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Hyperthyroid state or in vitro thyroxine treatment modulates $T_H1/T_H2$ responses during exposure to HSV-1 antigens

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Abstract

In recent years, thyroid hormones (THs) have been considered to be important regulators of the immune system. However, their roles in host defense against viral infections have not been clearly established. Therefore, this study was undertaken to examine proliferative activity and cytokine production by lymphocytes isolated from hyperthyroid and euthyroid Balb/c mice in response to herpes simplex virus-1 (HSV-1). Lymphocytes of hyperthyroid animals showed a significantly higher rate of proliferation and interferon (IFN)-γ production when compared with that by lymphocytes from euthyroid mice. In vitro thyroxine (T4) treatment was similarly effective in the potentiation of proliferation, but not IFNγ production, by euthyroid lymphocytes. Furthermore, the hyperthyroid state significantly attenuated ConA-, but not HSV-1-, induced interleukin (IL)-10 release; in vitro T4 treatment synergized this effect. These findings suggest that supra-physiologic TH levels (i.e. as occur in hyper-thyroid states) or in vitro TH treatment modulates T-helper (Th)1/Th2 lymphocyte responses and thereby amplify host defenses against viral infections. One may also conclude that THs may have a potential application in viral immunization and/or treatment of viral infections.

Introduction

It is now accepted that almost all tissues in the body are affected by thyroid hormones (THs). Although THs are best known for their indispensable roles in developmental process and tissue metabolism (Babu et al., 2012; Duncan Bassett & Williams, 2003; Schmalzt, 2012; Silva, 2001), their roles in immune system homeostasis are only beginning to be explored. In 1973, it was reported that removal of the thyroid gland leads to significant reduction in peripheral lymphocytes, impaired humoral immunity, and reduced sensitivity of lymphocytes to mitogen (Fabris, 1973).

Available data from several lines of in vivo and in vitro studies show that functions of almost all immune system cells, including monocytes, thymocytes, lymphocytes, natural killer (NK) cells, and splenocytes, are affected by THs (Csaba et al., 1977; Hodkinson et al., 2009; Mascalfroni et al., 2008; Savino et al., 1990).

Tang & Kaptain (1989) were perhaps the first to show that alterations in serum levels of THs modulate host defense against viral infection. THs are able to enhance sensitivity of lymphocytes to interferons (IFN) and promote IFN-mediated cytotoxic activity of NK cells (Provinciali & Fabris, 1990, 1994). Interferons (i.e., α, β, and γ) are small regulatory cytokines that were originally identified as anti-viral factors and play pivotal roles in immune responses to viral infection (Wang & Fish, 2012).

It has been shown that THs enhance anti-viral actions of these cytokines and are essential for execution of their actions (Lin et al., 1994, 1996; Ouyang et al., 2011). Data from recent studies have revealed that these hormones may also control the latency/reactivation and infectivity of viruses (HSV-1 in particular) via regulation of gene expression (Davis et al., 2011; Wang & Fish, 2012). Host immune responses to HSV-1 infection, like those to other viral infections, are complex. However, it is also quite likely that both IFNγ as a potent inducer of Th1 cells and anti-inflammatory IL-10 are certainly involved (Kubo & Motomura, 2012).

As T-lymphocytes are central to host defenses against viral infections, we hypothesized that THs may improve immune responses to HSV-1 by altering lymphocyte proliferation and cytokine production. Therefore, in this study, we examined the effects of a hyperthyroid state as well as of supra-physiologic levels of thyroxine (T4) on lymphocyte proliferation and cytokines (i.e., IFNγ and interleukin [IL]-10) production during exposure to HSV-1 antigens.

Materials and methods

Chemicals and reagents

Cell culture media, DMEM and RPMI 1640, bovine serum albumin, glutamate, pyruvate, and antibiotics were purchased from Gibco (Grand Island, NY). L-thyroxine sodium salt pentahydrate (T4) was obtained from Sigma (St. Louis, MO). A cell proliferation assay (BrdU ELISA) kit was bought from Roche Diagnostics (Penzberg, Germany). The mouse IL-10 and mouse IFNγ ELISA kits, with sensitivities of 13 pg/ml and 2 pg/ml,
respectively, were from Invitrogen (Carlsbad, CA). Radioimmunoassay (RIA) kits to measure thyroid hormone levels were obtained from DRG Diagnostics (Marburg, Germany). All other reagents were bought from Sigma.

Animals

Male Balb/c mice (4-weeks-old) were obtained from the Pasteur Institute (Tehran, Iran) for use in this study. Animals were housed individually in facilities maintained at 22 ± 2°C with 50 ± 5% relative humidity and 12-h on/off lighting. All mice were acclimatized for 1 week prior to initiation of any experiments. The mice had ad libitum access to a commercial rodent chow and filtered water throughout. Food and water intake of each mouse was monitored during the experimental period (4 weeks of T4 treatment [150 μg/kg body weight] to induce hyperthyroidism). Control mice were provided only with the vehicle (i.e., 4 M stock of ammonium hydroxide [in methanol], diluted 1:2000 in water). Mice were weighed weekly to allow for adjustments in preparation of the treatment water. One day after the final treatment course, each mouse was anesthetized, blood was collected from the aorta, and, after exsanguination, the thyroid and spleen were collected. The Ethics Committee of the Shiraz University of Medical Sciences approved all procedures utilized herein.

Evaluation of thyroid gland histomorphology and thyroid hormone measurements

Serum levels of tetraiodothyronine (T4) and triiodothyronine (T3) were determined using a RIA kit and following manufacturer instructions. To assess thyroid morphology, the thyroid tissues harvested at necropsy were immediately washed in phosphate-buffered saline and fixed in 4% paraformaldehyde for at least 48 h. After gross examination, the tissues were processed, embedded in paraffin blocks, and sectioned at 4 μm using standard techniques. The sections were mounted on glass slides, stained with hematoxylin and eosin (H&E), and then subjected to microscopic examination.

Splenocyte preparation

Lymphocytes from each harvested spleen were obtained using a previously-described protocol (Amirghofran et al., 2012). Briefly, the spleen was placed in a sterile plate containing 3 ml RPMI 1640 media and disrupted by gentle application of mechanical force (i.e., a flat syringe head). The suspension was then placed on top of 6 ml of NycoPrep™ 1.077 solution (Axis-Shield, Oslo, Norway) and centrifuged at 2600 rpm (at 4°C) for 25 min. The isolated lymphocytes were then collected from the interface, washed with PBS, re-suspended in 2 ml RPMI 1640 media, and then counted via a hemocytometer. Thereafter, the cells were diluted to a working concentration and cultivated in 96-well plates (see below).

Virus preparation

A plaque-purified strain of HSV-1 isolated from a patient was propagated in Vero cells. The Vero cells were provided by the Pasteur Institute (Tehran) and cultured in Dulbecco’s Modified Eagle Medium, with passaging every 2 days. The virus was titrated as plaque forming units (PFU). To isolate the virus for use in these experiments, infected Vero cells were collected and then subjected to four cycles of freeze–thawing. After centrifugation (2500 rpm, 10 min, 4°C), the supernatant was collected and the virus was heat-inactivated by incubation at 56°C for 30 min. The type of the virus was determined by neutralization assay using guinea pig anti-HSV-1 serum (NIH, Bethesda, MD) and monoclonal anti-HSV-1 antibodies against the virus glycoproteins D and G1 (Autogen Bioclear, Calne, UK), respectively. For further verification, polymerase chain reaction (PCR) analyses were performed with a pair of specific primers for HSV-1 protein G1.

Cell culture and treatments and conditioned media collection

Isolated lymphocytes were plated in 96-well plates at a cell density of 10^5 cells/well (in 80 μl complete media); thereafter, the cells (in triplicate) were either treated with 20 μl of Concanaavalin A solution (1 μg ConA/ml), 10^5 PFU inactivated HSV-1, T4 (60 ng/ml), or combinations (in 20 μl total) of these agents. Control wells received an equal volume of the various vehicles. The plates were then incubated at 37°C in a humidified chamber containing 95% air and 5% CO2 for up to a total of 72 h. For each sample, two plates were prepared in parallel for simultaneous assessment of proliferation and cytokine production. Wells in the plate designated ‘proliferation’ received 40 μl 5-bromo-2′-deoxyuridine (Brdu) solution (provided by kit manufacturer), 48 h after the plating. These wells were then incubated a further 24 h before the cells/wells were analyzed for proliferation. Wells in the plate designated ‘cytokines’ were allowed to incubate for the full 72 h before the medium in each was harvested for analyses of cytokine release.

Lymphocyte proliferation assay

Lymphocyte proliferation was assessed by nuclear incorporation of Brdu in proliferating cells that was, in turn, detected immunocytochemically according to the kit protocol. Briefly, 24 h after the Brdu was added to the wells, the medium was removed, the cells were fixed, and DNA denatured to improve antibody accessibility to the incorporated Brdu. After washing, kit-provided horseradish peroxidase (HRP)-conjugated anti-Brdu antibody was added to the wells and the plate was incubated at room temperature (RT) for 90 min. At the end of this period, the immune complex was detected by adding kit tetramethylbenzidine substrate; reaction product levels were quantified by measuring the absorbance of the samples at 450 and 690 nm reference wavelengths in a Elx 808 microplate reader (BioTek, Winooski, VT). The proliferation index for each sample was calculated as the ratio between the absorbance of the test wells vs that of the un-stimulated cells, and compared against the average among the untreated controls (which was then taken as 100%). At least three independent experiments in triplicate were carried out.

Cytokine assay

Concentrations of IFNγ and IL-10 in conditioned media recovered from the cells were determined using mouse ELISA kits and following manufacturer protocols. In brief, 100 μl test sample was added to a well pre-coated with primary antibody and the plate was then incubated at RT for 1 h. After gentle rinsing to remove non-bound antibody, the wells were then treated with 100 μl biotinylated anti-mouse secondary antibody and incubated for 1 h at RT. After washing, streptavidin-HPR was added and the plate was incubated for 30 min at RT. The wells then received 100 μl stop solution (1 M H2SO4) and the reaction product was then quantified at 450 nm in the microplate reader. Sample readings were applied to a plotted standard curve (generated in parallel using kit-provided standards) and the cytokine concentration present extrapolated. All samples were tested in duplicate.

Statistical analysis

All data are representative of at least three independent experiments. Data are expressed as means ± SE. Significant differences
Results

Alterations in histomorphology of thyroid gland and serum TH levels

The hyperthyroid state was assessed by histomorphological examination of the thyroid gland and serum levels of T4 and T3 at the end of the T4 treatment course. As shown in Figure 1 (Panel a) is hyperthyroid host, Panel a is control), microscopic examination of the corresponding thyroid tissue sections revealed large colloid-rich follicles with flat cells indicating that the exogenous T4 has acted strongly enough to suppress the hypothalamic-pituitary-thyroid axis. The observed alterations in thyroid gland histology are hallmarks of a suppressed secretion of thyroid stimulating hormone (TSH) from pituitary. The size of the gland in the hyperthyroid animals was also smaller than that in euthyroid animals, suggesting diminished stimulatory and trophic effects of TSH on the thyroid gland tissue. Moreover, a significant increase in water intake and a moderate increase in food intake of mice during the 4 weeks of T4 treatment, in conjunction with no changes in body weight (data not shown), were indications of a hyperthyroid state. Serum levels of T4 and T3 were affected significantly by the treatment as well. As shown in Figure 2, after 4 weeks of T4 treatment, animals showed significant increases in serum levels of T4 (6.65 ± 0.39 μg/dl, p < 0.001) and T3 (65.17 ± 8.23 ng/dl, p < 0.001) as compared to those in control (untreated) animals (2.31 ± 0.17 μg T4/dl, 33.38 ± 2.94 ng T3/dl).

Alterations in lymphocyte proliferation in response to antigen and mitogen

Figure 3 shows the results of the effects of hyperthyroid state and in vitro T4 treatment on the proliferative activity of lymphocytes in response to the HSV-1 particles (Ag) and mitogen (ConA). As the bars illustrate, the hyperthyroid state caused a significant increase in proliferative activities of the cells in response to the Ag as compared to that of a euthyroid state (euthyroid = 254.5 ± 15.0%, hyperthyroid = 484.3 ± 72.6%; p < 0.001). The in vitro presence of T4 induced a significant increase in antigen-induced proliferation of the cells of euthyroid animals (p < 0.05). However, it was ineffective for cells of the hyperthyroid animals (euthyroid = 498.4 ± 50.5%, hyperthyroid = 520.3 ± 75.1%). Response to mitogen was also affected by the hyperthyroid state. The effect on the response to mitogen was similar to that of the antigen. In response to ConA, the cells of hyperthyroid mice showed a significant increase in proliferation compared to that by cells of euthyroid counterparts (hyperthyroid = 15.0% euthyroid = 332.2 ± 17.1%; p < 0.05). The in vitro presence of T4 potentiated mitogen-induced proliferation of the cells of euthyroid mice (euthyroid = 691.9 ± 97.1%; p < 0.05). A similar effect was observed on cells from the hyperthyroid mice. However, in this case, the potentiation was not significant (hyperthyroid = 757.5 ± 86.8%).

Alterations in cytokine production in response to antigen and mitogen

To evaluate the effects of hyperthyroid state and in vitro T4 treatment on cytokine production, levels of IL-10 and INFγ released into the culture media by the cells in response to the antigen and the mitogen were measured. Figure 4 shows the results of IL-10 assessment. The hyperthyroid state resulted in a significant increase in cell IL-10 release in response to the Ag as
Figure 3. Effects on lymphocyte proliferation. The hyperthyroid state (dark grey) significantly potentiated antigen (Ag)- and ConA-induced proliferation of lymphocytes compared with that seen with the euthyroid state (light grey). In vitro treatment with T4 was similarly effective (see text for details). Data shown are mean (±SE) of eight mice/group. Values significantly different between spanned groups at **p < 0.01 or ***p < 0.001.

Figure 4. Effects on IL-10 formation/release. The hyperthyroid state (dark grey) significantly attenuated ConA-induced IL-10 release as compared with that seen with the euthyroid state (light grey). The in vitro T4 treatment was more effective in hyperthyroid than euthyroid. Antigen (Ag)-induced IL-10 release was moderately affected by the hyperthyroid state (see text for details). Data shown are mean (±SE) of eight mice/group. Values significantly different between spanned groups at *p < 0.05 or ***p < 0.0001.

Figure 5. Effects on IFNγ formation/release. The hyperthyroid state (dark grey) significantly increased both antigen (Ag)- and ConA-induced IFNγ release (more for ConA than for Ag) as compared with that seen with the euthyroid state (light grey). The in vitro T4 treatment was more effective in euthyroid than hyperthyroid (see text for details). Data shown are mean (±SE) of eight mice/group. Values significantly different between spanned groups at *p < 0.05 or **p < 0.001.

Discussion

Our data, collectively, show that supra-physiologic levels of serum THs (referred to as a hyperthyroid state in this study) may have profound effects on immune response to HSV-1 via alterations in T_{H1}/T_{H2} cytokines. The remarkable potentiating effects of hyperthyroid state on the mitogen- and antigen-induced lymphocyte proliferation in conjunction with intensification of IFNγ suggests that THs amplifies the T_{H1} response during host defense against HSV-1 infection. Moreover, significant attenuation of mitogen-induced IL-10 production by the lymphocytes of hyperthyroid animals (as compared to that by cells of their euthyroid counterparts) suggests to us that this amplification may have been mediated through attenuation of the T_{H2} arm.

The existence of polarized T-lymphocyte responses is well documented (Wang & Fish, 2012). The T_{H1} cells preferentially develop during infections by intracellular micro-organisms, such as occurs in viral infections (Chew et al., 2009). It is generally accepted that T_{H1} cells mainly produce IFNγ and are responsible for propagating cell-mediated immunity (Wang & Fish, 2012). There is evidence that, during T_{H1} responses, these cells can secrete IL-10 that—in a regulatory-feedback loop—will limit T_{H1} responses. A moderate increase in IL-10 secretion in response to Ag as was observed here in hyperthyroid state can be justified by this anti-inflammatory effect wherein IL-10 is used to down-regulate tissue damage during a T_{H1}-mediated inflammatory response (Ouyang et al., 2011). Nonetheless, based on these findings, it might be suggested that THs may have potential...
therapeutic implications in viral immunization and/or in the treatment of viral infections. Besides, the study shows that the presence of T4 in the culture media significantly reduced ConA-induced IFNγ release by the cells of euthyroid mice. This decrease in production of IFNγ during in vitro treatment of cells with T4 may also suggest a possible anti-inflammatory role of THs; the effect that may have an implication in immune-mediated disorders. The interaction of THs and immune system seems to be complex, and available data regarding the immunomodulatory roles of THs are contradictory. Almost all tissues in the body are affected by alterations in THs, which may add to the complexity of the interaction.

Given that T41 and T42 clones differentiate from a common pool of lymphocytes, one may ask about factors that affect the differentiation pathway. Nature of antigen, type of antigen presenting cells (professional or nonprofessional), and immunization route are shown to play roles. To our knowledge, this is the first report showing that increased levels of THs may also be involved. Alterations in the levels of THs have been shown in viral infections (Provinciali & Dabris, 1990). The cause or effect nature of these alterations is not yet known. At this time, we do not know whether hyperthyroid state-induced alterations are mediated solely through direct effects of THs on lymphocytes. However, the further reduction in IL-10 and sustained elevation of IFNγ by the cells of hyperthyroid animals, relative to that of the cells of the euthyroid animals, in the presence of in vitro T4 indicated that lymphocytes had been directly affected by the THs, and that the alterations in cytokines production were not a consequence of the global effects of the THs on body metabolism. These findings are in concert with the data reported by many investigators who showed that in vitro T4 treatment of lymphocytes alters proliferative and synthetic activities in lymphocytes. Likewise, we believe that the in vivo-activating effects of THs on cells in hyperthyroid mice may have led to a weaker antigenic response to in vitro T4 treatment as compared to that evidenced by cells in euthyroid mice. These findings are in agreement with data reported by other investigators (see Fricks et al., 2009; Karanikas et al., 2004; Klecha et al., 2006; Yao et al., 2007) who, in general, demonstrated that in vitro T4 treatment or alterations in the normal thyroid gland state impacted on the proliferative and synthetic activities of lymphocytes.

The selection of HSV-1 antigen for this study was based on our previous findings and some related reports by others. Epidemiologically speaking, herpes simplex virus type 1 (HSV-1) infection is one of the most common viral infections in humans and continues to be a major public-health problem (Ryan & Kinghorn, 2006). Besides, the results of a recent in vitro study conducted by Hsia et al. (2011) revealed that THs induces transcription of the HSV-1 latency associated transcript. The finding is rather interesting because nuclear receptors of THs often function as transcription factors and, therefore, may directly modulate viral gene transcription. In addition, the HSV-1 glycoproteins H/L (gH) can bind to a cell surface protein, αvβ3 integrins, which are receptors for THs as well (Parry et al., 2005).

Many actions of THs including in vitro enhancement of antiviral activity of IFNγ are believed to be mediated through these receptors (Bergh et al., 2005; Cheng et al., 2010; Cody et al., 2007; Davis et al., 2009). Of interest, Davis et al. (2013) have recently reported that THs act through αvβ3 integrins and modulate inflammatory response by altering expression of specific cytokines and chemokines.

We are confident that the hyperthyroid state was clearly established at the time of lymphocyte isolation. Blood levels of both T4 and T3 and parallel alterations in morphohistology of the thyroid gland tissue were good indications of this status. The atrophic gland in conjunction with remarkable histological changes in the thyroid tissue of the T4-treated mice confirmed that the raised serum level of THs was accompanied with a raise in their activities.

In summary, the data here allow us to conclude that supraphysiologic doses of THs (as in hyperthyroid states) or in vitro TH treatment may induce T41/T42 modulation through an increase in T41 responses and, thereby, could amplify host defenses against viral infections. Whether this effect is a direct effect or is potentially secondary to decreases in T-Regulatory (Treg) cell levels or due to reduced T42 responses still requires investigation. A better understanding of the molecular basis of the anti-viral actions of THs could eventually lead to a potential utilization of these hormones as anti-viral agents in clinical settings.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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