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MODULATION OF IMMUNE RESPONSES BY ANABOLIC ANDROGENIC STEROIDS

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Abstract — Anabolic androgenic steroids (AS) have recently been placed on the Food and Drug Administration's (FDA's) list of controlled substances, because of the adverse effects seen in athletes taking accelerated dosages in attempts to enhance performance. Reported deleterious effects on abusers include sterility, gynecomastia in males, acne, balding, psychological changes, and increased risks of heart disease and liver neoplasia. Considering the roles of the immune and neuroendocrine systems and their interactions in many of these pathologies, it is important to determine the effects of these derivitized androgens on this connection. Little is known in this respect. We therefore determined the effects of anabolic steroids on certain immune responses and their effects on the extrapituitary production of corticotropin by lymphocytes. We present evidence that (1) both 17-β and 17-α esterified AS, nandrolone decanoate and oxymethenelone, respectively, significantly inhibited production of antibody to sheep red blood cells in a murine abuse model; (2) the control androgens testosterone and dehydroepian-drosterone (DHEA) or sesame seed oil vehicle had no significant effects on antibody production; (3) nandrolone decanoate and oxymethenelone directly induced the production of the inflammatory cytokines IL-1β and TNF-α from human peripheral blood lymphocytes but had no effect on IL-2 or IL-10 production; (4) control androgens had no direct cytokine inducing effect; (5) nandrolone decanoate significantly inhibited IFN production in human WISH and murine L-929 cells; and (6) nandrolone decanoate significantly inhibited the production of corticotropin in human peripheral blood lymphocytes following viral infection. These data indicate that high doses of anabolic steroids can have significant effects on immune responses and extrapituitary production of corticotropin. Furthermore, the mouse model should provide an effective means by which to study other deleterious effects of anabolic steroid abuse in humans.

Keywords: androgens, anabolic steroids, immune system, neuroendocrine system.

Anabolic androgenic steroids (AS) are derivatives of testosterone (Murad & Haynes, 1980). Over 40 AS are available worldwide in both oral and injectable forms. Conventionally, AS have been used in treating bone marrow failure, hypogonadal states, hereditary angio-neurotic edema, renal disease and anemia, and in the late stages of breast cancer (Wilson & Griffin, 1982). Anabolic androgenic steroids have recently been placed on the FDA's list of controlled substances because of the adverse effects seen in athletes taking accelerated dosages in attempts to enhance perform-ance (Wilson, 1988). Some of the most popular oral forms include methandrostenolone and methyltestos-terone, while the most popular injectables include nandrolone and methenelone (Goldman et al., 1984). Known side-effects of AS abuse include testicular atrophy, sterility, gynecomastia, acne, folliculitis, enhanced erythropoiesis and increased risk of cardio-vascular disease. Investigators have reported major depression and psychosis as side-effects. In addition, increased aggressiveness and irritability are common. The potential for physical and psychological depend-ence has been observed. Additionally, AS abuse has been associated with peliosis hepatitis, hepatocellular carcinoma, hepatic angiosarcoma and Wilm's tumor (Strauss & Yesalis, 1991; Zimmerman, 1978; Ishak, 1979; Friedl, 1989). AIDS and hepatitis infections have also been reported in athletes who have shared needles when injecting AS (Yesalis et al., 1989).

While the effects of normal gonadal steroids on certain immune responses have been studied (Gross-man & Reselle, 1986; Grossman, 1984, 1985, 1989;

Grossman *et al.*, 1979; Sasson & Mayer, 1981; Sthoeger *et al.*, 1988; Ansar *et al.*, 1985; Coulson *et al.*, 1982), the effects of high doses of AS on immune system responses are unclear (Calabrese, 1989; Mendenhall, 1990). Considering this, we attempted to study more definitively their effects by using a chronic abuse model in a murine system and by determining their effects on inflammatory cytokine levels produced by human peripheral blood leukocytes *in vitro*. Here, we report that mice receiving AS in doses approximating those chronically abused in humans are significantly inhibited in generating antibody responses to sheep red blood cells. Both the 17-α and 17-β esterified forms of AS had similar effects. We also find that AS can directly induce the production of the inflammatory cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) in human peripheral blood lymphocytes (HPBLs) *in vitro*. Anabolic steroids also significantly inhibit interferon (IFN) production in both human and murine cell lines, and significantly inhibit the production of corticotropin by HPBLs following viral infection. These studies also indicate the potential utility of the murine model for the study of chronic AS abuse in humans.

EXPERIMENTAL PROCEDURES

Mice

Outbred Swiss ICR mice, 6–8 weeks of age, obtained from Harlan–Sprague–Dawley (Madison, WI), were used in these studies. Mice were allowed to acclimate for 1 week prior to use in experiments.

Preparation and use of human peripheral blood lymphocytes (HPBLs)

Buffy coats were obtained from the University of Texas Medical Branch Blood Bank. HPBLs were enriched for by centrifugation through Histopaque (Sigma Chemicals, St Louis, MO, U.S.A.) according to the manufacturer's instructions. For experimental purposes, cells in 24-well tissue culture plates (5×10^6 /well) were incubated in RPMI medium (Gibco/BRL, Bethesda, MD, U.S.A.) supplemented with antibiotics (penicillin/streptomycin, 100 U and 100 µg/ml, respectively) and 10% fetal bovine serum in a 4% CO_2, 37°C, 100% humidity atmosphere. Anabolic steroids, control steroids and other substances, at the required concentrations, were added directly to the cultures. Supernatant fluids were harvested 24–72 h following initiation of experimental procedures.

Anabolic steroid abuse model and in vitro sheep red blood cell antibody plaque-forming cell assay (SRBC-PFC)

To mimic anabolic steroid abuse in humans, mice (4–8/group) received 250 µg of nandrolone decanoate (Schein Pharmaceuticals, Port Washington, NY, U.S.A.) or oxymethenelone (Sigma Chemicals, St Louis, MO, U.S.A.) intraperitoneally (i.p.) every other day. This dose is roughly equivalent to the sustained levels obtained from a daily 200 mg dose in a 91 kg (200 lb) human, considering that an average mouse weighs 30 g during the experimental period. Control mice received the same dose of testosterone (Upjohn, Kalamazoo, MI, U.S.A.) or sesame oil (Sigma Chemicals), which is the vehicle in which some anabolic steroids are commonly suspended. Injections were done every other day for 10 days before and 6 days after i.p. administration of sheep red blood cells in 50% Alsever's solution (10^8–10^9 in 0.5 ml; University of Texas MD Anderson Cancer Center, Bastrop, TX, U.S.A.). Mice were then killed and splenocytes taken for *in vitro* SRBC-PFC on glass microscope slides as described elsewhere (Gronowicz *et al.*, 1976). Briefly, each spleen was dissociated and then resuspended in 1 ml of media from which 40 µl, 20 µl or 10 µl of spleen cells for each group of mice in duplicate were mixed with the indicator SRBC in agarose. Guinea-pig complement was then added and incubated for 2 h at 37°C and the resultant hemolytic plaques were counted after 24 h of incubation at 4°C. Viability counts were performed on all spleen cell preparations prior to use to ensure that an equivalent number of viable cells was used in each assay.

Enzyme-linked immunosorbent assays (ELISAs) for human IL-1 and TNF-α

ELISAs for IL-1β and TNF-α were performed on supernatant fluids from HPBL cultures by a four-stage procedure in microtiter plates coated with antibody specific for either IL-1β or TNF-α. Experimental or control solutions were placed in the plate wells and incubated according to the manufacturer's instructions (Genzyme, Cambridge, MA, U.S.A.). Plates were washed rigorously between each step of the assay. Optical densities of the indicator solutions were determined by automated spectroscopy at 492 nm. All values were adjusted for non-specific binding.

Interferon assays

Interferon assays were performed by a slightly modified microplaque assay as described elsewhere on

Table 1. Nandrolone decanoate inhibits the antibody plaque-forming cell response to SRBCs in an anabolic steroid abuse murine model

Treatment	Plaque count	% Inhibition from control
Control (sesame oil)	86 ± 15	—
Testosterone	78 ± 37	9.3[ns]
Nandrolone decanoate	9.6 ± 2.5	88.3*

Female outbred ICR mice were treated with sesame oil (equivalent dilution as nandrolone decanoate), testosterone, or nandrolone decanoate and administered SRBCs for determination of specific antibody production as described in Experimental Procedures. Representative of three experiments. [ns]Not significant ($P = 0.64$; by paired t-test). *$P = 0.00012$.

Table 2. Additive effects of 17-α (oxymethenolone) and 17-β (nandrolone decanoate) esterified androgens on the antibody plaque-forming cell response to SRBCs in an anabolic steroid abuse murine model

Treatment	Plaque count	% Inhibition from control
Control (sesame oil)	76.4 ± 8.1	—
Testosterone	80.5 ± 8.0	+6[ns]
Nandrolone decanoate	56.5 ± 13	26*
Oxymethenolone	49.5 ± 10	35'
Nandrolone/Oxymethenolone	41.8 ± 3.0	45[‡]

Experiments were performed as described in Table 1. [ns]Not significant ($P = 0.34$; by paired t-test). '$P = 0.029$; [†]$P = 0.005$; [‡]$P = 0.0006$.

mouse L-929 or human WISH cells (Campbell et al., 1975).

Immunofluorescence staining

Twenty-four to forty-eight hours following treatment with medium, Newcastle disease virus, and/or nandrolone decanoate, PBLs were fixed to glass cover slips with 100% ethanol, and following rehydration, treated with polyvalent rabbit anti-ACTH (1–13). After washing in PBS, fluorescene isothiocyanate conjugated goat anti-rabbit immunoglobulin was applied, and the cells were scored for immunofluorescence by ultraviolet light microscopy.

RESULTS

These studies were designed to determine the effects of anabolic steroid abuse on certain immune responses. We initially determined their effects on antibody formation utilizing a mouse model. In three separate experiments, mice were given 250 mg nandrolone decanoate (AS esterified at the 17-β position), oxymethenolone (AS esterified at the 17-α position), testosterone, or sesame seed oil vehicle every other day for 10 days prior to and 6 days following SRBC. As shown in Table 1, nandrolone decanoate significantly inhibited the SRBC-PFC response, compared with the control. Testosterone had a minor effect; however, this was not significantly different from the control. Table 2 indicates that oxymethenolone also significantly inhibited the SRBC-PFC response, and shows that nandrolone decanoate together with oxymethenelone resulted in a 45% additive inhibition.

We next determined the direct effects of AS on the production of the inflammatory cytokines IL-1β and TNF-α in HPBL cultures in vitro. Thus, HPBLs were treated with nandrolone decanoate, oxymethenolone, the control androgens testosterone and dehydroepiandrosterone (DHEA), or sesame oil for periods of up to 72 h, following which the supernatant fluids were tested by ELISA for IL-1β or TNF-α. Table 3 shows that both of the AS, but not the control androgens or sesame oil, induced the production of IL-1β and TNF-α. Induction occurred in a dose-dependent fashion with the maximum levels detected 24 h following exposure to the AS. In addition, AS had no effect on induction of these cytokines by the conventional inducer, lipopolysaccharide (LPS). Nandrolone decanoate had no effect on the levels of two other cytokines tested; IL-2 induced by staphylococcal enterotoxin A, and IL-10 induced by LPS (data not shown).

We also tested whether nandrolone decanoate could affect another early non-specific immune response, the production of interferon in response to viral infection. To perform these studies, mouse L-929 or human WISH cells were treated with nandrolone decanoate prior to infection with Newcastle virus (NDV), a known inducer of IFN in these cells. Supernatant fluids were tested for IFN levels 24 h later. As shown in Table 4, nandrolone decanoate significantly inhibited the production of IFN by mouse L-929 and human WISH cells in response to NDV infection.

Finally, the effects of AS on lymphocyte production of ACTH were determined. PBLs were infected with NDV with or without nandrolone decanoate and scored by immunofluorescence for ACTH levels. Table 5 shows that while there was a 33% increase in PBL derived ACTH following infection with NDV, those treated with 20 μg/ml of nandrolone decanoate had only a 14% expression. Thus, it appears that AS significantly inhibited the production of ACTH by PBLs following viral infection.

Table 3. Anabolic steroids (nandrolone decanoate and oxymethenolone) directly induce the production of inflammatory cytokines (IL-1β and TNF-α) in human peripheral blood leukocyte cultures

| | Experiment 1 | | Experiment 2 |
| Treatment | IL-1β   TNF-α | | IL-1β (pg/ml)‡ |
	(pg/ml)*†		
Control (sesame oil)	<20	125	<20
Testosterone	<20	157	—
DHEA	<20	70	—
Nandrolone decanoate	145	>1000	90
Oxymethenolone			160
LPS			915
LPS + nandrolone decanoate			700
LPS + oxymethenolone			660

HPBLs were obtained as described in Experimental Procedures. One million cells/well in microtiter plates were cultured in the presence of the indicated reagents (androgens at 3–10 µg/ml). Twenty-four hours later supernatant fluids were harvested and subjected to ELISA for IL-1β and TNF-α. Representative of three experiments. DHEA, Dehydroepiandrosterone; LPS, lipopolysaccharide (0.1 µg/ml).
*Maximum S.D. IL-1β 10 pg/ml; †maximum S.D. TNF-α 20 pg/ml; ‡maximum S.D. IL-1β 8.2 pg/ml.

Table 4. Nandrolone decanoate inhibits interferon production in NDV-infected mouse L-929 and human WISH cells

Treatment	Cell line	IFN (U/ml)	% Inhibition VC	% Inhibition IFN
CC	WISH	<3	—	—
VC	WISH	—	—	—
NDV	WISH	3000	0	—
Nandrolone decanoate (10 µg/ml)	WISH	<3	100	0
NDV/Nandrolone decanoate	WISH	500	100	83
CC	L-929	<3	—	—
VC	L-929	—	—	—
Nandrolone decanoate	L-929	<3	0	—
NDV	L-929	300	100	0
NDV/Nandrolone decanoate	L-929	75	100	75

Mouse L-929 or human WISH cells were treated with the indicated reagents and/or NDV for 24 h following which supernatant fluids were harvested and assayed for IFN. Representative of two experiments. CC, Cell control; VC, virus control; NDV, Newcastle virus. A three-fold or greater reduction in IFN titer represents a significant difference.

DISCUSSION

These studies were performed to determine the effects of anabolic steroids, approximating those levels used during abuse in humans, on certain immune responses. We have shown that (1) both 17-β and 17-α esterified AS, nandrolone decanoate and oxymethenelone, respectively, significantly inhibited production of antibody to SRBCs in a murine abuse model; (2) the control androgens testosterone and DHEA or sesame seed oil vehicle had no significant effects on antibody production; (3) nandrolone decanoate and oxymethene-lone directly induced the production of the inflammatory cytokines IL-1β and TNF-α but had no effect on IL-2 or IL-10 production; (4) control androgens had no direct cytokine inducing effect; (5) nandrolone decanoate significantly inhibited IFN production in human WISH and L-929 cells; and (6) nandrolone decanoate significantly inhibited the production of corticotropin by human peripheral blood lymphocytes following viral infection. Our studies also indicate that the murine model we describe may provide an effective means by which to study anabolic steroid abuse in humans.

Previous studies provided conflicting results as to

Table 5. Nandrolone decanoate inhibits ACTH production by NDV-infected HPBLs

Treatment*	% Fluorescing cells/field[†]	$X \pm$ S.D.[‡]	% Decrease from NDV-infected cells
None	6.7, 8.5, 7.1, 0, 9.6	6.4% ± 3.8	80.5%
NDV[§]	38.7, 34.3, 31.3, 26.7, 33.3	32.9% ± 4.4	—
Nandrolone decanoate (20 μg/ml)[‖]	4.5, 6.7, 3.3, 7.4, 0	4.4% ± 3.0	86.6%
NDV/Nandrolone decanoate[*]	15.6, 11.5, 14.3, 16.7, 13.8	14.4% ± 2.0	56.2%

*PBLs were infected with 100 hemagglutinating units/ml of NDV. Twenty-four hours later, the cells were stained for ACTH specific immunofluorescence as described in Experimental Procedures.
[†]Each microscopic field represents approximately 50 cells.
[‡]Average ± standard deviation.
[§]$P=0.0001$; [‖]$P=0.4$; and [*]$P=0.003$ by ANOVA when compared to control.

the effects of AS on immune system responses. One early report indicated that AS enhanced antibody responses and resistance to infection. In contrast, there have been reports on reductions in both humoral and cell-mediated responses in both animals and humans (Calabrese et al., 1989; Mendenhall et al., 1990). Considering our results, the parameters of modulation of this system by AS remain to be completely determined. Little has been published on the impact of AS on the actual incidence and prevalence of disease in users. However, considering the side-effects described above, it would appear that the immune system is playing a substantial role.

An abundance of data has accumulated which strongly suggests that a linkage exists between the immune and neuroendocrine systems (Blalock, 1985; Blalock & Smith, 1985; Weigent & Blalock, 1987; Smith, 1992; Hughes & Chin, 1994). Many of the reports demonstrate that both systems, in communicating with each other, produce and respond to similar signal molecules, i.e. cytokines and neuropeptides. The data also suggest that there is an interplay between these substances in the autoregulatory process of the immune system. More specifically, neuropeptides have been shown to affect immune responses through their influence on cytokine production and action. Conversely, cytokines are known to induce or influence the induction of peptidergic messenger substances such as hypothalamic hormones (Hughes & Chin, 1994). From our studies it appears that AS may affect both arms of these pathways by inducing and/or possibly altering production of these messenger substances. It remains to be determined, however, whether responses of the immune and neuroendocrine system are altered, perhaps owing to down-regulation or up-regulation of specific receptors. In addition to the peptide hormones and cytokines, it is possible that AS might also modulate the immunomodulatory and antiviral activities of endogenous androgens such as dehydroepiandrosterone. This adrenal androgen has been shown to have

numerous immunomodulatory activities (Blauer et al., 1991; Daynes et al., 1990; Lucas et al., 1985; Schwartz, 1985; Weindruch et al., 1984; Merrill et al., 1989), including enhancement of interleukin-2 production, prevention of autoantibody production, and inhibition of virus infection and cancer. The proposed mechanism for DHEA's antagonism has been attributed to its ability to reverse the immunosuppressive effects of glucocorticoids, especially cortisol (Blauer et al., 1991). The molecular mechanism(s) of this effect is presently unknown; however, DHEA and cortisol do not compete for a similar cytosolic receptor (Blauer et al., 1991; Kalimi & Regelson, 1988). In contrast, AS and cortisol do compete for receptors (Snochowski et al., 1981). However, both DHEA and androgens such as AS modulate the immunosuppressive effects of cortisol, suggesting that interactions between DHEA and AS and their immunoregulatory activities should be investigated.

Overall, given the known effects of AS abuse, it is easy to think that they could play a major role in disrupting the immune and neuroendocrine network that we have described. Indeed, at the supraphysiologic concentrations at which the AS are commonly used during abuse, feedback mechanisms that affect the hypothalamus and anterior pituitary gland could virtually abolish such hormones as ACTH, luteinizing hormone (LH) and follicle stimulating hormone (FSH), among others, from the circulation (Rogol & Yesalis, 1992). In addition, AS can block and even reverse the catabolic effects of glucocorticoids at the level of the glucocorticoid receptor. Glucocorticoids down-regulate the immune system (Munck et al., 1984). In addition, certain levels of AS may be immunoenhancing; however, the effects of long-term use of high levels of AS on this system remain to be elucidated. Overall, there appears to be a mechanism by which AS may modulate the extra-hypothalamic–pituitary production and action of hormones such as those produced by the immune system and those of the adrenal glands.

Likewise, we have shown that a mechanism may exist by which AS could affect production of antibodies and cytokines. In summary, as indicated by these studies, it will be important to ascertain further the effects of AS abuse on immune system activities. It is also apparent that these studies will have to be extended into their effects on the immune and neuroendocrine connection.

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