Testosterone: the culprit for producing splenocyte immune depression after trauma hemorrhage

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Angele, Martin K., Alfred Ayala, William G. Cioffi, Kirby I. Bland, and Irshad H. Chaudry. Testosterone: the culprit for producing splenocyte immune depression after trauma hemorrhage. Am. J. Physiol. 274 (Cell Physiol. 43): C1530–C1536, 1998.—Studies indicate that, whereas immune functions in males are depressed, they are enhanced in females after trauma hemorrhage. Moreover, castration of male mice (i.e., androgen depletion) before trauma hemorrhage prevented the depression of cell-mediated immunity. Nonetheless, it remains unknown whether or not testosterone per se is responsible for producing the immune depression. To study this, female C3H/HeN mice (n = 7 animals/group) were pretreated with 5α-dihydrotestosterone (DHT) or vehicle for 19 days, then subjected to laparotomy (e.g., trauma) and hemorrhagic shock (blood pressure 35 ± 5 mmHg for 90 min) followed by fluid resuscitation or sham operation. Nontreated males underwent either trauma hemorrhage or sham operation. Twenty-four hours thereafter, splenocyte immune functions as well as plasma DHT, estradiol, and corticosterone levels were measured. DHT-pretreated females had significantly (P < 0.05) increased DHT levels, comparable to those seen in males. Conversely, estradiol levels in such females were similar to control males. Splenocyte proliferation as well as interleukin-2 and interleukin-3 release were not depressed in vehicle-treated females, whereas it was in DHT-treated females after trauma hemorrhage, comparable to hemorrhaged males. Thus high testosterone and/or low estradiol levels appear to be responsible for producing splenocyte immune depression in males after trauma hemorrhage. Agents that block testosterone receptors or increase estradiol levels may therefore be helpful in improving depressed immune functions in male trauma patients.

Furthermore, female mice maintained splenocyte functions after the induction of sepsis as compared with depressed immune responses in male mice (21). Different immune responses with regard to gender have also been observed after trauma hemorrhage. Proestrus female mice show enhanced immune function after trauma hemorrhage compared with a depressed immune response in male mice (19). Additional studies have also shown that testosterone depletion by castration of male mice 2 wk before trauma hemorrhage prevents the depression of splenocyte as well as macrophage immune functions in those animals (18).

The above studies indicate that sex hormones are involved in the modulation of normal immune functions as well as during disease processes such as autoimmune diseases and also after trauma hemorrhage. Despite this available information, it remains unknown whether or not testosterone per se is responsible for producing the depressed immune response after trauma hemorrhage in males. The aim of the present study, therefore, was to determine whether or not administration of testosterone to female mice could mimic the depression of splenocyte functions seen in male mice after trauma hemorrhage. The testosterone derivative 5α-dihydrotestosterone (DHT) was chosen for pretreatment, since DHT is considered to be the primary androgenic hormone in males.

MATERIALS AND METHODS

Animals

Inbred female and male C3H/HeN mice (Charles River Laboratories, Wilmington, MA), 7 wk old (24–26 g body wt), were used in this study. All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" [Department of Health and Human Services Publication No. (NIH) 85–23, Revised 1985]. This project was approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital and Brown University.

DHT Procedure

A 21-day release DHT pellet (Innovative Research of America, Sarasota, FL) or a placebo pellet containing the vehicle was injected subcutaneously with a 10-gauge trochar (Innovative Research of America) in female mice, i.e., 19 days before the initiation of the experiment.

Experimental Groups

Female mice were randomized into four groups: groups 1 and 3 received placebo; groups 2 and 4 received DHT. Groups 5 and 6 were untreated male mice. Each group contained

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SEVERAL CLINICAL AND epidemiological studies indicate gender differences in the susceptibility to and morbidity from sepsis (4, 7). Suppressive effects of androgens on B-cell function in autoimmune diseases have been reported (10). This is reflected in a predominance of autoimmune diseases such as lupus erythematosus, Hashimoto thyroiditis, rheumatoid arthritis, and primary biliary cirrhosis in females as compared with males (10). Furthermore, androgen administration in a murine model of lupus erythematosus prolonged survival of female mice (13).

Cell-mediated immune responses also appear to exhibit sexual dimorphism. Increased interleukin (IL)-2 and interferon-γ (IFN-γ) release by peripheral T-cells from normal mice has been reported after castration (17). Studies indicate that female mice tolerate sepsis better than male mice as demonstrated by increased survival rate of females after septic challenge (21).
seven animals. Groups 1, 2, and 5 served as sham-operated animals. The animals in groups 3, 4, and 6 were subjected to the trauma hemorrhage procedure. To initially synchronize the estrus cycle of the females, the animal cages were kept together during the treatment period. The state of the estrus cycle was determined by examination of a vaginal smear before the trauma hemorrhage procedure.

**Trauma Hemorrhage Procedure**

Mice in the hemorrhage groups were lightly anesthetized with methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL) and restrained in a supine position. A 2.5-cm midline laparotomy (e.g., trauma induced) was performed, which was then closed aseptically in two layers using 6-0 Ethilon sutures (Ethicon, Somerville, NJ). After this, both femoral arteries were aseptically cannulated with PE-10 tubing (Clay-Adams, Parsippany, N.J.) using a minimal dissection technique. Heparin (2 U/25 g body wt. beef lung heparin, Upjohn Labs, Kalamazoo, MI) was then administered, and the animals were allowed to awaken. Blood pressure was constantly monitored by attaching one of the catheters to a blood pressure analyzer (Digi-Med, Louisville, KY). Upon awakening, the animals were bled rapidly through the other catheter to a mean arterial blood pressure of 35 ± 5 mmHg (blood pressure prehemorrhage was 95 ± 5 mmHg), which was maintained for 90 min. At the end of that period, the shed blood was returned and lactated Ringer solution (2 times the shed blood volume) was infused to provide adequate fluid resuscitation. Lidocaine was applied to the incision sites, the catheters were then removed, the vessels were ligated, and the groin incisions were closed. Sham-operated animals in groups 1 and 2 underwent the same surgical procedure, which included heparinization and ligation of both femoral arteries, but neither hemorrhage nor fluid resuscitation was carried out. There was no mortality observed in this trauma hemorrhage model within the first 24 h.

**Blood, Tissue, and Cell Harvesting Procedure**

The animals were killed by methoxyflurane overdose at 24 h after the completion of the experiment to obtain the spleen and whole blood from the heart. The mice were killed at the same time of day to avoid fluctuations of plasma hormone levels due to circadian rhythm.

**Plasma Collection and Storage**

Whole blood was obtained by cardiac puncture and placed in microcentrifuge tubes (Microtainer, Becton Dickinson, Rutherford, N.J.). The tubes were then centrifuged at 16,000 g for 15 min at 4°C. Plasma was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen, and stored (−80°C) until assayed.

**Preparation of Splenocyte Culture**

The spleens were removed aseptically and placed in separate petri dishes containing cold (4°C) PBS. Splenocytes were isolated as previously described (20), and the ability of the splenocyte cultures to produce lymphokines in response to a mitogenic challenge was assessed by incubation for 48 h at 37°C, 5% CO2 and 90% humidity in the presence of 2.5 µg/ml concanavalin A (con A; Pharmacia Biotech, Piscataway, N.J.). After that period, the cell suspension was centrifuged at 300 g for 10 min, and the supernatants were harvested, aliquotted, and stored at −80°C until assayed for IL-2 and IL-3. A second portion of the splenocyte suspension was placed in a 96-well microtiter plate (Corning Glass, Corning, NY) in aliquots of 100 µl. The cell's ability to proliferate in response to mitogenic stimulation with 0 (negative control) or 2.5 µg/ml con A was determined by incubation for 48 h at 37°C in a 5% CO2 atmosphere with 90% humidity. The extent of proliferation was measured by [3H]thymidine incorporation technique, previously described by Stephan et al. (15).

**Cell Line Maintenance**

The IL-2-dependent CTLL-2 cells were obtained from the American Type Culture Collection and maintained according to their directions. The IL-3-dependent FDC-P1 cells (gift from Dr. Charles J aneway) were maintained as previously described (20).

**Assessment of Lymphokine Release**

The capacity of the mixed splenocyte culture to produce IL-2 was assessed by determining the amount of IL-2 in the collected culture supernatant. Serial dilutions of the supernatants were added to CTLL-2 cells (1 × 105 cells/ml) and incubated for 48 h at 37°C and 5% CO2. At the end of this period, 1 µCi [3H]thymidine (sp act 6.7 Ci/mmol, New England Nuclear, Wilmington, DE) was added to each well, and the cultures were incubated for an additional 16 h. The cells were then harvested onto glass-fiber mats, and the β-decay was detected by liquid scintillation radiography as previously described (20).

IL-3 activity in the culture supernatants was detected by adding serial dilutions of the supernatants to FDC-P1 cells (2.5 × 105 cells/ml) (20). After a 24-h incubation period at 37°C in a 5% CO2 atmosphere, the cultures, similar to the IL-2 assay, were pulsed with [3H]thymidine, incubated for another 16 h, harvested, and counted. All samples were tested in triplicate.

Relative units per milliliter of lymphokine activity present in the unknown samples were determined by comparison of the curves produced by dilutions of the unknown samples to those generated by a dilution of recombinant murine lymphokine standard (IL-2 or IL-3 standard: 100 U/ml, Genzyme, Cambridge, MA) as previously described (20).

**RIA's**

Plasma DHT concentration was determined using a commercially available coated-tube RIA kit (Diagnostic Systems Laboratories, Webster, TX). In this antibody coated-tube RIA, 100-µl plasma samples were assayed in duplicate. The cross-reactivity of the RIA for testosterone was found to be 100%. Testosterone levels of the unknowns were assigned by interpolation against a testosterone standard curve. The lowest detectable level of testosterone in this RIA was 4 pg/ml.

Plasma 17β-estradiol concentration was determined using a commercially available RIA kit specifically designed for rats and mice (ICN Biomedicals, Costa Mesa, CA). In this Immuchem double-antibody RIA kit, 50-µl plasma samples were assayed in duplicate. The cross-reactivity of the RIA for 17β-estradiol was found to be 100%. Estradiol levels of the unknowns were assigned by interpolation against an estradiol standard curve.

Plasma corticosterone was determined using a commercially available RIA kit specifically designed for mice and rats (ICN Biomedicals). In this Immuchem double-antibody RIA kit, 10-µl plasma samples were assayed in duplicate. The cross-reactivity of the RIA for corticosterone was found to be 100%. Corticosterone levels of the unknowns were assigned by interpolation against a corticosterone standard curve.
Statistical Analysis

The results are presented as means ± SE. One-way ANOVA method followed by the Student-Newman-Keuls test as a post hoc test for multiple comparisons was used to determine the significance of the differences between experimental means.

RESULTS

Splenocyte Proliferation

There was no significant difference in the splenocyte proliferation between the DHT- and vehicle-treated female shams (Fig. 1). The proliferation capacity of splenocytes from female sham animals was found to be comparable to male shams. However, splenocyte proliferative capacity in female animals receiving vehicle and subjected to trauma hemorrhage was found to be similar to that seen in female sham animals. In contrast, the splenocyte proliferation from DHT-pretreated female hemorrhaged mice was significantly depressed (−50.4% compared with vehicle-treated female shams, \( P < 0.05 \)) and was comparable to splenocyte proliferation from male mice subjected to trauma hemorrhage (−52.5% compared with sham males, \( P < 0.05 \)).

IL-2 Release

Splenocyte IL-2 release was similar in the three groups of sham-operated animals (Fig. 2A). IL-2 release by splenocytes was unaltered in female mice receiving vehicle and subjected to trauma hemorrhage. However, splenocytes harvested from DHT-pretreated female hemorrhaged animals exhibited a significant depression of IL-2 release (−38.5% compared with shams receiving vehicle, \( P < 0.05 \)). Male animals also showed significantly decreased splenocyte IL-2 release after hemorrhagic shock (−40.2% compared with male sham animals, \( P < 0.05 \)), and the release was comparable to the release by splenocytes from DHT-pretreated female hemorrhaged mice.

IL-3 Release

No significant difference in the splenocyte IL-3 release was evident between the sham animals (DHT- and vehicle-treated female and male mice) (Fig. 2B). Furthermore, splenocytes from female mice receiving vehicle and subjected to trauma hemorrhage showed levels of IL-3 release comparable to sham animals. In contrast, IL-3 release by splenocytes from female mice pretreated with DHT 19 days before trauma hemorrhage was significantly decreased (−36.3% compared with vehicle-treated female shams, \( P < 0.05 \)), and the release was comparable to the levels seen in male mice subjected to trauma hemorrhage (−34.9% compared with male shams, \( P < 0.05 \)).

Plasma Hormone Levels

Plasma DHT levels. There was no significant difference in DHT levels between the vehicle-treated female
mice subjected to trauma hemorrhage or sham operation (Fig. 3A). Administration of DHT-releasing pellet produced a 10-fold increase in plasma DHT levels compared with the plasma DHT levels of female animals receiving vehicle. Trauma hemorrhage did not significantly change DHT plasma levels in the pretreated female animals. The DHT levels in these animals were comparable to the DHT plasma levels in male animals. In male mice subjected to trauma hemorrhage, circulating DHT levels were slightly decreased; however, this decrease was not statistically significant.

Plasma 17β-estradiol levels. In female mice receiving vehicle, plasma estradiol levels were not altered by trauma hemorrhage (Fig. 3B). However, administration of DHT significantly decreased plasma estradiol levels. In male mice, plasma estradiol levels were not affected by trauma hemorrhage, and the levels were comparable to the levels observed in DHT-treated female mice.

Plasma corticosterone levels. There was no significant difference in plasma corticosterone levels either between sham and trauma hemorrhaged animals or between females and males at 24 h after resuscitation (Fig. 3C).

DISCUSSION

As early as 1898, Calzolari (5) reported a connection between reproduction and immunology. He observed that the thymus of rabbits castrated before sexual maturity was larger than that of the male controls (5). In this regard, IL-2 and IFN-γ release by peripheral T cells is increased after castration of male mice (17). Moreover, female mice in the proestrus state have been shown to tolerate sepsis better than male mice as demonstrated by maintenance of normal immune functions and increased survival rates after septic challenge (21).

Previous studies from our laboratory have also shown immunologic dimorphism with respect to gender after hemorrhagic shock, with the immune function being enhanced in proestrus female mice, as opposed to depressed responses in males (19). Although these findings provide some basis for the perceived immunologic advantage female mice exhibit in response to shock or septic insult, they do not provide the explanation for the suppression of splenocyte function that has been demonstrated in male mice under such conditions. Studies indicate that these reproductive-immunologic interactions appear to be hormonally regulated, and the hormones involved originate from the thymus, from the hypothalamus-pituitary unit, and, in particular, from the gonads (8). In this respect, studies examining the effects of castration of male mice 2 wk before hemorrhagic shock on immune responsiveness showed that testosterone depletion prevented the depression of cell-mediated immunity in males after hemorrhage (18).

The above findings suggest that male sex hormones contribute to the different immune responses in males compared with females. Nevertheless, it remains unclear whether testosterone per se is responsible for initiating the divergent immune response in male and female mice after trauma hemorrhage. In attempting to address this issue, we administered testosterone to female mice to determine whether or not elevating plasma testosterone in female mice to levels comparable to male mice depresses their splenocyte function after trauma hemorrhage and, if so, whether the depression of splenocyte functions was similar to that observed in normal male mice subjected to trauma hemorrhage. To accomplish this, 21-day release pellets of DHT were implanted in female mice to attain consistently elevated plasma DHT levels before trauma hemorrhage. Administration of DHT was chosen in this study, since DHT is considered to be the primary androgenic hormone in males. Moreover, other derivatives of testosterone might act directly via their binding to the androgen receptor in the cytoplasm or alterna-
tively might be metabolized to steroids that are more androgenic, estrogenic, or inactive. Furthermore, females lack the enzyme 5α-reductase, which in males is responsible for the conversion of testosterone to DHT. To ensure that the pellets were releasing DHT, plasma levels of DHT were monitored in females and found to be comparable to the DHT levels in males.

Before hemorrhage, female animals underwent vaginal lavage, and the phase of the estrus cycle was determined by cytology. The DHT pellets had a fixed release period of 21 days, and because the time course of the estrus cycle is not constant in mice, it was not possible to include only female mice in the proestrus and diestrus states. However, all animals were housed together during the pretreatment period, and thus their cycle was synchronized at the time of hemorrhage. Therefore, differences in the immune response due to estrus cycle variations were similar in all vehicle-treated female groups, since an equal number of females were subjected to trauma hemorrhage or sham operation every day. Nonetheless, the different phase of the cycle in vehicle-treated female mice as well as the physiological variations of plasma estradiol levels within the same state of the estrus cycle are probably responsible for the large standard error observed for the plasma estradiol levels in these animals. DHT administration caused disruption of the estrus cycle.

Our previous study demonstrated enhanced splenocyte proliferation and IL-3 release and unchanged IL-2 release in proestrus female mice after trauma hemorrhage (19). The present study, however, showed that the splenocyte function from female mice receiving vehicle and subjected to trauma hemorrhage was comparable to splenocyte function from female sham animals. Our previous study, however, utilized female mice in the proestrus or diestrus state only. Thus the differences in our present study and the previous findings (19) are most likely due to the fact that the majority of the female mice receiving vehicle were in the metestrus and estrus state of the cycle in the present study, whereas they were in the proestrus state in the previous study.

Administration of DHT for 19 days did not alter splenocyte function in female sham animals. In contrast to female shams, administration of DHT before trauma hemorrhage resulted in significantly decreased splenocyte function after trauma hemorrhage comparable to levels observed in untreated male mice subjected to trauma hemorrhage. These findings therefore suggest that high testosterone levels appear to be harmful only in an immunologically compromised host but not in normal animals. Because the capacity of splenocytes to release IL-2 and to proliferate correlate closely in DHT-treated females after trauma hemorrhage, it is possible that the depressed proliferation is due to decreased IL-2 release. Whether or not the addition of exogenous IL-2 to splenocyte cultures from DHT-treated female mice after trauma hemorrhage restores their capacity to proliferate, as observed in splenocytes harvested from burned mice (11), remains to be determined. It could be argued that the decrease in cell proliferation in the DHT-treated female hemorrhaged mice might be because of the release of anti-inflammatory and immunosuppressive mediators directly or indirectly by DHT. This, however, appears to be unlikely, since DHT-treated female shams did not show any decrease in splenocyte proliferative capacity.

Previous studies indicate that blockade of testosterone by testosterone receptor blockade in male mice restored the depressed splenocyte functions and decreased the susceptibility to sepsis after trauma hemorrhage (3). Furthermore, androgen receptor blockade also decreased the mortality of male mice after trauma hemorrhage and subsequent sepsis (3). In light of these findings, we propose that the depressed splenocyte functions in DHT-pretreated females after trauma hemorrhage might increase the mortality rates of animals after the induction of subsequent sepsis.

Several in vitro and in vivo studies demonstrate suppressive effects of androgens on T- and B-cell function. For instance, leukocytes of chinook salmon were cultured in the presence of testosterone, cortisol, and 17β-estradiol. Testosterone and cortisol, but not 17β-estradiol, were found to significantly decrease immunocompetence of leukocytes as evidenced by decreased plaque-forming response in vitro (14). In vivo studies also demonstrate a reduced lymphoproliferative response in cells harvested from testosterone-treated animals that were infected with Mycobacteria tuberculosis (2). The above findings to some extent may be considered contrary to our results, since in our study DHT treatment did not significantly decrease splenocyte functions in sham animals. However, in our study, female animals were pretreated with DHT for only 19 days, whereas in other studies discussed above (12, 13) prolonged administration of DHT for 3 mo was carried out. Support for our findings comes from studies of Ansar Ahmed et al. (1), which showed that administration of testosterone to normal C57BL/6 mice for 2–4 wk did not alter splenocyte IL-2 release.

In contrast to DHT, in vivo administration of estrogen alters total peripheral T-cell activity in a manner that suggests either an enhancement of helper/inducer or a reduction in suppressor/cytotoxic cellular activity by estrogen (6, 12, 14). In vitro studies have also documented increased cytotoxic T-lymphocyte activity after addition of estrogen to the culture media. However, other studies have demonstrated immunosuppressive effects of long-term estrogen treatment in female mice, including a decreased response of spleen lymphocytes to con A and lipopolysaccharide in diethylstilbestrol-treated animals (9). Studies from Ansar Ahmed et al. (1) demonstrate reduced IL-2 production in estradiol-treated animals. However, estradiol levels in treated mice in the above study indicating immunosuppressive effects of estrogen were found to be well above the physiological estradiol levels (22).

A number of immunologic responses are suppressed by glucocorticoid administration, such as lymphoid
organ weight, mitogen-induced lymphocyte proliferation, T cell-mediated cytotoxicity, natural killer cell activity, cytokine production, and leukocyte migration (8). Our findings showed at 24 h after trauma hemorrhage and resuscitation no differences in plasma corticosterone among all experimental groups. Therefore, at the time point examined, the different splenocyte responses between hemorrhaged animals receiving DHT or vehicle do not appear to be corticosterone mediated. However, previous studies indicate that plasma corticosterone levels are elevated 2 h after hemorrhage only in males and decreased in females (19). Therefore, it might be possible that different levels of plasma corticosterone earlier than 24 h after trauma hemorrhage and resuscitation contribute to the observed differences in the immune responses in females treated with DHT or vehicle and males.

Our previous studies suggest that testosterone may play an important role in causing depression of splenocyte and macrophage functions after trauma hemorrhage in males, since castration prevented the depression of immune responses under those conditions (18). In this study, splenocyte function in DHT-pretreated female mice was found to be comparable to males after trauma hemorrhage. However, it remains unknown whether or not inhibition of splenocyte functions would have been evident earlier than 19 days after implantation of the DHT pellet. Moreover, the minimum concentration of plasma testosterone that depresses splenocyte functions after trauma hemorrhage remains to be determined.

The underlying mechanisms by which sex hormones mediate their effect on immune cells remain unclear at present. Studies, however, have shown the presence of nuclear testosterone receptors on various immune cells such as thymocytes and peripheral T cells (16). Furthermore, these studies indicate that androgen receptors are equally expressed in males and females (16). Because DHT did not significantly suppress splenocyte functions in DHT-pretreated female sham animals, it appears that the immunodepressive effects of testosterone after trauma hemorrhage are mediated via the release of secondary mediator(s). However, to what extent the higher levels of estradiol in female mice receiving vehicle contribute to the maintenance of splenocyte functions after trauma hemorrhage remains to be determined.

In summary, the present study demonstrates that DHT pretreatment of female mice for 19 days before trauma hemorrhage produces immunodepression that was comparable to that seen in male mice after trauma hemorrhage. These results further support our previous findings that suggested that testosterone might be the culprit for causing splenocyte depression after trauma hemorrhage in males. To what extent the decreased estradiol levels in DHT-pretreated female mice contribute to the immune depression in those animals after trauma hemorrhage remains to be determined. Nonetheless, the results of this study suggest that treatment with an androgen receptor blocker, such as flutamide, could be a novel and useful adjunct for the treatment of immune dysfunction encountered in male trauma patients. Whether or not increasing estradiol levels pharmacologically in males after trauma hemorrhage has any beneficial effects on the immune functions remains to be determined.

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REFERENCES


