

MEASURING THYROGLOBULIN CONCENTRATIONS IN PATIENTS WITH DIFFERENTIATED THYROID CARCINOMA

MERENJE KONCENTRACIJE TIREOGLOBULINA KOD PACIJENATA SA DIFERENTOVANIM KARCINOMIMA ŠTITASTE ŽLEZDE

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Summary: Thyroid carcinomas are the most common malignant endocrine tumors. Thyroglobulin (Tg), a specific thyroid protein, is the most important tumor marker in thyroid oncology. After total thyroidectomy or radioiodine therapy, detectable or increasing serum Tg levels in patients with differentiated thyroid carcinoma indicate persistence of active thyroid tissue or cancer recurrence. Serum Tg concentration primarily reflects three variables: the mass of differentiated thyroid tissue present; the degree of thyrotropin receptor stimulation and the intrinsic ability of the tumor to synthesize and secrete Tg. Measurement of serum Tg by current immunometric (IMA) and radioimmunological (RIA) assays encounters some methodological problems which can diminish its clinical importance. Discrepancy between the results for Tg using different methods may be caused by: different reference materials, specific properties of the primary and secondary antibodies for antigenic determinants on Tg and diverse binding affinities of these epitopes, together with interference by serum factors (usually antibodies to Tg (TgAb)) with the primary and secondary Tg antibodies from the diagnostic set. In the presence of endogenous TgAb, Tg values measured by immunoradiometric assay (IRMA) and similar assays are usually lower than the real concentrations, while in RIA apparently lower or higher results can be obtained. Falsely low values may lead to delay in necessary treatment, while an inappropriately high Tg value can cause patient anxiety and unnecessary scans. Despite current methodological limitations, serum Tg measurement is a useful test for determining worsening disease and monitoring the effects of therapy in patients who have undergone surgery for differentiated thyroid carcinoma.

Keywords: antithyroglobulin autoantibodies, differentiated thyroid carcinoma, immunometric assay, thyroglobulin

Kratak sadržaj: Tiroidni karcinomi su najčešći maligni endokrini tumori. Tireoglobulin (Tg), specifični protein štitaste žlezde, najvažniji je tumorski marker u tireoidnoj onkologiji. Kod pacijenata sa diferentovanim karcinomima tireoideje, nakon operativnog lečenja, koncentracija Tg određuje se radi otkrivanja rezidualnog tumorskog tkiva ili postojanja lokalnih, odnosno udaljenih metastaza. Na koncentraciju Tg u serumu utiču: masa prisutnog tireoidnog tkiva (benignog ili malignog), intenzitet stimulacije receptora za tireostimulišući hormon (TSH) i sposobnost tumorskih ćelija da sintetišu i luče Tg. Savremene metode, imunometrijske (IMA) i radioimunološke (RIA), kojima se određuje koncentracija Tg u serumu ispitanika, imaju određena ograničenja koja mogu da umanje klinički značaj dobijenih rezultata. Usled metodoloških razlika, koncentracije Tg u istim uzorcima seruma, izmerene različitim testovima, mogu se razlikovati. Faktori koji mogu prouzrokovati razlike u izmerenim koncentracijama Tg su brojni: različiti referentni materijali, razlike u specifičnosti primarnih i sekundarnih antitela za antigenske determinante Tg, različit afinitet vezivanja tih antitela za epitope Tg, i interferencija serumskih faktora. Princip testa, kao i eventualno prisustvo TgAt u serumima ispitanika, može uticati na izmerenu koncentraciju Tg. Svako odstupanje izmerenih koncentracija Tg od stvarnih vrednosti može imati ozbiljne posledice: lažno niske vrednosti Tg mogu odložiti neophodni tretman pacijenata, dok lažno povećane vrednosti Tg mogu prouzrokovati nepotrebnii stres, ili čak tretman pacijenata. I pored ograničenih mogućnosti savremenih metoda, određivanje koncentracije Tg u serumu pacijenata operisanih od diferentovanog tiroidnog karcinoma je koristan test za otkrivanje pogoršanja bolesti i za praćenje efekata terapije.

Ključne reči: diferentovani tireoidni karcinom, imunometrijski test, tireoglobulin, tireoglobulinska antitela

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Introduction

Thyroid carcinomas are the most common malignant tumors of the endocrine system and their incidence is on the rise (1). Most thyroid carcinomas are differentiated tumors: 88% papillary and 8% follicular (1). Differentiated thyroid carcinoma (DTC) can appear at any time of life with peaks in the third and sixth decade and a median at 44 years (2). Epidemiological data indicating an increase in the number of thyroid carcinomas can partially result from the application of advanced diagnostic methods that can detect micropapillary carcinomas (smaller than 1 cm) or more detailed research of autopsy material which showed a 5–24% incidence of thyroid carcinoma (2).

If thyroid carcinoma is diagnosed in a patient, the physician may decide on operative treatment (with or without application of an ablative dose of radioactive iodine – (I^{131}) (3, 4) or clinical follow-up depending on the tumor size and other characteristics (5). Most (90%) thyroid tumors are smaller than 2 cm in diameter and are considered low risk, so less aggressive treatment and monitoring are applied, in accordance with current protocols (3, 4).

In disease diagnostics and monitoring of patients with thyroid carcinoma, determining the serum concentration of thyroglobulin (Tg) is very important. Tg is a glycoprotein with a molecular weight of 660 kDa, synthesized exclusively by thyroid follicular cells – thyrocytes. The fact that the thyroid gland is the only organ synthesizing Tg (6) makes this molecule a marker of differentiated thyroid carcinoma relapse or the occurrence of metastasis in the postoperative period, if the thyroid gland tissue was completely removed by a surgical procedure with eventual use of an ablative dose of radioactive iodine (I^{131}). Namely, there is currently no method that can establish whether circulating Tg originates from normal or malignant tissue.

However, due to malignant transformation of thyroid follicular cells, the structure of Tg (especially the carbohydrate component) can be changed (7) together with alterations in the secretion mechanism (8). Epitope mapping on the Tg molecule has shown the existence of six different antigenic regions to which different thyroglobulin-specific antibodies (TgAb) can bind (9, 10), most probably due to differences in Tg molecules secreted from malignantly changed thyroid cells (9, 11–13).

The Tg concentration in serum is a reflection of: thyroid tissue mass presence, disruption of thyroid follicle integrity and degree of thyrostimulating hormone (TSH) receptor stimulation. For newly diagnosed and treated patients with DTC, where the thyroid tissue has not been completely removed, there are combinations of normal remnant and/or tumor tissue, though one must have in mind that not

all thyroid tumors can synthesize and secrete Tg (14, 15). Injuries to the thyroid tissue, i.e. disruption of thyroid follicle integrity (fine-needle aspiration biopsy, surgery, radioactive iodine therapy or inflammation) can lead to the release of Tg from follicles where it is stored. Finally, besides the secretion of thyroid hormones, stimulation of TSH receptors releases Tg (endogenous TSH, recombinant human TSH (rhTSH), human chorionic gonadotropin in pregnancy or antibodies that stimulate the TSH receptor in Graves' disease) (15). For patients with differentiated thyroid carcinoma the Tg concentration in sera is determined before and after surgery before applying the ablative dose of radioactive iodine, and also during regular checkups after therapy is completed.

Even though Tg is mainly determined in sera, it can be measured in the needle washout after aspirative biopsy of lymph nodes suspected of DTC metastasis.

Preoperative Tg measurement

The level of increase of Tg concentration prior to thyroidectomy has no predictive value, although it has been shown that patients with malignant tumors have (on average) higher concentrations of Tg in serum (if it is calculated in relation to the node size) in comparison with patients with benign nodules (18, 19). If biopsy has enabled detection of malignant tissue, high levels of Tg before surgery indicate that the tumor cells produced Tg. Approximately two thirds of patients diagnosed with DTC have high Tg levels in sera prior to surgery (>40 ng/mL). For these patients measuring Tg levels after surgery will be a useful, specific marker for the continuation or worsening of the disease. The sensitivity of Tg measurements after surgery will be higher if the tumor is relatively small (2 cm or smaller) and the Tg value before surgery high. If the Tg value is low before surgery, a non-detectable Tg value after surgery is not a convincing confirmation of the absence of tumor tissue, as it is not clear whether the tumor produced Tg or not (20, 21). For such patients a detectable level of Tg after surgery might indicate the presence of a large tumor mass or the appearance of metastases.

Measuring Tg after surgery

No detectable Tg in the sera of patients from whom the thyroid gland has been completely removed is usually a confirmation of success of the surgical treatment. However, after surgery for thyroid carcinoma, detectable serum Tg in some patients may originate from a small amount of normal thyroid tissue that has not been removed (near total thyroidectomy). Thus, detectable Tg does not definitely indicate the presence of carcinoma, as it can also

originate from normal (benign) thyroid gland tissue. In such cases patients are given an ablative dose of radioactive iodine (^{131}I) to destroy the remaining thyroid tissue. After this tissue is eliminated (that might or might not be malignant), serum concentrations of Tg should be reduced, which will enable higher sensitivity of later measurements of Tg as a marker of relapse or metastasis.

In patients with DTC (papillary or follicular), Tg, which has an average half life of about 65 hours, completely disappears from the circulation within a month after total thyroidectomy (22). Only after this period can one measure the Tg concentration in serum in order to evaluate the disease course, i.e. success of the surgical treatment of thyroid carcinoma.

Determining the concentration of serum Tg as a specific tumor marker of thyroid tissue has eased the postoperative follow-up of patients with thyroid carcinoma and enabled earlier discovery of residual tumor tissue or relapses. Measuring Tg can also be useful in patients where total thyroidectomy was not performed, but interpretation of the results is harder (23, 24). Independently of the type of surgery, it is important to monitor changes in Tg values at certain time intervals (3–6 months).

Measuring Tg during substitution therapy with thyroid hormones

After surgical treatment of patients with differentiated thyroid carcinoma and removal of the tumor together with healthy functional thyroid tissue, the patients are left without endogenous secretion of thyroid hormones. This slowly leads to clinical hypothyroidism. Due to the lack of thyroid hormones and their inhibitory effect on TSH secretion, TSH secretion increases in these patients, followed by elevated TSH levels in serum. Besides stimulating the thyroid gland to produce thyroid hormones, it has been shown that TSH releases Tg and increases its concentration in the circulation (25, 26) and it is believed to stimulate the growth of most thyroid tumors. During introduction of substitution therapy with thyroid hormones (most often levothyroxine), the dose is increased until suppression of TSH is achieved. If during thyroid hormone therapy TSH levels are suppressed and stable, an increase of Tg concentration in serum (at regular patient checkups) will best reflect changes in tumor size. As there are no reference values for serum concentrations of Tg in patients with DTC after surgery, the relation between Tg levels in serum and thyroid tissue mass can indicate the existence of a tumor and its size. Namely, one gram of normal thyroid tissue releases in the circulation approximately 1 ng/mL Tg, if the TSH level in serum is within reference values (0.4–4.0 IU/L), or 0.5 ng/mL Tg if the TSH level in serum is suppressed

below 0.1 IU/L. As the remaining thyroid tissue after total thyroidectomy should not be larger than 2 g, it is expected that the Tg concentration in such a patient's serum should be lower than 2 ng/mL. Some data indicate that patients with high Tg levels after total thyroidectomy almost always have residual thyroid carcinoma (27, 28) or relapses will appear (29).

Measuring Tg after stimulation with TSH

The most sensitive method for determining residual thyroid carcinoma is measuring Tg concentrations in serum after stimulation with endogenous or exogenous TSH. The term »non-detectable« Tg value refers to disease-free patient status, but it depends on the conditions under which samples are taken for analysis and also test characteristics. Several studies have shown that Tg concentrations will increase after TSH stimulation in 20–25% of patients with undetectable serum Tg (during substitution therapy and TSH suppression) (30–32). Before scintigraphy, patients are taken off thyroid hormone therapy to induce hypothyroidism, until TSH concentrations increase above 25 mIU/L. This increase in TSH will stimulate the release of Tg, when tumor and/or normal thyroid tissue is present and increase accumulation of radioactive iodine in the thyroid gland. Thus, stimulation with TSH increases the test sensitivity for Tg in determining tumor relapses and metastasis (33, 34) and is at the same time an indicator of tumor sensitivity to TSH stimulation. After stimulation with TSH well-differentiated thyroid tumors (and also the remaining normal thyroid tissue) give a tenfold increase in serum Tg concentrations, while the increase of Tg level is usually less than threefold with less differentiated carcinomas. Factors that also influence the elevation of Tg serum concentrations are the duration and level of increased TSH in serum. Patients from whom levothyroxine therapy is withdrawn for several weeks do not bear hypothyroidism easily, so instead of withdrawing the substitution therapy and stimulating endogenous TSH, an alternative method is now used. This includes intramuscular application of recombinant human TSH (rhTSH, Thyrogen), a few days before determining Tg concentrations. According to Mazzaferri et al. (30) both approaches have equivalent sensitivity, though other authors (35, 36) consider that suspending substitution therapy gives higher sensitivity for detection of residual thyroid carcinoma. Current recommendations of the American Thyroid Association are that 6 months after ablation Tg concentrations should be measured after suspending levothyroxine therapy (due to better sensitivity in detecting recurrent or persistent disease), but if Tg values are obtained pointing to a low risk, then further monitoring can be done using rhTSH (3).

Methodological problems in determining Tg

Measuring Tg in serum is associated with a series of methodological problems that can reduce its clinical significance (37). Nevertheless, there are many commercial diagnostic kits for its determination. According to the detection principle (competitive binding of labelled and unlabelled Tg for a limited number of TgAb or Tg in a sandwich between labelled and unlabelled antibodies), they can be categorized into two groups: radioimmunoassay (RIA) and immunoradiometric assay (IRMA) or broader (IMA) tests. The test principle, its sensitivity and also the eventual presence of serum factors: thyroglobulin antibodies (TgAb) or heterophilic anti-mouse antibodies (HAMA) in patient serum (38, 11), can influence the measured Tg concentrations. Thus, determining Tg in the same serum sample in different laboratories can give different values. Factors causing differences in the serum concentrations of Tg obtained using different tests are numerous: different reference materials, non-harmonized standards for the same reference material, different specificity and also the affinity of primary and secondary antibodies for Tg epitopes, interference of serum factors, primarily thyroglobulin autoantibodies (39–41) with primary and secondary TgAb in the diagnostic kits (42–45). These differences between methods do not allow comparison of results and practically prevent interpretation of changes in Tg levels noted using different methods, such as changes in tumor size or the appearance of metastatic tissue. Therefore, in order to be able to compare the results of control measurements of Tg concentrations in patient serum, Tg measurements must be done using the same method and, if possible, in the same laboratory.

Influence of test sensitivity on monitoring a patient with DTC

The sensitivity of Tg tests is important for the detection of small DTC. If a test cannot detect Tg in individuals with an intact thyroid gland, then such a test has insufficient (suboptimal) sensitivity for monitoring patients with DTC after thyroidectomy. In principle, IMA tests are more sensitive than RIA tests. Modern guidelines, instead of the term »ultrasensitive«, have defined standard protocols for measuring Tg test sensitivity. Functional sensitivity is defined as the thyroglobulin concentration that can be measured with a 20% coefficient of variation under clinically relevant conditions. Namely, assays of TgAb-negative human sera are repeated over at least a 6–12 month period using at least two lots of reagents and calibrators (46). Conventional tests for measuring Tg have a functional sensitivity of 1 µg/L, while for newer generation tests it is lower than or equal to 0.1 µg/L. Advocates for the application of these tests consider that this will eliminate the need for expensive rhTSH stimulation tests.

There is no agreement concerning Tg cut-off values for detection of a relapse or recurrent disease in patients with DTC. Current guides recommend values of 2 µg/L after stimulation (endogenous or rhTSH) as a risk factor for DTC (3), but instead of a fixed cut-off value the method of monitoring the trend of measured concentrations of basal Tg (without stimulation) can be used (47). It is a good practice in some laboratories to freeze and store sera after determining Tg, so the samples can be used again in parallel with the next sample from the same patient. Results obtained in this way can confirm that changes in Tg levels are the result of alterations in tumor mass and not due to differences in test performance. This approach assists the doctor to conclude with greater certainty whether the disease has worsened or not.

Reference Tg

Due to differences between methods, a project has started at the European level, supported by the Committee for Reference Materials of the European Union Commission, for isolation and characterization of reference Tg. The result of this project is a certified reference material for thyroglobulin – CRM-457 (48, 49). Most tests are now calibrated, directly or indirectly, according to this standard. However, regardless of standardization according to CRM 457, there is still great variability between different tests for determining Tg. This could be a reflection of diverse specificities in individual tests for Tg isoforms (37, 50–54) and interference by serum factors, primarily autoantibodies (TgAb), with reagent antibodies in the assays. When six diagnostic kits standardized according to CRM 457 (2 RIA and 4 IRMA) were analyzed, almost twofold differences for Tg concentrations in serum without TgAb were obtained (15). The coefficient of variation for these six tests was 37%, which is more than double the biological variability in healthy euthyroid individuals (55) or in individuals with DTC and suppressed TSH (15).

The results obtained using IMA techniques varied more than for RIA (37), most probably because polyclonal antibodies with wider epitopic specificities are used in RIA and they can detect tumor-modified Tg isoforms better than IMA techniques that employ monoclonal antibodies with limited epitopic specificities. This can be significant, as the posttranslatory changes of Tg are altered in tumor cells, leading to conformation changes in secreted Tg (53). Due to the type of conformation of Tg epitopes, it is not surprising that larger differences have been shown between individual IRMA methods than for RIA tests.

Interference with serum factors

Interference with antibodies against human immunoglobulin (HAMA) occurs in about 3% of sera sent for determination of Tg concentration (56). For

IMA tests HAMA mainly produces a false increase (56) and more rarely false negative Tg values (57). One should consider interference caused by HAMA if the measured Tg concentration does not correspond to the patient's clinical status and, if possible, Tg in the presence of HAMA should be measured by RIA.

According to data in the literature, increased concentrations of TgAb are present in 20–30% of patients with DTC (58, 59). Successful removal of the tumor leads to a decline in TgAb concentration and antibodies disappear from the circulation after about 3 years (60). Disappearance of circulating TgAb indicates a positive outcome, i.e. an insignificant risk for the occurrence of relapse or metastasis in patients with DTC. On the other hand, some studies have indicated a higher incidence of residual thyroid carcinoma in patients with high TgAb values compared to patients with low or non-detectable TgAb values (61, 62).

The presence of Tg-specific autoantibodies influences the measured Tg concentration in most assays and can depend on their affinity and capacity on the one hand, and the properties of reagents in the tests for determining Tg concentrations on the other hand (37, 41, 43).

Depending on the type of method used, samples with TgAb can give inappropriate higher or lower Tg values. It has generally been accepted that RIA tests are less sensitive to the presence of TgAb than IRMA tests (63). Measured serum Tg concentrations using RIA tests can sometimes be lower, but are often higher than the true values (39, 40). Nowadays, many laboratories employ immunometric tests, as they have certain technical advantages such as shorter incubation time and process automation (37, 41, 46).

The measured values of Tg using IRMA (or similar) tests are usually lower than the actual levels (63), even though TgAb concentrations may be very low, even below positive limits (59, 64, 65). It seems that values for Tg measured by RIA tests correspond more closely to physiological values, as both free Tg and Tg complexed with TgAb are detected (15). The principle of RIA tests is competitive binding by polyclonal antibodies, which have greater possibilities of recognizing epitopes exposed on Tg molecules bound to TgAb (9, 11–13). The method of disrupting binding of the TgAb-Tg complex to antibody in IMA tests could be twofold: steric inhibition or masking of the epitope that recognizes the monoclonal antibody in the diagnostic kit, but there are no experimental data for this mechanistic concept (9, 11, 66).

In the presence of TgAb, the measured values of Tg should be cautiously interpreted regardless of the applied test. Falsely low values for serum Tg concentration can postpone the required patient treatment. In contrast, falsely increased Tg values

measured using RIA tests can initiate additional screening or treatment that is an unnecessary stress for the patient.

For now there is no expert agreement on the question of selection of the method best suited for Tg measurement in sera where, using a sensitive enough method, the presence of TgAb has been shown. With RIA methods there is less interference with Tg specific autoantibodies and some laboratories in the USA consider that they provide more clinically accurate values, and so give the advantage to RIA methods for measuring serum Tg (37). Moreover, it is considered that IMA methods should not be used when TgAb is present in patient serum, as falsely low Tg values are a greater problem than falsely high ones. For example, a falsely low Tg value obtained due to the interference of endogenous TgAb can lead to postponement of necessary treatment, while a falsely high value of Tg obtained in the presence of TgAb in the patient's serum will only direct the doctor's attention to this patient.

Identification of the influence of TgAb

As the presence of TgAb in the patient's serum can significantly influence the measured Tg value, it is important to use a precise and sensitive method for determining TgAb. Unfortunately, the results obtained using different methods for determining TgAb concentrations differ even more than the results for Tg measurements. Thus, one assay can show that there is an increased concentration of anti-thyroglobulin autoantibodies in a certain patient's serum, while another detects no TgAb in the same serum (67, 68).

Besides measuring the presence of TgAb with an adequately sensitive method, the presence of these autoantibodies in a patient's serum can be established by adding a known amount of Tg to serum samples (»recovery test«) and also by comparison of the degree of agreement of Tg results using tests based on different analytical principles (IRMA/RIA). If TgAb is present in the samples, the values for Tg measured using RIA are usually higher than the values obtained by IRMA (34, 59). Thus, if the results obtained using IRMA and RIA methods show good agreement, there is a large possibility that the finding is acceptable for clinical diagnostics. This approach enables identification of samples where interactions occur between autoantibodies and Tg specific reagent antibodies in the test for determining Tg, but it does not direct the doctor's actions, as it cannot answer the question of how the different values obtained relate to the real concentration of Tg in the sample. To evaluate the influence of TgAb on Tg concentrations measured by RIA and IRMA tests, it is necessary to do a »recovery test« for individual samples where disagreement between measured Tg

values has been shown. Though not ideal, the »recovery test« does indicate whether the Tg value measured using a certain method is lower or higher than the real one.

Of course, the serum concentration of Tg is only one parameter in the postoperative monitoring of patients with differentiated thyroid carcinoma, and if there is a difference in the results obtained using methodologically different tests, a clinical practitioner should take into account that neither result is true and undertake additional clinical investigations.

Measuring TgAb in serum

It is necessary to determine TgAb concentrations in every serum sample where Tg is measured, for two reasons: first, the patient's TgAb status can change from positive to negative or vice versa, and secondly, to follow the trend of TgAb concentration change over a long time period in order to evaluate how the tumor reacts to therapy (for example, when applying ablative ^{131}I doses). Serial determination of TgAb is sometimes recommended as a surrogate tumor marker for DTC (59, 60, 69). The TgAb concentration strives to follow the Tg trend (specific antigen) that is recognized by the immune system. When TgAb is present in the serum of a patient with DTC before surgery and the antigen source is removed, TgAb cannot be detected after 3 years (60, 70) and its concentration is reduced by 50% after 6 months, in accordance with the half-life of circulating endogenous immunoglobulin (49, 71). However, the concentration of TgAb can increase (or become positive in patients negative before the ablative dose) 6 months after applying doses of ^{131}I in response to the release of Tg from destroyed thyroid tissue (72). Interpretation of TgAb concentrations as a surrogate tumor marker is possible if tests from the same manufacturer are used during the monitoring of a patient, if possible in the same laboratory. Despite standardization according to the international

reference material, MRC 65/93, commercial tests for TgAb have different sensitivities, specificities and measured absolute values (45, 46, 59, 69, 73), most probably due to differences in test specificities for different conformation epitopes of TgAb (69, 71, 74). A declining trend for TgAb over a long time period is a good sign of treatment efficacy (surgery/radioiodine ablation). In contrast, if TgAb levels increase with time, this can be an early sign of worsening disease (56, 61, 75). Thus, measuring TgAb concentrations in serum may serve as a surrogate marker for evaluating disease progress (besides measuring Tg concentrations). However, one study (76) based on data obtained by monitoring changes of TgAb levels in the serum of patients who had had surgery for DTC, disputes the value of Tg-specific antibodies as a surrogate tumor marker. Namely, during a three year follow-up no difference in TgAb concentrations was detected between patient groups with or without a relapse of thyroid carcinoma.

Conclusion

To conclude, we can say that measuring Tg in the serum of patients who had undergone surgery for differentiated thyroid carcinoma is a useful test for determining worsening disease and monitoring the effects of therapy. Patients should be continually monitored using one selected method and if possible in the same laboratory. In order to obtain optimal results it is necessary to understand the limitations of modern methods used in clinical practice and the pathophysiology of Tg secretion.

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