Selective macrophage depletion in the liver does not prevent the development of the sick euthyroid syndrome in the mouse

Anita Boelen. Marianne C Platvoet-ter Schiphorst, Nico van Rooijen1 and Wilmar M Wiersinga

Department of Endocrinology, Academic Medical Center, University of Amsterdam, The Netherlands; Department of Cell Biology and Immunology1, Faculty of Medicine, Free University, Amsterdam, The Netherlands


A decreased serum triiodothyronine (T3) level is one of the main characteristics of the sick euthyroid syndrome, caused mainly by a decreased 5\(^{-}\)deiodination of thyroxine (T4) in the liver. Cytokines have been implicated in the pathogenesis of the changes in thyroid hormone metabolism during illness. We therefore investigated the role of cytokines produced by the liver macrophages (Kupffer cells) in the development of the sick euthyroid syndrome, which was induced in mice by a single injection of bacterial endotoxin (lipopolysaccharide) or by 24-h starvation. Experiments were carried out with or without previous selective depletion of liver macrophages by intravenous administration of liposome-encapsulated dichloromethylene diphosphonate. Relative to saline-injected pair-fed controls, the administration of lipopolysaccharide caused a decrease of serum T3 and T4 and liver 5\(^{-}\)deiodinase mRNA. Selective depletion of liver macrophages did not affect these changes. Starvation for 24 h decreased serum T3 and T4, associated with a slight decrease of liver 5\(^{-}\)deiodinase mRNA. There were no differences between macrophage-depleted and non-depleted animals in this respect. In summary, selective depletion of liver macrophages did not affect the decrease in serum T3, T4 or liver 5\(^{-}\)deiodinase mRNA induced by lipopolysaccharide or 24-h starvation in mice. We conclude that cytokines produced by Kupffer cells are not involved in the pathogenesis of the sick euthyroid syndrome in this experimental model.

A Boelen, Department of Endocrinology, F5-171 Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

The sick euthyroid syndrome (SES) refers to changes in thyroid hormone metabolism and regulation in patients with non-thyroidal illness (NTI). The general term SES comprises more than one entity because it includes components of reduced food intake, of medication and of illness itself. These changes include a decrease in serum T3 and an increase in serum rT3 levels. The TSH level usually remains unchanged, and serum T4 decreases in severe cases of NTI. The lower T3 and higher rT3 serum values are caused by a lower T3 production rate and a decreased rT3 metabolic clearance rate due to diminished iodothyronine 5\(^{-}\)deiodinase activity (1). Eighty percent of the daily T3 production rate is generated by 5\(^{-}\)-deiodination of T4 (2), which occurs predominantly in the liver. The liver, therefore, seems to be an important organ in the development of SES but the pathogenesis of this syndrome is incompletely understood.

Recent studies suggest a role for cytokines because administration of tumor necrosis factor alpha (TNF-\(\alpha\)), interleukin 1\(\alpha\) (IL-1\(\alpha\)) or IL-6 to human subjects or experimental animals induces changes in thyroid hormone metabolism strongly resembling SES. Recently, we have shown that administration of the bacterial endotoxin lipopolysaccharide (LPS) to mice results in a decrease in serum T3 and T4 accompanied by a decrease in liver 5\(^{-}\)-deiodinase mRNA (3). Cells of the macrophage lineage respond to LPS by producing a variety of cytokines, including IL-1, TNF-\(\alpha\) and IL-6 (4, 5). Liver macrophages (Kupffer cells) are considered to be the main source of cytokine production in the liver (6).

The aim of the present study, therefore, was to investigate the role of cytokines generated by liver macrophages in the development of SES in mice. Sick euthyroid syndrome was induced by a single injection of LPS or by 24-h starvation. Caloric deprivation also results in a decrease of serum T3 and T4 and is one of the factors contributing to SES (1, 7, 8). Cytokine production in liver macrophages during these experimental procedures was eliminated by the intravenous administration of liposome-encapsulated dichloromethylene diphosphonate (Cl\(_2\)MDP). The liposomes are selectively ingested by macrophages; subsequent disruption of the multilamellar liposomes is mediated by phospholipase; Cl\(_2\)MDP is then released, resulting in macrophage destruction. Selective elimination of macrophages from liver (Kupffer cells) and spleen is accomplished.
by the intravenous route of administering liposome-encapsulated Cl2MDP, which in contrast to intraperitoneal or subcutaneous administration only affects macrophages in liver and spleen (9–12).

Materials and methods

Materials

Endotoxin. Lipopolysaccharide (LPS, E. coli 127:B8) was obtained from Sigma Chemical Co. (St Louis, MO).

Liposomes. Multilamellar liposomes were prepared according to van Rooijen et al. (12). Briefly, 86 mg of phosphatidylcholine and 8 mg of cholesterol (molar ratio 6:1) were dissolved in 20 ml of methanol–chloroform (1:1) in a round-bottom flask. The thin film formed on the interior of the flask after low-vacuum rotary evaporation at 37°C was dispersed in 10 ml of 10 mmol/l phosphate-buffered saline (PBS, pH 7.4) containing 2.7 g of Cl2MDP (a gift from Boehringer Mannheim, Mannheim, Germany) by gentle rotation for 10 min. The liposomes were resuspended in PBS after removing free Cl2MDP by rinsing the liposomes with PBS and centrifuging 30 min at 100 000g and 16°C.

Animal experiments

Female Swiss mice (Harlan Sprague-Dawley, Inc., Zeist, The Netherlands) were used at a weight of about 25 g. The animals were kept in 12-h light/dark cycles, in a temperature-controlled room (22°C) and received food and water ad libitum. A week before the experiment the mice were housed in groups. The LPS was diluted in sterile 0.9% NaCl immediately before injection. All LPS injections were given intraperitoneally in a volume of 0.5 ml. In order to deplete macrophages selectively in liver and spleen, mice were injected intravenously with 0.1 ml of Cl2MDP-liposomes, as described in detail previously (9–12). To confirm macrophage depletion, histological examination of liver and spleen from mice injected with saline or Cl2MDP-liposomes was performed by staining unfixed cryostat sections of liver and spleen, thaw-mounted on slides, for acid phosphatase and counter-staining with hematoxylin (13).

Two sets of experiments were performed. In experiment A, SES was induced by 75 μg of LPS. Because food deprivation as a result of illness per se affects thyroid function, the effects of LPS were compared to a pair-fed control group (PFC). Two days before LPS administration, 0.1 ml of liposomes or 0.1 ml of PBS was given intravenously in the tail vein. This results in four different experimental conditions, i.e. PBS/LPS, PBS/PFC, liposomes/LPS and liposomes/PFC. In experiment B, SES was induced by 24-h starvation; the control group received food ad libitum; fed controls (FC). Two days before the start of the starvation period, 0.1 ml of liposomes or 0.1 ml of PBS was given intravenously. This also results in four experimental conditions: PBS/starvation, PBS/FC, liposomes/starvation and liposomes/FC.

Body weight was recorded in all animals at the start and the end of the experiment. At different time points (1, 2, 8 and 24 h) after LPS administration (Exp. A) or after 24-h starvation (Exp. B), six to eight mice were sacrificed after diethyl ether anesthesia. Blood was taken by cardiac puncture (puncture was performed with 250 IU of heparin in the needle) and serum was stored at −20°C. The liver and spleen were obtained and stored immediately in liquid nitrogen.

Assays

Thyroid function tests. Serum T3 and T4 were measured with in-house RIAs (14).

Cytokine assays. Tumor necrosis factor alpha was measured by an ELISA, as described previously (15). The detection limit of the assay was 80 pg/ml TNF. Interleukin 6 was measured with the murine hybridoma B-cell line B9 (16). The detection limit of the bioassay was 0.5 U/ml. To prevent intra-assay variation, all cytokine samples of one experiment were measured within the same assay (3).

Liver 5'-deiodinase mRNA. The RNA was isolated using the guanidiniumthiocyanate method (17). Concentrations were determined from the absorbance at 260 nm (A260/A280 > 1.6). Total RNA was electrophoresed on 1% agarose/formaldehyde gels and blotted onto nylon membrane (NY 13, Schleicher and Schuell, Dassel, Germany) in 20 × SSC (sodium chloride/sodium citrate; 3 mol/l NaCl and 0.3 mol/l Na2HPO4 • 2H2O), using a Posiblot (Stratagene, La Jolla, CA). Total RNA was also spotted onto nylon (NY13, Schleicher and Schuell, Dassel, Germany) in 20 × SSC using a vacuum manifold (dot-blot). The RNA on the blots was fixed using UV cross-linking (1 J/cm2), and probed with digoxigenin (DIG)-labeled 5'-deiodinase type 1 cDNA (plasmid kindly provided by Dr PR Larsen (Boston, MA) (18). A DIG-labeled 28S probe was used as internal standard (plasmid kindly provided by Dr A Das, University of Amsterdam, The Netherlands). Messenger mRNA was isolated from a pool of RNA using the polyAT tract mRNA isolation system of Promega (Madison, WI). A 5-μg aliquot of poly A+ RNA was electrophoresed and blotted as described above. The blot was probed with DIG-labeled α2M RNA (cDNA kindly provided by Dr T Hakford, University of Amsterdam). A DIG-labeled β-actine RNA probe was used as internal standard (Boehringer Mannheim Biochemical, Mannheim, Germany). The DIG-labeling and hybridization conditions were according to the manufacturer's protocols (DIG) Luminescent Detection Kit, Boehringer Mannheim Biochemical, Mannheim, Germany). After hybridization, filters were washed twice at room temperature
with 2 × SSC 0.1% SDS for 5 min and twice at 65°C with 0.1 × SSC 0.1% SDS for 15 min. Specific mRNA was detected by chemiluminescent detection (DIG Luminescent Detection Kit, Boehringer Mannheim Biochemica, Mannheim, Germany). Filters were exposed at room temperature for X-ray film (5 min to 2 h). Quantification of 5′-deiodinase mRNA levels was performed by scanning the dot-blot X-ray films by densitometry using an XRS/3CX scanner (X-ray Scanner Corporation, Torrance, CA) and Whole Band analysis (version 2.4 1991) from Millipore Corporation (Ann Arbor, MI). Liver 5′-deiodinase mRNA levels were normalized for liver 28S mRNA expression.

Statistics
The data of serum thyroid hormones of experiment A were analyzed by analysis of variance with two grouping factors (time and treatment) using the BMDP package (BMDP Statistical Software, Inc., Los Angeles, CA). The data of liver 5′-deiodinase mRNA and serum thyroid hormones of experiment B were analyzed by Student’s t-test (Statgraphics 3.0, Statistical Graphics Corporation and STSC, Inc., Rockville, MD) (19).

Results
To verify macrophage depletion in our study, liver and spleen sections from mice injected with PBS or Cl2MBP-liposomes were investigated histologically for the absence of macrophages. The intravenous administration of Cl2MBP-liposomes resulted in an almost complete disappearance of acid phosphatase staining in the liver and in the red pulp and marginal zone of the spleen, which can be taken as evidence of effective depletion of macrophages in both organs (9–12). These results are in accordance with previously published data on the efficacy of Cl2MBP-liposome treatment on macrophage depletion in liver and spleen (9–12). Because serum TNF-α and IL-6 levels were undetectable at time point zero, it is unlikely that destruction of liver macrophages by the liposomes in the previous 2 days resulted in release of cytokines; moreover, there were no baseline differences in serum T3 and T4 between macrophage-depleted and non-depleted animals. Macrophage depletion resulted in a more than 50% decrease of serum TNF-α (p < 0.05) and IL-6 (p < 0.05) concentrations within 2 h after LPS injection (Fig. 1) and in a reduction of 32% of α2-macroglobulin mRNA in the liver 24 h after LPS administration. Starvation did not result in an increase of serum cytokine levels (data not shown).

The administration of LPS was associated with a decrease in serum T3 (p < 0.001) and T4 (p < 0.01) concentrations as compared to saline-treated pair-fed controls. This decrease was significant after 8 h (p < 0.005) and 24 h (p = 0.04). The LPS-induced decrease was also observed in macrophage-depleted

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Fig. 1. Tumor necrosis-factor alpha and IL-6 concentrations (values as mean ± SEM. N = 6) in mice after administration of lipopolysaccharide (LPS) without selective macrophage depletion (left) or with selective macrophage depletion (right). Both TNF-α and IL-6 levels were lower in the macrophage-depleted animals (p < 0.05).

Fig. 2. Serum T3 and T4 levels in mice after administration of 75 μg of lipopolysaccharide (LPS) (●) or saline in pair-fed controls (○) without (left panels) or with previous selective macrophage depletion (right panels). Mean values ± SEM (N = 6) are depicted; p values indicate the statistical significance between series of time points by ANOVA.

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mice (T<sub>3</sub>; p < 0.001; T<sub>4</sub>, p < 0.05). No differences in serum thyroid hormone were observed between liver macrophage-depleted and non-depleted mice (Fig. 2).

Administration of LPS also resulted in a decrease of liver 5′-deiodinase mRNA. The decrease in liver 5′-deiodinase mRNA again was independent of selective macrophtage depletion in the liver (Fig. 3).

The effects of starvation on serum thyroid hormones and liver 5′-deiodinase mRNA are shown in Fig. 4. Starvation during 24 h resulted in a decrease of serum thyroid hormone levels associated with a decrease in liver 5′-deiodinase mRNA, but this decrease was not significant. The depletion of Kupffer cells in the liver caused by the intravenous administration of liposomes did not affect the observed changes in serum thyroid hormones induced by 24-h starvation. Liver 5′-deiodinase mRNA, however, was decreased significantly in macrophtage-depleted animals compared to accompanying fed controls but was not different from mRNA of non-depleted mice.

**Discussion**

Acute and chronic illness induces a wide range of changes in thyroid hormone regulation and metabolism. These changes can be observed at the pituitary level (down-regulation of TSH secretion), at the thyroid level (diminished secretion of thyroid hormones), at the level of the serum proteins (decreased thyroid hormone binding capacity of T<sub>4</sub>-binding globulin) and at the level of peripheral tissues (decreased transport of thyroid hormones across the plasma membrane, decreased iodothyronine-5′-deiodination and decreased T<sub>3</sub> binding capacity of nuclear T<sub>3</sub> receptors) (1). Cytokines might play a role in these adaptive changes at each of the mentioned levels.

Hypothalamic pro-TRH mRNA and hypophysial TSH-β mRNA are decreased after IL-1 infusion in rats (20). Interleukin 1α also dose-dependently inhibits thyroglobulin (TG) and cAMP in human thyroid cells in vitro (21), and IL-6 inhibits thyroid peroxidase mRNA expression and T<sub>3</sub> secretion in a primary culture of human thyrocytes (22). Tumor necrosis factor alpha inhibits TSH-induced 5′-deiodinase activity in FRTL-5 cells (23), which is also found by Pekary et al. (24). A study of Tang et al. showed that TNF-α and interferon gamma (IFN-γ) decreased gene expression of type 1 5′-deiodinase in FRTL-5 cells (25). It is also shown that IFN-γ inhibits TSH-stimulated TG gene expression and secretion in vitro (26). Interleukin 6 causes a dose- and time-dependent decrease in the secretion of T<sub>3</sub>-binding globulins by human hepatoblastoma-derived (HepG2) cells (27). Administration of IL-1α to experimental animals decreases liver 5′-deiodinase mRNA (3). Lastly, it has been reported recently that IL-1β, TNF-α and IL-6 all decrease nuclear thyroid hormone receptor capacity in a liver cell line (28). These findings support the hypothesis that cytokines indeed are involved in the observed changes in thyroid hormone regulation and metabolism during illness. The proof of such a proposed mediatory role of cytokines is hampered by the fact that administration of cytokines to experimental animals makes the animals sick and reduces their food intake. The effect of diminished food intake (which also affects thyroid hormone metabolism) can be ruled out by the use of pair-fed controls. However, even when pair-fed controls are used, the observed changes in thyroid hormone metabolism after administration of cytokines in vivo can be a direct effect of the cytokines or an indirect effect via the illness induced by the cytokines, and it is difficult to discriminate between these two possibilities.

In the present study we set up an experimental model to induce SES in mice by administration of LPS, characterized by a decrease in serum T<sub>3</sub>, T<sub>4</sub> and liver 5′-deiodinase mRNA and unchanged serum TSH levels (3). We sought to evaluate the role of cytokines produced by the Kupffer cells in the liver on peripheral thyroid hormone metabolism. The Kupffer cells are an important source of cytokine production during inflammation; the cytokines stimulate the hepatocytes to express several proteins involved in the early stages of the acute phase response (29). In agreement with the literature, intravenous administration of liposome-encapsulated C<sub>12</sub>MDP resulted in effective depletion of liver and spleen macrophages (9–12), which was shown by complete reduction of acid phosphatase staining in liver and spleen and by a reduction of 32% of α<sub>2</sub>-macroglobulin mRNA in the liver. Sick euthyroid syndrome was effectively induced by LPS, as evident from the decrease in serum T<sub>3</sub> and T<sub>4</sub> and liver 5′-deiodinase mRNA as compared to pair-fed controls. The decrease in serum thyroid hormones is partly due to extrathyroidal inhibition of 5′-deiodinase and partly by direct thyroidal inhibition (3). The observed decrease in 5′-deiodinase mRNA levels could be the result of a change either in gene transcription or mRNA stability.
Selective depletion of liver macrophages, however, did not affect the occurrence of SES: the quantitative changes in serum thyroid hormones and liver 5'-deiodinase mRNA were similar whether or not liposomes had been given previously. It has been shown recently that liposome treatment resulted in an almost complete inhibition of cytokine production in the liver (30). The study of Salkowksi et al. demonstrated that the induction of liver IL-1β, IL-6, IL-10 and IL-12 mRNA by LPS was reduced by more than 95% and liver TNF-α and IFN-γ mRNA by 50–75% in macrophage-depleted mice, implicating macrophages as the primary producers of these cytokines (30). We conclude that cytokine production by Kupffer cells in the liver does not contribute to the generation of LPS-induced SES. This is not to say that any role of cytokines in this respect is excluded. Liver endothelial cells are capable of producing substantial amounts of IL-6 in response to LPS (31). A paracrine effect of cytokines derived from liver endothelial cells on the gene expression of 5'-deiodinase in hepatocytes thus remains a possibility. Also, an endocrine effect of cytokines in the circulation on hepatocytes is feasible: the increase of serum TNF-α and IL-6 in response to LPS in the macrophage-depleted animals (although approximately 50% lower than in non-depleted animals) is still considerable and is probably generated by LPS in non-phagocytic liver cells or macrophages outside the liver and the spleen. Our studies, however, do allow, by inference, IL-1 to be excluded as a major factor in the pathogenesis of LPS-induced SES, because animals who received intravenous liposome-encapsulated Cl₂MDP lacked an increase of serum IL-1 in response to a pyrogenic dose of LPS (32) and showed an almost complete reduction of IL-1β mRNA in the liver after LPS administration (30). This is in agreement with a recent study in human volunteers in whom SES was also induced by LPS: concomitant administration of the IL-1 receptor antagonist did not prevent the occurrence of SES (33).

The effect of food intake in our LPS experiments was excluded by the use of pair-fed controls. We therefore looked for the effect of reduced food intake in a separate experiment, and showed that the decrease in serum T₁ and T₄ after 24-h starvation was not affected by depleting the liver of Kupffer cells. The decrease in liver 5'-deiodinase mRNA after starvation was of marginal significance and might be secondary to the decreased serum T₃ levels (in contrast to the marked decrease after LPS, which precedes the decrease of serum T₃). This is in agreement with the results of O’Mara et al., who found only a significant decrease in 5'-deiodinase mRNA in rats after 48-h starvation but not after 24 h, while serum T₃ was already significantly decreased (34). Also, the observed decrease in liver 5'-deiodinase mRNA was not affected by selective macrophage depletion. Different mechanisms appear to account for the changes in thyroid hormone metabolism after LPS or during starvation. In line with previous studies, the prime effect of starvation might be a reduction of thyroidal secretion of T₁ and T₄ (8) and reduced synthesis and release of TRH and TSH (35). Considering that considerable amounts of T₂ are produced from deiodination of T₄ in the thyroid, which appears to be an important source of T₃ production in rats (36), it might be plausible that decreased levels of thyroidal 5'-deiodinase contribution to the observed LPS-induced decrease of serum T₃.

In summary, we have found no evidence that cytokine production by Kupffer cells in the liver is involved in the pathogenesis of SES.

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Fig. 4. Serum T₁ and T₄ levels (N = 8) and liver 5'-deiodinase mRNA concentrations (N = 4, normalized for 28S expression) in mice after 24-h starvation with or without previous selective liver macrophage depletion. Starved animals are represented by dark bars and the control group by light bars. Difference between groups is indicated by: * p < 0.05.
References


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