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Experimental Chagas disease: the influence of sex and psychoneuroimmunological factors

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Abstract The effects of gender and psychoneuroimmunological factors resulting from the social environment and status of males were investigated with regard to the concentrations of testosterone and corticosterone and the course of *Trypanosoma cruzi* infection in mice. Hormone concentrations varied considerably; and only testosterone concentrations showed a tendency to be higher in dominant males. Females kept singly developed lower and more similar parasitaemias than males kept singly or together with a female. This difference was significant when comparing groups of females or males. Within groups of male mice, parasitaemia was strongly correlated with the social position, being high in inferior males and low in dominant ones. The importance of these findings is that they clearly prove that chronic social stress in males strongly affects the course of infection with *T. cruzi*.

Introduction

In many infectious diseases, females are more resistant than males (Alexander and Stimson 1988; Brabin and Brabin 1992; Roberts et al. 1996). This has been attributed to the immune system's sexual dimorphism and the action of steroid sex hormones (Eidinger and Garrett 1972; Ahmed et al. 1985; Grossman 1985; Araneo et al. 1991). In general, oestrogen enhances humoral immunity but lowers cell-mediated immune responses, whereas both types of immune response seem to be suppressed by male hormones (Alexander and Stimson 1988; Zhang et al. 2001). The reaction cascade is regulated via specific oestrogen- and androgen-binding sites in the thymus (Alexander and Stimson 1988; Gaillard and Spinedi 1998).

Besides the different action of sex hormones on the immune reaction, stress-steroid interactions with the immune system also differ between males and females, as shown in the pituitary/adrenal function by different baseline levels of plasma corticosterone and the different diurnal rises of plasma corticosterone concentrations in males and females (Gaillard and Spinedi 1998). During investigations into the effects of stress, mainly acute and not chronic stress have been considered, although both potentially can lead to disease (Kradin and Benson 2000).

Psychoneuroimmunological factors, e.g. the social environment, can represent a major source of stress (Koolhaas et al. 1997a), not only directly affecting cortisol and/or corticosteroid levels, but also testosterone concentrations in males. Once having experienced a potent stressor, animals tend to become more susceptible to subsequent stressors (Koolhaas et al. 1997b). In dominant male Guinea pigs, rats and mice, an acute stress by a confrontation with another sexually experienced, unfamiliar male considerably increases the level of testosterone and slightly increases that of plasma glucocorticoids. In the losers, the testosterone levels drop – even below baseline levels for a couple of days after confrontation – and their stress hormone titres increase remarkably (Sachser 1987; Sachser and Lick 1989, 1991; Koolhaas et al. 1997a, b).

Social stress directly affects the immune system. In Guinea pigs, the activity of the alternative pathway of the complement system seems to be correlated with behavioural patterns, because less aggressive animals show higher complement activity (Stefanski et al. 1989). Complement activity drops during periods of stress, not only in subordinate but also in dominant males. In confrontation experiments between male Guinea pigs, this complement-activity response to social stress is stronger in individually housed and less experienced males than in socially experienced ones (Stefanski and Hendrichs 1996). Social environment also influences

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splenic lymphocyte sub-populations. Non-stressful environments lead to an increase in the number of T-helper cells while, as a consequence of social defeat, the number of $CD8^+$ T cells rises (Bohus et al. 1993).

Social stress as a factor in disease has been considered in detailed investigations of a rodent protozoan parasite, *Babesia microti*. A previous social stress by maintenance in groups and an infection of the subsequently isolated males caused a higher parasitaemia in dominant males (Barnard et al. 1993, 1996). In *Schistosoma*-infected male hamsters kept single or pairwise, the latter contained more worms and thereby a higher egg burden (Rashed et al. 1996).

In infections of laboratory mammals with *Trypanosoma cruzi* – the causative agent of Chagas disease – not all investigators found a higher susceptibility of males than females (summarised in Tay et al. 1978; Prado et al. 1998). In addition, administration of sex hormones and/or orchidectomy gave no consistent results (see Discussion). However, various investigators used different but only easily obtainable parasite stages from in vitro cultures or the blood of experimental animals and used different but rather high infection doses. Psychoneuroimmunological factors have never been investigated as a possible reason for these conflicting results or as factors to be considered, but corticosteroid applications caused an increase of parasitaemia in patients with chronic Chagas disease (Rassi et al. 1997). Therefore and for the first time, we used vector-derived metacyclic trypomastigotes and a natural infection dose to investigate the influence of sex and chronic social stress on the course of parasitaemia during the acute phase of *T. cruzi* infection in mice.

Materials and methods

Mice

The stock of Balb/c mice was kindly provided by Dr. H. Mossmann from the Max Planck Institute of Immunobiology, Freiburg. The mice were bred at our institute, using a 16 h/8 h light/dark rhythm. Commercial rodent diet and water were available ad libitum. Mice about 5 weeks old were separated from their mothers and kept singly or in groups. In groups, offspring of different females were mixed immediately so as to ensure that brothers were separated. Females were kept either singly in cages 24×14×13.3 cm, or in groups of three or five individuals in cages 43×26.5×15 cm. Males were housed either singly in cages of 24×14×13.3 cm, or paired together with one female, or in groups of three non-sibling males in cages 26.5×21×14 cm, the latter to increase the social stress in this strain of mice, which is usually not a very aggressive strain (Eskola and Kaliste-Korhonen 1999).

In total, 6 series of experiments were performed, comprising 9 single and 8 grouped females and 55 single, 44 paired and 81 grouped males.

Determination of social rank

At the age of approximately 10 weeks, the social rank was determined within the 27 groups of males. Since the aggressive biting behaviour is strongly reduced in long-term established groups (Barnard et al. 1993), another procedure was chosen, giving a female to the group of males. Then, the highest level of ranking was usually reflected by making the initial copulation. By excluding the α-male and repeating the same procedure, the middle ranking male (β-male) and the inferior animal (γ-male) also could be classified. In some groups, the γ-male could also be recognised by the occurrence of single, small skin wounds. The behavioural experiments were performed prior to infection and after the animals had survived the acute phase of infection. Groups in which social ranking could not be determined were omitted from the analysis.

Determination of concentrations of testosterone and corticosterone

To measure the testosterone and corticosterone levels of males 3–5 days prior to infection with *Trypanosoma cruzi* in two series (17 single, 16 paired, 33 grouped males), approximately 200 µl of blood were collected from each animal by puncturing the retro-orbital plexus within 3 min of handling. The samples were centrifuged at 400 *g* and the sera stored at –80 °C.

At the end of the experiments, the animals were killed by cervical dislocation; and blood was taken immediately by heart puncture. The heparinised samples were centrifuged at 1,000 *g*. To exclude an infection risk during measurement of the concentrations of hormones, the plasma was filtered through 0.22-µl filters (Eppendorf, Germany) and stored at –80 °C.

The concentrations of testosterone and corticosterone were determined in the laboratories of Prof. Dr. D. von Holst (University of Bayreuth), using radioimmunoassays (Fenske 1988).

Parasites

The "Tulahuén" strain of *T. cruzi* was kindly provided by Dr. S. Croft (London School of Hygiene and Tropical Medicine, London, UK) and maintained cyclically between bugs and mice. First instars of the natural vector *Triatoma infestans* [a stock originating from Cachiyuyu/Chile (Schaub and Schottelius 1984), fed regularly on hens and reared at about 26 °C, 60–70% relative humidity and on a 16 h/8 h light/dark cycle] were fed on mice at the peak of parasitaemia. An aliquot of infectious faeces of fifth instars was mixed with human serum and incubated at 37 °C for 30 min to kill all non-metacyclic trypomastigotes by complement lysis (Chao et al. 1985). The metacyclic trypomastigotes were counted in a Neubauer chamber and their concentration in the original faeces suspension was adjusted to 1×10^3/ml by dilution with 0.9% NaCl.

Infection of mice

At the age of approximately 12 weeks, the animals were transferred to the infection area. Each mouse was anaesthetised with a mixture of Rompun (Bayer, Germany), Ketavet (Upjohn, Germany) and 0.9% NaCl (2:6:17). To conform with the natural infection dose of *Trypanosoma cruzi* in infections via the triatomine's bite puncture in the skin (Heide 1999), about 100 trypomastigotes/mouse (i.e. 5 flagellates/g body weight) were injected subcutaneously into the animal's back. Starting with day 16 post-infection, parasitaemia was determined at 2-day intervals by examination of 100 microscopic fields of fresh blood preparations (magnification ×400). If 1 flagellate/field was counted, the number of flagellates was additionally determined using diluted blood and a low-volume Neubauer chamber (Schuster and Schaub 2000). Although both methods to determine the parasitaemia showed a good correlation (regression: $y=0.0099x$, $r=0.964$), in the presentation of the data we have consistently used the data obtained by counting the parasites under a coverslip, since the regression line only covers concentrations of >100 parasites/100 fields, i.e. 1×10^6 parasites/ml.

Evaluation of data

In classifying data within a group into a high, middle or low level, pairs of data were considered to be not different if the lower value

was more than 90% of the higher value. Hormone concentrations below the detection limit were considered to be at the limit, in order to calculate the mean values and standard deviations.

Both the statistical comparison of hormone concentrations with vaginal-smear data and the comparison of prepatent periods were performed using the unpaired *t*-test (Graph Pad Prism; Graph Pad Software, San Diego, Calif.). Statistical comparison of the parasite burden was performed by first calculating the areas under the individual parasitaemia curves, followed by a Mann–Whitney *U*-test (Graph Pad Prism). Since some mice died earlier than others, the means could not be calculated for the parasitaemia or parasite burden of all α-, β- and γ-males. Therefore, we determined for each mouse the percentage of the total parasite burden within that respective group until the death of the first animal; and we then calculated the mean and standard deviation.

Results

Social rank and hormone concentrations

In 15 of 27 groups, the rank of each male could be determined; but, in an additional three groups, only the α-male could be classified and, in another three groups, only the γ-male. After having survived the acute phase of infection, very often males lost libido, so that the social position of the co-inhabitants within their groups could only be determined for ten individuals in six groups, out of a total of 51 individuals from 21 groups: For seven of these ten animals (three α-, two β-, two γ-males), the social position within the group had not changed. As for the other three, an α- and a β-male had exchanged their social position in one group, and in another the γ-male had become the α-male.

In uninfected males, testosterone concentrations ranged from below the detection limit (0.2 ng/ml) to 29 ng/ml. There was considerable variation in hormone levels for individuals of the same social position in different groups. In the 11 groups, the mean values for the different ranking animals did not differ (mean ± SD: α-male: 4.74 ± 5.14 ng/ml, β-male: 1.05 ± 0.84 ng/ml, γ-male: 4.34 ± 4.06 ng/ml) and also did not differ from those of the singly kept males (5.7 ± 8.1 ng/ml) or single males kept together with a female (5.87 ± 8.16 ng/ml). The ranking according to behavioural parameters sometimes correlated positively with testosterone levels: Prior to infection, four of the eight α-males showed the highest testosterone level in the group (and none showed the lowest value). However, two γ-males also showed the highest testosterone-level and one showed the lowest value. The tendency was that about 50% of the α-males had the highest testosterone level within a group and the β- and γ-males together made up the other 50%; and this was also seen in a second, larger investigation encompassing 20 groups of males (data not shown). After the acute phase of infection, the mean testosterone concentration increased slightly but not significantly.

The corticosterone concentrations of uninfected males ranged from the detection limit (6 ng/ml) to 80 ng/ml. The mean values of the different ranking animals (α-male: 29.89 ± 15.56 ng/ml, β-male: 17.38 ± 16.88 ng/ml, γ-male: 13.56 ± 10.55 ng/ml), singly kept males (30.5 ± 40.6 ng/ml) and males kept together with a female (11.41 ± 9.16 ng/ml) only differed significantly between the α-male and γ-male or between paired males (unpaired *t*-test: $P < 0.05$). Prior to infection, four of the eight α-males showed the highest corticosterone level and two showed the lowest value and, of the γ-males, one showed the highest and none the lowest value. After the acute phase of infection, the mean corticosterone concentration was significantly increased (about five-fold), but again with no significant differences for the different ranking males or the singly kept ones.

Development of *Trypanosoma cruzi*

The prepatent periods of 18–28 days after subcutaneous infection with about 100 metacyclic trypanosomes were very similar in all groups, with the highest congruence for mice infected on the same day.

In contrast to the prepatent period, the course and level of parasitaemia were affected by sex and social environment. In females, the development of *Trypanosoma cruzi* was reduced and thereby the outcome of the disease was less severe, as compared with males. Housing females and males singly resulted in slight differences in the mean course of infection and the different parasite burdens of males and females were not statistically significant (Fig. 1A). There was a high standard deviation in males, with five of the ten males showing a much stronger increase in parasitaemia than the others. Similarly, in five other independent experiments using nine or ten males kept singly or paired with a female, as a rule two to four of the ten males developed high parasitaemias. For males maintained in groups, parasitaemia was much higher than for mice kept singly or in pairs and was significantly higher than in females. Standard deviations of means were the highest for grouped males (Fig. 1B; Mann–Whitney *U*-test: $P < 0.05$).

If each individual course of infection was compared for grouped males, the level of parasitaemia in individuals in the same cage was negatively correlated with their social status. The mean percentage of the total parasite burden within a group differed significantly between the α-male and the γ-male and also between β-males and γ-males (Mann–Whitney *U*-test: $P < 0.001$). For the 18 groups in which the α-male could be reliably identified, in 12 groups this male possessed the lowest parasitaemia/parasite burden; and in only one group did it possess the highest. For γ-males which could be identified definitively, in 15 of 18 groups they showed the highest parasitaemia within the group; and in two they showed the lowest. In 10 of 15 groups, α-males developed the lowest parasitaemias, β-males expressed parasitaemias intermediate between α- and γ-males, and those of the γ-males were highest. In one group, parasitaemia did not follow this rule; instead, the α-male showed the highest parasitaemia and died first, whereas the inferior animal survived the acute phase of infection.

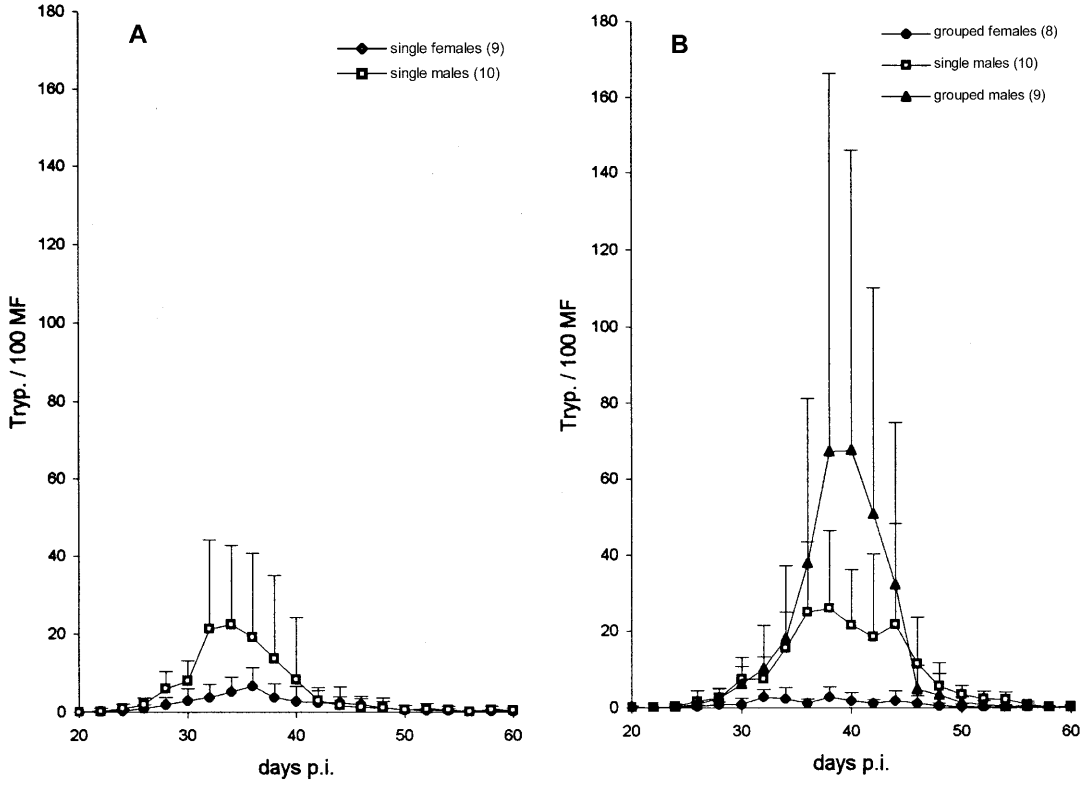

Fig. 1A, B Average courses of *Trypanosoma cruzi* parasitaemia in male and female Balb/c mice kept under different housing conditions. *Tryp./100 MF* Number of trypanosomes/100 microscopic fields (magnification ×400; 100 Tryp./100 MF are approximately 10^6 trypanosomes/ml blood)

Parasitaemia levels could not be correlated with testosterone or corticosterone concentrations. Rarely did mice die and only males, without any correlation to the social rank.

Discussion

Gender and the corresponding sex steroids have been shown to affect the immune response in a wide range of different animal species, including humans, rodents and birds (Schuurs and Verheul 1990). To elucidate the interactions of sex and parasites in humans, investigations have focused on sex-dependent differences in diseases for which the parasite developing in animals is strongly related to one occurring in humans or for which the parasite affects not only animals but also humans.

In the major tropical disease, malaria, there are several indications that female patients are more efficient in clearing peripheral parasitaemia (summarised in Brabin and Brabin 1992). In a test model for this disease, rodent malaria, this sexual dimorphism has been studied in detail; and the male sex hormone testosterone has been proved to be pivotal for the higher susceptibility of males. As a rule, female C57Bl/10 mice are able to self-cure a *Plasmodium chabaudi* malaria, whereas males succumb to this infection. Orchidectomised males become resistant to *P. chabaudi*, but a subsequent testosterone administration promotes the disease (Benten et al. 1992), as does testosterone administration to females; and this persists even after withdrawal of the androgen (Benten et al. 1997; Mossmann et al. 1997).

In human infections with *Trypanosoma cruzi*, the age-specific distribution of seropositive individuals and/or the total infection rates were similar in both sexes (e.g. Laranja et al. 1956; Mott et al. 1976; Hoff et al. 1979). However, more marked abnormalities in autopsies were evident in males (Laranja et al. 1956), as well as more abnormal electrocardiogram tracings (summarised in Brabin 1992). In experimental *T. cruzi* infections, most investigations showed a sex-related effect (summarised in Tay et al. 1978). The prepatent period did not differ between males and females, but the peak of parasitaemia of males was about twice that found for females and the males died earlier (Hauschka 1947; Solari et al. 1998). This was also evident in the present investigation, in which for the first time vector-derived metacyclic trypomastigotes and a natural infection dose were used. In this work, using singly kept animals, the peak of parasitaemia of males was about four-fold higher than that of females. Comparing groups of females and males, these peaks for males were significantly different and 14-fold higher. In preliminary experiments, the higher parasitaemia and/or stronger pathological effects in males were also evident in NMRI and C57Bl/6 mice.

The role of testosterone in *T. cruzi* infection is not as unequivocal as in murine malarial infection. Whereas, after infection, the daily administration of 1 mg of

testosterone/kg to females and of the same dose of progesterone, oestrone or diethystilbesterol to males did not change the course of infection (Goble 1952), an administration of very high doses (0.1 mg progesterone or 0.02 mg testosterone) three times per week, starting 2 weeks before infection with *T. cruzi*, reduced the peak of parasitaemia in males by 70% and in females by 50% for testosterone and 70% for progesterone; and the mortality rate was even more strongly reduced in the latter group (Tay et al. 1978). The effect of gonadectomy on the course of infection also differed: While castration did not change the course of parasitaemia and the mortality rate of male *Mus musculus* (Chapman et al. 1975), parasitaemia was reduced in *Callomys callosus* and was increased by a testosterone administration to castrated mice (Prado et al. 1999). However, ovariectomy induced similar effects in both systems, strongly increasing parasitaemia and mortality rate. This effect could be counteracted in *C. callosus* by the subsequent administration of progesterone, oestrogen or a combination of both (Chapman et al. 1975; Prado et al. 1998).

In the present investigation, the levels of parasitaemia were not correlated with testosterone levels. One reason for this may be that plasma concentrations in different male mice consistently show strong variations, linked to the fact that this androgen is not secreted constantly (Bartke and Dalterio 1975). However, male Guinea pigs living in colonies had higher testosterone and cortisol levels than males housed singly for a long period of time but with visual and olfactory contact to other animals of their species (males and females; Sachser 1986). This represents exactly the circumstances of the housing facilities of the present study, in which only a tendency towards higher testosterone levels in α-males was recognisable. There was a significantly lower stress hormone concentration in paired males, compared with grouped and isolated males, which did not differ much. In other investigations, the plasma corticosterone levels of isolated male mice did (Benton et al. 1978) or did not (Brain and Nowell 1971) differ from grouped animals. *T. cruzi* may represent a very serious subsequent stressor, since plasma-corticosterone concentrations have been reported to show elevated levels after the animals pass the acute phase of infection (Leite de Moares et al. 1991). This was confirmed in the present investigation.

Investigations about the interactions between testosterone, corticosterone, social ranking and immune response either induced artificially or by parasites – namely *Heligmosomoides polygyrus* and *Babesia microti* – have revealed complex interrelationships between these parameters (Barnard et al. 1993, 1994, 1996, 1997a, b, 1998). *B. microti*, a sporozoan parasite which develops in erythrocytes like the sporozoan *Plasmodium* and causes a transient infection typically resolving 2–3 weeks after exposure, more strongly affects α-males. This is in contrast to the present results, but *T. cruzi* is more pathogenic and the period of confrontation is much longer. As in the present study, there was no direct cause-and-effect relationship between sex- and/or stress-hormones and resistance to *B. microti* (Barnard et al. 1996). In the present investigation, we used groups of males kept together for about 6 weeks before starting the tests. In such colonies, the long-term social hierarchical order is set and actual fights do not appear as often and as intensively as in short-term confrontation experiments with unfamiliar males. In these long-term hierarchies, the individual characteristics of coping with stress influence the immunological consequences (Bohus et al. 1991, 1993). This may explain why, in the present investigation, parasitaemias of β-animals in particular strongly varied in relation to the parasitaemia of cohabitants. Some α-males were very aggressive, whereas in other groups the cohabitants did not fight at all. Hence, it is not surprising that hormone levels differed considerably between individuals of the same social position in different groups. Since in the present investigation, some singly kept males and some of those kept paired with a female consistently developed high parasitaemias, which were not correlated with hormone concentrations, additional disease-influencing factors must exist.

In the light of these data, we emphasise the necessity of a very careful experimental design when working with animal models, since the social environmental conditions (at least for male mice) definitely affect the course of the experimental *T. cruzi* infection. Although the psychoneuroimmunological factors cannot be attributed directly to testosterone and/or corticosteroid levels, we clearly show that chronic stress contributes to a severe course of the acute phase in experimental and thus presumably also in human Chagas disease.

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