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SEX DIFFERENCES IN IMMUNE RESPONSES AND VIRAL SHEDDING FOLLOWING SEOUL VIRUS INFECTION IN NORWAY RATS

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Abstract. In the field, male rodents are more frequently infected with hantaviruses than females. This study examined whether patterns of immune responses against hantavirus differed between the sexes. Male and female Long Evans rats (*Rattus norvegicus*) were inoculated with Seoul virus, and antibody and cytokine responses, as well as virus shedding were assessed. Males were more likely to shed virus in saliva, to shed virus through multiple routes (saliva, urine, and feces), and to have viral RNA in the spleen than females. Anti-Seoul virus IgG responses were higher in males than females. In both sexes, splenic IFNγ and IL-4 production increased following infection. After infection, males had higher Th1 immune responses (i.e., IgG2a, IFNγ, and IL-2) than females; in contrast, Th2 immune responses (i.e., IgG1, IL-4, and IL-10) were similar between the sexes. These data suggest that immune responses to Seoul virus differ between the sexes.

INTRODUCTION

Hantaviruses are negative-sense RNA viruses (Family: Bunyaviridae) that are primarily maintained by rodents.[1] For example, in the Americas, Seoul virus mainly infects Norway rats (*Rattus norvegicus*), Sin Nombre virus infects deer mice (*Peromyscus maniculatus*), New York virus infects white footed mice (*Peromyscus leucopus*), and Black Creek Canal virus primarily infects cotton rats (*Sigmodon hispidus*).[1] Field observations of several rodent species, including brush mice, deer mice, harvest mice, bank voles, and cotton rats, indicate that males are more likely to be infected than females.[2–7] In contrast to other rodent species, sex differences in hantavirus prevalence are not reported consistently among natural populations of Norway rats. Among adult Norway rats, however, males (90%) are infected with Seoul virus more often than females (75%).[8,9] Seoul virus is hypothesized to be transmitted through wounding, and the prevalence and severity of wounding is higher in adult male rats than either females or juvenile males.[8,9] Thus, sex differences in hantavirus prevalence may reflect interactions between behavior and physiology.[8–10]

Several studies suggest that host immunity plays an important role in protection against hantavirus infection. Humoral immune responses are initiated at the onset of infection and persist for the duration of infection. Although neutralizing antibody is important for reducing plasma viremia, antibody does not eliminate virus replication in tissue.[11] Cell-mediated immunity is important for elimination of hantavirus in the blood and in tissue.[11] Cytotoxic T-cells and helper T-cells (Th) are involved in the control of hantavirus replication and patients diagnosed with hantavirus have elevated Th1 (i.e., IFNγ, IL-2) and Th2 (i.e., IL-4, IL-6, IL-10) responses after infection.[11–15]

Reliance on Th1 or Th2 cells to overcome infection often differs between males and females, with males generating high Th1 phenotypic responses (i.e., elevated IFNγ, IL-2, and IgG2a) and females exhibiting high Th2 responses (i.e., increased IL-4, IL-5, IL-6, and IL-10 production).[10,16–19] Sex differences in Th1 and Th2 responses appear to be mediated by sex steroid hormones, such that androgens promote Th1 responses and estrogens facilitate Th2 responses.[18] After inoculation with Seoul virus, male rats have higher IgG2a responses than females, suggesting that males have elevated Th1 responses against hantavirus.[10] The extent to which hantavirus shifts the contribution of Th1 and Th2 cytokine responses during the course of infection in rodents, and how these responses differ between infected males and females has not been examined and is the primary goal of this study. Because sex differences in Th1 versus Th2 responses to infection are well-documented[10,16–19], male rats were hypothesized to have elevated Th1 responses and females were hypothesized to have elevated Th2 responses against Seoul virus.

MATERIALS AND METHODS

Animals. 70–80 day old male (n = 75) and female (n = 75) Long Evans rats (*Rattus norvegicus*) were purchased from Charles Rivers Laboratories (Raleigh, NC) and housed individually in polypropylene cages covered with polyester filter bonnets. All animals were housed in a Biosafety Level (BSL) 3 animal facility. Animals were maintained on a constant light-dark 16:8 cycle with lights on at 0600 hr EST. Food and sterile tap water were available *ad lib.* The Johns Hopkins Animal Care and Use Committee (protocol # RA98H536) and the Johns Hopkins Office of Health, Safety, and Environment (registration # A9902030102) approved all procedures described in this paper.

Procedures. After a two-week acclimation period, 60 males and 60 females were inoculated intraperitoneally (ip) with 10^4 plaque forming units (pfu) of purified Seoul virus (strain SR-ll) suspended in 0.2 cc Minimum Essential Medium Eagle (with Earle's salts; Mediatech Cellgro, Herndon, VA).[10] Seoul virus was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Ft. Dettrick, MD) where virus was isolated from neonatal rat brains and passaged 4 times in Vero E6 cells. The remaining 15 males and 15 females were used as controls (Day 0 group). Infected animals were assigned to have blood, saliva, urine, feces, lung, kidney, and spleen samples collected at 10, 20, 30, or 40 days post-infection. Animals were anesthetized with methoxyflurane vapors (Metofane, Schering Plough, Union, NJ), and blood samples were collected from the retro-orbital sinus into heparinized tubes, centrifuged at 2,500 rpm for 30 min, and plasma was removed and stored at

−80°C. Saliva samples were collected from anesthetized rats after injecting animals ip with 2.5 mg/kg of pilocarpine HCl (Sigma, St. Louis, MO) suspended in 0.9% sterile saline.[20] After saliva was collected, animals were killed, and urine, feces, lung, kidney, and spleen samples were collected.

Tissue culture and cytokine enzyme-linked immunosorbent assays (ELISA). Spleens were placed in sterile RPMI-1640 medium (Mediatech Cellgro), and splenocytes were recovered by pressing the whole spleen between sterile frosted glass slides. Separated cells were suspended in culture medium (RPMI-1640/Hepes supplemented with 1% penicillin [5,000 U/ml]/streptomycin [5,000 μg/ml], 1% L-glutamine [2 mM/ml], 0.1% 2-mercaptoethanol [5×10^{-2} M/ml], and 10% heat-inactivated fetal bovine serum [FBS]), layered on Ficoll-Histopaque (Sigma), and centrifuged at $900 \times g$ for 40 min. The buffy layer of cells was removed, washed twice with RPMI-1640, and re-suspended in culture medium. Lymphocyte counts and viability were determined using a hemacytometer and trypan blue exclusion (Sigma). Viable cells were adjusted to 2×10^6 cells/ml by dilution with culture media, and 200 μl were added to the wells of sterile round-bottom 96-well tissue culture plates. Con A (Sigma) was added at final concentrations of 0.5, 1, or 3 μg/well for IFNγ, IL-2/IL-10, and IL-4, respectively. Plates were incubated at 37°C with 5% CO_2 for 48 hr to assess IL-2, IL-4, and IL-10 production and 72 hr for IFNγ production. Mitogen concentrations and incubation times were based on pilot data (Klein SL and Glass GE, unpublished data). At the end of the incubation, cell supernatant was removed from each well, centrifuged at $5{,}000 \times g$ for 5 min, re-suspended in sterile tubes, and stored at −80°C.

IL-2 and IL-10 concentrations were assayed by ELISA using the manufacturer's protocols for the OptEIA IL-2 and IL-10 kits (Pharmingen, San Diego, CA). To assess IFNγ and IL-4 concentrations matched antibody pairs and recombinant cytokines were used with the manufacturer's protocol (IFNγ: Biosource International, Camarillo, CA; IL-4: Pharmingen). For each ELISA, microtiter plates were coated with capture antibody, for the respective cytokine, diluted in coating buffer (0.1 M carbonate, pH 9.5, for IL-2, IL-4, and IFNγ; 0.2 M sodium phosphate, pH 6.5, for IL-10). Undiluted supernatants were added in duplicate to anti-cytokine antibody-coated wells, and serially diluted to 1:10 and 1:100 in PBS-Tween (PBS-T) with 10% FBS. Horseradish peroxidase-conjugated secondary antibody diluted in PBS with 10% FBS was added, and enzyme reactions were developed using tetramethylbenzidine substrate buffer (Pharmingen). Plates were protected from light during the enzyme-substrate reaction, which was terminated after 15–30 min by adding 2 N H_2SO_4. The optical density (OD) was measured at 450 nm. The sample dilution used in data analyses was based on the concentration that yielded values within the linear range of the standard curves. Undiluted samples were used to assess IL-4 and IL-10 concentrations, and samples diluted 1:10 were used to assess IL-2 and IFNγ production.

Antibody ELISA. Microtiter plates were coated overnight at 4°C with 100 μl of gamma-irradiated Vero E6 cells infected with Seoul virus or gamma irradiated uninfected Vero E6 cells diluted 1:500 in carbonate buffer. Plasma samples were diluted 1:100 in PBS-T with 2% FBS, and 100 μl of each plasma dilution were added in duplicate to antigen-coated wells containing either infected or uninfected Vero E6 cells. Positive control samples (i.e., pooled plasma from rats previously determined to have anti-Seoul virus IgG) and negative control samples (i.e., pooled plasma from Seoul virus-naive rats) were included. The plates were sealed, incubated at 37°C for 1 hr, and then washed with PBS-T. Secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD; alkaline phosphatase-conjugated anti-rat IgG (H+L), horseradish peroxidase-conjugated anti-rat IgG1, or horseradish peroxidase-conjugated anti-rat IgG2a diluted 1:400 in PBS with 2% FBS) was then added and plates were sealed and incubated for 1 hr at 37°C. Plates were washed with PBS-T, and substrate buffer (0.5 mg/ml p-Nitrophenyl phosphate diluted in diethanolamine substrate buffer for alkaline phosphatase reactions or tetramethylbenzidine for horseradish peroxidase reactions) was added to each well. Plates were protected from light during the enzyme-substrate reaction which was terminated after 30–45 min by adding 25 μl of 1.5 M NaOH to each well for alkaline phosphatase reactions or 25 μl of 2 N H_2SO_4 to each well for horseradish peroxidase reactions. The OD was measured at 405 nm for alkaline phosphatase reactions, and 450 nm for horseradish peroxidase reactions and the average OD for each set of uninfected Vero E6 duplicates was subtracted from the average OD for each set of infected Vero E6 duplicates. Samples were considered positive if the average adjusted OD was ≥ 0.100 OD units. To minimize intra- and inter-plate variability, the average adjusted OD for each sample was expressed as a percentage of its plate positive control OD for statistical analyses.[21]

RNA isolation. Virus RNA was isolated using a guanidine isothiocyanate procedure.[22] For RNA isolation from saliva and urine, samples were collected from each rat and added to Trizol LS reagent (Life Technologies, Rockville, MD) at a 3:1 ratio. RNase-free glycogen (10 μg) also was added to saliva and urine samples as a carrier. For feces, lung, kidney, and spleen samples, 50–100 mg of tissue were homogenized in Trizol LS, centrifuged at $12{,}000 \times g$ for 10 min, and supernatants were transferred to clean (RNase-free) tubes. The manufacturer's protocol was used to separate, precipitate, and re-suspend viral RNA (Trizol LS).

Nested RT-PCR. Viral RNA was assessed in saliva, urine, feces, lung, kidney, and spleen samples using nested reverse transcriptase polymerase chain reaction (RT-PCR) and the presence of virus in saliva, urine, or feces was used to determine virus shedding. A 280 base pair (bp) nucleotide sequence of the SR-11 S genome was amplified using two 20 bp primers, HTN-S4 (5′ GATAGGTGTCCACCAACATG 3′) and HTN-S6 (5′ AGCTCTGGATCCATGTCATC 3′), that amplified positions 979–1259.[22] The DNA fragment obtained from the RT-PCR was further amplified using primers, HTN-S3 (5′ GCCTTCTTTTCTATACTTCAGG 3′) and HTN-S5 (5′ CCAGGCAACCATAAACATAAC 3′), designed to amplify a 176 bp nucleotide sequence (positions 1031–1207).[10]

First strand synthesis cDNA was prepared using the GeneAmp RNA PCR kit protocol (Applied Biosystems, Foster City, CA). A 20 μl reaction mixture containing 0.3 μM of the HTN-S4 primer and 1 μl of sample RNA was incubated in a DNA thermocycler (Genius, Techne, Princeton, NJ) at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min,

TABLE 1
Plasma anti-Seoul virus antibody†

		Day post-inoculation				
		0	10	20	30	40
IgG	M	0.0 ± 0.0	5.1 ± 1.0	74.4 ± 13.5	147.2 ± 36.2	202.3 ± 38.7*
	F	0.0 ± 0.0	11.4 ± 3.0	50.2 ± 10.69	94.2 ± 25.8	120.2 ± 40.7
IgG2a	M	0.0 ± 0.0	0.68 ± 0.4	9.44 ± 3.5	27.8 ± 6.0	61.7 ± 15.6*
	F	0.0 ± 0.0	2.65 ± 1.1	6.47 ± 2.2	39.1 ± 12.4	23.2 ± 15.5
IgG1	M	0.0 ± 0.0	2.09 ± 1.7	0.29 ± 0.2	8.65 ± 6.0	30.1 ± 20.8
	F	0.0 ± 0.0	1.68 ± 1.7	2.30 ± 2.3	2.99 ± 1.7	89.6 ± 79.1

† Plasma anti-Seoul virus antibody responses (mean ± SE) in male (M) and female (F) rats. Blood samples were collected 0, 10, 20, 30, or 40 days following inoculation with Seoul virus. Data are presented as IgG, IgG2a, and IgG1 units in which the average adjusted optical density (OD) of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. Asterisk (*) indicates time points where males had higher antibody responses than females, based on a two-way ANOVA ($P < 0.05$).

and then held at 4°C. The reaction mixture contained 5 mM $MgCl_2$, 1 mM dNTPs, 1 unit RNase inhibitor, and 2.5 units MuLV reverse transcriptase. The positive control was SR-11 RNA isolated from virus stock and the negative control was diethyl pyrocarbonate (DEPC) water.

The 280 bp sequence was amplified in a 100 μl reaction mixture containing 20 μl of the cDNA, 0.3 μM of the HTN-S6 primer, and 2.5 units of polymerase (AmpliTaq, Applied Biosystems). The nested 176 bp sequence was amplified in a 100 μl reaction mixture containing 2 μl of the first DNA amplification product, 20 μM HTN-S3 primer, 20 μM HTN-S5 primer, 10 mM $MgCl_2$, 1 mM dNTPs, and 2.5 units of polymerase. Primary and secondary reactions were amplified for one cycle at 94°C for 3 min and 40 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 60 sec, followed by 10 min at 72°C. The PCR products were electrophoresed on a 4% gel (3% NuSieve + 1% SeaKem; FMC Bioproducts, Rockland, ME), stained with ethidium bromide, and examined for bands of the appropriate size.

Statistical analyses. Sex differences in cytokine and antibody production following Seoul virus infection were analyzed using ANOVAs with two between subject variables (sex and day post-inoculation). Because males were hypothesized *a priori* to have elevated Th1 responses (i.e., IgG2a, IFNγ, and IL-2) and females were hypothesized to have elevated Th2 responses (i.e., IgG1, IL-4, and IL-10) following infection, responses were analyzed using one-tailed ANOVAs.[23] Logarithmic transformations were used in cases where the data violated the assumptions of a normal distribution.[23] Significant interactions were further analyzed using the Tukey method for pairwise multiple comparisons. Virus and antibody prevalence (i.e., the number of animals with detectable virus or antibody, respectively) were compared between males and females using Chi-square analyses. Mean differences were considered statistically significant if $P < 0.05$.

RESULTS

Antibody responses. Based on the presence of detectable IgG (i.e., ≥ 0.100 OD units), there was no difference in the proportion of males (29/60) and females (23/60) producing anti-Seoul virus IgG after inoculation (i.e., Days 10–40 post inoculation) ($P > 0.05$). Because seroconversion in rodents typically occurs by Day 30 post-inoculation,[3,10,24] antibody prevalence 30–40 days after inoculation was compared and also did not differ between males (17/30) and females (14/30) ($P > 0.05$). Males, however, had higher anti-Seoul virus IgG titers than females 40 days post-inoculation (F [3, 146] = 2.77, $P < 0.05$; Table 1). After inoculation with Seoul virus, male rats also had higher IgG2a responses than females 40 days post-inoculation (F [3, 145] = 2.76, $P < 0.05$; Table 1). In contrast, sex differences in anti-Seoul virus IgG1 responses were not observed ($P > 0.05$; Table 1).

Virus shedding. Not all animals with measurable antibody (Days 10–40 post-inoculation) shed virus in saliva, urine, or feces (shedding = 33/52, not shedding = 19/52) ($P > 0.05$). There were, however, sex related differences in virus shedding. Following inoculation, more males (28/58) shed virus in saliva than females (15/54) ($X^2 = 4.14$, $df = 1$, $P < 0.05$; Table 2). Although there was no sex difference in the number of animals shedding virus in either urine or feces ($P > 0.05$ in each case; Table 2), more males (14/55) shed virus through multiple routes (i.e., a combination of at least two routes, including saliva, urine, and feces) than females (5/53) ($X^2 = 2.74$, $df = 1$, $P < 0.05$; Figure 1).

The prevalence of Seoul virus in the spleen also differed between the sexes, with more males (31/57) having detectable virus than females (20/59) ($X^2 = 4.14$, $df = 1$, $P < 0.05$; Table 2). A similar number of males and females had viral RNA present in lung and kidney ($P > 0.05$ in each case; Table 2). There also was no sex difference in the number of animals with viral RNA present in multiple organs (males = 33/58, females = 27/59; $P > 0.05$; Figure 1).

Cytokine responses. Splenic IL-2 concentrations did not change significantly following Seoul virus infection ($P > 0.05$; Figure 2a). After infection, IL-2 concentrations were higher in males than females 10 days and 40 days post-inoculation (F [1, 98] = 10.49, $P < 0.05$; Figure 2a). IFNγ production increased after infection in both male and female rats (F [4, 128] = 4.29, $P < 0.05$; Figure 2b). Males also had higher concentrations of splenic IFNγ than females early during infection (F [1, 98] = 3.47, $P < 0.05$; Figure 2b).

IL-4 concentrations increased in both sexes following infection and peaked 10 and 20 days post-infection (F [4, 113] = 3.76, $P < 0.05$; Figure 2c). Sex differences in splenic IL-4 production were not observed ($P > 0.05$; Figure 2c). IL-10 concentrations in the spleen did not change following infection ($P > 0.05$; Figure 2d). Sex differences in IL-10 production also were not apparent ($P > 0.05$; Figure 2d).

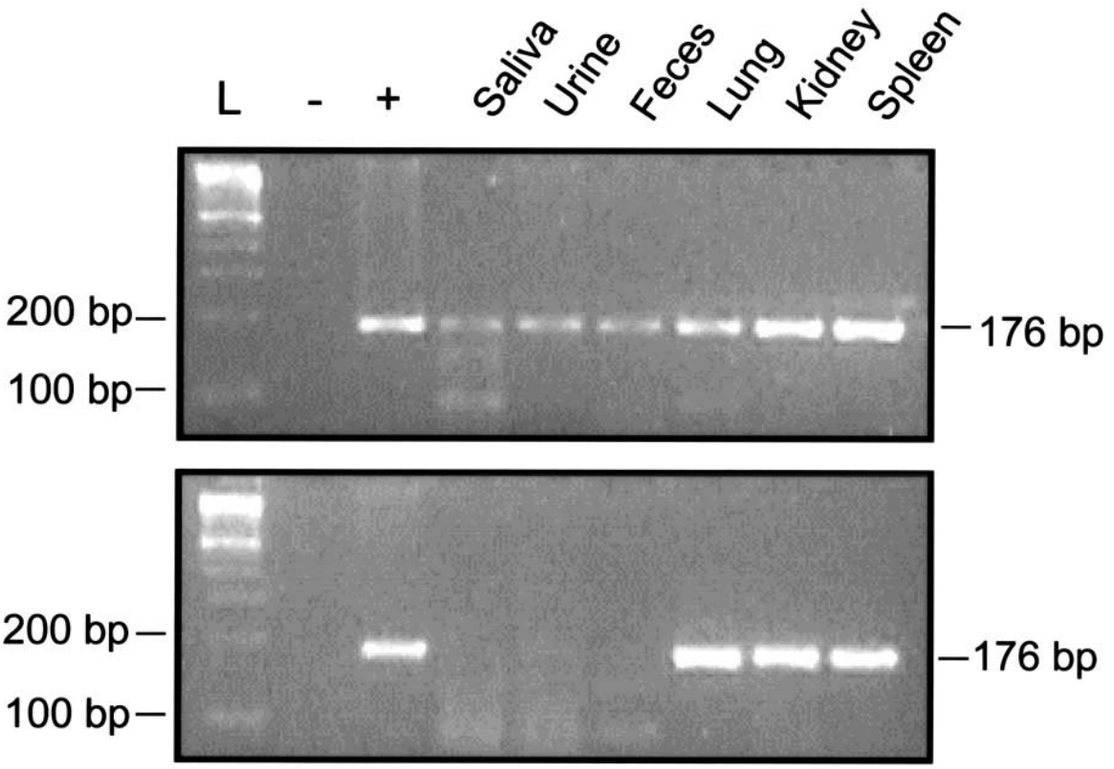

FIGURE 1. Seoul virus detection in saliva, urine, feces, lung, kidney, and spleen samples. Viral RNA was examined in samples collected 0, 10, 20, 30, or 40 days post-inoculation using nested reverse transcriptase polymerase chain reaction (RT-PCR) (see Methods). These data represent samples collected from a male and female rat 40 days post-inoculation. Lane 1 is a 100 bp ladder, lane 2 is the negative control (−) (i.e., diethyl pyrocarbonate [DEPC] water), lane 3 is the positive control (+) (i.e., cDNA from SR-11 RNA), lanes 4–9 are samples collected from a male (top panel) and a female (bottom panel) rat. The 176 bp PCR products were electrophoresed on an agarose gel (4%) and visualized with ethidium bromide.

DISCUSSION

Sex differences in the prevalence of hantavirus infection have been observed in several natural rodent reservoir species.[2–7] In each case, males are infected more often than females. In the present study, although males did not shed virus longer than females, males were more likely to shed virus through multiple routes than females. Increased virus shedding among males may explain why males are more likely to acquire Seoul virus infection following aggressive encounters among natural populations of Norway rats.[9] High circulating androgen concentrations increase the probability of engaging in aggressive encounters in several vertebrate species.[25] In addition to modulating aggression, sex steroid hormones can affect immune responses against infection and may contribute to increased hantavirus prevalence among male rodents.[10,26]

TABLE 2

Viral RNA in samples collected from male and female rats after Seoul virus inoculation†

		Days post-inoculation					
		0	10	20	30	40	Total‡
Saliva	M	0/11	9/13	7/15	4/15	8/15	28/58*
	F	0/11	4/13	3/14	4/14	4/13	15/54
Urine	M	0/14	6/14	0/15	6/13	3/15	15/57
	F	0/13	4/13	3/13	6/14	1/13	14/53
Feces	M	0/14	3/15	6/15	1/15	4/15	14/60
	F	0/14	2/15	4/15	2/15	2/15	10/60
Lung	M	0/14	9/14	8/12	11/14	11/15	39/55
	F	0/14	10/14	10/12	10/14	8/15	38/55
Kidney	M	0/14	10/15	6/14	6/15	10/15	32/59
	F	0/14	9/14	7/14	7/15	6/15	29/58
Spleen	M	0/14	7/15	7/13	9/14	8/15	31/57*
	F	0/14	5/15	6/14	6/15	3/15	20/59

† Viral RNA was examined in samples collected from male (M) and female (F) rats 0, 10, 20, 30, or 40 days after inoculation with Seoul virus. Data are presented as the proportion of animals with detectable viral RNA relative to the number of animals tested. Unequal numbers of animals per group are due to technical difficulties.

‡ Total virus prevalence in males and females day 10–40 post-inoculation. An asterisk (*) indicates that virus prevalence was higher in males than females, based on chi-square analyses ($P < 0.05$).

In the present study, Th1 responses differed between the sexes whereas Th2 responses did not. Following infection, males had higher IgG2a, IL-2, and IFNγ concentrations and similar IgG1, IL-4, and IL-10 responses as compared to females. A comparable pattern of immune responses against Seoul virus has been reported for Norway rats.[10] Studies of other infections in rodents suggest that females often have higher Th2 responses than males, and this is due in part to the effect of estrogens on cytokine production.[17,18] In contrast to estrogens, androgens promote differentiation of Th cells to a Th1 phenotype.[18] In the present study, males had higher IgG2a, IFNγ, and IL-2 responses than females suggesting that androgens may facilitate Th1 responses against hantavirus infection. Previous data, however, reveal that castrated

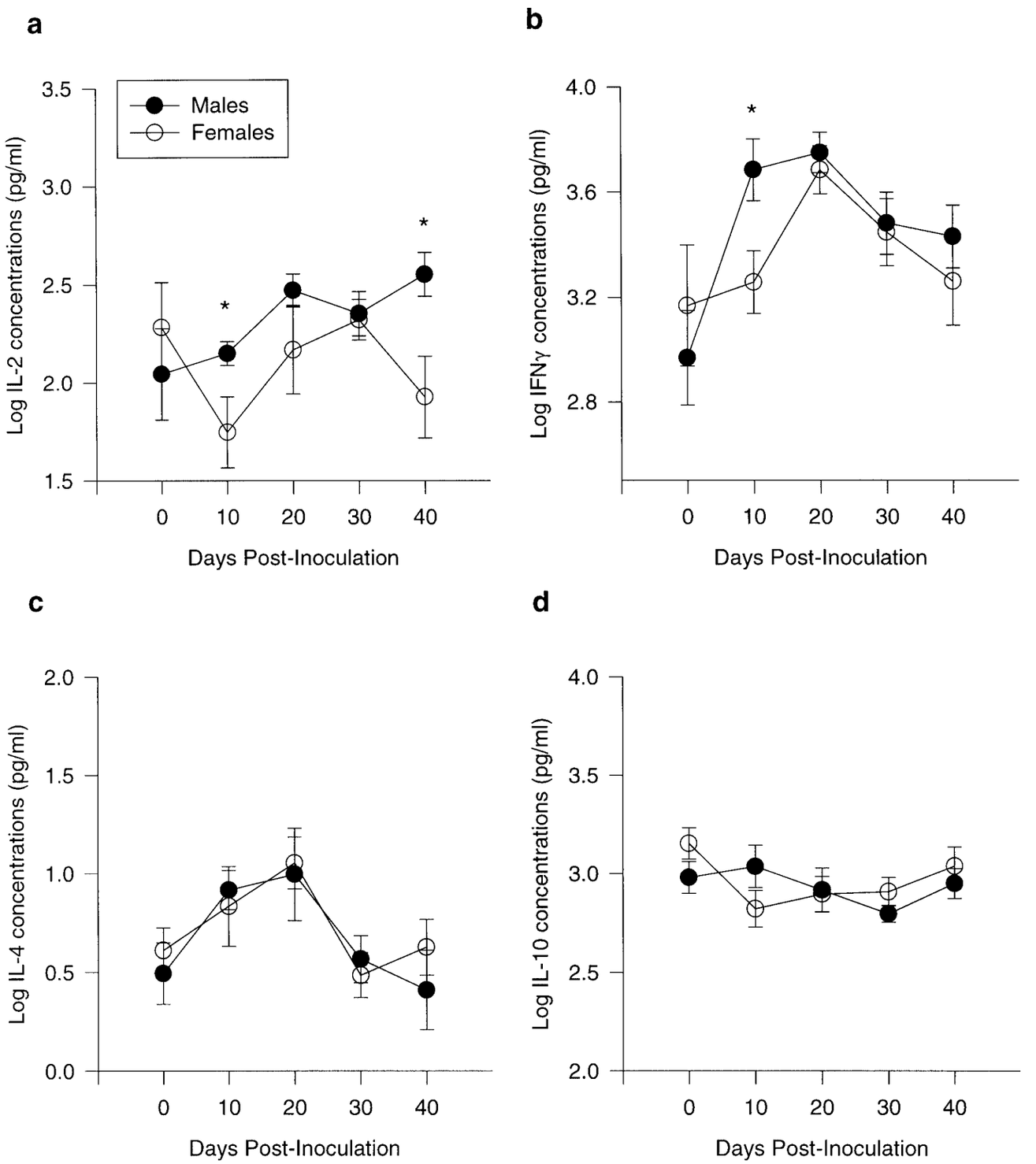

FIGURE 2. Concentrations (pg/ml) (mean ± SE) of IL-2 (**a**), IFNγ (**b**), IL-4 (**c**), and IL-10 (**d**) from splenocytes that were isolated from male and female rats 0, 10, 20, 30, or 40 days post-inoculation. Data are presented as the natural logarithm. An asterisk (*) indicates time points where concentrations differed between the sexes based on a two-way ANOVA ($P < 0.05$).

and intact male rats have similar anti-Seoul virus IgG2a responses,[10] suggesting that increased Th1 responses against Seoul virus may not be due to a direct effect of circulating androgens. In contrast, sex differences in immune responses against Seoul virus may be organized by sex steroids earlier during ontogeny.[26] The organizational effects of sex steroids on Seoul virus infection are currently under investigation.

Higher Th1 responses in males may indicate that males have more efficient immune responses against infection. Alternatively, males may have higher antibody and cytokine responses than females because virus replication is increased in males. Higher Th1 responses, including increased production of IgG2a, IFNγ, and IL-2, are associated with increased susceptibility to infection with coxsackievirus and Sindbis virus in mice.[18,27] Although quantitative analyses were not conducted, males were more likely to shed Seoul virus through multiple routes than females, suggesting that increased Th1 responses among males may be a consequence of increased virus replication. Studies using quantitative PCR will determine if there are sex-steroid mediated differences in virus replication.

In addition to increased shedding of Seoul virus in males, more males had viral RNA in splenic tissue than females. When viral RNA was examined in all target organs collectively (i.e., lung, kidney, and spleen), females were as likely to have Seoul virus present in multiple organs as males, suggesting that the sexes are equally susceptible to hantavirus infection. Studies of Norway rats and cotton rats reveal that after inoculation with Seoul virus or Black Creek Canal virus, respectively, antibody prevalence is similar between the sexes.[10,28] Although males and females may be equally susceptible to hantavirus, the pattern of their immune responses differs and may explain why males often shed virus longer than females.[10] In the present study, however, male rats did not shed virus longer than females. This discrepancy may be an artifact of the experimental design used in the present study (i.e., between-subjects design) that did not permit individual animals to be followed over the course of infection. Smaller sample sizes in this study as compared to previous studies (i.e., 15 animals/sex as compared to 30 animals/sex) also may contribute to why certain trends in viral shedding were not statistically significant.[10]

The present study revealed that cytokine concentrations increased in both sexes following infection. Specifically, concentrations of IFNγ and IL-4 increased and peaked 10–20 days after inoculation with Seoul virus. These data suggest that both Th1 and Th2 responses are facilitated following infection. Patients diagnosed with hantavirus infection often have elevated Th1 (i.e., IFNγ, IL-2) and Th2 (i.e., IL-4, IL-6, IL-10) responses in blood and in target organs, including the lungs and spleen.[13–15] The extent to which increased cytokine production modifies virus replication still is unclear. Future studies should determine the precise role of Th1 and Th2 responses in hantavirus infection by using monoclonal antibodies to deplete cytokines in vivo and shift the Th1 and Th2 responses to hantavirus infection. Studies utilizing quantitative RT-PCR to examine cytokine production also are required to determine if changes in cytokine responses are due to the non-specific effects of viral burden, or are due to specific changes in host immune function.

The data from the present study support and extend previous findings suggesting that males have higher Th1 immune responses and are more likely to shed Seoul virus than females. Sex differences in Th1 versus Th2 responses to infection are hypothesized to be related to sex steroid hormones.[18,19] Although several studies illustrate a relationship between sex steroids and immune responses against infection,[26,29] other studies have been unable to document a direct relationship among androgens, immunocompetence, and infection status in adult males.[10,30–32] Although endocrine-immune interactions may play a fundamental role in Seoul virus infection, sex steroids also may modulate genes and behaviors that make males more susceptible to infection.[26] In summary, the present study illustrates that males and females mount different immune responses against infection, and that males are more likely than females to shed Seoul virus following infection. Whether sex steroid hormones modulate responses against hantavirus infection remains to be determined.

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REFERENCES

1. Mertz GJ, Hjelle BL, Bryan RT, 1997. Hantavirus infection. *Adv Intern Med 42:* 369–421.
2. Bernshtein AD, Apekina NS, Mikhailova TV, Myasnikov YA, Khlyap LA, Korotkov YS, Gavrilovskaya IN, 1999. Dynamics of Puumala hantavirus infection in naturally infected bank voles (*Clethrionomys glareolus*). *Arch Virol 144:* 2415–2428.
3. Childs JE, Ksiazek TG, Spiropoulou CF, Krebs JW, Morzunov S, Maupin GO, Gage KL, Rollin PE, Sarisky J, Enscore RE, Frey JK, Peters CJ, Nichol ST, 1994. Serologic and genetic identification of *Peromyscus maniculatus* as the primary reservoir for a new hantavirus in the southwest United States. *J Infect Dis 169:* 1271–1280.
4. Glass GE, Livingstone W, Mills JN, Hlady WG, Fine JB, Biggler W, Coke T, Frazier D, Atherley S, Rollin PE, Ksiazek TG, Peters CJ, Childs JE, 1998. Black Creek Canal virus infection in *Sigmodon hispidus* in southern Florida. *Am J Trop Med Hyg 59:* 699–703.
5. Mills JN, Ksiazek TG, Ellis BA, Rollin PE, Nichol ST, Yates TL, Gannon WL, Levy CE, Engelthaler DM, Davis T, Tanda DT, Frampton JW, Nichols CR, Peters CJ, Childs JE, 1997. Patterns of association with host and habitat: antibody reactive with Sin Nombre virus in small mammals in the major biotic communities of the southwestern United States. *Am J Trop Med Hyg 56:* 273–284.
6. Mills JN, Johnson JM, Ksiazek TG, Ellis BA, Rollin PE, Yates TL, Mann MO, Johnson MR, Campbell ML, Miyashiro J, Patrick M, Zyzak M, Lavender D, Novak MG, Schmidt K, Peters CJ, Childs JE, 1998. A survey of hantavirus antibody in small-mammal populations in selected United States national parks. *Am J Trop Med Hyg 58:* 525–532.
7. Weigler BJ, Ksiazek TG, Vandenbergh JG, Levin M, Sullivan

WT, 1996. Serological evidence for zoonotic hantaviruses in North Carolina rodents. *J Wildl Dis 32:* 354–357.
8. Childs JE, Korch GW, Glass GE, LeDuc JW, Shah KV, 1987. Epizootiology of hantavirus infections in Baltimore: isolation of a virus from Norway rats, and characteristics of infected rat populations. *Am J Epidemiol 126:* 55–68.
9. Glass GE, Childs JE, Korch GW, LeDuc JW, 1988. Association of intraspecific wounding with hantaviral infection in wild rats (*Rattus norvegicus*). *Epidemiol Infect 101:* 459–472.
10. Klein SL, Bird BH, Glass GE, 2000. Sex differences in Seoul virus infection are not related to adult sex steroid concentrations in Norway rats. *J Virol 74:* 8213–8217.
11. Asada H, Tamura M, Kondo K, Okuno Y, Takahashi Y, Dohi S, Nagai T, Kurata T, Yamanishi K, 1987. Role of T lymphocyte subsets in protection and recovery from Hantaan virus infection in mice. *J Gen Virol 68:* 1961–1969.
12. Groen J, Gerding M, Jordans JGM, Clement JP, Osterhaus ADME, 1994. Class and subclass distribution of hantavirus-specific serum antibodies at different times after the onset of nephropathia epidemica. *J Med Virol 43:* 39–43.
13. Krakauer T, LeDuc JW, Morrill JC, Anderson AO, Krakauer H, 1994. Serum levels of alpha and gamma interferons in hemorrhagic fever with renal syndrome. *Viral Immunol 7:* 97–101.
14. Linderholm M, Ahlm C, Settergren B, Waage A, Tärnvik A, 1996. Elevated plasma levels of tumor necrosis factor (TNF)-α, soluble TNF receptors, interleukin (IL)-6, and IL-10 in patients with hemorrhagic fever with renal syndrome. *J Infect Dis 173:* 38–43.
15. Mori M, Rothman AL, Kurane I, Montoya JM, Nolte KB, Norman JE, Waite DC, Koster FT, Ennis FA, 1999. High levels of cytokine-producing cells in the lung tissues of patients with fatal hantavirus pulmonary syndrome. *J Infect Dis 179:* 295–302.
16. Bijlsma JWJ, Cutolo M, Masi AT, Chikanza IC, 1999. The neuroendocrine immune basis of rheumatic diseases. *Trends Immunol 20:* 298–301.
17. Daniels CW, Belosevic M, 1994. Serum antibody responses by male and female C57Bl/6 mice infected with *Giardia muris. Clin Exp Immunol 97:* 424–429.
18. Huber SA, Kupperman J, Newell MK, 1999. Hormonal regulation of CD4+ T-cell responses in coxsackievirus B3-induced myocarditis in mice. *J Virol 73:* 4689–4695.
19. Huber SA, Pfaeffle B, 1994. Differential Th1 and Th2 cell responses in male and female BALB/c mice infected with coxsackievirus group B type 3. *J Virol 68:* 5126–5132.
20. Bodner L, Baum BJ, 1985. Characteristics of stimulated parotid gland secretion in the aging rat. *Mech Ageing Dev 31:* 337–342.
21. de Savigny D, Voller A, 1980. The communication of ELISA data from laboratory to clinician. *J Immunoassay 1:* 105–128.
22. Arthur RR, Lofts RS, Gomez J, Glass GE, LeDuc JW, Childs JE, 1992. Grouping of hantaviruses by small (S) genome segment polymerase chain reaction and amplification of viral RNA from wild-caught rats. *Am J Trop Med Hyg 47:* 210–224.
23. Howell DC, 1992. *Statistical Methods for Psychology*, Third edition. Belmont, CA: Duxbury Press.
24. Nuzum EO, Rossi CA, Stephenson EH, LeDuc JW, 1988. Aerosol transmission of hantaan and related viruses to laboratory rats. *Am J Trop Med Hyg 38:* 636–640.
25. Nelson RJ, 2000. *An Introduction to Behavioral Endocrinology*, Second edition. Sunderland, MA: Sinauer Associates, Inc.
26. Klein SL, 2000. The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehavioral Rev 24:* 627–638.
27. Wesselingh SL, Levine B, Fox RJ, Choi S, Griffin DE, 1994. Intracerebral cytokine mRNA expression during fatal and nonfatal alphavirus encephalitis suggests a predominant type 2 T cell response. *J Immunol 152:* 1289–1297.
28. Hutchinson KL, Rollin PE, Shieh W-J, Zaki S, Greer PW, Peters CJ, 2000. Transmission of Black Creek Canal virus between cotton rats. *J Med Virol 60:* 70–76.
29. Lindström K, Krakower D, Lundström JO, Silverin B, 2001. The effects of testosterone on a viral infection in greenfinches (*Carduelis chloris*): an experimental test of the immunocompetence-handicap hypothesis. *Proc R Soc Lond B 268:* 207–211.
30. Evans MR, Goldsmith AR, Norris SRA, 2000. The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). *Behav Ecol Sociobiol 47:* 156–163.
31. Hasselquist D, Marsh JA, Sherman PW, Wingfield JC, 1999. Is avian humoral immunocompetence suppressed by testosterone? *Behav Ecol Sociobiol 45:* 167–175.
32. Klein SL, Gamble HR, Nelson RJ, 1999. Sex differences in *Trichinella spiralis* infection are not mediated by circulating steroid hormones in voles. *Am J Physiol 277:* R1362–R1367.