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Chronic stress influences the immune system through the thyroid axis

Graciela A. Cremaschi[a,b,*], Gabriela Gorelik[a], Alicia J. Klecha[b], Alexis E. Lysionek[b], Ana M. Genaro[a]

[a]*Centro de Estudios Farmacológicos y Botánicos (CEFYBO) – CONICET, Serrano 669–3P. (1414) Buenos Aires, Argentina*

[b]*Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina*

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Abstract

The aim of the present work was to analyze the effect of chronic stress on thyroid axis and its influence on the immune response. For this purpose a murine model of chronic stress was developed to evaluate and to correlate thyroid hormone levels with humoral alloimmune response. Results show a reduction in serum levels of thyroid hormones, specially a significant decrease in serum levels of triiodotyronine (T_3) in stressed animals. On the other hand, alloimmunization was not able to induce an early increment in T_3 and thyroxine (T_4) levels as it was previously reported in normal animals. In addition, lower titers of alloantibodies were obtained in animals under stress conditions as compared to normal mice. The sustitutive T_4 treatment in stressed animals increased significantly alloantibody production as well as the early increment in thyroid hormones after antigenic challenge. These findings suggest that chronic stress induces an alteration of the function of thyroid axis that alters the immune response.

Keywords: Streess; Thyroid hormones; Antibody production; Alloimmune response

Introduction

Over the past several years, many studies have suggested that stress has a profound effect on immune function in both animals and humans [1,2]. The impact of stressor exposure on the development of an immune response depends on a variety of factors such as the duration and type of stressor and the type of immunological challenge. Thus, it has been described that acute stress may activate acute phase immune responses which are critical for rapid and

* Corresponding author. Fax: (541) 4-856-2751.

E-mail address: grace@cefybo.edu.ar (G.A.Cremaschi)

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effective pathogen clearence in both systemic and local tissue infections [3,4]. On the contrary, chronic stress has been associated to diminished immunity. In accordance, alterations of cellular immunity including suppression of lymphocyte responses to the mitogenic stimulation of concanavalin A and phytohemagglutinin, with increased antibody titers to Epstein-Barr virus have been described [5,6]. Higher antibody titers to Epstein-Barr virus and altered percentages of T lymphocytes subsets have been observed in family caregivers of Alzheimer patients [7,8].

There are a variety of potential mechanisms by which stressors can alter immune function. One of these mechanisms would include the alteration of the hypothalamus-pituitary axis and the corresponding target endocrine glands (namely thyroid, gonads and adrenal) that in turn modulate the immune function [9,10]. The most common mechanism studied is the activation of both, the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system. Less attention has been given to the role of the hypothalamic-pituitary-thyroid axis, although evidence of an important relationship between stress and thyroid function has a long history [11].

On the other hand, a great bulk of experimental and clinical data has been accumulated in the past several years on the interaction between thyroid axis and the immune system on the basis of either the existence of receptors for thyrotrophic and thyroid hormones on lymphocytes as well as the frequent immune alterations in physiological and pathological fluctuations of thyroid hormones [12]. Previously, we have reported the influence of thyroid hormones on the course of an alloimmune response in a murine model [13]. We showed that triiodothyronine (T_3) and thyroxine (T_4) levels were increased few days after immunization. Besides *in vivo* treatment with T_4 increased alloantibody titers during the early stages of alloimmunization [13]. Conversely, lowering thyroid hormone serum levels by propylthiouracil treatment, down modulated the humoral response [13].

The aim of the present work was to study the participation of thyroid axis in chronic stress induced alterations of a murine alloimmune reponse. For this purpose, thyroid hormone levels were evaluated and correlated to the humoral alloimmune response in animals under chronic stress.

Methods

Animals

Inbred BALB/c (H-2^d) and C3H (H-2^k) mice were obtained from the Instituto de Oncología “A. H. Roffo”. All animals were used at the age of 60 to 70 days.

Stress model

Forty female BALB/c mice, weighing 29–31 g at the start of the experiment were used. All the animals were singly housed, maintained on a 12 h light/dark cycle under controlled temperatures 18–22° C. Except as described below, food and water were freely available. Firstly, the animals were trained to consume a 1% sucrose solution. Sucrose consumption was monitored throughout the experiment. After this phase (2 weeks), the animals were distributed into two groups (n=20). One group was subjected throughout the experiment to chronic un-

predictable mild stress. The stress schedule was slightly modified from that used previously in mice [14], and consisted of: three 3 h periods of food and water deprivation, immediately prior to the sucrose test; one additional 16h period of water deprivation; two periods of continuos overnight illumination; two periods (7 and 17 h) of 45° degree cage tilt; one 17 h period in a soiled cage (100 ml water in sawdust bedding); one period (8 h) of food deprivation; one 17 h period of paired housing (animals are always housed in the same pairs, but the location alternates between the home cages of each member of the pair). The stressors were scheduled through the week for ten weeks, in a similar manner to that previously described in rats [15]. Finally, sucrose consumption tests were continued for a further week, following the withdrawal of stress. A depression-like state was induced by this chronic exposure to unpredictable mild stress, which led to diminished food consumption and diminished preference for sweet drink (anhedonia). These parameters reached their maximum in the sixth week, and stayed between these levels in the subsequent weeks (sucrose consumption: week 1, normal animal: 300 ± 30 μl, depressed animal: 295 ± 28 μl; week 6, normal: 291 ± 27 μl, depressed: 30 ± 3 μl. Weight: week 1, normal: 30 ± 2 g, depressed: 31 ± 2 g; week 6, normal: 30 ± 3 g, depressed: 23 ± 2 g).

Alloimmunizations

BALB/c mice were immunized with C3H lymphoid cells. All immunizations were carried out between animals of the same sex (female) to avoid immune responses directed to the sex related minor histocompatibility complex antigen (H-Y), according to the following schedule: one i.d. injection of 1×10^7 lymphoid cells (pooled spleen, lymph node and thymus suspension), followed at 7 days intervals by one immunization with 3×10^7 lymphoid cells i.p. Non immunized mice were administered with the same volumes of RPMI 1640 medium alone.

In vivo thyroxine hormone replacement treatment

BALB/c stressed mice received an intraperitoneal injection of 50 ng T_4 (purchased from Sigma Chemical Co.) (dissolved in 0.1 N NaOH, diluted 1: 10 with phosphate-buffered saline (PBS), pH 7.2, and Millipore filtered) 5 times per week for one month. Mice injected with the vehicle alone were used as controls.

Hormone determinations

Blood from animals under different experimental conditions was collected into plain tubes (without anticoagulant). Serum T_3 and T_4 levels were determined using highly sensitive double antibody radioimmunoassay kit (Diagnostic Products Corporation, U.S.A.).

Antibody titers

Alloantibody titers were determined by enzyme-linked immunoadsorbent assay (ELISA) over C3H fixed cells on 96 plat bottom wells (Falcon 3912, Beaton Dickinson), with 0.5 glutaraldehyde in PBS. A goat anti-mouse IgG phosphatase alkaline conjugated (Sigma Chemical Co.) and p-nitrophenylphosphate (Sigma Chemical Co.) as substrate were used for developing coloration that was read at 405 nm. It is worth noting that all the dilutions of sera were

made in PBS containing 1 mM Na azide and 15% of normal goat sera to manage the blockade of Fc receptors on C3H fixed cells. Staining were considered positive when the optical density (O.D.) values were above the mean value plus 2 S.D. of normal sera (sera from non-immunized and/or vehicle alone injected mice that give non statistical differences among them).

Statistical analysis

Student's t-test for unpaired values was used to determine the level of significance. When multiple comparison were necessary, after analysis of variance, the Student-Newman-Keuls test was applied. Differences between means were considered significant if $p \leq 0.05$.

Results

Stress reduces basal and allostimulated T_3 and T_4 serum levels

In order to analize if stress is able to modify basal and allostimulated serum thyroid hormone levels, normal and stressed animals were bled before and seven days after one or two challenges with allogenic cells. As can be seen in Table 1, stressed mice showed significant lower levels of T_3 and some lowering of T_4 that was not significant as compared to normal animals. Moreover T_3 and T_4 serum levels were not increased in stressed animals after alloimmunization in contrast to the increment observed in control animals (Table 1).

Stress induces a lower antibody response

To analyze the influence of stress on alloantibody production, we evaluated alloantibody titers in sera from control and stressed alloimmunized mice by ELISA assay with C3H fixed cells as coating antigen. Results are shown in Figure 1. As can be seen alloantisera from control mice gave positive reaction with higher dilutions than those from stressed animals.

Table 1
Seric thyroid hormone levels in normal and stressed mice. Effect of allogenic stimulation

Animal[a]	Allogenic stimulation number[b]	T_3 (ng/dl)[c]	T_4 (ug/dl)[c]
Normal	0	74.9 ± 5.6	3.04 ± 0.26
	1	118.5 ± 9.2*	4.41 ± 0.48*
	2	92.3 ± 8.5	4.67 ± 0.59*
Stressed	0	57.1 ± 4.2*	2.66 ± 0.15
	1	59.2 ± 5.3	2.52 ± 0.16
	2	46.8 ± 4.2	2.25 ± 0.13

[a] BALB/c mice housed in control conditions (normal) or submitted to the chronic mild stress model described in Methods (stressed). [b] Animals were bled 7 days after one or two boosters with C3H lymphoid cells as allogenic challenge. For basal non-immunized condition (0) animals were injected only with RPMI medium. [c] T_3 and T_4 seric levels were determined by RIA as indicated before. Results shown are mean ± SEM of 5 determinations performed by duplicate.

* differ from normal non-immunized mice with $p \leq 0.05$.

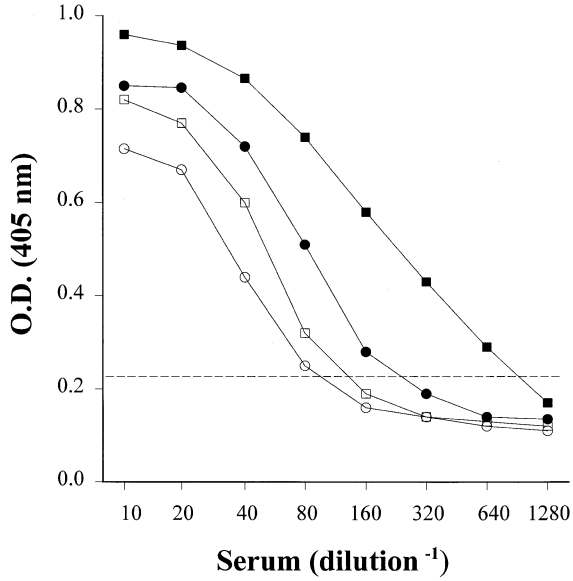

Fig. 1. Titration of normal and stressed mice alloantisera. Normal (white symbols) or stressed (dark symbols) animals were bled 7 days after one (circle) or two (square) alloimmunizations and alloantisera were diluted and assayed by ELISA as described in Methods. The curves shown are representative of 5 experiments performed by duplicate.

In vivo sustitutive T_4 treatment improved the alloantibody response

To evaluate if the decreased T_3 and T_4 serum levels observed in mice under stress conditions were correlated to the down regulation of alloantibody production in these animals, the action of *in vivo* sustitutive T_4 therapy was studied. For this purpose, alloimmunization scheduled was performed on stressed mice injected daily with 50 ng T_4 for 3 weeks, and hormone titers serum levels as well as antibody titers were evaluated. As shown in Table 2, this therapeutic schedule was able to rise T_3 and T_4 levels up to normal values. It is worth noting

Table 2
Basal thyroid hormone levels and alloantibody titers in normal, stressed or stressed animals submitted to *in vivo* T_4 therapy

	Seric basal thyroid hormone levels[b]		Alloantibody titer[c]	
Animals [a]	T_3 (ng/dl)	T_4 (μg/dl)	1	2
Normal	74.8 ± 5.1	3.11 ± 0.28	1/160	1/640
Stressed	57.1 ± 4.2*	2.66 ± 0.15	1/80	1/160
Stressed + T_4	77.8 ± 6.3	3.15 ± 0.27	1/160	1/640

[a] Normal, stressed or T_4 treated stressed mice were obtained as indicated in Methods. [b] Basal levels of seric T_3 and T_4 were determined in the indicated mice before alloimmunization. Results shown are the mean ± SEM of 5 experiments performed by duplicate. [c] Alloantibody titers in sera from the indicated mice, seven days after 1 or 2 boosters of allogenic C3H cells, were determined by ELISA assay. Results shown are the mean of 5 alloantisera in each group.

* differ from control values obtained in normal animals with $p \leq 0.05$.

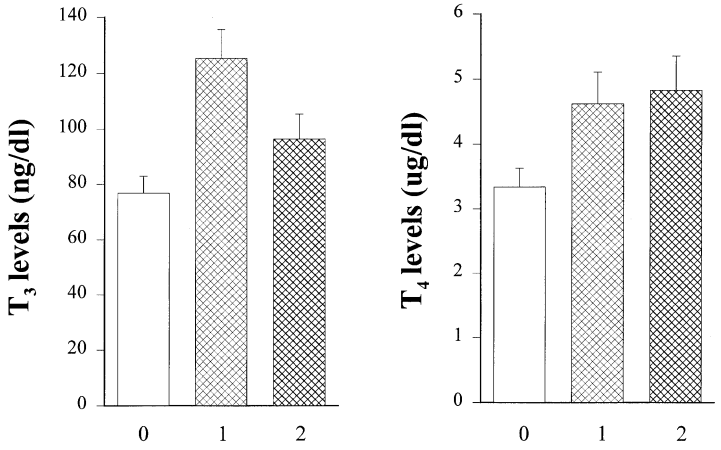

Fig. 2. Modulation of thyroid hormone levels in T_4 treated-stressed animals by alloimmunization. T_3 levels (left panel) and T_4 levels (right panel) were determined in sera before (0) and seven days after one (1) or two (2) alloimmunizations. Results shown are the mean ± SEM of 5 determinations performed by duplicate.

that following *in vivo* T_4 treatment, an increment of both T_3 and T_4 levels after one week alloimmunization was obtained similar to that observed in control animals (Fig. 2). In addition, under these conditions the alloantibody response was similar to that obtained in normal animals (Table 2).

Discussion

There are a variety of mechanisms by which stressors can alter immune function. It is well established that glucocorticoid, the end hormone of the HPA axis, plays a major role in the stress-induced suppression of the immune reaction. Growing evidence points to the modulation of the immune system by thyroid hormones [12,13], as well as to the stress-mediated alteration of thyroid activity [11]. However the relationship among stress, thyroid axis and immunity has not yet been defined. Therefore, in the present work the effect of chronic stress on thyroid axis and its association with an allogenic immune response was studied.

We found that stress was able to affect the thyroid gland function: a reduction in serum levels of thyroid hormones, specially those of T_3, was observed. Differences in adaptative responses to stress would be related to either activation or suppression of the thyroid system. Some reports described that traumatic stress was accompanied by activation of thyroid function. Thus, the relationship between traumatic stress and thyroid function was reviewed by Bram [16] who reported that a clear history of traumatic stress was found in 85 % among more than 3000 cases of thyrotoxicosis. More recent research continue to support the observation that patients with hyperthyroidism report a history of more stressful life events than do members of a control population [17–20]. In contrast, chronic stress has been generally associated with suppression of thyroid axis function. The mediators of these changes in thyroid function include glucocorticoids, somatostatin and cytokines [21,22]. Furthermore, chronic stress has been demonstrated to be the major cause leading to depressive disorders [23,24]

and accordingly, patients with melancholic depression, as well as anorectics and highly trained athletes have significantly lower thyroid hormone concentrations than controls [22,25]. In fact, the chronic mild model of stress applied in this work was developed to mimic depressive disorders [15,24]. During stress, a suppressed secretion of TSH and a decreased conversion of the relatively inactive T_4 to the potent T_3 in peripheral tissues were described [26]. Even though, the levels of serum T_4 and T_3 were measured for monitoring the thyroid state, which would reflect the serum TSH level. An attempt was made to measure the TSH by RIA with a rat anti-TSH antibody. However, this antibody was poorly cross-reactive to the mouse TSH, as was also described by Nishida and Kawada [27]. Since our results showed a major decrease in T_3 levels, it seems that the most important mechanism altered by this model of stress is the conversion of T_4 to T_3 more than the lowering in TSH values, but this could not be ruled out conclusively.

When studying the humoral immune response in alloimmunized stressed animals lower titers of alloantibodies were obtained. Modulation of antibody titers by thyroid hormone serum levels was previously reported during the course of an alloimmune response [13]. In fact, *in vivo* treatment with thyroxine was able to increase alloantibody titers. On the contrary, low levels of thyroid hormones, induced by propylthiouracil treatment, down regulated the alloimmune response. Moreover early after one or two boosters of an alloantigenic challenge, increment in both T_3 and T_4 hormone levels were observed [13]. Interestingly, our results showed that animals under stress conditions did not show increment in thyroid activity. The interactions of thyroid hormones in the immune development and function has been studied [for review see 12 and 28]. Briefly, the association of hypothyroidism with thymic growth depression and with a decrease in circulating lymphocyte number, has been demonstrated. Both are restored by parenteral or oral supplementation of T_3 or T_4. Moreover, the presence of thymus hypertrophy in human hyperthyroid diseases, as well as its reduction after treatment, has been demonstrated. On the other hand, a clear decrease in lymphocyte proliferative responses to mitogens and a depression of primary humoral immune responses in hypothyroid animals has also been reported. However, the effect of hyperthyroidsm provoked by T_3 or T_4 administration on humoral and cellular immunity is still controversial. Futhermore, an immunoregulatory role has also been suggested for TSH, owing to its ability to bind directly to lymphocytes and modulate murine antibody responses, lymphocyte proliferative responses and NK cytotoxicity.

To further analyze if low thyroid hormone levels would be related to the low humoral specific immune response in stressed animals we investigated whether sustitutive *in vivo* T_4 treatment would restored the normal alloimmune response. T_4 is usually used in hormone replacement therapies in hypothyroid patients as its half-life is higher than that of T_3, allowing a constant potency and prolonged action being converted to T_3 in peripheral tissues. For inducing normal levels of thyroid hormones in stressed mice, the schedule of replacement therapies for hypothyroid patients was used. These treatment was able to increase significantly alloantibody production resembling those obtained in control animals. Also, after alloimmunization of T_4-treated stressed animals, an early increment in thyroid hormone levels was obtained, resembling that observed in control mice. These effects would be explained by the restablishment of normal thyroid hormone levels that would be neccessary for the additional activation of thyroid axis after antigenic challenge, perhaps through a mechanism involving

central brain structures. In fact, thyroid hormones therapy has been proposed as a coadyuvant of antidepressants in psychiatric patients [29].

Taking together these results it could be assumed that stress conditions can alter thyroid axis function that in turn affects the immune response. Despite the numerous effects on immune function exerted by thyroid hormones, the mode through which they mediate these effects has remained uncertain. In view of the demonstrated presence of nuclear thyroid hormone receptors within lymphocytes [30], the possibility of a direct action on these cells must be considered. Alternatively, thyroid hormones may mediate immune effects by the modulation of other endocrine mediators. Among these, the connections between thyroid axis and HPA is specially important since it has been described that both T_3 and T_4 exert a clear influence on the levels of plasma and adrenal corticosterone [31] as well as the modulation of the immune function by glucocorticoids [32].

Finally, to our knowledge, the present work would be the first experimental evidences that point to the stress-thyroid axis-immune response interaction. The mediators and the molecular mechanism involved in this interaction are under study.

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References

1. Kielcolt-Glaser JK, Glaser R. Stress and Immune Function in Humans. In R. Ader, D.L. Felten and N. Cohen (Eds). Pshychoneuroimmunology. Academic Press, San Diego, 1991. pp. 849–67.
2. Ackerman SH, Keller SE, Schleifer SJ, Shindledecker RD, Camerinno M, Hofer MA, Weiner H, Stein M. Brain Behav. Immun. 1988; 2: 161–5.
3. Tilg H, Dinarello C, Mier J. Immunol. Today 1997; 18: 428–32.
4. H. Ali, B. Haribaby, Richardson R, Snyderman R. Adv. Rheumatol. 1997; 81:1–29.
5. Kielcolt-Glaser JK, Fisher LD, Ogrocki P, Stout JC, Speicher CE, Glaser R. Psychosom. Med. 1987; 49:13–34.
6. Pike JL, Smith TL, Hauger RL, Nicassio PM, Patterson TL, Mc-Clintick J, Costlow C, Irwin MR. Psychosom. Med. 1997; 59:447–57.
7. Kielcolt-Glaser JK, Dura JR, Speicher CE, Traskk OJ, Glaser R. Psychosom. Med. 1991; 53:345–62.
8. Mills PJ, Yu H, Ziegler MG, Patterson T, Grant I. Psychosom. Med. 1999; 61:168–74.
9. Besedovsky HO, Del Rey A. Neth. J. Med. 1991; 39:274–80.
10. Savino W, Dardene M. Immunology Today 1995; 16:318–22.
11. Manson JW. Psychosom. Med. 1968; 30:666–81.
12. Fabris N, Mocchegiani E, Provinciali M. Horm. Res. 1995; 43:29–38.
13. Klecha AJ, Genaro AM, Lysionek AE, Caro RA, Coluccia AG, Cremaschi GA. Int. J. Immunopharmacology 2000; 22:491–500 (in press).
14. Monleon S, D'aquila P, Parra A, Simon VC, Brain PF, Willner P. Psychopharmacology 1995; 117:453–7.
15. Willner P, Muscat R, Papp M. Neurosci Biobehav Rev 1992; 16:525–34.
16. Bram I. Endocrinology 1927; 11:106–16.
17. Winsa B, Adami HO, Bergstrom R, Ganstedt A, Dahlerg PA, Adamson U, Jansson R, Karlsson A. Lancet 1991; 338:1475–9.
18. Harris T, Greed F, Brugha TS. Br. J. Psychiatry 1992; 161:535–41.
19. Radosavljevic VR, Jankovic SM, Marindovic JM. Eur. J. Endocrinol. 1996; 134: 699–701.

20. Wang S, Manson J. Psychosom. Med. 1999; 61:131–8.
21. Crhousos GP, Gold PW. JAMA 1992; 267:1244–52.
22. Dom LD, Chrousos GP. Endocrinnol. Metab. Clin. N. Am. 1993; 22:685–700.
23. Anisman H, Zacharko RM. Br. J. Psychiatry 1992; 160:36–43.
24. Overstreet DH, Steiner M. Stress med. 1998; 14:261–8.
25. Sullivan PF, Wilson DA, Mulder RT, Joyce PR. Acta Psychiatr.Scand. 1997; 95:370–8.
26. Stratakis CA, Crhousos GP. Neuroendocrinology and pathophysiology of the stress system. In: Chrousos GP, McCarty R, Pacak K, Cizza G, Sternberg E, Gold PW, Kavetnansky R, editors. Stress. Basic Mechanisms and Clinical Implications. The New York Academy of Sciences, New York, 1995. pp. 1–18.
27. Nishida M, Kawada J. Experientia 1992; 48:262–5.
28. Chandratilleke D, Hala K, Marsh JA. Int. J. Immunopharmac. 1995; 18:203–210.
29. Hennessey JV, Jackson IMD. The Endocrinologist 1996; 6:214–23.
30. Lemarchand-Berraud T, Holm AC, Scazziga BR. Acta endocrinol. 1977; 85:44–51.
31. Lo MJ, Kau MM, Chen YH, Tsai SC, Chiao YC, Chen JJ, Liaw C, Lu CC, Lee BP, Chen SC, Fang VS, HO LT, Wang PS. Am. J. Physiol. 1998; 274:238–45.
32. Chrousos GP. N. Engl. J. Med. 1995; 332: 1351–62.