The pathogenic role of anti-thyroglobulin antibody on pregnancy: evidence from an active immunization model in mice

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BACKGROUND: The presence of antibodies to thyroglobulin (Tg) is associated with fetal loss even in the absence of thyroid dysfunction. The aim of this study was to examine whether active immunization with Tg could elicit anti-Tg autoantibodies and reproductive failure without interfering with thyroid function. METHODS: BALB/c mice that were immunized with human Tg in complete Freund’s adjuvant (CFA) or injected with only CFA were studied for the development of antibodies to Tg, T4, dsDNA, ssDNA and cardiolipin. Total T4, free T4 and thyroid-stimulating hormone (TSH) levels were also assessed before and during pregnancy. Percentages of resorbed fetuses (the equivalent to human missed abortion) were compared and autoantibody presence on the placenta and fetuses was examined. RESULTS: Following immunization, high levels of anti-Tg were observed in mice immunized with Tg, compared with mice injected with CFA [0.83 ± 0.23 versus 0.012 ± 0.016 respectively; mean ± SD optical density (OD) at 405 nm; P < 0.001]. The specificity of binding to Tg was confirmed by competition assay. Although total T4 levels were increased in comparison with control mice, this was associated with the presence of antibodies to T4. Indeed, free T4 levels and TSH were similar to control mice. Mice were killed after 14 days of pregnancy. The thyroid function and the histology of the thyroid glands were normal. Increased fetal wastage was found among the Tg-immunized mice compared with the CFA-injected mice (P = 0.04), with lower fetal and placental weights (fetal weights: 194 ± 4 mg versus 240 ± 6 mg; placental weights: 105 ± 2 mg versus 130 ± 3; P < 0.001 for both). Antibodies to Tg were demonstrated only on the placenta of Tg-immunized mice. CONCLUSION: Immunization with Tg results in the production of Tg antibodies and fetal resorption. These effects occur in the absence of thyroid dysfunction.

Key words: animal model/autoimmunity/fetal loss/thyroglobulin/thyroid

Introduction

Many different conditions may cause pregnancy loss, such as chromosomal abnormalities, anatomical malformations and autoimmune diseases including systemic lupus erythematosus or the antiphospholipid syndrome (Branch, 1990; Cowchock, 1991). Abnormal hormone levels such as hypothyroidism or hyperthyroidism can also cause fetal demise (Gardiner-Hill, 1929; Hamburger and Stoffer, 1981; Davis et al., 1988), and fetal loss has been demonstrated in mice with thyroiditis (Imaizumi et al., 2001). Several studies have also found an association between spontaneous abortions and autoantibodies to the thyroid gland, such as thyroid peroxidase or thyroglobulin (Tg) (Davis et al., 1988). As thyroid dysfunction is not always detected in the presence of thyroid abnormalities (Lejeune et al., 1993; Pratt et al., 1993a;b; Geva et al., 1997), the higher miscarriage rate in women with autoimmune thyroiditis could be due to the autoantibodies rather than an impaired hormonal state. However, as other studies have not demonstrated an association between the presence of thyroid antibodies and pregnancy loss (Esplin et al., 1998; Rushworth et al., 2000), the direct role of thyroid autoantibodies in fetal loss is debatable. In order to study the specific role of...
autoantibodies to the thyroid in pregnancy loss, we utilized a murine model of active immunization with human Tg. The aim of the study was to induce the production of Tg autoantibodies without thyroid hormone abnormalities or histological evidence of thyroiditis, in order to study the effects on pregnancy outcome.

Materials and methods

Active immunization with thyroglobulin
BALB/c female mice (8–10 weeks old) were purchased from the Sackler Faculty of Medicine, Tel-Aviv University, Israel. Human Tg (10 μg) (Biogenesis, Poole, UK) was emulsified in complete Freund’s adjuvant (CFA) and injected intradermally into the hind foot-pads of 27 mice. The control group included 29 mice that were injected with CFA alone. The mice were bled 3 weeks after immunization and prior to mating. Both mouse groups were mated overnight, and the presence of a vaginal mucus plug the following morning was taken as an evidence of mating, and designated as day 0 of pregnancy. Mice were bled on the 14th day of pregnancy and killed. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees of the Sheba Medical Center and Tel-Aviv University.

Screening of mice sera for autoantibodies

Anti-Tg
ELISA plates were coated with human Tg (10 μg/ml in carbonate buffer) and blocked with 5% BSA. Following extensive washing, mouse sera [diluted 1:200 in tris-buffered saline (TBS)] were added to the wells. Bound antibodies were detected using anti-mouse IgG rabbit serum conjugated to alkaline phosphatase (Jackson Immunoresearch 115±055±146) and the intensity of the colour in the wells was read in a Titertrek ELISA reader using a 405 nm filter and a 620 nm reference filter.

Anti-dsDNA, anti-ssDNA and anti-cardiolipin
ELISA plates were coated with poly-L-lysine, calf thymus DNA (250 ng/ml in TBS) and cardiolipin (50 μg/ml in ethanol), for the detection of double stranded DNA (dsDNA), single stranded DNA (ssDNA) and cardiolipin (Cl) respectively, as previously described (Blank et al., 2002). Mouse sera [diluted 1:200 in TBS] were added to the wells and bound antibodies were detected as described above.

Anti-thyroxine (T4)
T4 antibodies were tested prior to pregnancy in 10 mice injected with CFA and 12 mice immunized with Tg, and during pregnancy in 18 mice injected with CFA and 19 immunized with Tg (all of which were randomly chosen). The percentage of T4 bound to IgG was measured. Serum (20 μl) was incubated with 15 000 cpm of 125I-thyroxine in 30 μl of barbital buffer for 6 h at 20°C. Bound 125I-thyroxine was precipitated with 450 μl of 20% PEG 6000 at 4°C for 30 min. The amount of labelled thyroxine in the pellet was quantified and is expressed as the percentage of the total.

Thyroid function tests
T4 levels were determined 3 weeks after injection/imunization and also immediately prior to killing. Total T4 levels were measured in each mouse from blood spotted on filter paper using the neonatal kit (Diagnostic Products Corporation, Los Angeles, USA) according to the manufacturer’s instructions. Free T4 was measured by equilibrium dialysis using a kit (Quest Diagnostics, Nichols Institute, San Juan Capistrano, USA). Pools of sera with antibodies to T4 >15% (range 15.8–55.0) and <2% (range 0.6–1.8) were prepared. Serum TSH was measured in 50 μl of serum using a sensitive, heterologous, disequilibrium, double antibody precipitation RIA as described elsewhere (Pohlzen et al., 1999). The sensitivity of this assay was 5–10 mIU/l. The intra- and inter-assay coefficients of variations were 16 and 27% at 20 mIU/l, 6.3 and 8.2% at 200 mIU/l, 5.4 and 9.8% at 850 mIU/l and 10 and 24% at 2000 mIU/l respectively. Thyroid-stimulating hormone (TSH) levels were tested prior to pregnancy in 12 mice injected with CFA and 14 immunized with Tg, and also in 18 randomly selected pregnant mice from both groups.

Histology of the thyroid gland
Thyroid glands of 10 Tg-immunized and 10 CFA-injected mice were excised after killing. The thyroids were fixed in 4% paraformaldehyde and stained with haematoxylin and eosin. Three slices were taken from each gland, the slices contained three or more sections separated by >50 μm apart, and these were evaluated by an observer unfamiliar with the study protocol and the different mouse groups.

Evaluation of pregnancy outcome
The uterine horns were examined to determine the number of live fetuses and number of resorbed pregnancies in order to calculate the percentage index of fetal resorption. Fetal resorption was identified as growth arrest and regression into an oval-shaped mass smaller than expected for the 14th day of pregnancy (with a size similar to 7–8 days pregnancy). Placental and fetal weights of randomly chosen mice (15 Tg and 15 CFA immunized mice) were determined. The person who selected the mice from both groups was blinded regarding the treatment groups.

Elution of antibodies from the placentae and the embryos
Six placentae and embryos were separated, hand-homogenized and washed five times with 50 ml phosphate buffer saline (PBS). The last wash was retained in order to confirm that no IgG was found in them. Immunoglobulins were eluted from the washed homogenates with glycine HCl buffer (0.1 mol/l pH 2.7, 15 min, room temperature) that were later neutralized with TBS. Following centrifugation, the supernatants were separated and dialyzed against glycine buffer and maltose. The supernatants were analysed for the presence of antibodies to Tg and Cl.

Statistical analysis
Data were analysed by Student’s t-test and the $\chi^2$-test. $P < 0.05$ was considered statistically significant.

Results

Autoantibody production in mice immunized with Tg
Mice immunized with human Tg developed high levels of anti-Tg autoantibodies (Figure 1). The levels of anti-Tg on day 14

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of pregnancy were 0.83 ± 0.23 (mean ± SD OD at 405 nm) in the mice immunized with human Tg, compared with 0.012 ± 0.016 in the mice injected with CFA (P < 0.001). No mice from either group developed high levels of antibodies to dsDNA, ssDNA or cardiolipin.

**Inhibitory effect of Tg on binding of anti-Tg**

The specificity of anti-Tg autoantibodies to Tg was demonstrated by the dose-dependent inhibition of binding of anti-Tg to Tg-coated plates by the human Tg (Figure 2; three separate experiments). Whereas 50 μg/ml of ovalbumin resulted in 20% inhibition of anti-Tg binding to Tg, the same concentration of Tg resulted in 80% inhibition of anti-Tg binding to Tg.

**Thyroid function and histology**

Tg immunization was associated with increased serum T4 levels compared with mice injected with CFA (mean ± 2 SD: 6.5 ± 3.6 μg/dl versus 5.1 ± 1.2 μg/dl respectively, P = 0.03). During pregnancy, serum T4 levels decreased in both groups (Table I), but remained higher in the Tg-immunized group than in the controls (4.7 ± 3.0 μg/dl versus 2.3 ± 0.5 μg/ml respectively, P < 0.001). Tg immunization was associated with increased binding of T4 to IgG in non-pregnant (8.5 ± 2.3%) and pregnant mice (20.3 ± 3.3%), while this was very low in CFA-injected animals, which suggests that the presence of the elevated serum T4 may be due to anti-T4. Indeed, when free T4 was assessed in pooled sera from mice with a high percentage of T4 bound to IgG (>15%, range 15.8–55.0) and in those with a low percentage of T4 bound to IgG (<2%; range 0.6–1.8), no significant difference was found (8.8 ng/ml versus 8.1 ng/ml respectively). Further evidence that the Tg-immunized mice were euthyroid was shown in the normal serum TSH levels and there was no significant difference in serum TSH levels between mice immunized with Tg or with CFA (Table I).

**Pregnancy outcome in Tg immunized mice**

Mice were killed on day 14 of pregnancy. There were differences in fetal resorption rate between the Tg-immunized mice and the CFA-injected mice (Table II). The mice were divided into categories according to the fetal resorption rate of each mouse (<10, 10 to <20, 20 to <30, and >30%; Table III), yielding a significant difference between the two groups (P = 0.04).

There were also lower mean fetal and placental weights in the Tg-immunized mice (Figure 4) compared with the control group (fetal weights: 194 ± 4 mg versus 240 ± 6 mg; placental weights: 105 ± 2 mg versus 130 ± 3 mg; P < 0.001 for both). All of the placentae and fetuses from the 15 mice were measured, there were 105 embryos in the Tg group and 122 embryos in the CFA group and a similar number of placentae.

**Elution of antibodies from placentae and embryos**

After separating the placentae from the embryos, anti-Tg autoantibodies were detected in increasing concentrations of

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**Table I.** T4, thyroid-stimulating hormone (TSH) and antibodies to T4 in mice immunized with thyroglobulin (Tg) or complete Freund’s adjuvant (CFA) before and during pregnancy.

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<th></th>
<th>Tg</th>
<th>CFA</th>
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<tr>
<td></td>
<td>Non-pregnant</td>
<td>Pregnant</td>
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<tr>
<td>T4 levels (mg/dl)</td>
<td>6.5 ± 3.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>4.7 ± 3.0&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>TSH (mU/L)</td>
<td>35.0 ± 4.9</td>
<td>17.3 ± 3.7</td>
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<td>T4 bound to IgG (%)</td>
<td>8.5 ± 2.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>20.3 ± 3.3&lt;sup&gt;f&lt;/sup&gt;</td>
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P-values: <sup>a</sup>0.04; <sup>b</sup>0.03; <sup>c</sup>< 0.001; <sup>d</sup>0.001; <sup>e</sup>0.009; <sup>f</sup>< 0.001.

Histological examination of the thyroids did not reveal any pathological changes in either the mice immunized with Tg or those injected with CFA (Figure 3). There were no lymphocytic infiltrates, which typically develop in experimental thyroiditis.
the supernatant eluted only from the placentae of the Tg-immunized mice (Figure 5). These autoantibodies were not found on embryos of Tg-immunized mice, nor were they eluted from the placentae or embryos of CFA-injected mice. No other autoantibodies could be detected on the placentae or embryos (data not shown).

Discussion

This study shows that active immunization of mice with human Tg results in the production of anti-Tg autoantibodies and pregnancy failure manifested by an increased fetal resorption rate (equivalent to human missed abortions). In order to examine the isolated effect of anti-Tg on pregnancy outcome, we utilized BALB/c female mice which have been previously shown to develop high titres of anti-Tg following immunization with Tg, without accompanying thyroiditis or thyroid hormone abnormalities (Vladestine and Rose, 1971; Tomer et al., 1996). The specificity of anti-Tg was confirmed by competition assays, in which the antibody binding to Tg-coated plates was inhibited by soluble Tg, but not by ovalbumin. Although the mice immunized with Tg developed higher T4 levels than the mice injected with CFA, the increased T4 levels were due to an increased amount of T4-bound to IgG. Indeed, free T4 levels did not differ between mice with variable total T4 levels, and there was no significant difference in serum TSH levels between mice immunized with Tg or with CFA. Furthermore, thyroid histology was completely normal in mice immunized with Tg, indicating that this murine model is suitable to assess the isolated effect of anti-Tg.
Some reports support an association between Tg antibodies and fetal loss (Stagnaro-Green et al., 1990; Gilnoe et al., 1991; Pratt et al., 1993b; Takashi et al., 1997; Kutteh et al., 1999a; Abramson and Stagnaro-Green, 2001; Matalon-Tartakover et al., 2001); however, a causative role has not been established. Fetal loss among patients with thyroid antibodies could be induced by several putative mechanisms. The most obvious one is thyroid dysfunction, as is commonly seen in Hashimoto’s thyroiditis. However, the increase in miscarriages cannot always be explained by thyroid dysfunction alone (Dendrinos et al., 2000). This suggests that the higher rate of miscarriages observed in women with autoimmune thyroid disturbances reflect primarily an autoimmune phenomenon, rather than, or in addition to, a consequence of overt thyroid hormone abnormalities. Increased pregnancy loss is found among patients suffering from different autoimmune diseases, and organ specific antibodies to the thyroid have been found in parallel to non-organ specific autoantibodies (Magaro et al., 1997). Therefore, the presence of antibodies to the thyroid could represent a secondary marker of a predisposition for an autoimmune disease rather than the actual cause of fetal loss (Coulman et al., 1999; Sherer and Shoenfeld, 1999). However, we failed to identify other non-organ specific autoantibodies among the mice that developed anti-Tg and increased fetal resorption rates. Other studies have also reported discordance between the presence of thyroid autoantibodies and non-organ specific autoantibodies (Pratt et al., 1993; Bussen and Steck, 1997; Mecacci et al., 2000), thus excluding polyclonal activation as the cause for thyroid autoantibody production.

Immunization with Tg was associated with lower placent al and embryonic weights. The finding of anti-Tg binding to the placenta and not to the embryo-body might suggest that these autoantibodies have a direct effect on the placenta. It is well known that autoantibodies can pass into the amniotic fluids (Cohen et al., 2000) and interact with the syncytiotrophoblast and cytotrophoblast (Kutteh et al., 1999b). However, it is possible that anti-Tg bound passively to the placenta but exhibited no harmful effect, as many other antibodies and immune complexes do. Moreover, a direct pathogenic effect of anti-Tg antibodies is only a possible explanation for the reproductive failure, as other possibilities which interfere with reproduction can occur following immunization, such as a switch to Th1. Further studies will clarify the precise effect that anti-Tg may exert on placental function, and whether these antibodies have a pathogenic role on pregnancy. These should include, for example, passive transfer of anti-Tg antibodies, and transfer of sera from mice immunized with Tg into naive mice.

In conclusion, mice immunized with Tg developed high titres of Tg antibodies without thyroid dysfunction, had a higher incidence of fetal resorptions and reduced placental and embryo weights. Hence, anti-Tg might exert a direct pathogenic effect on pregnancy outcome in the absence of thyroid dysfunction, but more studies are required in order to confirm this assumption.

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