Immunoreactive thyroglobulin-like material derived from saliva


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Abstract. The presence of an immunoreactive thyroglobulin-like material in saliva from normal subjects and from 35 patients with thyroid carcinoma was detected by radioimmunoassay. The levels of this material in saliva were markedly elevated in patients with extensive metastases.

Concentrated saliva samples from normal subjects and from patients with thyroid carcinoma were fractioned on Sepharose-6B and each fraction was assayed for thyroglobulin content by RIA. Several protein peaks of varying molecular size with thyroglobulin-like immunoreactivity were observed. The physiological significance of these molecules in saliva remains to be established.

Quantitation of serum thyroglobulin (Tg) in patients with proved differentiated thyroid carcinoma is considered a reliable biochemical marker for early detection of recurrence of disease or subsequent metastases (Van Herle & Uller 1975; LoGerfo et al. 1977; Shah et al. 1978, 1981; Schlossberg et al. 1979; Botsch et al. 1979). Observations from our laboratory have further shown the presence of Tg-like material in saliva from normal subjects and from patient with thyroid carcinoma by a radioimmunoassay procedure (Shah et al. 1978).

The aim of the present work was to study some of the biochemical and physiochemical properties of immunoreactive Tg-like material in saliva from patients with thyroid carcinoma and from normal subjects.

Materials and Methods

Study subjects

Spot samples of saliva were collected from 11 normal subjects and from 35 patients with differential thyroid carcinoma (24 patients with predominantly follicular variety, 6 with papillary variety, 5 with mixed type). Of these 35 patients, 4 were free of disease at the time of investigation as evidenced by the scintiscan after oral administration of 5 mCi of radioiodine (131I obtained from Isotope Division, Bhabha Atomic Research Centre, Bombay, India). 3 had a residual mass in the neck region while 28 patients had evidence of metastases. Metastatic patients were further classified according to the site of metastases; 3 patients had involvement of lymphnode(s), 1 had metastatic spread in lungs, 4 in lymphnode(s) and lungs, 15 in bones, while the remaining 5 patients had metastases in lung(s) as well as bone(s).

Serum samples from all these patients as well as from normal subjects were also analysed simultaneously for circulating Tg levels.
**Collection of saliva samples**
A spittow was given to each patient for collection of saliva over a period of 2 to 4 h. No stimulation was given for this purpose. The volume of the saliva varied between 4.0 to 8.0 ml. Occasionally, these samples contained some sputum also. Saliva from one of these patients was collected on 2 successive days over a period of 4 h each. These saliva samples were mixed and used for chromatographic studies.

**Processing of saliva sample**
Each saliva sample was mixed with (1/10th of the volume) phosphate buffer saline (0.0055 M phosphate buffer, pH 7.0, containing 0.15 M NaCl) containing 0.1% sodium azide. The samples were stirred with a magnetic stirrer for 2 to 4 h at 4°C, centrifuged and the supernatant was analysed for Tg content.

**Radioimmunoassay (RIA) of Tg**
The RIA procedure followed for quantitation of Tg was essentially the same as that described by Van Herle et al. (1973) and has been detailed in our previous publication (Shah et al. 1981) with the following difference: 1) 225 μl of saliva sample was used in place of 100 μl of test serum, 2) 25 μl of sheep serum was added to each tube containing saliva sample. The samples which contained higher amounts of Tg were diluted in 1:10 sheep serum prior to analysis.

**Validity of the assay**
All the saliva samples were analysed individually in different RIA (as they were collected at different times dependent upon the availability of the patient) as well as collectively in one batch in a single RIA.

The non-specific binding with saliva sample without the addition of first antibody ranged between 3.0–5.0% which was comparable to that obtained with blanks without addition of sample. The degree of variation inter-assay was 11.8% and intra-assay was 9.6%. The recovery of the added standard h-Tg 1 and 2 ng to saliva sample was 103–107 and 106–110%, respectively. The addition of serum containing a known amount of Tg to saliva sample gave a recover of 103–106%.

**Parallelism of samples with standard h-Tg**
One serum sample from patient K and 5 samples of saliva (one saliva sample from a normal person which was concentrated by negative pressure dialysis and 4 saliva samples from patients with thyroid carcinoma) were assayed at various dilutions for Tg content to validate the procedure further.

**Fractionation of concentrated saliva on Sepharose-6B**
Saliva samples from one of the patients (patient P) with metastases in bones having a serum Tg level of 40 000 ng/ml and a normal subject having a serum Tg value of 8.0 ng/ml were concentrated (15 to 2 ml for the patient and 15 to 2.4 ml for the normal subject) by negative pressure dialysis. The concentrated samples were dialysed against PBS, 0.6 ml of each sample (3.04 and 2.59 mg of protein content in the saliva of the patient and the normal subject, respectively) was chromatographed on Sepharose-6B (Pharmacia Fine Chemicals, Uppsala, Sweden, column size 1.7 x 94 cm at 8°C) and 3.3 ml fractions were collected. An aliquot of 225 μl from each fraction was analysed for Tg content as described earlier. The Sepharose-6B column was calibrated with known molecular weight proteins (bovine thyroglobulin 660 000; gamma globulin 160 000; bovine serum albumin 69 000; egg albumin 40 000).

**Antithyroglobulin antibodies in samples**
All the serum and saliva samples reported in the present study were found to be negative for the presence of antithyroglobulin and antimicrosomal antibodies as tested by a haemagglutination technique (kits obtained from Fujizoki Pharmaceutical Co., Japan).

**Results**

**Tg-like material in saliva**
Tg levels in saliva of 11 normal subjects and 35 patients with differentiated thyroid carcinoma are shown in Fig. 1. The levels of this molecule in saliva varied from undetectable to 10 ng/ml in 11 normal subjects, with a mean value of 3.27 ± 3.42 (SD).

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**Fig. 1.**
Tg content in saliva of patients with differentiated thyroid carcinoma.

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Thirty-five samples of saliva from patients with differentiated thyroid carcinoma showed a value ranging between undetectable to 70 ng/ml with a mean of 15.89 ± 19.16 (D). The patients who had extensive metastases showed very high levels of salivary Tg-like material while patients with little metastatic spread and free of disease at the time of investigation were within the normal range.

A scatter diagram of Tg in serum and Tg-like material in saliva is shown in Fig. 2. The levels of Tg-like immunoreactive material in saliva were raised in the patients where serum Tg levels were also increased. The correlation coefficient (r) observed with these two parameters was only 0.41.

Parallelism of serum and saliva samples with standard h-Tg

The identity of salivary immunoreactivity Tg-like material was further validated using various sample sizes for the estimation.

Serum from patient K and saliva from 4 patients were assayed at different dilutions for the estimation of Tg. A serum sample with 500 ng/ml was assayed with a dilution range of 1:55 to 1:550. The slope obtained with the serum sample was the same as that of the standard h-Tg preparation (Fig. 3).

Various sizes (25 to 225 µl) of saliva samples were used in the assay. The curves obtained with these samples were parallel to that obtained with standard h-Tg. The saliva sample from a normal person was concentrated prior to the assay. It also gave a parallel curve to the standard h-Tg preparation when assayed at various dilutions (Fig. 3).

Content of Tg-like material in the fractions of concentrated saliva separated on Sepharose-6B

Concentrated saliva from a normal subject, total protein (2.59 mg) and patient P with thyroid carcinoma, total protein (3.04 mg) were chromatographed on a Sepharose-6B column. The Tg-like material in each fraction eluted from the Sepharose-6B column is shown in Fig. 4. Apart from 19S Tg protein, larger and smaller molecular weight proteins also exhibited reactivity like Tg. There were at least 4 major and 2 minor protein peaks which contributed to the Tg measurement in the saliva obtained from the patient with thyroid carcinoma. Moreover, the summation of the Tg con-
tent in each fraction obtained on the Sepharose-6B column exceeded the Tg content of the saliva measured as a whole sample. Concentrated saliva from the normal subject showed negligible Tg content in these fractions.

Discussion

Serum Tg levels are raised in several disorders of the thyroid gland. Nonetheless, it is valuable in monitoring the course of disease in patients with proved differentiated thyroid carcinoma. Patients with bone metastatic lesions tend to show much higher Tg level than those with pulmonary metastases (Shah et al. 1978, 1981; Hagemann & Schneider 1977). While exploring various possible mechanisms of this differential behaviour of metastatic lesions, we showed the presence of Tg-like material in salivary secretions from these patients as well as in normal subjects (Shah et al. 1978) as measured by RIA.

The data presented in this report show that saliva from normal subjects and from patients with differentiated thyroid carcinoma contain immuno-reactive Tg-like material as detected by RIA. Also, its levels are raised in patients with extensive metastases. An attempt was further made to correlate the Tg content in serum and Tg-like material in saliva. However, a poor correlation (r = 0.41) was obtained. It is difficult to correlate precisely the serum Tg content and Tg-like material in saliva for several reasons. In the present study, spot samples of saliva were used for estimation of Tg and the rate of salivary secretion may vary with the individual. The mean saliva Tg value obtained in 11 normal subjects ranged between undetectable and 10 ng/ml. The serum Tg levels obtained in our laboratory in 40 normal subjects varied between undetectable and 20.0 ng/ml. A relation between the two parameters appears unlikely, at least in

![Dilution curves with saliva and serum from patients with thyroid carcinoma along with concentrated saliva from normal subjects.](image-url)
normal subjects. However, a larger sample analysis is needed to derive a statistical relation, if any.

Saliva samples from 2 patients RX and CH were collected on 2 different days to study the variation in the level of Tg-like material, if any. The values obtained were 16.0 and 16.6 ng/ml with RX and 11.5 and 15.5 ng/ml with CH, respectively, indicating no significant variations in the biomolecule assayed.

Saliva with a high content of Tg-like material when assayed at various dilutions exhibited slopes which are similar to that obtained with a standard h-Tg preparation as well as serum Tg. Establishment of parallelism between the saliva and standard h-Tg preparation is not necessarily conclusive evidence of identity. Nonetheless, it could be considered as an additional operational criterion.

Fractionation of concentrated saliva from patient P (serum Tg 40,000 ng/ml; saliva Tg 42.0 ng/ml) on Sepharose-6B, showed several protein peaks with different mobility containing Tg-like material (Fig. 4). The molecular weight of these proteins varied between larger molecules eluting along with void volume to molecules smaller than 59,000 daltons. Similar findings of low molecular weight Tg circulating in the plasma of patients who have undergone total thyroidectomy have been reported by Feldt-Rasmussen et al. (1978).

The total Tg content of the saliva sample from patient P as a whole was much lower than the summation of Tg content in each fraction of saliva obtained on Sepharose-6B. This discrepancy could be explained by the difference in the antigenicity of each fraction. The increased antigenicity of smaller molecular weight proteins in plasma has been reported by Feldt-Rasmussen et al. (1978). The Tg content of a concentrated saliva sample from a normal subject was low and hence the levels of Tg obtained in each fraction obtained on chromatography also showed a lower amount of Tg.

In any event, the physiological significance of the presence of immunoreactive 19S Tg and Tg-like smaller molecular weight proteins in saliva remains unclear. Whether this immunoreactivity reflects
true biological activity remains to be established. The isolation, purification and characterization of these biomolecules from saliva may reveal their biological importance which is currently being investigated.

References


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