Gender Is a Major Determinant of the Clinical Evolution and Immune Response in Hamsters Infected with *Leishmania* spp.

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In regions where leishmaniasis is endemic, clinical disease is usually reported more frequently among males than females. This difference could be due to disparate risks of exposure of males and females, but gender-related differences in the host response to infection may also play a role. Experimental studies of the influence of gender on *Leishmania* infection have not included parasites of the subgenus *Viannia*, which is the most common cause of cutaneous leishmaniasis in the Americas. Mice are not readily susceptible to infection by *Leishmania* (*Viannia*) spp., but cutaneous infection of hamsters with *L. (V.) panamensis* or *L. (V.) guyanensis* resulted in chronic lesions typical of the human disease caused by these parasites. Strikingly, infection of male hamsters resulted in significantly greater lesion size and severity, an increased rate of dissemination to distant cutaneous sites, and a greater parasite burden in the draining lymph node than infection in female animals. Two lines of evidence indicated this gender-related difference in disease evolution was determined at least in part by the sex hormone status of the animal. First, prepubertal male animals had smaller and/or less severe cutaneous lesions than adult male animals. Second, infection of testosterone-treated female animals resulted in significantly larger lesions than in untreated female animals. The increased severity of disease in male compared to female animals was associated with significantly greater intralesional expression of interleukin-4 (IL-4) ($P = 0.04$), IL-10 ($P = 0.04$), and transforming growth factor β (TGF- β) ($P < 0.001$), cytokines known to promote disease in experimental leishmaniasis. There was a direct correlation between the expression of TGF- β mRNA and lesion size (Spearman's correlation coefficient = 0.873; $P < 0.001$). These findings demonstrate an inherent risk of increased disease severity in male animals, which is associated with a more permissive immune response.

The outcome of *Leishmania* infection depends on several biological traits of both the host and the infecting parasite strain. A number of epidemiological studies indicate that leishmaniasis occurs more frequently among adult males than females (3, 13, 38). It is unclear whether this difference is due merely to dissimilar risks of exposure because of the distinct activities of males and females or whether gender-related differences in the host immune response play a role in resistance and susceptibility to infection.

Epidemiological data for children <15 years of age, where both genders would seem to have similar risks of infection, indicate that boys are threefold more likely to develop visceral leishmaniasis than girls (35). Similarly, the disease rate for cutaneous leishmaniasis in an area of Brazil where the disease is endemic was shown to be 50% higher in males than females in all age groups, including children, who are expected to have comparable risks of exposure for both sexes (13). In studies of several different endemic foci in both the New and Old Worlds, regardless of cultural behaviors and occupational risks, men were reported to acquire cutaneous or visceral leishmaniasis more frequently than women (19, 38).

Experimental studies of animal models focusing on the influence of gender in *Leishmania* infection are scarce and have

not included *Leishmania* species of the subgenus *Viannia*, which is the most common cause of leishmaniasis in the Americas. In mice infected with *Leishmania major*, disease evolution was found to be different in males and females according to the route of inoculation, i.e., the intradermal route was more severe in females and the intravenous route was more severe in males (1, 26). In contrast, male DBA2 mice were more susceptible to subcutaneous *Leishmania mexicana* infection than were female mice (1). Other studies, comparing pregnant or castrated mice to normal controls, demonstrated that susceptibility to *L. major* or *L. mexicana* strongly depended on hormone levels, which in turn regulated the expression of different cytokines (2, 16, 17, 32, 33). The relative resistance of female mice to *L. mexicana* infection compared to male mice was related to increased expression of gamma interferon (IFN- γ) (32, 33). Although the study of mice infected with *Leishmania* spp. (especially *L. major*) has contributed to the understanding of the cellular immune response associated with a protective (Th1) and susceptible (Th2) phenotype (34), this rodent species is not readily susceptible to infection by *Leishmania* (*Viannia*) spp. Instead, the hamster is the model of choice because of its susceptibility to all species of the subgenus *Viannia* (12, 30). Cutaneous infection of hamsters results in chronic, but controlled, lesions, including the appearance of cutaneous metastases following chronic infection with some strains (20, 21). The immune response of hamsters is not as well characterized as that in mice, but the recent development of molecular

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probes has enabled the determination of cytokine patterns associated with experimental visceral leishmaniasis in the hamster model (23, 24). In the present study, using the hamster experimental model, we demonstrated that gender has a significant influence on the clinical evolution of, and immunological response to, *Leishmania (Viannia)* infection. These results are relevant to the design of preclinical and clinical trials of *Leishmania* vaccines and therapies for American cutaneous leishmaniasis.

MATERIALS AND METHODS

Animals. Recently weaned (3- to 4-week-old 40- to 50-g) or adult (3-month-old 100- to 110-g) Syrian hamsters of both genders, derived from the inbred Chester Beaty line (Charles River Laboratory), were used in all of the experiments. The animals were maintained under standard caging conditions and were provided with commercial rodent food and water ad libitum, according to the Guiding Principles for Biomedical Research Involving Animals (Council for International Organizations of Medical Sciences) and law 84 of the Estatuto Nacional de Protección de los Animales-Colombia of 1989.

Infection. *Leishmania (Viannia) panamensis* (MHOM/COL/84/1099) and *Leishmania (Viannia) guyanensis* (WHI/BR/78/M5313) promastigotes were cultured in Seneckje's medium (1984; Difco). Promastigotes (10^3 or 10^6) from the stationary phase of culture were washed, suspended in phosphate-buffered saline, and inoculated intradermally (50 μ l) in the hind foot of hamsters.

Hormone treatment. To evaluate the influence of sex hormones on the clinical evolution of *Leishmania* infection, male or female hamsters ($n = 8$ per group) were treated with an estrogen or androgen, respectively. Recently weaned male hamsters each received a subcutaneous implant (Compudose 200; Eli Lilly Laboratories) that released approximately 240 μ g of 17 β -estradiol per day throughout the study period. Individual female animals of a similar age received intramuscular injections of 1 mg of testosterone enantate (Testoviron-Depot; Schering) twice per week until the end of the experiment (3 months postinfection [p.i.]). The hormone was diluted 1:10 with sterile corn oil in order to inject 40 μ l per dose. After 20 days of hormone treatment, the animals were inoculated with 10^6 *L. (V.) panamensis* organisms as described above.

Clinical and parasitological evaluations. The animals were evaluated for lesion size and severity every 15 days from the fourth to the eighth week p.i. and monthly until 4 months p.i. These evaluations were carried out by measuring the thickness of the inoculated foot with a caliper (Digimatic; Mitutoyo Corp., Kawasaki, Japan) and subtracting the thickness of the contralateral foot; the lesion size was expressed in millimeters. The severity of the lesions in each group was estimated by the proportion of animals with necrosis and by ascribing a numerical value to represent the extent of cutaneous ulceration (0, no ulcer; 1, small; 2, medium; 3, large). The appearance of cutaneous metastases, which presented as swollen, depilated nodules in the toes, tail, or ears, were also recorded on a monthly basis until the end of the study (360 days p.i.).

The relative densities of parasites in the lymph nodes draining the sites of cutaneous inoculation were assessed as follows. The lymph nodes were aseptically removed, weighed, and triturated in Schneider's medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with penicillin (100 U/ml), streptomycin (10 mg/ml; Gibco BRL, Grand Island, N.Y.), and 10% heat-inactivated fetal calf serum. The tissue samples were adjusted to a concentration of 0.1 mg/ml, and 130 μ l per well was distributed in flat-bottom microplates (Becton Dickinson, Franklin Lakes, N.Y.) containing a layer (slant) of Seneckje's medium overlaid with a final volume of 260 μ l of Schneider's medium. Samples were serially twofold diluted up to 1:40,960. Also, 200 μ l of the same undiluted samples per tube was seeded in culture tubes containing Seneckje's medium.

In situ cytokine determination. Assessment of cytokine expression at the primary lesion site was carried out at 3.5 months p.i. in male and female hamsters infected with *L. (V.) panamensis* ($n = 7$ per group). Skin samples from uninfected age-matched animals were used as controls to determine baseline cytokine expression. The level of expression of hypoxanthine phosphoribosyltransferase (HPRT), IFN- γ , interleukin-10 (IL-10), IL-12p40, IL-4, and transforming growth factor β (TGF- β) mRNAs was evaluated by PCR amplification of reverse-transcribed mRNA followed by hybridization with a specific dUTP-digoxigenin-labeled oligonucleotide probe and quantification by densitometry. Briefly, the tissue was rapidly excised and snap frozen in liquid nitrogen. The frozen tissue was homogenized with a mortar, and the RNA was isolated and treated with DNase I to eliminate any contaminating genomic DNA by using the SV total RNA isolation system (Promega, Madison, Wis.) according to the manufacturer's

instructions. Approximately 2 μ g of RNA was reverse transcribed into cDNA using 200 U of Moloney murine leukemia virus reverse transcriptase, 80 U of RNase inhibitors (RNasin; Promega), 200 μ M deoxynucleoside triphosphates, 0.2 mM dithiothreitol, and 1 μ g of random hexamers (Promega). The cDNA was amplified by PCR as described previously (25) and subsequently dot blotted to a nylon membrane and hybridized with 0.2 pmol of dUTP-digoxigenin-labeled specific probes/ml. The hybridization product was detected by autoradiography (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions, and the intensity of the signal was quantified by densitometry (GEL-DOC 2000; Bio-Rad, Richmond, Calif.). The ratio of the level of cytokine mRNA expression to the level of HPRT expression in the same sample (the mRNA expression index) was determined in order to semiquantitatively compare the different groups. The absence of genomic DNA in the RNA preparation was confirmed by a lack of amplification product in a PCR using RNA that had not been reverse transcribed. The primers and internal probes used for this study were derived from the published sequences (24) and are as follows: HPRT (forward, CTTGCGATGTCATGGTAGAG; reverse, GTTGAGATATCATCCGCACC; internal, ATCTACAGTTATGGGAGTGG), IL-4 (forward, TCCTATCACTGACGGTAGAG; reverse, TGCAAATGAGGTCTTTCTCC; internal, GTACTGCTAGAAAGGCAGAG), IL-10 (forward, GGACAACATACTACTCACTG; reverse, ACAGGGGAGAAATCGATGAC; internal, CTCTGCCTGGGGCATCAC), IL-12p40 (forward, ACTGCTGCTTCACAAGAAGG; reverse, CTTCTCTGCAGACAGACG; internal, CGTCCAGAGTAATTTGCTGC), IFN- γ (forward, TCATTGAGAGCCAGATCGTC; reverse, GGCTAAGTTTCGTGACAGG; internal, ACAGGTCTGCCTTGATGGTG), and TGF- β (forward, GAGAAGAACTGCTGTGTGCG; reverse, ACCCACGTTAGTACACGATGG; internal, CCTTACTGTACTGTGTGTCC).

DTH and antibody responses. Delayed-type hypersensitivity (DTH) was determined in hamsters infected with *L. (V.) panamensis* at 45 days p.i. by intradermal injection in the forefoot of 10^8 formalin-inactivated promastigotes. The size of the induration was determined at 48 and 72 h by subtracting the thickness of the contralateral foot (injected with vehicle alone) from the thickness of the foot injected with inactivated promastigotes.

Total immunoglobulin G antibodies were determined in serum (diluted 1:100) at 3 months p.i. by enzyme-linked immunosorbent assay (ELISA) using a soluble *L. (V.) panamensis* antigen (1 μ g per well) and protein A labeled with peroxidase (Kirkegaard & Perry Laboratories).

Statistical analysis. Multiple comparisons between groups were made with a one-way analysis of variance (Duncan test). Paired comparisons between groups were carried out by Student's *t* test. Correlations among parasite burden, cytokine expression, and lesion size were determined by the Spearman rank correlation test. Significance was established at a *P* value of <0.05 . All of the analyses were carried out with SPSS, Inc., SPSS 7.5 Base for Windows 98.

RESULTS

Clinical evolution of primary lesions and development of skin metastases. Evaluation of the clinical evolution of primary lesions produced by infection with 10^6 stationary-phase promastigotes of *L. (V.) panamensis* or *L. (V.) guyanensis* demonstrated that male hamsters were significantly more susceptible than female hamsters (Fig. 1; $P < 0.05$ at all time points p.i.). Males infected with *L. (V.) panamensis* showed conspicuous lesions by the 30th day p.i., while females had very small lesions that barely reached the detection level (0.5-mm diameter) between the third and fourth months p.i. (Fig. 1A). The gender difference in lesion size persisted throughout the experiment. The difference in lesion size between male and female hamsters was greater for *L. (V.) panamensis*- than for *L. (V.) guyanensis*-infected animals (Fig. 1A and C). Skin necrosis, another measure of lesion severity, was more frequent and of greater extent in males than in females (Fig. 1B and D). Animals infected with *L. (V.) guyanensis* developed larger lesions ($P < 0.05$) and had a higher frequency of necrosis than animals of the same gender infected with an equal number of *L. (V.) panamensis* promastigotes (Fig. 1).

The influence of the inoculum size was most evident in male animals and during the early stage of infection, where infection

FIG. 1. Lesion size and severity of disease in male and female hamsters infected with *L. (V.) panamensis* or *L. (V.) guyanensis*. Age-matched male and female hamsters were infected with 10^6 stationary-phase promastigotes and evaluated every 15 days from the fourth to the eighth week p.i. and then monthly until 4 months p.i. The lesion size was determined by measuring the thickness of the inoculated foot with a caliper and subtracting the thickness of the contralateral foot. The lesion size is expressed as the mean ($\pm$ standard error of the mean [SEM]). The severity of lesions in each group was determined by calculating the proportion of animals with necrosis (bars) and giving a numerical lesion severity score representative of the size of the ulcer (0, no ulcer; 1, small; 2, medium; 3, large). The severity score is expressed as the mean of the lesion severity score (lines). (A) Lesion sizes of *L. (V.) panamensis*-infected hamsters. The difference in lesion size between the two groups was significant ($P < 0.05$) at all time points. (B) Lesion severities of *L. (V.) panamensis*-infected hamsters. The differences in frequency of dermal necrosis and lesion severity score between the two groups were significant ($P < 0.05$) at all time points. (C) Lesion sizes of *L. (V.) guyanensis*-infected hamsters. The difference in lesion size between the two groups was significant ($P < 0.05$) at all time points. (D) Lesion severities of *L. (V.) guyanensis*-infected hamsters. The differences in frequency of dermal necrosis and lesion severity score between the two groups were significant ($P < 0.05$) at all time points.

with 10^6 *L. (V.) panamensis* promastigotes resulted in larger lesions at 30 days p.i. than infection with 10^3 promastigotes ($P < 0.05$) (data not shown). This difference was not evident beyond the 30-day observation point, although at 4 months p.i. the lesions resulting from the small inoculum were still increasing in size, while lesions resulting from the large inoculum had peaked and were decreasing. The effect of inoculum size on the extent and proportion of animals with dermal necrosis was similarly most evident in male animals during the initial phase of infection (data not shown). Female animals showed no significant differences in lesion evolution upon infection with inocula of different sizes. Inoculation of male animals with the

high and low doses of *L. (V.) guyanensis* produced results similar to those obtained with *L. (V.) panamensis*. Female hamsters had a tendency to develop more severe lesions with the largest inoculum, but these differences were not statistically significant (data not shown).

There was no significant difference between the metastatic capacities of *L. (V.) guyanensis* and *L. (V.) panamensis*. Male hamsters were more prone to develop skin metastases than female hamsters after infection with either *L. (V.) panamensis* ($P = 0.05$; Fisher exact test) or *L. (V.) guyanensis* ($P = 0.018$; Fisher exact test) (Table 1). Only 1 of 27 female hamsters (4%) infected with *L. (V.) panamensis* showed cutaneous metastases

TABLE 1. Effect of gender on the frequency of cutaneous metastases in hamsters infected with promastigotes of *Leishmania (Viannia)* spp.

Dose	Frequency (%) of metastases ^a					
	<i>L. (V.) panamensis</i>			<i>L. (V.) guyanensis</i>		
	Adult	Juvenile	Total	Adult	Juvenile	Total
Female						
High ^b	0/6	1/7 (14)	1/13 (8)	0/7	0/7	0/14
Low ^c	0/7	0/7	0/14	0/6	0/6	0/12
Total	0/13	1/14 (7)	1/27 (4)	0/13	0/13	0/26
Male						
High ^b	0/6	5/7 (71)	5/13 (39)	0/7	2/6 (33)	2/13 (15)
Low ^c	1/7 (14)	0/6	1/13 (8)	2/3 (66)	1/7 (14)	3/10 (30)
Total	1/13 (8)	5/13 (39)	6/26 (23)	2/10 (20)	3/13 (23)	5/23 (22)

^a Evaluated at 360 days p. i.

^b Infected intradermally in the hind foot with 10^6 parasites.

^c Infected intradermally in the hind foot with 10^3 parasites.

compared with 6 of 26 male hamsters (23%). Similarly, none of 26 females infected with *L. (V.) guyanensis* developed skin metastases, while 5 of 23 males (22%) showed this pathological manifestation. The prevalence ratio indicated that the odds that males would develop skin metastases due to *Leishmania (Viannia)* spp. was 12-fold higher than females ($\chi^2 = 8.7$; $P = 0.001$). In general, the age of the animal at the time of infection

or the size of the inoculum did not have a significant influence on the proportion of animals that developed skin metastases.

DTH and antibody responses. At 45 days p.i., there was no difference in the DTH response, as measured by foot induration 48 h after challenge with killed *L. (V.) panamensis* promastigotes, between males (0.35 ± 0.14 -mm diameter [mean $\pm$ standard deviation]) and females (0.31 ± 0.18 -mm diameter). Similarly, by ELISA, no differences in *Leishmania*-specific antibody titers (total immunoglobulin G at a 1:100 serum dilution) were observed between male (optical density, 0.38 ± 0.15) and female (optical density, 0.32 ± 0.14) hamsters.

Effect of exogenous administration of hormones. The administration of the opposing sex hormones to male and female hamsters for 20 days prior to and throughout the course of *L. (V.) panamensis* infection altered the course of disease evolution. Female hamsters treated with testosterone developed larger cutaneous lesions than untreated females (Fig. 2; $P < 0.05$ at all time points p.i.) and in fact developed larger lesions than male animals ($P < 0.05$). This difference in lesion evolution was observed from the 30th to the 90th day p.i., when the experiment was terminated. Androgens had a more pronounced effect on females than estrogens did on males. Male animals treated with estrogens showed a tendency to develop smaller lesions than their untreated controls, but this was not statistically significant (Fig. 2).

FIG. 2. Effects of the administration of opposing sex hormones to male and female hamsters infected with *L. (V.) panamensis*. Recently weaned male hamsters ($n = 8$) received a subcutaneous implant (Compudose 200) that released approximately 240 μ g of 17 β -estradiol/day throughout the study period. Recently weaned female hamsters ($n = 8$) received intramuscular injections of 1 mg of testosterone enantate (Testoviron-Depot) per hamster twice per week until 3 months p.i. Twenty days after initiation of the sex hormone treatment, the hamsters were inoculated with 10^6 *L. (V.) panamensis* stationary-phase promastigotes as described in Materials and Methods. Lesion evolution was determined as described in the legend to Fig. 1, and the results are expressed as the mean ($\pm$ standard error of the mean) of the lesion size. Female animals treated with testosterone had significantly larger lesions than untreated females ($P < 0.05$) at all time points. There was no significant difference in lesion size between the estradiol-treated and untreated male animals.

FIG. 3. Lesion size and severity of disease in juvenile and adult male and female hamsters infected with *L. (V.) panamensis*. Adult (3-month-old) and prepubertal juvenile (3-week-old) male and female hamsters were infected in the footpad with 10^6 *L. (V.) panamensis* stationary-phase promastigotes. The lesion size and severity were determined, and the results are expressed as described in the legend to Fig. 1. (A) Lesion sizes in adult and prepubertal juvenile male hamsters. Differences in lesion size were significant ($P < 0.05$) at 30 days p.i. (B) Lesion severities in adult and prepubertal juvenile male hamsters. Differences in lesion severity were significant ($P < 0.05$) at 30 and 45 days p.i. (C) Lesion sizes in adult and prepubertal juvenile female hamsters. There were no significant differences in lesion size at any of the time points. (D) Lesion severities in adult and prepubertal juvenile female hamsters. There were no significant differences in lesion severity at any of the time points.

Influence of age at the time of infection. Because of the pronounced effect of testosterone administration on lesion size, we reasoned that the clinical course of infection might also be influenced by the age-related hormonal state of the animal. Juvenile (prepubertal) male hamsters were less susceptible to *L. (V.) panamensis* than adult (postpubertal) male hamsters, developing smaller lesions at 30 days p.i. (Fig. 3A; $P < 0.05$). Also, in the early phase of infection, the proportion of juvenile animals with dermal necrosis was less than that observed in the adult animals (Fig. 3B). By 90 to 120 days p.i., at a time when the juvenile male animals would have matured to have adult levels of androgens, the sizes of the lesions in the animals infected as adults and juveniles were equivalent. In contrast, there were no age-related differences in lesion size or severity among the female animals (Fig. 3C and D).

The difference in susceptibility of adult male hamsters and juvenile males was less in hamsters infected with *L. (V.) guyanensis* than in those infected with *L. (V.) panamensis*. There was no difference in lesion size, but there was a higher proportion and extent of necrosis early during the course of infection in the adult than in juvenile animals infected with *L.*

(V.) guyanensis (Fig. 4A and B). On the other hand, juvenile female hamsters were more susceptible to *L. (V.) guyanensis* infection than adult females, which had significantly smaller and less necrotic lesions early in the course of infection (Fig. 4C and D; $P < 0.05$). There was no difference in lesion size or severity later in the course of infection in the female animals.

Parasite burden. Parasitological results corroborated the clinical observations that females were more resistant than male animals to primary infection with *L. (V.) panamensis* or *L. (V.) guyanensis*. Limiting-dilution assays from homogenates of popliteal lymph nodes obtained 360 days p.i. showed that males had the highest number of parasites, while female animals either had a lower density of amastigotes or were negative by conventional culture techniques (Fig. 5). In chronic primary lesions, a positive correlation was found between the size of the lesion and the parasite burden of hamsters infected with *L. (V.) panamensis* (Spearman's correlation coefficient = 0.772; $P = 0.025$) or *L. (V.) guyanensis* (Spearman's correlation coefficient = 0.941; $P = 0.05$).

Immune response. The in situ profile of cytokine expression in well-established lesions (3.5 months p.i.) of male and female

FIG. 4. Lesion size and severity of disease in juvenile and adult male or female hamsters infected with *L. (V.) guyanensis*. Adult (3-month-old) and prepubertal juvenile (3-week-old) male and female hamsters were infected in the footpad with 10^6 *L. (V.) guyanensis* stationary-phase promastigotes. The lesion size and severity were determined, and the results are expressed as described in the legend to Fig. 1. (A) Lesion sizes in adult and prepubertal juvenile male hamsters. Differences in lesion size were not significant. (B) Lesion severities in adult and prepubertal juvenile male hamsters. Differences in lesion severity were significant ($P < 0.05$) at 30 to 90 days p.i. (C) Lesion sizes in adult and prepubertal juvenile female hamsters. Differences in lesion size were significant ($P < 0.05$) at 30 to 60 days p.i. (D) Lesion severities in adult and prepubertal juvenile female hamsters. Differences in lesion severity were significant ($P < 0.05$) at 30 and 60 days p.i.

hamsters infected with *L. (V.) panamensis* showed both type 1 and type 2 cytokine mRNAs in the lesions. In general, the level of cytokine expression was higher in male than in female animals. Uninfected animals expressed baseline levels of IL-10 and TGF- β that were not significantly different between genders. The primary lesions of male hamsters had significantly higher levels of IL-10 ($P = 0.04$), IL-4 ($P < 0.04$), and TGF- β ($P < 0.001$) than female hamsters (Table 2). In infected animals, there was a direct correlation between the expression of TGF- β mRNA and lesion size (Spearman's correlation coefficient = 0.873; $P < 0.001$). No differences were found between genders in the expression of IFN- γ and IL-12p40 (Table 2).

DISCUSSION

Although epidemiological studies indicate that American cutaneous leishmaniasis is more frequent in males than in females, it has been unclear whether this reflects a gender-related difference in the host response to the parasite or

merely different intensities of exposure among men and women. By controlling for the genetic background and size of the parasite inoculum in this model of infection with *Leishmania (Viannia)* spp., we have demonstrated a primary role of the host gender in the outcome of infection. Experimental infection of inbred age-matched male and female hamsters demonstrated that male animals were more susceptible to infection with *Leishmania (Viannia)* spp. than female animals. This difference was evident for strains of both *L. (V.) panamensis* and *L. (V.) guyanensis* when either primary lesion size or severity or frequency of dissemination (cutaneous metastases) was assessed. In addition, the exogenous administration of the opposing sex hormone to male and female hamsters demonstrated that testosterone had a disease-promoting effect, possibly through a direct effect on the immune response or by blocking a protective effect of estrogen. The possibility that estrogens could be responsible for the relative resistance of adult female hamsters to *L. (V.) panamensis* and *L. (V.) guy-*

FIG. 5. Parasite burden in the lymph node draining the primary lesions of adult or juvenile male and female hamsters infected with *L. (V.) panamensis* or *L. (V.) guyanensis*. The hamsters were infected as described in the legends to Fig. 3 and 4, and the draining (popliteal) lymph nodes were harvested 360 days after the primary infection. The lymph node tissue samples were adjusted to a concentration of 0.1 mg/ml, and twofold serial dilutions were cultured in Seneckje's medium. The parasite burden is expressed as the reciprocal of the last dilution positive for parasite growth.

anensis infection is supported by a previous observation that the increased susceptibility of ovariectomized female DBA/2 mice to *L. mexicana* infection could be abrogated by estrogen replacement (2). Nevertheless, the protective effect of estrogens does not apply to all experimental models of *Leishmania* infection. For example, DBA/2 and B10.129 (10 M) ScSn male mice infected with *L. major* developed benign lesions that healed spontaneously, whereas females showed ulcerated chronic lesions (1).

The finding of gender-related differences in the clinical outcome of *Leishmania (Viannia)* spp. infection, and the disease-promoting role of testosterone, was supported by the observation of age-related differences in clinical disease. Juvenile (prepubertal) male hamsters infected with *L. (V.) panamensis* or *L. (V.) guyanensis* at 21 to 28 days of age developed smaller and/or less severe lesions than did adult male hamsters infected at 120 days of age. Prepubertal juveniles have circulating androgen (primarily testosterone) levels that are approximately 20% of the level in adult male animals (31, 36, 37). A dramatic increase in circulating androgens occurs at 30 to 50 days of age (37), after the time of infection of the juvenile animals in our study. The role of estrogen in disease outcome was less clear. In *L. (V.) panamensis*-infected animals, there was no difference in lesion size or severity between pre- and postpubertal female hamsters. In contrast, juvenile female hamsters infected with *L. (V.) guyanensis* had larger and more severe lesions than did adults. This was corroborated by the finding of fewer parasites in the lesions of adult than of juvenile females. There was no significant protective effect observed when adult male hamsters were treated with estrogen, suggesting that the presence of high levels of androgens rather

than lower levels of estrogens is responsible for the more severe disease observed in the male animals.

Studies of murine cutaneous leishmaniasis caused by *L. major* infection have determined that Th1 cytokines (principally IFN- γ) mediate a protective immune response whereas Th2 cytokines (IL-4, IL-5, and IL-10) are disease promoting (10, 11). Studies of humans have shown a more heterogeneous pattern of cytokine expression, without the strict cytokine dichotomy observed in the classical mouse model of *L. major* infection. Although IL-4 expression has not been consistently found in all studies of humans with cutaneous leishmaniasis (18, 22), in general, the more severe forms of American cutaneous leishmaniasis were associated with a more prominent Th2 response at the site of infection (7, 29).

Because parasites of the subgenus *Viannia* do not readily induce lesions in mice, the immunopathogenic mechanisms related to experimental American cutaneous leishmaniasis had not been studied previously. The recent development of molecular probes specific for hamster cytokines (24) enabled us to determine the cytokine patterns associated with infection in this experimental model and, in particular, to explore a possible immunological basis for the increased susceptibility of male compared to female hamsters. By means of reverse transcription-PCR, we found that the greater severity of lesions in male hamsters was not related to decreased IFN- γ expression but was associated with a higher intralesional expression of the counterprotective cytokines IL-4, IL-10, and TGF- β . This contrasts with the observation by Satoskar et al. that the gender-related difference in susceptibility of DBA/2 mice to *L. mexicana* infection was related to increased IFN- γ production in the more resistant female mice but not to increased Th2 cytokine production in the more susceptible male mice (32, 33). The mixed type 1-type 2 cytokine pattern found in the more susceptible male hamsters was also observed in the lesions of hamsters infected with *L. (V.) panamensis* in a highly permissive site (the snout) (Y. Osorio, P. Melby, C. Pirmez, B. Chandrasekar, N. Guarín, and B. L. Travi, submitted for publica-

TABLE 2. Effect of gender on cytokine mRNA expression in lesions of hamsters infected with *L. (V.) panamensis*^a

Cytokine	Group ^b	mRNA expression index		<i>P</i> ^a
		Males	Females	
IFN- γ	Infected	1.24 $\pm$ 0.25	1.0 $\pm$ 0.32	NS
	Uninfected	0	0	
IL-10	Infected	0.84 $\pm$ 0.34	0.46 $\pm$ 0.12	0.04
	Uninfected	0.44 $\pm$ 0.07	0.52 $\pm$ 0.13	
IL-12p40	Infected	0.44 $\pm$ 0.08	0.44 $\pm$ 0.27	NS
	Uninfected	0	0	
IL-4	Infected	0.41 $\pm$ 0.30	0.08	0.04
	Uninfected	0	0	
TGF- β	Infected	0.18 $\pm$ 0.05	0	0.001
	Uninfected	0.68 $\pm$ 0.27	0.57 $\pm$ 0.23	

^a Hamsters were infected in the hind footpad, and at 3.5 months p.i., the skin tissue at the site of infection was harvested. Skin samples from uninfected age-matched animals were used as controls to determine the baseline cytokine expression. The data are expressed as the mean ($\pm$ standard deviation) of the ratio of the level of cytokine mRNA expression to the level of HPRT expression in the same sample (the mRNA expression index).

^b *n* = 7 animals per group.

^c *P* value determined by the Student *t* test; NS, not significant at 95% confidence interval.

tion), in humans and hamsters with progressive visceral disease (14, 15, 24), and in humans with mucosal and diffuse cutaneous leishmaniasis (7, 29). IL-4 expression has been associated with lesion severity in the murine *L. major* model (27), but it should be recognized that the disease-promoting role of IL-4 is somewhat strain dependent and that IL-4 production is not essential for susceptibility (28). Although IL-4, IL-10, and TGF- β are known to have a suppressive effect on type 1 cytokine synthesis, IFN- γ and IL-12 were prominently expressed in the face of these suppressive cytokines in the male animals. IL-4, IL-10, and TGF- β can also directly inhibit macrophage activation (6, 8, 9). It should be noted that TGF- β is posttranscriptionally regulated, so mRNA levels must be interpreted with caution (4). The down regulation of TGF- β mRNA expression (below the baseline levels of uninfected hamsters) following infection was observed previously in hamsters infected with *Leishmania donovani* (23, 24), but the underlying mechanisms are unknown.

Collectively, these cytokine data underscore the concept that the impaired elimination of *Leishmania* parasites in the more susceptible male hamsters is not mediated by inhibition of type 1 cytokine production but more likely by the macrophage-deactivating effects of IL-4, IL-10, and TGF- β . Once neutralizing antibodies against hamster IL-4 and IL-10 are available, we will be able to better define the role of these cytokines in disease evolution.

The role of sex hormones in the development of the immune response has been previously demonstrated. Studies of C57BL/6 mice infected with *L. major* demonstrated that pregnancy, which is accompanied by a decrease in estrogen levels, is associated with an increased susceptibility to the parasite. This was attributed to the high expression of Th2 cytokines (IL-4, IL-5, and IL-10) that help maintain pregnancy and to the Th2 cytokine-mediated diminution of IFN- γ and IL-2, which promote fetal resorption and implantation failure (16, 17). Recent work has demonstrated that there are gender-dependent differences in the secretion of IL-10 and IL-12 by antigen-presenting cells (APCs) (39). APCs from male mice secreted IL-10 but not IL-12 during T-cell activation, and this pattern was reversed in APCs from female mice. Similarly, T-cell lines selected in the presence of exogenous androgens secreted more IL-10 and less IFN- γ than T-cell lines selected in the absence of androgens (5). Thus, the high IL-10 production in the skin of male compared to female hamsters in our study may be related to the effect of androgens on APCs or activated T cells, and this may be the driving force behind the difference in clinical outcome.

In summary, we have demonstrated that gender is a major determinant of the host immune response and clinical outcome of *Leishmania (Viannia)* sp. infection in a novel hamster model. Cutaneous infection in this model results in chronic but controlled clinical lesions and persistent parasitism, much like the disease in humans. Strikingly, male hamsters had significantly more-severe disease than female animals when lesion size, lesion severity (degree of tissue necrosis), parasite burden in the draining lymph node, and rate of parasite dissemination were evaluated. Associated with the increased severity of disease in the male animals was a significantly greater intraleisional production of IL-4, IL-10, and TGF- β , cytokines known from other studies to exacerbate experimental *Leishmania* sp.

infection. The notion that the gender-related differences in disease evolution were the result of the sex hormone milieu of the animal is supported by two findings. First, prepubertal male animals, which would have significantly lower androgen levels than adult males, had smaller and/or less severe lesions than the adults until late in the course of infection, when the androgen levels would be equivalent. Second, administration of testosterone to female animals resulted in a dramatic increase in lesion size. These findings underscore an inherent increase in disease susceptibility in male animals and suggest that an androgen-related permissive immune response may contribute to the increase in disease prevalence among men in endemic areas. These findings have potential bearing on future preclinical and clinical evaluations of antileishmanial therapeutic agents and vaccines. Care should be taken in the design of such studies so that both genders are represented and appropriate controls are included.

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