Spontaneous Autoimmune Thyroiditis in Bio Breeding / Worcester (BB / W) Rat

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Abstract

We investigated the serial changes in the plasma levels of anti-thyroglobulin antibody (ATA) by solid-phase enzyme immunoassay, thyroid hormones and blood glucose, since spontaneous occurring lymphocytic thyroiditis (LT) has been found in spontaneously diabetic Bio Breeding/Worcester (BB/W) rat. We also observed the correlation between these levels and histological findings in the thyroid gland. The incidence of diabetes was 0% in 5 week old rats (group A), 70% in 11 week old rats (group B), and 86% in 20 week old rats (group C), while LT was observed in 0% in group A, 20% in group B and 48% in group C. Although the incidence of both increased with age, there was no link between LT and diabetes. Plasma ATA levels were 91.4 ± 28.5 ($OD_{492} \times 1,000$, mean $\pm$ SEM) in the control (14 week old Wistar Furth) rats. 49.5 ± 15.4 in group A, 197.8 ± 41.5 in group B, and 376.7 ± 48.7 in group C, again showing a clear increase with age. In group C, the plasma levels of ATA in rats with LT were significantly higher than those without LT. In addition, 6 out of 11 rats without LT had abnormally high ATA levels. In group C, the plasma levels of free 3,5,3'-triiodothyronine (FT3) and total thyroxine (TT4), and also the FT3/TT4 ratio were significantly lower and the plasma levels of blood glucose were higher than in the other groups. There was no difference between the plasma thyroid hormone levels in rats with LT and those without LT. These studies suggest that (1) LT may occur independently of insulinitis, namely diabetes, (2) ATA levels and the incidence of LT increase with age, (3) the site of ATA production may not be confined to the thyroid gland, and (4) the derangement of glucose metabolism may be one of the factors in the decrease in plasma thyroid hormone. The BB/W rat is not only a useful animal model to use in exploring the pathogenesis of human insulin-dependent diabetes mellitus, but also spontaneous autoimmune thyroiditis.

The Bio Breeding/Worcester (BB/W) rat has been developed as the model animal

for human insulin-dependent diabetes mellitus (IDDM) since it was discovered in 1974. Spontaneous diabetes occurs at between 60-120 days of age and results in hyperglycemia,

ketoacidosis, hypoinsulinemia, weight loss, dehydration, and eventual death (Nakhooa *et al.*, 1978; Kawazu *et al.* manuscript in preparation). At the time of diagnosis, morphologic changes, such as lymphocytic infiltration and destruction of the pancreatic beta-cells, are observed. Like the increased prevalence of thyroid auto-immunity in patients with IDDM (Gray *et al.*, 1980), in some instances, lymphocytic thyroiditis (LT) was also found in BB/W rat (Sternthal *et al.*, 1981). Moreover many organ specific antibodies, such as islet cell surface antibody (ICSA), gastric parietal cell antibody (PCA), smooth muscle antibody (SMA) and thyroidal colloid antibody (TCA) have been also found in BB/W rat (Dyrberg *et al.*, 1982; Elder *et al.*, 1982; Like *et al.*, 1982). Therefore it was suggested that BB/W rat represents a new model of multiple autoimmune endocrinopathy (Sternthal *et al.*, 1981).

Through active immunization with thyroid extract or thymectomy with subsequent whole body irradiation, experimental autoimmune thyroiditis (EAT) can be induced in many animals (Witebsky *et al.*, 1956; Rose *et al.*, 1971; Miescher *et al.*, 1961; Penhale *et al.*, 1973). However, spontaneously occurring LT in experimental animals is rare and has been reported only in Beagle dog (Tucher *et al.*, 1962), in obese strain chicken (Coie *et al.*, 1968), in monkey (Biggazi *et al.*, 1975) and in Buffalo strain (BUF) rat (Hajdu *et al.*, 1969). The BB/W rat may thus be valuable in the elucidation of the basic pathogenesis (Colle *et al.*, 1981), prevention (Like *et al.*, 1979; Naji, 1981; Like *et al.*, 1982; Rossini *et al.*, 1984) and treatment of auto-immune thyroidal disease in man.

In previous reports on BB/W rat, the relationship between serum antithyroid antibody and histological changes in the thyroid gland were not studied. In the present study in BB/W rat, we investigated serial changes in the plasma levels of ATA by enzyme immunoassay (EIA), thyroid hormones, and

blood glucose, and also studied the relationship between these parameters and histological changes in the thyroid gland.

Materials and Methods

Animals and treatment

BB/W rats were kindly donated by Dr. Arthur A. Like (Massachusetts Medical School, Worcester, Mass., USA). This strain of BB/W rats was maintained by brother-sister mating at Animal Research Center in Tokyo Medical College and used for the experiments. All BB/W rats were tested for glycosuria (Tes-Tape, Eli Lilly and Co., Indianapolis, Indiana) and ketonuria (Ketostix, Ames Division, Miles Laboratories, Inc., Elkhart, Indiana) between 1300–1500 h every day. Rats were considered to have diabetes if they had 2(+) glycosuria. Diabetic rat were maintained on long acting insulin (Ultralente, Novo, Vagevard, Denmark) given daily by subcutaneous injection in the following doses: 4U, 5U, or 6U for 2(+), 3(+), or 4(+) glycosuria, respectively. If ketonuria was more than 1(+), additional insulin was administered as follows: 2U for 2(+), 3U for 3(+) and 4U for 4(+). Diabetic rats were permitted to have 1(+) glycosuria in order to avoid hypoglycemic shocks.

The BB/W rats were sacrificed at three different ages: 5 week old (group A; n=10, 5 males and 5 females), 11 week old (group B; n=10, 5 males and 5 females), and 20 week old (group C; n=21, 14 males and 7 females). Their plasma were separated and stored until assay. As the control, 14 week old Wistar Furth rats (n=5) were used.

Histology

Thyroid tissues were fixed in buffered formalin and stained with hematoxylin-eosin. Three sections from each thyroid were examined for histological evidence of lymphocytic thyroiditis (LT). LT was judged to be positive when there was clear lymphocytic infiltration along with the destruction of some thyroid follicles. In this series there was no case which showed severe, diffuse thyroiditis. Photomicrographs of sectioned thyroids from 5 and 20 week old BB/W rats are shown in Figs. 1 and 2.

Preparations of rat thyroglobulin (Tg)

Thirty Wistar Furth rat thyroids (0.54 g) stored at -20°C were gently homogenized in

Fig. 1. Photomicrograph of the thyroid section obtained from 5 week old BB/W rat, showing no lymphocytic infiltration. (hematoxylin-eosin, $\times 100$)

Fig. 2. A typical lymphocytic thyroiditis with destruction of some thyroid follicles obtained from 20 week old BB/W rat. (hematoxylin-eosin, $\times 100$)

12 ml of saline on ice. The homogenate was centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant was centrifuged again at 100,000 g for 60 minutes at 4°C. Rat Tg was precipitated with 1.5 M-1.9 M of ammonium sulfate and further purified by gel filtration on Sephacryl S 300 (Pharmacia Fine Chemicals, Uppsala, Sweden). Finally, 6 mg of rat Tg was obtained and stored in aliquots at 500 µg/ml at -20°C. The protein concentration was measured by Lowry's method (Lowry *et al.*, 1951).

Assay for ATA

Rat ATA was measured by solid-phase EIA using the biotin-avidin-peroxidase system based on the methods of Hara *et al.*, 1984. The protocol for the solid-phase EIA is briefly: Two hundred µl of Rat Tg at 50 µg/ml in 0.01 M carbonate buffer, pH 9.5 was coated onto 96 well plastic micro-ELISA plates (Nunc-Immuno-plate II, Nunc, Kamstrup, Denmark) by incubation for 30 min at room temperature (RT). After removing uncoated Tg, 250 µl of 2% BSA in 1/60 M phosphate buffered saline, pH 7.4 (PBS), was coated on to the plates and incubated for 30 min at RT. The plates were washed with PBS-Tw20 (PBS containing 0.1% Tween 20 and 2% BSA). In triplicate 200 µl of serum sample diluted 1:200 in PBS-Tw20 were applied to the wells and incubated overnight at 4°C. After washing, 200 µl of biotinylated antirat IgG (Vector Lab. Inc., Buirmingham, CA) diluted 1:1,000 in PBS-Tw20 was added and allowed to incubate for 2 hr RT. After washing, 200 µl of avidin peroxidase diluted 1:1,000 was added and allowed to incubate for an additional 2 hr at RT. After washing, 250 µl of substrate solution (o-phenylenediamine and hydrogen peroxide) was then added. The reaction was stopped with the addition of 6N H₂SO₄ to each well. The optical density of the reaction mixture was read at 492 nm on a spectrophotometer (Titertek Multiscan, Flow Laboratory, McLean, VA).

The results for the samples were expressed as optical density (O.D.₄₉₂ × 1,000) of the Tg coated wells minus that of BSA coated wells.

Thyroid hormones and blood glucose

Plasma free 3,5,3'-triiodothyronine (FT3) and total thyroxine (TT4) levels were determined using Amerlex FT3 RIA and TT4 RIA kits (Amersham Japan Co., Tokyo). In the preliminary experiments, plasma TT3 levels of some normal and young BB/W rats were too low to be measured accurately with a commercial kit and plasma FT4 levels were much more influenced by plasma albumin levels. Therefore we chose TT4 levels as indices for thyroidal hormone release and FT3 levels as indices for peripheral conversion of thyroid hormone.

Blood glucose was measured by a glucose oxidation method using the Glucose B-test (Wako, Osaka).

Statistics

Statistical analyses were performed by unpaired Student's *t*-test when standard deviations (SD) were not different statistically (F test). When SD were different unpaired Wilcoxon's rank test was used. For some analyses χ^2 test was also used.

Results

Incidence of diabetes and LT

As shown in Table 1, the incidence of diabetes was 0% in group A, 70% in group B and 86% in group C. On the other hand, LT was observed in 0%, 20%, and 48% in group A, B and C, respectively. The relationship between the occurrence of diabetes and LT was examined in the rats in groups B and C. LT was observed in 32%

Table 1. Incidences of diabetes and lymphocytic thyroiditis (LT) in the three groups of BB/W rat and the control rats

Group	Age (weeks)	Number	Incidence of diabetes	Incidence of LT
BB/W rat				
A	5	10	0/10 (0%)	0/10 (0%)
B	11	10	7/10 (70%)	2/10 (20%)
C	20	21	18/21 (86%)	10/21 (48%)
Control	14	5	0/5 (0%)	0/5 (0%)

Fig. 3. Plasma anti-thyroglobulin antibody (ATA) levels measured by the solidphase EIA in the control rats and three groups of BB/W rats. ATA levels were expressed as absorbance $\times 1,000$.

(8/25) of the diabetic rats and in 67% (4/6) of non-diabetic rat ($\chi^2=2.51$, not significant). Although both of the two diseases occurred with increasing frequency with age, we could not demonstrate the correlation between diabetes and LT. There was also no sex-related difference in the incidence of diabetes or LT (data not shown).

ATA levels

The results are shown in Figs. 3 and 4. The mean $\pm$ SEM value for the ATA level was 91.4 ± 28.5 in the control, 49.5 ± 15.4 in group A, 197.8 ± 41.5 in group B and 376.7 ± 48.7 in group C, showing a clear increase with age (Fig. 3). In this study, 83% (10/12) of BB/W rats with LT had ATA levels higher than the mean ± 2 SD in the controls, whereas only 34% (10/29) of BB/W rats without LT had such high ATA levels. This result suggest a strong association of ATA with LT ($\chi^2=8.11$, $p < 0.01$). When the mean levels of ATA were compared between rats with and without LT in group C (Fig. 4), the ATA level in the rats with LT ($n=10$, 492.0 ± 71.4) was significantly higher ($p < 0.02$) than in those without LT ($n=11$, 271.3 ± 53.0). In addition, even in the rats without LT, the ATA level was also significantly higher than that in the control rats ($p < 0.01$).

Fig. 4. Plasma anti-thyroglobulin antibody (ATA) levels in the group C BB/W rats with or without lymphocytic thyroiditis (LT).

The ATA levels were correlated negatively with FT3 levels ($r=0.3470$, $p < 0.05$) and positively with plasma glucose levels ($r=0.4301$, $p < 0.01$), but not with TT4 levels. Again, there was no difference attributable to sex in the ATA level (data not shown).

Thyroid hormone levels

As shown in Fig. 5, the mean $\pm$ SEM of FT3 value was 1.78 ± 0.15 pg/ml in the control rats, 2.04 ± 0.16 in group A, 2.30 ± 0.15 in group B, and 1.06 ± 0.08 in group C. When compared with the value in the control rats, group B showed an increase in FT3 ($p <$

0.05), whereas group C demonstrated a decrease in FT3 ($p < 0.01$). The amount of albumin did not seem to be the major cause of these changes, because FT3 did not correlated with albumin in the whole body of the samples ($r = 0.0394$, not significant) and the albumin levels in both group B (3.15 ± 1.06 g/dl) and group C (3.11 ± 1.53) were significantly lower (both, $p < 0.05$) than in normal controls (4.22 ± 1.26).

The TT4 levels, shown in Fig. 6, were 2.80 ± 0.25 μ g/l in the control rats. 3.34 ± 0.07 in group A, 3.28 ± 0.18 in group B and 2.10 ± 0.11 in group C. Group C showed a significantly lower TT4 level than the other groups ($p < 0.01$).

The FT3/TT4 ratio (pg/ μ g), shown in Fig. 7, was also significantly lower in group C than in the other groups ($p < 0.01$).

There was no difference between the

Fig. 5. Plasma free T3 (FT3) concentrations in the control rats and three groups of BB/W rats.

Fig. 6. Plasma total T4 (TT4) concentrations in the control rats and three groups of BB/W rats.

plasma thyroid hormone levels in LT positive and LT negative BB/W rats.

Blood glucose levels

As shown in Fig. 8, blood glucose levels were 166 ± 17 mg/dl in the control rats, 159 ± 15 in group A, 182 ± 9 in group B, and 318 ± 35 in group C. Despite daily insulin injections, the blood glucose level was significantly higher in group C than in

the other groups ($p < 0.05$). The plasma glucose levels were correlated positively with ATA levels (as mentioned above) and negatively correlated with FT3 levels ($r = -0.5052$, $p < 0.01$) and TT4 levels ($r = -0.4590$, $p < 0.01$).

Then, in order to see the effects of the control status of diabetes on plasma thyroid hormones and ATA levels, we compared these levels in the two groups of BB/W rats

Fig. 7. Plasma FT3 to TT4 ratios (pg/μg) in the control rats and three groups of BB/W rats.

Fig. 8. Blood glucose concentrations in the control rats and three groups of BB/W rats.

Table 2. Comparison of thyroid hormones and anti-thyroglobulin antibody (ATA) levels in BB/W rats with normal and high plasma glucose (PG) concentrations

BB/W rats	N	FT3	TT4	FT3/TT4	ATA
		mean (SD)	mean (SD)	mean (SD)	mean (SD)
PG<250	21	1.90 (0.95)	2.72 (0.83)	69.0 (22.9)	280.1 (152.4)
PG≥250	10	0.93 (0.37)	2.30 (1.09)	47.5 (23.9)	410.7 (294.4)
		p<0.00	NS	p<0.025	NS

Notes: Group B and C were combined. Fifteen of PG<250 group and all of PG≥250 group were on insulin therapy. NS=not significant. Units; PG (mg/dl), FT3 (pg/ml), TT4 (μg/dl), FT3/TT4 (pg/μg), ATA (OD₄₉₂ × 10³)

divided according to plasma glucose levels (Table 2). In the poor control group (PG ≥ 250), FT3 and FT3/TT4 were significantly lower than in the good control group.

Discussion

In BB/W rat, the onset of diabetes is usually between the ages of 60 and 120 days (Nakhoo *et al.*, 1978), while the appearance of TCA was found at 45 days of age and increased intensely above the age of 80 days (Like *et al.*, 1982). Since the age of 11 weeks, thus, appeared to be a critical period for observing the occurrence of LT and the production of ATA, we separated our BB/W rats into three different ages, i.e. before, around and after 11 weeks.

Sternthal *et al.* (1981) reported for the first time about spontaneous LT in 8–10 month old BB/W rat, in which the incidence of LT was 59% in diabetic rats and 11% in nondiabetic rats. In the present study, LT was found in 0%, 20% and 47.6% in each group of BB/W rats, showing a clear increase with age. However, the occurrence of LT did not seem to be closely linked to diabetes, which is in striking contrast to Sternthal's results. This difference may be due to the relative shortness of the period of our observation (20 weeks) and the fact that the BB/W rat is not always an inbred strain. There could be a subtle differences in the genetic backgrounds of BB/W rats

in the two facilities. In our study, the incidence of diabetes was very high (91.3%) at the age of 120 days, whereas the incidence of diabetes in BB/W rats reported elsewhere was around 40–60% (Butler *et al.*, 1983).

Although LT in man and BUF rat is clearly more common in females (Silverman and Rose, 1971), there was no sex-related difference in our study in BB/W rat. This is consistent with the data reported by other groups (Sternthal *et al.*, 1981; Colle *et al.*, 1985). IDDM in BB/W rat also shows no sex-related difference (Nakhoo *et al.*, 1978). These results suggest that the autoimmune phenomena in BB/W rat are related to the profound immunologic abnormalities (MacLaren *et al.*, 1983; Jackson *et al.*, 1983; Prud'homme *et al.*, 1984) which are common to both sexes and are not influenced by sex hormones or sex chromosomes (Colle *et al.*, 1983).

In this study, a strong association of ATA with LT ($\chi^2=8.11$, $p<0.01$) was demonstrated. In addition, even in the rats without LT, ATA level was increased in 24% (10/41). However, 3 sections of each thyroid do not seem to be enough to conclude the absence of LT. In fact, we examined the whole thyroid glands of two such cases by 5 μm sectioning and found a small focus of lymphocyte infiltration in one case and no LT in the other (unpublished observation). Therefore, it might be concluded that, in some cases, the occurrence of ATA is seen

before that of LT. Such a situation seems to indicate that ATA could be produced in lymphoid tissues other than the thyroid gland. Weetman *et al.*, 1984 have also shown in rats with experimental autoimmune thyroiditis that the iliac lymphnodes draining the immunization site are important in anti-thyroid autoantibody production.

It has been reported that the presence in severe LT in BB/W rat is not accompanied by abnormalities in serum T4, T3, or TSH levels or in the TSH response to thyrotropin releasing hormone (TRH) (Sternthal *et al.*, 1981). On the other hand, in BUF rat (Kieffer *et al.*, 1978) and in its F₁ crossed with BB rat (BUF×BB) (Colle *et al.*, 1985), the rats with severe LT have been shown to have lower mean T4 and higher TSH levels than rats without LT. In the present study, the levels of FT3 and TT4 were significantly lower in group C, in which the incidence of LT was the highest, than in the other groups. Decreased FT3 and TT4 in this group could not be attributed to the decreased thyroïdal secretion of thyroid hormones, because the mean thyroid hormone level in the rats with LT was not different from that in the rats without LT. On the other hand, there was a relatively good, negative correlation between the plasma glucose levels and the thyroid hormone levels in this study. This is in agreement with the reports on human diabetes (Pittman *et al.*, 1979). Therefore it seems more likely that low FT3 and TT4 levels in group C rats were due to the consequences of impaired glucose metabolism. The effects of diabetes on serum thyroid hormone concentrations have been extensively studied in streptozotocin treated rats (Zaninovich *et al.*, 1977; Gonzalez *et al.*, 1980) and in diabetic patients (Naeije *et al.*, 1978; Fujii *et al.*, 1981), in which the most consistent changes were a decrease in the serum T3 and T3/T4 ratio and a rise in the serum reverse T3 level. These alterations have been supposed to be due to decreased T4-

5'-monodeiodinase activity. In this study the FT3/TT4 ratio was also significantly lower in group C than in the other groups, suggesting the involvement of such a mechanism in the low FT3 levels. Low TT4 levels were also reported in streptozotocin-induced diabetic rats (Zaninovich *et al.*, 1977) and in diabetic patients (Fujii *et al.*, 1981), although the precise mechanisms have not been fully clarified yet. Decreased TRH secretion (Gonzalez *et al.*, 1980), intrinsic pituitary abnormality (Naeije *et al.*, 1978), and impaired functions in the thyroid itself (Bagchi *et al.*, 1981) have all been implicated.

In group B the mean FT3 level was higher than in the other groups. The reason for this remains to be clarified. Albumin was not increased. Some such mechanism as in transient thyrotoxicosis in human chronic or subacute thyroiditis might be involved. However, histological findings did not reveal such destructive processes.

It should be noted that BB/W rat is valuable for observing the relationship of thyroid hormone metabolism to spontaneous diabetes mellitus as well as for studying the mechanism of autoimmune thyroid diseases.

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