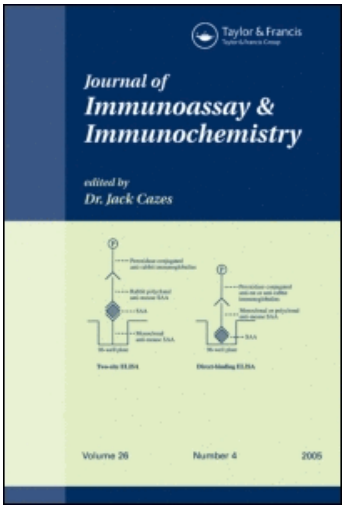

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VALIDATION OF CYTOCHEMICAL SECTION BIOASSAY FOR THYROID-STIMULATING IMMUNOGLOBULINS IN TREATED AND UNTREATED GRAVES' DISEASE

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ABSTRACT

The cytochemical section-bioassay of thyroid stimulating activity is described. Plasma of thyrotoxic patients as well as those on block-replace treatment with carbimazole and thyroid hormones was used. Linear parallel responses were obtained over the range 1.5×10^{-7} to 1.5×10^{-5} mU/l MRC LATS-B standard. The index of precision was 0.19 $\pm$ 0.028. The fiducial limits (p=0.95) of a sample tested on ten separate occasions were 52-192%. Specificity was investigated using time course studies, and the effect of anti-TSH or anti-IgG antisera. No effect of methimazole or a variety of other drugs was detected. The assay is accordingly validated for measurement of TSI in patients both untreated and on block-replace therapy.

INTRODUCTION

It is now widely accepted that the overstimulation of the thyroid gland in Graves' disease is due to the presence of circulating immunoglobulins which act as abnormal thyroid stimulators, but are not under feed-back control by the thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4). Carbimazole is one of the most commonly used drugs in the medical management of thyrotoxicosis. Originally this drug was thought to act solely through its action in blocking the synthesis of thyroid

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hormones. In a previous study (1) patients were treated by "block-replace" regimen of carbimazole 10 mg q.d.s. and T_3 20µg q.d.s. Technetium-99 (^{99m}TC) trapping by the thyroid which is unaffected by this drug consistently fell, implying that stimulation of the thyroid gland was reduced. Accordingly, the authors suggested that carbimazole might also act by depressing the levels of thyroid stimulating immunoglobulins (TSI). Although several methods for studying the TSI levels after such therapy have been proposed, the conventional bioassays (2,3) are not particularly sensitive, and binding assays (4,5) are not necessarily measuring thyroid stimulating activity. Because it was considered helpful to have a sensitive bioassay with which to monitor changes in TSI levels, it seemed appropriate to investigate whether the cytochemical bioassay (6) could be adapted to meet these requirements. So far this assay has been used to report the titre of TSI, that is the highest dilution of the sample capable of causing a response (7). It was therefore necessary to evaluate whether the cytochemical section assay would be sufficiently accurate, precise and specific for this new application to a clinical study, and whether the active metabolite of carbimazole, methimazole, or other drugs likely to be used by such patients, would interfere with the assay of TSI.

MATERIALS AND METHODS

The long acting thyroid stimulator MRC Research Standard B (LATS-B; 65/122) diluted in normal plasma was used as the

standard. Plasma samples were taken from patients with thyrotoxic Graves' disease before treatment and at intervals after commencing block-replace treatment with carbimazole 10 mg and T_3 20μg six hourly. Brief clinical details of these patients are given in Table 1. One sample of plasma, taken from patient GN before treatment, was used to assess the assay in detail. This sample was divided into about 50 separate aliquots and was designated the quality control sample (QC). The plasma was separated immediately after venesection and was snap-frozen in small aliquots to 70°C. The aliquots were stored at this temperature for at least three months before they were used in the assay.

In some experiments, the effect of either anti-human TSH antiserum or anti-human IgG antiserum was tested. The rabbit anti-human TSH antiserum (kindly supplied by Dr. B. Richards, University College Hospital, London) binds 20% of the labelled hTSH at a dilution of 1:175,000; for these studies it was used at a dilution of 1:100,000. The rabbit anti-human IgG antiserum (Dako Immunoglobulins, Copenhagen: 1 ml binds 1200 mg human IgG) was used at a dilution of 1:100.

To test the effect of drugs likely to be present in the plasma of the study group, the following compounds were added to the test plasma: methimazole, 10^{-5}nmol/l; prednisolone, 500 nmol/l; ethinyloestradiol, 500 pg/ml; levonorgestrel, 5ng/ml; norethisterone, 5ng/ml. Except for methimazole, the compounds were dissolved in absolute ethanol and added to the test plasma to give a concentration of alcohol of 0.001%. The samples were

TABLE 1

Clinical Details of Patients whose Plasma was used for Validation Studies

Patient	Age	Sex	Contra-ceptive Pill	Ophthal-mopathy	Thyroid microsomal antibody titre	Duration of Treatment Studied (weeks)	Treatment			Anti-TSH Studies	Anti-Ig Studies	Parallelism Studies
							CBZ	T_3	T_4			
PM	38	F	0	+	$1/20^2$	42	+	+	0	+	+	+
GD	33	M	–	+	$1/80^2$	55	+	*	+	+	+	+
JR	21	F	0	0	$1/80^2$	16	+	+	0	+	+	+
GN	35	F	+	+	$1/20^2$	21	+	+	0	+	+	+
JD	38	M	–	+	$1/80^2$	38	+	+	0	0	0	+
DC	22	F	+	0	$1/160^2$	18	+	+	0	0	0	+
GL	24	F	+	+	$1/80^2$	14	+	+	0	0	0	+

CBZ = Carbimazole 10 mg qds. T_3 = Triiodothyronine 20 µg qds. T_4 = L-thyroxine 2 mg om.
*Patient GD received T_3 from week 11 to week 17 and T_4 thereafter.

assayed at a dilution of $1:10^5$. As a control the effect of 0.001% alcohol in the carrier medium was tested.

The section assay was performed as previously described (6). In outline, the thyroid was removed from a female guinea-pig, each lobe was bisected and each half was maintained in organ maintenance culture for 5 hours. The half lobes were chilled to $-70^{o}C$ and sectioned in a cryostat at 12 μm. Duplicate sections were exposed for 180 seconds either to graded concentrations of the MRC LATS-B standard (1.5×10^{-5} - 1.5×10^{-8} mU/l) or to dilutions of the test plasmas (usually diluted $1:10^4$ or $1:10^5$) in a medium consisting of Trowell's T8 culture medium (Gibco Europe) containing 0.02% gum tragacanth and 0.05M sodium acetate. After this time, the sections were transferred to a bath containing the chromogenic reaction medium for demonstrating lysosomal naphthylamidase activity (6). The coloured reaction product in individual thyroid follicle cells was measured by means of a Vickers M85 scanning and integrating microdensitometer at 550 nm with an X100 oil immersion objective. By suitable calibration the measurements were converted into units of mean integrated extinction (MIE x 100).

RESULTS

Reproducibility of measurement of enzyme activity

The mean percentage difference in enzyme activity measured in duplicate sections that had been exposed to the carrier medium alone was 2.3% ± 1.0 (mean ± SEM; n=7). Similar results were found in sections exposed either to different concentra-

tions of the MRC LATS-B standard or to two dilutions of the test plasma. Thus in fourteen assays, the percentage difference between duplicate sections exposed to the standard ranged from 1.2 to 2.2%, while the mean difference in ten assays between sections exposed to the dilutions of the quality control plasma was 1.1% ± 0.19 (range 0-2%).

Index of precision, slope and fiducial limits.

There was a consistent linear response in enzyme activity to increasing concentrations of the MRC LATS-B standard over the range 1.5×10^{-7} to 1.5×10^{-5} mU/l. The correlation coefficient was 0.9? ± 0.004 (mean ± SEM; n=12) with a slope of 3.7 ± 0.3; the index of precision was 0.19 ± 0.028 (n=8). In all experiments exposure of sections to 1.5×10^{-4} mU/l of the MRC LATS-B standard caused a decrease in enzyme activity compared with those treated with 1.5×10^{-5} mU/l. In only two experiments was there a significant difference between the activity in sections treated with the carrier medium and those exposed to 1.5×10^{-8} mU/l of the standard. Thus the working range of the assay was 1.5×10^{-5} to 1.5×10^{-7} mU/l.

The response to two dilutions (10^{-4} and 10^{-5}) of the quality control plasma was measured in ten assays. The mean percentage variation in the potency estimate of each of these concentrations was 20.5%; there was no significant deviation from parallelism (8) between the response to these concentrations of this plasma and that to the standard. (Fig. 1). The calculation of the fiducial limits of the estimation of potency

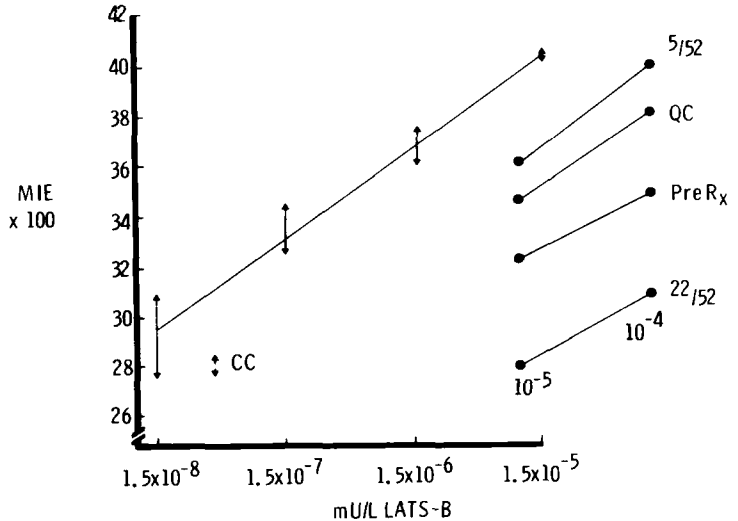

FIGURE 1

A typical dose-response graph to logarithmic concentrations of the standard preparation of MRC LATS-B: triangles show the activity (MIE x 100) measured in each of duplicate sections. The mean responses in each of duplicate sections to two dilutions (10^{-4} and 10^{-5}) of three different plasma samples (filled circles) were not significantly non-parallel to the response to the standard QC: quality control, test plasma from an untreated thyrotoxic patient; Pre R_X 5/52 and 22/52: samples from a patient before treatment, after five weeks of carbimazole treatment only, and after a further 17 weeks of block-replace therapy with carbimazole and T_3 respectively. CC: carrier medium control. Sample 22/52 was assayed again at lower dilutions (not shown) and gave a parallel response.

was made on the results of ten assays, each of a different aliquot of the same quality control plasma (9). The mean estimate of potency of this plasma, assayed at dilutions of 10^{-4} and 10^{-5} was 55.6 mU/l LATS-B equivalents. The fiducial limits ($p = 0.95$) were 52 - 192%. The coefficient of variation between assays calculated on the same results was 29%.

Specificity

The specificity of the response of sections of thyroid exposed to plasma was tested in two ways. It is known (6) that the same response can be elicited by TSH, although at appropriate concentrations of the stimulator, the response to TSH occurs earlier than that evoked by TSI (that to TSH being maximal at 90 sec whereas the peak stimulation by TSI occurs at 180 sec). In agreement with these published reports, there was no significant change in enzyme activity in sections exposed to 1.5×10^{-5} mU/l LATS-B standard for 90 sec, and the peak activity was seen at 180 sec. Normal plasma which had been stored for three months and which was used to dilute the standard preparation, was tested at a dilution of 10^{-5}. This evoked no response at any of the times tested (from 30-210 sec).

When excess of anti-human IgG antiserum was added to the LATS-B standard (1.5×10^{-5} mU/l), the response fell to that of sections exposed to 1.5×10^{-8} mU/l of the standard. There was no change in the activity of sections exposed to this antibody added to the carrier medium. Additions of the anti-human IgG

antiserum to the quality control plasma reduced the potency estimate from 46.2 mU/l to 0.05 mU/l. (See Fig. 2).

In contrast, excess of the anti-human TSH antibody added either to the LATS-B standard or to the test plasma caused no change in the activity produced. The antibody added to the control carrier medium produced no effect (See Fig. 3). This neutralisation by anti-human IgG and not by anti-human TSH antiserum was consistent in plasma for thyrotoxic patients on carbimazole alone as well as for euthyroid patients on the block-replace regime (See Figs. 2 and 3).

Effect of drugs on the assay of TSI in plasma

When added at the maximum circulating concentration (10), methimazole, the active metabolite of carbimazole, did not affect the apparent potency of the quality control plasma. The addition of prednisolone, ethinyloestradiol, or the progestagens norethisterone or levonorgestrel, also at the maximum circulating levels (11,12,13,14) did not affect the potency of this plasma. Thus the potency estimations of the TSI in this plasma whether assayed alone or in the presence of these compounds were within the fiducial limits of the assay. There was no significant difference in the enzyme activity of sections exposed to the carrier medium containing alcohol at the concentration that was used in the test plasma and the sections exposed to the medium alone.

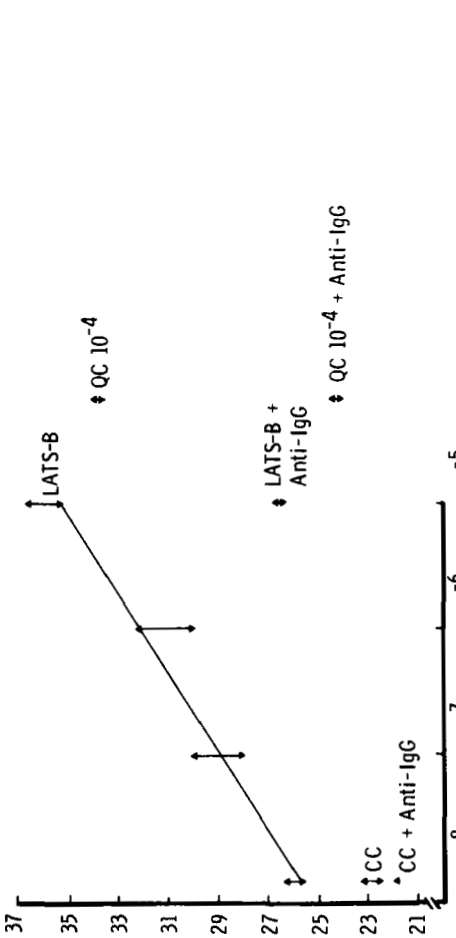

FIGURE 2

The addition of anti-human IGg antiserum to the highest concentration of the standard preparation of MRC LATS-B, or to a dilution of the quality control plasma (QC 10^{-4}) caused a reduction in the activity of the sections (duplicate sections shown as triangles) whereas there was no significant change when the antiserum was added to the carrier medium (CC).

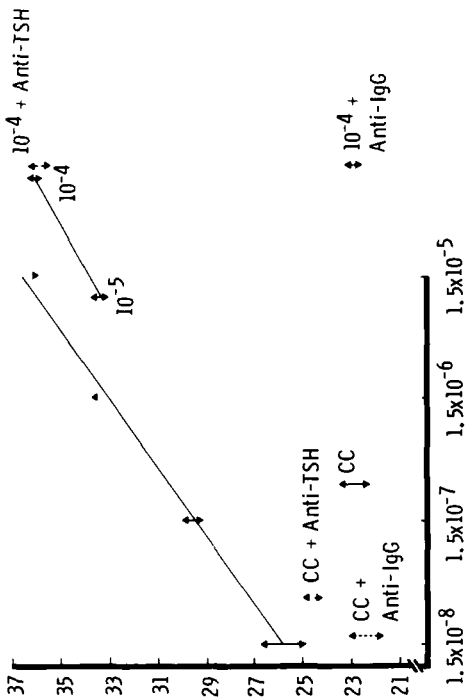

FIGURE 3

In contrast to the inhibition caused by the addition of anti-human IGg antiserum to the highest concentration of plasma (patient PM after 5 weeks of treatment), the addition of anti-human TSH antiserum caused no change (triangles show activity in individual sections). Neither antiserum added to the carrier medium (CC) caused any significant change.

DISCUSSION

The purpose of this study was to determine whether the cytochemical section-bioassay for thyroid stimulators would be sufficiently accurate and specific to be used in clinical studies on the circulating levels of TSI. The results show that, using the MRC LATS-B as the standard, the inter-assay reproducibility and the fiducial limits of the assay are of the same order as those of other cytochemical bioassays (8). The limit of consistent sensitivity was 1.5×10^{-7} mU/l, there being no significant difference between the activity of sections exposed to 1.5×10^{-8} mU/l and to the carrier medium alone. Although Ealey and Smyth (15) reported that the sensitivity of their section assay was 1.5×10^{-8} mU/l, the higher limit of sensitivity identified in the present study was not considered a limitation to its use since it was necessary to dilute plasma from untreated patients to $1:10^4$ and $1:10^5$ to obtain responses parallel to those of the LATS-B standard. Even if therapy were to reduce the circulating level of TSI to one-hundredth of the initial potency, this could still be assayed by using dilutions of the plasma of 1:10 and 1:100.

It is now widely accepted that the abnormal thyroid stimulator in Graves' disease is an IgG. The decision to use plasma and not a relatively crude or purified IgG fraction for these studies was taken for a number of reasons: firstly because Ealey (16) found that the assay of TSH in serum frequently but not invariably gave results that were different from those using

plasma, and Ealey and Smyth (15) reported similar findings in the assay of TSI. Secondly, the LATS-B standard is itself not an IgG preparation. Thirdly, other materials that might be present in the plasma and which might influence the potency of TSI could not be ignored.

A possible and largely theoretical disadvantage in using plasma was the presence of TSH. This was considered unlikely to be a realistic disadvantage for two reasons. In thyrotoxic Graves' disease, the secretion of TSH is suppressed; while on block-replace therapy, this suppression is maintained by treatment with exogenous thyroid hormone. The levels of TSH in active Graves' disease that have been assayed by the cytochemical assay (8) have been of the order of 0.1 to 0.3 mU/l, so that these levels would only be detectable at plasma dilutions of 1:100 or less. Secondly, it has been shown (16) that TSH in plasma loses activity at the rate of 1% per day, even if the plasma is stored at $-70^{\circ}C$. The plasma samples used in this study, both for the test plasmas and that used as a vehicle for the standard, were deliberately aged by storage for three months before being used in the assay. Under these conditions it is likely that only about 10% of any original TSH-activity would still be present. This is borne out by the fact that no TSH-like response was produced by the aged normal plasma diluted $1:10^3$, acting for 90 sec., the time at which concentrations of TSH, which are not supramaximal, produce a change in enzyme activity (6). Furthermore, anti-TSH antiserum

added to a test plasma (eg. patient PM, after 5 weeks of treatment; Fig 3) did not alter the apparent potency of the plasma.

On the other hand, the addition of anti-human IgG antiserum, which in these studies almost abolished the activity of the LATS-B standard, reduced the potency of the test plasmas to about one-hundredth of the original estimate. Thus it is almost certain that the thyroid stimulating activity measured in these studies is indeed due to TSI.

Graves' disease is much more common in young women of child-bearing age, the bias towards the female being about six to one (17). Thus it is likely that many of the patients being treated by the block-replace regimen will also be taking some form of contraceptive therapy. For this reason, the possible interference by an oestrogen and two commonly-used contraceptive progestagens as well as the active metabolite of carbimazole were examined. Possible interference of prednisolone was also evaluated, because this steroid is often used for the treatment of progressive ophthalmopathy. None of these compounds added to the quality control plasma at the maximum concentrations likely to be achieved in plasma under therapeutic conditions, affected the apparent potency of the TSI.

This study indicates that the cytochemical section-bioassay for thyroid stimulators should be sufficiently precise and specific for investigating the circulating levels of TSI before and during block-replace therapy with carbimazole and thyroid hormones in the doses given.

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REFERENCES

1. Wise, P.H., Marion, M. and Pain, R. Mode of Action of Carbimazole in Graves' Disease. Clin. Endocrinol 1979; **10**:655-64.

2. Onaya, T., Kotani, M., Yamada, T. and Ochi, Y. New in vitro Tests to Detect the Thyroid Stimulator in Sera from Hyperthyroid Patients by measuring Colloid Droplet Formation and Cyclic AMP in Human Thyroid. J. Clin. Endocrinol. Metab. 1973; **36**:859-66.

3. McKenzie, J.M. and Zakarija, M. A Reconsideration of a Thyroid-Stimulating Immunoglobulin as the cause of Hyperthyroidism in Graves' Disease. J. Clin. Endocrinol. Metab. 1976; **42**:778-81.

4. Smith, B.R. and Hall, R. Thyroid-Stimulating Immunoglobulins in Graves' Disease. Lancet 1974; **11**:427-30.

5. Hardisty, C.A., Hanford, C., Humphries, H. and Munro, D.S. Long Acting Thyroid Stimulator and Long Acting Thyroid Stimulator Protector in untreated Thyrotoxicosis. Clin. Endocrinol. 1981; **14**:631-39.

6. Chagen, J., Gilbert, D.M., Robertson, W.R., Bitensky, L. and Besser, G.M. A Cytochemical Section - Bioassay for Thyrotrophin. J. Immunoassay 1980; **1(1)**:1-13.

7. Smyth, P.P.A., Neylan, D. and O'Donovan, D.K. Prevalence of Thyroid-Stimulating Antibodies in Goitrous Disease Assessed by Cytochemical Section Bioassay. J. Clin. Endocrinol. Metab. 1982; **54**:357-61.

8. Chayen, J. The Cytochemical Bioassay of Polypeptide Hormones. Monographs on Endocrinology Vol. 17. Heidelberg: Springer-Verlag, 1980.

9. Statistical Analysis of Results of Biological Assays and Tests. In: European Pharmacopoeia 1977; **2**:441-98.

10. Skellern, G.G., Stenlake, J.B., Williams, W.D. and McLarty, D.G. Plasma concentrations of Methimazole, a Metabolite of Carbimazole in Hyperthyroid Patients. Br. J. Pharmac. 1974; **1**:265-69.

11. Tanner, A., Bochner, F., Caffin J., Halliday, J. and Powell, L. Dose-dependant Prednisolone Kinetics. Clin. Pharmacol. Ther. 1979; **25(5)**:571-78.

12. Hunter, D.J.S., Julier, D., Franklin, M., Green, E. Plasma levels of Estrogen, Luteinizing Hormone, and Follicle Stimulating Hormone Following Castration and Estradiol Implant. Obst. and Gynaecol. 1977; **49(2)**:180-5.

13. Saxena, B.N., Shrimanker, K. and Grudzinskas, J.G. Levels of Contraceptive Steroids in Breast Milk and Plasma of Lactating Women. Contraception 1977; **16(6)**:605-13.

14. Back, D.J., Bates, M., Breckenridge, A.M. et al. The Pharmacokinetics of Levonorgestrel and Ethinylestradiol in Women - Studies with Ovran and Ovranette. Contraception 1981; **23(3)**:229-40.

15. Ealey, P.A. and Smyth, P.P.A. Validation of the Cytochemical Section Bioassay for Thyroid Stimulating Antibodies. J. Immunoassay 1980; **1(2)**:175-94

16. Ealey, P.A. The Validation of the Cytochemical Bioassay of Thyrotrophin and its Application to Selected Clinical Problems. 1979. PhD Dissertation. University of London.

17. Mornex, R. and Orgiazzi, J.J. Hyperthyroidism. In: De Visscher, M., ed. The Thyroid Gland. New York. Raven Press, 1980:279-362.