Evidence for Extrathyroidal Formation of 3-Iodothyronamine in Humans as Provided by a Novel Monoclonal Antibody-Based Chemiluminescent Serum Immunoassay

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Context: Thyronamines are thyronergic metabolites of thyroid hormones. Lack of reliable and sensitive detection methods for endogenous 3-iodothyronamine (3-T_1AM) has so far hampered progress in understanding their physiological action and role in endocrine homeostasis or pathophysiology of diseases.

Objective: We characterized newly generated mouse monoclonal 3-T_1AM antibodies and established a monoclonal antibody-based chemiluminescence immunoassay as a powerful tool for monitoring 3-T_1AM levels in investigations addressing altered serum profiles and potential sites of origin and action of 3-T_1AM in humans.

Design and Setting: Our exploratory study on 3-T_1AM serum levels in humans measured 3-T_1AM concentrations in comparison with thyroid hormones.

Patients or Other Participants: Thirteen adult healthy subjects, 10 patients with pituitary insufficiency, and 105 thyroid cancer patients participated.

Interventions: Interventions included L-T_4 withdrawal in patients with pituitary insufficiency as well as TSH-suppressive T_4 substitution in thyroid cancer patients.

Results: 3-T_1AM was reliably quantified in human serum and stable after storage at room temperature and 4 C overnight as well as after four freeze-thaw cycles. The median serum concentration in healthy subjects was 66 $\pm$ 26 nM. 3-T_1AM was also detected in T_4-substituted thyroid cancer patients. Although free T_4 and T_3 significantly decreased during T_4 withdrawal, 3-T_1AM levels remained constant for 6 d.

Conclusion: Because higher 3-T_1AM levels are detectable in T_4-substituted thyroid cancer patients after thyroidectomy/radioiodine treatment compared with healthy controls, we concluded that 3-T_1AM is mainly produced by extrathyroidal tissues. The serum profile during T_4 withdrawal suggests either a long half-life or persisting 3-T_1AM release into serum from intracellular thyroid hormone precursors or stores. **(*J Clin Endocrinol Metab* 96: 1864–1872, 2011)**

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Abbreviations: EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; fT_4, free T_4; KLH, keyhole limpet hemocyanin; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LIA, chemiluminescence immunoassay; MAb, monoclonal antibody; HRP, horseradish peroxidase; PBST, PBS with 0.05% Tween 20; TAc, iodothyroacetic acid; 3-T_1Ac, 3-iodothyroacetic acid; TAM, thyronamine; 3-T_1AM, 3-iodothyronamine; Tg, thyroglobulin; TH, thyroid hormone.

Thyronamines (TAM), a novel class of potent endogenous thyronergic signaling compounds, might fine-tune or even antagonize classical thyroid hormone (TH) effects (1–4), which are mainly exerted by the thyromimetically active hormone T_3 (5). Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques, two representatives of nine possible TAM isomers, namely 3-iodothyronamine (3-T_1AM) and the iodine-free TAM T_0AM, have recently been detected *in vivo* in serum and tissues (2, 6, 7). Pharmacological animal studies have shown that a single high dosage treatment (50 mg/kg body weight) with 3-T_1AM resulted in a rapid and drastic decrease in body temperature (2), drop in heart rate (8), reduction in cardiac output (8, 9) and an induction of behavioral inactivity in mice (2, 10). Furthermore, the decreased respiratory quotient indicated a shift from carbohydrate to fat metabolism (7) and glucoregulatory hormones (hyperglycemia, hypoinsulinemia, hyperglucagonemia) were influenced whereas suppressed serum TH (decreased TSH, T_4, and T_3) indicated a perturbation of the normal feedback paradigm (11, 12). On the other hand, dose-dependent stimulation of food intake and neuropeptide Y release after ip and intracerebroventricular 3-T_1AM administration at lower doses has also been reported (10). These recent observations significantly expand the potential molecular and metabolic repertoire of TAM (for detailed information, see recent reviews in Refs. 3 and 13–15). Therefore, the biosynthesis, metabolism, and action of 3-T_1AM could be relevant factors in physiological and pathophysiological states like the low-T_3 syndrome observed in nonthyroidal illness (16), severe general and neurological diseases, or adaptation to food and carbohydrate restriction. Extensively described variations of the T_3 and rT_3 serum levels cannot yet adequately explain the observed metabolic changes in relation to serum TH status (17–19).

3-T_1AM has already been confirmed as an endogenous compound (2, 6, 20). In 2008, the group of Scanlan (21, 22) presented for the first time 3-T_1AM concentrations in human serum and organs (21) using a previously published LC-MS/MS method (22). The physiological concentration (~60 nM, n = 16) in human serum interestingly reaches that of the thyroid prohormone T_4 (70–150 nM). Similarly high tissue concentrations were reported for human thyroid, suggesting 3-T_1AM biosynthesis in this organ, and furthermore, comparable 3-T_1AM levels were reported for putatively responsive organs like muscle and white adipose tissue, indicating either uptake from serum and/or local generation (21). Nevertheless, controversies still exist about the appropriate methods of detection as well as about serum and tissue levels of endogenously present and exogenously administered 3-T_1AM in body fluids and tissues using LC-MS/MS-based analytics (6, 20, 23, 24).

Hypotheses have been proposed for thyroidal or extrathyroidal *de novo* synthesis of TAM or thyroidal and/or extrathyroidal formation from T_4 and/or T_4 metabolites. *In vitro*, sequential deiodination of TAM has been demonstrated by incubation with homogenates of various human cell lines, each expressing one of the three individual deiodinase selenoproteins (25), and furthermore, incubation of H9C2 cardiomyocytes with L-T_3 but not with L-T_4 was shown to generate 3-T_1AM (6). The essential step of iodothyronine decarboxylation yielding TAM has not been identified or biochemically characterized so far, but in analogy to the biosynthesis of other biogenic amines originating from the amino acid tyrosine, Dratman's group (4, 26) proposed a similar thyronergic pathway for TAM formation from iodothyronines. Although tyrosine-derived biogenic amines require a hydroxylation step to yield catecholamines, such a reaction might not apply to iodothyronines, which already are iodinated in ortho-positions to the 4′-phenolic hydroxyl group.

Clinical assessment of patients' thyroid status relies on the precise determination of serum levels of the classical TH T_4 and T_3 as well as the TSH, typically based on immunological methods (*e.g.* RIA or ELISA) (24, 27). Up to now, no poly- or monoclonal antibodies (MAb) for construction of a TAM and especially a 3-T_1AM immunoassay have been published. Therefore, we generated new mouse MAb targeting 3-T_1AM and established a MAb-based chemiluminescence immunoassay (LIA) as a powerful tool for basic and clinical investigations on biosynthesis, metabolism, function and monitoring of 3-T_1AM levels in serum, body fluids and tissues.

Materials and Methods

Reagents and equipment

Tween 20 and 40, BSA, dimethylsulfoxide, T_4/T_3-deficient serum, NaN_3, Tris-(hydroxymethyl)-aminomethane, diethylenetriaminepenta-acetic acid, Hunter's Titer Max, keyhole limpet hemocyanin (KLH), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and horseradish peroxidase (HRP) were supplied by Sigma Chemical Co. (St. Louis, MO). 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer was purchased from Carl Roth GmbH Co. KG (Karlsruhe, Germany). Rabbit anti-mouse antibody (Z109) was acquired from Dako Deutschland GmbH (Hamburg, Germany). Phosphate buffer (Na_2HPO_4), NaOH, and NaCl were obtained from Merck & Co., Inc. (Darmstadt, Germany). Polyethylene glycol 1500 was acquired from Roche Diagnostic (Mannheim, Germany). 3-T_1AM for the immunization of mice was synthesized by Dr. R. Smits and Dr. B. Koksch (Freie Universität Berlin, Germany). All other TH-related substances for cross-reactivity studies were obtained from Dr. R. Thoma (Formula GmbH Pharmaceutical and Chemical

Development Company/Henning Berlin Arzneimittel GmbH, Berlin, Germany) or kindly provided by Dr. T. Scanlan (Oregon Health and Science University, Portland, OR). Lyphochek drug-free serum was purchased from Bio-Rad Laboratories, Clinical Diagnostics Group (Hercules, CA). Fast-performance liquid chromatography equipment, AMDEX HRP-labeled streptavidin and rProtein A-Sepharose were procured from GE Healthcare (Freiburg, Germany). Fetal calf serum, DMEM, horse serum, and protein-free II medium were purchased from Invitrogen GmbH (Karlsruhe, Germany). Mini-PERM devices were obtained from Heraeus (Hanau, Germany). The ImmunoPure MAb isotyping kit and the Slide-A-Lyzer dialysis cassettes were obtained from Pierce Chemical Co. (Rockford, IL). The chemiluminescent substrate for the LIA was purchased from Diagnostic Systems Laboratories (Webster, TX). Europium-labeled streptavidin, enhancement solution, and the VICTOR3 multilabel counter were acquired from PerkinElmer (Waltham, MA). Ninety-six-well flat-bottom microtiter plates (Maxisorp plates) were obtained from Nunc (Roskilde, Denmark).

Conjugation of 3-T_1AM to BSA, KLH, and HRP

Briefly, BSA, KLH, HRP, and EDC were dissolved in demineralized water. 3-T_1AM was dissolved in dimethylsulfoxide and diluted in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid buffer. Then, KLH and BSA were mixed with 3-T_1AM solution to form the 3-T_1AM-BSA and 3-T_1AM-KLH conjugate, respectively, using EDC as a coupling reagent. The Eppendorf tubes containing the conjugates were wrapped in aluminum foil and incubated at room temperature for 120 min. Finally, the conjugates were dialyzed overnight at 4 C against PBS using Slide-A-Lyzer Dialysis cassettes and stored frozen in aliquots at −80 C. The same procedure was used to couple 3-T_1AM to HRP.

Production of MAb against 3-T_1AM

Two-month-old female BALB/c mice were immunized with BSA-coupled 3-T_1AM dissolved in Hunter's TiterMax adjuvant according to the manufacturer's recommendations and injected intradermally. Immunized mice were boosted twice by the same treatment at approximately 4-wk intervals, and then a last injection was given 3 d before the harvesting of spleen cells. The anti-3-T_1AM antibody titer within the serum of immunized mice was monitored using the same methods as used for the screening of hybridoma supernatants. For selection of specific MAb, supernatants of hybridoma cells were examined simultaneously with both BSA- and KLH-coupled 3-T_1AM. Hybridoma cells producing MAb with high affinity to 3-T_1AM and lowest cross-reactivity to other TAM, TH, and iodothyroacetic acids (TAc) were selected, subcloned, and expanded.

ELISA-based screening of hybridoma supernatant and mouse serum

The 96-well high binding microplates were coated with 100 ng/100 μl 3-T_1AM-KLH, 3-T_1AM-BSA, and BSA diluted in Na_2HPO_4 buffer (pH 7.4) and incubated overnight at 4 C. After washing the plates three times with PBS with 0.05% Tween 20 (PBST), fresh serum dilutions from immunized mice or hybridoma supernatant were added to the plates (100 μl/well) for 1 h at room temperature under agitation. Plates were washed again three times with PBST before a rabbit antimouse antibody (10 ng/100 μl) diluted in assay buffer [50 mmol/liter Tris-(hydroxymethyl)-aminomethane, 154 mmol/liter NaCl, 20 μmol/liter diethylenetriaminepenta-acetic acid, 0.01% Tween 40, and 0.05% NaN_3 (pH 7.75)] was added to the plates for 1 h at room temperature under agitation. After another wash step, 10 ng europium-labeled streptavidin per well in 100 μl PBST was added and incubated for 30 min at room temperature under agitation. After six washes with PBST, 0.2 ml enhancement solution was added to each well and incubated for 10 min. The signal was then read by VICTOR3 multilabel counter. Concentrations were automatically calculated according to a standard curve fitting by the Multicalc program.

Isotyping and purification of MAb

MAb were isotyped using a mouse MAb isotyping kit according to the manufacturer's recommendations. The resulting MAb were purified on a rProtein A-Sepharose column. The purified antibodies were exchanged into PBS using Slide-A-Lyzer dialysis cassettes overnight at 4 C, sterile filtered, and stored in aliquots at 4 C.

LIA procedure

White microtiter plates were coated overnight at 4 C with a rabbit antimouse antibody (100 ng/0.1 ml per well). The plates were washed three times with PBST, and 25-μl 3-T_1AM standard samples (0–500 nM) diluted in T_3/T_4-deficient serum or 7% BSA-PBST, respectively, control sera as well as duplicate serum samples were pipetted into wells together with 25 μl HRP-labeled 3-T_1AM (10 ng) diluted in 0.5% BSA-PBST. Then 50 μl MAb 9C5 (2 ng) diluted in 0.5% BSA-PBST was added before the plates were incubated at room temperature under agitation for 90 min. After a final washing step (six times with PBST), 0.1 ml substrate solution was added to each well and incubated for 10 min. The signal was then analyzed by a multilabel counter, and concentrations were automatically calculated according to a standard curve.

As internal control samples, we used the commercially available standard serum Lyphochek as a positive control and T_4/T_3-deficient serum as a negative control.

Thyroid hormones, TSH, and thyroglobulin (Tg) measurement

Free T_4 (fT_4), fT_3, TSH, and Tg were measured by commercially available immunoassays (Advia Centaur for fT_4, fT_3, and TSH and Siemens Immulite 2000 for Tg; Siemens Healthcare, Frimley, Camberley, UK).

Clinical subjects

We obtained serum from 13 healthy volunteers (nine females and four males) to study the normal concentration range of 3-T_1AM. The clinical sample panel consisted of 10 patients with pituitary insufficiency [seven female and three male patients, age 28–57 yr, with complete anterior pituitary deficiency (panhypopituitarism) in all but two patients where the corticotropic axis was preserved] under full T_4 replacement therapy and after 6 d of L-T_4 withdrawal as well as of 105 thyroid cancer patients on TSH-suppressive L-T_4 therapy (80 female and 25 male patients, age 18–84 yr: 77 with papillary, 21 with follicular, three with Hurthle cell, and four with medullary thyroid carcinomas). Patients provided their informed consent, and both studies were approved by the local ethical committee.

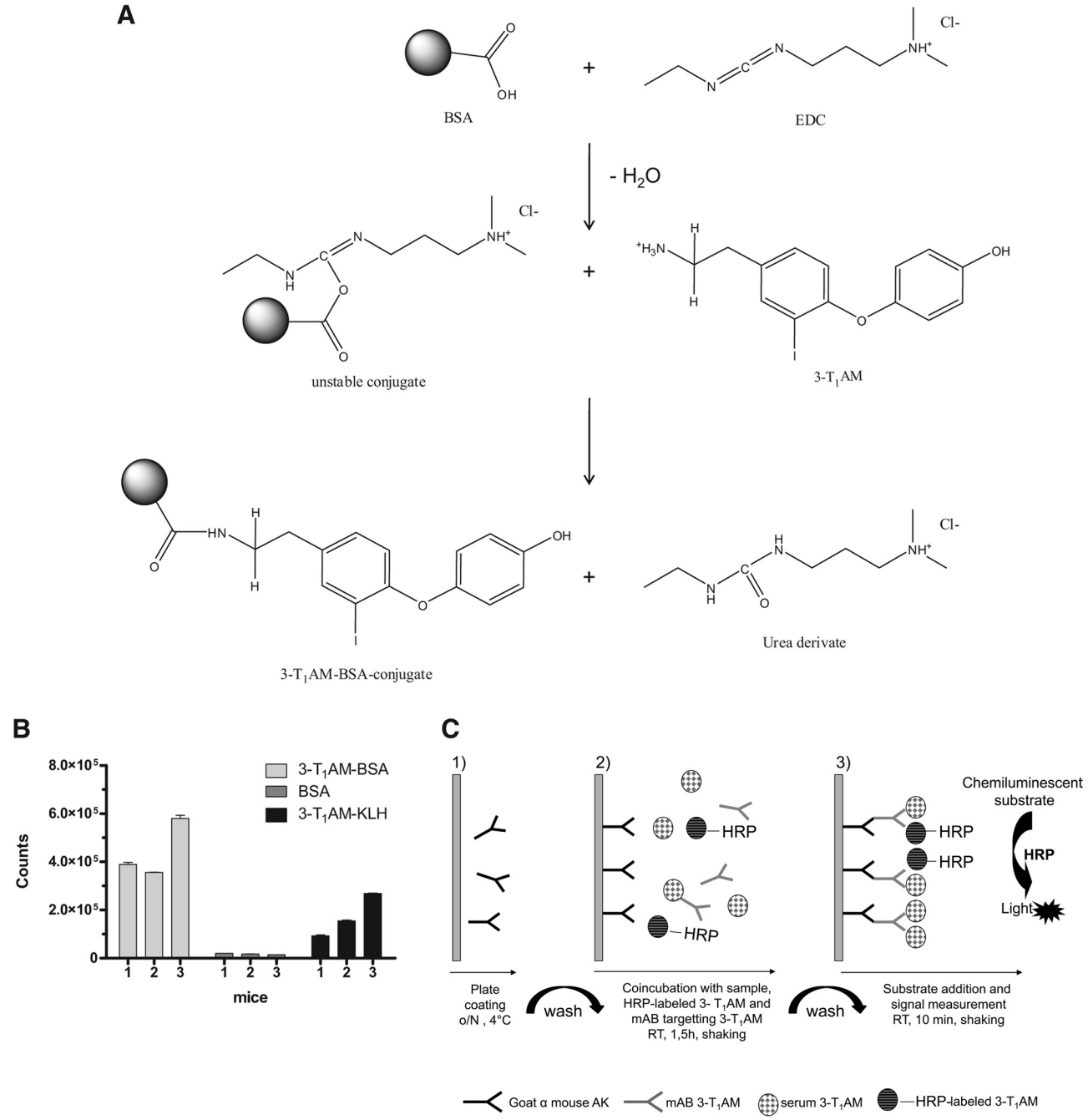

FIG. 1. A, Principle of the 3-T_1AM conjugation to BSA to form the hapten for immunization. B, Proof of the specificity of the polyclonal antisera against 3-T_1AM. Mouse three was used for the production of hybridomas. C, Schematic diagram of the developed 3-T_1AM chemiluminescent immunoassay.

Statistical analysis

GraphPad Prism version 4 software was used for all computations with the tests indicated in the figure legends. Data are expressed as mean ± SD. Statistical significance was defined as $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Results

Production of MAb targeting 3-T_1AM

MAb-producing cells were generated using the hybridoma technology by Köhler and Milstein (28). To generate MAb targeting 3-T_1AM, we used BSA-coupled 3-T_1AM (Fig. 1A) for immunization of three BALB/c mice. After three immunization injections, a test assay indicated that all three immunized mice responded with high antibody titers that displayed a high antibody avidity for 3-T_1AM-BSA, particularly that from the mouse number three. BSA as the hapten conjugation partner does not interfere with the assay (Fig. 1B). Hybridomas are produced by fusion of the antibody-producing cells from the mouse's spleen and immortalized myeloma cells PAI.

More than 30 wells of hybrids were detected to produce antibodies binding to 3-T_1AM with high affinity. Eight

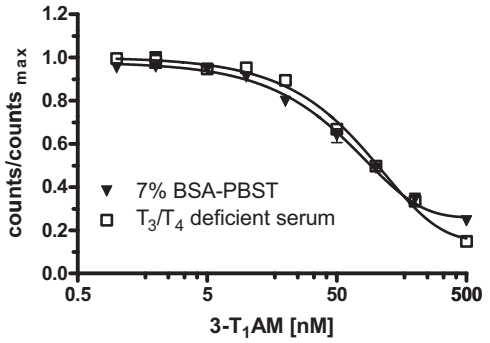

FIG. 2. Standard curves for 3-T_1AM. Increasing concentrations of nonlabeled 3-T_1AM were added to 7% BSA-PBST (▼) or T_3/T_4-deficient serum (□) to displace binding of the 3-T_1AM-HRP tracer and its specific antibody.

MAbs were examined for their cross-reactivity to T_1, T_3, and T_4, and three MAbs called 5C11, 10D1, and 9C5 with high affinity and specificity to 3-T_1AM were chosen for cloning, expansion, and further characterization. The MAb 9C5 was selected for construction of the 3-T_1AM LIA due to its high specificity and low cross-reactivity with other TAM. In the competitive assay procedure, HRP-labeled 3-T_1AM competes with 3-T_1AM in serum for binding to a limited amount of anti-3-T_1AM MAb (Fig. 1C). To obtain the optimal assay conditions, all assay parameters and incubation times were optimized.

T_3/T_4-deficient serum was identified as the matrix of choice lacking 3-T_1AM. Therefore, we also used T_3/T_4-deficient serum to construct the assay standard curve ranging from 0–500 nM 3-T_1AM. A typical standard curve using T_3/T_4-deficient serum and 7% BSA-PBST as an artificial serum matrix for the present assay is shown in Fig. 2. The ratio of counts to $counts_{max}$ (max, maximum) was used for calculation of the standard curve. Both standard curves are highly parallel, suggesting comparable matrix effects.

Analytical characteristics

MAb 9C5 showed less than 0.1% cross-reactivity with T_4 and T_3 and less than 1% with most of the other TH, TAM, and TAc by competitive binding assay. Only 3,5-T_2AM and 3-iodothyroacetic acid (3-T_1Ac), which so far have not been detected in human serum or tissue by chromatographic or LC-MS/MS methods, showed a cross-reactivity of 12.4 and 5.1%, respectively (Table 1). These data clearly indicate that this particular MAb against 3-T_1AM was highly specific.

In human serum, 3-T_1AM was reliably detected and quantified in the nanomolar range by the LIA. The dilution linearity was assessed by assaying 3-T_1AM standard samples after serial dilution with T_3/T_4-deficient serum. Measured concentrations were multiplied by the dilution factor and compared with the original concentration. The results show a good agreement between the expected and

TABLE 1. Relative cross-reactivities (percent) and physiological concentrations (nanomolar) of several 3-T_1AM-related molecules

	Cross-reactivity (%)	Physiological concentration (nM)	Ref.
Iodothyronamines (TAM)			
T_0AM	<0.1	NQ	
3-T_1AM	100	~60	21, this study
3′-T_1AM	<0.1	ND	
3,5-T_2AM	12.4	ND	
3,3′-T_2AM	<0.1	ND	
3′,5′-T_2AM	<0.1	ND	
T_3AM	<1	ND	
rT_3AM	<1	ND	
T_4AM	<0.1	ND	
Iodothyronines (TH)			
T_0	<0.1	NQ	
3-T_1	<0.1	0.075	33
3′-T_1	<0.1	0.059	29
3,5-T_2	<1	0.055	29
3,3′-T_2	<1	0.058	29
3′,5′-T_2	<1	0.085	29
T_3	<0.1	2.1	29
rT_3	<0.1	0.62	29
T_4	<0.1	110	29
Iodoacetic acids (TAc)			
Tetrac	<0.1	7.2	29
Triac	<0.1	2.8	29
Diac	<1	ND	
3-T_1Ac	5.3	ND	

ND, Not detected so far in human serum; NQ, not quantified so far in human serum; Tetrac, 3,3′,5,5′-tetraiodothyroacetic acid; Triac, 3,3′,5-triiodothyroacetic acid; Diac, 3,3′-diiodothyroacetic acid.

the observed values in the steep part of the standard curve (Fig. 2). The working range of the assay is estimated between 10 and 500 nM. We tested the recovery of 3-T_1AM measurement by comparing the data of five sera in different combinations of equal-volume mixtures of two sera each (Fig. 3A). Measured concentrations from each mixture of two sera were added up to obtain the theoretical value for the measurement of the equal-volume mixtures. Results fairly agreed with the theoretically expected arithmetic means for the corresponding samples and yielded a mean recovery of 105%.

Furthermore, we studied the recovery of 3-T_1AM spiked to serum in raising concentrations (0–50 nM) (Fig. 3B). The mean recovery of the theoretical values to the measured data determined by the assay was 104.7% for all samples.

The stability of the substance was tested in serum. 3-T_1AM was stable at room temperature and 4 C overnight (83 and 93%, respectively) as well as after four freeze-thaw cycles (98%) (Fig. 3C).

The interassay precision of the assay was measured by using a sample with 3-T_1AM concentration of 38 nM and was 18.5%, whereas the intraassay precision was calculated by using a sample with 3-T_1AM concentration of 29 nM and was 10.8%.

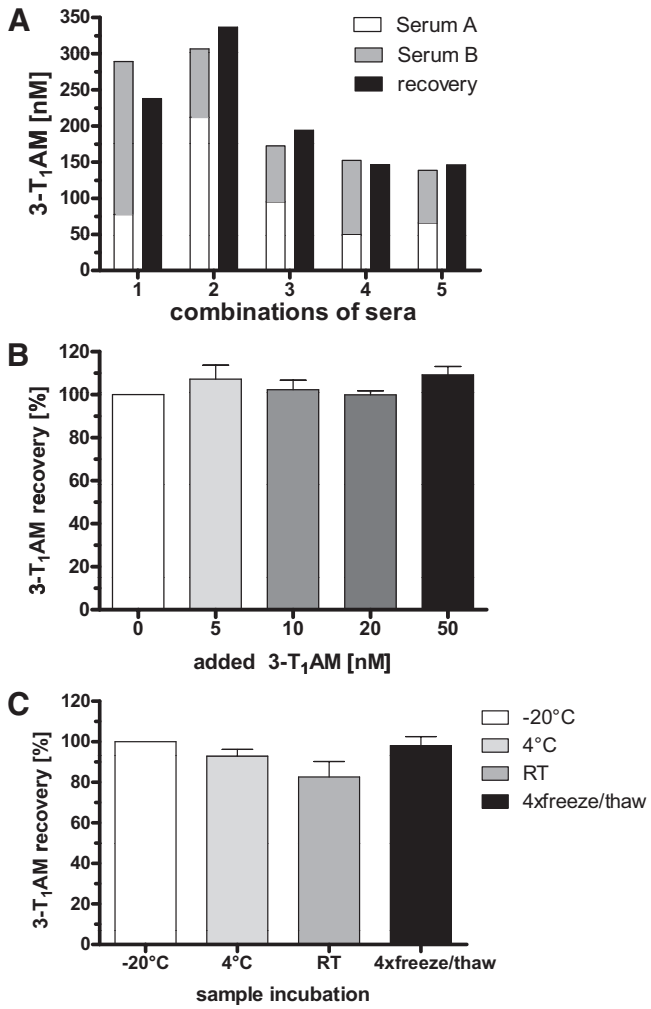

FIG. 3. Assay validation. In this recovery study, different combinations of mixtures of two sera were measured; results were summed up [*two superposed (white and grey) columns*, *left*) and are presented along with the calculated concentration (*black bar*, *right*) (A), the recovery of 3-T_1AM spiked into serum in raising concentrations (B), stability of 3-T_1AM stored at room temperature or 4 C overnight and after 4 freeze/thaw cycles (C).

Analysis of human samples

The median serum concentration of 3-T_1AM in 13 adult healthy subjects was 66 ± 26 nM and therefore comparable with previous LC-MS/MS results (21) and in the same nanomolar concentration range as the thyroid prohormone T_4 (Fig. 4A). In sera of thyroid cancer patients on TSH-suppressive L-T_4 therapy (n = 105, Fig. 4A), 3-T_1AM levels were significantly higher than in controls and a correlation was not observed between 3-T_1AM and fT_4 levels (Fig. 4B) or with TSH levels. In the whole group of thyroid cancer patients, 3-T_1AM was age independent (Fig. 4C), but a trend toward slightly higher levels in sera of females (125 nM, n = 80) than in those of males (110 nM, n = 25) was observed although not reaching statistical significance.

3-T_1AM was also detected in 10 T_4-substituted patients with pituitary insufficiency. Although fT_4 [18.1 *vs.* 11.4 nM; Fig. 5A) and fT_3 (4.9 *vs.* 3.7 nM; $P < 0.001$ in both; Fig. 5B) significantly decreased during T_4 withdrawal after 6 d in these patients, 3-T_1AM levels remained constant (97 *vs.* 92 nM) (Fig. 5C) and did not correlate with serum TSH, fT_3, and fT_4 (data not shown).

Discussion

Several LC-MS/MS methods have been reported for the detection of 3-T_1AM in serum and tissues of various species, but no MAb-based immunoassay to measure 3-T_1AM in body fluids such as serum has been developed. Furthermore, there is no clear evidence on the tissue sources or sites of its production, and no reference values for 3-T_1AM levels in human serum have been presented till now.

Here, we present a rapid and highly specific MAb-based quantitative chemiluminescent immunoassay for the reliable quantification of 3-T_1AM in human serum. We generated a highly specific MAb targeting 3-T_1AM. Among known T_4-derived TH metabolites, this MAb only showed higher cross-reactivity to 3,5-T_2AM and 3-T_1Ac. Both compounds have not been detected in human or animal serum so far. In addition, the concentration of endogenous 3,5-T_2 in human serum is around 0.055 nM (29). Therefore, the cross-reactivity to 3,5-T_2AM as a potential confounder seems to be insignificant. Our new assay allows precise measurement of 3-T_1AM over a broad concentration range with an interassay coefficient of variation of less than 18.5% and an intraassay coefficient of variation of less than 10.8%. Currently observed assay variability most likely relates to matrix differences between calibrators and human sera as well as in reagents supplied by the manufacturer.

Controversies still exist about the physiological 3-T_1AM serum concentration in humans using LC-MS/MS (6, 20, 23). Using the newly established LIA, 3-T_1AM serum concentrations are found in the same nanomolar concentration range as the thyroid prohormone T_4, whereas most studies using LC-MS/MS measured 3-T_1AM levels in the picomolar range. One reason might be the preanalytical sample preparation in LC-MS/MS studies, which requires protein precipitation, and/or liquid- or solid-phase extraction using organic solvents and frequently are followed by sample concentration and its preadsorption to the LC precolumn before elution to the separation columns. A high fraction of 3-T_1AM, tightly bound to serum apolipoprotein B100 (>99% with a high-affinity binding site and a K_D value of 15 nM) as proposed by T. S. Scanlan (30), might be precipitated during the sample workup. The strong binding to apolipoprotein B100 would also explain

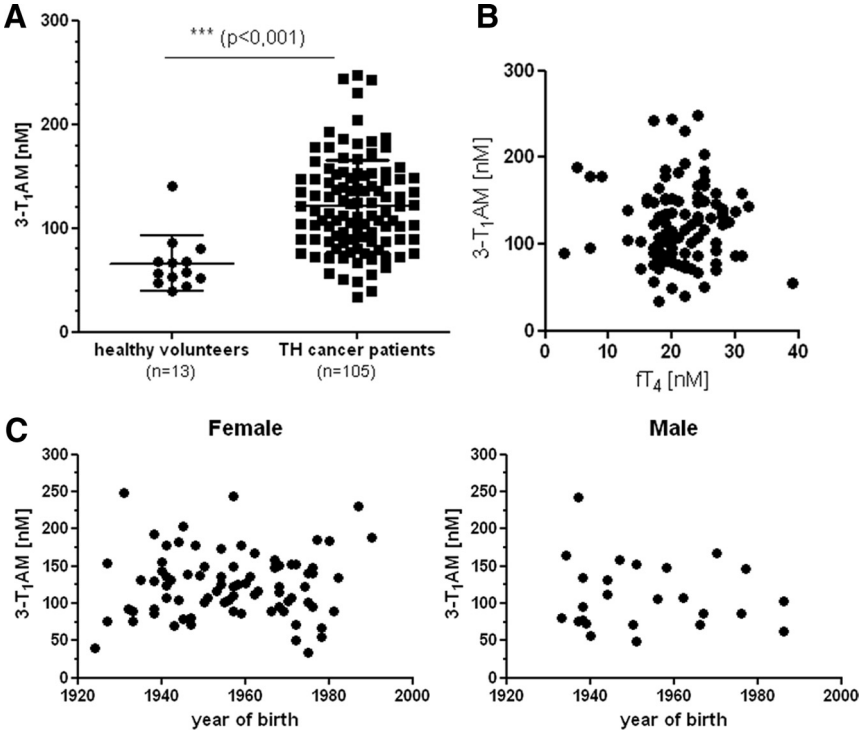

FIG. 4. A, 3-T_1AM concentrations in 13 healthy individuals and 105 thyroid (TH) cancer patients. Median values are indicated by *horizontal lines*. There was a significant difference in median values between the two groups (unpaired *t* test: ***, $P < 0.001$). B, Correlation of 3-T_1AM and fT_4 concentration in thyroid cancer patients. There was no negative correlation. C, Correlation of 3-T_1AM and age in the thyroid cancer cohort. There was no negative correlation.

the extreme stability of 3-T_1AM in serum after storage at room temperature and 4 C overnight as well as after four freeze-thaw cycles. The amino group of the thyronergic amine 3-T_1AM seems to be protected from oxidation or modification when 3-T_1AM is bound to apolipoprotein B100. Moreover, another inconsistency between the published LC-MS/MS methods is the used matrix. Some groups work with plasma (in most cases, it is not stated which kind of plasma is used) (23), whereas others use serum (6, 22). A matrix effect on 3-T_1AM detection in human samples cannot be excluded at the moment. Finally, the published LC-MS/MS methods also differ in the preanalytical sample preparations. Extensive offline (6, 22) and online solid phase extraction methods (20, 23) for the detection of 3-T_1AM using 1 ml serum (6) and 0.2 ml serum (20, 22), respectively, were reported. Some groups tried to dissociate protein-bound 3-T_1AM with 8 M urea (22) or with sodium chloride (6), and others used proteinase K (23). Summing up, all published LC-MS/MS methods differ considerably in their preanalytical procedure, resulting in different 3-T_1AM concentrations.

Compared with LC-MS/MS methods, our newly developed immunoassay has several advantages. First, it is more convenient, because no preanalytical sample preparation is necessary using our highly specific MAb. Second, only 50 μl serum is required to measure 3-T_1AM in human serum in duplicates. Third, the investment or presence of expensive LC-MS/MS equipment requiring a dedicated operator is avoidable, whereas immunoassays are routinely used in diagnostic and research labs.

Our estimate in comparison with the studies by Saba *et al.* (6) and Soldin *et al.* (20) would indicate a free fraction of only 1‰ detected by LC-MS/MS, whereas the LIA taking advantage of a high-affinity and -avidity MAb might be able to efficiently compete with apolipoprotein B100 (and other serum-binding proteins) and thus quantify total 3-T_1AM. The free fraction of 3-T_1AM in serum might be as low as that of T_4, which is bound to the high-affinity binding proteins T_4-binding globulin and transthyretin in addition to albumin.

In one study by Ackermans *et al.* (23), the authors could not detect endogenous 3-T_1AM in human serum using LC-MS/MS. In this case, the online solid-phase extraction procedure employed as preanalytical step in combination with the sensitivity of the LC-MS/MS equipment used could be limiting the detection of free 3-T_1AM.

In our study, 3-T_1AM serum levels are detectable at equal or even higher levels compared with healthy controls in TSH-suppressed T_4-substituted patients all of whom lack functional thyroid tissue after surgery and/or radioiodine therapy. Therefore, we concluded that 3-T_1AM is mainly produced extrathyroidally from exogenous T_4 supplements, albeit the 3-T_1AM serum profile is distinct from that of T_4 and T_3 during T_4 withdrawal in patients with hypopituitarism. This assumption is supported by the fact that under TSH-suppressive therapy, where higher than normal T_4 serum values are reached, also 3-T_1AM

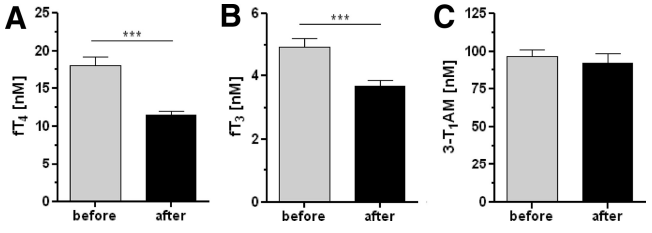

FIG. 5. Exploratory study of 10 T_4-substituted patients with pituitary insufficiency before and after 6 d of L-T_4 withdrawal. A–C, Serum concentrations of 3-T_1AM (A), fT_3 (B), and fT_4 (C) in this cohort. Statistical significance was calculated using paired *t* test: ***, $P < 0.001$.

levels are higher than in controls. This is further supported by the lack of change in the hypopituitary patients where peripheral fT_4 and fT_3 significantly decreased during T_4 withdrawal, but 3-T_1AM remained unaltered. The ratio of 3-T_1AM to T_4 and/ or T_3 might also indicate a balance (or imbalance) in homeostatic regulation of the TH metabolism. Deiodination of various TAM by the three deiodinase selenoproteins has been demonstrated *in vitro* in human cell lines (25), and slow but measurable 3-T_1AM production occurs from T_3 but not T_4 in rat H9C2 cardiomyocytes (6). Also, Ackermans *et al.* (23) suggest a low conversion of exogenous ^{13}C-labeled T_4 to 3-T_1AM. Enzymes catalyzing decarboxylation of TH to generate TAM have not been identified so far. Only the most probable candidate for this reaction, the aromatic amino acid decarboxylase, might be excluded (31) (unpublished data). Still, an alternative hypothesis on *de novo* generation of 3-T_1AM and T_0AM cannot be excluded by these and other published data. However, the complex biosynthesis of diphenlyether-coupled (iodo-)thyronine molecules has so far been shown *in vivo* only in the thyroid gland, which still remains the major or even only source of these classes of hormonally active iodinated biomolecules in humans and higher animals.

In addition, in T_4-substituted patients with pituitary insufficiency, 3-T_1AM is still detected without a decrease 6 d after withdrawal, whereas fT_4 and fT_3 levels are already clearly diminished as expected. The 3-T_1AM serum profile during T_4 withdrawal suggests either a rather long half-life or persisting 3-T_1AM release into serum from intracellular TH precursors or stores. Similar to classical iodothyronines (with the exception of rT_3), but in contrast to other amines, the half-life of 3-T_1AM in mice is in the range of 5 h (32). Moreover, the half-life of 3-T_1AM in the incubation medium of 3-T_1AM exposed H9C2 rat cardiomyoctes is about 22 min (6). But of course, the half-life of 3-T_1AM in mice, conditioned cell culture medium, and human serum differs considerably due to different *in vivo* metabolism and binding properties to serum (lipo)proteins, similar to major differences in half-life of iodothyronines in humans and rodents, which lack, *e.g.* high-affinity TH-binding thyroxine-binding globulin.

Additional kinetic studies after administration of classical TH *vs.* 3-T_1AM need to be done and might also give an indication of the source of 3-T_1AM biosynthesis.

In T_4-substituted cancer patients, neither age-dependent variations of serum 3-T_1AM serum were observed nor did females and males show different 3-T_1AM levels, which is compatible with the fact that mean age, body weight, and body mass index were not significantly different between women and men.

However, we provide strong evidence that 3-T_1AM is formed in extrathyroidal tissues as illustrated by normal to elevated 3-T_1AM serum levels in thyroid cancer patients lacking functional thyroid tissue. Additional thyroidal production can, however, not be totally excluded by our data.

This novel 3-T_1AM immunoassay constitutes an important new tool for addressing unresolved questions about the physiology of 3-T_1AM as a potential biomarker and its role in highly prevalent human diseases.

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