PAPERS AND ORIGINALS

Evaluation of a New In-vitro Blood Test for Determining Thyroid Status: The Effective Thyroxine Ratio

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Summary

Estimation of serum thyroxine concentration in test serum was performed in the presence of test serum itself and expressed as a fraction (Effective Thyroxine Ratio, E.T.R.) of serum thyroxine concentration similarly determined in normal serum. From studies of serum obtained from euthyroid, hyperthyroid, and hypothyroid patients and from euthyroid pregnant women and women taking contraceptive (oestrogen) medication, the E.T.R. estimation has been shown to have a diagnostic accuracy (99%) in defining thyroid status comparable to that shown by the free thyroxine index (98%). Because of the ease and rapidity with which the procedure can be completed, estimation of E.T.R. shows promise as a single, accurate measure of thyroid status.

Introduction

Since Ekins (1960) first utilized competitive protein-binding analysis to measure the concentration of thyroxine (T-4) in serum, considerable evidence has shown a precise correlation between serum T-4 concentration and thyroid status provided the concentration of serum thyroxine-binding proteins is normal (Murphy *et al.*, 1966). However, it is well appreciated that alterations in the concentration of serum thyroxine-binding proteins—namely, thyroxine-binding globulin (T.B.G.)—are often associated with parallel deviations of serum T-4 concentration even though thyroid status is normal. Thus the euthyroid woman who is found to have a raised concentration of serum T.B.G. as a consequence of pregnancy or oestrogen medication (most often as a contraceptive) often shows a T-4 concentration that is raised above normal (Murphy *et al.*, 1966; Howorth and MacLagan, 1969). Although other causes of both increased and decreased concentrations of T.B.G. are occasionally encountered

as a cause of an abnormally deviated serum T-4 concentration (Oppenheimer, 1968), the categories of pregnant women and women taking contraceptive medication constitute the main obstacle in utilizing serum T-4 by itself as a reliable measure of thyroid status.

From theoretical considerations and laboratory evidence, estimation of the unbound or free serum T-4 concentration seems to be the most precise index of thyroid status in the presence of a binding protein abnormality. However, currently accepted dialysis and ultrafiltration techniques used to measure directly serum-free T-4 are too cumbersome to have clinical application (Oppenheimer, 1968). Similarly the calculation of a free T-4 index or free T-4 factor from serum T-4 (or protein-bound iodine) and the resin uptake of either T-4 or triiodothyronine (T-3) (Clark and Horn, 1965; Goolden *et al.*, 1967; Howorth and MacLagan, 1969), though correlating well with thyroid status in the presence of a binding protein abnormality, is cumbersome in that two separate procedures involving many steps are required for the calculation.

In recent studies (Mincey *et al.*, 1971, 1972) we have described the basis of and our experience with a new method of measuring serum T-4 concentration. Because the procedure quantifies only that concentration of T-4 that determines thyroid status, and because the quantification is expressed as a ratio of T-4 similarly determined in normal serum rather than absolute units ($\mu\text{g}/100\text{ ml}$), we have chosen to call this measurement the "Effective Thyroxine Ratio (E.T.R.)*". The purpose of the present study is to assess an extended clinical experience with the E.T.R. procedure in terms of generally available laboratory tests of thyroid status—serum T-4 concentration, T-3 uptake, and free T-4 index calculation.

Materials and Methods

Radioactive reagent (Mallinckrodt/Nuclear) was prepared to consist of 0.5 ml of pooled normal serum, 2.5 μCi of ^{125}I -T-4 with specific activities approximating 100 mCi/mg, and sufficient 0.1-M barbitone buffer pH 8.6 to make 100 ml of solution. The radioactive reagent was dispensed into individual phials by using automatic dispensing equipment with sufficient accuracy

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* Effective Thyroxine Ratio (E.T.R.) is a registered trademark of Mallinckrodt Chemical Works, St. Louis, Missouri, U.S.A.

so that initial counting of phials was unnecessary. To bind displaced ¹²⁵I-T-4, fibre strips impregnated with a quaternary ammonia base ion exchange resin (Mallinckrodt ResOMat strips) were used. Reconstituted freeze-dried normal serum (Monitrol I—lot No. LTD-107CD, Dade Division, American Hospital Supply Corp.) was used as normal reference serum.

All of the following was completed at room temperature (see flow sheet shown in Fig. 1). Ethanol extraction of T-4 from both

FIG. 1—Flow chart of the procedure followed to determine the effective thyroxine ratio.

test and reference serum was carried out by the addition of 2 ml of 95% ethanol to test-tubes containing 1 ml of serum followed by vortex mixing and centrifugation at 500 g for five minutes. From each tube 0.3 ml of supernate was transferred to stoppered phials containing 4 ml of radioactive reagent. To each phial 0.005 ml of test serum was added by putting into the phial one entire 0.005-ml Microcap pipette (Drummond Scientific Co., Broomall, Pennsylvania) filled with serum (reproducibility $\pm$ 2%). After insertion of a resin strip into each phial, the phials were placed on a rotary mixer (12-14 r.p.m.) for 60 $\pm$ 5 minutes. At the end of this time the resin strips were removed and discarded. Residual radioactivity in test and reference phials was then determined by counting the phials in a well counter to accumulate a total of 10,000 counts. From these counts the E.T.R. was calculated from the formula:

E.T.R. = $\frac{\text{c.p.m. reference serum phial}}{\text{c.p.m. test serum phial}}$

Duplicate E.T.R. values were determined in serum obtained from a total of 410 adult patients and normal subjects of both sexes and compared with serum T-4 concentrations, T-3 uptakes, and calculated free T-4 indices which were determined in all but a few instances in the same serum specimens. Serum T-4 concentrations were determined with commercially available test components (Mallinckrodt ResOMat T-4 test) in a simplified procedure described in a previous study from this laboratory (Thorson *et al.*, 1970). T-3 uptakes were determined also with commercially available test components (Mallinckrodt ResOMat T-3 uptake test) (Brookeman and Williams, 1971) and expressed as percentage uptake from a nomogram supplied by the manufacturer. Free T-4 indices were calculated

from the products of the serum T-4 concentrations and T-3 uptakes (expressed as fractions rather than percentages).

The 410 patients and normal subjects studied were each evaluated as regards thyroid status by history and examination. Thyroid status was confirmed by serum T-4 concentration, T-3 uptake, serum free T-4 concentration (McIntosh and Thorson, 1970), and thyroidal uptake of ¹³¹I (in some instances following T-3 suppression) as needed. Of the 324 patients classed as being euthyroid, 38 were women taking contraceptive medication containing oestrogen, 52 were women in middle or late pregnancy, and 36 were critically ill from non-thyroidal disease. The remaining 198 euthyroid patients consisted of 26 healthy laboratory personnel and 172 patients who presented as outpatients for assessment of thyroid function or thyroid gland abnormality. These latter two categories were considered collectively as representing "normal" in the present study. All 63 patients classed as being hyperthyroid and all 23 patients classed as being hypothyroid were unequivocally so by clinical and laboratory criteria.

Results

In-run reproducibility of the E.T.R. procedure was found to compare well with other blood criteria of thyroid status as shown by the finding that the mean difference $\pm$ S.D. of duplicate E.T.R. estimations determined in serum from all patients studied was 0.026 $\pm$ 0.020. Day-to-day variation was similarly found to be small, for when the E.T.R. value of reconstituted freeze-dried normal serum was determined daily for 31 days a mean $\pm$ S.D. value of 0.997 $\pm$ 0.024 was found.

Fig. 2 shows the average E.T.R. value determined in serum from each patient studied. The 198 euthyroid (normal) patients

FIG. 2—Effective thyroxine ratios determined in different patient categories.

showed values ranging from 0.87 to 1.12 (95% confidence range 0.89-1.09). All E.T.R. values of euthyroid pregnant women and euthyroid women taking contraceptive medication (birth control pill, B.C.P.) and of all but two euthyroid sick patients were within the actual range determined for the euthyroid normal

Mean $\pm$ S.D. Values of Serum T-4 Concentrations, T-3 Uptakes, Free T-4 Indices, and Effective Thyroxine Ratios (Figures in Parentheses Indicate Numbers of Patients Studied)

Patient Category	Serum T-4 Concentration (μ g/100 ml)	Serum T-3 Uptake (%)	Serum Free T-4 Index Serum T-4 $\times$ Fractional T-3 Uptake	Effective Thyroxine Ratio
Euthyroid { Normal	8.32 $\pm$ 1.90 (191)	30.0 $\pm$ 2.5 (191)	2.50 $\pm$ 0.57 (191)	0.99 $\pm$ 0.05 (198)
Euthyroid { B.C.P.	12.1 $\pm$ 1.7 (38)	22.9 $\pm$ 3.2 (38)	2.80 $\pm$ 0.64 (38)	1.01 $\pm$ 0.05 (38)
Euthyroid { Pregnant	11.1 $\pm$ 2.4 (52)	19.9 $\pm$ 2.2 (52)	2.20 $\pm$ 0.41 (52)	0.99 $\pm$ 0.05 (52)
Euthyroid { Sick	9.0 $\pm$ 3.2 (29)	31.9 $\pm$ 3.3 (27)	2.78 $\pm$ 0.77 (27)	1.01 $\pm$ 0.06 (36)
Hyperthyroid	17.2 $\pm$ 2.53 (56)	37.5 $\pm$ 4.2 (59)	6.68 $\pm$ 1.48 (58)	1.31 $\pm$ 0.11 (63)
Hypothyroid	1.9 $\pm$ 1.7 (23)	24.8 $\pm$ 2.2 (22)	0.48 $\pm$ 0.44 (23)	0.79 $\pm$ 0.04 (23)

group. In contrast, all but one each of 63 hyperthyroid and 23 hypothyroid patients showed values that were respectively above and below the actual range of normal.

The free T-4 indices calculated from serum T-4 concentrations and T-3 uptakes determined in the same serum specimens are shown in Fig. 3. As with the E.T.R. values shown in Fig. 2,

FIG. 3—Free thyroxine indices (serum T-4 × fractional T-3 uptake) determined in different patient categories.

the free T-4 indices of all pregnant women and all but two each of the sick patients and women taking contraceptive medication were within the actual range determined for the normal group. In contrast, only one hyperthyroid and two hypothyroid patients showed free T-4 indices that were within the actual range of normal.

Although Fig. 4 shows that only one each of 56 hyperthyroid and 23 hypothyroid patients and all but 4 of 29 sick patients

FIG. 4—Serum thyroxine concentrations determined in different patient categories.

showed serum T-4 concentrations that were within the 95% confidence limits of the normal group (4.5-12.1 μg/100 ml), 19 of the 38 women taking contraceptive medication (50%) and 20 of the 52 pregnant women (38%) showed T-4 concentrations that were above the 95% confidence range of normal.

Fig. 5 shows the T-3 uptakes determined in the same study group. Fourteen of 59 hyperthyroid patients (23%) and 10 of 22

FIG. 5—Serum T-3 uptakes determined in different patient categories.

hypothyroid patients (45%) showed T-3 uptakes that overlapped the 95% confidence range of normal (25-35%). Only 2 of the 52 pregnant women (4%) and 7 of the 38 women taking contraceptive medication (18%) showed T-3 uptakes that were within the normal range. Five of 27 sick patients (19%) showed T-3 uptakes that were raised above normal.

The means of the above values are given in the Table.

Discussion

As previously reported when using the same method of estimating serum T-4 (Thorson *et al.*, 1970), the T-4 concentrations

shown in Fig. 4 correlate well with thyroid status in the absence of a binding protein abnormality. The significant overlap of T-4 concentrations into the hyperthyroid range found in pregnant women and women taking contraceptive medication (43% of all values in these categories were raised above the 95% limits of normal) illustrate well the deficiency of the estimation of serum T-4 as a single screen of thyroid status in these circumstances.

Although others (Mitchell *et al.*, 1960) have appreciated that the T-3 uptake test poorly discriminates hypothyroid from normal patients (45% of the hypothyroid patients in this study showed normal T-3 uptakes), the poor discrimination of hyperthyroid from normal patients apparent in Fig. 5 (23% of the hyperthyroid patients studied showed normal T-3 uptakes) implies that the present method of estimating T-3 uptake is uniquely inaccurate compared with results obtained with resin sponges and charcoal (Mitchell *et al.*, 1960; Braverman *et al.*, 1967). Our own experience with resin sponge and charcoal procedures, however, indicates that a similar order of overlap of hyperthyroid and euthyroid T-3 uptakes obtains with all three procedures. Although the discriminatory function of the T-3 uptake test in our hands has been found to be grossly inadequate as a single screen of thyroid status, Fig. 5 does show that confirmation of a raised T-4 concentration being related to the "hyperTBGaemia" of oestrogens and pregnancy can be obtained by measuring a T-3 uptake (89% of all patients in these categories showed T-3 uptakes below the normal range). Of interest was the finding that 21% of the euthyroid sick patients showed T-3 uptakes that were raised above normal, which observation has been noted previously (Mitchell *et al.*, 1960), and probably relates in part at least to decreased binding of thyroxine-binding prealbumin (T.B.P.A.).

It is accepted that the concentration of free T-4 in serum is involved in an equilibrium reaction involving thyroxine-binding proteins (T.B.G., T.B.P.A., and albumin) according to the mass action equation

$$I. [T-4] [T.B.P.] = K [T-4.T.B.P.]$$

where T.B.P. represents the total concentration of T.B.G., T.B.P.A., and albumin and T-4.T.B.P. represents the concentration of T-4 bound to these proteins (Robbins and Rall, 1960). According to this concept, on rearranging equation I to

$$II. [T-4] = \frac{K [T-4.T.B.P.]}{[T.B.P.]}$$

it follows that the ratio of T-4.T.B.P. to T.B.P. is constant within a certain range in all euthyroid subjects regardless of the binding protein concentration. Thus in euthyroid pregnant serum the increased concentration of T-4.T.B.P. (as measured by estimation of serum T-4) will be attended by a proportionately increased concentration of T.B.P., but the concentration of free T-4 will be normal. Conversely in euthyroid T.B.G.-deficient serum a decreased concentration of T-4.T.B.P. will be attended by a proportionately decreased concentration of T.B.P., but again the concentration of free T-4 will be normal.

These concepts can be applied to describe the basis of the E.T.R. estimation described, for when a certain volume of test serum containing T.B.P. is added with a concentration of extracted T-4 (proportionate to T-4.T.B.P.) from the same test serum to the E.T.R. competitive protein-binding analysis system, the concentration of T-4 available for competitive binding is determined by the ratio of the concentration of T-4.T.B.P. to the concentration of T.B.P. as in equation II. It follows that the volume of serum required to effect a constant concentration of T-4 available for competitive binding will be the same in all euthyroid subjects irrespective of serum binding protein concentrations.

Fig. 6 shows the results of a preliminary experiment in which serum T-4 concentrations (expressed as precount/postcount ratios instead of μg/100 ml) were determined in five sera when increasing quantities of serum (T.B.P.) from each serum specimen were added to the present competitive protein-binding analysis

system containing also T-4 extracted from the same serum. For example, in the case of pregnancy serum, microlitre quantities of serum containing T.B.P. were added to phials containing a constant concentration of T-4 (contained in 0.3 ml of ethanol extract) previously extracted from the same pool of pregnancy serum. The sera studied included pooled serum from normal subjects, pregnant women, hyperthyroid and hypothyroid patients, and serum from one euthyroid patient showing significant deficiency in T.B.G. When no serum and only extracted T-4 was added to the test system, serum T-4 concentrations of both hyperthyroid and pregnancy serum were significantly raised above the value determined for normal serum, and T-4 concentrations of hypothyroid and T.B.G.-deficient serum were significantly decreased.

FIG. 6—Serum T-4 concentrations (expressed as ratio of initial to final c.p.m.) determined in normal, hyperthyroid, hypothyroid, pregnancy, and T.B.G.-deficient sera when different volumes of test serum are added with 0.3 ml of ethanol extract of the same serum to the competitive protein-binding analysis system.

With the addition of increasing quantities of serum to 0.1 ml, T-4 concentrations of hyperthyroid and hypothyroid sera remained respectively increased and decreased relative to that of normal serum, but the T-4 concentration of pregnancy serum became less than, and the T-4 concentration of T.B.G.-deficient serum greater than, that determined for normal serum. The basis for the present E.T.R. test system can be understood from the T-4 concentration determined in each serum pool when approximately 0.005 ml of serum was added, for with this quantity of added serum the serum T-4 concentrations of normal, pregnancy, and T.B.G.-deficient serum are seen to approximate each other while T-4 concentrations of hyperthyroid and hypothyroid serum are respectively increased and decreased.

It is apparent from Figs. 2 and 3 that the E.T.R. procedure and free T-4 index determination obviate the increased T-4 concentrations relating to contraceptive medication and pregnancy and discriminate hyperthyroid and hypothyroid from normal patients equally well. The overall diagnosis compatibility of the E.T.R. procedure (99%) compares precisely with that of the free T-4 index (98%). As might be anticipated, a linear relationship exists between E.T.R. values and corresponding free T-4 indices determined in different patient categories. This is shown in Fig. 7 in which randomly selected E.T.R. values of euthyroid, hypothyroid, and hyperthyroid patients have been plotted against corresponding free T-4 indices. A straight-line relationship ($r = 0.98$) is apparent which is in keeping with our previous finding of a precise relationship between E.T.R. values and serum free T-4 concentrations (Mincey *et al.*, 1972).

In addition to the conditions of increased T.B.G. concentration (pregnancy and oestrogen medication) and decreased T.B.P.A. concentration (sick patients), euthyroid patients deficient in T.B.G. have been shown to have normal E.T.R. values (Mincey *et al.*, 1972). Very few data have been gathered to determine whether or not other situations associated with

binding protein abnormalities—for example, patients receiving diphenylhydantoin—cause significant deviation of the E.T.R. from normal. It seems safe to predict from the evidence gathered thus far, however, that any abnormality of binding protein concentration will not adversely affect the diagnostic accuracy of the E.T.R. Limited studies of hypothyroid patients rendered euthyroid with T-4 replacement indicate that the E.T.R. is

FIG. 7—Correlation of effective thyroxine ratios and free thyroxine indices determined in the same serum specimens from normal, hyperthyroid, and hypothyroid patients. Broken lines indicate the 95% confidence limits of the two procedures.

within normal limits in this patient category although many values are in the upper range of normal. E.T.R. values of three hypothyroid patients rendered euthyroid with T-3 replacement showed values that were below the normal range. This finding implies that the E.T.R. will be insensitive to selective changes of serum T-3 concentration such as occur in T-3-toxicosis (Hollander, 1968).

The accuracy of the E.T.R. estimation as an index of thyroid status compares favourably with that shown by the free T-4 index, as would be predicted from our previous demonstration of a precise correlation between E.T.R. values and serum free T-4 concentrations in different patient categories. In comparison with the index and free T-4 determinations, however, the E.T.R. procedure is notable for both the ease and rapidity with which the procedure can be completed. Indeed, using automatic counting equipment, one technician in our laboratory can complete 100 E.T.R. determinations during the course of one day. Although further studies are required to determine whether or not the E.T.R. procedure differentiates borderline cases of abnormal thyroid status more precisely than currently available criteria, the findings of the present study indicate that the E.T.R. estimation shows promise as an accurate, single measure of thyroid status.

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Distinction between Infection and Rejection in Lung Transplantation

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Summary

In distinguishing between infection and rejection after human lung transplantation clinical and radiological features were unhelpful, and even confusing. However, incipient rejection could be predicted and distinguished from infection by monitoring alterations in lymphocyte activity by the rosette inhibition test. Earlier prediction seems possible by detecting circulating lung-binding antibody. The ability to detect changes in the immunological status of a patient, even before clinical deterioration, has fundamental implications for the management of patients after transplantation.

Introduction

Human lung transplantation has yet to achieve a status comparable with that of certain other organs. All except one of the 27 lung allografts reported in man have failed within one month of surgery, usually resulting in the death of the patient (Organ Transplant Registry, 1971). Among the major obstacles to success are the particular problems of transplantation, especially the risk of infection and the absence of a simple test or tests to predict rejection. The importance of distinguishing between the two is increased in lung transplantation as the clinical features of each are similar, yet their treatment is in so many ways opposed (Wildevuur and Benfield, 1970). Destruction of lung parenchyma during rejection will promote infection in an organ particularly exposed and vulnerable to it, yet increased immunosuppression to treat rejection will inhibit the natural mechanisms of defence against bacterial, fungal, or viral invaders. A minimal but nevertheless effective immunosuppressive regimen should be the object of therapy.

We detail here the relevant clinical, radiological, and immunological findings which we found helpful in preventing and dis-

tinguishing between infection and rejection in a patient with fibrosing alveolitis who received a lung transplant. Although some of this information has been reported (Hugh-Jones *et al.*, 1971) the additional considerations given here have important implications, not only for lung transplantation but for transplantation generally.

Methods

Diagnosis of Incipient Rejection.—The clinical features, oxygen requirements, and daily chest x-ray films were compared with changes in the patient's immunological status. The rosette inhibition test was used to assess alterations in peripheral blood lymphocyte activity as an indication of adequate immunosuppression (Munro *et al.*, 1971). In addition, an increase in the number of rosettes per thousand lymphocytes was noted. The presence of circulating lung-binding antibody was measured retrospectively by immunofluorescent techniques. Frozen sections of normal lung were incubated in the patient's serum and treated with fluorescein-labelled antihuman gammaglobulin.

Prevention of Infection.—A sterile unit for postoperative care (Barlow Leslie & Partners, Croydon CR9 1PB) was used, which had air counts comparing favourably with those suggested for operating theatres (Williams *et al.*, 1966), and which allowed daily electrocardiographic and radiological examinations without entry across the sterile barrier. All supplementary gas was administered through a 0.3-micron filter (Foramoflow) without humidification through an MC face-mask. Later the same face-mask, delivering at least 20 litres of air per minute, was used by the patient as a protection against aerial contamination during transit within the hospital to other departments or outdoors. Monitoring of the patient for bacterial, fungal, and viral pathogens was performed on most days, and bacterial organisms were continually studied for changes in their antibiotic sensitivity. In addition, staff carriers of multiresistant staphylococci were excluded from the sterile area.

Anatomical Pathology.—Shortly after death tissue was taken by needle puncture from the donor lung for electron microscopy. Further material for electron microscopical study was sampled at the routine necropsy, which was performed 20 hours after death. Both donor and residual lungs were removed en bloc and inflated with formalin for subsequent examination.

Details of clinical progress have already been presented (Hugh-Jones *et al.*, 1971). The relevant features are compared with the immunological changes that occurred (Fig. 1).

Infection and Rejection

PATIENT SEPSIS

Pulmonary sepsis was clinically evident between the ninth and fourteenth postoperative days. At first the patient was ill, his

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