Testosterone receptor blockade restores cellular immunity in male mice after burn injury

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Abstract

Males are known to have increased risk for septic complications after traumatic injury, which appears to be mediated by the inhibitory effects of testosterone on immune function. The role of testosterone in immunity after burn injury, however, remains unclear. Herein, we examined the effects of a testosterone receptor antagonist, flutamide, on delayed type hypersensitivity response (DTH), splenocyte proliferation, interleukin (IL)-2 secretion, and IL-2 receptor (IL-2R) expression in male BALB/c mice subjected to a 15% total body surface area burn or sham injury. Burn- or sham-injured mice were given flutamide s.c. at 30 min and 24 h after injury. At 48 h, burn injury caused a 48% ($P < 0.001$) decrease in DTH response; however, mice that received flutamide treatment did not demonstrate significant suppression of DTH. Likewise, splenocyte proliferation and IL-2

production were depressed in burned animals in comparison with sham-injured controls, and flutamide treatment resulted in a partial restoration of these responses. *In vitro* studies indicated that splenocytes from sham- and burn-injured mice were equally sensitive to the suppressive effects of 5 α -dihydrotestosterone in regard to proliferation and IL-2 production. Further evaluation revealed a decrease in IL-2R expression on splenocytes from burned mice and a partial restoration of this expression with flutamide treatment. Thus blocking testosterone receptor activation improves the cellular immunity in thermally injured mice, possibly through restoration of IL-2 production and IL-2R expression. It remains to be determined whether the effects of testosterone in this injury model are direct or indirect.

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Introduction

More than 100 000 people are admitted to hospital because of burn injury each year in the United States (Smith & Kraus 1988). For these patients, septic complications are the greatest risk for those who survive the initial injury (Dinkel & Lebok 1994). The susceptibility to infection, and resultant mortality, is greatly increased after burn, as a result of suppression of cell-mediated immunity (Moss *et al.* 1988, Dinkel & Lebok 1994). The immune defects observed after burn include anergy to delayed type hypersensitivity (DTH) antigens (Tchervenkova *et al.* 1988), decreased activation and proliferation of lymphocytes in response to mitogenic (concanavalin A (Con A)) or antigenic stimulation and decreased cytokine secretion (Wolfe *et al.* 1982, Teodorczyk-Injeyan *et al.* 1986, 1987, Moss *et al.* 1988).

Interleukin-2 (IL-2) is the primary cytokine required for T lymphocyte activation and proliferation. It has been well established that one of the main factors contributing to decreased T lymphocyte function, and thus cellular immunity, after burn injury is a dramatic decline in IL-2 production (Wolfe *et al.* 1982, Wood *et al.* 1984, Horgan *et al.* 1994a, b, O'Riordain *et al.* 1995). Clinically, the ability of peripheral blood mononuclear cells to produce IL-2 *in vitro* is positively correlated with survival after burn injury (Teodorczyk-Injeyan *et al.* 1986, Sparkes 1993). In addition to the production and secretion of IL-2, T lymphocyte function is also dependent on expression of functional IL-2 receptor (IL-2R) on the cell surface. The IL-2R is a dimer composed of an inducible, high-affinity α -chain (p55) and a constitutive, low-affinity β -chain (p70/75) (Waldman 1986). Expression of the α -chain is increased after antigenic or other stimuli at the initiation of

an immune response. Thus the p55 IL-2R is a critical regulator of lymphocyte proliferation and resultant cell-mediated immunity. There is conflicting evidence regarding the effect of burn injury on the expression of the IL-2R (Teodorczyk-Injeyan *et al.* 1987, Horgan *et al.* 1994a, O'Riordain *et al.* 1995, Meert *et al.* 1996). However, in studies in which the IL-2R was decreased after burn, inability to recover receptor expression was correlated with mortality after injury (Teodorczyk-Injeyan *et al.* 1986, Sparkes 1993).

Nearly 80% of patients admitted for burn or other traumatic injury are male (Offner *et al.* 1999). Clinically, males are at increased risk for suffering morbidity and mortality from sepsis after burn than females (Schroder *et al.* 1998, Offner *et al.* 1999). A variety of experimental trauma models also reveal a sex difference in immunity and survival after injury, and suggest that testosterone mediates some of the immune suppression observed in males (Wichmann *et al.* 1996, 1997, Angele *et al.* 1997, 1998). In these studies either chemical (androgen receptor blockade) or surgical (gonadectomy) castration protected mice from the decreased lymphocyte proliferation and cytokine secretion and increased mortality after sepsis, which are normally observed in intact male mice. The mechanism by which testosterone mediates this suppression of immunity remains to be determined. In addition, the role of testosterone in immune suppression after burn trauma has not previously been explored.

Our current studies aim to determine whether the presence of testosterone influences the suppression of cellular immunity in male mice after burn injury, and to examine the role of testosterone in the regulation of cellular immunity as determined by the DTH and splenocyte proliferative responses, IL-2 production and IL-2R expression by splenic lymphocytes isolated from burn-injured mice.

Materials and Methods

Induction of thermal injury

Male BALB/c mice 10 weeks of age (Charles River Laboratories, Portage, MI, USA) were subjected to dorsal scald injury as previously described (Gregory *et al.* 2000a). Briefly, mice were anesthetized (40 mg/kg sodium pentobarbital in 0.9% normal saline, i.p.; Nembutal, Abbott Laboratories, Abbott Park, IL, USA), and shaved with animal clippers to expose their dorsal surface. Animals were then placed into a plastic template designed to expose 15% of their total body surface area. The mouse and template were then immersed into a water bath at room temperature (sham), or at 100 °C (burn), for 8 s. All animals were thoroughly dried with a towel to prevent further scalding and received intraperitoneal (i.p.) resuscitation with 1.5 ml 0.9% normal saline. They were then placed under warming lamps until recovery from

anesthesia, after which they were returned to their cages. To avoid complications arising from daily cortisol fluctuations, all procedures were performed between 0830 and 1030 h, when circulating cortisol is at its lowest. All animal studies described herein were performed in strict accordance with the guidelines set forth by the Loyola University Chicago Institutional Animal Care and Use Committee.

Flutamide treatment

The effects of testosterone were inhibited by means of the administration of flutamide, a non-steroidal androgen receptor antagonist (Kempainen *et al.* 1992), dissolved in sesame oil. Both sham- and burn-injured mice were treated with oil vehicle or flutamide (25 mg/kg, s.c.) at 30 min and 24 h after the burn (Wichmann *et al.* 1997).

Circulating testosterone

At the time the animal was killed, whole blood was collected via cardiac puncture and allowed to clot at room temperature for 2 h. The blood was centrifuged and the serum was collected, aliquoted and stored at -80 °C until required for analysis. Total testosterone of duplicate samples was determined using a commercially available radioimmunoassay kit (DSL-4900, Diagnostic Systems Laboratories, Inc. Webster, TX, USA).

Determination of DTH responses

DTH responses were induced as described previously (Hansbrough *et al.* 1984, Faunce *et al.* 1997, Gregory *et al.* 2000a). Briefly, 5 days before thermal injury all groups of experimental mice were sensitized to the hapten 2,4-dinitrofluorobenzene (DNFB) (Acros Organics, New Jersey, NJ, USA) by the application of 20 µl of a 0.5% solution in acetone:olive oil (4:1) directly to the shaved skin of the abdomen. Twenty-four hours after thermal injury, ear thickness measurements were made with a micrometer and then an eliciting dose (20 µl 0.2% DNFB) was applied to the pinna of the right ear. At 24 h after elicitation (48 h after the burn), both the unelicited and the elicited ear were measured by an observer blinded to the study group. The magnitude of ear swelling was expressed as % change in ear thickness using the following formula: (change in thickness/pre-elicitation thickness) × 100%, where change in thickness = post-elicitation - pre-elicitation ear thickness. Measurements on the left (completely unmanipulated) ear were obtained and served as internal controls for each animal. A group of naïve animals were not subjected to either sham or burn injury and received only the elicitation dose of DNFB, to allow for the determination of non-specific ear swelling caused by application of the hapten in oil. The naïve mice routinely showed a 3–9% increase in ear thickness in response to the

eliciting dose of DNFB; furthermore, splenocyte proliferation and cytokine secretion of naïve mice were not different from those in sham-injured, oil control-treated mice (data not shown).

Analysis of splenocyte proliferation

At the time of killing, spleens were aseptically removed and single cell suspensions of splenocytes were plated into 96-well microtiter plates at a density of 250 000 cells per well in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) supplemented with L-glutamine (2 mM), penicillin-G (100 U/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum. The viability of the cells was confirmed to be >94% by trypan blue exclusion. Triplicate splenocyte cultures were incubated for 72 h at 37 °C, 5% CO₂ in the presence or absence of concanavalin A (Con A; 2 µg/ml). A colorimetric assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT); Sigma Chemical Co., St Louis, MO, USA) was utilized to measure proliferation as described previously (Gregory *et al.* 2000a). Briefly, after 68 h of incubation, plates were centrifuged, the supernatant was removed, and 50 µl of a 1 mg/ml solution of MTT in phenol red-free RPMI 1640 was added to each well. Plates were then incubated for the remaining 4 h at 37 °C, 5% CO₂. After the incubation and after centrifugation, the untransformed MTT was removed by careful inversion and blotting. Next, 50 µl isopropanol was added and the optical density of each well was measured using an automatic microplate reader at a wavelength of 540 nm. The adjusted mean absorbance for each animal was determined by averaging values obtained from triplicate wells containing medium alone or Con A for each animal and then subtracting the unstimulated absorbance (medium alone) value from the Con A-stimulated absorbance. The adjusted means were then averaged to obtain an overall mean absorbance ± S.E.M. for each experimental group.

Evaluation of splenocyte IL-2 production

Splenocyte cultures were set up in a manner identical to that of the proliferation assay. After a 20-h incubation (37 °C, 5% CO₂), the plates were centrifuged and triplicate supernatants were pooled, aliquoted, and frozen at -80 °C prior to cytokine determination. IL-2 secretion was determined by ELISA using a commercially available sandwich ELISA kit using paired capture and detection antibodies and standard specific for murine IL-2 (Endogen Inc., Cambridge, MA, USA). The minimum level of detection of this assay was 15 pg/ml.

Evaluation of cell-surface antigen expression

Splenocyte cell populations were analyzed via direct immunofluorescence to determine the percentage of

Table 1 Circulating testosterone concentrations. Data are expressed as mean testosterone concentration in ng/ml ± S.E.M. (n=3–5 per group)

Treatment group	Testosterone (ng/ml)
Sham injury	
Oil vehicle	15.4 ± 1.2*
Flutamide	1.42 ± 0.9
Burn injury	
Oil vehicle	0.6 ± 0.5
Flutamide	4.0 ± 3.7

Mice were given a 15% total body surface area sham or burn injury and whole blood was obtained from them 48 h after injury, via cardiac puncture. Serum was evaluated for the presence of unbound testosterone by RIA.

**P*<0.05 compared with all other groups (ANOVA and Fisher's least significant difference post-hoc analysis).

macrophages (Mac-3), T cells (CD4 or CD8a), B cells (CD45/B220) and IL-2R (p55/CD25) receptor expression, using flow cytometry (Pharmingen, San Diego, CA, USA). After blocking for 30 min (PBS/2% BSA, 4 °C), 1.0×10^6 splenocytes were incubated for 30 min with 0.5 µg antigen-specific or isotype control antibody on ice in the dark. Cells were then washed twice (PBS/2% BSA, 4 °C) and fixed in 2% paraformaldehyde (Sigma Chemical). All flow cytometry was performed using a Becton-Dickinson FACSCalibur and Cell Quest software. Data are expressed as mean percent positively staining cells ± S.E.M. of 10 000 events.

Statistical analysis

Data are expressed as mean ± S.E.M. of each group unless otherwise indicated. Analysis of variance (ANOVA) was performed using a statistical analysis package (GB-STAT, Dynamic Microsystems) to assess differences among all experimental groups. If ANOVA indicated a significant main or interactive effect, Fisher's protected least significant difference was used to make *post-hoc* comparisons. *P*<0.05 was considered to be significant.

Results

Circulating testosterone concentrations

Burn and other traumatic injuries have been shown to decrease circulating testosterone concentrations in males (Lephart *et al.* 1987). To evaluate whether our burn injury altered testosterone concentrations, and if flutamide treatment had any effect on these values, circulating total testosterone was measured by RIA. As shown in Table 1, sham-injured mice treated with oil vehicle had normal circulating concentrations of testosterone, and flutamide

Figure 1 DTH response in sham- and burn-injured mice treated with oil vehicle or flutamide (25 mg/kg, s.c.) 30 min and 24 h after the burn. Mice were killed 48 h after burn and DTH response was evaluated as an *in vivo* measure of cell-mediated immunity. Data are shown as the mean % change in ear thickness $\pm$ S.E.M. ($n=9-11$ mice per group). * $P<0.001$ compared with all other groups.

treatment of sham-injured mice resulted in a significant ($P<0.01$) decrease in testosterone concentration in the circulation. As expected, burn injury (oil vehicle) resulted in a significant ($P<0.01$) decrease in testosterone concentration, but flutamide treatment of burned mice did not further reduce hormone concentrations in this group.

DTH response

The DTH response was used as an *in vivo* measure of cellular immunity. Mice were sensitized 5 days before injury to ensure that antigen exposure occurred when the animals were immunologically intact. Twenty-four hours after sham or burn injury, DTH responses were elicited via a second application of antigen to the pinna of each mouse's right ear. The percentage change in ear thickness is proportional to the immunological competence of the mouse. As shown in Fig. 1, treatment of sham-injured mice with oil resulted in a robust increase in ear thickness. Administration of flutamide had no effect on sham-injured animals in comparison with sham-injured mice given an oil injection. Burn injury (plus oil vehicle) resulted in a dramatic decrease ($P<0.001$) in the amount of ear swelling in comparison with that in both groups of sham-injured mice. Flutamide treatment of burned mice resulted in a complete restoration of the DTH response to the levels observed in sham-injured mice (treated with oil vehicle or flutamide).

Splenocyte proliferation

Previous studies have shown that, coincident with the suppression of DTH responses, splenocyte proliferation was decreased in male BALB/c mice at 2 and 4 days after burn injury (Gregory *et al.* 2000a). To evaluate the role of testosterone in the control of cellular immunity after burn,

Figure 2 Total splenocyte proliferation in sham- and burn-injured mice treated with oil vehicle or flutamide (25 mg/kg, s.c.) 30 min and 24 h after the burn. Mice were killed 48 h after burn and splenocytes were cultured in the presence of medium alone or Con A (2 μ g/ml) for 72 h. The MTT assay was used to assess proliferation. The data are expressed as mean absorbance at 540 nm (Con A – medium) $\pm$ S.E.M. from one experiment ($n=4-6$ mice per group). * $P<0.05$ compared with sham-injured mice treated with oil or flutamide.

splenic lymphocyte proliferative responses were evaluated in response to the T cell mitogen, Con A, as shown in Fig. 2. In agreement with the findings of the DTH assay, sham-injured mice treated with oil or flutamide demonstrated similar proliferative capacity after culture with Con A. Burn injury (and oil vehicle) resulted in a significant suppression of mitogen-induced proliferation ($P<0.05$). Mice treated with flutamide after burn demonstrated a partial restoration of lymphocyte proliferation, although this recovery was not as marked as was seen with the DTH response.

Splenocyte production of IL-2

It has been well established that burn injury results in decreased IL-2 production, which contributes to the observed failure of T lymphocyte proliferation observed in both clinical and experimental burn models of injury (Wood *et al.* 1984, O'Riordain *et al.* 1993). To evaluate the role of testosterone in the regulation of IL-2 production, and to examine the possibility that administration of flutamide restores cellular immunity by increasing production of this cytokine, IL-2 secretion by cultured splenocytes was determined. As shown in Fig. 3, there was no difference in IL-2 production by splenocytes isolated from sham-injured mice treated with oil vehicle (1120 ± 120 pg/ml) or flutamide (1082 ± 189 pg/ml). As expected, burn injury resulted in a significant decrease ($P<0.05$) in splenic production of IL-2 (741 ± 123 pg/ml) in comparison with that in sham-injured mice. Flutamide

Figure 3 IL-2 production by total splenocytes isolated from sham- and burn-injured mice treated with oil vehicle or flutamide (25 mg/kg, s.c.) 30 min and 24 h after the burn. Mice were killed 48 h after burn and splenocytes were cultured in the presence of medium alone or Con A (2 µg/ml) for 20 h. IL-2 was measured in the culture supernatants by ELISA. Unstimulated cells (medium alone) did not produce any detectable IL-2. Data are expressed as mean concentration of IL-2 $\pm$ S.E.M. for each group ($n=6-8$ mice per group). * $P<0.05$ compared with sham-injured mice treated with oil or flutamide.

treatment only partially restored splenocyte secretion of IL-2 (923 ± 94.5 pg/ml) by lymphocytes isolated from burned animals, which was similar to the findings in the proliferation assay.

Characterization of splenocyte subpopulations

The splenocyte suspensions evaluated for their proliferative responses to mitogen were unfractionated, thus they

included primarily T and B lymphocytes and macrophages. The proportion of each cell type present in the cultures could influence not only the mitogen-induced proliferation and IL-2 production *in vitro*, but also the *in vivo* DTH response, as this assay depends on systemic lymphocyte recruitment and proliferation. To explore the possibility that a change in cell number or proportion, rather than function, was affecting these assays, the percentage and total number per spleen of macrophages (Mac-3⁺), T cells (CD4⁺ or CD8⁺) and B cells (CD45R/B220⁺), were analyzed using direct immunofluorescence and flow cytometry. As shown in Table 2, there were no significant differences in the percentages of CD4⁺ T, CD8⁺ T or B cells with regard to the various treatment groups. Both groups of burned mice showed an increase in the percentage of splenic macrophages (Mac-3⁺), in comparison with sham mice. This finding was statistically significant ($P<0.05$) in the group of mice treated with oil vehicle after burn, but not in those treated with flutamide. When the absolute number of each cell population was determined, burn injury resulted in a significant ($P<0.05$) reduction in the number of CD4-, CD8- and B220-expressing cells. There was no reduction, however, in the number of Mac3⁺ cells, which resulted in an increased percentage of this cell type in comparison with others.

The effects of *in vitro* testosterone on proliferation

The mechanism by which testosterone mediates its immune suppressive effects after traumatic injury is unknown. Although flutamide treatment does not alter testosterone concentrations in this model (Table 1), it is possible that burn injury could alter hormone receptor expression or cell signaling pathways, resulting

Table 2 Immunofluorescent staining of splenocyte subpopulations and IL-2 receptor. All values are represented as percent (number) of positively immunostained cells minus positively stained isotype control cells $\pm$ S.E.M. ($n=4-6$ animals per group)

Treatment	Immunopositive cells (% (absolute number $\times 10^6$))				
	CD4 ⁺	CD8 ⁺	B220 ⁺	Mac-3 ⁺	IL-2R
Sham injury					
Oil vehicle	16.8 $\pm$ 2.7 (20.0 $\pm$ 3.2)	7.9 $\pm$ 0.3 (9.8 $\pm$ 0.6)	54.7 $\pm$ 1.1 (68.4 $\pm$ 2.6)	14.3 $\pm$ 2.9 (17.8 $\pm$ 4.3)	63.9 $\pm$ 2.6 (79.8 $\pm$ 4.3)
Flutamide	14.6 $\pm$ 3.2 (17.5 $\pm$ 1.9)	11.1 $\pm$ 1.6 (13.3 $\pm$ 2.4)	54.1 $\pm$ 6.6 (64.8 $\pm$ 8.9)	15.3 $\pm$ 6.2 (18.4 $\pm$ 8.1)	62.6 $\pm$ 3.5 (75.1 $\pm$ 5.8)
Burn injury					
Oil vehicle	16.8 $\pm$ 0.1 (3.3 $\pm$ 0.1)*	8.7 $\pm$ 2.5 (6.9 $\pm$ 1.9)†	52.3 $\pm$ 3.5 (41.5 $\pm$ 2.4)*	23.1 $\pm$ 0.4* (18.3 $\pm$ 0.3)	53.6 $\pm$ 1.3† (47.5 $\pm$ 0.9)*
Flutamide	15.8 $\pm$ 0.2 (13.4 $\pm$ 0.1)*	9.7 $\pm$ 0.7 (8.2 $\pm$ 0.5)*	53.7 $\pm$ 3.5 (45.6 $\pm$ 2.9)*	21.5 $\pm$ 1.4 (18.3 $\pm$ 1.2)	60.4 $\pm$ 2.7 (51.3 $\pm$ 2.4)*

Mice were given a 15% total body surface area sham or burn injury and splenocytes were obtained from them 48 h after injury and evaluated for expression of surface antigens (CD4, CD8, B220 and Mac-3). To evaluate IL-2R (p55/CD25), cells were cultured overnight in the presence of 2 µg/ml Con A then washed and stained for receptor expression. * $P<0.05$ compared with sham-injured mice; † $P<0.05$ compared with all other groups (ANOVA and Fisher's least significant difference post-hoc analysis).

Figure 4 Non-adherent splenocyte (lymphocyte) proliferation from sham- and burn-injured animals in response to *in vitro* exposure to Con A (2 µg/ml) alone or Con A plus 3, 10 or 30 ng/ml 5α-dihydrotestosterone (DHT). Mice were killed 48 h after the burn and splenocytes were adherence-purified and cultured for 72 h. The MTT assay was used as an indicator of proliferation. The data are expressed as mean absorbance at 540 nm (Con A – medium) ± s.d. of triplicate cultures from one experiment. * $P < 0.05$ compared with lymphocytes from sham-injured mice treated with 0 ng/ml DHT; # $P < 0.05$ compared with lymphocytes isolated from burn-injured mice and cultured with 0 ng/ml DHT.

in a differential susceptibility to the effects of testosterone in burned animals. To examine this possibility, non-adherent splenocytes (lymphocytes) from sham- and burn-injured mice were cultured with increasing concentrations (0, 3, 10 and 30 ng/ml) of a non-aromatizable form of testosterone, 5α-dihydrotestosterone (DHT), and proliferation was evaluated using the MTT assay. As shown previously (Fig. 2), the lymphocytes from burned mice demonstrated significantly ($P < 0.05$) decreased (34.8%) proliferation in response to Con A (0 ng/ml DHT) compared with those from sham-injured mice (Fig. 4). Lymphocytes from sham and burn mice also revealed a dose-dependent decrease in proliferation in response to *in vitro* DHT. Addition of 3, 10 or 30 ng/ml DHT resulted in a 30.8%, 45.1% and 62.1% decrease in proliferation lymphocytes isolated from sham-injured mice and a 17.1%, 33.5% and 50.2% decrease in proliferation of lymphocytes isolated from burn injured mice respectively.

The effects of *in vitro* testosterone on IL-2 production

Because differences in testosterone sensitivity could also affect cellular immunity by influencing cytokine production, the effect of increasing concentrations (0, 3, 10 and 30 ng/ml) of DHT on splenocyte IL-2 production was evaluated. In agreement with the splenocyte proliferation data shown in Fig. 4, the secretion of IL-2, in the absence

Figure 5 IL-2 production by non-adherent splenocytes (lymphocytes) adherence-purified from sham- and burn-injured animals in response to *in vitro* exposure to Con A (2 µg/ml) alone or Con A plus 3, 10 or 30 ng/ml 5α-dihydrotestosterone (DHT). Mice were killed 48 h after the burn, splenocytes were cultured for 20 h, and the supernatant IL-2 was determined by ELISA. * $P < 0.05$ compared with lymphocytes isolated from sham-injured mice treated and cultured with 0 ng/ml DHT; # $P < 0.05$ compared with lymphocytes isolated from burn-injured mice and incubated with 0 ng/ml DHT.

of DHT, was suppressed by 36.4% in burned mice in comparison with that in sham-injured mice (Fig. 5). IL-2 production was further suppressed by the addition of increasing doses of DHT: addition of 3, 10 or 30 ng/ml DHT resulted in a 19.8%, 36.2%, and 51.0% decrease in proliferation of lymphocytes isolated from sham-injured mice and a 30.8%, 39.5% and 51.8% decrease in proliferation of lymphocytes isolated from burn-injured mice respectively.

IL-2 receptor expression

In addition to IL-2 production, the level of IL-2R (p55/CD25) expression is critical for a productive cellular immune response. Therefore, the p55/CD25 receptor levels were measured on mitogen-stimulated (2 µg/ml Con A, 18 h) splenocytes from sham and burn mice treated with oil or flutamide. IL-2R was not detectable on freshly isolated cells from any experimental group of mice. Flutamide treatment did not affect IL-2R expression in sham-treated mice (Table 2). Burned mice treated with oil vehicle demonstrated a decrease in the percent IL-2R⁺ splenocytes in comparison with sham-injured mice treated with oil (53.6 ± 1.3 compared with 63.9 ± 2.6), which was restored by treatment with flutamide after burn injury (60.4 ± 2.7). Interestingly, when absolute cell numbers expressing IL-2R were examined, burn injury (oil treatment) resulted in a decrease (79.8 ± 4.3 sham compared

with 47.5 ± 0.9 burn) in the number of IL-2R expressing cells; this was not fully restored by flutamide treatment (51.3 ± 2.4 in the burn-injured, flutamide-treated group).

Discussion

Males are at a significantly increased risk for developing septic complications after burn or other traumatic injury (Offner *et al.* 1999), and suffer from increased mortality in comparison with females that are matched for age, type and severity of injury (Schroder *et al.* 1998). These findings suggest that immunity after injury is influenced by the gonadal hormones, estrogen and testosterone. In support of this, injured male mice have decreased proliferation of lymphocytes and secretion of cytokines (including IL-2) that are associated with increased susceptibility to sepsis, in comparison with females (Wichmann *et al.* 1996). In addition, castration of males before injury, or flutamide treatment after injury, restores immune responses, and improves survival after septic challenge (Wichmann *et al.* 1996, 1997, Angele *et al.* 1997). Likewise, treatment of female mice with DHT before injury results in defects in immunity that are similar to those seen in intact injured males (Angele *et al.* 1998). Thus it is likely that the increased morbidity and mortality observed in males after traumatic injury are due to the presence of the immune-suppressive androgenic hormone, testosterone. The current studies confirm this hypothesis and extend the previous experimental findings in traumatic injury models to include burn injury.

The suppressive effects of testosterone on immune function are widely accepted; however, the mechanism for this androgen control of immunity is not well understood. Both *in vivo* and *in vitro* testosterone exposure results in decreases in B and T lymphocyte expansion in the bone marrow, spleen, and thymus (Weinstein & Berkovich 1981, Olsen *et al.* 1994, Olsen & Kovacs 1996, Viselli *et al.* 1997). Burn injury is also known to alter splenocyte subpopulations within these organs (Kupper *et al.* 1985, O'Mahony *et al.* 1985), which could influence the DTH and splenocyte proliferative responses. In the current studies, evaluation of the absolute number of each cell type revealed a decrease in T and B cells, but no change in splenic macrophages, thereby resulting in an increase in the overall percentage of macrophages (Table 2). Flutamide treatment did not affect these observations, further suggesting that androgen receptor blockade after burn is altering the function of immune cells resulting in restoration of the DTH response *in vivo*.

The macrophage is known to have a key role in mediating injury-induced immune suppression via the production of factors such as prostaglandin E₂ (PGE₂), transforming growth factor (TGF)- β and IL-6 (Miller-Graziano *et al.* 1993, Olsen *et al.* 1994, Gregory *et al.* 2000b). The production of these factors after burn is

associated with decreased lymphocyte functions including proliferation, IL-2 production and IL-2R expression (Miller-Graziano *et al.* 1991, 1993, Fukushima *et al.* 1994, Horgan *et al.* 1994a, Meert *et al.* 1996, Gregory *et al.* 2000c). Furthermore, exposure of macrophages to testosterone can increase production of TGF- β directly (Benten *et al.* 1999), and thus that of IL-6 and PGE₂ (Miller-Graziano *et al.* 1991, 1993, Olsen *et al.* 1994). Therefore, an increase in the percentage of macrophages after injury would result in an exaggerated effect of these factors on lymphocyte function, which could be ameliorated by the administration of flutamide.

Both burn and other traumatic injury alter circulating concentrations of gonadal steroid hormones. Specifically, estrogen concentrations are enhanced in both males and females, whereas testosterone is decreased in males (Woolf *et al.* 1985, Lephart *et al.* 1987, Christeff *et al.* 1992, Fourrier *et al.* 1994, Sharma *et al.* 1996). Despite the finding that injury decreases the immune-suppressive androgenic hormones, flutamide treatment consistently results in restoration of cellular immune function after trauma (Wichmann *et al.* 1996, 1997, Angele *et al.* 1997). The current studies also demonstrate a decrease in circulating testosterone after burn injury, which was further reduced in sham-injured mice, but not in burn-injured mice, by flutamide treatment. *In vivo* flutamide treatment has variable effects on testosterone concentrations in non-injury models (Falsetti *et al.* 1999, Zaccheo *et al.* 1999, Chertin *et al.* 2000); however, injury models utilizing a hemorrhagic shock reveal a further decrease in serum testosterone after flutamide treatment in both control and hemorrhaged mice (Samy *et al.* 2000). In the current study, the lack of any affect of flutamide on testosterone concentrations in burned mice is likely to be the result of the very low concentrations of testosterone in the burned animals.

The suppressive nature of testosterone after injury, in the presence of decreased circulating concentrations of the hormone, is admittedly counterintuitive. Whereas increased glucocorticoids after injury are believed to regulate the decrease in testosterone after injury (Lephart *et al.* 1987), glucocorticoid concentrations are not affected by flutamide treatment (Wichmann *et al.* 1997). Thus the beneficial effects of flutamide treatment on immunity after injury are not the result of decreased circulating testosterone. It has been suggested that even a small concentration of testosterone is enough to produce immune depression in an immunologically compromised host (Angele *et al.* 1997). It is also possible that burn injury results in increased androgen receptor expression, which would allow for the increased influence of low circulating testosterone concentrations.

The current studies reveal that *in vivo* testosterone blockade completely restores the DTH response, but only partially restores lymphocyte proliferation and IL-2 production. In addition, *in vivo* flutamide treatment

modulated immunity only in burned mice, whereas *in vitro* testosterone had approximately equal suppressive effects on splenocytes of burn- and sham-injured animals (proliferation and IL-2 secretion). Taken together, these findings suggest that flutamide may have testosterone-independent effects on splenocytes. In fact, androgen depletion by castration or flutamide treatment has been shown to increase estrogen receptor expression in a variety of cells, including T cells (West *et al.* 1990, Bodker *et al.* 1994, Kruithof-Dekker *et al.* 1996, Samy *et al.* 2000). Furthermore, preliminary studies suggest that, not only are estrogen concentrations increased after burn as previously reported (Woolf *et al.* 1985, Lephart *et al.* 1987), but flutamide treatment further augments this increase (K A N Messingham, M A Emanuele and E J Kovacs, unpublished observation). Such flutamide-induced increases in estrogen receptor expression would have little function *in vitro* (in the absence of estrogen); however, increased estrogen receptor expression on lymphocytes, in combination with increased circulating estradiol after burn injury, would probably stimulate lymphocyte functions *in vivo*.

There are conflicting reports regarding the effects of injury on IL-2R expression (Teodorczyk-injeyan *et al.* 1986, 1989, Horgan *et al.* 1994b, O'Riordain *et al.* 1995). It is unknown whether the small decrease in IL-2R expression after burn, and its restoration by flutamide, is biologically relevant (Table 2). It is possible that a small restoration of receptor expression, in combination with a partial restoration of IL-2 production, could provide a mechanism for the recovery of splenocyte proliferation in flutamide-treated mice. However, as the IL-2R expression was fully recovered, but proliferation and IL-2 production were only partially restored, it is unlikely that this change in IL-2R expression is relevant. In addition, in experiments demonstrating reduced IL-2R after injury, lymphocyte proliferation could be restored by the addition of exogenous IL-2 (Teodorczyk-Injeyan *et al.* 1986, Sparkes 1993, Horgan *et al.* 1994b). Thus it appears that the lack of IL-2, not IL-2R, is the critical defect in lymphocyte function after burn.

The current studies are the first to demonstrate that blocking testosterone in male mice after burn injury results in recovery of immunity. The involvement of gonadal steroids in the morbidity and mortality after burn injury suggests that sex-specific injury therapies may be necessary. In support of this, studies by Gregory *et al.* (2000a) found that estrogen concentrations in females reach immune-suppressive pregnancy levels, whereas hormone concentrations in males are similar to those found in cycling females (immune stimulatory). Furthermore, ovariectomy of female mice resulted in restoration of immunity after burn. Recently, the administration of estrogen to male mice was shown to improve immunity after injury (Knofler *et al.* 2000). Thus, in males, administration of low (stimulatory) concentrations of estrogen could prove to be beneficial to lymphocyte functions

whereas, in females, administration of testosterone, or blocking of estrogen, may result in improved immunity and decreased susceptibility to sepsis after burn injury.

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