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Comparison of radioassay and haemagglutination methods for anti-thyroid microsomal antibodies

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SUMMARY

Parallel measurements of circulating anti-thyroid microsomal (anti-M) antibodies by radioassay and haemagglutination were performed on subjects with or without thyroid disorders. Three-quarters (75·4%) of control subjects had undetectable antibody levels (< 10 u/ml) by radioassay and only 3·1% had concentrations of ⩾ 75 u/ml. Abnormally elevated levels (⩾ 75 u/ml) were found in most of the patients with Hashimoto's thyroiditis (94·1%) or idiopathic myxoedema (86·7%), in the majority (75·0%) of those with Graves' disease and only in a minority of those with other thyroid disorders. The percentage of positive sera by haemagglutination was very similar in all groups to that of abnormal values observed in the radioassay.

Direct comparison of parallel tests on a total of 631 sera revealed a highly significant correlation ($r = 0{\cdot}91$, $P < 0{\cdot}001$) between the two methods, but elevated antibody titres by haemagglutination were found in some sera with negative radioassays. All these sera were from a single patient with thyroid carcinoma associated with Hashimoto's thyroiditis and had elevated levels of anti-thyroglobulin (anti-Tg) antibodies. Evidence that such discrepancies were due to anti-Tg antibodies reacting with microsomal-bound Tg was provided by the demonstration that the haemagglutination produced by these sera could be completely inhibited by the addition of Tg. A similar inhibition was observed with two rabbit antisera to human Tg, but not with sera from patients with thyroid autoimmune disorders containing high levels of anti-microsomal anti-bodies.

INTRODUCTION

Circulating antibodies to thyroid microsomal antigen, Tg and other thyroidal components are frequently present in patients with thyroid autoimmune disorders (Doniach, 1975). Recently sensitive and quantitative competitive binding radioassay methods for measurement of serum anti-Tg and anti-microsomal (anti-M) antibodies have been introduced by Mori, Fisher & Kriss (1970) and Mori & Kriss (1971), using Tg or thyroid microsomes coated on plastic cups and radioiodinated anti-Tg or anti-M antibodies respectively. The clinical usefulness of these assays has been confirmed in this laboratory (Fenzi *et al.* 1973; Pinchera *et al.*, 1976a; Pinchera *et al.*, 1976b). In a previous study (Pinchera *et al.*, 1977) we have compared the results of parallel measurements of anti-Tg antibodies by passive haemagglutination (Fulthorp *et al.*, 1961) and radioassay. Good correlation was found between the two methods, but discrepancies were observed in several sera. Evidence was obtained that these were due to the presence of elevated levels of serum Tg producing false positive results in the radioassay. In the present study we have compared the radioassay method for anti-M antibodies with the haemagglutination technique developed by Fujita *et al.* (1970) utilizing tanned sheep red blood cells coated with human thyroid microsomal antigen. A highly significant correlation was observed and clear discrepancies were consistently found only in sera from a single patient with thyroid carcinoma associated with Hashimoto's thyroiditis. Evidence has been provided that this was due to the presence of elevated levels of anti-Tg antibodies, producing false positive results in the haemagglutination tests.

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MATERIALS AND METHODS

Microsomal preparations. Microsomes were prepared by differential centrifugation (Pinchera *et al.*, 1970) from frozen specimens of toxic diffuse goitres or human control tissues (lung, liver and placenta). These preparations were washed five times with PBS (pH 7·8) and stored at −20°C in small aliquots.

Thyroglobulin. Purified specimens of human Tg were gifts of Dr G. B. Salabè and Dr F. Monaco (Centro di Fisiopatologia Tiroidea, C.N.R., Roma, Italy). These materials were prepared from saline extract of non toxic goitres by repeated precipitation in 1·8 M ammonium sulphate or by ammonium sulphate fractionation followed by sucrose density centrifugation (Andreoli, Califano & Viscidi, 1966; Monaco *et al.*, 1974). Analytical ultracentrifugation showed that 19S accounted for virtually all the protein material of the two preparations used in this study.

Preparation and radioiodination of IgG. IgG was prepared according to Sober & Peterson (1958). The degree of purity was checked by immunoelectrophoresis (Scheidegger, 1955). IgG concentration was determined by an agar gel diffusion method (Tri-Partigen and LC Partigen, Beringwerke AG, Marburg/Lahn, Germany). Serum from a patient with Graves' disease with undetectable anti-Tg antibodies and very high levels of anti-M antibodies (as assessed by haemagglutination) was used as the source of the anti-M IgG preparation employed in the radioassay. Normal IgG (N-IgG) was prepared from pooled sera of healthy volunteers. Radioiodination of anti-M IgG with ^{125}I was performed by the lactoperoxidase method (Marchalonis, 1969), as previously described (Fenzi *et al.*, 1972). Prior to use, radioiodinated anti-M IgG was pre-incubated with mixed control microsomes from human placenta and liver (100 μg of tissue protein/μg of IgG), and then centrifuged at 105,000 *g* for 90 min to remove non-specifically reacting components.

Radioassay for anti-M antibodies. The solid state radioassay procedure described by Mori *et al.* (1970) was used with minor modifications. U-shaped cups of plastic Microtiter® plates (Cooke Engineering Co., Alexandria, Virginia, USA) were coated with human thyroid microsomes in PBS (pH 7·8). Assay was performed by overnight incubation at room temperature with a mixture containing: 100 μl of normal rabbit serum (NRS) or test specimen (diluted with NRS when needed), 10 μl of [^{125}I]anti-M IgG (250,000 ct/min) and PBS up to 250 μl. Graded amounts of unlabelled anti-M IgG ranging between 40 and 2,500 ng in 1 to 10 μl of PBS were added to the cups used for the standard curve. Cups coated with NRS were used as blanks. All tests were performed in triplicates. After removal of unbound material by extensive washing, individual cups were cut out and separately counted in plastic counting tubes. Preliminary experiments showed that binding of radiolabelled anti-M IgG increased progressively from 0·2 to 5% by augmenting the protein concentration of the suspension of thyroid microsomes from 0·125 to 50 μg/ml, no significant change being observed when greater amounts were used. Therefore, a

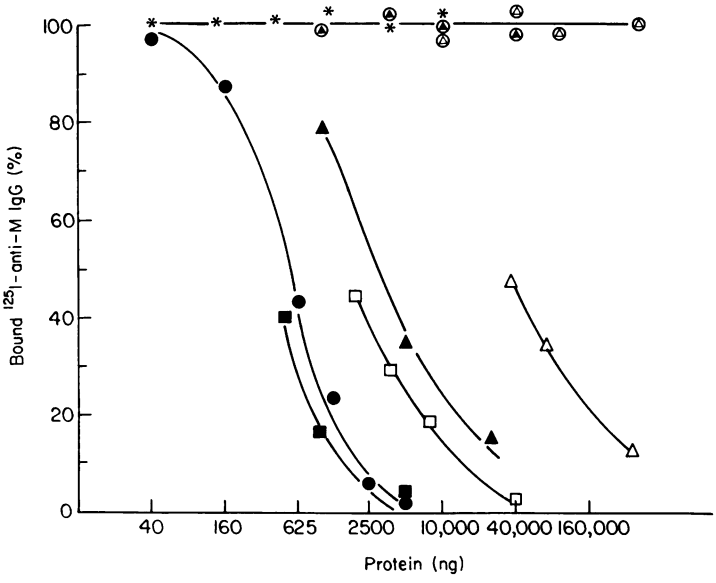

FIG. 1. Binding of [^{125}I]anti-M IgG to human thyroid microsomes coated on plastic cups. Effects produced by the addition of graded amounts of cold anti-M IgG, pooled normal human serum, representative sera from a patient with Graves' disease (2277) or Hashimoto's thyroiditis (2798), their IgG fractions and purified human Tg (hTg). Amounts of all these test materials are expressed in terms of ng of proteins. A dose-dependent inhibition of binding was observed with cold anti-M IgG, serum samples 2277 and 2798 and their IgG fractions. No effect was produced by normal serum, normal IgG or hTg. (●———●) Anti-M IgG; (◬———◬) normal serum; (◬———◬) normal IgG; (△——— △) sample 2277 serum; (▲———▲) sample 2277 IgG; (□——— □) sample 2798 serum; (■———■) sample 2798 IgG; (*———*) hTg.

concentration of 50 μg/ml was employed in subsequent experiments. Non-specific binding (i.e. radioactivity found in the blank) was less than 0·1%. Results were expressed in percentage of maximal binding, after subtraction of the blank. As shown in Fig. 1, a dose-dependent inhibition of binding of [^{125}I]anti-M IgG was observed by adding graded amounts of cold anti-M IgG, while no effect was produced by normal human serum, N-IgG or Tg. Parallelism with the standard curve was observed with two representative sera from patients with Graves' disease or Hashimoto's thyroiditis and virtually all the activity was recovered in the IgG fraction. Tests for specificity performed with plastic cups coated with microsomes of control tissues or purified human Tg showed no significant binding of radioiodinated anti-M IgG. Antibody content was expressed in units (u) per ml, 1 u being defined as the amount equivalent to 100 ng of the standard anti-M IgG. The minimum detectable amount was usually 1 u corresponding to a concentration of 10 u/ml when 100 μl of test serum were used.

Radioassay for anti-Tg antibodies. Measurements of anti-Tg antibodies were performed by a modification of the method of Mori *et al.* (1970), using partially purified anti-Tg antibody obtained by affinity chromatography, as previously described (Pinchera *et al.*, 1977).

Haemagglutination tests. Anti-M and anti-Tg antibodies were determined using commercial kits (Sera-Tek, Thyroid Microsomal Antibody Test and Thyroglobulin Antibody Test, Miles Laboratories Inc., Elkhart, Indiana, USA). Serial two- or four-fold dilutions starting with 1 : 100 were performed by means of the Takatsy microtitration apparatus (Scientific Shandon Instruments Co., London). Several sera producing no agglutination at low dilutions were found to be positive when tested at higher dilutions. In agreement with Amino *et al.* (1976) this was mainly observed in the anti-M antibody system. Therefore, sera were considered negative only if agglutination failed to occur at any dilution ranging from 1 : 100 to 1 : 102,400.

Rabbit antisera to human Tg. Two rabbit antisera to human Tg were used in this study. These were kindly prepared by Dr G. F. Fenzi (Patologia Speciale Medica II, University of Pisa, Italy) and Dr J. Torresani (Laboratoire de Biochimie Médicale, University of Marseille, France), using a technique previously described (Lissitzky *et al.*, 1973).

Storage of sera. Sera were kept frozen at −20°C until used. Repeated thawing and freezing was avoided.

Patients. Included in this study were 160 patients with Graves' disease (forty-three males, 117 females; age range: 12–80 years), seventeen with Hashimoto's thyroiditis (all females; age range: 19–62 years), fifteen with idiopathic myxoedema (six males, nine females; age range: 34–80 years), twenty-six with toxic adenoma (three males, twenty-three females; age range: 13–76 years), fifty-two with non-toxic goitre (ten males, forty-two females; age range: 15–65 years), forty-five with thyroid carcinoma (seven males, thirty-eight females; age range: 18–81 years). The control group consisted of 130 subjects (fifty-one males, seventy-nine females; age range: 12–83 years), including sixty-two healthy volunteers and sixty-eight patients with various conditions without evidence of thyroid disease or autoimmune disorders. The diagnosis was based on clinical criteria and was confirmed by appropriate laboratory tests. Histological confirmation of the diagnosis was available in nine out of the seventeen cases of Hashimoto's thyroiditis. In the remaining eight patients the diagnosis was made on the basis of euthyroid or hypothyroid goitre and serological evidence independent of radioassay results: anti-M and/or anti-Tg antibody titre of ⩾1 : 1600 by haemagglutination. Three of the forty-five cases of thyroid carcinoma had histological evidence of associated lymphocytic thyroiditis.

RESULTS

Anti-M antibodies by radioassay

Results of tests for serum anti-M antibodies by radioassay are shown in Table 1. Sera from patients tested more than once were chosen by a table of random numbers. Of the 130 control subjects, ninety-eight (75·4%) had undetectable (< 10 u/ml) antibody levels by this method and only four (3·1%) had con-

TABLE 1. Serum anti-M antibody levels by radioassay in subjects with and without thyroid disorders

Subjects	Total number of cases	<10 u/ml	10–74 u/ml	75–299 u/ml	300–999 u/ml	1000–9999 u/ml	⩾10,000 u/ml
Control	130	98 (75·4)*	28 (21·5)	4 (3·1)	0	0	0
Graves' disease	160	27 (16·9)	13 (8·1)	20 (12·5)	21 (13·1)	41 (25·6)	38 (23·8)
Hashimoto's thyroiditis	17	0	1 (5·9)	0	2 (11·8)	10 (58·8)	4 (23·5)
Idiopathic myxoedema	15	0	2 (13·3)	3 (20·0)	1 (6·7)	4 (26·7)	5 (33·3)
Non-toxic goitre	52	25 (48·1)	21 (40·4)	6 (11·5)	0	0	0
Toxic adenoma	26	15 (57·7)	6 (23·1)	5 (19·2)	0	0	0
Thyroid carcinoma	45	24 (53·4)	12 (26·7)	2 (4·4)	3 (6·7)	2 (4·4)	2 (4·4)

* Numbers in parentheses indicate percentage.

centrations of ⩾ 75 u/ml. This value was considered as the upper limit for the normal range. The highest level found in this group was 195 u/ml. The results were unrelated to sex and age.

A large majority (75·0%) of the 160 patients with Graves' disease had abnormally elevated (⩾ 75 u/ml) values. Thirty-eight subjects (23·8%) had very high levels (⩾ 10,000 u/ml) and the highest serum concentration was 148,300 u/ml.

All but one (94·1%) of the seventeen patients with Hashimoto's thyroiditis had abnormally elevated values and four (23·5%) had levels of ⩾ 10,000 u/ml, including one case with a concentration of 375,000 u/ml.

Most (86·7%) of the patients with idiopathic myxoedema also had abnormal antibody levels, and the highest concentration found in this group was 30,000 u/ml. Both patients with low antibody levels (< 75 u/ml) had been on thyroid replacement therapy for several years.

Only a minority of the fifty-two patients with non-toxic goitre (11·5%) and of the twenty-six with toxic adenoma (19·2%) had abnormally elevated levels, and none of the patients of these groups had concentrations of ⩾ 300 u/ml. A similar percentage (19·9%) of the forty-five patients with thyroid carcinoma had abnormally elevated values, but seven subjects of this group had values exceeding 300 u/ml. Two of the three patients with histological evidence of coexistent Hashimoto's thyroiditis had values of ⩾ 1000 u/ml, but one had undetectable antibody levels by this method.

Anti-M antibodies by haemagglutination

The results of haemagglutination tests performed on the same sera are reported in Table 2. It is apparent that the distribution of anti-M antibody titres was similar in all groups to that found in the radioassay.

Direct comparison of haemagglutination and radioassay results

Parallel tests were performed on a total of 631 sera. For the purpose of this study specimens drawn from the same patients on different occasions were also considered. In spite of some overlapping, there was a highly significant correlation between the mean antibody concentration by radioassay and the haemagglutination titre ($r = 0{\cdot}91$, $P < 0{\cdot}001$). As shown in Fig. 2, of the 352 sera with negative haemagglutination tests, only twenty-three had abnormally elevated levels by radioassay and none had concentrations of ⩾ 300 u/ml. Similarly, only twenty-five of the 354 sera with undetectable or low (< 75 u/ml) radioassay values had positive haemagglutination tests. However, while low to moderate haemagglutination titres (1 : 100–1 : 400) were found in eighteen of these sera, elevated titres ranging between 1 : 1600 to 1 : 25,600 were observed in seven sera with undetectable antibody by radioassay. All these serum samples derived from a single patient with differentiated thyroid carcinoma associated with Hashimoto's thyroiditis, who also had elevated anti-Tg antibody levels both by radioassay and

TABLE 2. Serum anti-M antibody titres by haemagglutination in subjects with and without thyroid disorders*

Subjects	Total number of cases	Neg.	1 : 100	1 : 400	1 : 1600	1 : 6400	⩾ 1 : 25,600
Control	130	123 (94·6)†	5 (3·9)	2 (1·5)	0	0	0
Graves' disease	160	36 (22·5)	20 (12·5)	19 (11·9)	25 (15·6)	28 (17·5)	32 (20·0)
Hashimoto's thyroiditis	17	1 (5·9)	1 (5·9)	3 (17·6)	3 (17·6)	3 (17·6)	6 (35·3)
Idiopathic myxoedema	15	2 (13·3)	2 (13·3)	3 (20·0)	4 (26·7)	2 (13·3)	2 (13·3)
Non-toxic goitre	52	51 (98·1)	1 (1·9)	0	0	0	0
Toxic adenoma	26	22 (84·6)	3 (11·5)	1 (3·9)	0	0	0
Thyroid carcinoma	45	37 (82·2)	2 (4·4)	2 (4·4)	1 (2·2)	0	3 (6·8)

* Tests were performed on the same serum samples reported in Table 1.

† Numbers in parentheses indicate percentage.

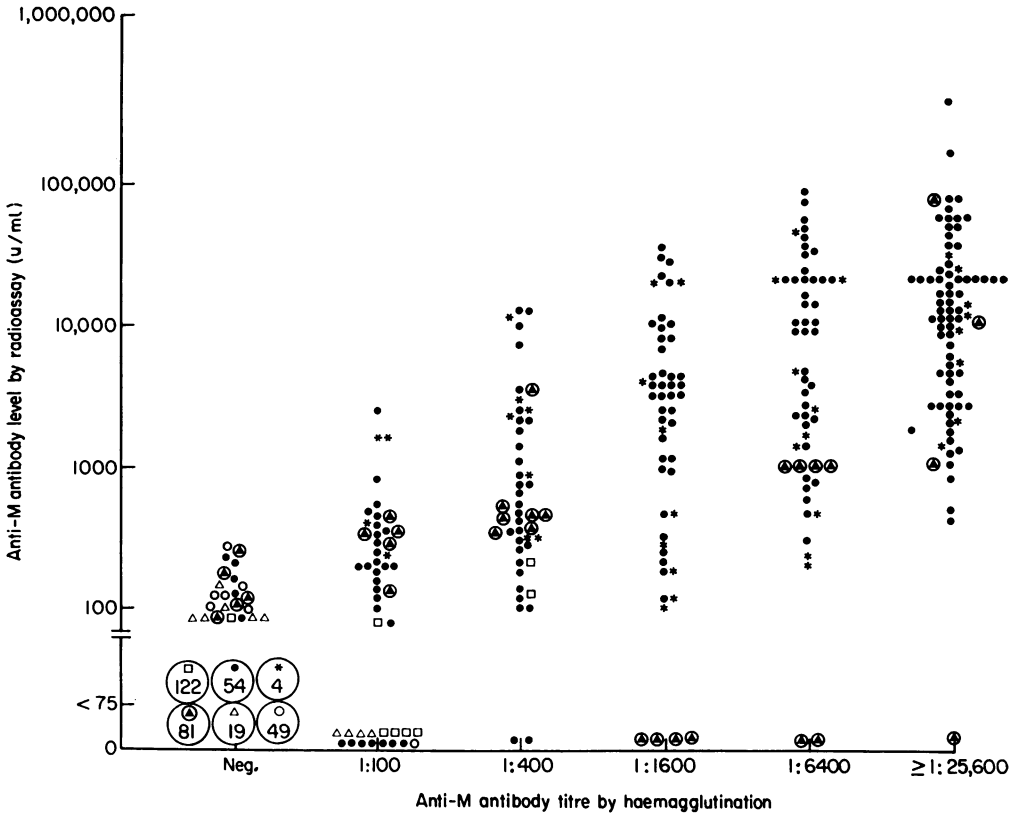

FIG. 2. Comparison of parallel measurements of anti-M antibodies by competitive binding radioassay and by passive haemagglutination. Of the 631 sera, 130 were from control subjects, 261 from treated or untreated Graves' disease, forty-six from Hashimoto's thyroiditis or idiopathic myxoedema, 113 from thyroid carcinoma, twenty-nine from toxic adenoma and fifty-two from non-toxic goitre. For convenience, sera of each category with negative haemagglutination titres and radioassay values of 75 u/ml were grouped together. A highly significant positive correlation ($r = 0{\cdot}91$, $P < 0{\cdot}001$) was found between the mean antibody concentration by radioassay and the haemagglutination titre. (□) Controls; (●) Graves' disease; (*) Hashimoto's thyroiditis or idiopathic myxoedema; (▲) thyroid carcinoma; (△) toxic adenoma and (○) non-toxic goitre.

haemagglutination. Since Tg antigenic determinants are present in thyroid microsomal preparations (Konno, Murthy & McKenzie, 1970; Pinchera *et al.*, 1976a; Pinchera *et al.*, 1976b), the question was raised whether such discrepancies could be due to the interference of anti-Tg antibodies in the haemagglutination test for anti-M antibodies.

Interference of anti-Tg antibodies in the anti-M haemagglutination test

To study this problem, parallel measurements of anti-M and anti-Tg antibodies by haemagglutination were performed with and without pre-incubation for 1 hr at 4°C of test sera with an excess of purified human Tg (100 μg/10 μl of serum). Representative results are reported in Table 3. Two samples (2544 and 3417) had elevated anti-M antibody levels and undetectable anti-Tg both by radioassay and by haemagglutination; no effect of Tg was observed in the haemagglutination tests. Two samples (2242 and 3387) had elevated anti-Tg and anti-M antibody concentrations by both methods; complete inhibition of haemagglutination in the system for anti-Tg antibodies was produced by excess Tg, while no change in the anti-M antibody titre was observed. Sera 2720 and 3287 were selected from among the above seven samples in which anti-M antibody levels were elevated by haemagglutination but undetectable by radioassay, whereas anti-Tg antibodies were elevated by both methods. In these cases complete inhibition of haemagglutination by Tg was also observed in the system for anti-M antibodies. Similar results

TABLE 3. Effect of human Tg on measurements of anti-Tg and anti-M antibodies by haemagglutination

Serum number	Anti-Tg Antibodies			Anti-M Antibodies		
	By haemagglutination		By radioassay (u/ml)	By haemagglutination		By radioassay (u/ml)
	No Tg added	Tg added (100 μg)		No Tg added	Tg added (100 μg)	
2544	Neg†	Neg	<20	1 : 102,400	1 : 102,400	61,600
3417	Neg	Neg	<20	1 : 102,400	1 : 102,400	84,666
2242	1 : 409,600	Neg	25,750	1 : 25,600	1 : 25,600	29,600
3387	1 : 409,600	Neg	33,750	1 : 102,400	1 : 102,400	27,750
2720	1 : 102,400	Neg	12,500	1 : 12,800	Neg	<10
3287	1 : 6400	Neg	9566	1 : 3200	Neg	<10
Anti-Tg (1)*	1 : 102,400	Neg	8333	1 : 12,800	Neg	<10
Anti-Tg (2)*	1 : 1,638,400	Neg	260,000	1 : 102,400	Neg	<10

* These were rabbit antisera to human Tg.
† Negative tests.

were obtained with two rabbit antisera to human Tg. These data indicate that anti-Tg antibodies may interfere in the anti-M antibody haemagglutination test producing false positive results.

DISCUSSION

Measurements of circulating anti-M antibodies by the competitive binding radioassay (Mori *et al.*, 1970) in a large series of subjects with and without thyroid disorders confirmed the clinical usefulness of this procedure. As reported previously by Mori & Kriss (1971) and by this laboratory (Fenzi *et al.*, 1973; Pinchera *et al.*, 1976a; Pinchera *et al.*, 1976b), a consistent proportion of the sera from control subjects who had no clinical evidence of thyroid disorders had detectable anti-M antibody levels by this method. Most of these sera had only low concentrations (< 75 u/ml) of questionable significance, but the possibility that these subjects had focal thyroiditis should be considered. This would be consistent with the finding of focal lymphoid infiltration of the thyroid in up to 25% of women and 7% of men at post-mortem examination (Doniach, 1975). The presence of low antibody titres in non-toxic goitre and thyroid carcinoma has been shown to parallel the extent and intensity of thyroiditis lesions (Senhauser, 1964). Population surveys indicate that the incidence of thyroid antibodies increases with age, and is higher in females (Doniach, 1975). This was not observed in the present series, but a much larger control sample would be required to study this problem.

Abnormally elevated values (⩾ 75 u/ml) by radioassay were found in most patients with Hashimoto's thyroiditis (94·1%) or idiopathic myxoedema (86·7%) and in the majority (75·0%) of those with Graves' disease. These groups also had a high incidence of markedly elevated antibody levels (⩾ 1000 u/ml) ranging between 49·4% in Graves' disease and 82·3% in Hashimoto's thyroiditis. Only a minority of the subjects with toxic adenoma (19·2%) or non-toxic goitre (11·5%) had anti-M antibody levels of ⩾ 75 u/ml and none had serum concentrations exceeding 300 u/ml. A similar incidence of abnormally elevated values (19·9%) was observed in thyroid carcinoma, but seven patients (15·5%) of this group had antibody levels of ⩾ 300 u/ml. At least two of these subjects had histological evidence of coexistent thyroiditis; in the other cases, lymphoid infiltration in areas not examined at the time of operation could not be excluded, since tissue specimens were not available for further examination.

Tests performed on the same sera by the haemagglutination technique were in agreement with previous reports (Bird & Stephenson, 1973; Perrin & Bubel, 1974; Aoki *et al.*, 1976; Abreau *et al.*, 1977) and showed a distribution of anti-M antibodies in all groups of patients that was strikingly similar to that observed in the radioassay. A highly significant correlation between the two methods was found when

results of parallel tests on 631 sera were compared. In a previous study (Pinchera *et al.*, 1977), comparison of measurements of anti-Tg antibodies by radioassay and haemagglutination showed that several sera with negative haemagglutination tests had an apparently elevated level of anti-Tg antibodies by radioassay. This occurred more frequently in patients with differentiated thyroid carcinoma. Evidence was obtained that this was due to increased serum Tg levels producing false positive results in measurements of anti-Tg antibodies by radioassay. Discrepancies of this type, i.e. markedly elevated anti-M antibodies by radioassay with negative haemagglutination tests, have not been observed in the present study. Preliminary data indicating that this might occur (Vitti *et al.*, 1976) could not be confirmed in subsequent experiments. In fact, sera with elevated anti-M antibodies by radioassay and negative haemagglutination tests at low dilutions were found to be positive in the latter procedure too when tested at higher dilutions. Such a phenomenon has previously been reported by Amino *et al.* (1976) and was attributed to the presence of incomplete 'blocking' antibodies which compete with haemagglutinating anti-M antibodies.

In a few serum samples elevated anti-M antibody titres by haemagglutination were associated with negative radioassay results. All these sera were obtained from a single patient with differentiated thyroid carcinoma associated with Hashimoto's thyroiditis and contained elevated anti-Tg antibody concentrations. Since Tg antigenic determinants are known to be present in thyroid microsomal preparations (Pinchera *et al.*, 1976a; Pinchera *et al.*, 1976b; Konno *et al.*, 1970), the question was raised whether such discrepancies could be due to the interference of anti-Tg antibodies in the haemagglutination test. Evidence supporting this explanation was provided by the finding that pre-incubation with an excess of Tg completely inhibited the agglutination of tanned red cells coated with thyroid microsomes produced by these sera. This inhibition was also observed with rabbit antisera to human Tg, but not with sera from patients with autoimmune thyroid disorders containing anti-M antibodies, irrespective of the presence or the absence of anti-Tg antibodies. The latter finding suggests that anti-Tg antibodies interfere in the haemagglutination test for anti-M antibodies only when present in excess. This may explain the discrepancy with results previously reported by Aoki *et al.* (1975) who observed only a partial inhibition of haemagglutination by Tg in this system, while Bird & Stephenson (1973) and Amino *et al.* (1976) found no effect of Tg. The present data suggest that haemagglutination tests for anti-M antibodies in sera with elevated anti-Tg antibodies should be performed after absorption with purified human Tg, in order to exclude the interference of anti-Tg antibodies. It should be pointed out that this interference may be of little practical importance, since, in agreement with others (Aoki *et al.*, 1975), we rarely observed the presence of markedly elevated anti-Tg antibody concentrations in the absence anti-M antibodies. In the present series this occurred only in a single patient, whereas as much as 71% of the patients with detectable anti-M antibodies had undetectable anti-Tg antibodies as assessed by haemagglutination.

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