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Immune sexual dimorphism: Effect of gonadal steroids on the expression of cytokines, sex steroid receptors, and lymphocyte proliferation

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ABSTRACT

The aims of this study were, first, to explore the differences in the expression of Th1/Th2 cytokines and of steroid receptors in spleen of intact and gonadectomized mice of both sexes; second, to evaluate the effect of estradiol (E2), progesterone (P4) and testosterone (T) on cytokine production and lymphocyte proliferation, and third, to determine the percentage of spleen cell subpopulations in both sexes. Results indicated dimorphic expression of IFN-γ and IL-4, which was affected by gonadectomy. CD4+ T lymphocytes were the most frequent type of cell in the spleen, followed by B lymphocytes (CD19+). Interestingly, there was no dimorphic pattern of cell subtypes, and gonadectomy had no effect. Regarding lymphocyte proliferation, E2 inhibited both cells of male (19.51%) and female (24.62%). P4 diminished lymphocyte proliferation by 22% in cells of female and had no effect on cells of male. It is very interesting to note that the sex steroid receptors mRNA was highly expressed in all splenocytes, and that this expression was dimorphic. However, flow cytometry analysis confirmed that only expression of progesterone receptor was dimorphic. This dimorphic pattern was, however, only seen in lymphocytes. Present evidence indicates that sex steroids are capable of affecting crucial immune system functions dimorphically.

1. Introduction

It has been repeatedly shown that sex, and its associated steroids, significantly influence various aspects of the immune system. A number of reports on immune and neuroendocrine system interactions indicate that hormones are capable of affecting immune functions [1].

The importance of the interaction between the immune and endocrine systems becomes evident in circumstances such as pregnancy, autoimmune diseases and some infectious diseases. In all cases, the available evidence underscores the importance of sex steroids as immunoregulators [2,3].

Although much information remains to be elucidated, these hormones are able to regulate processes implicated in the immune response, including the maturation and selection of thymocytes, cellular transit expression of the major histocompatibility complex class II molecules, lymphocyte proliferation and cytokine production [4]. These functions involve a large repertoire of highly specialized cells that perform different functions with precision and efficacy. Molecules secreted by components of the immune system delicately regulate these cells, but they are also susceptible to regulation by hormones, neurohormones and/or neurotransmitters apparently distant from the immune system. Thus, the system was originally thought to be largely autoregulated, however, it has become increasingly clear that, together with the neuroendocrine system, both systems are directly and bidirectionally interconnected [5]. In this way, physiological systems that integrate complex organisms interact forming networks of mutual control, which favor the correct achievement of their specific functions and of the more general requirements of the complete organism [6]. This interaction plays a relevant role for evolution of vertebrates.

Sex hormones apparently play an important role in the differences in susceptibility associated to sex in certain infectious and autoimmune diseases [7]. Females of different species are known to produce higher levels of circulating immunoglobulins than males, and display a more pronounced humoral immune response against infection. The production of a variety of autoreactive antibodies is also more frequent in females [8]. Estrogens have been found to increase the B cell response both *in vivo* and *in vitro*, while androgens and progesterone diminish antibody production. However, the mechanisms of action of these hormones remain largely unknown. According with these observations, it has been suggested that estrogens potentiate immunity mediated by B cells and suppress some

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mechanisms dependent on T cells [9]. Testosterone seems to suppress both the T and the B cell mediated response [10,11]. In addition to the different immune factors implicated in the regulation of the complex cytokine network, evidence has been found that gender is an important factor in the determination of the secretion pattern of these proteins [12], which suggests that sex steroids may be responsible for these differences. For these hormones to have an effect on immune system cells, the presence of hormone receptors in these cells is necessary. Although steroid hormones also exert effects by non-genomic mechanisms by acting on cell surface receptors and triggering signaling cascades [13,14], it is currently accepted that the main route of biological activity occurs by means of specific nuclear receptors (NR) that function as transcription factors, and coordinate, after binding to their ligand, the expression of target genes [15,16]. The following NR are mediators of these effects: estrogen receptors (ER), ER-α, ER-β, each coded for by an individual gene, whose predominating ligand is E2; progesterone receptor (PR), which has variants A and B generated from the same gene by alternative splicing, whose main ligand being P4, and androgen receptors (AR), coded for by a single gene, its ligands being estosterone (T) and dehydrotestosterone (DHT). One effect of steroids, crucial in the regulator and effector functions of the immune system, and which has not been fully explored yet, is its action on cytokine production and lymphocyte proliferation. According with experimental evidence that supports that host sex is relevant, the object of the present study was to explore the sexual dimorphism of these important aspects of the immune response *in vivo* and*in vitro*.

2. Materials and methods

2.1. Mice and surgical processes

Six week-old BALB/c AnN mice of the two sexes were used in this study. They were fed *ad libitum* with Purina Diet 5015 and water, and kept in light/dark cycle (14 h light:10 h dark). When mice were four weeks old, they were castrated under pentobarbital anesthesia, as reported previously [17] other group received control surgeries (sham) and intact animals were used as controls. Mice were allowed a two-week recovery period before tissue extraction. After this period, mice were sacrificed by cervical dislocation after deep pentobarbital anesthesia and spleen, lymph nodes and gonads were obtained.

Animal care and experimentation practices at the Biomedical Research Institute are frequently evaluated by the University Animal Care and Use Committee and by governmental agencies to ensure compliance with established international regulations and guidelines.

2.2. RNA extraction

Total RNA was isolated from mouse testes, uterus, ovary (positive expression control tissues for sex steroid receptors), spleen and splenocytes by the extraction method using TRIzol reagent (Gibco-BRL, USA). Briefly, each tissue was removed and immediately disrupted in TRIzol reagent (1 mL/0.1 g tissue), and 0.2 mL of chloroform were added per mL of TRIzol. The aqueous phase was recovered after 10-min centrifugation at 15,000 × *g*. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and re-dissolved in RNase-free water. RNA concentration was determined by absorbance at 260 nm and its purity was verified after electrophoresis in 1% denaturing agarose gel in the presence of 2.2 M formaldehyde. Total RNA from all extracted tissues was reverse-transcribed, followed by specific PCR amplification of cytokines (IFN-γ, IL-2, IL-4, IL-6) and sex steroid receptors (ER-α, ER-β, PR-A, PR-B, AR) as well as the constitutively expressed gene β-actin.

Table 1
Sequences of the primers used for PCR amplification of total spleen RNA reverse transcribed.

Gene	Sense primer	Antisense primer
IL-2	5′-tgatggacctacaggagctcctgag	5′-gagtcaaatccagaacatgccgcag
IL-4	5′-cgaagaacaccacagagagtgagct	5′-gactcattcatggtgcgacttatcg
IL-6	5′-atgaagttcctctctgcaagag	5′-cactaggtttgccgagtagat
IFN-γ	5′-agcggctgactgaactcagattgtag	5′-gtcacagttttcagctgtataggg
PR-A	5′-cagtggtggatttcatccatg	5′-cttccagagggtaggtgcga
PR-B	5′-ggaggcagaaattccagacc	5′-gacaacaacccttggtagc
ER-α	5′-agactgtccagcagtaacgag	5′-tcgtaacacttgcgcagccg
ER-β	5′-catctgggtatcattacggtg	5′-ggcacttctctgtcttcg
AR	5′-gaatgtcagcctatctttctta	5′-tgcctcatcctcacacactggc
β-Actin	5′-gggtcagaaggattcctatg	5′-ggttctaaacatgatctggg

Primers were designed based on these mouse sequenced genes (Gene databank, NCBI, NIH).

2.3. Retrotranscription-polymerase chain reaction (RT-PCR)

Nucleotide sequences of primers used for amplification are shown in Table 1. Briefly, 4 μg of total RNA from each tissue were incubated at 37 °C for 1 h with 400 units of M-MLV reverse transcriptase (Applied Biosystems, Boston, MA, USA) in 15 μL of reaction volume containing 10 mM of each dNTP and 0.01 μg oligo (dt) primer (Gibco, long Island, NY). Five μL of the cDNA reaction were injected to PCR to amplify specific sequences of the specified genes. The 50 μL PCR reaction included 5 μL of previously synthesized cDNA, 5 μL of 10× PCR-buffer (Biotecnologías Universitarias, UNAM, México City) 30 mM $MgCl_2$, 10 mM of each dNTP, 15 μM of each primer, and 0.5 units of Taq DNA polymerase (Biotecnologías Universitarias, UNAM, Mexico City). Twenty-five μL of the total PCR reaction products of each sample were electrophoresed on 2% agarose gel. PCR products were visualized by staining with ethidium bromide. A single band was detected in all cases, as expected. In order to determine that progesterone receptor (A and B), estrogen receptor (α and β), androgen receptor and each cytokine (IFN-γ, IL-2, IL-4, IL-6) as well as β-actin were in the exponential phase of amplification, and to be assured that changes in these molecules are not artifactual (such as β-actin being in the stationary phase), we performed RNA, cycling and temperature curves for each treatment and tissue studied. The expression of β-actin was used as an internal control at all times and in all tissues studied. In all samples, a single product corresponding to the amplification fragment expected for PR-A (197 nt), PR-B (198 nt), ER-α (251 nt), ER-β (239 nt), AR (365 nt), IFN-γ (247 nt), IL-2 (168 nt), IL-4 (181 nt) and IL-6 (638 nt) and β-actin (238 nt) expression was obtained. After an initial denaturation step at 95 °C for 5 min, temperature cycling was initiated as follows: 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min during 25 cycles for PR-A, PR-B and β-actin, 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min during 30 cycles for ER-α and ER-β, 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min during 25 cycles for AR, 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min during 30 cycles for IFN-γ, 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min during 25 cycles for IL-2, and 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min during 30 cycles for IL-4 and IL-6. An extra extension at 72 °C was done during 10 min for every gene.

2.4. Densitometric analysis

Hybridization signals were quantified by densitometric scanning of multiple autoradiograms of various exposures, and were represented as the ratio of the signal from the problem gene, relative to the expression of β-actin, a constitutively expressed gene used as internal control (relative expression). The expression of all genes is numerically presented as the ratio of the optical den-