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Preliminary report

Antibody responses in hyperthyroid rats

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Abstract

This study evaluated antibody production against sheep red blood cells (SRBC) in hyperthyroid rats during treatment with triiodothyronine (T_3). The immune response was evaluated by measuring plaque forming cells (PFC) in the spleen and by enzyme-linked immunosorbent assay (ELISA) in serum of male Wistar rats (180±10 g) treated with 25 μg/day of triiodotironine (T_3) during 7–12 days and immunized with SRBC at the 8th day of treatment. The results showed that anti-SRBC antibody production was significantly decreased in animals treated for 12 days when compared to normal rats immunized with the same antigen, as evaluated by the two assays. These results show that in this experimental model hyperthyroidism decreases antibody response. We previously observed the opposite effect, that is, an increase in this response in hypothyroid rats resulting from the treatment with propylthyouracil, a blocker of thyroid hormone biosynthesis. It is suggested that antibody production is affected by thyroid hormone levels.

Keywords: T_3; Thyroid hormones; Hyperthyroidism; Antibody response

1. Introduction

Triiodothyronine (T_3) and thyroxine (T_4) are secreted by the thyroid gland and T_4 converted to T_3 in peripheral tissues. The hormones have many important functions in the body such as binding to cell nucleus receptors and regulating gene expression through changes in DNA transcription and mRNA stabilization. Thyroid dysfunction due to autoimmune processes is associated to suppressive or stimulatory events of the immune response. Thyroid stimulating (TSH) and thyroxine releasing (TRH) hormones, the regulators of thyroid function as well as T_3 and T_4 are related with the immune system as lymphocytes and thymus cells have nuclear receptors for thyroid hormones [1–3]. Reports in the literature show that cell functions may be modified minutes or seconds after administration of these hormones indicating effects also at the cell membrane [4]. TRH and TSH increase antibody syntheses, and affect B lymphocyte differentiation [5,6]. Activation of T lymphocyte sub-classes, reduction of natural killer (NK) cells and decrease in responsiveness of T CD4 lymphocytes to thyroid and other antigens occur in severe hyperthyroidism [7]. On the other hand, Pacini et al. [8] verified that induced hyperthyroidism in rats did not affect the ratio of helper/suppressor T lymphocytes.

Observations that TSH production in response to some pathogens may increase antibody response to thymus-dependent antigens in this condition also point to an association between thyroid function and the immune system [9]. TSH was shown to increase anti-sheep SRBC

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antibodies response [6] but a decrease of antibody production was observed in stress caused by high levels of T_4, T_3 and corticotrophin [10].

Kai et al. [11] showed that in hypothyroid, propylthyouracil (PTU) treated chickens the antibody production and the cellular immune response could be increased, not affected or decreased depending on drug concentration. In a previous study in our laboratory hypothyroid rats treated with the same drug showed increased antibody production against SRBC, 2–3 weeks after treatment [12]. As reported in the literature patients with thyroid disease, generally of autoimmune origin involving genetic, environmental or other endogenous factors, are evaluated not only by thyroid hormone levels but also by their immune responses. For example, in Grave's disease produced autoantibodies stimulate the gland TSH receptor (TSH-R) due to many factors. The major histocompatibility complex (MHC) of classes I and II and genetic polymorphism seem to be involved [13].

Recent investigations relating thyroid hormone to the immune response in the absence of interference resulting from pathologic conditions or other hormone alterations are scarce in the literature. Cope and Kapnic [14] correlated nutritional status to hypo- and hyperthyroidism but did not observe alterations in the immune response to pathogens in these conditions. However, their methods and results cannot be interpreted according to the knowledge now available. In this context the present work was undertaken to investigate the production of antibodies against SRBC in hyperthyroid rats treated with T_3.

2. Material and methods

2.1. Materials and reagents

Triiodothyronine (T_3) from Purifarma® (Brazil) was used in the treatment of rats. Kits for T_4 and T_3 determination were from Diagnostic Products Corporation (DPC, USA); low melting point agarose from Gibco (USA); Tween-20 from Merck (Germany); goat anti-rat IgM (μ-chain specific) labeled with alkaline phosphatase were from Southern Biotechnology Associates (USA); *p*-nitrophenylphosfate (*p*-NPP) tablet sets, alkaline phosphatase substrate was from Sigma (USA); microtitration plates, Immulon-2HB, were from Thermo Labsystems (Finland).

2.2. Determination of protein concentration

Proteins were quantified by the method of Bradford [15].

2.3. Animals and treatments

The SPF ("*specific pathogen free*") animal breeding house from the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of S. Paulo supplied young male adult Wistar rats, weighing 180±10 g, which were maintained under the same conditions. During the treatment animals received food ration and water *ad libitum*. The T_3 vehicle was a suspension (CMC) consisting of carboxymethylcellulose (CMC) 0.5 g; ethyl 4-hydroxybenzoate, 0.18 g; propyl 4-hydroxybenzoate, 0.02 g and water to 100 ml. Rats were divided into an experimental and a control group receiving daily 1 ml of the T_3 suspension or 1 ml of CMC suspension, respectively, both by gavage. Both groups were treated during 12 days and in the 8th day the rats were immunized with 0.6 ml of a 10% suspension of SRBC in phosphate-buffered saline (PBS), given intraperitoneally. The primary immune response was evaluated in the 12th day of treatment. Rats were euthanized according to criteria approved by the Committee of Ethics in the Use of Animals of the Campus of Ribeirão Preto, University of S. Paulo, USP, CEUA, process number 05.1.1160.53.9.

2.4. Samples

Twenty-four hours after the end of each period of treatment, the animals were decapitated, blood samples collected and serum prepared and stored at −70 °C. The spleen was excised and used to evaluate the number of PFC, which is the antibody-producing response.

2.5. Determination of serum levels of thyroid hormones

Total levels of T_3 and T_4 in serum were determined by chemiluminescence assays (Immulite model 1000, DPC) using commercial kits.

2.6. Preparation of spleen cells

Spleen cells were obtained from the excised organ, suspended in HBSS (Hanks' balanced salt solution), washed and finally suspended in 3 ml of HBSS per spleen. The number of cells was determined using Trypan blue in a Neubauer chamber and the suspension standardized at two concentrations: 2.5×10^5 cells/ml and 2.5×10^6 cells/ml.

2.7. Assay of PFC

The number of anti-SRBC antibody (IgM) secreting cells was determined by PFC assay. Briefly, 100 μl of

a standardized suspension of spleen cells (2.5×10^5 cells/ml or 2.5×106 cells/ml) was added to glass tubes containing 400 μl of agarose 0.5% in HBSS, previously heated to 37 °C. To the tubes maintained in a water bath at 37 °C was added 50 μl of 5% SRBC in sterile PBS, the contents of the tubes mixed and poured onto glass slides of 2.5×7.5 cm (previously covered by 0.5% agarose in water and dried for 48 h). Following agarose solidification, the slides were inverted and placed in acrylic holders containing HBSS-filled troughs, and incubated for 150 min in an atmosphere of 5% CO_2 at 37 °C. Following addition of 1:5 (v/v) HBSS-diluted guinea pig serum the slides were incubated in the same conditions for 40 min. Lysis sites corresponding to plaques were counted by visual inspection under a magnifying glass.

2.8. ELISA

Sheep blood of two animals was washed thrice with saline solution (NaCl 0.9%) to obtain red cells and the membranes, which were prepared according to Temple et al. [16,17]. Briefly, red cells previously washed were lysed by Tris–ethylenediaminetetraacetic acid (EDTA) buffer (0.05 M Tris–HCl, 0.1 mM EDTA, pH 7.6), and centrifuged at 25,100×*g* for 30 min. The process was repeated until the supernatant was colorless. The membranes were suspended in Tris–EDTA buffer, filtered and resuspended in 0.1% sodium dodecyl sulfate (SDS) in PBS (with 0.02% sodium azide). The suspension of membrane antigens was dialyzed for 48 h against 0.1% SDS in PBS at room temperature and stored at 4 °C.

2.9. Preparation of the microtitration plates for the ELISA assay

Microtitration plates were coated with membrane antigen by incubation with 125 μl of the antigen suspension in PBS (5 μg/ml) at 4 °C overnight. The plates could then be used immediately or kept in this condition for up to 30 days. One column in each plate was kept uncoated to serve as a control.

2.10. Assay

The plates were washed thrice with PBS-T (phosphate-buffered saline, 0.5% Tween-20), incubated at room temperature for 2 h with 5% powdered milk solution, and again washed. Serial dilutions (1:16 for IgM) of treated and control rat serum was added to each well (125 μl/well) and plates incubated at room temperature. After 2 h of incubation plates were washed thrice with PBS-T, and goat anti-rat IgM, at optimal dilution (1:1000, v/v) were added to the wells. Following incubation for 1 h at room temperature, the plates were thrice washed with PBS-T and 125 μl of substrate solution prepared according to the manufacturer's instructions were added/well. After 15 min of incubation at room temperature in the absence of light, the content of each well was analyzed at 405 nm (EIA multi-well reader, Sigma diagnostics). The assay had the following controls: (a) coating control, where all the steps of ELISA were excluded; (b) only substrate was added to the plate for the substrate control; (c) for control of nonspecific antibody absorption serum samples were excluded; (d) for the positive control the serum sample used was a pool from rats immunized with SRBC; (e) for the negative control the serum sample used was a pool from non-immunized rats.

2.11. Statistical analyzes

Results for the control and treated animals were compared by the Mann–Whitney test using the software GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, California [18]. Statistical significance was considered for $p < 0.05$.

3. Results

Normal rats were immunized with SRBC (Fig. 1) as a preliminary PFC assay to select an adequate period for antibody evaluation. The highest number of PFCs was observed 4 days after antigen injection and this period was then utilized in the subsequent assays.

Groups of rats were treated daily for with 25 μg of T_3 for 12 days and immunized in the 8th day with a suspension of SRBC. As expected, treatment of rats

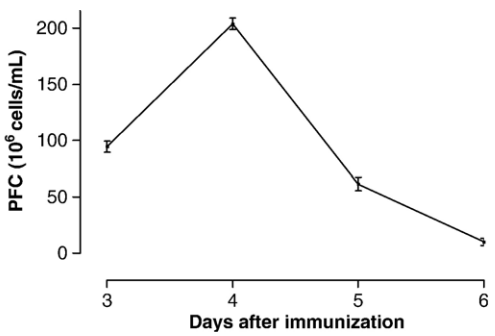

Fig. 1. PFC numbers at different time intervals after immunization of normal rats with 0.6 ml of a 5% SRBC suspension. Results obtained with 10^6 spleen cells/ml; $n = 3$ animals/day.

with T_3 increased serum T_3 levels (Fig. 2) and decreased TSH. T_4 concentrations were undetectable in this condition. Table 1 shows that hyperthyroidism leads to a significant decrease in the number of PFC in approximately 71% of the animals as compared to rats immunized but not treated with T_3 (controls).

As controls to our assay conditions, antibodies were evaluated by ELISA in a *pool* of serum from rats immunized with SRBC but not treated with T_3 (control) and in a *pool* of normal rats not treated with T_3 and not immunized (negative control) (Fig. 3). A significantly decreased production of IgM antibodies anti-SRBC in rats treated with 25 μg of T_3 when compared to controls ($p=0.0286$) was observed (Fig. 3). The levels of IgM anti-SRBC in treated rats were decreased to values similar to that of rats not immunized with SRBC (negative control).

4. Discussion

The production of antibodies against SRBC was reduced in rats treated with T_3 as evaluated by the PFC assay and ELISA indicating that high levels of this hormone may decrease immune responses. The effect was observed after the animals were treated with T_3 (25 μg/day) for 12 days and immunized at the 8th day of treatment. In these conditions serum T_3 concentration is high and TSH is low, characterizing a state of induced hyperthyroidism. T_4 levels were undetectable in our conditions. This effect does not occur if the T_3 treatment is stopped at the 7th day, i.e., before immunization. Thus, the build up of the immune response seems to require maintenance of altered levels of T_3 (and/or of TSH and probably T_4).

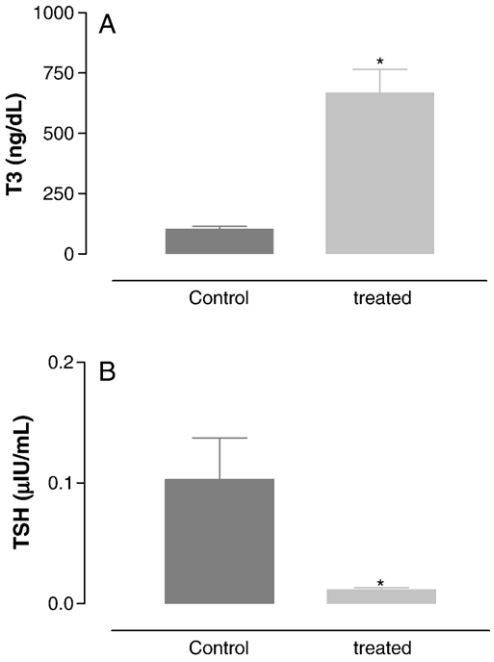

Fig. 2. Serum levels of thyroid hormones in controls and rats treated with 25 μg of T_3/day for 12 days ($n=10$). (A) Levels of T_3, *$p=0.0043$. (B) Levels of TSH, *$p=0.0022$ (Mann–Whitney test).

Table 1
Primary immune response estimated by the number of spleen PFCs in rats treated with T3 [a] and immunized with SRBC

Number of experimental animals [b]	Experimental animals showing decreased PFCs	
	Number	Percentage (%)
32	23	71.87

[a] Treated with 25 μg T3/day for 12 days and immunized at the 8th day with SRBC.
[b] An equal number of controls were evaluated.

Reports in the literature correlate supra normal levels of thyroid hormones to alterations in the immune response. Hassman et al. [19] observed response suppression in an animal model of autoimmune hyperthyroidism induced by T_4 treatment and immunization with SRBC. Keast and Ayre [20] verified that the primary immune response in chickens is regulated by T_3. A review on the subject [7] reinforces the fact that hyperthyroidism in humans has several effects on cellular and humoral immune responses.

We have previously shown that thyroidectomized rats have increased production of anti-SRBC antibodies [21]. Similarly, significant increases of this response were observed in treatments with the anti-thyroid drug

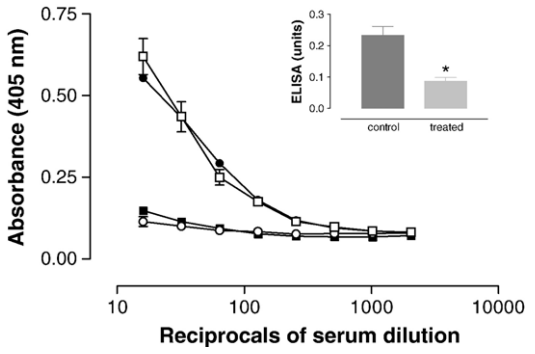

Fig. 3. Immune response (IgM) against SRBC evaluated by ELISA. Rats ($n=8$) were treated with 25 μg of T_3/day for 12 days and immunized at the 8th day of treatment with 0.6 ml of a 5% SRBC suspension (○). (□) Controls receiving CMC suspension and immunized. Same control is presented in the insert. (●) Positive control: pool of serum from normal rats immunized with SRBC; (■) negative control: pool of serum from normal rats (NRS). Insert: results as expressed in ELISA units. *$p=0.0286$ (Mann–Whitney test).

propylthyouracil, but the effect was dose-dependent and according to the period of treatment [12]. Together, these results and the ones obtained in the present study suggest that in this experimental model thyroid hormones influence the build up of the antibody response. These data are important considering that these alterations were observed in the absence of pathological processes such as thyroid autoimmune diseases, and therefore may add to the understanding of the role of thyroid hormones in these diseases.

The mechanisms underlying thyroid hormone effects on the immune response are not completely understood. T_3 treatments in mice lymphocyte cultures alter DNA synthesis and antibody production stimulated by PHA [3]. In mice deficient in anterior pituitary hormones (Snell dwarf mice) bone marrow cellularity in thyroxine-treated dw/dw animals was comparable to that in control, and both the frequency and absolute number of B lineage cells increased to normal. Zabelina et al. [22] showed that thyroid hormone levels positively correlate with concentrations of immunocytes and negatively with immunoglobulins. Our laboratory has previously shown that complement system activity is increased in hypothyroid rats and suggested that these alterations could be related to the increased antibody response observed in this condition [23]. Considering our present results and the role of the CS in the immune response, future studies will focus on this system in our model of experimental hyperthyroidism.

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