidence of Human Infection with Rat-Associated Hantaviruses

Table 9 summarizes the serological findings from 13 individuals from Baltimore which unequivocally demonstrate infection with a rat strain of Hantavirus. The consistently higher neutralizing titers against Baltimore Rat virus strongly implicate this strain of Hantavirus as the infecting agent. A subsample (N=4) of these sera were tested against Prospect Hill virus with uniformly negative results. The majority of these individuals (10/13) report no history of foreign travel, so we infer that their infections were acquired in Baltimore. A portion of this data is currently in press (Childs et al. Am. J. Epidemiol.).

Table 9. Evidence of Human Infection in Baltimore with a Rat Associated Hantavirus.

Individual*	Sample	ELISA	PRNT	
			Baltimore	Hantaan
STD 1	1	200	160	0
STD 2	1	16000	2048	128
JHH 1	1	100	2048	40
JHH 2	1	1700	1024	160
	2	2100	1024	160
JHH 3	1	300	40	10
	2	300	40	0
	3	300	40	0
	4	300	40	10
JHH 4	1	3000	40	10
	2	3000	40	0
	3	2600	40	10
	4	9000	160	40
JHH 5	1	3400	160	0
JHH 6	1	16000	1024	128
JHH 7	1	1800	1024	64
DIA 1	1	100	80	0
DIA 2	1	1800	640	20
DIA 3	1	1600	1280	40
DIA 4	1	130	80	0

* JHH-Johns Hopkins Hospital; STD-Sexually Transmitted Disease Clinic; DIA-Dialysis Units.

Charts from nine seropositive patients and 27 matched seronegative patients were reviewed. A significantly higher proportion ($X^2 = 14.95$; $df = 1$, $p < 0.005$) of seropositive individuals, (7/9) were reported chronic renal failure compared to seronegative individuals (3/27). The two seropositive individuals without renal failure were a boy who died as a result of myocarditis secondary to viral cardiomyopathy from an unknown virus, and a man with diabetes mellitus and hypertension. Efforts are underway to obtain tissue samples from the autopsy of the child to test for the presence of antigen and/or viral RNA.

The prevalence of diabetes mellitus also tended to high in seropositive individuals (62.5%) but not significantly higher ($X^2 = 0.80$; ldf , $p = 0.05$) than the seronegative population (44.4%). There was no evidence in the charts describing any acute illness similar to that seen in prototypical HFRS cases. The only acute illness associated with the seropositive individuals was the boy reported above.

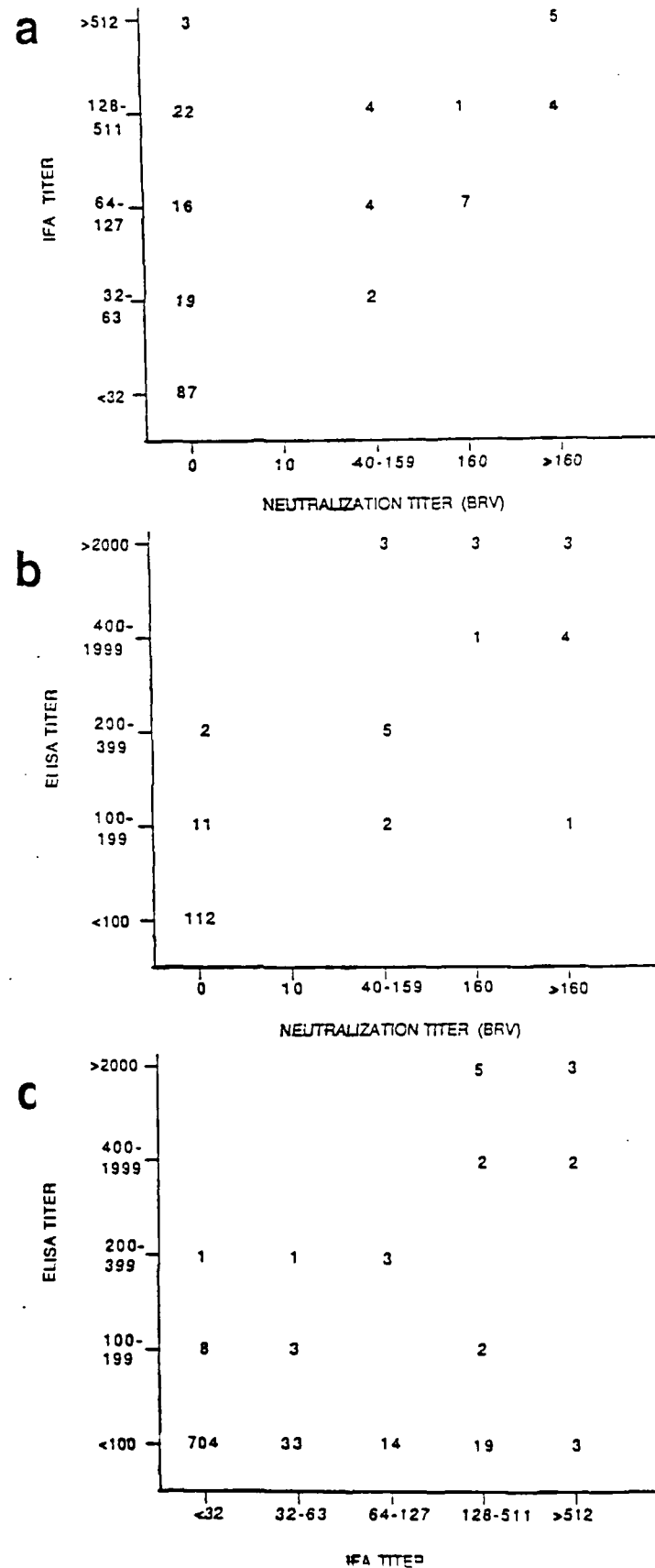


Figure 3. Comparison of serological tests for samples collected from Baltimore residents. a. IFA titers (Hantaan antigen) versus neutralization titers against Baltimore rat virus (BRV). b. ELISA titers (Hantaan antigen) versus neutralization titers against Baltimore rat virus (BRV). c. ELISA titers versus IFA titers.

The association between renal failure, and exposure to a rat borne hantavirus is interesting in light of Balducci, et al's (1987) observation that 4/14 cases of HFRS in Italy due to a rats-associated Hantavirus developed chronic renal insufficiency within the study period. The absence of acute illness is not necessarily surprising, as the onset of renal insufficiency for many of the patients was several years (5-10) ago and charts were often incomplete that far into the past. In addition, there may be many cases of inapparent infection for each illness in endemic areas so that using serodiagnostic techniques to identify exposed individuals may identify many of the subclinical exposures.

Antigen and RNA detection

Antigen and RNA distributions in mice experimentally infected with Hantaan 76-118 (HTN) and Baltimore rat (BRV) viruses were similar but differed in some aspects of tissue distribution. BRV RNA and antigen were abundant in salivary glands, pancreatic islets and the renal distal tubules while HNTV was sparse or absent in these regions. BRV RNA was particularly abundant in the adrenal medulla while HTNV RNA was present but markedly reduced. BRV RNA and antigen was found in both heart muscle and spleen to a minor degree, while HTNV RNA was not detected.

Both viruses showed very strong affinities for various regions in the brain. Major localizations of RNA and antigen were in the hippocampus, cerebellum and pituitary. Antigen and RNA were concentrated in the Purkinje cells, lining of the cortex, and in the stellate cells of the molecular layer. Viral RNA, and antigen were also concentrated along the border of the hippocampus and in the cortical amygdylodid nucleus. Antigen, of both viruses, but not RNA, was noted in the lungs of mice but this may have been due to poor histological preparation rather than a true absence of virus.

In situ hybridization and avidin-biotin complex methods appear to offer promise as techniques for aiding our understanding of the pathogenesis and pathology of hantaviruses. These approaches permit us to identify the specific target organs to the cellular level affected by these viruses. Differences in the antigen and RNA distributions and intensities between BRV and HTNV may reflect variation in titers of the infecting viruses or other factors rather than true patterns of tissue tropisms. These differences will be investigated in future studies.

CONCLUSION

Information gathered to date indicate that Norway rats and meadow voles are the major reservoirs of hantaviruses within Baltimore. There is little evidence that other species serve as

reservoirs, at this time. Each of the two major reservoirs circulates one type of virus independently of the other and there is no evidence for cross-infection.

Seroprevalence to hantaviruses among humans is approximately 1.3%, occurring primarily in individuals of lower socioeconomic status areas. Exposures are to a Baltimore rat virus strain of Hantavirus, rather than Prospect Hill or prototype Hantaan viruses. Interviews indicate the human population is exposed to wild rats at a high frequency. Seropositive individuals have higher rates of chronic renal difficulties (78%) than age and sex matched controls (11%).

Comparative serological testing indicates that IFA tests produce a high rate of positives that are not confirmed by other tests. Although no 'false negatives' have been found by IFA, the rate of false positives may inflate the seroprevalence in humans 3-4 times (4.5%), above that found by ELISA. The continued use of IFA, in the absence of other tests is not recommended. A Western blot test was developed and was found to be very sensitive and specific (at rates comparable to PRN tests). It is easier, and more rapid than PRN tests, and does not use live virus. However, it does not distinguish between hantaviruses. It is useful as a confirmatory test especially when knowledge of the particular Hantavirus is not important.

In situ hybridization of viral RNA, and Avidin-Biotin Complex labeling of viral antigen in experimentally infected animals show that BRV and HTN have strong affinities for the cerebellum, hippocampus, and pituitary. Differences in tissue tropisms were noted but may be due to experimental protocols that are under review. In situ hybridization and ABC techniques may prove useful in understanding the pathogenesis of hantaviruses in experimental and naturally occurring infections.

REFERENCES CITED

- Balducci, M., P. Verani, and L. Nicoletti. 1987. Epidemiological study of HFRS in Italy. 29th Colloquium Inst. Trop. Med., Belgium.
- Childs, J.E., G.W. Korch, G.A. Smith, A.D. Terry and J.W. LeDuc. 1985. Geographical distribution and age related prevalence of antibody to Hantaan-like virus in rat populations of Baltimore, Maryland, USA. Am. J. Trop. Med. Hyg., 34: 385-387.

- Childs, J.E., G.W. Korch, G.E. Glass, J.W. LeDuc and K.V. Shah. 1987a. Epizootiology of Hantavirus infections in Baltimore: Isolation of a virus from Norway rats, and characteristics of infected rat populations. *Am. J. Epidemiol.* 125:55-68.
- Childs, J.E., G.E. Glass, G.W. Korch and J.W. LeDuc. 1987b. Prospective seroepidemiology of hantaviruses and population dynamics of small mammals in Baltimore, MD. *Am. J. Trop. Med. Hyg.* 37: 648-667.
- Childs, J.E., G.E. Glass, G.W. Korch, R.R. Arthur, K.V. Shah, D. Glasser and J.W. LeDuc. 1988. Evidence of human infection with a rat-associated Hantavirus in Baltimore, Maryland. *Am. J. Epidemiol.* 127: 875-878.
- Forthal, D.N., S.P. Bauer and J.B. McCormick. 1987. Antibody to hemorrhagic fever with renal syndrome viruses (hantaviruses) in the United States. *Am. J. Epidemiol.* 126: 1210-1213.
- French, G.R., R.S. Foulke, O.A. Brand, G.A. Eddy, H.W. Lee and P.-W. Lee. 1981. Korean hemorrhagic fever: propagation of the etiologic agent in a cell line of human origin. *Science*, 211:1046-1048.
- Korch, G.W., J.E. Childs, G.G. Glass, C.A. Rossi and J.W. LeDuc. 1988. Spatial and temporal analyses and host range of hantaviral infections within small mammal communities of Baltimore, Maryland, USA. *Am. J. Trop. Med. Hyg.*, (in press).
- LeDuc, J.W., G.A. Smith and K.M. Johnson. 1984. Hantaan-like viruses from domestic rats captured in the United States. *Am. J. Trop. Med. Hyg.*, 33: 992-998.
- LeDuc, J.W., G.A. Smith, L.R. Bagley, S.E. Hasty and K.M. Johnson. 1982. Preliminary evidence that Hantaan or a closely related virus is enzootic in domestic rodents. *N. Eng. J. Med.* 307:624.
- Lee, H.W., L.J. Baek and K.M. Johnson. 1982. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. *J. Infect. Dis.*, 146: 638-644.
- Lee H.W., I.W. Seong, L.J. Baek, D.A. McCleod, J.S. Seo, C.Y. Kang. 1984. Positive serological evidence that Hantaan virus, the etiologic agent of hemorrhagic fever with renal syndrome, is endemic in Canada. *Can. J. Microbiol.*, 30:1137-1140.
- Lee, P.-W., H.L. Amyx, D.C. Gajdusek, R.T. Yanagihara, D. Goldgaber and C.J. Gibbs Jr. 1982. New haemorrhagic fever with renal syndrome-related virus in indigenous wild rodents in United States. *Lancet*, 2: 1045.
- Lee, P.-W., H.L. Amyx, R. Yanagihara, D.C. Gajdusek, D. Goldgaber, D and C.J. Gibbs Jr. 1985. Partial characterization of Prospect Hill virus isolated from meadow voles in the United States. *J. Infect. Dis.*, 152: 826-829.

- Schmaljohn, C.S., S.E. Hasty, J.M. Dalrymple, J.W. LeDuc, H.W. Lee, C.-H. von Bonsdorff, M. Brummer-Korvenkontio, A. Vaheri, T.F. Tsai, H.L. Regnery, D. Goldgaber and P.-W. Lee. 1985. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. *Science*, 227: 1041-1044.
- Schmaljohn, C.S., H.W. Lee, and J.M. Dalrymple. 1987. Detection of hantaviruses with RNA probes generated from recombinant DNA. *Arch. Virol.*
- Tang, Y.W., Z.Y. Xu, Z.Y. Zhu, and T.F. Tsai. 1985. Isolation of HFRS virus from Suncus murinus, an insectivore. *Lancet*, 1:513-514.
- Tsai, T.F., S.P. Bauer, D.R. Sasso, J.B. McCormick, H. Bradford, C.T. Caraway, L.M. McFarland, O. Medrano and G. Soulie. 1982. Preliminary evidence that Hantaan or a closely related virus is enzootic in domestic rodents. *N. Eng. J. Med.*, 307:624.
- Tsai, T.F., S.P. Bauer, D.R. Sasso, S.G. Whitfield, J.B. McCormick, T.C. Caraway, L. McFarland, H. Bradford and T. Kurata. 1985. Serological and virological evidence of a Hantaan virus-related enzootic in the United States. *J. Infect. Dis.*, 152: 126-136.
- Yanagihara, R., C.-T. Chin, M.B. Weiss, D.C. Gajdusek, A.R. Diwan, J.B. Poland, K.T. Kleeman, C.M. Wilfert, G. Meiklejohn and W.P. Glezen. 1985b. Serological evidence of Hantaan virus infection in the United States. *Am. J. Trop. Med. Hyg.*, 34:396-400.
- Yanagihara, R., and D.C. Gajdusek. 1987. Hemorrhagic fever with renal syndrome: global epidemiology and ecology of Hantavirus infections. *Med. Virol.*, 6:171-214.
- Yanagihara, R., L.J. Baek, C.J. Gibbs, Jr, et al., 1987. Epizootiology of Hantavirus infections in the United States. Third Int. Symp. on Ecology of Arboviruses. 78-79.
- Zhao-zhaung, L., H. Hao and L. Yue-xian. 1985. Characterization of EHF virus isolated from cats in endemic area of Anhui province. *Chin. J. Microbiol. Immunol.*, 5:81-83.

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