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Hormonal influence on the secretory immune system of the eye: androgen control of secretory component production by the rat exorbital gland

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Summary. Androgens are known to regulate the level of secretory component (SC) in tears of male rats. The purpose of the present study was to explore the underlying mechanism of this hormone action by (i) identifying the ocular tissue(s) involved in SC production; and (ii) determining whether androgens increase SC production by this tissue. We also examined whether androgen administration influenced the concentration of SC in tears of female rats. Ocular tissues from adult Sprague-Dawley rats were cultured in the presence or absence of cycloheximide in the incubation medium. Secretory component in the culture media was measured by an RIA which detects primarily free SC. Analysis of media obtained after incubation of exorbital (lacrimal) glands, 'lid' tissues, globes, and Harderian glands revealed that only exorbital glands released substantial amounts of SC. This exorbital gland production of SC, which was significantly greater in tissues from male rats, as compared to those of female rats, was reduced by approximately 50% when cycloheximide was present in the culture medium. To determine whether SC production by exorbital glands was influenced by androgens, orchiectomized rats were administered either saline or testosterone (2·0 mg/day for 4 days), and exorbital glands were cultured 24 hr after the last injection. Testosterone treatment *in vivo* induced a significant, cycloheximide-sensitive increase in SC production *in vitro*, compared to the glandular SC output of saline-injected controls. It is interesting that similar androgen treatment of ovariectomized females also resulted in elevated tear SC concentrations and enhanced output of SC by their exorbital glands *in vitro*. These findings indicate that the exorbital gland is primarily responsible for SC production in the rat eye and that androgens may modulate the synthesis of SC in this gland.

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INTRODUCTION

Secretory component (SC) is an important regulatory factor in the secretory immune system. This glycoprotein, which is synthesized by mucosal epithelial cells and hepatocytes (Crago *et al.*, 1978; Socken *et al.*, 1979; Zevenbergen *et al.*, 1980), appears to control the transfer of polymeric IgA antibodies into external secretions (Brandtzaeg, 1981), to clear IgA-immune complexes from the blood (Stokes, Swarbrick & Soothill, 1980; Peppard *et al.*, 1982; Socken *et al.*, 1981) and to modulate several parameters of lymphocyte function (Crago *et al.*, 1981; South, 1979; Stafford, Knight & Fanger, 1982). The absence or deficient

production of SC may lead to mucosal immune dysfunction (Engstrom *et al.*, 1978; Ogra, Ogra & Coppola, 1975; Strober *et al.*, 1976).

Recently, we demonstrated that the level of free SC in rat tears is regulated by hormones (Sullivan, Bloch & Allansmith, 1983a). The SC concentration in tears of male rats was approximately five-fold greater than that measured in tears of females and underwent a significant, time-dependent decrease following orchiectomy. Administration of testosterone to castrated male rats reversed this decline and induced a marked increase in tear SC levels. This response appeared to be due specifically to androgens; progesterone, oestradiol and cortisol had no effect on tear SC content.

The mechanism by which androgens enhance the tear SC concentration remains unclear. Amounts of free SC in external secretions most probably reflect a balance between local production, the quantity of polymeric IgA available for transfer and mucosal clearance. The possibility that androgen action is mediated through effects on clearance is unlikely, because the volume and total protein level of tears were relatively unchanged after testosterone treatment (Sullivan *et al.*, 1983a). An alternative possibility is that testosterone stimulates the synthesis and/or secretion of SC by an ocular tissue, such as the exorbital (lacrimal) gland, which is a known target organ for androgens (Cavallero, 1967; Hahn, 1969; Lauria & Porcelli, 1979).

The purpose of the present study was to extend our earlier findings by identifying the rat ocular tissue(s) that produces SC and by evaluating whether this production is increased by exposure to testosterone. We have also examined whether androgen administration influences the concentration of SC in tears of female rats.

MATERIALS AND METHODS

General procedures

Adult male and female Sprague-Dawley rats (Charles River Breeding Laboratories; 8–12 weeks old) were housed in temperature-controlled rooms with light and dark intervals of 12 hr length. Orchiectomies or ovariectomies were performed 13–14 days prior to experimentation.

Tears were collected from the eyes of anaesthetized (ether) rats as previously described (Sullivan *et al.*, 1983a). Briefly, the tip of a graded, capillary micropipette (Fisher) was positioned at the inner canthus and then gently moved along the palpebral conjunctiva. After this procedure was repeated twice on each eye, tear volumes were measured and transferred to 1·5 ml polypropylene tubes (Sarstedt) containing 100 μl sodium phosphate buffer (0·1 M, pH 7·0). Tubes were centrifuged at 10,000 ***g*** for 4 min and resulting supernatants were stored at $-20°$.

Protein content in tears was determined by the Hartree method (Hartree, 1972), and bovine serum albumin (BSA; Calbiochem-Behring) was utilized as the standard.

The Student's *t*-test was used to statistically analyse the data.

Ocular tissue cultures

Ocular tissue obtained for incubation studies included the exorbital gland, globe, Harder's gland, and lid with adjacent structures (hereafter termed 'lid'). The bulbar conjunctiva was left attached to the globe. Tissues were removed immediately after exsanguination of etherized rats, then rinsed in phosphate-buffered saline (0·01 M, pH 7·4; PBS), blotted on surgical gauze, halved or quartered (not the globe) and weighed. Globes or tissue segments were placed in 20 ml glass vials containing 2 ml of prewarmed incubation medium, which consisted of RPMI-1640 (Gibco), 10% foetal bovine serum with glutamine (Gibco) and garamycin (25 μg/ml medium; Schering Corporation). Vials were gassed with 95% O_2–5% CO_2, capped and transferred to a shaking rack in a 37° water bath. At designated times during incubation, followed by regassing, 100 μl aliquots of media were removed and centrifuged at 10,000 ***g*** for 4 min. Supernatants were stored at $-20°$.

To distinguish between synthesis of SC by ocular tissues and the release of pre-formed or adsorbed SC, cycloheximide (Sigma Chemical Company), an inhibitor of protein synthesis, was added to selected vials in all incubation experiments. With the exception of globe samples, tissues were divided and combined so that approximately half of each tissue was incubated in the presence of cycloheximide.

To determine the concentration of cycloheximide to use throughout these studies, exorbital gland segments were incubated with varying amounts of cycloheximide and 10 μCi of [^{3}H]-L-amino acids (New England Nuclear). After 21 hr of incubation, centrifuged aliquots (50 μl) of media were mixed with 50 μl cold 20% trichloroacetic acid (TCA), incubated for 1 hr at 4° and then centrifuged at 10,000 ***g*** for 2 min.

Supernatants were discarded and pellets were washed with 500 μl cold 10% TCA. Following an additional centrifugation, wash fluid was removed and pellets were dissolved in 100 μl 3N sodium hydroxide; 50 μl aliquots from each sample were added to 4 ml hydrofluor and counted in a Packard scintillation spectrometer. Quench determination of sample radioactivity was performed by internal standardization.

Measurement of SC

The SC level in tears and incubation media were measured with a previously described radioimmunoassay (Sullivan & Wira, 1983a), which detects primarily free SC. Briefly, the procedure involved the transfer of 10 or 20 μl aliquots of sample or purified rat SC standards (prepared by Dr B. Underdown, Toronto, Canada) to 1·5 ml polypropylene tubes. Next, 30 μl of ^{125}I-SC, which was iodinated with ^{125}NaI (New England Nuclear) by using IODO-GEN (Markwell & Fox, 1978; Sullivan & Wira, 1983b), and 20 μl of 0·02% rabbit anti-rat SC antiserum (antibody prepared by Dr Underdown) were added. Tube contents were mixed and incubated for 1 hr at room temperature. Following incubation, 30 μl of goat anti-rabbit IgG antiserum (1/50 dilution; Miles Laboratories) were placed in the tubes. After mixing and a further 1 hr incubation period, tubes were centrifuged at 10,000 *g* for 4 min. Supernatants were discarded by suction, pellets were washed with 800 μl PBS and tubes were recentrifuged. Pellet radioactivity was measured in a Beckman scintillation spectrometer. Standard curves were included with each assay, and levels of SC standards ranged from 0·5 to 100 ng. Diluents for the standards were either sodium phosphate (0·1 M, pH 7·0), containing BSA (1 mg/ml), or incubation medium, depending upon the sample to be analysed. First and second antibodies, as well as the ^{125}I-SC, were diluted in BSA/TKM buffer (1 mg/ml; TKM: 50 mM Trizma, 25 mM KCl, 5 mM $MgCl_2$, pH 7·5).

Steroid preparation

Testosterone was purchased from Calbiochem-Behring and suspended in saline by glass–glass homogenization. Orchiectomized or ovariectomized rats were injected for 4 days with either testosterone (2 mg/day) or normal saline (controls) and killed 24 hr after the last injection. All injections (200 μl volume) were administered subcutaneously.

RESULTS

Production of SC by ocular tissues

To determine which ocular tissue(s) produced SC, exorbital glands, 'lids', globes, and Harder's glands from male rats were incubated in vitro in the presence or absence of cycloheximide (100 μg/ml medium). As shown in Fig. 1, media from exorbital gland cultures contained at least a nine-fold greater amount of SC than incubation media from other ocular tissues. This SC production by exorbital glands was significantly ($P < 0·05$) reduced by the addition of cycloheximide to the incubation medium. In contrast, accumulation of SC in 'lid' cultures was not influenced by the presence of cycloheximide. No SC could be detected in globe incubation media, and the SC level in media from Harder's gland cultures was very low.

We also examined the output of SC by various ocular tissues from female rats. The pattern of SC accumulation in incubation media was analagous to the profile presented in Fig. 1.

The concentration of cycloheximide utilized in these experiments had been determined by monitoring the effect of various doses on the accumulation of SC and TCA-precipitable [^{3}H]-protein in exorbital gland cultures. As demonstrated in Table 1, a cycloheximide

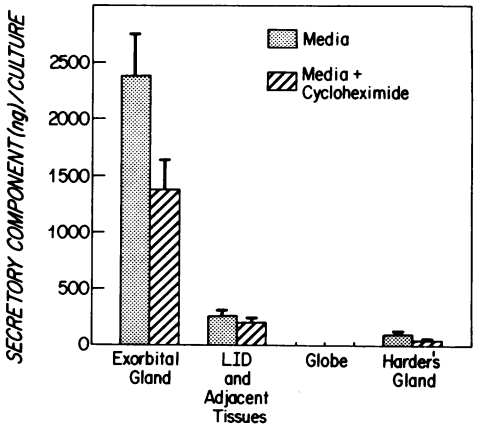

Figure 1. Variations in the amount of SC released by ocular tissues during incubation *in vitro*. Tissues from male rats ($n = 6$) were incubated for 22 hr in the presence or absence of cycloheximide (cyc; 100 μg/ml medium). Tissue weights (mg) were as follows: exorbital gland, 101 ± 10 and 95 ± 8 (cyc); 'lid', 119 ± 12 and 118 ± 5 (cyc); globe, 120 ± 1 and 122 ± 1 (cyc); Harder's gland, 107 ± 9 and 88 ± 6 (cyc). Bars and vertical lines represent the mean ± SE of the total SC content in media from six cultures.

Table 1. Effect of varying concentrations of cycloheximide on the accumulation of SC and TCA-precipitable [^{3}H]-protein in exorbital gland cultures

Cycloheximide (μg/ml medium)	Exorbital gland weight (mg)	Total media SC (ng/mg tissue)	Media TCA-precipitable [^{3}H]-protein (c.p.m./mg tissue)
0	93±11	41·0±2·7*	4757±690†
50	92±7	18·3±0·7	1117±122
100	87±11	17·2±1·7	678±185
200	103±10	18·1±2·0	541±146

Numbers represent the mean±SE of three values per experimental condition. The procedure utilized in this study involved removal of four exorbital glands from two rats, quartering each of the tissues and then adding ¼ of each gland to an incubation vial, which contained 0, 50, 100, or 200 μg/ml cycloheximide. Thus, each vial had approximately one gland equivalent from the same animals. This process was repeated twice to obtain the three samples/group.

* Significantly ($P<0·001$) greater than the SC content in other groups.

† Significantly ($P<0·005$) greater than the TCA-precipitable [^{3}H]-protein levels in other groups.

concentration of 100 μg/ml medium reduced SC and [^{3}H]-protein levels by approximately 50 and 85%, respectively. No further reduction in these parameters was achieved by increasing the level of cycloheximide in media to 200 μg/ml. Therefore, the 100 μg/ml dose was used.

Comparison between the production of SC by exorbital glands from male and female rats

A comparison between the SC accumulation in incubation media of exorbital glands from male and female rats is shown in Fig. 2. Levels of SC, whether expressed per weight of tissue or in total amounts, were significantly ($P<0·001$) greater in the media from male glands. Addition of cycloheximide to incubation media reduced by approximately one-half the accumulation of SC in cultures of tissues from both sexes.

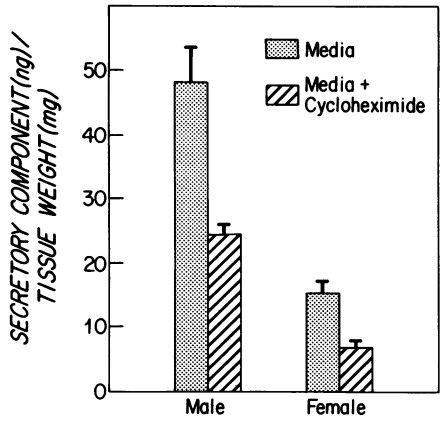

Figure 2. Influence of sex on the secretion of SC by exorbital glands *in vitro*. Exorbital glands from male ($n=6$) and female rats ($n=6$) were incubated for 21·3 hr in media with or without added cycloheximide (100 μg/ml). Tissue weights (mg) from males were 101±7 and 102±3 (cyc), and from females were 87±8 and 95±11 (cyc). The level of SC in incubation media was normalized to exorbital gland weight. Bars equal the mean±SE of six values.

Effect of testosterone exposure *in vivo* on SC production by exorbital glands from orchiectomized rats *in vitro*

To determine whether the androgen-induced increase in tear SC concentrations (Sullivan *et al.*, 1983a) might be due to hormonal stimulation of SC production by the exorbital glands, orchiectomized rats were treated for 4 days with either testosterone (2 mg/day) or saline. Twenty-four hours after the last injection, exorbital glands were removed and cultured. The concentration of SC in the media was measured at varying times after the start of incubation. As demonstrated in Fig. 3, exorbital glands from androgen-injected rats produced significantly ($P<0·005$) more SC *in vitro* than glands from control animals. This hormonal effect on SC output was found throughout the time course of the incubation experiment. The presence of cycloheximide in tissue cultures resulted in a decrease in SC production by glands from both testosterone- and saline-injected rats.

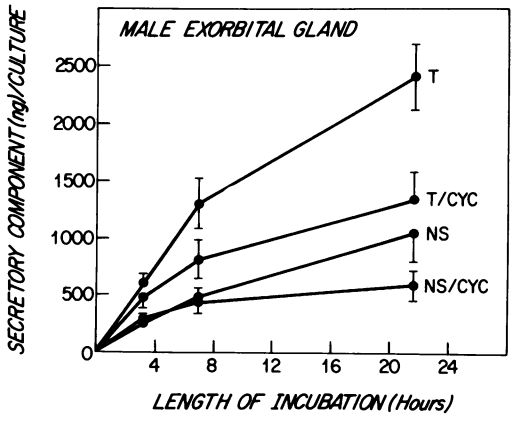

Figure 3. Effect of testosterone exposure *in vivo* on the SC production by male exorbital glands *in vitro*. Orchiectomized rats were injected with normal saline (NS; $n = 5$) or testosterone (T; 2·0 mg/day; $n = 6$) and killed 24 hr after the fourth treatment. Exorbital glands were placed in either incubation medium alone or medium containing cycloheximide (100 μg/ml). Tissues in the various experimental groups weighed 116 ± 7 (T), 120 ± 6 (T, cyc), 129 ± 18 (NS) and 142 ± 17 mg (NS, cyc). Each point represents the mean ± SE of five or six determinations of total SC content in incubation media.

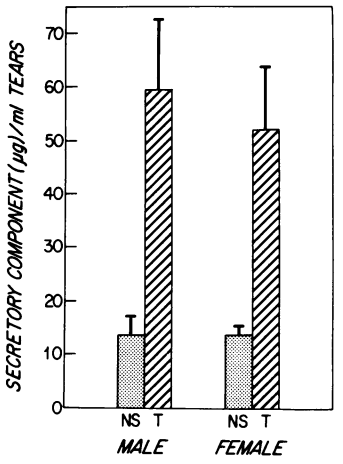

Figure 4. Influence of testosterone on the SC concentration in tears of male and female rats. Orchiectomized or ovariectomized rats were injected for 4 days with either saline (NS) or testosterone (T; 2·0 mg/day). Tears were collected 24 hr following the fourth injection. Bars equal the mean ± SE of five or six values.

Influence of testosterone on the SC concentration in tears of ovariectomized rats

To test whether androgens influence the SC levels in tears of female, as well as male, rats, testosterone or saline was administered for 4 days to ovariectomized or orchiectomized animals. Tears were collected 24 hr after the last injection. Testosterone treatment induced a significant ($P < 0·005$) elevation in the tear SC concentration of ovariectomized rats (Fig. 4). This response was similar to that observed after androgen administration to orchiectomized rats (Fig. 4), as previously described (Sullivan *et al.*, 1983a). The effect of testosterone on the SC level in tears of females could not be explained by alterations in either the volume or total protein content of tears. As shown in Table 2, androgen treatment had no significant effect on tear volume or the total protein level of tears. Consequently, the SC/protein ratio in tears of testosterone-treated females was significantly increased, compared to the ratio of saline-injected controls.

Effect of testosterone exposure *in vivo* on SC production by exorbital glands from ovariectomized rats *in vitro*

To examine whether testosterone treatment of ovariectomized rats also enhanced the SC production of exorbital glands, these tissues were cultured after 4 days of androgen exposure *in vivo*. As demonstrated in Fig. 5, SC levels were significantly ($P < 0·01$ at 7 and 22 hr) increased in cultures of exorbital glands from testosterone-treated female rats. Addition of cycloheximide to the incubation medium diminished the production of SC by exorbital glands from both groups.

DISCUSSION

The present studies indicate that the androgen regulation of tear SC levels in rats is mediated through effects on the exorbital gland. This conclusion is based upon the following results. (i) Comparison of SC content in media of various ocular tissue cultures showed that the exorbital gland is the primary source of SC in rat tears. Substantial quantities of SC were found in exorbital gland cultures, and these levels were significantly reduced by the presence of cycloheximide. In contrast, media in which Harder's glands, 'lids' and globes had been incubated contained either low or undetectable amounts of SC. These findings correlate well with our previous immunofluorescence observations on rat ocular tissues, in which SC was identified only in the exorbital gland (Gudmundsson *et al.*, submitted). (ii) Exorbital glands from intact male rats produced

Table 2. Influence of testosterone on the tear volume, SC and total protein levels and the SC/protein ratio in ovariectomized rats

Treatment	Number	Tears (μl)	SC (ng)	Total protein (μg)	SC (ng)/total protein (μg)
Saline	6	3·0±0·4	38±4	85±11	0·45±0·04
Testosterone	6	2·9±0·6	122±13*	100±14	1·77±0·39†

Values represent the mean ± SE of six determinations. Ovariectomized rats received four daily injections of either testosterone (2·0 mg/day) or saline (controls); tears were collected 24 hr after the last treatment. The SC and protein levels reflect total amounts in tear samples.

* Significantly ($P < 0.001$) greater than SC content in tears from saline-injected rats.

† Significantly ($P < 0.005$) greater than the SC/protein ratio of control animals.

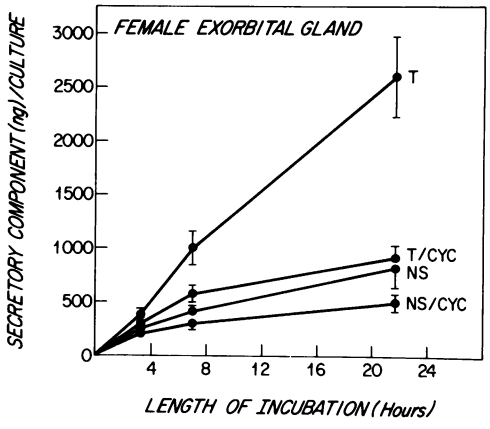

Fig. 5. Effect of testosterone exposure *in vivo* on the SC production by female exorbital glands *in vitro*. Exorbital glands were cultured 24 hr after the fourth daily injection of saline (NS; $n = 6$) or testosterone (T; 2·0 mg/day; $n = 6$) to ovariectomized rats. Cycloheximide (100 μg/ml) was present in the incubation media of designated samples. Tissues weighed 109 ± 7 (T), 110 ± 10 (T, cyc), 113 ± 7 (NS) and 98 ± 7 mg (NS, cyc). Bars represent the mean ± SE of six values.

significantly greater quantities of SC than glands from female rats. This difference would appear to account for the higher SC concentrations in tears of males compared to females (Sullivan *et al.*, 1983a). (iii) Exposure of exorbital glands *in vivo* to androgens resulted in an enhanced production of SC *in vitro*. This hormonal response occurred irrespective of gender and correlated well with the testosterone-induced increase in tear SC levels of castrated male or female rats.

Our finding that testosterone stimulated SC production by the exorbital gland represents another example of the hormonal control of SC. In the rat uterus, synthesis and/or secretion of SC appears to be regulated by oestradiol (Sullivan, Underdown & Wira, 1983b), whereas in the human cervix, SC content may be increased by progesterone (Murdoch, Buckley & Fox, 1982). Similarly, the positive influence of prolactin, progesterone and oestradiol on the accumulation of IgA by mouse mammary epithelial cells (Weisz-Carrington *et al.*, 1978; Weisz-Carrington, Dientsman & Lamm, 1980) may also be mediated through SC. One consequence of hormone action on SC is that levels of IgA in external secretions may rise. For example, the oestrogen-induced increase in the free SC content of rat uterine secretions was accompanied by a parallel increase in polymeric IgA levels (Sullivan *et al.*, 1983b; Sullivan & Wira, 1983c). Preliminary data in our laboratory also indicate that androgen treatment enhances IgA, as well as SC, concentrations in tears of orchiectomized rats (Sullivan & Allansmith, unpublished observations). These studies raise an intriguing, but unanswered, question: by what regulatory mechanism do different hormones control SC production at various mucosal sites?

Our observation that testosterone influences the exorbital gland is consistent with the previous findings of others, which demonstrated that this ocular tissue is an androgen target organ (Cavellero, 1967; Hahn, 1969; Lauria & Porcelli, 1979). The adult rat exorbital gland, like the rat liver (Roy & Chatterjee, 1983), is sexually dimorphic; after puberty, the glandular morphology and histochemistry are different in male and female rats (Cavallero, 1967; Hahn, 1969; Lauria & Porcelli, 1979; Paulini, Beneke & Kulka, 1972; Paulini *et al.*, 1972). This difference appears to be due principally to androgens, because castration of male rats results in a lacrimal appearance analogous to that of females (Cavallero, 1967; Hahn, 1969). Treatment of orchiectomized rats with testosterone restores the intact male characteristics of the exorbital gland,

which include: irregularly shaped acini with indistinct cell borders; centrally located, large and polymorphic nuclei that contain prominent nucleoli; granular PAS-positive material in the epithelial cell cytoplasm; increased uptake of ^{35}S-labelled sulphate; and the absence of leucine aminopeptidase activity (Cavellero, 1960; Cavellero *et al.*, 1960; Cavellero, 1967; Hahn, 1969; Lauria & Porcelli, 1979). These androgen effects are specifically antagonized by estrogens (Cavallero, 1967; Lauria & Porcelli, 1979). Of interest, treatment of female rats with testosterone also induces a shift of lacrimal gland appearance to that of the male pattern (Lauria & Porcelli, 1979). This response may account for the increased output of SC by exorbital glands from ovariectomized females after androgen administration.

The mechanism of action of testosterone on exorbital tissue may involve local steroid receptors. Hypophysectomy does not abolish the androgen effects on lacrimal morphology of orchiectomized rats (Cavallero, 1960), whereas treatment with cyproterone acetate, an anti-androgen, does (Hahn, 1969; Lauria & Porcelli, 1979). Whether androgen action on SC production by the exorbital gland interrelates in some fashion with the known stimulators of lacrimal protein secretion such as the autonomic nervous system (Bromberg, 1981) or cyclic AMP (Jahn *et al.*, 1982), remains to be determined.

Hormonal influence on the immune system of the acrimal gland may have clinical relevance. For example, Sjögren's syndrome, which is an immunological disorder encountered almost exclusively in females, is characterized by a lymphocytic infiltration into the lacrimal gland, destruction of acinar and ductal tissues and generation of keratoconjunctivitis sicca (Bloch *et al.*, 1965; Tabbara, 1983). This disease is associated with an abnormal distribution of T subsets (Adamson, 1983; Momimoto, 1982) and appears to involve the secretory immune system in its expression (Elkon *et al.*, 1982, 1983). Because androgens are known to increase the production of T cell modulators—e.g. interleukin-2 (Talal, Dauphinee & Wofsy, 1982), thymic regulatory factors (Stimson & Crilly, 1981)—to improve T cell function (Michalski *et al.*, 1983), and to effect the lacrimal gland (this study), these hormones may have therapeutic value in this syndrome. In fact, several clinical reports on the androgenic treatment of Sjögren's patients (Appelmans, 1948; Bruckner, 1945) support this hypothesis. Clearly, further research is required to examine endocrine involvement with the ocular immune system.

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