Immunology 1995 **84** 337–342

Castration alters peripheral immune function in normal male mice

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SUMMARY

While it is generally recognized that females show enhanced cell-mediated and antibody responses to antigenic stimulation, the physiological basis for this observed sexual dimorphism of the immune response is not well understood. We report here studies on the effects of androgen deficiency on the peripheral immune system. Intact male mice were compared to animals castrated 3–4 months previously. Phenotypic characterization of thymocyte and lymphocyte subpopulations was carried out using dual-colour flow cytometry. *In vitro* production by spleen cells of interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ), and levels of total immunoglobulin and autoreactive antibodies was measured by specific immunoassays. In addition to thymic hypertrophy, castrated animals showed significant splenic enlargement, which was largely owing to expansion of the B-cell population. The castrated spleens contained relatively fewer mature T cells than intact controls ($P \leq 0{\cdot}001$), but culture supernatants from these spleen cells contained higher levels of IL-2 and IFN-γ than control cultures ($P < 0{\cdot}04$). Levels of *in vitro* antibody synthesis (IgM, IgG, IgA) were not higher in castrated animals compared to controls, but the castrate spleen cell cultures showed increased levels of production of two autoreactive antibodies, anti-IgG (rheumatoid factor) and anti-thyroglobulin. These data suggest that androgen deprivation results in a relative decrease in the number of mature peripheral T cells, but those which reach the spleen have functional characteristics suggestive of enhanced activation. Dysregulation in the B-cell compartment may be the result of altered effects of T-cell-mediated control.

INTRODUCTION

The sexual dimorphism of immune function in humans and animals is believed to be the consequence of the actions of gonadal steroid hormones.[1] Females have more exuberant antibody and cell-mediated responses to exogenous antigens than males[1–3] and relative numbers of circulating T cells in helper or suppressor/cytotoxic subsets have been reported to be regulated by gonadal steroids.[4,5] The disproportionate incidence of human autoimmune diseases in females and the well-documented modulation of animal models of such autoimmune diseases by gonadal steroids also support a role for these hormones in the development of immune responses. The evidence from these clinical observations and experimental models suggests that androgens exert a tonic suppressive effect on immune function and that this effect is mediated through the thymus.

We have previously characterized changes in thymocyte proliferation and development that ensue after manipulation of androgen levels in normal adult male mice.[6] In the present study we have examined the consequences of prolonged androgen deficiency for peripheral immune system function. We report here that androgen-deficient mice show evidence for an expansion of the peripheral B-cell compartment, enhanced autoantibody production, and increased levels of production of interleukin-2 (IL-2) and interferon-γ (IFN-γ) by peripheral T cells.

Received 30 June 1994; revised 25 September 1994; accepted 16 October 1994.

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MATERIALS AND METHODS

Mice

C57Bl/6 male mice were obtained from Harlan Laboratories (Indianapolis, IN). Castration of some mice was performed at 6 weeks of age. The animals were kept in the Vanderbilt animal care facilities and studies were carried out at 18–24 weeks of age. Intact age-matched male mice were used as controls. In some experiments, an intact control animal 8–10 weeks of age was included. These younger controls showed no significant differences in any of the measured parameters compared to the age-matched control mice. All procedures and animal care were approved by the Vanderbilt University Animal Care Committee.

Antibodies

Fluorochrome-conjugated monoclonal antibodies were obtained from Pharmingen (San Diego, CA). These included:

fluorescein isothiocyanate (FITC)–anti-CD3 ε chain (clone 145-C11); FITC- and allophycocyanin (APC)–anti-CD8a (clone 53-6.7); phycoerythrin (PE)–anti-CD4 (GK1.5); PE-anti-Ly1 (CD5). Goat anti-mouse immunoglobulin conjugated with FITC was obtained from Sigma Chemical Co. (St Louis, MO).

Cell preparations and staining

Thymuses and spleens were removed at the time of death and weighed. Single-cell suspensions were prepared by mincing the organ and grinding the mince between microscope slides. Cells were washed in RPMI-1640 (Gibco, Grand Island, NY). Aliquots of 1×10^6 cells were washed and resuspended in fluorescence-activated cell sorting (FACS) buffer [2% bovine serum albumin (BSA), 0·1% azide, phosphate-buffered saline (PBS)] for staining. Appropriate concentrations of fluorochrome-conjugated monoclonal antibodies were added to each aliquot in a total reaction volume of 0·20 ml. Cells were incubated for 30 min at 0°, washed through a layer of fetal calf serum (FCS), resuspended in 0·10 ml FACS buffer and fixed by dropwise addition of ice-cold 70% ethanol while vortexing. DNA staining for cell cycle analysis with 7-amino actinomycin D (7-AAD) was performed according to the method of Rabinovitch.[7] The fixed cells were washed twice in serum-free FACS buffer, resuspended in 1 ml of 7-AAD (25 μg/ml; Molecular Probes, Eugene, OR) and incubated for 1 hr.

Flow cytometric analysis

Cells were analysed for surface fluorescence and DNA content using a dual-laser FACStar™ Plus (Becton Dickinson, San Jose, CA) as previously described.[8] All data were collected in list mode for later off-line analysis using Winlist (Verity Software House, Inc., Topsham, ME). The combined cell surface staining and DNA content data were collected while recording logarithmic amplification of the surface staining with linear fluorescence of 7-AAD above a forward angle light scatter trigger to exclude cellular debris. Doublets were excluded by analysing correlated area against width signals of the 7-AAD fluorescence. A minimum of 5×10^4 cells from each animal was analysed. Cell cycle data for the overall populations and for cell subsets were expressed as the percentage of cells in the S and G_2 or M phases of the cell cycle using algorithms found in Modfit™ (Verity). For two-colour phenotype analysis, FITC and PE fluorescence were excited at 488 nm and log-amplified signals were collected at 525 nm (FITC) and 575 nm (PE). APC fluorescence was excited at 633 nm and collected at 680 nm. A minimum of 5×10^3 cells was analysed for each animal.

Quantification of cytokine production by cultured splenocytes

Cells were cultured at 5×10^6 cells/ml in 0·20 vols with and without added concanavalin A (Con A) (2 μg/ml; Sigma) and/or phorbol 12-myristate 13-acetate (PMA; 10 ng/ml; Sigma) in replicate round-bottom microtitre wells. Cultures were incubated for 20–48 hr, as indicated. Supernatants were harvested and stored at −70° until assayed for cytokine content. Levels of IL-2, IL-4 and IFN-γ in culture supernatants were measured in solid-phase enzyme-linked immunosorbent assay (ELISA) using paired monoclonal antibodies (Pharmingen), according to the manufacturer's protocol. For each assay, plastic microwells were coated with 2 μg/ml of the primary antibody. Wells were blocked with PBS containing 3% BSA and 100 μl vols of each sample were added in duplicate. Plates were incubated at 4° overnight and then washed and blocked once again. Biotinylated anti-cytokine antibody (1 μg/ml) was added (100 μl/well) and the plates were incubated at room temperature for 1 hr. Avidin–phosphatase (1 : 400 dilution of 1 mg/ml solution; Sigma Chemical Co.) was prepared according to the manufacturer's recommendation and added to each well. Colour development was detected at 405 nm using a Titertek II plate Reader (Flow Laboratories, Bethesda, MD). Results are expressed as U/ml, calculated from a standard curve prepared for each cytokine using recombinant IL-2, IL-4 and IFN-γ (Genzyme, Boston, MA). Preliminary results indicated optimum production of IL-2 at 20–24 hr and of IFN-γ at 24–48 hr. Very low levels of IL-4 were found at all times tested.

Quantification of immunoglobulin production by cultured splenocytes

Immunoglobulin production was measured in supernatants from spleen cells cultured at 1×10^6/ml in round-bottom microtitre wells. Cells were cultured without added mitogen, and with pokeweed mitogen (PWM; 10 μg/ml; Sigma) or lipopolysaccharide (LPS; 25 μg/ml; Sigma). Supernatants were harvested after 7 days and stored at −70° until assayed. IgG, IgA and IgM were measured by ELISA, as previously described.[6] Results were expressed in ng/ml using standard curves prepared from purified mouse immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, AL). Immunoglobulins reactive with three different putative auto-antigens were also measured by ELISA using human IgG (Sigma; 50 ng/ml), calf thymus DNA (Sigma; 1 mg/ml) and bovine thyroglobulin (Sigma; 1 μg/ml). For DNA, plates were precoated with poly-L-lysine (50 μg/ml) prior to adding the antigen.[9] Bound immunoglobulins were detected with alkaline phosphatase-labelled anti-mouse immunoglobulin (Southern Biotechnology) and *p*-nitrophenyl phosphate substrate (Sigma). Data were expressed as net OD units at 405 nm, subtracting values for background wells containing buffer in place of the sample.

Data analysis and statistics

Results are expressed as mean values for each group ± SEM. Levels of IgM were compared after logarithmic transformation to approximate a normal distribution. Data from intact and castrated animals in each experiment were compared using Student's *t*-test. Production of IFN-γ was analysed in two groups using Fisher's exact test. $P < 0{\cdot}05$ was considered significant.

RESULTS

Effects of long-term castration on the size and phenotypic composition of the thymus

Castrated mice showed changes in the thymus similar to those observed after short-term androgen deprivation.[6,8] Thymuses from castrated animals were larger than those of age-matched intact controls (96 ± 5 mg versus 56 ± 6 mg; $P \leq 0{\cdot}001$) (Fig. 1), showed a decrease in the relative proportion of the $CD4^- CD8^+$ thymocyte subpopulation (1·8 ± 0·2% versus

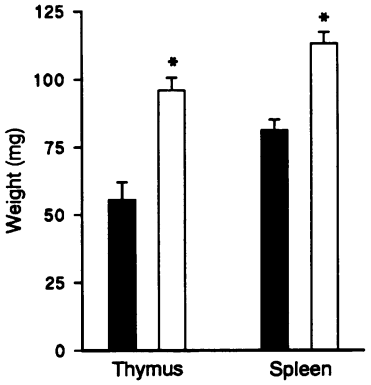

Figure 1. Mean ± SEM thymus and spleen weights in intact (■) and castrate (□) mice. *$P < 0.001$ versus intact; $n = 12–16$ animals.

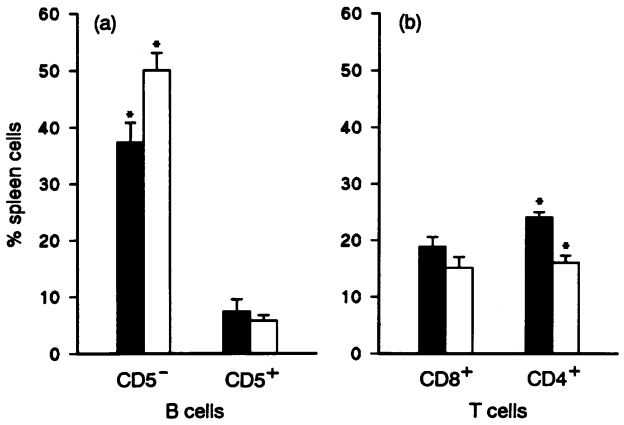

Figure 2. Subpopulations of lymphocytes in spleens in intact (■) and castrate (□) mice. (a) Splenic B cells, identified by the presence of surface immunoglobulin, are further defined by the presence or absence of CD5. The per cent of total splenocytes (mean ± SEM) in each B-cell subset is shown; $P = 0.01$. (b) Subsets of T cells, identified by the expression of CD3, are analysed in two subsets, defined by co-expression of $CD4^+$ or $CD8^+$: $P \leq 0.001$; $n = 6–8$ animals.

2·7 ± 0·2% for intact controls; $P = 0.008$) (Table 1), and had fewer cells expressing the T-cell receptor marker CD3 (15·9 ± 1·0% versus 20·4 ± 1·6% for intact controls; $P = 0.041$).

Splenic changes associated with long-term androgen deprivation

Spleen weight was significantly greater in the castrated group (113 ± 5 mg) than in the intact control group (81 ± 4 mg; $P < 0.0001$; Fig. 1). Flow cytometric analyses of spleen cell populations showed the relative number of $CD5^-$ B cells to be significantly greater in castrated spleens (50·7 ± 3·0%) than in intact controls (37·5 ± 3·4%; $P = 0.015$; Fig. 2a). However, the $CD5^+$ subpopulation of B cells was not significantly different between the two groups (7·4 ± 2·1% versus 5·8 ± 0·9%; $P > 0.3$). The percentage of cells bearing T-cell markers was reduced in spleens from castrated animals (Fig. 2b). This was most remarkable for the $CD4^+$ $CD3^+$ subset, which constituted 16·1 ± 1·2% of castrated spleen cells compared to 24·1 ± 0·9% of normal spleen cells ($P \leq 0.001$). The sum of the $CD4^+$ $CD3^+$ and $CD8^+$ $CD3^+$ subsets, representing the total number of mature T lymphocytes in the spleen, was also reduced in castrated animals (31·59 ± 3·46% versus 53·8 ± 3·5% in controls; $P \leq 0.001$).

Measurements of cell proliferation

Flow cytometric cell cycle analysis showed that 9·5 ± 0·4% of cells in thymuses from castrated animals were in phases G_2, S, or M of the cell cycle, compared to 8·2 ± 0·2% in these phases in intact animals ($P = 0.036$). In spleens, relative numbers of cells in active phases of the cell cycle were also higher in castrated mice than in intact controls (2·0 ± 0·2% versus 1·4 ± 0·1%; $P = 0.019$). Unstimulated [^{3}H]thymidine uptake by spleen cells in these experiments was significantly higher in the castrated group than in intact controls (2390 ± 330 c.p.m. versus 980 ± 95 c.p.m.; $P = 0.015$), consistent with the cell cycle data.

Table 1. Phenotypic profiles of thymocytes from intact and long-term castrate C57Bl/6 mice determined by flow cytometry

Phenotype	Intact* (%)	Castrate* (%)	P†
$CD8^+$ $CD4^-$	2·7 ± 0·2‡	1·8 ± 0·2	0·008
$CD8^+$ $CD4^+$	81·3 ± 1·7	84·2 ± 1·0	0·174
$CD8^-$ CD4	4·9 ± 0·8	4·9 ± 0·9	>0·3
$CD8^-$ $CD4^+$	11·1 ± 1·0	9·0 ± 0·4	0·071
$CD3^+$	20·4 ± 1·6	15·9 ± 1·0	0·041

*$n = 5–7$ animals.
†P values compare intact and castrate groups by Student's t-test.
‡Means ± SEM.

Production of IL-2 and IFN-γ by spleen cells

Splenic T lymphocytes from castrated animals produced significantly more IL-2 in response to Con A than lymphocytes from intact controls (36·5 ± 5·4 U/ml versus 23·4 ± 4·2 U/ml; $P = 0.026$; Fig. 3). Spleen cell cultures stimulated with the combination of Con A and PMA produced similar levels of IL-2 for castrated animals and controls (54·8 ± 5·1 U/ml versus 50·1 ± 3·6 U/ml; $P = 0.20$). Levels of Con A-induced IFN-γ in excess of 5 U/ml (the limit of detectability in the assay) were

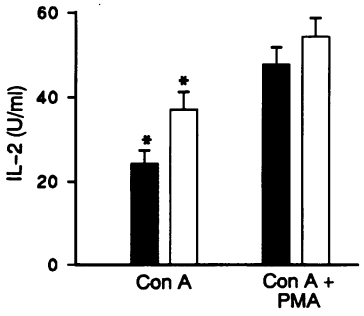

Figure 3. Mean ± SEM levels of IL-2 measured by ELISA in spleen cell supernatants from control (■) and castrate (□) mice. Unseparated splenocytes were cultured with either Con A (2 μg/ml) or con A + PMA (10 ng/ml) for 20–24 hr: $P = 0.03$; $n = 6–7$ animals.

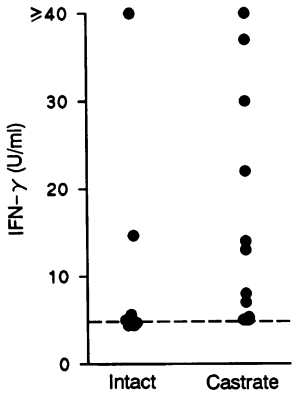

Figure 4. Levels of IFN-γ measured by ELISA in supernatants from Con A-stimulated unseparated spleen cells from intact and castrate mice. Results from individual animals are shown. Numbers of animals in the two groups with significant IFN-γ levels (> 5 U/ml, dashed line) were compared using Fisher's exact text, with a P-value of 0·036.

measured in only two of 11 intact animals compared to eight of 12 castrated mice (Fig. 4; $P = 0{\cdot}036$). These differences are more remarkable when the relative number of splenic T cells, which was lower in castrates, was considered. Using this adjustment, castrate T cells can be estimated to produce 2·6 times more IL-2 than controls; for IFN-γ, the calculated increase was 3·7-fold. No significant IL-4 production was measured in cultures stimulated with Con A or Con A/PMA for time periods ranging from 12 to 48 hr.

Antibody and autoantibody synthesis by spleen cells

IgM was the predominant immunoglobulin secreted into spleen cell supernatants. Levels of total IgM produced were generally lower in cultures of spleen cells from castrated animals compared to controls (Table 2). Nevertheless, levels of anti-IgG (rheumatoid factor) and anti-thyroglobulin were significantly higher in PWM-stimulated cultures from castrated animals than in controls ($P \leq 0{\cdot}026$; Table 3). Almost all of the rheumatoid factor and anti-thyroglobulin produced was IgM, with little or no IgG detected (data not shown). Considering levels of autoantibodies relative to total IgM in the same culture, a significantly higher proportion of LPS-induced anti-IgG was produced in cultures from castrated mice compared to controls (0·013% versus 0·004%; $P = 0{\cdot}021$). For anti-thyroglobulin, the proportion was also higher in castrated animals, at a borderline level of significance ($P = 0{\cdot}05$).

Table 2. Antibody synthesis by spleen cells from intact and long-term castrate mice

Specificity	Stimulus	Intact mice* (ng/ml)	Castrate mice* (ng/ml)	P†
IgG	None	42 ± 13‡	53 ± 14	> 0·3
	LPS	390 ± 75	487 ± 69	> 0·3
	PWM	163 ± 24	106 ± 9	0·067
IgM	None	2014 ± 911	363 ± 72	0·062
	LPS	18 353 ± 3952	9363 ± 2198	0·045
	PWM	4616 ± 2514	1650 ± 585	> 0·3
IgA	None	184 ± 31	245 ± 34	0·174
	LPS	205 ± 18	234 ± 18	0·109
	PWM	533 ± 204	194 ± 207	0·139

*n = 5–14 animals.
†P values calculated by Student's t-test.
‡Means ± SEM.

Table 3. Autoantibody synthesis by spleen cells from intact and long-term castrate mice

Specificity	Stimulus	Intact mice*	Castrate mice*	P†
Anti-IgG	None	0·187 ± 0·017‡	0·185 ± 0·026	> 0·3
	LPS	0·575 ± 0·037	0·610 ± 0·058	> 0·3
	PWM	0·253 ± 0·025	0·328 ± 0·034	0·026
Anti-TgB§	None	0·162 ± 0·013	0·173 ± 0·024	> 0·3
	LPS	0·484 ± 0·030	0·508 ± 0·049	> 0·3
	PWM	0·219 ± 0·023	0·292 ± 0·026	0·013
Anti-DNA	None	0·178 ± 0·014	0·204 ± 0·035	0·30
	LPS	0·625 ± 0·040	0·576 ± 0·046	0·206
	PWM	0·309 ± 0·035	0·355 ± 0·049	> 0·3

*n = 5–14 animals.
†P values calculated by Student's t-test.
‡Mean net OD value ± SEM.
§Tgb, thyroglobulin.

DISCUSSION

These studies indicate that androgen deprivation has significant effects on the peripheral immune system, including enhanced production of IL-2 and IFN-γ by peripheral T cells, expansion of the peripheral B-cell compartment and increased capacity for production of autoreactive immunoglobulins.

Spleen cells from female mice produce higher levels of IL-2 and IFN-γ *in vitro* in response to Con A or anti-CD3 than cells from age-matched males.[10,11] Both cytokines are produced by the Th1 subset of helper T cells.[12] In our current experiments, relative numbers of mature T cells were decreased in the spleens of castrated animals, suggesting that increased IL-2 and IFN-γ production was not the consequence of an expanded pool of helper cells. Oestrogen has been shown to up-regulate the IFN-γ promoter in lymphoid cells, a response which was shown to be dependent upon the presence of the oestrogen receptor.[13] Whether androgens exert negative direct effects on the transcriptional activity of the IFN-γ gene in peripheral T cells is unknown. Studies of androgen receptor expression in murine spleen cells which are currently in progress may clarify whether the effects of androgens are exerted directly on spleen cells or by indirect effects through the thymus.

Castration of males has been shown to result in a relative increase in splenic B cells in previous studies of normal and autoimmune mice. In non-obese diabetic (NOD) mice, castration at either 3 or 8 weeks of age resulted in a significant increase in absolute numbers of splenic B cells measured 2 weeks later.[14] Similarly, spleens from normal C57Bl/6 male

mice, which were castrated at 4 weeks of age and studied 3 months later, contained a mean of 59 ± 1% B cells, compared to 50 ± 2% in sham-operated controls ($P < 0{\cdot}05$).[15] However, relative numbers of $CD5^+$ B cells were not different in these animals, as also noted in this study. Although the $CD5^+$ B-cell subset has been implicated as the source of autoantibody production in murine and human systems,[16,17] other studies suggest that $CD5^-$ B cells may also produce autoreactive immunoglobulins.[18] Data from the present study support this possibility. The finding that B-cell expansion may occur with androgen withdrawal is of interest in view of other data suggesting that cytokines that stimulate B-cell growth and differentiation are produced in androgen-suppressible murine lupus-like syndromes.[19]

Oestrogen treatment of castrated male mice, including those of the C57Bl/6 strain, has been shown to augment production of autoantibodies.[15,20] However, in these studies, castration alone did not appear to have an effect on autoantibody levels, which contrasts with the results obtained in this study. Differences in approach may account for these disparate results. Other studies have examined spontaneously arising autoantibodies which were present in serum[20] or antibodies produced by spleen cells in response to *in vitro* stimulation with bromelain-treated mouse erythrocytes.[15] In the present study, only PWM stimulation was associated with a significant difference in the absolute levels of autoantibodies produced. Although the mechanism of action of PWM in murine systems is not completely understood, it is likely that T cells are required for optimal B-cell responses to occur, as in human systems.[21] In contrast, LPS induces terminal B-cell differentiation and antibody production in the absence of T-cell factors.[22] The results from castrated animals therefore suggest that the observed differences in autoantibody production are attributable to B cells which are dependent upon T cells or T-cell-derived factors for terminal differentiation. These findings are consistent with other data indicating that effects of gonadal steroids on B cells are dependent upon T-cell factors *in vitro* and *in vivo*.[23,24]

While primary triggers of the effects of castration on the immune system remain obscure, two testable hypotheses can be derived from the present data. First, B-cell expansion *in vivo* may be owing to the relative lack of normal mechanisms of T-cell control. Alternatively, enhanced T-cell activation may drive B-cell proliferation and differentiation. The first of these possibilities would be expected to result in increased production of polyreactive, or 'natural' autoantibodies, with characteristics of the neonatal repertoire, while the second would predict a more mature repertoire of specific, high-affinity antibodies. Further analyses of the cellular and molecular characteristics of the expanded B-cell population and associated autoantibodies may be useful in differentiating between these possibilities.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of M. Turney and K. Reese. Dr G. Stelzer (Cytometry Associates, Brentwood TN) provided helpful support for flow cytometric studies. This work was supported in part by grants from the National Institutes of Health (DK 41053 to N.J.O.) and the United States Department of Veterans Affairs (to W.J.K.). S.M.V. was the recipient of an NIH postdoctoral fellowship under training grant HD07043 and S.S. was supported by the Vanderbilt University Diabetes Research and Training Center (DRTC) Summer Student Program. The Hormone Assay Core Lab of the Vanderbilt University Center for Reproductive Biology Research (HD05797) provided immunoassays for androgens.

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