Neonatal stimulation of the thyroid gland with iodine or suppression during adolescence with triiodothyronine changes the prevalence of autoimmune thyroiditis in BB rats

M-L Hartoft-Nielsen1,2, Å K Rasmussen1, A Kaas2, U Feldt-Rasmussen1 and K Buschard2
1Department of Endocrinology PE-2131 and 2Bartholin Institutet, Copenhagen University Hospital, Copenhagen, Denmark

(Correspondence should be addressed to M-L Hartoft-Nielsen, Department of Medical Endocrinology, Rigshospitalet, National University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark; Email: ma-lars@dadlnet.dk)

Abstract

Objective: Changes in the functional state of beta cells by neonatal stimulation or adolescent suppression have reduced the incidence of type 1 diabetes mellitus in animal models. The aim of this study was to evaluate the effect of manipulation of the activity of the thyroid gland by neonatal stimulation or by adolescent suppression on the prevalence of spontaneous autoimmune thyroiditis (AIT) in rats.

Methods: Bio-Breeding/Worcester (BB) rats were treated neonatally with sodium iodine (NaI) or thyroid stimulating hormone (TSH), or during adolescence by triiodothyronine (T3), and the lymphocytic infiltration in the thyroid gland was evaluated.

Results: Neonatal treatment with NaI decreased the prevalence of AIT to 32±9% compared with 66±5% in the controls (P<0.002), mainly caused by a reduction among the female rats (13±9% vs 52±8%, P<0.006). TSH had no effect. Post neonatal suppression of the thyroid gland by T3 had a biphasic response. Early in adolescence the overall prevalence was 14±7% compared with 66±5% in the controls (P<10^-2); for female rats AIT was prevented (0±0%) compared with 52±8% in the controls (P<0.0003) and in male rats the values were 29±13% compared with 80±6% in the controls (P<0.001). Treatment with T3 later in adolescence increased the overall prevalence to 81±7% compared with 66±5% in the controls (not significant). For female rats the prevalence increased to 78±9% compared with 52±8% in the controls (P=0.04). The degree of thyroiditis among the affected animals was similar in all groups.

Conclusion: Neonatal stimulation of the thyroid gland by iodine or early adolescent suppression by T3 reduced the prevalence of AIT whereas T3 given later increased the prevalence of thyroiditis in rats. Thyroid activity at various ages seems to be of importance for the development of autoimmune thyroiditis.
In humans, the influence of thyroid status on thyroid autoimmunity is controversial (9, 10). However, the comparable pathogenetic mechanisms of AIT and T1DM and the reduction in the incidence of T1DM by changed β-cell activity (11–13) caused us to examine whether changed thyroid activity (i.e. antigen expression) by neonatal stimulation with iodine or thyroid stimulating hormone (TSH), or adolescent suppression of the thyroid gland by triiodothyronine (T₃), could change the incidence of AIT in BB rats.

Materials and methods

BB rats of the subline NB were bred in our facility and were obtained originally from the University of Massachusetts, Worcester, MA, USA. The study consisted of two parts. In the first part, the influence of sodium iodine (NaI) and TSH administered neonatally on the prevalence and degree of AIT was evaluated at the age of 19 weeks. The influence of the chosen doses of NaI and TSH on serum thyroxine (T₄) levels at the age of 7 and 18 days, respectively, the day after the end of treatment, was also evaluated. In the second part, the influence of T₃ administered during adolescence on the prevalence and degree of AIT was evaluated at the age of 19 weeks, and the effect of T₃ on serum TSH levels the day after the end of treatment at the age of 9 weeks was evaluated.

All rats were kept in sterile stables under constant temperature, humidity and light/darkness cycle of 9/15 hours, respectively. All rats had free access to food and water. The animals were observed daily and examined twice a week for glucosuria (Test-Tape, Lilly, Indianapolis, IN, USA) after the age of 60 days. If positive for glucosuria, blood glucose was measured (Glucometer Elite, Bayer Diagnostics, Tokyo, Japan) and diabetes was diagnosed when blood glucose was 15 mmol/l and above. Diabetic rats were treated with 15-20 IU Insulatard (Novo Nordisk, Bagsværd, Denmark) every second day.

Adolescent suppression by T₃

T₃ for suppression was administered in the drinking water. This avoided stressing the rats by repeated injections and since it was present continuously, it may eliminate fluctuating serum levels. Since the timing for this suppression is important, we started the treatment at two different times. Two groups of BB rats were given T₃ (T-2752, Sigma Chemical Co., St Louis, MO, USA) dissolved in PBS and administered i.p. immediately post partum and daily for the first 3 days in each of the first 3 weeks - 0.5 IU per day for the first week and 1 IU per day for the first 3 days in the second and third weeks. Control groups were treated with corresponding medium only.

To evaluate the influence of the NaI and TSH treatment on the function of the thyroid gland, two groups of newborn BB rats bred in our facility and originating from Mollegaard, Lille Skensved, Denmark, were included and treated as described previously with NaI and TSH; two groups of rats served as control groups. All the rats in this part of the study were killed after the end of treatment at the age of 7 and 18 days, respectively, the day after end of treatment. Serum was collected and T₄ was measured. The dose regimen of NaI significantly increased serum T₄ to 48.5 ± 2.6 (mean ± S.E.M.) from 42.6 ± 1.3 nmol/l. The dose regimen of TSH did not increase T₄ (44.1 ± 2.9 compared with 44.6 ± 1.3 nmol/l in controls) while an increase has been shown for lower doses of TSH in rats (15). The doses were chosen to avoid thyrotoxicosis or tissue damage induced by the drug itself and after 3 days of treatment the doses were adapted to the increased body weight of the rats.

Neonatal stimulation by NaI and TSH

To stimulate the thyroid gland neonatally we chose to use iodine and TSH. NaI and TSH were administered by injection because the pups were fed by the mother and were unable to take treatment orally. Since treatment for 6 days with glucose is known to reduce the incidence of T1DM in BB rats and NOD mice (11, 12) we chose to give NaI (6523, Merck, Darmstadt, Germany) dissolved in sterile PBS and administered s.c. immediately post partum and daily for 6 days - 60 μg NaI daily for the first 3 days and 120 μg for the last 3 days.

The plasma concentration of TSH is high neonatally (14) and since the hypothalamo–pituitary–thyroid axis changes dramatically in the neonatal period (15) TSH was administrated periodically for a longer period. TSH (T-3538, Sigma Chemical Co., St Louis, MO, USA) was dissolved in PBS and administered i.p. immediately post partum and daily for the first 3 days in each of the first 3 weeks - 0.5 IU per day for the first week and 1 IU per day for the first 3 days in the second and third weeks. Control groups were treated with corresponding medium only.

To stimulate the thyroid gland neonatally we chose to use iodine or thyroid stimulating hormone (TSH), or adolescent suppression of the thyroid gland by triiodothyronine (T₃), could change the incidence of AIT in BB rats.

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disappeared within 3 weeks after the end of treatment (4.7 (4.4–8.3) μg/l and 3.9 (2.5–5.7) μg/l respectively). The body weight of male Wistar rats treated with T3 was lower than in the control group (275 ± 5 vs 304 ± 7 g (mean ± S.E.M.), P < 0.005) just after the end of treatment. This difference was not found 3 weeks after the end of treatment. The dose was chosen so as not to induce thyrotoxicosis. T3 in the given dose induced a resting state in the thyroid gland as evaluated by light microscopy on hematoxylin and eosin (HE)-stained sections (Fig. 1).

**T4 measurement**

T4 was measured by Coat-A-Count Canine T4 assay (TKC41, Diagnostic Products Corporation, Los Angeles, CA, USA). The sensitivity was 2.1 nmol/l, the intra-assay variation was less than 4.3% at the level of our samples and the reference range was 18–50 nmol/l.

**TSH measurement**

TSH was measured by the Amersham Pharmacia Biotech Rat TSH enzyme immunoassay system (code RPN 2564, Amersham Pharmacia Biotech, UK Limited). The sensitivity was 0.6 μg/l. The within-assay coefficient of variation was less than 6% and the reference range was 3-30 μg/l.

**Histopathological evaluation**

After killing at the age of 19 weeks the thyroid glands were dissected out, together with the trachea, and placed in formalin. Randomly rotated, assuming isotopic tissue destruction by autoimmune processes, the thyroids were embedded in paraffin, cut into 5 μm sections and stained with HE. On average 12 (range 4 to 26) sections from each gland were randomly taken at different levels throughout the thyroid gland from each animal and were examined by two examiners who were unaware of the treatment groups for mononuclear cell infiltration and follicle disruption. A Leitz Leica Dialux 20 EB microscope was used with objectives of 2.5, 10 and 40. On each section the extent of infiltration and tissue damage was estimated as a percentage and the final score for each gland was the mean of all the estimates. Finally, the degree of thyroiditis for each gland was expressed on a scale from 0 to 4 as previously described (16, 17) depending on the percentage of histological alteration related to the tissue area without knowledge of eventual tissue shrinkage: 0, normal histology; 1, less than 10%; 2 approximately 10–30%; 3, approximately 30–50%; 4, more than 50% (Fig. 2).

The thyroids from the rats killed at the age of 7 and 18 days respectively did not show any signs of necrosis or other toxic effects or thyroiditis on HE-stained sections evaluated by light microscopy compared with the corresponding control groups. It was not possible to detect cell stimulation or cell proliferation on the HE-stained sections evaluated by light microscopy.

**Ethics**

The study complies with the European Committee guidelines for the use of experimental animals and was approved by the local Ethical Committee.

**Statistics**

Unless otherwise stated the results are presented as means ± S.E.M. Serum levels of T4 were compared by use of the unpaired t-test. The measured values of TSH were log-transformed and compared using analysis of variance. To compare the prevalence of thyroiditis between the experimental groups and the control group the χ²-test was used for the combined groups of male and female rats, whereas Fisher’s exact t-test was used to compare the separate groups of male and female rats because of the lower number of rats in each group. The degree of thyroiditis was compared in the animals with histological signs of thyroiditis.
using Mann-Whitney’s test. Analysis of variance was applied to compare the body weights of the adult BB rats and unpaired t-test was used to compare the body weights of Wistar rats. In respect of the prevalence and degree of AIT and body weight of the adult BB rats the control groups were homogenous and were combined to serve as one control group (n = 82).

Results

Neonatal treatment with NaI

NaI given immediately post partum and daily for the first 6 days reduced the prevalence of AIT to 32±9% (mean±S.E.M.) compared with 66±5% in the control animals (P < 0.002) (Table 1). This reduction was mainly accounted for by female rats the prevalence of whom was reduced to 13±9% compared with 52±8% in the control group (P < 0.006). No difference was found for the group of male rats (53±1 vs 80±6, P = 0.054). No differences in the degree of thyroiditis were found among the affected animals (Table 1).

Neonatal stimulation by TSH

Neonatal stimulation by TSH given immediately post partum and for the first 3 days in the first 3 weeks did not change the prevalence or degree of AIT compared with the control group (Table 1).

Adolescent suppression with T3

Suppression of the thyroid gland during adolescence by T3 administered daily in the drinking water had a biphasic response (Table 1). Treatment from the age of 3 to 9 weeks reduced the prevalence to less than one fourth of the prevalence in the control group (14±7 vs 66±5%, P < 10\(^{-3}\)). This more than halved the prevalence of AIT to 29±13% for the group of male rats compared with 80±6% in the control group (P < 0.001) whereas for female rats AIT was completely eliminated (0±0%) while it was present

Table 1  Prevalence and degree (graduated from 0-4) of thyroiditis (AIT) in BB rats at the age of 19 weeks after neonatal stimulation with NaI or TSH or adolescent suppression with T3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prevalence in percent (mean±S.E.M.) (n)</th>
<th>Degree of thyroiditis (mean±S.E.M.) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Males</td>
</tr>
<tr>
<td>Control group</td>
<td>66±5 (82)</td>
<td>80±6 (40)</td>
</tr>
<tr>
<td>Neonatal stimulation</td>
<td>NaI 32±9 (31)+</td>
<td>53±13 (15)#</td>
</tr>
<tr>
<td>TSH 63±9 (32)</td>
<td>80±9 (20)</td>
<td>35±14 (12)</td>
</tr>
<tr>
<td>Adolescent suppression</td>
<td>T3(^a) 14±7 (28)++</td>
<td>29±13 (14)##</td>
</tr>
<tr>
<td>T3(^b) 81±7 (36)</td>
<td>85±10 (13)</td>
<td>78±9 (23)!!!</td>
</tr>
</tbody>
</table>

NaI was given for the first 6 days post partum and TSH for the first 3 days in the first 3 weeks post partum. T3 was given from the age of 3 to 9 weeks or 2 to 9 weeks. The degree of thyroiditis in BB rats at the age of 19 weeks was scored from 0 to 4 in animals positive for thyroiditis. All the treated groups were compared with the corresponding control group. The combined groups of male and female rats were compared with the control group by Chi-square test: + + P < 0.002, ++ P = 2.4 x 10\(^{-5}\), the groups of male and female rats taken separately were compared with the corresponding control group by Fisher’s exact test; #P = 0.053, ##P < 0.001, *P < 0.006, **P < 0.0003 and ***P < 0.035.

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in 52±8% of the female control group (P < 0.0003). No changes in the degree of thyroiditis were seen (Table 1).

Conversely, treatment of female rats with T3 from the age of 5 to 9 weeks increased the prevalence ofAIT to 78±9% compared with 52±8% in the control group (P = 0.035) (Table 1). For the combined group of male and female rats and for the group of male rats only the tendency was the same although the differences were not significant.

Adolescent suppression by T3 did not change the degree of AIT. None of the Wistar rats developed AIT.

**Body weight**

The weight of the BB rats at the time of death (19 weeks) was not affected by the different treatments (Table 2) or the presence of thyroiditis (data not shown).

**Serum levels of TSH**

Serum levels of TSH, measured in six male rats with and six male rats without AIT from each group of BB rats at the time of death at 19 weeks old, were identical in all the groups (Table 2).

Despite the loss of weight of the Wistar rats killed just after the end of T3 treatment, none of the rats showed any clinical signs of hyper- or hypothyroidism.

**Discussion**

In a spontaneous animal model of AIT, the influence of the development of AIT by neonatal stimulation with NaI and TSH and adolescent suppression with T3 was examined. Neonatal treatment with iodine was found to reduce the prevalence of thyroiditis particularly in female BB rats, which to our knowledge has not been described before. By contrast, it is well known that iodoine given in excess in adult life usually provokes development of AIT in several species including humans and rats (18). Acute changes in iodine intake seem to be especially important (19, 20). The reduced prevalence of thyroiditis found in this study is in line with the results of two other studies, Buschard et al. (12) and Bock et al. (11), who similarly found that the incidence of diabetes was reduced after neonatal stimulation of the β-cells by arginine and/or glucose in both BB rats and NOD mice. The theoretical explanation for the reduced prevalence of AIT following neonatal stimulation is suggested to be the formation of a strong ‘self’ and induction of immunotolerance due to early increased organ activity and exposure of organ-specific antigens to the immune system. In this connection thyroglobulin (Tg) could be an interesting antigen, since highly iodinated Tg is found to be highly antigenic in adult rats and chickens (21, 22) and thus an early iodination of Tg may induce tolerance instead.

Neonatal stimulation with TSH did not reduce the prevalence of AIT. TSH was given during a period when the gland is susceptible to TSH (15) so the different outcome compared with iodine treatment is peculiar and without obvious explanation. In the evaluation of the effect of TSH on thyroid function it failed to change serum T4 concentrations. Although speculative, this could be due to a relative iodine deficiency resulting in a shift to T3 production or a rebound effect because the effect of TSH is much faster and the maximum response in serum T3 concentration is found already 2–3 h after TSH administration (15). Finally, TSH is secreted in a pulsatile manner and consequently administration only once a day may not be enough to stimulate the gland sufficiently (23). Experimentally, it has previously been shown that TSH increased the expression of thyroperoxidase (TPO) on the surface of human thyrocytes in vitro with an over-representation of epitopes recognized by autoantibodies against TPO (24), and thyroid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g) (mean±S.E.M.) (n)</th>
<th>TSH (mU/l) (median and range) (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal stimulation NaI</td>
<td>452±7 (40)</td>
<td>255±4 (41)</td>
</tr>
<tr>
<td>TSH</td>
<td>446±11 (14)</td>
<td>262±6 (16)</td>
</tr>
<tr>
<td>Adolescent suppression T3</td>
<td>431±9 (19)</td>
<td>256±7 (11)</td>
</tr>
<tr>
<td>T3a</td>
<td>425±11 (14)</td>
<td>261±7 (14)</td>
</tr>
<tr>
<td>T3b</td>
<td>436±12 (13)</td>
<td>260±5 (22)</td>
</tr>
</tbody>
</table>

NaI was given for the first 6 days post partum and TSH for the first 3 days in the first 3 weeks post partum. T3 was given from the age of 3 to 9 weeks or 5 to 9 weeks. The body weights of the BB rats in all treated groups were compared with the corresponding control group by use of analysis of variance and finally the groups of rats with thyroiditis were combined with the groups of rats without thyroiditis (data not shown). Serum values of TSH were measured in six randomly selected male rats with and six without thyroiditis from each group of BB rats at death (19 weeks) and the log-transformed values were compared with the control groups. No statistical significances were found.

Nd, not done.
In female rats, the thyroiditis was completely prevented (9, 10). Thyroid hormones might enhance an ongoing immune process at this age. The biphasic time-dependent effect of T3 on the incidence of thyroiditis has been described in previous studies in spontaneous and induced AIT in rats (26–28) and hypothyroid cats (29) where the incidence, severity and level of antibodies were found to be reduced after treatment with T4. In these previous studies, T4 and not T3 was used and it was administered in supraphysiological doses and for periods of up to 3 months (26–28). Only in one previous study was no effect of T4 on the prevalence of AIT found (30). Our finding also parallels studies in type 1 diabetes in which a lower β-cell activity after insulin treatment of BB rats in adolescence was found to reduce the incidence of diabetes (13). A recent study has found a preventive effect of insulin treatment on the progressive β-cell failure in slowly progressive T1DM in a Japanese population (31).

The underlying mechanism of action has yet to be determined but a possible mechanism could include an induction of thyroid cell rest, and due to this rest a reduced antigen expression on the thyrocytes and less risk of autoimmune aggression in parallel with the described mechanism for the reduced diabetes incidence (5, 32, 33). Break of tolerance might be more difficult if the antigens are weakly exposed. In addition, the resting β-cell is found to be less vulnerable to interleukin 1β-mediated impairment (7).

Furthermore, we found that late adolescent suppression of the thyroid gland by T3 increased the prevalence of AIT in female BB rats. This effect of thyroid hormones has not, to our knowledge, been shown before and seems to be closely related to the time window of the treatment. A possible mechanism behind this effect of T3 could be stimulation of an already ongoing immune process at this age. The biphasic time-dependent effect of T3 on the prevalence of thyroiditis may reflect the dilemma of whether or not treatment with thyroid hormones might enhance an ongoing immune process of human autoimmune thyroiditis (9, 10).

It is unknown whether the biphasic effect of T3 on AIT is due to the start-point of treatment or the different duration of treatment (in this case 4 and 6 weeks respectively). If duration of treatment is the important cause, differences in maturation of the endocrine system, sexual development or the immune system itself could be the explanation. For the importance of start-point as a crucial factor, it has been found that complete removal of thymic tissue by thymectomy at the age of 5 weeks and irradiation after 2 weeks in Wistar rats increased the incidence of thyroiditis (34) and reconstitution with normal lymphoid cells restored the animals (35). However, timing in reconstitution was also crucial since only early reconstitution could abrogate the autoimmunity while later reconstitution had no effect (35). Although these experiments are different from ours they may provide a possible explanation for the difference in response to T3 found in early and late adolescence where thymic maturity might be the key. This will need to be clarified by further study.

We found that after neonatal treatment by NaI and early T3 administration female rats were better protected against thyroiditis than male rats and were more affected after later T3 administration. Usually females are affected more often than males by autoimmune diseases which is also found in experimental models of AIT in rats (36). Loci on the X chromosome seem to be of importance in the pathogenesis of Graves’ disease which could be related to the female preponderance (37–41). The lack of female preponderance in the control group has also been described by others (42) and could be due to inbreeding of the rats and thus increased influence of the genetic factor in disease development (43) or it could be due to a stronger genetic influence in animals with polyendocrine diseases where the environmental influence, in this case the sex hormones, may disappear (44). In this study we found that the different treatments changed the prevalence of thyroiditis but not the degree of lymphocytic infiltration and tissue destruction. These results are original and we can still only speculate on the possible mechanisms. The histological grading of the lesions is semi-quantitative and able to detect significant differences in prevalence. It is not yet known how the degree of tissue destruction correlates to the clinical and biochemical disease manifestations. We did not measure anti-Tg antibodies in these rats but previous studies have found that the presence and the level of anti-Tg antibodies correlate with the presence of AIT in both spontaneous and induced rat models of AIT also after T4 treatment (16, 18, 28, 45, 46) although one study found no difference after T4 treatment (27) and another study could not find a correlation between the degree of AIT and the level of anti-Tg antibodies after induction of AIT by iodinated Tg (21).

In conclusion, neonatal stimulation of the thyroid gland with iodine as well as early adolescent suppression with T3 reduced the prevalence of AIT in BB rats, whereas T3 administered later increased the prevalence. Our results indicate that thyroid activity at various ages of development seems to influence the development of autoimmune thyroid diseases.
Acknowledgements
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