J. Steroid Biochem. Molec. Biol. Vol. 37, No. 1, pp. 71–76, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

ANABOLIC STEROID EFFECTS ON IMMUNE FUNCTION: DIFFERENCES BETWEEN ANALOGUES

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(Received 6 February 1990)

Summary—As an untoward effect of chronic anabolic steroid use, immunologic alterations may be induced. To evaluate this possibility five commercially available steroids with various types of structural differences were studied in male Sprague–Dawley rats. Animals were divided into five groups and treated with testosterone (Group 1), testosterone propionate (Group 2), testolactone (Group 3), oxandrolone (Group 4), and stanozolol (Group 5). Androgenic anabolic steroids were administered daily, subcutaneously dissolved in oil, at a dose of 1.1 mg/kg. Immune alterations were assessed by skin-test reponses to phytohemagglutinin. After five days of treatment (1.1 mg/kg/day) a significant immuno-suppression was observed with all groups. However, by day 10, groups 3, 4, and 5 showed an immuno-stimulation. Using oxandrolone as the model stimulant, serum testosterone levels were significantly suppressed, while castration abolished the stimulatory effect. These observations indicate that immune alterations do occur with anabolic steroids which are immuno-suppressive when the steroid nucleus is intact and immuno-stimulatory with nuclear alterations. It appears that these changes are associated with altered gonadal testosterone release.

INTRODUCTION

Due to their considerable anabolic activity, the gonadal steroid hormones, testosterone and analogues, have come into increased use and abuse in athletics to attain maximal muscle mass and strength [1, 2]. Clinically, they have been used to stimulate anabolism in a variety of medical conditions where the reversal of catabolism is desired [3], such as in renal failure, aplastic and refractory anemia, post-traumatic or surgical catabolic states, and severe alcoholic hepatitis [4].

One of the biological actions of gonadal steroid hormones is the modulation of the immune system through down-regulation of T-lymphocyte activities [5]. Testosterone, directly, or indirectly through conversion to estradiol, has been shown to participate in the process. The clinical use or athletic abuse of testosterone or testosterone analogues could therefore have significant consequences on immune function. Until now, however, the differences in the biological effect on the immune function of the different anabolic compounds have not been established. We report here our observations with testosterone and four of its derivatives.

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METHODS

Male Sprague–Dawley rats (Taconic Farms, Germantown, N.Y., U.S.A.) were used in two sets of experiments to evaluate the androgenic anabolic steroid modulation of immune function. The initial experiment compared immune alterations [delayed cutaneous hypersensitivity (DCH) responses to phytohemagglutinin (PHA)] induced by testosterone and four of its analogues. Thirty animals (220–225 g) were randomly placed into five groups and individually housed in climate and light controlled rooms maintained on a 12-h light cycle (lights on from 7:00 to 19:00 hours) during the course of the experiments. Treatment groups received respectively: (1) Testosterone (Rugby Lab, Inc., Long Island, N.Y., U.S.A.), (2) Testosterone propionate (Geneva Generics, Broomfield, Col., U.S.A.) (3) Testolactone (Squibb, Princeton, N.J., U.S.A.), (4) Oxandrolone (G. D. Searle,

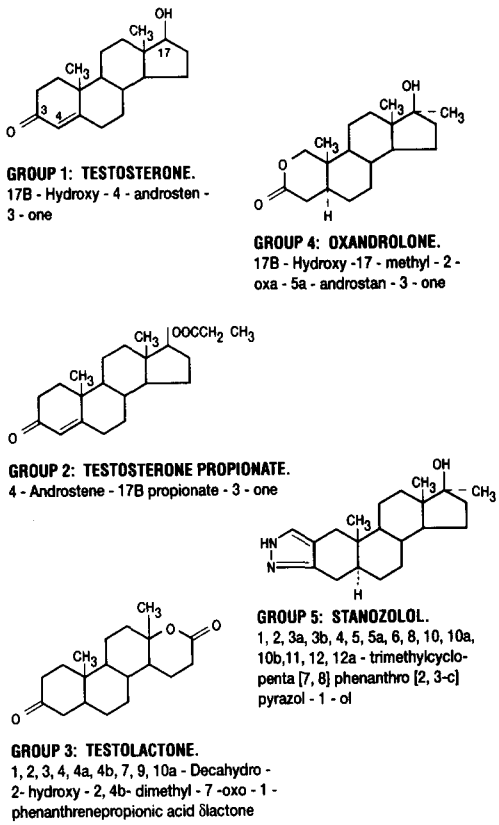

Fig. 1. Structures of the androgenic anabolic steroids used in the five treatment groups. All agents were dissolved in corn oil and administered intramuscularly, 1.1 mg/kg/day, into the nuchal area.

Skokie, Ill., U.S.A.), (5) Stanozolol (Sterling-Winthrop Research Institute, Rensselaer, N.Y. U.S.A.). Steroids were dissolved in corn oil and administered intramuscularly into the nuchal area, 1.1 mg/kg/day, for ten days. Since most of these are not naturally occurring compounds, we wished to mimic their pharmacologic effects at therapeutic levels. Therefore, doses were selected empirically similar to those used therapeutically in patients with malnutrition [6, 7], alcoholic liver disease [4, 8, 9], and aplastic anemia [10], and exceeding that required for replacement therapy in pituitary gonadal insufficiency [11].

All of the compounds used in this study are available commercially for specific therapeutic indications. They were generously donated for research purposes in a pure form by the respective manufacturers. Several are among those more widely abused by athletes (i.e. stanozolol and oxandrolone [2, 12]). For the purposes of this study, these analogues were selected because they offered a spectrum of compounds with a diversity of structural changes (Fig. 1). Groups 1 and 2 represent an intact steroid nucleus, with Group 2 containing a propionate ester at the 17 carbon of the D-ring. Groups 3, 4, and 5 have significant alterations in the steroid nucleus; in Group 3 the D-ring contains an oxygen substituted at the 17 position, forming a lactone bridge; in Group 4 the A-ring is substituted at the 2 position with a similar oxygen bridge and in Group 5 a pyrazol ring is attached at the 2, 3 position of the A-ring.

Immune function was assessed as described previously [13], using DCH responses to intradermal PHA, 0.5 mg/0.1 ml. These prior studies established that the intradermal injection of PHA produces an area of induration which histologically represents a diffuse predominantly lymphocytic infiltration involving the entire skin thickness and which is consistent with a delayed cutaneous hypersensitivity (Type 4) response [4]. These studies confirmed that vehicle alone produces no reaction but combined with PHA-M produces a response which is dose-dependent and linear with 0.5 mg/0.1 ml producing a measurable area of induration $\geqslant 20\ mm^2$, in 90% of the animals but without tissue necrosis. Area of induration measured at 4 h intervals, was observed to peak at 24 h and was progressively diminished in size by 48 and 72 h [13]. Hence, the recorded responses for this study represent peak areas of induration (24 h). This technique permits longitudinal testing in the same animal, offers good reproducibility and correlates well with other *in vitro* and *in vivo* measures of immune status [5,15]. Sequential measurements were made over a 2 week baseline period prior to treatment and at 5 and 10 days following initiation of steroid treatment. Each animal

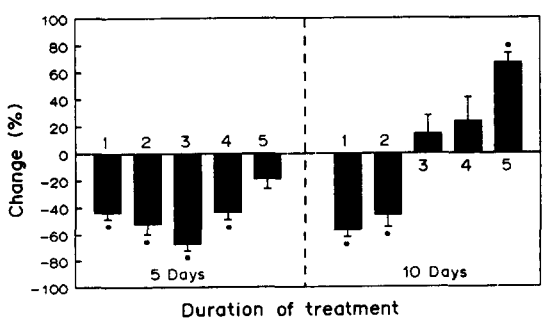

Fig. 2. DCH responses to intradermal PHA, 0.5 mg/0.1 ml. Response represents the area of induration (mm^2) that develops 24 h after injection. Responses were measured twice in the two weeks prior to treatment (pre-treatment) and at days 5 and 10 during treatment. 1 = testosterone, 2 = testosterone propionate, 3 = testolactone, 4 = oxandrolone, and 5 = stanozolol. Results are expressed as percent change from pre-treatment. *Treatments that were significantly different from pre-treatment ($P < 0.05$).

represented his own baseline control. Results are expressed as percentage change from baseline.

RESULTS

After treatment for 5 and 10 days, peak immune (DCH) responses in each of the treatment groups were compared to their pre-treatment levels by paired Student's t-tests.

Baseline immune responses prior to steroid treatments were not significantly different between groups (overall mean $\pm$ SEM; $47 \pm 2\ mm^2$). Treatments with androgenic anabolic steroids did not effect the development of the area of induration, (i.e. peak response was still seen at 24 h). However, by day 5 of steroid treatments a depression in the maximal (24 h) DCH response as compared to baseline was observed in all of the groups. Figure 2 illustrates the percentage change from baseline for each steroid: testolactone (Group 3) showed the greatest depression and stanozolol (Group 5) the smallest (depression ranging from 67 to 17% of baseline). Continued treatment, however, produced differences in responses. By day 10, treatment Groups 3, 4, and 5 (testolactone, oxandrolone and stanozolol, respectively) were all higher than baseline; while Groups 1 and 2 (testosterone and testosterone propionate) maintained their depressed response. None of the groups showed significant change in either body weight or fat stores as measured by skin fold thickness.

To test the effects of endogenous gonadal hormones on these responses a second experiment was then performed. Ten animals maintained in a similar manner to the initial experiment were either treated intact or were castrated and then treated for 8 days, with oxandrolone (1.1 mg/kg/day), testosterone (1.1 mg/kg/day) or oxandrolone combined with physiologic amounts of testosterone (15 μg/day). Oxandrolone was selected because it has the greatest anabolic activity of all testosterone analogues as compared to testosterone (androgenic/anabolic activity = 1:13) [16].

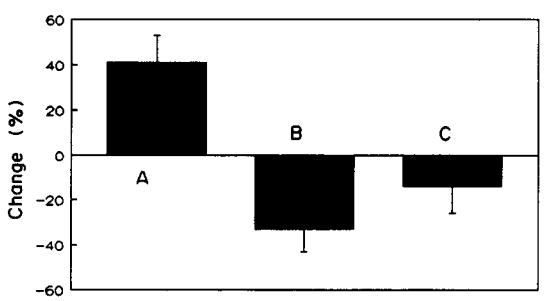

Fig. 3. Immune (DCH) responses after treatment (A), oxandrolone, 1.1 mg/kg/day, or (B) testosterone, 1.1 mg/kg/day or (C) combined treatment with (A) plus physiologic amounts of testosterone, 15 μg/day for 8 days in intact rats. DCH responses are expressed as percentage change from pre-treatment responses (baseline). (A) produced a significant increase in immune response ($P = 0.04$) while (B) produced a significant immuno-suppression ($P = 0.001$). (C) abolished the stimulatory effect of (A) and returned the responses to pre-treatment levels.

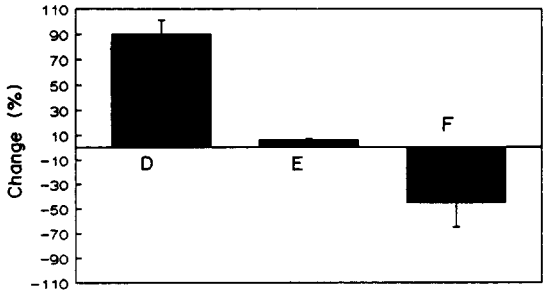

Fig. 4. DCH responses to intradermal PHA, 0.5 mg/0.1 ml after (D) surgical hypogonadism (castration) (E) surgical hypogonadism plus oxandrolone, 1.1 mg/kg/day, for 8 days; or (F) surgical hypogonadism plus replacement with physiological amounts of testosterone, 15 μg/day, plus oxandrolone, 1.1 mg/kg/day, for 8 days. DCH responses are expressed as percentage change from pre-treatment responses (baseline). Oxandrolone in surgically induced hypogonadal animals (Group E) is immuno-suppressive abolishing the immuno-stimulation of castration. The replacement of physiologic amounts of testosterone (Group F) produces an additive immuno-suppressive effect.

In the intact animals after 8 days of treatment with oxandrolone, serum testosterone levels were measured by RIA on tail vein blood. Levels were either undetectable or very low (mean $\pm$ SEM; 0.03 ± 0.02 ng/ml, normal range 5–10 ng/ml). Immune function (DCH responses) measured at the same time revealed a 41% increase over baseline (Fig. 3, response A). Testosterone produced the anticipated reduction (36%, response B). Further treatment for 8 days with oxandrolone combined with physiologic amounts of testosterone abolished this stimulation and returned the DCH responses to levels that were not significantly different from baseline (response C).

Results were different in the castrated animals. Castration, as previously reported [17] resulted in an increase in immune (DCH) responses (mean observed change 90% greater than intact (pre-castration) baseline; (Fig. 4, response D). Eight day administration of oxandrolone to these animals had an immunodepressive effect returning the DCH response to baseline (response E). Eight day treatment with oxandrolone combined with physiologic doses

of testosterone produced, in these castrated animals, an even greater suppression, 45% change from baseline (response F).

DISCUSSION

The PHA skin-test used here to assess immune function is similar in many respects to the Classical Type IV Delayed Hypersensitivity response [13] although in the PHA skin response it is unnecessary to pre-sensitize the subject with the antigen prior to introduction of the antigen into the skin site. This is because PHA is a mitogen and therefore the responding T-effector cells already possess constitutive PHA membrane receptors which do not require induction by antigen. Classical Type IV delayed hypersensitivity reactions involve stimulation of classes of helper T-lymphocytes by foreign substances called antigen [18]. The T-cells are then thought to stimulate other effector cells such as macrophages by release of lymphokines. This results in the release of factors from the macrophages localized at the site of the antigen injection in the skin. Specifically, with 12–24 h of the introduction of an antigen into a sensitized individual T-lymphocytes can be found in the peripheral vascular sites and this will disrupt the collagen bundles in the connective tissues of the dermis. T-helper (Th) cells (CD4) outnumber T-suppressor (Ts) cells (CD8) by a factor of 2:1 in this infiltrate along with Langerhans-like cells. By 24–48 h some Th-cells also are found in the epidermis along with-macrophages. The outcome is therefore a localized area which appears indurated. Since the DCH response is dependent on the function of Th-cells it must follow that factors that regulate the Th-cell function would be likely to effect the outcome of a DCH response. Furthermore, the presence of Ts-cells probably act as a limiting influence on the magnitude of the response. We have reported on a variety of factors that can modulate the action of Th and Ts-cells including androgenic steroids that may function both directly at the effector T-cell and indirectly through the release of factors such as thymic, adrenal and gonadal hormones from other sites [19–21].

While the mechanism of action of the androgen analogues on immune function is still not entirely defined, from previous studies by our groups as well as by others a variety of mechanisms can be hypothesized. Firstly, receptors that bind androgen have been reported to be present both in the reticuloendothelial (RE) cells of thymus [22–25] as well as in specific classes of T-lymphocytes [26–30]. Since the thymic androgen receptors may function to modulate the release of thymic hormones such as thymosin or thymulin from the RE cells [19–21], it is possible that binding of the analogue to the RE cell receptor could alter release of thymic factors that would effect the function of effector T-lymphocytes. This would in turn result in changes observed in the PHA skin-test which is a form of DCH response modulated by the T-effector cells in the skin. It is also conceivable that the analogue might act through competitive inhibition at the steroid receptor site to block binding of the true androgens (testosterone or dihydrotesterone) and thus alter the release of thymic factors. Secondly, such agonistic or antagonistic effects of androgen analogues might function directly at the effector T-lymphocyte and result in the observed changes on the DCH response as measured in the skin by PHA. Thirdly, androgens have been reported to block the effects of glucocorticoid

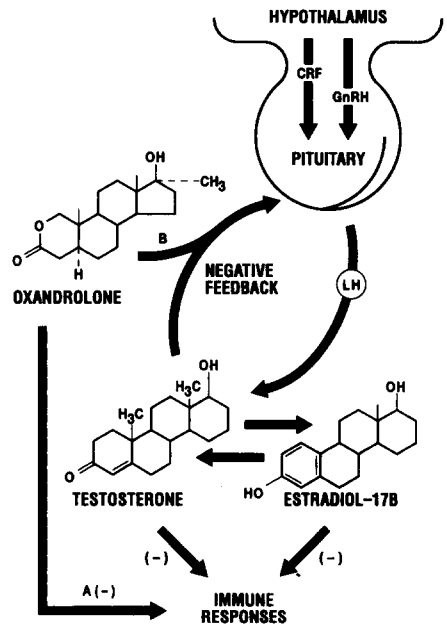

Fig. 5. Hypothesis to explain observed immune responses after oxandrolone treatment. Exogenous androgenic anabolic steroids produce two effects on immune function: (A) a direct early effect on immune function which is suppressive and, (B) an indirect delayed stimulatory effect mediated through the negative feedback on the pituitary. (B) results in inhibition of gonadal testosterone through diminished LH release. A decrease in the synthesis of testosterone is manifest by a low serum testosterone level and immune stimulation. Castration, by abolishing the modulation of testosterone secretion eliminates the effect of (B) but leaves (A) intact.

binding to androgen receptors on rat lymphocytes [26,31,32] and since glucocorticoids are potent immunological inhibitory agents it follows that blocking the glucocorticoid binding may derepress the effector T-lymphocyte and increase the PHA skin-test. To further complicate the possible outcome the stimulatory or inhibitory effects might be expressed either on T-helper cells or T-suppressor cells, or both. Therefore, depending on the net result, the outcome may be either stimulatory or inhibitory on the PHA skin test as was observed in this study (i.e. the depressors were testosterone and testosterone propionate, while stimulation was observed with testolactone, oxandrolone, and stanozolol). These differences in immunologic response appeared to be related to the integrity of the steroid nucleus. Analogues with an intact steroid nucleus exhibited a persistent immunosuppression effect while those with nuclear alterations elicited a delayed immuno-stimulatory effect.

Another possible mechanism for the diversity of responses is through altered hypothalamic–pituitary–gonadal function in the intact animal. Clerico and associates have reported [33] that anabolic steroids suppress serum levels of gonadotropins (FSH and LH) and testosterone. This would mimic a medical castration and would result in immune stimulation.

Figure 5 shows graphically a possible endocrinologic explanation for the observed changes. There appears to be two processes involved. One early effect is immuno-suppression seen at 5 days and manifested by all steroids. This presumably represents a direct modulation of the immune DCH response by the steroid itself at the Th, Ts, or receptor level.

The second effect occurs later and is observed only with certain analogues after 10 days of treatment. This could result from the negative feedback suppression by the analogs of the natural immuno-inhibitor, testosterone. This effect was seen only with analogues in which the steroid nucleus was altered so that the compound cannot be converted to testosterone (our treatment Groups 3, 4, and 5). In this situation in intact (non-castrate) animals, a net immunostimulation was seen which was abolished by the addition of physiological amounts of testosterone. In castrated animals, the modulation of testosterone secretion being abolished, the second stimulatory effect is no longer present. Only the early direct immuno-suppressive effect is observed.

These observations indicate that after anabolic steroid therapy differences in immune function do occur which must be considered when such therapy or abuse is present.

Acknowledgement—We wish to acknowledge the assistance given by Squibb in supplying testolactone; by G. D. Searle in supplying oxandrolone; and Sterling-Winthrop Research Institute in supplying stanozolol. We wish to thank Ms D. Day for manuscript preparations, Ms S. Rouster for technical assistance and Mr G. Schmitt for the graphic illustrations.

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