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Strongyloides ratti: additive effect of testosterone implantation and carbon injection on the susceptibility of female mice

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Abstract A sex-related difference in host susceptibility to *Strongyloides ratti* was previously known. Male mice were more susceptible to *S. ratti* infection and the difference was seen against migrating larvae under the regulation of testosterone. Against migrating larvae, macrophages were assumed to play important roles in host natural immunity. On the basis of these findings, to examine the effect of testosterone on macrophages we treated female mice with testosterone and/or carbon to block the function of macrophages. Mice were then infected with third-stage larvae of *S. ratti*. By counting of the migrating larvae in the cranial cavity at 36 h after infection the effect of each treatment was assayed. Testosterone treatment alone (Te) or carbon injection alone (Ca) effectively increased the worm recovery. Given together, Te and Ca (Te + Ca) significantly increased the worm recovery to levels almost equal to the sum of those achieved with Te and Ca. The serum testosterone concentration was elevated in mice that had undergone Te and Te + Ca at the time of worm recovery. Surprisingly, the serum testosterone concentration reached after Te + Ca was elevated more than that attained by Te. The same experiment with a half-dose of Te and Ca (Te half + Ca) resulted in the same testosterone concentration achieved with Te and resulted in a worm recovery almost equal to the sum of that achieved with Te and Ca. These results clearly showed that Te and Ca had an additive effect on the recovery of migrating *S. ratti* larvae. Testosterone had an effect after macrophages had been blocked. The relationship between testosterone and macrophage function during *S. ratti* infection is discussed.

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Introduction

There are sex-related differences in susceptibility to some parasites in mice. Male jirds were highly susceptible to *Brugia* infection (Ash 1971), as were mice to *Plasmodium chabaudi* infection (Benten et al. 1993). Kiyota (1984) showed that male C57BL/6 mice were more susceptible to *Strongyloides ratti* than were females. The sex-related difference in susceptibility to parasites was attributed to the serum testosterone. *S. ratti* migrates from the inoculated site to the gastrointestinal tract mostly via the head and partially through the lung after subcutaneous infection (Tada et al. 1979; Dawkins et al. 1982). Furthermore, Kiyota (1984) clarified that the testosterone effect was expressed during this migrating phase of the larvae. On the other hand, neither the kind of mechanism(s) involved in the testosterone effect nor the kind of cell(s) associated with the effect has been clarified.

Abe et al. (1985) have shown that mononuclear phagocyte blockade at the time of infection increases the susceptibility of mice to *S. ratti*. In beige mice, natural immunity to migrating larvae of *S. ratti* was impaired (Nawa et al. 1988). This impaired immunity was restored by the transfer of peritoneal cells from syngeneic normal mice (Abe et al. 1992). In that context, the investigators considered macrophages to be important in the natural immunity to migrating larvae of *S. ratti*.

Recently it has been shown that testosterone affects many types of functions of cells. For example, testosterone can regulate the production of reactive oxygen intermediates and nitrates from rat macrophages (Miller and Hunt 1996). Castration of male mice increased the production of interleukin 1 (IL-1) and IL-6 from Kupffer cells, known as liver macrophages, after trauma hemorrhage (Wichmann et al. 1997). In the present study we examined the effect of testosterone treatment on worm recovery after macrophage blockade to determine whether testosterone would change the susceptibility through macrophages.

Materials and methods

Animals

Male and female C57BL/6 mice (8–12 weeks old) raised in the Laboratory for Animal Experiments of Kyushu University under specific pathogen-free (SPF) conditions were used.

Parasitological examination

Strongyloides ratti, TMDU strain, has been maintained in our laboratory by serial passage in retired Wistar rats. Infective larvae (L3) were obtained by filter-paper culture of the feces of infected Wistar rats (Tada et al. 1979). In all, 2,000 L3 were suspended in 0.2 ml of saline and injected subcutaneously into the lower abdomen of each mouse. *S. ratti* L3 migrate from the infection site to the intestine mostly via the head (Tada et al. 1979). The numbers of worms recovered from the heads of mice were examined at the indicated times after infection. Each mouse was killed by decapitation using ether anesthesia. After removal of the head skin, the skull, the brain, and the nasofrontal portion were cut into small pieces. These parts were incubated in petri dishes with saline at 37 °C for 3 h, after which the minced tissues were removed and worms were counted under a dissecting microscope.

Testosterone implantation

The backskin of each female mouse was opened with scissors 2 cm longitudinally and 10 mg methyltestosterone (Nacalai Teskque Co. Japan) was implanted subcutaneously. The wound was closed by wound clip (Becton Dickinson Co. Japan). The testosterone was implanted every 2 weeks for a total of three times. At 7 days after the last implantation these mice were subcutaneously infected with 2000 L3. Sham-operated mice were used as controls.

Macrophage blockade by carbon injection

For blockade of macrophages, carbon was injected twice, at 24 h and at 3 h before infection. Carbon was prepared as follows: 100 µl Rotring ink (Art. 591017, Rotring, Germany) containing 10% carbon was centrifuged with 900 µl Phosphate-buffered saline (PBS) in a 2-ml plastic container at 15,000 rpm for 20 min. The supernatant was discarded, and the pellet was resuspended in 250 µl PBS and sonicated before use. This solution was used as the carbon preparation, containing 10 mg carbon.

Serum testosterone measurement

At the time of worm recovery, blood was taken from the retroorbital venus plexus of each mouse by capillary tube. The serum testosterone level was measured at the SRL laboratory (Hachioji, Japan) by radioimmunoassay (RIA).

Results

Kinetics of migrating worm numbers in mice

First we tried to determine the optimal time of worm recovery. Male mice were infected subcutaneously with 2000 *S. ratti* L3. Worm numbers in the heads and lungs were examined every 12 h after infection. The result is shown in Fig. 1. Worm numbers in the head began to increase at 24 h and peaked at 36 h after infection (255.5 ± 70.9 worms). Therefore, we examined worm recovery at 36 h after infection.

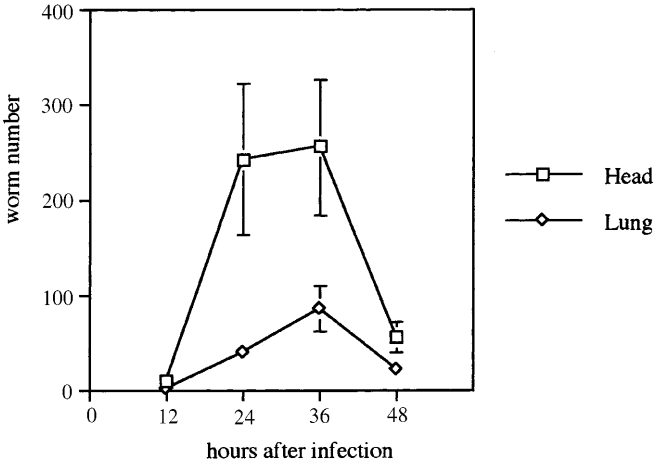

Fig. 1 Kinetics of migrating worm numbers in the head and the lung of mice. Four groups of mice, each containing four male, were infected with 2,000 *Strongyloides ratti* L3. All mice were killed at the indicated hours after infection and worm numbers in the head and the lung were examined. Data represent mean worm numbers ± SD (n = 4 mice)

Numbers of worms recovered from the heads of mice treated with carbon

Next we tried to determine the optimal dose of carbon required for maximal inhibition of the function of macrophages. For this purpose we injected carbon into female mice twice, at 24 h and at 3 h before infection. Three groups of mice were injected with either 1 or 10 mg of carbon intravenously or 50 mg of carbon intraperitoneally at 24 h before infection.

At 3 h before infection, mice were also intraperitoneally injected with 1, 10, or 50 mg carbon, respectively. After carbon injection, mice were infected subcutaneously with 1,500 L3. The numbers of migrating larvae in the head were determined at 36 h after infection (Fig. 2). The mice injected twice with 1 mg carbon showed the same worm recovery as did control mice (control 48.3 ± 36.5 worms, 1-mg-injected mice: 47.3 ± 26.6 worms). The mice injected twice with 10 mg carbon showed enhanced worm recovery, which was 4–5 times as high as that of control mice (10-mg-injected mice 243 ± 56.7 worms). Two treatments of mice with 50 mg carbon resulted in the same recovery attained in the mice treated with 10 mg carbon (50-mg-injected mice 215.7 ± 65.6 worms). On the basis of these findings, we used 10 mg carbon treatment to inhibit the macrophages.

Numbers of worms recovered from the heads of mice treated with testosterone

We tried to ascertain the effect of testosterone on the susceptibility of female mice as follows: 10 mg methyltestosterone was implanted every 2 weeks for a total of three times. At 7 days after the last implantation, female

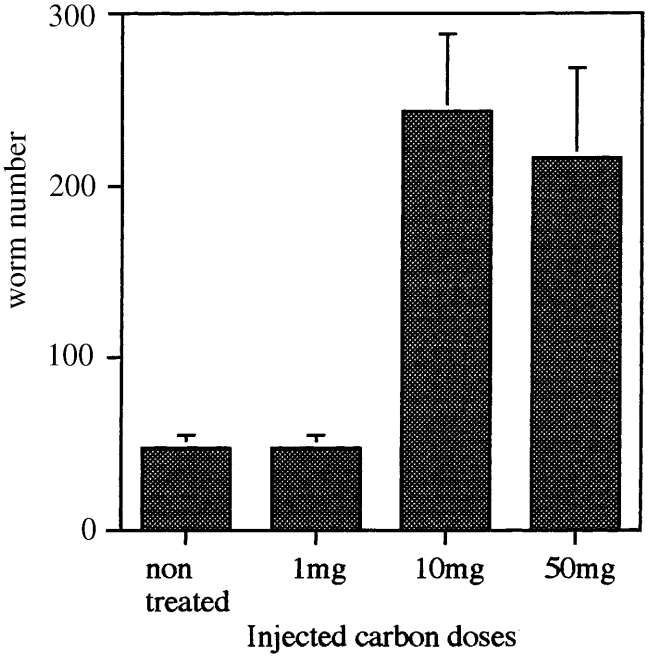

Fig. 2 Effect of various doses of carbon injection on worm recovery. The indicated doses of carbon were injected intravenously (0, 1, 10 mg) or intraperitoneally (50 mg) at 1 day before infection and the same doses were injected intraperitoneally at 3 h before infection with 1,500 *S. ratti* L3. Worm numbers in the head were examined at 36 h after infection. Each *column* represents the mean worm recovery from 3 female mice, and each *bar* represents the SD

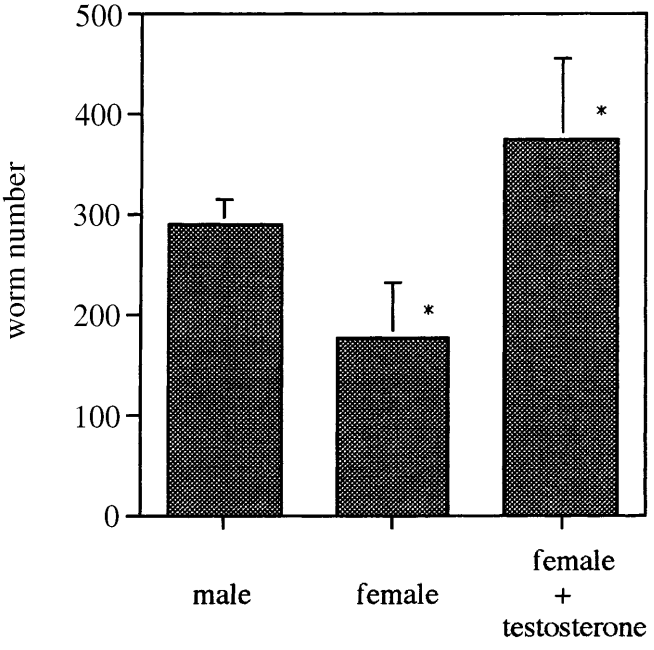

Fig. 3 Effect of testosterone implantation in female mice. Methyltestosterone at 10 mg was implanted in the backs of mice every 2 weeks for a total of 3 times. At 7 days after the last implantation, mice were infected with 2,000 *S. ratti*. Male mice and nontreated female mice were also infected as controls. Worm numbers in the head were examined at 36 h after infection. Each column represents the mean worm recovery from 4 mice, and each bar represents the SD. $*P < 0.05$ (Students *t*-test) compared with male

mice were subcutaneously injected with 2,000 L3. We counted the numbers of migrating larvae in the heads of mice at 36 h after infection (Fig. 3). This experiment revealed that the implantation of 10 mg testosterone could render female mice as susceptible as male mice (female control mice: 177.0 ± 55.7 worms, testosterone-implanted female mice 376.0 ± 78.5 worms, male control mice 290.5 ± 24.7 worms).

Effect of testosterone implantation and carbon injection on the recovery of migrating worms

We tried to examine whether testosterone would affect the susceptibility of female mice to infection with *S. ratti* L3 through macrophages. Female mice were treated with testosterone and their macrophages were then blocked by carbon injection. The detailed protocol was as follows: testosterone was implanted in female mice every 2 weeks for a total of three times. At 6 and 7 days after the last implantation, mice were injected with carbon and subcutaneously infected with 2,000 L3 at 3 h after the last carbon treatment. Worm numbers in the head were counted at 36 h after infection (Fig. 4). Simultaneously, serum was collected and the testosterone concentration was assayed (Table 1).

The mice treated with testosterone (Te) or carbon (Ca) alone showed significant increases in worm recovery as compared with control mice (Fig. 4; Te:

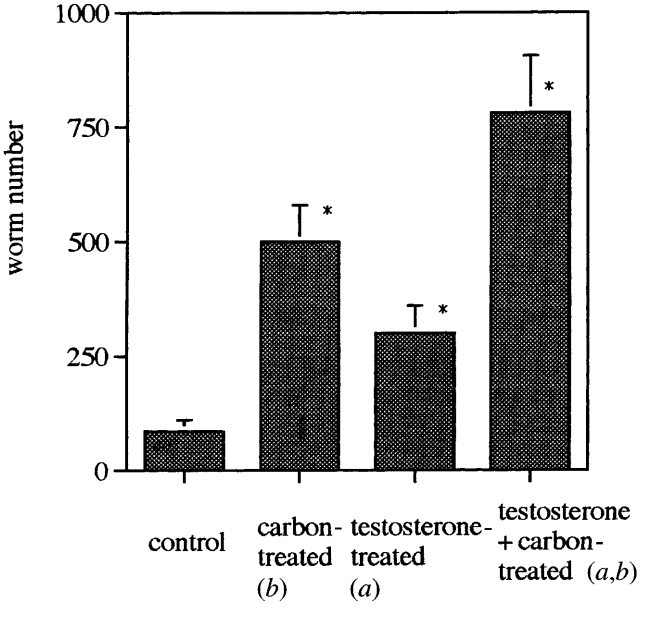

Fig. 4 Worm recovery at 36 h after infection from the heads of mice treated with testosterone and/or carbon injection as follows: *a* 10 mg testosterone implantation every 2 weeks for a total of 3 times, followed at 7 days after the last implantation by infection with 2,000 *S. ratti* L3; or *b* carbon injection at 1 day before infection and at 3 h before infection. Each *column* represents the mean worm recovery from each group of mice, and each *bar* represents the SD. Each group consisted of 3–4 mice. $*P < 0.05$ (Students *t*-test) compared with control

Table 1 Serum testosterone concentration as determined at the time of worm recovery[a]

Treatment	Serum testosterone (ng/dl)
Nontreated control	10.2 ± 5.2
Carbon	7.4 ± 2.5
Testosterone 10 mg[b]	28.2 ± 5.6
Carbon[c] + testosterone 10 mg[b]	63.9 ± 8.5

[a] Mice were bled from the retroorbital plexus at 36 h after infection with *Strongyloides ratti* L3. Each value represents the mean concentration ± SD; each group contained 4 mice
[b] 10 mg testosterone was implanted every 2 weeks for a total of 3 times and at 7 days after the last implantation, mice were infected with 2,000 *S. ratti* L3
[c] Carbon was injected at 1 day and at 3 h before infection

302 ± 58.1 worms, and Ca: 541 ± 120 worms, respectively). The worm recovery from mice treated with testosterone plus carbon (Te + Ca) increased in comparison with the recovery from those treated with Te or Ca alone (Te + Ca: 778 ± 126 worms). The worm recovery from mice treated with Te + Ca was almost equal to the sum of the numbers of worms recovered from those treated with Te alone and Ca alone. The serum testosterone level of each group is shown in Table 1. The mice treated with Te (10 mg, three times) + Ca showed a higher level of serum testosterone than did those treated with Te at 10 mg (Te+Ca: 63.9 ± 8.5 ng/dl, Te: 28.2 ± 5.6 ng/dl). As judged from this result, it might be possible that the increased worm recovery from mice treated with Te + Ca was due to increased serum testosterone concentrations.

To examine whether this increased worm recovery from mice treated with Te + Ca was due to an additive effect of testosterone implantation and carbon injection, rather than to increased serum testosterone levels, we carried out another experiment. In this experiment, female mice were treated with 5 mg testosterone every 2 weeks for three times and then either were injected with carbon or were not additionally treated. Mice were then infected with *S. ratti* L3. Another group of mice were treated the same as before. The results of this experiment are shown in Fig. 5. Serum testosterone levels are shown in Table 2. The worm recovery from mice treated with Te (5 mg, three times) + Ca was higher than that observed in mice treated with Te (10 mg) or Ca alone (Te 5 mg, three times + Ca: 533 ± 58.5 worms, Te 10 mg, three times: 323 ± 64.3 worms, and Ca: 173 ± 18.1 worms, respectively).

The worm recovery from mice treated with Te (5 mg, three times) + Ca was almost equal to the sum of the numbers of worms recovered from mice treated with Te (10 mg, three times) alone and Ca alone. Serum testosterone levels determined in the Te (5 mg, three times) + Ca-treated group and the Te (10 mg, three times)-treated group were at the same level (23.1 ± 3.9 and 25.6 ± 8.2 ng/dl respectively). These results showed that the increased worm recovery observed in mice treated with Te + Ca was due to an additive effect of testosterone implantation and carbon injection, not to increased testosterone levels. Testosterone and carbon treatment had an additive effect on worm recovery.

Table 2 Serum testosterone concentration as determined after various doses of testosterone treatment[a]

Treatment	Serum testosterone (ng/dl)
Nontreated control	13.0 ± 2.9
Carbon	11.8 ± 0.8
Testosterone 5 mg[b]	9.9 ± 3.6
Testosterone 5 mg[b] + carbon[c]	23.1 ± 3.9
Testosterone 10 mg[b]	25.6 ± 8.2
Testosterone 10 mg[b] + carbon[c]	50.8 ± 14.2

[a] Mice were bled from the retroorbital plexus at 36 h after infection with *S. ratti* L3. Each value represents the mean concentration ± SD; each group consisted of 4 mice
[b] 5 or 10 mg testosterone was implanted every 2 weeks for a total of 3 times and at 7 days after the last implantation, mice were infected with 2,000 *S. ratti* L3
[c] Carbon was injected at 1 day and at 3 h before infection

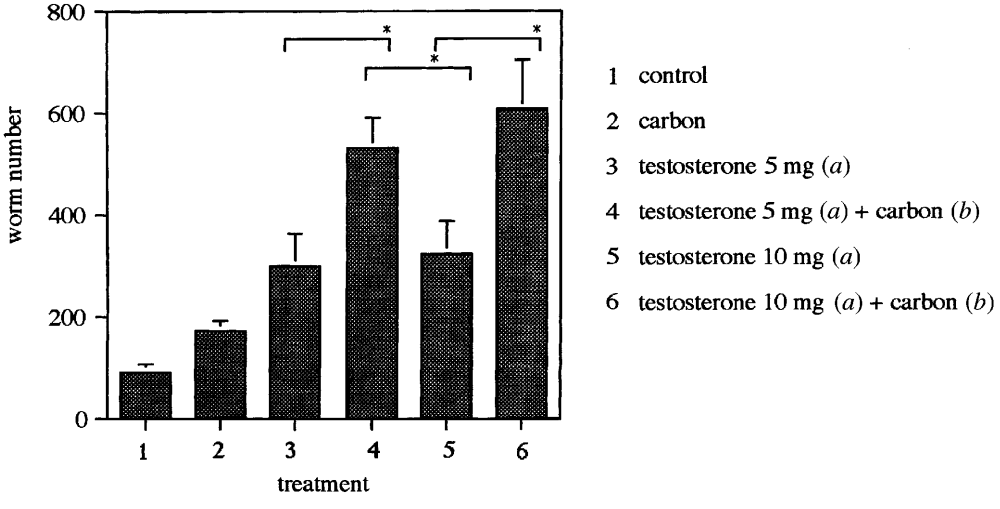

Fig. 5 Worm recovery from the heads of female mice treated with different doses of testosterone and 10 mg carbon as determined at 36 h after infection with *S. ratti* as follows: *a* 5 or 10 mg testosterone implantation every 2 weeks for a total of 3 times, followed at 7 days after the last implantation by infection with 2,000 *S. ratti* or *b* carbon injection at 1 day before infection and at 3 h before infection. Each *column* represents the mean worm recovery from each group of mice, and each *bar* represents the SD. Each group consisted of 3–5 mice. *$P < 0.05$ (Students *t*-test)

Discussion

It has been shown that natural immunity against migrating larvae of *Strongyloides ratti* is chiefly regulated by macrophages (Abe et al. 1985, 1992; Nawa et al. 1988). In the small intestine, host mast cells were related to adult worm expulsion (Olson and Schiller 1978; Nawa et al. 1985). In the *S. ratti*-mouse model, male mice are more susceptible to *S. ratti* than are female mice (Kiyota 1984; Kiyota et al. 1984). This sex-related difference is clearly mediated by testosterone during the migration of larvae, suggesting that testosterone renders mice susceptible to migrating larvae by modulating their natural defense mechanisms (Kiyota 1984).

Recently some investigators have shown that testosterone affects various cell functions (Araneo et al. 1991; Benten et al. 1993; Mohan and Jacobson 1993; Wichmann et al. 1997). In the present study we tried to examine whether the effect of testosterone on natural immunity against *S. ratti* migrating lavae could be mediated through macrophages. Our experiments showed that Te + Ca treatment increased worm recovery more than did Te or Ca treatment alone (Figs. 4, 5) and that this effect was not due to an increased testosterone level. This finding means that testosterone can affect natural defense mechanisms after macrophage blockade. We did not examine the effect of testosterone on macrophages directly. Therefore, we cannot conclude whether testosterone was taken up by macrophages, resulting in suppression of its function, in this model. Since testosterone and carbon treatment had an additive effect on worm recovery, testosterone could affect either other cells contributing to natural immunity against migrating larvae or, directly, L3 of *S. ratti*. The relationship of other host cells, i.e., granulocytes, to susceptibility is now under investigation in our laboratory.

Testosterone treatment in mice affects the development and growth of another nematode, *Heterakis spumosa*, by prolonging the period of infection (Harder et al. 1992). It has also been reported that *S. ratti* L3 migration is affected by the temperature and sodium concentration to which the parasites are exposed (Tobata and Shimada 1996; personal communication). Considering these findings and our results, testosterone treatment may affect the migrating ability of *S. ratti* L3.

In conclusion, our data show that testosterone and carbon treatment in mice has an additive effect on numbers of *S. ratti* migrating larvae and that testosterone can alter the susceptibility of female mice to *S. ratti* infection after macrophages have been blocked. Therefore, the effect of testosterone on worm recovery is partially independent of macrophage function.

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