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Effects of testosterone, oestradiol and progesterone on immune regulation

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SUMMARY

Clinical observations on differences in the sexual incidence of diseases associated with defects of immune regulation, and of the occasional beneficial effects of pregnancy on disease course suggest that endocrine mechanisms may be important in the immunopathogenesis of these disorders. To investigate this possibility the *in vitro* effects of testosterone, oestradiol and progesterone on selected aspects of immune regulation were studied in normal adults. We observed the effects of these hormones on a mitogen-induced suppressor T-cell system and a monocyte mediated prostaglandin-producing suppressor cell system. The addition of progesterone but not oestradiol or testosterone to the Concanavalin A (Con A) generation of T lymphocyte suppressor cells produced significantly increased suppressor cell activity ($P < 0{\cdot}005$). Pre-incubation of lymphocytes with testosterone but not oestradiol or progesterone in the absence of Con A resulted in the generation of modest, but highly significant suppressor cell activity ($P < 0{\cdot}005$). No effect on the prostaglandin-producing suppressor cell activity was observed. These findings suggest that certain endocrine changes may alter immunoregulatory function and account for some of the clinical observations previously noted in diseases associated with defects of immune regulation.

INTRODUCTION

Clinicians have long realized that several connective tissue diseases are much more frequently encountered in females than in males and that pregnancy can dramatically alter the disease course. Pregnancy is a time of considerable endocrine change and it is possible that alterations in the circulating levels of various sex hormones may account, in part, for some of these observations. An important role for sex hormones is further suggested by a number of other observations including the increased resistance of females to some bacterial infection (Wheater & Hurst, 1961), higher immunoglobulin levels in young women than in either older women or men (Rhodes *et al.*, 1969), and the observation that oral contraceptives result in increased levels of circulating IgM (Bole, Friedlaender & Smith, 1969). Augmentation of the antibody response in women, compared to men, to *E. coli* (Paty *et al.*, 1976), measles (Michaels & Rogers, 1971), rubella (Spence *et al.*, 1977), brucella (Rhodes *et al.*, 1969) and hepatitis B virus (London & Drew, 1977) has also been described. Auto-antibodies are much more frequent in women than in men (Hooper *et al.*, 1972) and sex differences in *in vitro* lymphocyte cytotoxicity (Santoli *et al.*, 1976) and proliferation (Barnes *et al.*, 1974) have been described.

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Perhaps the best studied disease known to be associated with abnormal immunoregulation is systemic lupus erythematosus (SLE), in which the sex ratio in adults is usually about nine females to one male (Kornreich, 1976) but is only about 3:1 in children. SLE is also more common in Klinefelter's syndrome (Stern *et al.*, 1977) than in normal males. In both children and patients with Klinefelter's syndrome, hormonal differences are much less marked than in normal adults. Studies of the NZB mouse, an animal model for SLE, have also shown that female mice have more severe disease than males and that the prognosis in females is improved by the administration of testosterone (Roubinian, Papoian & Talal, 1977). Similarly castration or oestrogen therapy results in male mice dying earlier of the disease (Melez, Reeves and Steinberg, 1978). Both in the animal model and in humans with the disease, defects in suppressor cell function have been described (Talal, 1976) and may be a factor in the immunopathogenesis of the disease.

The purpose of this study was to investigate possible actions of sex hormones on aspects of *in vitro* immunoregulatory function. We have therefore studied the effects of testosterone, progesterone, and oestradiol on the Con A-induced suppressor cell system which is mediated by T lymphocytes and on the prostaglandin-producing suppressor cell system which is thought to be mediated by monocytes.

PATIENTS AND METHODS

Subjects. A total of 17 healthy hospital and laboratory staff were studied. Nine were male (age range 28–55) and eight were female (age 23–55). All gave informed consent and the study conformed to the guidelines of the Committee on Human Experimentations at the University of Vermont.

Preparation of peripheral blood mononuclear cells (PMBC). PBMC were obtained by density centrifugation after layering fresh heparinized (10 units/ml) venous blood on Ficoll–Hypaque. Mononuclear cells were recovered and washed twice with phosphate buffered saline. Cell suspensions were made in RPMI 1640 with 25 mM HEPES (GIBCO) and supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 mM glutamine (Microbiological Associates) (RPMI–PSG). Viability was assessed by exclusion of trypan blue and preparations less than 95% viable were discarded.

Concanavalin A (Con A) suppression of T-cell activity (ConASST). Con A suppression of T cell activity was based on a method (Shou, Schwartz & Good, 1976) which measures the degree of inhibition of Con A-induced ^{3}H-thymidine incorporation into responder T-lymphocytes by Con A generated suppressor T cells. In order to generate suppressor T cells, peripheral blood mononuclear cells were adjusted to a concentration of 5×10^6/ml in RPMI-PSG and incubated with 20% human AB serum in 1 ml aliquots in round bottomed tubes with 60 μg/ml Con A (Calbiochem) for 48 hr. Identical cultures were incubated without Con A for subsequent addition to responder lymphocytes so that the 'non-suppressed' Con A-induced ^{3}H–thymidine incorporation of the latter could be assessed. Following the 48-hr incubation, aliquots were recombined, pelleted and the cells suspended in fresh RPMI-PSG plus 50 μg/ml mitomycin C (Sigma), and incubated for 30 min. The cells were washed three times in 30 mM alpha methyl-d-mannoside (Sigma) in Hanks' balanced salt solution (Microbiological Associates) to remove cell-bound Con A and then resuspended in RPMI-PSG prior to reincubation with responder lymphocytes.

Throughout, cryopreserved cells isogeneic to the suppressor cells (ie) autologous cells were used as Con A responders to avoid possible HLA restriction of cell interactions. Established techniques were used for cryopreservation as described elsewhere (Karpovitch *et al.*, 1980).

As noted, suppressor activity was measured by the inhibition of Con A (7 μg/ml) stimulated ^{3}H–thymidine incorporation in peripheral blood mononuclear responding cells by Con A generated suppressor cells. Suppressor cells (10^5) and responder cells (10^5) were incubated in microtitre plates at 0·2 ml/well with 20% human AB serum for 3 days at 37°C in a humidified 5% CO_2 atmosphere. Cells were labelled with 1 μCi/well ^{3}H–thymidine (NEN spec. act. 6·7 mCi/ml) 18 hr prior to harvesting on glass-fibre filters in a Titertek Cell Harvester (Flow Lab). Dried filters were counted in minivials by liquid scintillation spectrometry in a Packard Tri-Carb.

The data are expressed as mean c.p.m. from triplicate cultures and the degree of suppression calculated according to the following formula:

$$\% \text{ suppression} = 1 - \frac{\text{c.p.m.}_s\text{.Con A} - \text{c.p.m.}_s\text{.0}}{\text{c.p.m.}_c\text{.Con A-c.p.m.}_c\text{.0}} \times 100$$

in which c.p.m.$_s$.Con A is a mean c.p.m. of ^{3}H–thymidine incorporation in responder cell cultures containing Con A generated suppressor cells (in addition to responding cells) and Con A, c.p.m.$_s$.0 is ^{3}H–thymidine incorporation in cultures containing Con A generated suppressor cells, but no Con A, c.p.m.$_c$.Con A is ^{3}H–thymidine responder cell incorporation in responder cell cultures containing preincubated control cells (in addition to responding cells) and Con A, and c.p.m.$_c$.0 is ^{3}H–thymidine incorporation in responder cell cultures containing preincubated control cells but no Con A.

Prostaglandin suppressor system (PgSS). Suppression of T cell activity by prostaglandin-producing mononuclear cells is based on a method (Goodwin, Bankhurst & Messner, 1977) which utilizes indomethacin in its role as a prostaglandin synthetase inhibitor to enhance phytohaemagglutinin (PHA) stimulated ^{3}H–thymidine incorporation *in vitro*. Peripheral blood mononuclear cells were adjusted to 10^5 cells/well in microtitre plates and incubated with 1 μg/well PHA-P (Burroughs Welcome HA–16) with and without 250 ng/well indomethacin (Merck, Sharp & Dohme). Wells contained a final volume of 0·25 ml/well, 20% AB serum and RPMI-PSG medium. Cultures were incubated for 3 days at 37°C in a humidified 5% CO_2 atmosphere before labelling with 1 μCi/well ^{3}H–thymidine 18 hr prior to harvesting on glass-fibre filters in a Titertek cell harvester (Flow Lab). Dried filters were counted in minivials by liquid scintillation spectrometry in a Packard Tri-Carb.

The data are expressed as mean c.p.m. from triplicate cultures with suppression calculated according to the following formula:

$$\% \text{ suppression} = 1 - \frac{\text{CPM–PHA}}{\text{CPM–PHA–IND}} \times 100$$

in which CPM–PHA is the mean c.p.m. in the absence of indomethacin and CPM–PHA–IND is the mean c.p.m. in the presence of indomethacin.

The effects of hormones. The concentrations used for testosterone was 12 ng/ml, for oestradiol was 40 ng/ml and for progesterone was 20 ng/ml. These concentrations were chosen because they approximate upper normal values seen in adults. The original stock solutions were made in ethanol but the final concentration of alcohol was only 0·0001 mg/ml which, in preliminary experiments, had no effect on lymphocyte responses. We studied the effects of these three hormones on (1) the response of lymphocytes to the mitogen PHA, (2) the generation of suppressor cells by Con A (1st incubation period of the ConASST) and (3) the proliferative response of the responder cells (the second incubation period of the ConASST). The effect of incubation of cells during the first incubation period with the three hormones was also studied in the absence of Con A. These cells were incubated for 48 hr in the presence of the hormones and treated exactly as those cells in the conASST assay and added to isogeneic responder cells. The effects of the hormones on the PgSS were studied by addition of the hormones (at the same time as indomethacin) and comparing the results with the normal response.

Statistical analysis. Statistical analysis was performed by the paired Student's *t*-test. Probability values of less than 5% were considered significant.

RESULTS

The results of the effects of testosterone, progesterone and oestradiol on the responses of lymphocytes to the various manipulations are summarized in Tables 1, 2 & 3. At the concentrations used, these hormones did not significantly alter the response of the lymphocytes to the mitogen PHA. Similarly, addition of the hormones to the second incubation period of the ConASST did not

Table 1. The effects of addition of progesterone (Prog) on mitogen response and the two incubation periods of the ConASST. Values expressed as c.p.m. and % suppression (mean ± s.d.)

	Mitogen response		Manipulation of the 1st incubation period of the ConASST				Manipulation of the 2nd incubation period of ConASST	
	PHA alone	PHA +Prog	Medium alone	Prog alone	Con A alone	Con A +Prog	Con A alone	Con A +Prog
Male	139,386	149,760	47,441	43,905	19,963	14,813	19,963	21,136
$n=8$	±38,478	±54,297	±21,805	±18,399	±13,109	±8,374	±13,109	16,942
					$n=4$	$n=4$	$n=4$	$n=4$
					58%±13·5	67%±14	58%±13·5	59%±10
Female	182,292	176,259	61,798	48,479	20,179	14,613*	20,179	18,843
$n=7$	±45,498	±43,052	±38,489	±19,969	±10,773	±8,673	±10,773	±9,352
					$n=4$	$n=4$	$n=4$	$n=4$
					68%±18	78·4%±10·2	68·8%±18	75%±5
Total	155,889	159,697	53,182	45,734	20,044	14,738*	20,044	19,939
$n=15$	±44,229	±49,013	±29,219	±18,473	±11,462	±7,847	±11,462	±13,225
					$n=8$	$n=8$	$n=8$	$n=8$
					62%±15	71%±13	62%±15	65%±13

* $P<0·005$.

Table 2. The effects of addition of testosterone (Test) on mitogen response and the two incubation periods of the ConASST. Values expressed as c.p.m. and % suppression (mean ± s.d.)

	Mitogen response		Manipulation of the 1st incubation period of the ConASST				Manipulation of the 2nd incubation period of ConASST	
	PHA alone	PHA +Test	Medium alone	Test alone	Con A alone	Con A +Test	Con A alone	Con A +Test
Male	103,211	99,969	49,252	46,349	27,340	32,470	27,340	26,371
$n=9$	±55,264	±54,184	±14,693	±15,587	±15,230	±10,805	±15,230	±13,899
					$n=5$	$n=5$	$n=5$	$n=5$
				6·0%±18	44%±22	37%±14	44%±22	40%±23
Female	87,203	94,612	60,617	49,023*	25,551	25,079	25,551	30,145
$n=9$	±84,502	±87,316	±21,640	±19,328	±10,751	±20,242	±10,751	±10,124
					$n=5$	$n=5$	$n=5$	$n=5$
				15%±13	59%±21	60%±36	59%±21	52%±33
Total	95,482	97,763	54,600	47,606*	26,764	28,774	26,764	28,052
$n=17$	±66,679	±67,215	±19,398	±16,936	±9,521	±15,785	±9,521	±11,789
					$n=10$	$n=10$	$n=10$	$n=10$
				12%±22	51%±21	48%±29	51%±21	46%±24

* $P<0·005$.

Table 3. The effects of addition of oestradiol (Oest) on mitogen response and the two incubation periods of the ConASST. Values expressed as c.p.m. and % suppression (mean ± s.d.)

	Mitogen response		Manipulation of the 1st incubation period of the ConASST				Manipulation of the 2nd incubation period of ConASST	
	PHA alone	PHA +Oest	Medium alone	Oest alone	Con A alone	Con A +Oest	Con A alone	Con A +Oest
Male	103,211	102,908	49,252	46,972	27,340	26,614	27,340	26,371
$n=8$	±55,264	±56,065	±14,693	±15,799	±15,230	±6,909	±15,230	±13,897
					$n=5$	$n=5$	$n=5$	$n=5$
					44%±22	47%±16	44%±22	47%±17
Female	87,203	92,425	49,023	51,561	25,551	26,190	25,551	26,190
$n=9$	±84,562	±83,432	±19,328	±20,205	±10,751	±20,277	±10,751	±26,279
					$n=5$	$n=5$	$n=5$	$n=5$
					59%±21	50%±29	59%±21	60%±29
Total	95,482	98,592	47,606	49,131	26,764	26,320	26,764	26,281
$n=17$	±66,679	±66,383	±16,936	±12,578	±9,521	±14,303	±9,521	±16,389
					$n=10$	$n=10$	$n=10$	$n=10$
					51%±22	54%±24	51%±22	53%±24

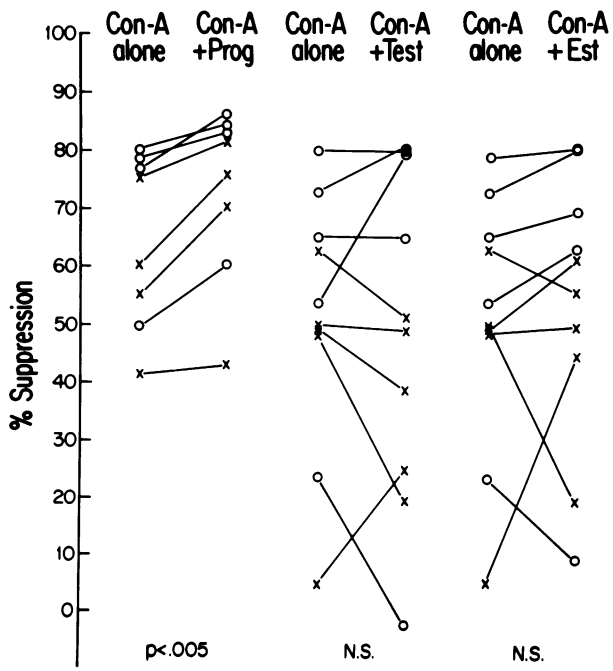

Fig. 1. The effect of adding progesterone (Prog), testosterone (Test) and oestradiol (Est) to the Con A generation of suppressor cells. Results indicate % suppression of responder cells. (× = Male, o = Female).

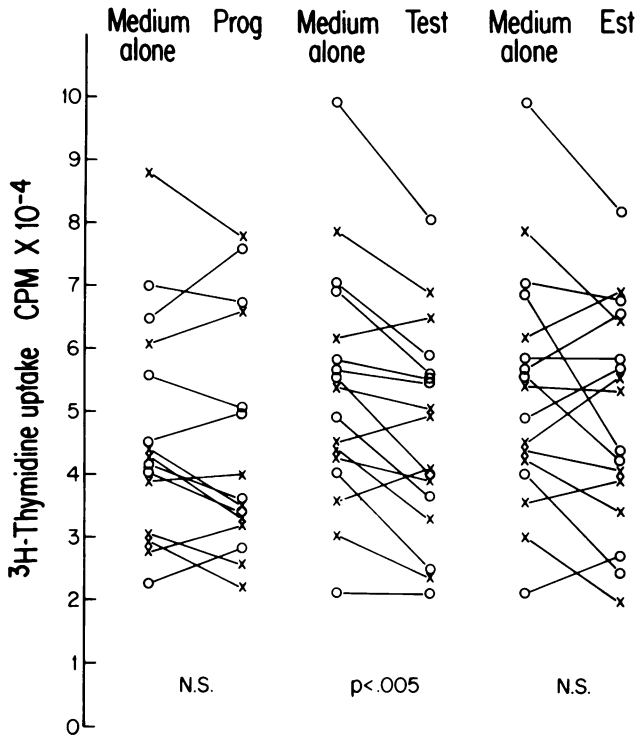

Fig. 2. The effects of preincubation with progesterone (Prog), testosterone (Test) and oestradiol (Est) in the absence of Con A. Results indicate the response of the responder cells (c.p.m.). (× = Male, o = Female).

have any effect on the proliferative response of the responder cells to Con A and the suppressor cell activity was unaltered. In contrast, the addition of progesterone to the Con A generation of suppressor cells (1st incubation) resulted in significantly greater suppression than that achieved by Con A alone ($P < 0{\cdot}005$), while neither of the other two hormones produced statistically significant differences (Fig. 1). The effects of cells preincubated with hormones in the absence of Con A on the isogeneic responder cells are shown in Fig. 2. Those cells pretreated with testosterone resulted in significant suppression ($P < 0{\cdot}005$) of the responder cells but neither of the other two hormones had any such effect. The Con A-induced suppression in our small group tended to be slightly greater in the female patients than in the male patients but this difference did not reach statistical significance.

No significant difference was observed when the hormones were added to the PgSS. The results for the PgSS were $3{\cdot}5\% \pm 11$, $0{\cdot}4\% \pm 18$, $2{\cdot}5\% \pm 9$ and $1{\cdot}5\% \pm 15$ in medium alone, testosterone, estradiol and progesterone respectively.

DISCUSSION

We have found that Con A generation of suppressor cells is enhanced by the addition of progesterone but not by testosterone or oestradiol. Pre-incubation with the hormones alone resulted in testosterone generating modest, but highly significant, suppressor cell activity, whilst progesterone and oestradiol had no such effect. There was no evidence that any of the hormones affected the prostaglandin-producing suppressor cell system.

Although at first sight the effects of the hormones alone, and on Con A-induced suppressor cell

activity, appear to be inconsistent, the results may reflect more than one process. Although the concentrations of the hormones used in our assays were not high enough to inhibit mitogen responses, all three hormones at higher concentrations are known to be inhibitory (Wyle & Kent, 1977). Hormones with these inhibitory properties might therefore be expected to reduce the generation of suppressor cell activity non-specifically, as has been shown for hydrocortisone (Knapp & Posch, 1980). If this were so, the findings of normal, and not reduced Con A-induced suppressor cell activity, in the presence of the hormones may still indicate a specific effect of the hormones on suppressor cells. The differences in these two systems remain unexplained but the effects of the hormones in the absence of Con A may well be more physiological, and of greater clinical relevance, as antigens encountered *in vivo* are unlikely to resemble Con A.

Although Con A-induced suppression in our small group tended to be greater in females than in males, this was not statistically significant and the individuals were not matched for age and other variables. Although other studies have not stressed differences between sexes, it may be of relevance that in a family study of patients with SLE, 12 of the 13 first degree asymptomatic relatives of patients with SLE found to have weak suppressor cell activity were women (Miller & Schwartz, 1979). It is also noteworthy that other diseases known to be associated with weak suppressor cell activity are also predominantly diseases of women.

To our knowledge there are no directly comparable studies on the effects of these hormones on immune regulation. The observation that testosterone increases suppressor cell function is consistent with the reduced immune responses that have been described in males compared to females and also with the observation that the heightened immune response in female mice can be reproduced by castrating male mice as long as the thymus gland is intact (Eidinger & Garret, 1972). The humoral response of castrated male mice is even greater than females and one interpretation of this is that testosterone resulted in either increased suppressor cell or reduced helper cell activity (Eidinger & Garret, 1972). A recent report showing that the incidence of auto-antibodies in alcoholic cirrhotics inversely correlates with testosterone levels is further supporting evidence (Gluud *et al.*, 1981). Another study investigating the immunoregulatory effects of oestrogens has shown that serum from rats treated with oestradiol contains a factor which inhibits most lymphocyte responses *in vitro* but that this effect can be abolished by thymectomy (Stimson & Hunter, 1980).

Our findings would, in part, explain some of the observed clinical manifestations in diseases in which defects of suppressor cell function have been noted. Thus the effects of testosterone could explain why adult males appear to be more resistent to developing autoimmune diseases than females and why children and patients with Klinefelter's syndrome behave differently. Less convincingly the increase of mitogen-induced suppressor cell activity found in the presence of progesterone could explain, in part, why patients with SLE may improve dramatically during pregnancy but relapse post partum (Garsenstein, Pollak & Kark, 1962), co-inciding with rising and falling progesterone levels respectively. It is also possible that 'weak' suppressor cell function in women with autoimmune disease might reflect on inability to generate a progesterone-dependent suppression of immune response. It should be noted, however, that we have studied only one aspect of the immunoregulatory changes which occur in pregnancy and the influence of foetal suppressor T lymphocytes which spill over into the maternal circulation may also be important (Olding & Oldstone, 1974).

These results do offer some support for the so far unconfirmed concern over the effects of the contraceptive pill on the late development of auto-immune disease (Bole *et al.*, 1969). Unfortunately the possibility of extension of these findings to clinical studies is limited because of the side effects associated with the use of these hormones *in vivo*.

In conclusion our findings indicate that sex hormones, at physiological dosage, have an effect on *in vitro* immunoregulation. These findings are compatible with some clinical observations and other previously reported *in vivo* and *in vitro* effects.

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