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The Release of Thyroglobulin from the Thyroid Gland into Thyroid Lymphatics; the Identification of Thyroglobulin in the Thyroid Lymph and in the Blood of Monkeys by Physical and Immunological Methods and its Estimation by Radioimmunoassay

P. M. DANIEL, O. E. PRATT, I. M. ROITT AND G. TORRIGIANI

Department of Neuropathology, Institute of Psychiatry, The Maudsley Hospital, Denmark Hill, London, S.E.5, and Rheumatology Research Department, Arthur Stanley House, The Middlesex Hospital Medical School, London, W.1

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Summary. The iodoprotein which was found in the lymph draining from the thyroid gland of monkeys has been identified as thyroglobulin, both by physical and by immunological techniques.

A sensitive and highly specific radioimmunoassay was developed by which thyroglobulin has been estimated in the thyroid lymph and in the blood of these animals.

Small but appreciable concentrations of thyroglobulin were found in thyroid venous and in peripheral blood. Non-thyroid lymph did not usually contain detectable concentrations of thyroglobulin but thyroglobulin was regularly found in thyroid lymph, sometimes in high concentrations. Thyroid stimulating hormone raised the concentration of thyroglobulin in the thyroid lymph still higher as did gentle massage of the tissues overlying the gland.

It was shown that the release of thyroglobulin into the thyroid lymph was a normal physiological process, for the possibility that it might have been released as a result of radiation or operative damage to the thyroid gland was excluded by experiments in which the need for administration of radioisotope to the animals was avoided and in which samples of lymph were obtained by cannulation of a cervical lymphatic trunk at some distance from the thyroid gland itself.

The implications of these findings are discussed in relation to the autoimmune phenomena seen in human thyroid disease.

INTRODUCTION

Previous work has shown that the concentration of organic radioactive iodine in the lymph draining from the thyroid gland of cats, sheep, rabbits and baboons previously given radioactive iodine is much higher than that in the peripheral or thyroid venous blood plasma and that the administration of thyroid stimulating hormone increases the concentration still further (Daniel, Excell, Gale and Pratt, 1962a; Daniel, Gale and Pratt, 1963c, d). The main radioactive iodine component in the lymph draining from the

thyroid gland of cats and rabbits was found to be an iodoprotein yielding iodotyrosine upon alkaline hydrolysis. When radioactive iodoprotein was obtained by precipitation from the thyroid lymph of monkeys and subjected to enzymic hydrolysis it yielded a pattern of iodoamino acids closely resembling that given by thyroglobulin (Daniel, Gale, Plaskett and Pratt, 1963a, b; Daniel, Plaskett and Pratt, 1966a).

There are now available a number of physical and immunological techniques for the study of iodoprotein which enable thyroglobulin to be identified by its characteristic reactions (Daniel, Pratt, Roitt and Torrigiani, 1966b). Most of the techniques depend upon labelling the iodoproteins *in vivo* with radioiodine (for example ^{131}I) a procedure which enables quantitative data to be obtained with ease, but which has the disadvantage that the thyroid gland may be damaged by radiation. If ^{125}I is used instead of ^{131}I the radiation dose to the thyroid gland is reduced by more than twenty times and consequently the likelihood of damage to the gland is lessened (Daniel, Gale and Pratt, 1962b). However, since it is desirable to avoid giving the animals any radioactive material at all a sensitive and specific immunoassay method for thyroglobulin was developed using radioactive labelling of thyroglobulin *in vitro* only. With this method it has been possible to show that thyroglobulin is present as a normal constituent of the thyroid lymph of animals which have not received any radioactive iodine. In addition, it has been possible in some of the present experiments to avoid surgical disturbance of the thyroid gland by cannulating cervical lymph trunks, rather than the lymphatics draining the thyroid gland directly.

MATERIALS AND METHODS

Animals and surgical techniques

Nineteen baboons and rhesus monkeys (of either sex) were used. Anaesthesia was induced with ether followed by chloralose, 70 mg/kg intravenously. A Magill intratracheal tube was inserted and an intravenous glucose–saline drip was set up. An intravenous catheter was passed up through the femoral vein and into the inferior vena cava. Blood samples were taken through this catheter.

All the work on the lymphatic vessels was done with the help of a Zeiss operating microscope. In the early stages of the work the thyroid gland was exposed and one of the twenty or so lymphatic vessels draining the gland itself entered with the tip of a micropipette made by drawing out glass capillary tubing. The lymph flowing along the lymphatic from the thyroid was sucked into the capillary tube during the course of several minutes. In this way samples of undiluted thyroid lymph weighing from 3 to 50 mg were obtained. Usually a non-thyroid lymphatic vessel (either the thoracic duct or a lymphatic in the groin, draining the leg) was cannulated so that control samples of lymph could be obtained. In some cases an inferior thyroid vein was cannulated at the end of the experiment to provide a sample of thyroid venous blood.

During the later stages of the work, when the radioimmunoassay for detecting thyroglobulin was available, the thyroid gland was not exposed. In these experiments either the right or the left cervical lymph trunk (Fig. 1) was exposed at the root of the neck and cannulated with fine polythene tubing. This trunk receives most of the lymph from the thyroid gland which is, however, greatly diluted by the non-thyroid lymph coming from the tissues of the head and neck. All the operative procedures were carried out 2–6 cm away from the thyroid gland which was never exposed or damaged. In some experiments,

after a series of preliminary lymph collections, the skin over the region of the thyroid gland was lightly massaged with a finger tip in order to increase lymph flow. This was a delicate procedure mimicking the effect of neck movements, the gland itself being protected by overlying skin, subcutaneous tissue and muscle. After the first collections of lymph and blood, thyroid stimulating hormone (TSH) was given intravenously and repeated 2-hourly as needed. The bovine TSH (Thytropar) was obtained from the Armour Pharmaceutical Co., Illinois, U.S.A.

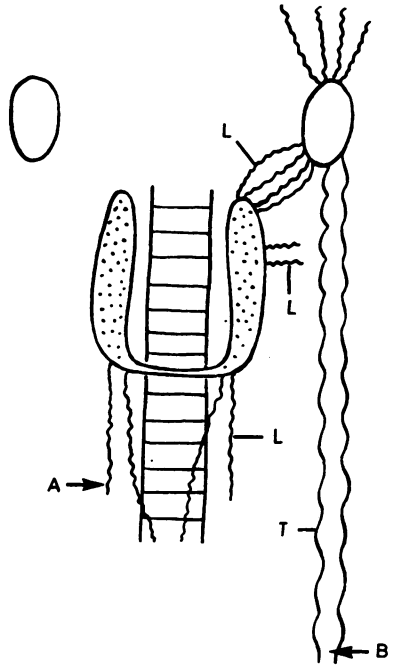

FIG. 1. A schematic diagram of the lymphatic drainage of the thyroid gland. A number of small lymphatic vessels (L) are shown leaving the gland. The large cervical lymph trunk (T) drains part of the lymph from the thyroid gland but also that from a considerable mass of the tissues of the head and neck. Lymph from the thyroid gland contributes only a small proportion of the total. Lymph was collected from two different sites. From A pure thyroid lymph was obtained. The lymph collected at B only contained a small proportion of thyroid lymph.

At the end of each experiment a small quantity (about 0·1 ml) of blue dye solution was injected into the substance of the thyroid gland, on the side on which the lymph collections were made. If the dye failed to appear in the cervical lymphatic cannula within a few minutes the experiment was rejected.

Two rhesus monkeys were submitted to total surgical thyroidectomy. In one animal, it was necessary to see whether there was any residual thyroid tissue. The animal was given 200 μc of ^{131}I and TSH (10 i.u.) intramuscularly and 36 hours later it was anaesthetized; the thyroid region was exposed and searched with a portable Geiger probe.

Preparation of thyroglobulin

Thyroglobulin was prepared from the thyroid glands of humans and monkeys by an ammonium sulphate precipitation method (Derrien, Michel and Roche, 1948).

For the radioimmunoassay, monkey thyroglobulin (5 μg) was iodinated with ^{131}I (2·0 mc) by the chloramine T method (Greenwood, Hunter and Glover, 1963). The free

iodine and the breakdown products of the reaction were removed by passing the solution through a Sephadex G-200 (obtained from Pharmacia, G.B., Ltd, London) column and pooling the eluted fractions constituting the first radioactive peak. This eluate contained the iodinated thyroglobulin but as a result of the treatment some of the labelled molecules tended to combine non-specifically with any antigen–antibody complex. Therefore, equivalent proportions of rat anti-ovalbumin serum (0·5 ml) and ovalbumin (100 μg) were added to 2 ml of the labelled thyroglobulin solution. The mixture was incubated at 37° for 1 hour and left overnight at 4°. The precipitate was centrifuged down and the supernatant was used as the labelled thyroglobulin preparation. Whenever possible, the immunoassay was set up immediately after this purification. Otherwise, the preparation was diluted in normal goat serum, but, even then, it was used not later than the following day.

In one experiment the specific activity of thyroglobulin in saline extracts of the thyroid gland of a monkey which had been given ^{131}I was determined. Samples were counted and then titrated by quantitative precipitation with anti-monkey thyroglobulin serum; comparison was made with a standard precipitin curve established by using a purified preparation of monkey thyroglobulin. The thyroid tissue contained about 12 per cent of its fresh weight of thyroglobulin.

Antisera

Antisera to human and monkey thyroglobulin were produced in rabbits by intramuscular and subcutaneous injection of 20 mg of protein in complete Freund's adjuvant, followed 4 weeks later by intravenous injection of a total of 20 mg of antigen–alum precipitate in divided doses. Antibodies to monkey thyroglobulin were purified from an antiserum which did not react with thyroxine or triiodothyronine, using the method of Metzger and Edelhoch (1962). For the precipitin experiments the antiserum to human thyroglobulin was absorbed with 0·2 volumes of normal monkey serum.

Antisera to rabbit γ-globulin were produced in goats by the intramuscular injection of 100 mg of sodium sulphate-precipitated rabbit γ-globulin in complete Freund's adjuvant followed by two injections at a week's interval of the same amount of antigen in incomplete Freund's adjuvant. These antisera showed a small degree of cross-reaction with human thyroglobulin and for this reason were absorbed before use with human thyroglobulin made insoluble by cross-linking with benzidine bis-diazonium salt (DeCarvalho, Lewis, Rand and Uhrick, 1964).

Crude preparations of thyroid autoantibodies were obtained from various human sera by salting-out with 18 per cent sodium sulphate solution and conjugated with fluorescein isothiocyanate (Marshall, Eveland and Smith, 1958). The conjugates were absorbed with guinea-pig liver powder before use in the immunofluorescent 'spot' test.

Radioimmunoassay of thyroglobulin

Radioactively labelled monkey thyroglobulin solution (0·02 ml) giving approximately 100 counts/sec was added to 0·1 ml of the unknown lymph or serum sample inactivated by heating at 56° for 30 minutes. Enough purified rabbit anti-monkey thyroglobulin antibody to combine with approximately 85 per cent of the labelled thyroglobulin was added and the mixture was incubated at 37° for 2 hours and left at 4° for 4 days. This complex of the rabbit antibody with thyroglobulin was co-precipitated by the further addition of 0·1 ml of a 1:200 dilution of normal rabbit serum and 0·2 ml of goat anti-rabbit γ-globulin.

This mixture was incubated for 30 minutes at 37° and left overnight at 4°. The mixture was centrifuged, the supernatant removed and the precipitate washed. The washings and supernatant were combined for radioactive counting. The supernatant, representing free thyroglobulin, and the precipitate, representing bound thyroglobulin, were counted separately.

A standard curve was constructed from assays carried out on a series of 0·1 ml portions of normal goat serum to which amounts of unlabelled thyroglobulin had been added to give concentrations ranging from 0 to 0·1 μg/ml (Fig. 2). The assay was not affected by the presence of thyroxine in concentrations up to 5000 ng/ml of serum.

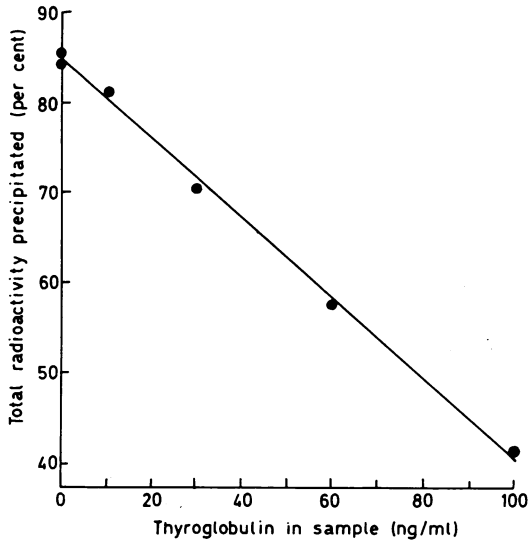

FIG. 2. Standard curve for the radioimmunoassay of thyroglobulin. Trace amounts of [^{131}I]thyroglobulin were added to samples of goat serum containing various concentrations of unlabelled thyroglobulin. After addition of rabbit anti-thyroglobulin, radioactive protein bound to antibody was co-precipitated with goat anti-rabbit γ-globulin and counted. In some assays, the standard curve was convex towards the axes and occasionally a marked shoulder occurred at the lower thyroglobulin concentrations.

Red cell agglutination

A method for the estimation of thyroglobulin was developed in collaboration with Dr N. R. Ling and Mr K. G. Couchman employing pyruvic aldehyde-treated cells coated with purified rabbit anti-monkey thyroglobulin antibody under conditions described by Ling (1961). The sensitivity lay between 10 and 40 ng/ml of thyroglobulin.

A variation of this method was to test the ability of different dilutions of lymph to inhibit the agglutination by monkey anti-thyroglobulin of thyroglobulin-coated, tanned red cells (cf. Hjort, 1962).

Immunofluorescent 'spot' test

A modification of the method of Crawford, Wood and Lessof (1959) was employed. Drops of antigen-containing solution were placed with a wire loop on acetone-cleaned microscope slides and the slides dried in air for 45 minutes. The dried spots were fixed in absolute methanol for 45 minutes and further dried by fan for 30 minutes. The slides were then dipped for 8 seconds into 70 per cent methanol and washed for 5 minutes with

0·01 M sodium diethylbarbiturate buffer, pH 7·2, containing 0·85 per cent of sodium chloride. The fluorescein-conjugated antibody was applied and left for 20 minutes before washing again in the same buffer solution. The unmounted slides were examined in ultraviolet light.

Precipitin tests

Samples of lymph or serum (0·3 ml) from monkeys given ^{125}I were incubated for 30 minutes at 37° with 0·2 ml of saline and 0·2 ml of a potent rabbit antiserum to human thyroglobulin and left to stand overnight at 4°. In order to co-precipitate any soluble complexes which might be present, 50 μg of human thyroglobulin in 0·2 ml of saline was added to the above mixture and incubation was continued for a further 30 minutes at 37°. The precipitate obtained was washed twice with saline and the radioactivity was counted in the washed precipitate and in the supernatant.

Electrophoresis

Samples of lymph (0·04 ml) were mixed with an equal volume of either rabbit anti-human thyroglobulin or anti-human γ-globulin and incubated for 15 minutes at 37°. Electrophoresis was carried out with a current of 1·25 mA for 16 hours on 4 cm wide strips of Whatman No. 1 paper, using sodium diethylbarbiturate buffer (pH 8·6; ionic strength = 0·1). The strips were stained with azocarmine. Under the conditions used the albumin ran off the paper and the globulin bands were scanned for radioactivity in a Nuclear Chicago strip counter (Model C100B).

Ultracentrifugation

Samples were layered over a discontinuous sucrose gradient (Torrigiani and Roitt, 1965) and centrifuged at 35,000 rev/min for 15 hours in the SW39 head of the Spinco Model L centrifuge at a temperature of 1–4°.

RESULTS

Thyroglobulin was found in the thyroid lymph of animals which had been injected with radioactive iodine, and in the cervical lymph (Fig. 1) both of animals which had been given the radioisotope and of those to which radioactive material had not been given.

THYROID LYMPH FROM ANIMALS WHICH HAD BEEN GIVEN RADIOACTIVE IODINE

In one experiment thyroid lymph (18·5 mg) was obtained from lymphatics close to the thyroid gland of a baboon which had been given ^{131}I 6 days before the experiment and to which TSH (10 i.u., intravenously) had been given 2 hours before the collection. This lymph was diluted to 0·3 ml with saline and a series of tests was carried out. Addition of rabbit anti-human thyroglobulin to one sample gave a visible precipitate containing 93 per cent of the radioactivity (Table 1). By contrast a non-specific immune complex obtained by successive addition of normal monkey serum and rabbit anti-human γ-globulin to a control sample of lymph failed to carry down any radioactive material. A further portion of this lymph was stained in the 'spot' test by fluorescein conjugates prepared from globulins isolated from the serum of a patient with Hashimoto's disease and containing anti-thyroglobulin antibodies. It was not stained by conjugates prepared from

normal serum or from the serum of a case of Hashimoto's disease which lacked anti-thyroglobulin but which had antibodies to the microsomal antigen and to the second auto-antigen of the colloid (Roitt and Doniach, 1960, 1965). A further 0·07 ml of the diluted lymph was mixed with 0·5 ml of a 1:5 dilution of normal monkey serum containing

TABLE 1

PRECIPITATION OF RADIOACTIVELY LABELLED PROTEIN IN THYROID LYMPH BY ANTI-THYROGLOBULIN

Incubation system				Radioactivity		
Lymph sample (ml)	Normal monkey serum 1:2 (ml)	Saline (ml)	Antiserum	In supernatant (counts/sec)	In precipitate (counts/sec)	(per cent)
0·06	0	0·4	0·1 ml rabbit anti-human thyroglobulin*	1·5	21·3	93
0·06	0·01	0·4	0·1 ml rabbit anti-human γ-globulin	20·7	0·0	0

* Previously absorbed with one-fifth of its volume of normal monkey serum.

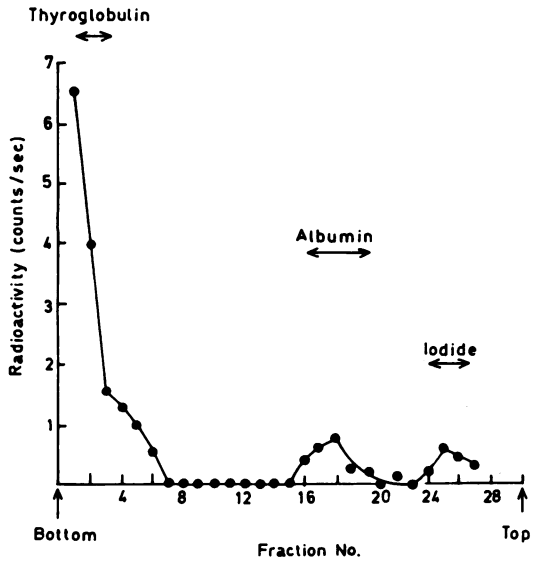

FIG. 3. Zone ultracentrifugation of thyroid lymph through a sucrose density gradient. Sample obtained from a monkey pre-treated with ^{131}I. The position of the albumin was indicated by bromophenol blue which was added before centrifugation. The thyroglobulin marker was a radioactively labelled preparation, run in parallel in a separate tube.

bromophenol blue as an albumin marker and subjected to discontinuous sucrose-gradient centrifugation. The major peak of radioactivity was recovered at the base of the tube in a region corresponding with that occupied by a thyroglobulin marker which had been centrifuged simultaneously in a separate tube (Fig. 3). The small peak of radioactivity in the albumin region probably represented protein-bound iodothyronines while the minor peak at the top of the gradient was presumably inorganic iodide. Further samples of the

diluted lymph were subjected to electrophoresis. In the presence of anti-human thyroglobulin all the radioactivity was held back at the origin, whereas, in the presence of anti-human γ-globulin the main radioactive component was found in the α_1–α_2-globulin zone (Fig. 4).

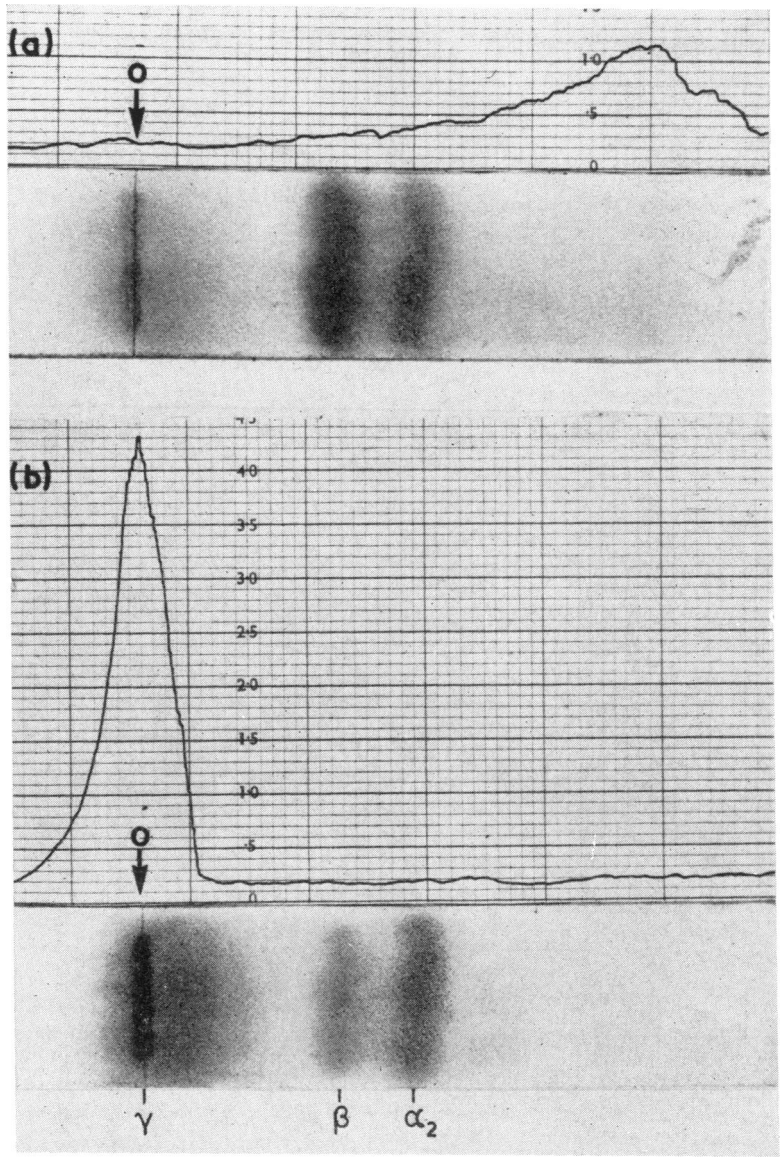

FIG. 4. Effects of antisera upon the distribution of radioactivity in the globulins of thyroid lymph after electrophoresis. The monkey had previously been given ^{131}I. (a) Electrophoresis in the presence of rabbit anti-human γ-globulin. The main radioactive peak has moved ahead of the α_2-globulins. (b) Electrophoresis in the presence of rabbit anti-human thyroglobulin. The radioactive material is retained at the origin. Each radioactive scan is shown above its corresponding stained paper electrophoretic strip. The positions of the α_2-, β- and γ-globulins are indicated. O = Origin.

The iodoprotein radioactivity in 1 mg of the undiluted lymph was equivalent to that in 20 μg of thyroid tissue obtained from the animal at the end of the experiment, suggesting that the thyroglobulin concentration in the lymph was of the order of 2 mg/ml.

CERVICAL LYMPH FROM ANIMALS WHICH HAD BEEN GIVEN RADIOACTIVE IODINE

Cervical lymph, abdominal lymph and peripheral blood were collected from a monkey which had been given ^{125}I 5 days before the experiment. Repeated injections of TSH (10 international units in all, intravenously) were given, starting 2 hours before the first lymph collection. Incubation of this cervical lymph with the rabbit anti-human thyroglobulin did not at first yield a visible precipitate, but addition of human thyroglobulin led to co-precipitation of some radioactivity, giving the results shown in Table 2. It will be seen that a significant count was found only in the precipitate from the cervical lymph.

TABLE 2

PRECIPITATION OF RADIOACTIVELY LABELLED PROTEIN BY RABBIT ANTI-HUMAN THYROGLOBULIN FROM LYMPH AND PLASMA OF A MONKEY GIVEN ^{125}I

Sample	Counts/second in precipitate	Per cent of total counts in precipitate
Cervical lymph (including thyroid lymph)	0·14*	3·0
Non-thyroid lymph	0·02	0·2
Peripheral plasma	0·01	0·1

The standard error of the sample counts was ± 2 per cent.

* Significantly different from background value of 1·44 counts/sec; $P < 0{\cdot}01$

At the end of another experiment in which cervical lymph collections had been made, the thyroid gland was excised and the specific activity of the thyroglobulin computed. On the assumption that the specific activity of the thyroglobulin in the lymph was the same as that in the colloid and taking the fraction of the lymph radioactivity which was not removed by repeated extraction with hot n-butanol as being due to thyroglobulin, the cervical lymph after TSH was calculated to have a thyroglobulin content of 0·8 μg/ml. After light massage over the thyroid gland, the concentration of thyroglobulin in the cervical lymph rose to 4·5 μg/ml.

ANIMALS WHICH HAD NOT BEEN GIVEN RADIOACTIVE IODINE

In order to confirm the validity of the radioimmunoassay method for lymph and blood serum of monkeys, this method was used to estimate the apparent thyroglobulin content of the serum of two monkeys which were then subjected to total surgical thyroidectomy. Further serial examinations were carried out. Pre-operative concentrations of 50 and 70 ng of thyroglobulin per ml of serum were found which dropped to undetectable levels by the 3rd day (Table 3). The reappearance of thyroglobulin in the serum of one animal by the 12th day was unexpected although this fell again to undetectable levels by the 21st day. This monkey was investigated for thyroid remnants by the injection of TSH and ^{131}I: at operation a small amount of thyroid tissue was located sublingually.

The results of a typical experiment are shown in Fig. 5. Successive increases in the thyroglobulin concentration in cervical lymph to the high level of 5000 ng/ml were produced by the injection of TSH and by local massage; as the experiment continued the concentration fell to a value of less than 10 ng/ml. Non-thyroid lymph obtained from the

TABLE 3

EFFECT OF THYROIDECTOMY ON THE CONCENTRATION OF THYROGLOBULIN IN THE BLOOD SERUM

Time of assay relative to operation	Serum thyroglobulin concentration (ng/ml)	
	Monkey 44	Monkey 45
− 30 minutes	50	70
3 days	< 10	< 10
5 days	< 10	< 10
12 days	100	< 10
21 days	< 10	< 10

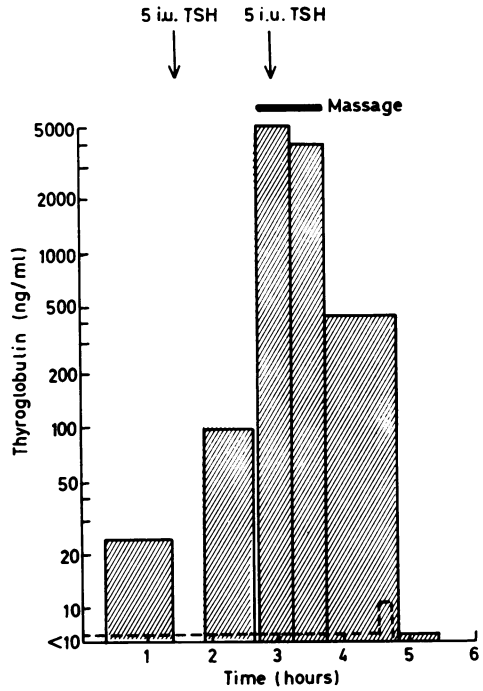

FIG. 5. The effect of TSH and also of local light massage over the thyroid gland on the concentration of thyroglobulin in the cervical (thyroid) lymph (cross-hatched areas) of a monkey, compared with the concentration of thyroglobulin in the peripheral (non-thyroid) lymph. Thoracic duct lymph, – – – –.

thoracic duct gave consistently negative results except for one sample with a positive result at the lowest limit of sensitivity of the test. The serum samples, including that from the thyroid vein, were steady throughout the experiment, giving values between 25 and 30 ng/ml of thyroglobulin.

In Fig. 6 are given the concentrations of thyroglobulin found in samples of peripheral

and thyroid venous blood, peripheral lymph and cervical lymph before and after TSH or local massage. There was no significant difference between the concentrations of thyroglobulin in thyroid venous blood and peripheral blood, the geometric mean values being $56 \times (1{\cdot}21)^{\pm 1}$ and $50 \times (1{\cdot}14)^{\pm 1}$ ng/ml respectively ($\times$ S.E. of mean). Thyroglobulin was not detectable in the peripheral lymph except in one instance where the concentration was the minimum demonstrable by the assay technique. The concentrations of thyroglobulin in the cervical lymph before TSH had been given were comparable with those present in blood. After the administration of TSH the mean concentration of thyroglobulin in the cervical lymph increased from $34 \times (1{\cdot}58)^{\pm 1}$ to $340 \times (1{\cdot}47)^{\pm 1}$ ng/ml. After massage still higher values were found and soon after massage even blood samples showed some increase in thyroglobulin concentration.

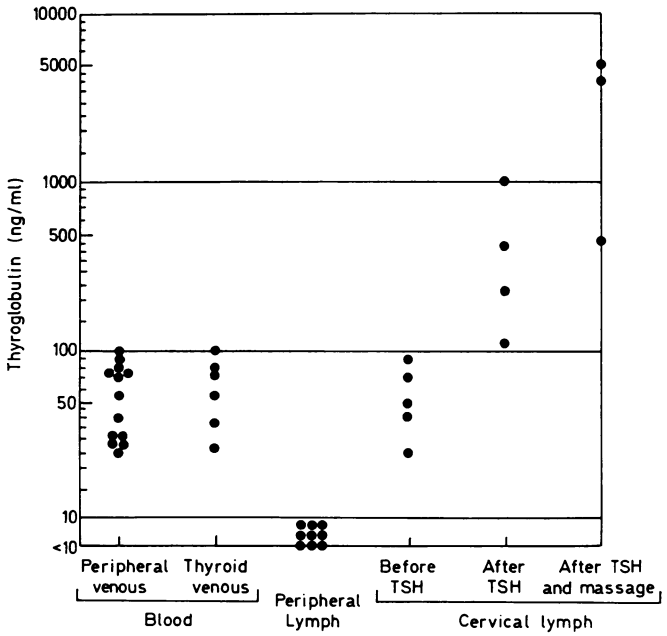

FIG. 6. Concentration of thyroglobulin in peripheral and thyroid venous blood, in non-thyroid (abdominal) lymph and thyroid (cervical) lymph of normal monkeys. The increases in the concentration of thyroglobulin in cervical lymph after TSH and after massage are shown.

A sample of cervical lymph and of peripheral blood serum were each subjected to sucrose-gradient centrifugation and the resulting fractions were tested by the radioimmunoassay method. In each case a positive result was obtained only in the 19S-fraction.

In one experiment the thyroglobulin content of cervical lymph was studied by the red cell agglutination method. This gave the very high value of 26 μg/ml which was increased to 200 μg/ml after the injection of 10 i.u. of TSH. The same samples were studied using the method based on the inhibition of the agglutination of thyroglobulin-coated red cells. These samples gave values of 60 μg/ml and, after TSH, 160 μg/ml respectively. Further work was not done on these agglutination assays because they were found to be subject to interference by non-specific factors in the body fluids, which varied from animal to animal.

CERVICAL LYMPH FLOW

The rate of flow from the right cervical lymph trunk always tended to be larger than that from the left. No appreciable change was detected after TSH but local massage always increased the flow rate, the average rise being about 45 per cent. The mean flow without massage was 0·65 ml/hr from the left and 1·2 ml/hr from the right cervical lymph trunk.

DISCUSSION

All the previous methods which have been used to study the iodoprotein in thyroid lymph are either too non-specific to enable its chemical nature to be established or they suffer from other disadvantages. For instance Dobyns and Hirsch (1956) in a letter, reported the finding of an iodoprotein in the thyroid lymph of dogs which had been given ^{131}I and suggested that it might be thyroglobulin. This work like our own earlier studies (e.g. Daniel *et al.*, 1966a) is open to the criticism that the thyroid gland may have been damaged by the radioactive iodine which had been given to the animal or by the surgical manipulation. A similar criticism may be made of the earlier series of the present experiments in which it was shown by specific immunological precipitation, sedimentation characteristics, electrophoresis and fluorescent antibody staining that the radioactive iodoprotein in lymph, obtained directly from thyroid lymphatic vessels, behaves as thyroglobulin.

The development of a sensitive and specific radioimmunoassay for thyroglobulin has meant that absolutely normal animals can be used and the risk of damage to the gland by radioactivity obviated. In previous work and in the earlier series of the present experiments, thyroid lymph was obtained from vessels draining directly from the thyroid gland, which had to be exposed at operation and therefore might be damaged and consequently leak thyroglobulin from the affected follicles. To avoid surgical trauma in the later experiments when the radioimmunoassay for thyroglobulin was available, lymph was collected from the cervical lymph trunk, at a considerable distance from the thyroid gland which was neither exposed nor in any way touched. In these experiments in which thyroglobulin was detected in cervical lymph from animals which had not been given radioactive iodine and in similar experiments carried out in the rat (Daniel *et al.*, 1966c, 1967b), there was no possibility that thyroglobulin could be released as a result of damage to the gland. The one disadvantage of using cervical lymph was that the thyroid lymph which was present in it was diluted to a considerable extent by lymph from non-thyroid tissues in the head and neck. Nevertheless, thyroglobulin could be easily detected in this cervical lymph by the radioimmunoassay.

The specificity of the radioimmunoassay method was confirmed by observing that the level of thyroglobulin which could be detected in the peripheral blood of monkeys before thyroidectomy, fell to zero 3 days after the operation. The subsequent unexpected reappearance of thyroglobulin in the blood of one of these animals was found to be due to a residual thyroid nodule. The validity of the findings was confirmed in another way by showing that when blood and lymph samples in which appreciable concentrations of thyroglobulin had been found by this method were subjected to ultracentrifugation, the substance which reacted in the radioimmunoassay had the sedimentation characteristics of thyroglobulin. Thus, the material that was being estimated by the radioimmunoassay method in the thyroid lymph was thyroglobulin which had not been appreciably degraded. Parallel experiments carried out with normal human sera showed that the material

detected by the radioimmunoassay behaved as undegraded thyroglobulin when subjected to differential sedimentation, electrophoresis or absorption with insoluble preparations of anti-thyroglobulin antibodies.

The iodoprotein found in the cervical lymph and in the blood was not derived from non-thyroid sources, such as the salivary glands, because iodoproteins from these other tissues differ considerably from thyroglobulin in their chemical and physical characteristics, containing, for example, mainly iodotyrosines. That the iodoprotein is solely derived from the thyroid gland is also shown by the disappearance of reacting material from the blood after thyroidectomy and the known organ-specificity of thyroglobulin.

By means of the radioimmunoassay method the presence of small but appreciable concentrations of thyroglobulin was regularly established both in peripheral and in thyroid venous blood. However, no evidence was obtained that thyroglobulin is released directly into the venous system since the concentration in the thyroid vein was not significantly higher than in peripheral blood.

Peripheral lymph (i.e. lymph obtained from completely non-thyroid sources) did not usually contain detectable levels of thyroglobulin, presumably because such a large protein molecule does not readily leave the vascular system. This suggests that the thyroglobulin in the cervical lymph originates from the thyroid lymph which it contains. An indication that the secretion of thyroglobulin into the thyroid lymphatics is a normal physiological process is provided by the striking increase in the concentration of thyroglobulin in the cervical lymph after TSH has been given. No comparable increase was seen in the peripheral blood, presumably because of the large dilution factor, although the high concentrations of thyroglobulin found in cervical lymph after thyroid massage were in fact reflected in a slight but appreciable rise in the concentration in the peripheral blood. These findings confirm that the thyroglobulin in the blood is derived from the cervical lymph and not the reverse. It may therefore be assumed that the thyroglobulin which is found in the peripheral blood of up to 70–80 per cent of normal human subjects (Hjort and Pedersen, 1962; Assem, 1964; Torrigiani, Roitt and Doniach, 1967) is derived mainly if not exclusively from the thyroid lymph.

It would be interesting to know the total rate of lymph flow from the thyroid gland and the rate at which thyroglobulin escapes, but these values are technically difficult to determine since not all of the thyroid lymph drains into the cervical trunk (a few lymphatics enter veins directly) and there is no readily available method for assessing the rates of flow. Such figures as we have only give a very rough indication of the quantities of thyroglobulin carried in the lymph (and are of necessity an underestimate). However, the mean concentration of thyroglobulin found in cervical lymph after TSH of 0·34 μg/ml together with the combined rate of cervical lymph flow from both sides of nearly 2 ml/hr suggests a daily output of the order of 16 μg of thyroglobulin. It is to be expected that in the normal unanaesthetized animal the constant movements of the head and neck must stimulate the flow of lymph from the thyroid gland. In those experiments in which gentle massage was applied over the thyroid region there was an increase in cervical lymph flow and a marked increase in its content of thyroglobulin probably as a result of an increase in the ratio of thyroid to non-thyroid lymph.

A close relationship between the secretion of thyroxine by the thyroid gland and the release of thyroglobulin into the thyroid lymph is suggested by the increase in both after TSH. Since the colloid stored in the thyroid follicles must be the source both of the thyroglobulin in the lymph and of the secreted thyroxine, the studies of Wollman, Spicer and

Burstone (1964) are of interest. They suggest that the initial event in the breakdown of thyroglobulin involves the formation of small vesicles containing colloid material which arise at the apical margin of the thyroid epithelial cells by a process akin to phagocytosis. At this stage the vesicles stain with periodic acid–Schiff reagent, but acid phosphatase activity is not demonstrable. Later, vesicles are seen which can be stained for acid phosphatase, as well as for colloid, and it is believed that the enzymes are acquired by transfer from lysosome-like dense granules originally present in the supranuclear region. As the vesicles move towards the base of the cell the periodic acid–Schiff reaction becomes progressively weaker and it may be assumed that during this time there is degradation of thyroglobulin with the formation of iodothyronines.

The finding of Roitt, Ballard, Holt, Doniach, Torrigiani and Shapland (1965) that an acid protease may be demonstrated within the follicular cells, rather than in the colloid, adds support to the view that thyroglobulin degradation is an intracytoplasmic process. It seems unlikely that complete degradation of thyroglobulin can occur during the 6 hours or so taken by these vesicles to reach the base of the cell, and if one may assume that the contents of the vesicles are extruded into the extracellular spaces, both iodothyronines and some remaining intact thyroglobulin molecules would appear outside the thyroid follicles. On this basis, one might expect the iodothyronines to be carried away largely by the thyroid venous blood, whose flow is much greater than that of the thyroid lymph, while the large thyroglobulin molecules (molecular weight 650,000) might pass into the lymphatic capillaries (Daniel, Plaskett and Pratt, 1967a). The present investigations are consistent with such a possibility. The increase in the concentration of thyroglobulin in the lymph draining from the thyroid gland which was observed after giving TSH can probably be explained by the stimulating effect of TSH on the phagocytosis of colloids.

It is not at present clear to what extent the thyroglobulin leaving the thyroid gland in the lymph fulfils a hormonal function, but it is of interest that Clutton, Harington and Yuill (1938) showed that injected thyroglobulin could increase the metabolic rate of rats.

It is also uncertain under what circumstances thyroglobulin in the human could become antigenic. The present studies imply that thyroglobulin can no longer be regarded as a protein secluded in the thyroid gland and out of reach of the lymphoid tissue. On the other hand it is not known to what extent the circulating thyroglobulin establishes full immunological tolerance. Certainly, it is extremely difficult to provoke autoantibody formation against homologous thyroglobulin without either the employment of suitable adjuvants (Rose, Kite and Doebbler, 1962) or the chemical alteration of the molecule with haptenic substituents (Weigle, 1965). Theoretically, thyroglobulin could become autoantigenic in a number of ways. There could be modification in the structure of the thyroglobulin molecule, although so far the evidence is against this (Torrigiani, Roitt and Doniach, to be published). A lysosomal abnormality leading to incomplete intracellular degradation of the thyroglobulin molecule could reveal new antigenic sites (Weissmann, 1964). There could be cross-reaction with bacterial or other exogenous protein, as has been demonstrated in the case of the heart (Kaplan and Svec, 1964) and colon (Perlmann, Hammarström, Lagercrantz and Gustafsson, 1965). Ineffective organisms could act as adjuvants (Hackett and Beech, 1960). There could be abnormalities in the immunological recognition processes (Irvine, 1964; Roitt and Doniach, 1965). Whichever of these possibilities is considered, it has to account for the known occurrence of autoantibodies to several organs in the same individual and the high familial incidence of autoimmunity.

Even when autoantibodies have developed, it is necessary to account for the initiation of the inflammatory process in the target organ, as in the production of experimental autoallergic thyroiditis by the immunization of animals with thyroglobulin in Freund's adjuvant. The presence of low concentrations of thyroglobulin in the extracellular spaces in the thyroid gland could account for this by providing a suitable target antigen, either for 'sensitized white cells' or for antibodies, whichever are involved in the initiation of the inflammatory process, for if the thyroglobulin were confined to the lumen of the follicles, it would be difficult to envisage how the immunological effector agents could reach it. Therefore, it is clear that both in man and in experimental animals the presence of thyroglobulin outside the thyroid follicles provides a ready source of antigen, both for provoking the autoimmune response, should appropriate conditions arise, and also for reaction with the products of such an immunological response.

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