Lymphocyte subset distribution and natural killer cell activity in men with idiopathic hypogonadotrophic hypogonadism

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Abstract. Sex-related differences in immune responsiveness are mediated at least in part by sex steroid hormones. Lymphocyte subset distribution in peripheral blood and natural killer cell function both have been reported to be under hormonal control. In order to gain more insight into sex steroid hormone action on the immune system, we have measured the lymphocyte subset distribution and natural killer cell activity in 18 men with idiopathic hypogonadotrophic hypogonadism before treatment, and after hormonal treatment had normalized plasma testosterone levels. In untreated patients, the mean plasma testosterone concentrations were significantly lower than those in the treated men (3.0 ± 0.5 nmol/l vs 16 ± 1.7 nmol/l, p < 0.001). The percentage of peripheral CD8+ lymphocytes, CD8+ cells, the CD4+/CD8+ ratio, and the natural killer cell activity of peripheral mononuclear cells measured in a ⁵¹Cr release assay against target K 562 cells did not differ between patients with idiopathic hypogonadotrophic hypogonadism and healthy adults, and most importantly, did not change during hormonal treatment which normalized plasma testosterone levels in the patients. In contrast, the percentage of peripheral CD4+ cells was significantly higher in untreated patients compared with normal adult subjects or patients with idiopathic hypogonadotrophic hypogonadism after hormonal treatment that resulted in normal plasma testosterone levels (53 ± 2 vs 47 ± 2, p < 0.05). It should be noted that the percentage of peripheral CD16+ cells was significantly lower in untreated men with low plasma testosterone levels than in normal controls. The percentage of CD16+ cells in peripheral venous blood rose significantly after hormonal treatment restored plasma testosterone levels to normal (6 ± 1 vs 11 ± 1, p < 0.001). In addition, the percentage of peripheral CD16+ cells correlated significantly with the plasma testosterone levels measured in men with idiopathic hypogonadotrophic hypogonadism (r = 0.534, p < 0.001). In conclusion, both the percentage of peripheral CD4+ cells (T-helper lymphocytes) and peripheral CD16+ cells (non-T-non-B cells) are related to the plasma testosterone levels in men with idiopathic hypogonadotrophic hypogonadism. These data suggest that in vivo human immune cells are under the regulatory influence of endogenous sex steroids.

Gonadal steroids such as estrogens, progestagens and androgens have long been recognized to participate in the regulation of the immune system. From the available data it has become apparent that sex hormones influence certain immune responses and modify the expression of autoimmunity in several mammalian species. Conversely, the immune system seems to be capable of exerting modulatory effects on the endocrine system (1-9). Clinically, such hormonal immunoregulation might account for the higher susceptibility to infection of males than of females, and for the fact that females develop autoimmune diseases more easily than males. For example, there is a correlation between serum estradiol levels during the menstrual cycle and the depression of cell-mediated immunity and concomitant lymphopenia (10). Furthermore, testosterone has been implicated in regulating immune functions and in particular the development and function of the thymus (11-14).

Specifically, it has been suggested in the litera-
ture that plasma testosterone levels correlate with the distribution of peripheral lymphocyte subsets: 1. Dunkel et al. reported a decrease of the CD4+/CD8+ ratio of peripheral lymphocytes in five boys after normalization of plasma testosterone levels following hCG administration (15). 2. An increase in the number of circulating T-suppressor cells (CD8+) was observed in patients with Klinefelter’s syndrome following testosterone treatment (16). To find out whether plasma testosterone levels would correlate with a certain pattern of peripheral lymphocyte subset distribution in healthy men, we have measured lymphocyte subset distribution in men with isolated sex hormone deficiency (i.e. idiopathic hypogonadotropic hypogonadism with low plasma testosterone levels and after normalization of plasma testosterone levels). Since natural killer (NK) cell activity, an immune function that is thought to play a role in vivo in the resistance to certain types of tumors and to viral infections (17-19), seems to be controlled by a number of hormones (i.e. catecholamines, growth hormone, estrogens) (8,9,19-21), we have also measured NK cell function in men with idiopathic hypogonadotropic hypogonadism to find out whether or not serum testosterone levels would influence NK cell function in vivo.

Subjects and Methods

Eighteen men (mean age 28 ± 6 years (± sd), range 17-40) with idiopathic hypogonadotropic hypogonadism (IHH) who had been referred to the National Institute of Child Health and Human Development (NICHD) between 1982 and 1986 for delayed sexual development, were studied. All of the patients fulfilled the following criteria: 1. failure to pubesce by age 17 years; 2. low plasma testosterone concentrations in the presence of low plasma LH and FSH levels; 3. normal pituitary-thyroid and pituitary-adrenal function as assessed by normal TSH, T4 and free T4 levels and a normal short ACTH stimulation test; 4. normal computed tomography of the sella, and 5. absence of underlying disease at the time of the study. Informed consent was obtained from the patients. The study protocol was approved by the Clinical Research Subpanel of the National Institute of Child Health and Human Development, NIH, Bethesda.

Reference ranges for lymphocyte subset distribution were obtained from a large number of healthy subjects (age range 22-35 years, male:female ratio approximately 2:1) (Clinical Immunology Services, Program Resources Inc, NCI, FCRF, Frederick, MD). Activity of NK cells obtained from healthy adult subjects (N=27, age range 21-35 years, 18 men, 9 women) was measured to obtain reference values for NK activity according to Maluish et al. (19,22).

Peripheral venous blood samples were drawn before the start of any therapy or after therapy had been discontinued for at least two months, and after at least four weeks of hormone treatment designed to normalize plasma testosterone and estradiol levels: the men received GnRH (Bachem,CA, 214-714 ng/kg sc, every two hours delivered via a portable minipump (Ferring, PRG)) (N=14), hCG (Ayerst, NY, 2000 IU) and hMG (Pergonal®, Serono, MA, 75 IU FSH and 75 IU LH) im every other day (N=9), or testosterone enanthate injections (2000 mg, im every other week) (N=3). The times of blood sampling were between 13.00-16.00 h. Blood was drawn into preservative-free heparinized syringes. Mean plasma testosterone levels did not differ between patients treated with GnRH, hCG/hMG, or testosterone. All three treatment protocols resulted in normal plasma testosterone levels. Certain patients were tested several times as they were placed on different treatments sequentially. Peripheral mononuclear cells were separated on Ficoll Hypaque (Pharmacia, Biotechnology Products, Piscataway, NJ) gradients according to the method of Boyum (23). The cells were then cryopreserved as described by Strong et al. (24) and stored frozen at −20°C until further testing. Cryopreservation under the conditions used does not interfere with NK cell function nor with the expression of lymphocyte surface antigens (24). The distribution of lymphocyte subsets was determined by using monoclonal antibodies (CD3, CD4, CD8, and CD16) (Becton Dickinson, Mountain View, CA) and an automatic fluorescent analyzer (Ortho Cytofluorograph, Model 30-H, Ortho Diagnostics). NK activity was determined as cytotoxicity against target cell K562 using a 51Cr release assay as described previously (17,19,22). Effector:target cell ratios were 50:1, 25:1, 12:1 and 6:1. Quantitation of NK activity was standardized by the calculation of a "set standard mean" of three normal standard samples (see control subjects) measured with each sample assay run. Data were calculated as "lytic units" as described (22).

Plasma total testosterone and estradiol (E2) concentrations were measured using specific radioimmunoassays after ether extraction and celite chromatography (24,25). The investigators who performed the immunologic measurements were blinded as to the results of the endocrine studies and the treatment schedule. Statistical analysis of the data was performed using least-square regression analysis, and paired and unpaired t-test, where appropriate. All results are expressed as mean ± sd unless stated otherwise.

Results

Plasma testosterone and E2 levels

The mean plasma testosterone level of the patients during therapy was significantly higher than that of
Fig. in higher ment.

Immunological panel). Estradiol was expressing the testosterone (E2) concentration in patients during therapy was significantly higher than that in untreated patients. The plasma E2 concentrations correlated significantly with the testosterone levels (r=0.60, p<0.001) (Table 1).

**Table 1.** Plasma testosterone and E2 levels before and after treatment in men with idiopathic hypogonadotropic hypogonadism.

<table>
<thead>
<tr>
<th></th>
<th>Without treatment</th>
<th>After treatment</th>
<th>Reference range*</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>3±0.5 (p&lt;0.001)</td>
<td>16±1.7</td>
<td>(8-40)</td>
<td>18 (44)</td>
</tr>
<tr>
<td>(nmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.07±0.001 (p&lt;0.05)</td>
<td>0.13±0.001 (&lt;0.18)</td>
<td>18 (40)</td>
<td></td>
</tr>
<tr>
<td>(nmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N indicates the number of individuals tested, number in parentheses gives the total number of determinations *in normal men. Values are mean ± SEM.

The untreated patients (Table 1) and was within the normal range for men as established in our laboratory (8-40 nmol/l). Plasma testosterone levels in all untreated men were below 8 nmol/l, whereas those in all men during therapy were higher than 8 nmol/l. The mean plasma E2 concentration in patients during therapy was also significantly higher than that in untreated patients. The plasma E2 concentrations correlated significantly with the testosterone levels (r=0.60, p<0.001) (Table 1).

**Immunological parameters**

The mean percentage of peripheral lymphocytes expressing the T-helper cell phenotype (CD4+) was significantly higher in untreated men (plasma

**Table 2.** Lymphocyte subset distribution and NK cell activity in men with idiopathic hypogonadotropic hypogonadism before and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>Without treatment</th>
<th>After treatment</th>
<th>(Standard range)*</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cells (CD3+)</td>
<td>77±1</td>
<td>75±1</td>
<td>(75±7)</td>
<td>15 (31)</td>
</tr>
<tr>
<td>T-helper cells (CD4+)</td>
<td>53±2</td>
<td>47±2</td>
<td>(45±10)</td>
<td>15 (31)</td>
</tr>
<tr>
<td>(p&lt;0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-suppressor cells (CD8+)</td>
<td>22±2</td>
<td>26±2</td>
<td>(28±9)</td>
<td>15 (31)</td>
</tr>
<tr>
<td>(NS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+/CD8+ ratio</td>
<td>2.7±0.3</td>
<td>2.1±0.3</td>
<td>(NS)</td>
<td></td>
</tr>
<tr>
<td>NK cells (CD16+)</td>
<td>6±1</td>
<td>11±1</td>
<td>(15±7)</td>
<td>15 (31)</td>
</tr>
<tr>
<td>(p&lt;0.001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK cell activity</td>
<td>31±5</td>
<td>41±5</td>
<td>(33±4)</td>
<td>18 (39)</td>
</tr>
<tr>
<td>(NS)</td>
<td></td>
<td></td>
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</table>

Lymphocyte subset distribution is expressed as % of total peripheral mononuclear cells expressing the indicated phenotype. NK activity is expressed as lytic units. Values represent mean ± SEM. N indicates the number of individuals tested, number in parentheses gives the total number of determinations. NS: not significant * in normal men.

**Fig. 1.**

Expression of the CD4+ antigen on peripheral mononuclear cells in patients with idiopathic hypogonadotropic hypogonadism before (●) and after (○) hormonal treatment. Correlation coefficient was -0.324, p = 0.075 (left panel). The percentage of CD4+ cells was significantly higher in patients with low plasma testosterone levels than in treated patients (p<0.05) (right panel).

**Fig. 2.**

Expression of the CD16+ antigen on peripheral mononuclear cells in patients with idiopathic hypogonadotropic hypogonadism before (●) and after (○) hormonal treatment. Correlation coefficient was 0.534, p < 0.001 (left panel). The percentage of CD16+ cells was significantly higher in patients with normal plasma testosterone levels than in untreated patients (p < 0.001).
testosterone below 8 nmol/l) than in men with normalized plasma testosterone (testosterone higher than 8 nmol/l) and in normal healthy adults (Table 2 and Fig. 1), whereas the mean value of the hormone-treated men was similar to that of normal controls. However, there was no direct correlation of CD4+ expression with either plasma testosterone or E2 levels (Fig. 1, and data not shown).

The percentage of circulating T-cells (CD3+) and T-suppressor cells (CD8+) in untreated men did not differ from the values measured in treated patients with normalized plasma sex steroid concentrations. Also, CD3+ and CD8+ phenotype expression was within the normal laboratory range in both treated and untreated patients. In addition, the ratio of T-helper/T-suppressor cells (CD4+/CD8+) in untreated men did not differ significantly from that in the treated patients, and all values were within the laboratory reference range (Table 2). Surprisingly and most importantly, the number of peripheral mononuclear cells expressing the CD16+ phenotype was significantly lower in untreated patients than in both treated patients and in normal adult control subjects (Table 2, Fig. 2). In addition, the number of circulating CD16+ cells correlated significantly with the plasma testosterone levels (Fig. 2). However, when NK cell activity was measured as cytotoxicity against target K 562 in a 51Cr release assay in untreated patients and men with normalized plasma testosterone and E2 levels, the NK activity was not different in the two groups (Table 2). In addition, NK cell activity did not correlate with plasma testosterone levels (data not shown). In seven patients, lymphocyte subset distribution and NK cell activity were measured before therapy and four weeks after hormonal treatment had normalized plasma testosterone levels (plasma testosterone before: 1.7 ± 0.6 nmol/l; after hormonal treatment: 18.5 ± 3.5 nmol/l; mean ± SEM. The numbers of peripheral mononuclear cells expressing the CD3+, CD4+, and CD8+ phenotypes did not change after four weeks of GnRH or hCG/hMG treatment (Fig. 3). In addition, the ratio of CD4+/CD8+ cells did not change with treatment (data not shown) in this small group of patients. However, the number of CD16+ cells in peripheral venous blood was significantly lower in untreated than in treated patients (Fig. 3, Panel B right). No significant difference in NK cell activity measured as 51Cr release from target K 562 was observed between treated and untreated men (Fig. 3, Panel B left).

**Discussion**

Men with idiopathic hypogonadotropic hypogonadism (IHH) have low plasma sex steroid levels owing to deficient stimulation of gonadotropin release from the pituitary gland secondary to hypothalamic GnRH deficiency. All the patients with IHH in this study (N=18) had low plasma testosterone levels before treatment. After they were placed on testosterone, gonadotropin or GnRH treatment, plasma testosterone levels normalized (>8 nmol/l). In addition, plasma E2 levels were significantly higher during than before treatment, but the levels were within the normal range for men (<0.18 nmol/l) both before and after treatment (24). Untreated patients with low plasma testoste-
rone levels (<8 nmol/l) had significantly higher percentages of circulating T-helper cells (CD4+) than treated men with normal levels (>8 nmol/l). In contrast, no significant difference between treated and untreated patients was observed with regard to the percentage of T-suppressor cells (CD8+), total T-cells (CD3+), and the CD4+/CD8+ ratio in peripheral blood.

A role of sex steroids in the development and function of the thymus has been suggested (3-5). Dunkel et al. reported a significant decrease in the CD4+/CD8+ ratio of peripheral blood lymphocytes of five prepubertal boys after normalization of plasma testosterone levels following hCG administration (15). In addition, an increase in the numbers of circulating T-suppressor cells (CD8+) in patients with Klinefelter's syndrome and associated autoimmune disease was observed following testosterone treatment (16). Thus, both studies indicate a possible role of testosterone in T-cell suppression in vivo. The reason for the lack of correlation between T-suppressor cell numbers and testosterone levels in peripheral blood in men with IHH in our study is not clear. It is possible that the differences in the results obtained in the three studies are due to the different patient populations studied. We found that the circulating CD4+ cell numbers in untreated patients were higher than those in men with normalized plasma testosterone levels. This observation is congruent with the study of Dunkel et al. in which a decrease of circulating T-helper cells (CD4+) was noted in 3 of their 5 patients after gonadotropin treatment.

It is of interest that androgen receptors have been isolated and characterized in the thymus (12,26). These data together suggest that thymus-derived lymphocytes might be androgen target cells. It should be noted that the percentage of CD16+ cells in peripheral blood of untreated patients with IHH with low testosterone plasma levels was significantly lower than in treated patients with normalized testosterone. The expression of the CD16+ antigen correlated significantly with the testosterone levels. Lymphocytes expressing the CD16+ phenotype are mainly non-T-non-B lymphocytes with NK cell activity (27). NK cells are thought to play a role in the resistance to certain types of tumours and to viral infections (17,18). However, in our study no significant correlation between testosterone levels and NK cell activity as measured in a $^{51}$Cr release assay against target K 562 was found.

Several possible factors might account for the lack of a significant correlation between NK cell activity of peripheral blood lymphocytes and plasma testosterone levels in men with IHH. First, the well known biologic variability of the NK cell activity might make detection of a small difference difficult. Second, the NK assay used might be relatively insensitive to detect such small differences in the patient population studied. Third, the treatment periods (4-6 weeks before testing) might be too short to cause detectable changes in NK cell activity. In addition, a subpopulation of immune cells bearing the CD16+ antigen, but not exerting NK activity, might be responsive to testosterone in vivo. Immune cells, bearing the CD16 antigen but not exerting NK activity have been described. There is evidence that such cells might have immunoregulatory functions (17,28-30). Since plasma E$_2$ levels were also significantly higher in treated men with IHH than in untreated men an opposite effect of E$_2$ on NK function could potentially mask a direct effect of testosterone on NK cell function.

Are sex steroid hormone-mediated differences in immunity of clinical relevance? IHH is generally not correlated with clinical immunodeficiency (i.e. increased susceptibility to infections). However, there is a large body of evidence that females develop autoimmune diseases more easily than males. In contrast, the weaker immune responses in males might contribute to the higher susceptibility to infection of males compared with females (1,2,4,5). It is therefore of considerable clinical relevance to gain more insight into the mechanisms of sex steroid hormone action on the immune system and to study such clinical phenomena which suggest the interaction between the hormonal and the immune system in more detail. Treatment of autoimmune disease might possibly include hormonal therapy.

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