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The effect of testosterone replacement treatment on immunological features of patients with Klinefelter's syndrome

I. H. KOÇAR, Z. YESILOVA, M. ÖZATA*, M. TURAN†, A. SENGÜL‡ & I. Ç. ÖZDEMIR* *Departments of Internal Medicine, *Endocrinology, †Hydroclimatology and ‡Immunology, Gülhane School of Medicine, Ankara Turkey*

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SUMMARY

Although the effects of androgen deficiency in the immune system have long been appreciated, little is known about the immunological features of patients with Klinefelter's syndrome (KS). On the other hand, interest in androgens as a possible treatment for some autoimmune diseases is growing. In the present study, some immunological parameters were evaluated in 26 patients with KS prior to androgen replacement treatment (ART) and the results were compared with those in 19 healthy control subjects. Patients were then treated with testosterone for 6 months and the pre- and post-treatment findings were compared. Serum levels of IgG, IgA, IgM, C3c and C4 were measured by nephelometry and lymphocyte subsets and $CD4^+/CD8^+$ ratios were examined by flow cytometry. IL-2 and IL-4 levels were measured by ELISA. Pretreatment levels of the serum IgA, IgG, IgM, IL-2 and IL-4 of the patients were higher than those of the controls and were all decreased significantly following ART. The pretreatment absolute numbers and percentages of $CD3^+$, $CD4^+$, $CD19^+$ cells and $CD4^+/CD8^+$ ratios of patients with KS were higher than those of the controls and were all decreased with ART. Percentages of $CD8^+$ cells were increased significantly, while C3 and C4 levels were both significantly decreased after ART. It is concluded that the lack of testosterone in patients with KS enhances cellular and humoral immunity and that ART may suppress this.

Keywords Klinefelter's syndrome immunoglobulins lymphocyte subsets complement interleukins

INTRODUCTION

The striking female predominance of a number of autoimmune and rheumatic diseases in both humans and animal models has long been recognized [1,2]. It is a general perception that oestrogen may play a role in the pathogenesis of many autoimmune diseases, whereas androgens reduce autoimmune sequelae [3].

The immune system differs between females and males, possibly due to the differing actions of oestrogens and androgens [4]. Women have a potent immune capability as manifested by their higher $CD4^+$ T cell numbers, and $CD4^+/CD8^+$ ratios, and higher plasma IL-1, IL-4, IgA, IgG and IgM concentrations [4–6]. Castration of male mice leads to an increase in peripheral B cells [7], thymic enlargement and a decrease in thymic $CD3^+$ and $CD8^+$ cell counts [8]. Androgen replacement reverses post-castration changes [7]. The levels of IL-2 and IL-4 are higher in mice with testicular feminization and in those with androgen resistance, respectively [9,10]. Androgen treatment enhances IL-2 production in animal models of systemic lupus erythematosus (SLE) [11].

In humans, androgen treatment inhibits B cell hyperactivity and immunoglobulin production by mononucleated cells in patients with SLE [12] and decreases $CD4^+$ cells in post-menopausal women [13]. These observations are supported by clinical data, which suggest a role for androgens in the immune modulation of autoimmune diseases [14–16].

Autoimmune diseases are not uncommon in patients with Klinefelter's syndrome (KS), which is characterized by gynaecomastia, under-androgenization, and elevated gonadotropins, and often with elevated oestrogens [17–29]. Elevated levels of IgG and IgM have been reported in some patients with KS [20]. Lower $CD3^+$, $CD8^+$ cell numbers and $CD4^+/CD8^+$ ratios, which were all normalized with androgen replacement treatment (ART), have been reported in KS patients who also had rheumatic disease [15]. However, little is known about the immunological features of KS in patients without any associated rheumatic diseases.

We have therefore studied some immunological parameters such as the serum levels of IgA, IgG, IgM, IL-2 and IL-4, total lymphocyte counts, counts of some subsets ($CD4^+CD8^+$, $CD3^+$, $CD19^+$) and the $CD4^+/CD8^+$ ratio in patients with KS, with no

Correspondence: Professor Dr Ísmail H. Koçar, GATA, Department of Internal Medicine, 06018, Etlik, Ankara, Turkey.

E-mail: ihkocar@gata.edu.tr

associated rheumatic disease, and we have determined the effect of ART.

PATIENTS AND METHODS

Patients

Twenty-six male patients with KS (mean age 21·77 ± 1·48 years, range 20–23 years), and 19 age-matched healthy males (mean age 21·74 ± 1·50 years, range 20–23 years) were enrolled in the study. Diagnosis of KS was based on clinical features, low levels of free and total testosterone, high levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the demonstration of a chromosomal anomaly (47/XXY) on karyotype analysis. Patients had no history of KS-associated disease, nor did they display any clinical stigmata indicative of it. All controls had a history of spontaneous puberty and their physical and biochemical findings were within the normal range.

Clinical and biochemical data were assessed prior to and 6 months after ART in the patient group. All patients and controls were informed about the study and their consent was obtained. The study was approved by the ethical committee of Gülhane Military Faculty of Medicine

Treatment and analyses

Twenty-six patients with KS were treated every 2 weeks for 6 months with an intramuscular injection of Sustanon 250 (NV Organon, Oss, The Netherlands), which contains 30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate, and 100 mg testosterone decanoate.

Fasting blood samples were collected from patients and controls between 08:00 and 08:30 am. Post-treatment blood samples were drawn 7 days after the final injection of Sustanon.

Serum FSH, LH, and prolactin were measured by immunoradiometric assay with reagents from Radim Techland (Angleur, Belgium). The detection limit for FSH was 0·18 U/*l*, for LH 0·20 U/*l*, and for prolactin 1 μg/l. The intra- and interassay coefficients of variation (CVs) were 4·4% and 6·0% for FSH, 4·8% and 5·4% for LH, and 4·6% and 6·0% for prolactin. Serum-free testosterone was determined by a solid-phase ^{125}I radioimmunoassay (RIA) with reagents from Diagnostic Product Corp. (Los Angeles, CA). The detection limit for free testosterone was 0·52 pmol/*l*. The intra- and interassay CVs were 3·8% and 4·2%. Serum total testosterone and dihydroepiandrostendione sulphate (DHEAS) were measured by RIA with reagents from Diagnostic Systems Labs (Webster, TX). The detection limit for total testosterone was 0·347 nmol/*l* and for DHEAS 0·0037 μmol/*l*. The intra- and interassay CVs were 9·3% and 11·0% for total testosterone, and 7·9% and 5·8% for DHEAS. Serum sex hormone binding globulin (SHBG) was measured by RIA with reagents from Radim Techland. The detection limit for SHBG was 10 nmol/*l*. The intra- and interassay CVs were 2·4% and 2·9%. The normal ranges in our laboratory are $<$15 U/*l* for FSH, $<$20 U/*l* for LH, 41·16–138·68 pmol/*l* for free testosterone, 9·3 −37·1 nmol/*l* for total testosterone, 3·17–16·42 μmol/*l* for DHEAS, and 9–55 nmol/*l* for SHBG. The upper limit for prolactin is 12 μg/*l*.

Lymphocyte counts were performed with an automatic haemocounter (Cell-Dyn 1700; Abbott, Santa Clara, CA).

Subsets of lymphocytes were identified by flow cytometry with antibodies from Becton Dickinson (San Jose, CA), serum immunoglobulin, C3c, and C4 levels were determined by nephelometry with reagents from Behring (Behringwerke AG, Marburg, Germany). Serum IL-2 and IL-4 levels were measured by ELISA, with reagents from Biosource Int. Inc. (Camarillo, CA).

Statistical analysis

All results are given as the mean ± s.d. The significance of the differences was determined between the means of the patients and the controls by unpaired Student's *t*-tests; data from the patient group obtained before and after ART were analysed by paired Student's *t*-tests. $P < 0{\cdot}05$ was considered to be statistically significant.

RESULTS

The mean levels of hormones in the control subjects and in the patients, before and after ART, are presented in Table 1. Following ART, while the mean levels of FSH, LH, and prolactin decreased, levels of free and total testosterone increased, all significantly at the $P < 0{\cdot}001$ levels. However, significant changes were not determined with regard to SHBG and DHEAS levels.

Immunological parameters

A comparison of immunological parameters of the patients and controls is given in Table 2.

Pretreatment levels of IgG, IgA and IgM were significantly higher in the KS patients than in the controls ($P < 0{\cdot}001$). Following ART, a significant decrease was observed in the levels of these immunoglobulins ($P < 0{\cdot}001$).

Although not significant, pretreatment total lymphocyte count was higher in the patients then in the control group ($P > 0{\cdot}05$) and it decreased significantly following ART ($P < 0{\cdot}05$).

The absolute numbers and the percentages of $CD3^+$ ($P < 0{\cdot}001$, $P < 0{\cdot}001$), $CD4^+$ ($P < 0{\cdot}001$, $P < 0{\cdot}001$), and $CD19^+$ cells ($P < 0{\cdot}01$, $P < 0{\cdot}005$), the absolute number of $CD8^+$ cells ($P < 0{\cdot}01$) and the $CD4^+/CD8^+$ ratio ($P < 0{\cdot}05$) were all significantly higher in the patients before ART than in the controls. A significant difference was not observed between the two groups with respect to percentage of $CD8^+$ cells ($P > 0{\cdot}05$).

After ART, a significant decrease in the absolute numbers and percentages of $CD3^+$, $CD4^+$, $CD19^+$ cells and in the $CD4^+/CD8^+$ ratio (all at the $P < 0{\cdot}001$ level), and a significant increase in the percentage of $CD8^+$ cells ($P < 0{\cdot}01$) were observed. However, the decrease in the absolute number of $CD8^+$ cells was found to be insignificant ($P > 0{\cdot}05$).

Pretreatment IL-2 and IL-4 levels of the patients were higher than those of the controls ($P < 0{\cdot}05$, $P < 0{\cdot}001$), and were significantly reduced after ART ($P < 0{\cdot}01$, $P < 0{\cdot}001$).

It was also seen that pretreatment C3c levels in the patient group were higher than those in the control group ($P < 0{\cdot}05$). The mean pretreatment C4 levels of the patients were higher than those of the controls, but did not reach significance ($P > 0{\cdot}05$). Following ART, the mean levels of C3c and C4 in the patients decreased significantly ($P < 0{\cdot}05$, $P < 0{\cdot}005$).

DISCUSSION

It is known that a number of autoimmune and rheumatic diseases are more prevalent in females than in males [2,3], and their association with KS is not uncommon [17–19]. This predilection is ascribed to the different actions of sex steroids on the immune

Table 1. Comparison of the hormonal levels of the patients and controls

Parameter	Patients		Controls	P1	95% CI	P2	95% CI
	Pretreatment	Post-treatment					
FSH (mU/ml)	45·51 ± 14·10	25·85 ± 13·71	3·74 ± 1·65	< 0·001	36·15–47·40	< 0·001	22·30–37·02
LH (mU/ml)	32·11 ± 8·82	22·58 ± 9·03	4·08 ± 1·62	< 0·001	24·46–31·58	< 0·001	13·85–25·20
FT (pg/ml)	2·72 ± 1·70	34·62 ± 6·89	28·96 ± 10·26	< 0·001	−31·24– −21·28	< 0·001	− 14·63– −9·18
TT (ng/ml)	1·96 ± 1·34	11·77 ± 2·37	10·51 ± 1·76	< 0·001	− 9·53– −7·57	< 0·001	− 7·71– −5·92
SHBG (nmol/*l*)	44·00 ± 14·61	37·77 ± 9·81	28·04 ± 5·47	< 0·001	9·70–22·20	NS	− 1·47–13·92
PRL (μg/*l*)	7·44 ± 4·98	7·14 ± 2·91	7·32 ± 1·62	< 0·001	6·02–10·21	< 0·001	2·33–6·27
DHEAS	457·81 ± 73·91	466·96 ± 103·02	455·55 ± 102·16	< 0·001	− 249·99– −145·49	NS	− 51·43–33·13

Values are mean ± s.d.

FSH, Follicle-stimulating hormone; LH, luteinizing hormone; FT, free testosterone; TT, total testosterone; SHBG, sex hormone binding globulin; PRL, prolactin; DHEAS, dihydroepiandrostendione sulphate.

P1, Pretreatment *versus* controls; P2, Pre- *versus* post-treatment.

system [21]. The effects of sex steroids in the immune system and their role in autoimmune disease have been the subject of many studies [4,14,22].

In this study, we observed elevated pretreatment levels of IgA, IgG and IgM in patients with KS**,** all of which decreased following ART. Studies of both the level of immunoglobulins in KS and the effect of androgens on immunoglobulin levels are scarce. Elevated levels of IgG and IgM, which were ascribed to associated malignancies, have been reported in patients with KS by Tsung *et al*. [20]. A decrease in rheumatoid factor with testosterone treatment has been documented in patients with rheumatoid arthritis [22]. Castration of male mice leads to an increase in peripheral B cells [23]. Oestrogen enhances antibody production by B cells [24,25], whereas testosterone inhibits *in vitro* immunoglobulin production [12]. Androgen deficiency appears to enhance B cell responses, leading to an increased production of immunoglobulins possibly by oestrogenic influences, and ART appears to inhibit immunoglobulin synthesis in KS.

The second parameter examined in this study was lymphocyte numbers. Pretreatment total lymphocyte count in the patients was higher than in the control group, and decreased following ART, although not significantly. Long-term testosterone treatment has been reported to lead to a decrease in circulating lymphocyte counts [26]. Bizzarro *et al*. reported a decrease in the total lymphocyte count with testosterone treatment in four out of five patients with KS-associated SLE or Sjögren's syndrome (SS) [15]. However, these changes were not significant.

In this study, pretreatment $CD4^+$ T cell numbers, and $CD4^+/CD8^+$ ratios in the patients were higher than in the controls. It may be suggested that oestrogenic influences predominate in androgen-deficient conditions, increase the number of $CD4^+$ cells and cause higher $CD4^+/CD8^+$ ratios [21]. Indeed, a higher $CD4^+/$

Table 2. Comparison of the immunological parameters of the patients and controls

Parameter	Patients		Controls	P1	95% CI	P2	95% CI
	Pretreatment	Post-treatment					
IgG (g/*l*)	21·32 ± 6·11	15·33 ± 3·46	14·93 ± 2·50	< 0·001	3·40–9·39	< 0·001	3·14–8·86
IgA (g/*l*)	2·98 ± 0·86	2·15 ± 0·78	1·96 ± 0·67	< 0·001	0·54–1·48	< 0·001	0·54–1·11
IgM (g/*l*)	3·43 ± 0·92	2·14 ± 0·78	1·79 ± 0·47	< 0·001	1·17–2·10	< 0·001	0·72–1·76
IL-2 (pg/ml)	18·77 ± 10·24	13·12 ± 6·59	12·70 ± 3·55	< 0·05	1·74–10·40	< 0·01	1·41–9·89
IL-4 (pg/ml)	8·10 ± 2·58	4·93 ± 2·32	4·91 ± 2·42	< 0·001	1·67–4·71	< 0·001	1·89–4·45
C3c (g/*l*)	1·01 ± 0·45	0·77 ± 0·21	0·75 ± 0·14	< 0·05	0·07–0·46	< 0·05	0·04–0·45
C4 (g/*l*)	0·28 ± 0·08	0·23 ± 0·07	0·27 ± 0·08	NS	− 0·04–0·07	< 0·005	0·02–0·09
Lymphocyte count (mm^3)	1966·66 ± 348·10	1644·44 ± 337·67	1836·31 ± 287·58	NS	− 99·50–360·20	< 0·05	101·58–542·86
$CD4^+$ count (mm^3)	900·16 ± 163·47	605·76 ± 126·68	716·10 ± 145·41	< 0·001	33·65–240·65	< 0·001	200·51–389·35
$CD4^+$ %	45·82 ± 2·58	36·91 ± 2·95	41·40 ± 3·34	< 0·001	2·46–5·98	< 0·001	7·11–10·69
$CD8^+$ (mm^3)	506·49 ± 121·86	483·43 ± 111·34	468·31 ± 114·02	< 0·01	− 33·76–110·19	NS	− 56·76–122·87
$CD8^+$ %	25·59 ± 2·63	29·41 ± 3·44	25·55 ± 2·93	NS	− 1·62–1·71	< 0·01	− 5·92– −1·70
$CD3^+$ count (mm^3)	1407·06 ± 263·45	1091·77 ± 232·55	1242·36 ± 278·25	< 0·001	− 0·09–329·39	< 0·001	152·49–478·10
CD3 %	71·47 ± 2·84	66·30 ± 2·63	67·65 ± 3·16	< 0·001	2·02–5·61	< 0·001	3·62–6·70
$CD19^+$ count (mm^3)	275·20 ± 69·30	178·94 ± 41·13	217·73 ± 52·21	< 0·01	14·62–99·75	< 0·001	60·59–129·76
CD19 %	13·92 ± 2·03	11·01 ± 1·63	11·84 ± 2·37	< 0·005	0·77–3·40	< 0·001	2·05–3·77
$CD4^+/CD8^+$	1·81 ± 0·22	1·27 ± 0·21	1·65 ± 0·25	< 0·05	0·02–0·30	< 0·001	0·38–0·69

Values are mean ± s.d.

P1, Pretreatment *versus* controls; P2, pre- *versus* post-treatment.

$CD8^+$ ratio is generally seen in females and in hypogonadal males due to lower numbers of circulating $CD8^+$ cells [27]

However, the number of $CD8^+$ cells was higher in the patients than in the controls. This finding may not be explained by oestrogenic influences, since oestrogen lowers the number of $CD8^+$ cells [7]. However, it may be considered to be in line with the demonstration of post-castration elevation of immunohistochemical staining for $CD8^+$ cells reported in rats [28]. Similar changes have been reported in patients with idiopathic hypogonadotropic hypogonadism (IHH) [29]. Kiess *et al*. reported that the number of $CD8^+$ cells before and after treatment did not change in patients with IHH [30].

Overall, the post-treatment decrease in $CD4^+$ cell numbers and in the $CD4^+/CD8^+$ ratio, as well as increase in the number of $CD8^+$ cells observed in this study, have been reported by others both *in vivo* and *in vitro* [13,15,22,31]. It may be suggested that androgens may cause $CD8^+$ predominance over $CD4^+$ T cells. Recently, Huber *et al*. reported that testosterone treatment enhances Fas-dependent apoptosis in $CD4^+$ Th2 cells [32].

The demonstration of the effects of androgens on B and T cell populations in the periphery, in this and other studies, does not imply that androgens act directly on these cells through androgen receptor (AR) expression. In fact, the expression of AR could not be demonstrated in peripheral B cells or in bone marrow B cell precursors [33–35], nor in T lymphocytes, which are important components of hormonal effects [35]. Nonetheless, a possible explanation may come from the demonstration of AR expression in thymocytes [21] and from the effects of castration and androgen replacement on the thymuses of mice [7,36–38]. It may be postulated that the thymuses of the KS patients might not have been involuted and intrathymic cells may account for our findings.

Furthermore, it has been demonstrated that androgen action on lymphocytes may be indirectly mediated through the control of immunoactive signals from epithelial cells or macrophages [39, 40]. Hence, in addition to the functional status of the thymus, AR expression in epithelial, stromal or mononucleated cells residing in spleen and/or bone marrow should be determined before making a considered suggestion about the cellular origins of our data. In this respect, the 'unconventional plasma receptors', which were described by Benten *et al*. on splenic T cells, need to be mentioned [41].

As for interleukins, IL-2 and IL-4 levels were higher in the patients than in the controls prior to treatment, and they decreased with ART. Although no study on the levels of interleukins in KS is available, interaction between interleukins and gonadal hormones has been studied both in mice and humans [23,42]. Briefly, our data are consistent with the findings that oestrogen increases IL-4 levels [3], that female mice produce more IL-2 than males [42], and that production of IL-2 is decreased by androgens [21]. The regulatory influences of the thymus on IL-2 and IL-4 production of mature peripheral T lymphocytes in mice demonstrated by Wiedmeier *et al*. [43] necessitate again the exploration of thymic functions in KS.

The last parameter of this study was complement factors C3c and C4. Pretreatment levels of C3c were elevated, while both C3c and C4 levels decreased following ART. This finding is contrary to the results of two previous studies [15,16]. It should be stressed that the number of patients was very small and the patients also had SLE or SS in both studies. Besides, pretreatment total haemolytic complement levels did not change in three patients with KS and SS [15]. The discrepancy between our and their results may be due to the fact that androgen metabolism in the liver may differ with the route of administration and associated disease in KS. Nevertheless, we observed similar findings in patients with IHH [29]. It is also of note that dehydroepiandrosterone may inhibit the activation of complement [44].

In conclusion, our pretreatment findings suggest that both humoral and cell-mediated immunity may be enhanced by oestrogen, the actions of which predominate in testosterone deficiency. Our results are consistent with immunological changes observed in castrated mice. Post-treatment findings may be explained by the immunosuppressive effects of androgens, and they support the suggestion that ART may induce immune changes. These data allowed us to propose that the potential effect of androgen administration on the immune system should not be disregarded in clinical practice. Longitudinal studies are needed to answer the question of whether ART may be protective against the development of autoimmune diseases in KS by decreasing autoantibody synthesis.

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