Male sex steroids are responsible for depressing macrophage immune function after trauma-hemorrhage

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Wichmann, Matthias W, Alfred Ayala, and Irshad H. Chaudry. Male sex steroids are responsible for depressing macrophage immune function after trauma-hemorrhage. Am. J. Physiol. 273 (Cell Physiol. 42): C1335–C1340, 1997.—Recent studies suggest beneficial effects of castration before soft tissue trauma and hemorrhagic shock on splenocyte immune functions. Nonetheless, it remains unknown whether this effect of testosterone depletion is limited to splenocytes or is a generalized effect on immune function. The present study was therefore carried out to determine whether androgen depletion before trauma-hemorrhage also has salutary effects on splenic and peritoneal macrophage as well as on Kupffer cell function, as indicated by interleukin (IL)-1 and IL-6 release. Male C3H/HeN mice were castrated or sham-castrated 2 wk before the experiment and were killed at 24 h after trauma-hemorrhage and resuscitation. Significant depression of macrophage IL-1 and IL-6 release was only observed in sham-castrated mice, as opposed to normal levels of cytokine release from castrated animals after trauma-hemorrhage. In addition, only sham-castrated animals showed significantly increased levels of IL-6 release from Kupffer cells, which is believed to contribute to the systemic inflammatory response to trauma-hemorrhage. These observations suggest that the beneficial effects of androgen depletion before trauma-hemorrhage are not limited to splenocyte immune functions but are more global in nature. These results in surgically castrated animals suggest that androgen-blocking agents should be studied for their potential to reverse the immunodepression associated with trauma-hemorrhage.

NUMEROUS STUDIES HAVE BEEN carried out in recent years to investigate the effects of hemorrhagic shock, alone or in conjunction with soft tissue trauma, on cell-mediated and humoral immunity (2, 7, 19–21, 28). Such studies have clearly indicated a marked depression of host immune functions following hemorrhagic shock, which were detectable immediately after the hypotensive period and persisted for a prolonged period of time (2, 19, 20). Soft tissue trauma per se is also known to cause a severe depression of cell-mediated and humoral immune function (1, 21). In view of the above, it is not surprising that the combination of soft tissue trauma and hemorrhagic shock produces a more sustained depression of immune functions (7, 21, 28).

Significant endocrine alterations have been reported following major blood loss, including increased release of adrenocorticotropic hormone, corticosterone, and β-endorphin (15). Despite the fact that gender differences in the susceptibility to and morbidity from sepsis have been observed in several clinical and epidemiological studies (5, 6, 14), the alterations in endocrine and immune functions have been investigated primarily using male laboratory animals. Immune function in normal males and females has been reported to be influenced by sex steroids (10). In this regard, it appears that better-maintained immune functions in females are due not only to physiological levels of female sex steroids typically present but also at least in part due to the absence of immunosuppressive male androgens (13). A number of clinical and experimental studies have shown the suppressive effects of androgens on immunity (13, 17, 22, 24). For instance, it has been reported that not only the peripheral B cell fraction is enlarged in androgen-deficient mice but that the production of interleukin-2 (IL-2) and interferon-γ is increased in peripheral T cells (22). Moreover, in a murine model of lupus erythematosus, survival was prolonged by androgen therapy (17). On the other hand, accelerated death from lupus was observed when the androgen receptor blocker flutamide was administered (24). Furthermore, recent immunological studies suggest beneficial effects of castration on splenocyte immune function after soft tissue trauma and hemorrhagic shock (26).

Nonetheless, it remains unknown whether androgens are also involved in depressing macrophage-dependent immune function following trauma-hemorrhage. It also remains unknown whether castration (i.e., testosterone depletion) before trauma-hemorrhage has any salutary effects on macrophage immune function following soft tissue trauma and hemorrhagic shock. This appears to be of importance, since antiandrogen therapy could have beneficial effects on immune functions following soft tissue trauma and/or hemorrhagic shock in the clinical situation. The aim of the present study, therefore, was to determine the effects of castration on splenic and peritoneal macrophage function following soft tissue trauma and hemorrhagic shock as indicated by IL-1 and IL-6 release. Furthermore, the release of IL-6 by Kupffer cells was measured. These cells are believed to contribute to the systemic inflammatory response following trauma-hemorrhage through the increased release of IL-6 under those conditions (3, 16).

MATERIALS AND METHODS

Animals

Inbred male C3H/HeN mice (9–11 wk old, 24–26 g body wt; Charles River Laboratories, Portage, MI) were used in this study. All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. This project was approved by the
Mice were subjected to sham-castration or castration at the age of 7 wk, i.e., 2 wk before the initiation of the experiment. The castration procedure was performed as previously described by Waynforth (24). All mice were then randomized into one of four groups. Groups 1 and 2 consisted of castrated animals. Animals in group 1 underwent sham-operated controls for the trauma-hemorrhage procedure, and the animals in group 2 underwent a combined model of soft tissue trauma and hemorrhagic shock. The animals in groups 3 and 4 were sham-castrated, with the animals in group 3 serving as sham-operated controls and the animals in group 4 undergoing the combined trauma-hemorrhage model. Mice in the trauma-hemorrhage groups were lightly anesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL) and restrained in a supine position, and a 2.5-cm midline laparotomy (e.g., trauma-induced) was performed. The abdominal incision was then closed aseptically in two layers using 6–0 Ethilon sutures (Ethicon, Somerville, NJ). After this, both femoral arteries were aseptically cannulated with polyethylene 10 tubing (Clay-Adams, Parsippany, NJ) using a minimal dissection technique. The animals were then heparinized (2 units beef of lung heparin/25 g body wt; Upjohn Labs, Kalamazoo, MI) and allowed to awaken. Blood pressure was constantly monitored by attaching one of the catheters to a blood pressure analyzer (Digil-Med, Louisville, KY). The areas of incision were then bathed with 1% lidocaine, and the animals were allowed to awaken. When they awakened, the animals were bled through the other catheter to a mean blood pressure of 35 ± 5 mmHg (blood pressure prehemorrhage was 95 ± 5 mmHg), which was maintained for 90 min.

At the end of the hypotensive period, the shed blood was returned to the hemorrhaged animals, and lactated Ringer solution (2 x the shed blood volume) was infused to provide adequate fluid resuscitation. 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 soft tissue trauma-hemorrhage and fluid resuscitation were not carried out. All animals were killed by methoxyflurane overdose at 24 h after initiation of the experiment, to obtain whole blood from the heart and macrophages from the peritoneal cavity, the spleen, and the liver. All mice were killed at the same time point to avoid artifacts due to marked circadian fluctuations of plasma hormone levels.

Plasma Collection and Storage

Whole blood was obtained by cardiac puncture and was placed in prechilled EDTA-containing microcentrifuge tubes (Microtainer; Becton Dickinson, Rutherford, NJ), which were kept on crushed ice. The tubes were then centrifuged at 16,000 g for 15 min in a refrigerated (4°C) room. Plasma was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen, and stored (−80°C) until the time of assay.

Preparation of Peritoneal Macrophage Culture

Resident peritoneal macrophages were obtained from mice, as previously described (4), and a monolayer was established as previously described (18). The macrophage monolayers were stimulated in vitro with 10 µg lipopolysaccharide W (LPS)/ml Click’s medium containing 10% fetal bovine serum (FBS) for 48 h at 37°C, 5% CO2, and 90% humidity, to assess the cells’ ability to release IL-1 and IL-6. At the end of the incubation period, the culture supernatants were removed, divided into aliquots, and stored at −80°C until assayed for IL-1 and IL-6. This protocol provided adherent cells that were >99% positive by nonspecific esterase staining and that exhibited typical macrophage morphology.

Preparation of Kupffer Cell Culture

After sterile collection of peritoneal macrophages, the peritoneal cavity was packed with sterile gauze. The liver was then blanched to remove cellular blood components by a retrograde perfusion with 30–40 ml of ice-cold Hanks’ balanced salt solution (HBSS) through the portal vein. This was immediately followed by perfusion with 10 ml of 0.05% collagenase class IV (Worthington Biochemical, Freehold, NJ) in HBSS at 37°C. The liver was removed en bloc and transferred to a petri dish containing warm enzyme-HBSS. The tissue was then minced finely, incubated at 37°C for 15 min, and passed through a sterile 150-mesh stainless steel screen into a beaker containing 10 ml of cold HBSS and 10% FBS (Biologics, Naperville, IL). The cell suspension was centrifuged at 1,200 g for 15 min at 4°C, the supernatant was removed, and the cell pellet was resuspended in HBSS and washed by centrifugation. The cell suspension was then layered over a 16% Metrizamide (Accurate Chemical, Westbury, NY) in HBSS and centrifuged at 3,000 g, 4°C, for 45 min in a preparative ultracentrifuge. This process separates the Kupffer cells (which form a band at the Metrizamide cushion interface) from the remaining parenchymal cells in the pellet. After removal of the nonparenchymal cells from the interface with a Pasteur pipette, the cells were washed twice by centrifugation (800 g, 10 min, 4°C) with HBSS. The pellet was then dispersed and resuspended in Click’s medium containing 10% FBS. The cells were transferred to a petri dish and incubated for 4 h at 37°C (5% CO2). Nonadherent and nonviable cells were then removed by three repeated washings of the dish. This protocol provides adherent cells that are >96% positive by nonspecific esterase staining and that exhibit typical macrophage morphology (3). The capacity of mouse Kupffer cells to produce IL-6 was determined by assaying the supernatants taken from these cells (3 x 106 Kupffer cells·ml−1·well−1) following 24-h incubation (37°C, 5% CO2) with or without 10 µg LPS/ml Click’s medium with 10% FBS.

Preparation of Splenic Macrophage Culture

The spleens were removed aseptically and placed in separate petri dishes containing cold (4°C) phosphate-buffered saline (PBS) solution. The splenocyte suspension was used to establish a macrophage culture as previously described (18). The splenic macrophage monolayer was stimulated to produce cytokines by incubation with 10 µg LPS (from Escherichia coli O55:B5; Difco Labs, Detroit, MI) per milliliter in Click’s medium with 10% FBS, 5% CO2, and 90% humidity. At the end of the incubation period, the culture supernatants were removed, divided into aliquots, and stored at −80°C until assayed for IL-1 and IL-6.

Cell Line Maintenance

The IL-1-dependent D10.G4.1 cells (a gift from Dr. Charles J aneway) were maintained as described by Ihle et al. (12). The IL-6-sensitive murine B cell hybridoma (7TD1; a gift from Dr. J jacques Van Snick) was maintained as previously described (12).
Assessment of Cytokine Release

IL-1 release by peritoneal and splenic macrophages was determined by adding serial dilutions of the supernatants to D10.G4.1 cells (2×10^4 cells/well) in the presence of concanavalin A (2.5 µg/ml; Pharmacia, Piscataway, NJ) as previously described (8). Proliferation of the D10.G4.1 cells was measured by [3H]thymidine incorporation.

IL-6 activity in culture supernatant was determined by the amount of proliferation of the murine B cell hybridoma cell line 7TD1, which only grows in the presence of IL-6 (11). Serial dilutions of macrophage supernatants were added to 4×10^4 7TD1 cells/ml, and the cells were incubated for 72 h at 37°C in 5% CO₂. For the last 4 h of incubation, 20 µl of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT; 5 mg/ml in RPMI 1640; Sigma Chemical, St. Louis, MO) were added to each well (only viable cells incorporate MTT). The assay was stopped by aspiration of 150 µl of supernatant from each well, with subsequent replacement by 150 µl of 10% sodium dodecyl sulfate solution in PBS (lauryl sulfate; Sigma) to dissolve the dark blue formazan crystals. With the use of an automated microplate reader (EL-311; Bio-Tek Instruments, Winooski, VT), the light absorbance was measured at 595 nm.

Cells for all populations were also incubated in parallel without stimulant as a negative control. No significant lymphokine or cytokine release was detected in these cultures.

Radioimmunoassay

Plasma testosterone concentration was determined using a commercially available radioimmunoassay (RIA) kit (ICN Biomedicals, Costa Mesa, CA). In this Immucchen double-antibody RIA kit, 50-µl plasma samples were assayed in duplicate. The cross-reactivity of the RIA for testosterone was found to be 100%. For other steroids, the cross-reactivity was as follows: 3.40% for 5α-dihydrotestosterone, 2.20% for 5α-androstanediol, 0.71% for 5α-androstane-3β,17β-diol, 0.63% for 5β-dihydrotestosterone, 0.56% for androstenedione, 0.20% for epipandrosterone, and <0.01% for all other tested steroids (including male and female sex steroids and their metabolites). Testosterone levels of the unknowns were assigned by interpolation against a testosterone standard curve. The lowest detectable level of testosterone in this RIA was 0.025 ng/ml.

Statistical Analysis

The results are means ± SE of each group (n = 6 animals sampled/group). One-way analysis of variance on the rank (for testosterone plasma level) and Student-Newman-Keuls methods were employed to determine the significance of the differences between experimental means. A value of P < 0.05 was considered significant.

RESULTS

Cytokine Release by Peritoneal Macrophages

IL-1. Both sham-operated groups and animals in the trauma-hemorrhage group with prior castration showed comparable levels of peritoneal macrophage IL-1 release, as opposed to significantly depressed IL-1 release in sham-castrated animals after trauma-hemorrhage (~53.8% compared with corresponding shams; P < 0.05; Fig. 1A).

IL-6. Peritoneal macrophages from sham-operated animals and castrated mice after trauma-hemorrhage were found to release similar levels of IL-6 (Fig. 1B). Sham-castrated mice showed significant depression of IL-6 release after trauma-hemorrhage (P < 0.05) compared with the corresponding shams (Fig. 1B).

Cytokine Release by Splenic Macrophages

IL-1. Castrated animals after trauma-hemorrhage demonstrated levels of splenic macrophage IL-1 release that were comparable with sham levels (Fig. 2A). Splenic macrophages from sham-castrated mice after trauma-hemorrhage showed significant depression of IL-1 release (~50.9% compared with the corresponding shams; P < 0.05).

IL-6. Sham-operated mice as well as castrated mice after trauma-hemorrhage were found to have similar levels of splenic macrophage IL-6 release (Fig. 2B). Sham-castrated mice after trauma-hemorrhage showed...
significant depression of IL-6 release from splenic macrophages compared with the corresponding sham-operated animals (\( P < 0.05 \)).

**Plasma Testosterone Levels**

Castration of male mice 2 wk before initiation of sham operation or trauma-hemorrhage reduced plasma testosterone to levels undetectable with the RIA used in the present study. Sham-castrated animals, on the other hand, had detectable levels of testosterone, which were not significantly different in sham-operated animals or animals undergoing trauma-hemorrhage (0.51 ± 0.29 and 0.35 ± 0.27 ng/ml, respectively).

**DISCUSSION**

It has been suggested that the sexual dimorphism of immune function in humans and animals is a result of the effects of gonadal steroid hormones (9). Female immune function during the proestrus and diestrus state has been found to be unchanged or stimulated following adverse circulatory conditions, as opposed to significant depression of immune functions in male mice after hemorrhagic shock (25, 27). In addition, survival in a polymicrobial sepsis model was significantly higher in proestrus female than in male mice (29). Thus it appears that female sex steroids may contribute to this sexual dimorphism. Alternatively, clinical observations and experimental studies also suggest an important suppressive effect of male sex steroids on immune functions (13, 17, 22, 23). Better maintenance of female immunity may, therefore, be in part due to the absence of immunodepressive androgenic hormones rather than the presence of physiological levels of estrogen or progesterone (13). The lack (or low level) of androgens in females also alters the ratio of androgen to estrogen, which is bound to sex hormone/
testosterone binding globin (13). Because this globin plays a key role in controlling the free plasma sex hormone levels (both testosterone and estrogen), it may also contribute to the effect castration had on hemorrhaged mouse immune responsiveness. However, the role of this agent or its levels in plasma following trauma-hemorrhage were not assessed in this study. Castration studies in animal models of autoimmune diseases have shown a potent protective role of androgens in suppressing the autoimmune disease process, which indicates a significant depression and/or control of the host immune system by androgens (for a review see Ref. 13). Moreover, recent studies indicate protective effects of castration before soft tissue trauma and hemorrhagic shock on splenocyte immune function, as indicated by splenocyte proliferative capacity and IL-2 and IL-3 release (26).

Despite the available information concerning the immunodepressive potential of male sex steroids on different aspects of immune functions in normal conditions as well as in autoimmune diseases, it remains unknown whether male sex steroids affect macrophage immune function following soft tissue trauma and severe hemorrhagic shock.

The results presented above clearly show that castration of male mice before the experiments maintains macrophage immune function after trauma-hemorrhage. This is evidenced by the restoration of normal levels of IL-1 and IL-6 release by peritoneal and splenic macrophages in response to stimulation in castrated mice, as opposed to significant depression of these immunological parameters in sham-castrated male mice after trauma-hemorrhage. Alternatively, IL-6 release from Kupffer cells was found to be significantly increased in sham-castrated mice after trauma-hemorrhage, whereas castrated mice after trauma-hemorrhage showed IL-6 release comparable with sham-operated mice. These observations of differential macrophage responsiveness seen following hemorrhage are in keeping with our earlier findings (3). The nature of the differential response of macrophage from the peritoneum and spleen (depressed inducible cytokine release) as opposed to Kupffer cells (augmented cytokine release) to exogenous stimulation appears to exist at a posttranscriptional level. This conclusion is based on our finding that macrophages obtained from all these tissue sites from hemorrhaged mice showed augmented cytokine mRNA expression in response to in vitro stimulation but not increased protein release (30). The actual nature of the translation and/or posttranslational block remains to be determined and is beyond the aim and scope of this study. Nonetheless, Kupffer cells are recognized to play an important role in the mediation of the systemic inflammatory response after trauma-hemorrhage (3, 16), and thus it was important to compare the responses of these varied macrophage populations. To the extent that this augmented Kupffer cell capacity to release IL-6 accounts for the rise in proinflammatory cytokine seen following hemorrhage, studies by O’Neill et al. (16) demonstrated that Kupffer cell depletion before hypotensive shock blunted the systemic IL-6 release in blood. To the best of our knowledge, the present study is the first to report the protective effects of androgen deficiency on depressed peritoneal and splenic macrophage immune functions seen following trauma-hemorrhage, as measured by the release of IL-1 and IL-6. In addition, the suppression of the augmented release of the proinflammatory cytokine IL-6 from Kupffer cells after soft tissue trauma and severe hypotension as observed here may serve to protect the traumatized host from the sequelae associated with systemic proinflammatory mediator release.

At 2 wk after castration, no detectable levels of testosterone were present in the plasma of male C3H/Hen mice when samples were obtained 24 h following trauma-hemorrhage or sham operation (see RESULTS). Alternatively, in mice that were not castrated, although testosterone levels between ~0.8 and 0.1 ng/ml plasma were detectable, trauma-hemorrhage did not produce any significant change in circulating testosterone levels compared with sham. Nonetheless, significant immunological alterations were observed when comparing sham-operated animals and animals after trauma-hemorrhage. This suggests that the immunological changes observed in the present study were not necessarily due to increased levels of male sex steroids after trauma-hemorrhage but might be due to the presence of normal physiological testosterone levels. We cannot, however, preclude the possibility that testosterone levels could have increased transiently during the hypotensive insult or within the initial hours following shock in the sham-castrated animals, as this period was not assessed.

Although our results support the notion that male sex steroids have immunosuppressive effects following trauma-hemorrhage, the mechanism(s) of androgenic suppression of the immune system is not known (23). Thus it remains to be determined whether the beneficial effects of androgen deficiency are due to the lack of testosterone interaction with immunocompetent cells or to the indirect effects of missing testosterone at receptor sites in the central nervous system or in other tissues. In addition, the present study cannot exclude the possibility that other hormonal alterations due to castration might also beneficially influence immune functions following trauma-hemorrhage. However, our preliminary studies indicate that administration of flutamide, a testosterone receptor blocker (25 mg/kg body wt sc) following trauma-hemorrhage in normal, i.e., noncastrated animals also appears to restore the depressed immune functions (unpublished observations). This would suggest that testosterone itself is involved in producing immunodepression, since testosterone depletion (by surgical castration) or testosterone receptor blockade prevented the immunodepression following trauma-hemorrhage.

Previous studies have shown that maximal immune suppression occurs within the 24-h period after hemorrhage, following which the immunological functions gradually return to normal over a period of 5–7 days (28). Because macrophage immune function was maintained at 24 h following trauma-hemorrhage by andro-
Gen depletion, it appears likely that normal immunological responses would be maintained at time points beyond the one used in this study. The present results, based on the measurement of macrophage immune function, therefore, indicate deleterious effects of male sex steroid on immune function after soft tissue trauma and severe hemorrhage. Short-term treatment with testosterone-blocking agents instead of castration following trauma-hemorrhage could therefore be a useful adjunct for maintaining host immune function under those conditions. Additional studies are, however, needed to demonstrate whether pharmacological testosterone antagonism/depletion following soft tissue trauma and severe hemorrhage with agents such as leuprolide and/or flutamide can provide any beneficial effects on immunity in these situations and by what mechanism they may act on these cells.

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