PHYSIOLOGY

Physiological Effects of Active Immunization with Triiodothyronine in Rats

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Immunization with triiodothyronine conjugated to a carrier protein through carbodiimide induces the production of specific antibodies interacting primarily with triiodothyronine and to a lesser extent with thyroxin. The immunization increases blood content of triiodothyronine, delays weight gain, increases the number of audiogenic seizures, disturbs thermoregulation in different thermal regimens, and sensitizes the animals to hypoxia. It can be hypothesized that immunization induces hyperthyroid shifts since anti-triiodothyronine antibodies stabilize the hormone in the circulation by preventing its inactivation and elimination and/or stimulate its synthesis.

Key Words: *triiodothyronin; active autoimmunization; autoantibodies; thermoregulation; hypoxia; behavior; audiogenic seizures*

Autoimmune damage to the thyroid gland is an important problem of endocrinology. The presence of autoantibodies to thyroglobulin, thyroid peroxidase, thyroid hormones, and other molecular components of the thyroid gland has been observed in humans and animals [6,9,11]. Since the titer of antithyroglobulin and antiperoxidase antibodies correlates with the degree of thyroid damage, it has been as a diagnostic test. However, the contribution of autoantibodies to triiodothyronine (T_3) and thyroxin (T_4) to thyroid pathology remains poorly understood. Moreover, autoimmune diseases of the thyroid gland are often associated with autoantibody production to different thyroid antigens, which determines a complex antibody spectra and the role of individual antibody types cannot be elucidated. Therefore, physiological effects of active immunization with thyroid hormones (conjugated to a protein carrier) seems to be an important experimental problem.

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MATERIALS AND METHODS

The study was carried out on random-bred male rats with an initial weight of 250 g.

The T_3-bovine serum albumin (BSA) and T_3- and T_4-rat serum albumin (RSA) conjugates were synthesized using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The protein concentration in the reaction medium was 3 mg/ml. T_3 (5 mg) was dissolved in 0.3 ml dimethylformamide and dropwise added to albumin solution in distilled water (3 mg) with gentle stirring; then 25 mg carbodiimide was added and the mixture was incubated for 2 h at room temperature with constant stirring and for 24 h in a refrigerator. The unbound hormone was removed by dialysis against distilled water (1:1000, 3 changes) and physiological saline (2

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changes). Protein content was measured by the method of Lowry.

The animals were immunized with the T_3-BSA conjugate in doses of 600 μg for primary immunization and 300 μg for boost injections (1 month after primary immunization with a 10-day interval) in Freund's complete adjuvant (1:1, v/v). Behavioral experiments were started several days after the last injection.

The titer of anti-T_3 antibodies was determined by enzyme-linked immunosorbent assay [3]. To this end, 100 μl T_3-RSA conjugate (50 μg/ml) in 0.15 M phosphate buffer (pH 7.4) containing 0.02% Tween-80 was transferred to microtitration wells and incubated overnight at 4°C; nonspecific binding sites were saturated with 0.1% gelatin in the same buffer (1.5 h, 4°C). The plates were washed, incubated with serum (1/500-1/64000, 100 μl per well) for 1.5 at 37.5°C, washed again, and incubated with anti-rat IgG antibodies conjugated with horseradish peroxidase for 1.0-1.5 h at 37°C. Then, o-phenylenediamine (4 mM) and hydrogen peroxide (0.03%) in 0.05M citrate-phosphate buffer (pH 5.0) were added, and after 25-min incubation in the dark at room temperature 50 μl 1 N H_2SO_4 was added and optical density was measured at 492 nm in a Titertek Multiscan spectrophotometer.

To evaluate serum specificity, the described procedure was modified as follows: the plates were precoated with T_3-RSA or T_4-RSA conjugates and T_3 was added to test sera (preliminary diluted 1:1000 and then to all dilutions on the plates) to a final concentration of 20 μM.

Serum concentration of T_3 was measured by radioimunoassay using RIO-T_3-PG kits (Belarus) with some modifications [6] to avoid errors caused by anti-T_3 autoantibodies.

The concentration of free T_4 in test sera was measured by enzyme-linked immunosorbent assay using Immunotekh kits (Russia) according to manufacturer's protocol.

The animals were weighed during the immunization period.

Behavioral experiments were carried out in a Rodeo automatic hole chamber (Ekran, Russia). Vertical (rearing) and horizontal activities were evaluated as well as the number of bottom and top holes visited.

Ninety-minute acoustic stimulation (115-dB ring) was used for evaluation of convulsive activity [2,4]; the reaction was assessed in points [4], and its latency was recorded.

The effect of immunization to T_3 on thermoregulation was studied in 3 temperature regimens characterized by certain functional state of the heat production and heat emission systems. The animals in individual plastic boxes were placed in a chamber with a programmed temperature regimen. Ambient temperature as well as rectal (6-cm depth) and tail skin temperatures were measured using copper-constantan thermocouples. The following temperature regimens were used:

1) 25-27°C, a thermoneutral medium characterized by a heat production/emission steady state;

2) 4-6°C, a cold medium characterized by enhanced heat production and reduced heat emission;

3) 32-33°C, a hot medium characterized by reduced heat production and enhanced heat emission.

The total duration of the experiments was 150 min in the cold and comfortable regimens and 180 min in the hot regimen.

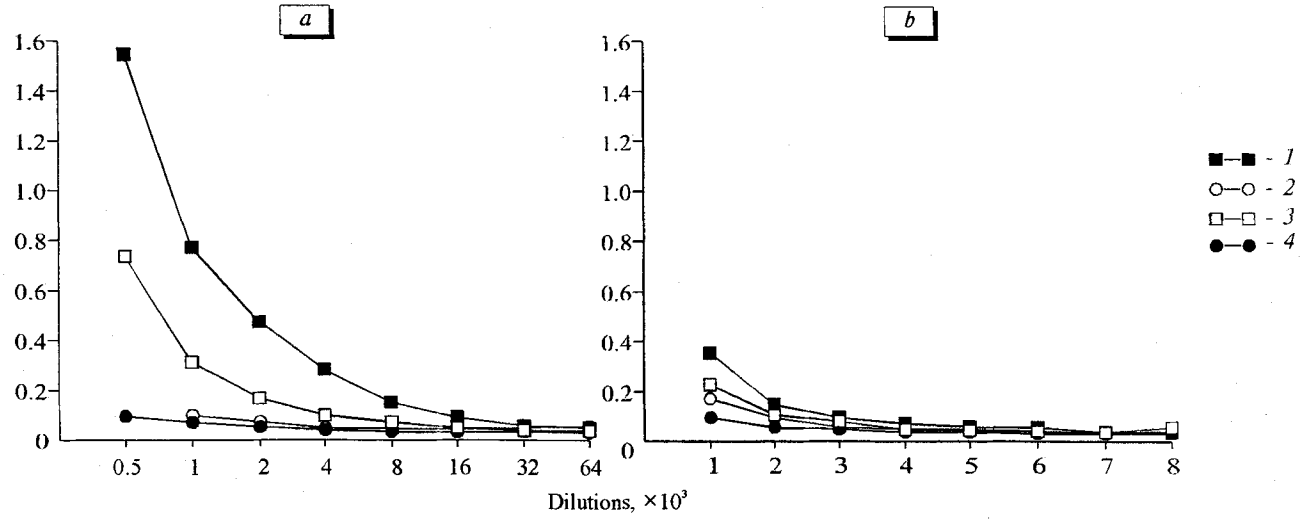

Fig. 1. Titration curves for rat sera after immunization with triiodothyronine-bovine serum albumin conjugate (T_3-BSA). Ordinate: extinction at 492 nm. Immobilized antigen is rat serum albumin conjugated with T_3 (*a*) and thyroxin (*b*). Sera were obtained from rats immunized with T_3-BSA conjugate (*1*, *3*) and BSA alone (*2*, *4*). Curves *3* and *4*: binding after addition of T_3.

The susceptibility to acute oxygen insufficiency was assessed by the method of extreme hypobaric hypoxia. The rats were placed in a glass jar from which air was evacuated for 1 min to a simulated altitude of 11,000 m above the sea level (oxygen content 5% of normal). The time of posture loss (lateral position), life time (until the last agonic inspiration), and the time of posture recovery after recompression were recorded.

The data were processed statistically using nonparametric Mann-Whitney and parametric Student *t* test.

RESULTS

Immunization with T_3-BSA conjugate induced production of anti-T_3 antibodies. The antibody titer varied from 1/1600 to 1/25600 (median 1/12800). Free T_3 added to test sera reduced antibody binding to immobilized T_3-BSA conjugate by 53.3%. The interaction between test sera and T_4-BSA constituted about 42.3% of that with T_3-BSA, while T_3 reduced this reaction by 38.5% (Fig. 1).

The immunization increased serum concentrations of T_3 and T_4. The total content of T_3 surpassed the control level almost 3-fold (3.195±0.557 *vs.* 1.098±0.140 nmol/liter, $p<0.01$). The concentration of free T_4 also increased: 71.8±5.0 *vs.* 58.9±4.3 nmol/liter in the BSA group ($p<0.01$) and *vs.* 56.1±3.9 nmol/liter in the NaCl group ($p<0.05$). The antibody titer positively correlated with the free T_4 content ($r=0.72$, $n=10$, $p<0.05$). Thus, immunization with the T_3-BSA conjugate induced generation of anti-T_3 antibodies that also bind T_4. Blood contents of T_3 and T_4 increased.

The dynamics of body weight throughout the experiment is shown in Fig. 2: in experimental group body weight increased more slowly during the first 7-10 days of immunization.

Behavioral experiments revealed decreased vertical activity in immunized animals, while other parameters remained unchanged (Table 1).

Convulsive activity was also affected by immunization to T_3. The score and latency of convulsive

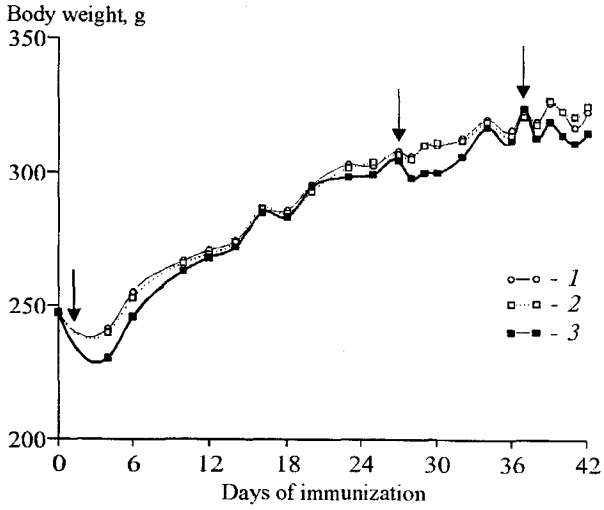

Fig. 2. Dynamics of body weight during immunization. *1*) NaCl, *2*) bovine serum albumin (BSA), 3) T_3-BSA conjugate. Arrows indicate days of injections.

reaction were practically the same in all groups, but more rats in the experimental group were susceptible to acoustic stimulation (Fig. 2). It can be hypothesized that the immunization makes the animals more susceptible to acoustic stimulation by increasing the nervous system excitability.

In cold chamber, initially higher rectal temperature in immunized rats decreased to the same values as in the control groups (Table 3). Initial skin temperature in experimental rats was significantly higher than in BSA-immunized rats, while final values were practically the same. In the thermoneutral medium, the initial rectal temperature was similar in all groups, while skin temperature was significantly higher in the experimental group; 2.5 h later, rectal temperature slightly increased, while skin temperature increased to a greater extent than in the control. In the hot medium, the initial skin temperature was also higher than in the control, 3 h later rectal temperature in all rats increased to 40°C and more, skin temperature in experimental animals increased to a lesser extent than in the control. Some animals died under these extreme conditions: 5 of 6 NaCl-treated rats, 3 of 7 BSA-immunized rats, and

TABLE 1. Rat Behavior in Rodeo Chamber during 10 min ($M\pm m$, $n=12$)

Group	Activity		Holes	
	horizontal	vertical	top	bottom
NaCl	293.3±27.0	33.5±3.4	15.2±3.5	72.9±9.2
BSA	296.0±34.7	38.3±4.1	12.3±1.7	66.1±9.8
T_3—BSA	267.6±24.7	24.9±3.3*	9.2±2.2	74.7±9.3

Note. *$p<0.05$ compared with BSA-immunized rats.

TABLE 2. Reaction to 115-dB Ring for 90 min (*M±m*, *n*=12)

Group	Latency, sec	Reaction score	Rats with convulsive reaction, %
NaCl	37.7±13.5 (3)	1.0±0.0 (3)	25.0
BSA	42.5±32.5 (2)	1.0±0.0 (2)	16.7
T_3—BSA	34.5±7.5 (8)	1.3±0.2 (8)	66.7

Note. Number of animals is shown in parentheses.

1 of 7 rats immunized with the T_3-BSA conjugate. Thus, the dynamics of rectal and skin temperature in different temperature regimens in rats immunized with the T_3-BSA conjugate differed from that in both control groups. It can be hypothesized that first, immunization to T_3 enhances both heat production and heat emission processes and second, anti-T_3 antibodies stimulate the thyroid axis and probably stabilize thyroid hormones in the circulation.

Lifetime under hypoxic conditions in experimental group was shorter then in the control (p=0.18 compared with BSA-immunized rats and p=0.1 compared NaCl-treated rats, Table 4). The time of posture recovery in experimental rats was increased in comparison with the control. Since the time of restitution strictly depends on the duration of hypoxia, the ratio of lifetime to the posture recovery time is usually taken into account as the most representative measure of susceptibility to hypoxia [1]. For instance, in animals highly resistant to hypoxia lifetime considerably surpassed the time of restitution, therefore their ratio was beyond 1, and otherwise in low-resistant animals it remained below 1. As seen from Table 4, this parameter in the experimental group is beyond 1, while in both control groups it is below 1 (p=0.05 compared with NaCl-treated rats and p=0.02 compared with BSA-immunized animals). Thus, rats immunized with the T_3-BSA conjugate are more susceptible to hypoxia than control animals.

TABLE 3. Core and Surface Temperature in Different Temperature Regimens (*M±m*, *n*=6-7)

Group	Rectal temperature		Tail skin temperature	
	initial	final	initial	final
Ambient temperature 4-6°C				
NaCl	38.25±0.12	37.43±0.23	15.3±0.2	8.8±0.2
BSA	38.01±0.28	37.89±0.11	14.9±0.1	8.5±0.2
T_3-BSA	38.46±0.10	37.59±0.21	15.9±0.2***	8.8±0.3
Ambient temperature 26-27°C				
NaCl	38.13±0.21	38.10±0.17	25.4±0.2	28.9±1.2
BSA	38.19±0.21	38.06±0.24	25.0±0.0	27.1±0.7
T_3-BSA	38.23±0.22	38.39±0.27	25.4±0.1**	32.3±0.5***
Ambient temperature 32-33°C				
NaCl	37.90±0.07	40.75±0.12*	29.5±0.6	37.6±0.2*
BSA	37.77±0.13	40.50±0.13*	27.7±0.4	36.9±0.1*
T_3-BSA	38.10±0.11	40.30±0.25*	31.8±1.2*	36.2±0.7*

Note. *Data correspond to the 120th min of the experiment, since later some animals died. Here and in Table 4: *p<0.05, **p<0.01, ***p<0.001 compared with BSA-immunized animals.

TABLE 4. Effects of Extreme Hypoxia (*M±m*)

Group	Time of posture loss, sec	Lifetime, sec	Time of posture recovery	Lifetime/posture recovery time
NaCl (*n*=10)	22.8±3.0	705.5±217.3	256.9±60.7	3.78±1.45
BSA (*n*=10)	16.4±2.6	511.7±143.9	198.5±33.5	2.52±0.75
T_3-BSA (*n*=7)	14.8±1.6	143.6±48.3	273.0±54.8	0.50±0.18*

Our findings can be attributed to the activation of thyroid function induced by immunization to T_3. Under these conditions hyperthyroidism is probably due to both compensatory activation of hormone synthesis and secretion in the thyroid gland (have to be experimentally verified) and stabilization and accumulation of thyroid hormones in the circulation without elimination, as it was described previously for grown hormone and other regulators [5,10], or due to formation of anti-idiotypic antibodies [7].

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