In vitro Tests of Thyroid Function in Active Thyroglobulin Immunity[1,2] (36541)

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The antibody binding of thyroxine (T_4) in active thyroid immunity, a phenomenon originally described in this laboratory based on electrophoretic distribution of radiothyroxine in immune sera (1), has now been confirmed in various other laboratories (2–6). More recent investigations in obese strain chickens have also shown binding of thyroxine by spontaneously forming thyroid autoantibodies (7). One of the consequences of antibody binding of thyroxine is the elevation in total plasma or serum T_4. In view of this, some investigators have wondered whether or not circulating total T_4 would serve as a parameter to reflect antibody–T_4 interaction in thyroid-immunized animals. However, in sera with a low thyroid antibody titer (experimentally induced), an increase in total T_4 over the control values is not always readily apparent. In order to further clarify the problem, as well as to assess the comparative sensitivity of the techniques in reflecting T_4–thyroid antibody interaction, we have carried out some *in vitro* tests of thyroid function simultaneously with paper electrophoretic studies in the sera of thyroglobulin-immunized animals. In addition, the effect of thyroglobulin immunization on antibody titer as well as on *in vitro* parameters of thyroid function have been followed for approximately 1 year. These investigations in thyroglobulin-immunized animals showed that *in vitro* thyroid function tests adequately reflected the presence of either moderate or high thyroxine-binding antibody titer. These tests, in contrast to electrophoretic methods, failed to consistently reflect a low grade antibody–T_4 interaction.

Materials and Methods. A total of 36 New Zealand white rabbits (1 month) were randomly assigned into three groups; thyroglobulin immunized, albumin immunized, and adjuvant-injected groups. The rabbits were housed in pairs in a room maintained at an ambient temperature of 75° ± 2°F and a relative humidity of 50 ± 10%. Lighting in the room was controlled by a time switch providing alternate periods of 12 hr of artificial light and 12 hr of darkness. The animals were fed Purina Rabbit Chow and were weighed once weekly. Prior to the initiation of experiments, the rabbits were acclimatized to our laboratory environment for a period of at least 2 weeks.

Animals were immunized with bovine thyroglobulin (Sigma Chem. Co.) emulsified in complete Freund's adjuvant (CFA). The controls were either immunized with bovine albumin (Fraction V, Sigma Chem. Co.) in CFA or treated with CFA alone. Procedures for antigen immunization were similar to those described previously from this laborato-

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[2] Throughout this article the use of the expression "active immunity" signifies artificial immunization, *i.e.*, exogenous inoculation followed by antibody synthesis *de novo*. Active thyroglobulin immunity, therefore, implies injection of an animal with thyroglobulin. In passive immunity there is no exogenous antigen challenge or *de novo* antibody synthesis, rather antibodies from an immune subject or an animal are merely transferred to another individual either naturally (*e.g.*, passage of antibody gamma globulin, IgG, through the placenta) or artificially through exogenous administration.

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ry (1). The hemagglutination technique was used to measure antibodies in thyroid-immune and albumin-immune sera. For this purpose tannic acid-treated sheep red cells were coated with either bovine thyroglobulin or bovine albumin by procedures similar to that of Fulthorpe *et al.* (8) with some minor modifications.

The *in vitro* tests of thyroid function consisted of (a) total serum thyroxine by the competitive protein-binding technique of Murphy (9), and in some instances by immunoassay as developed in this laboratory (10); (b) charcoal uptake of ^{125}I–T_3 (11). For studying the relative binding of thyroxine by proteins in animal sera, paper electrophoretic techniques, as described in detail previously (1, 12), were employed utilizing ^{125}I–T_4 as a tracer. Excepting the immunoassay, the procedures employed in all *in vitro* tests were akin to those of the authors cited in appropriate references above. The details of T_4 immunoassay as carried out in this laboratory are essentially similar to the recently described technique of Chopra *et al.* (13) with the exception that in our procedure free and bound thyroxine were separated by resin rather than by the use of a second antibody.

Results. Investigations with sera of animals immunized against thyroglobulin. Thyroglobulin antibody titer. Thyroglobulin antibodies were detected 2 weeks after immunization as indicated by a low tanned red cell (TRC) agglutinating antibody titer (1/405). Following this period, there was a logarithmic increase in antibody titer up to 8 weeks after immunization, followed by a plateau until 16 weeks, after which the antibody titer began to decline. At 48 weeks after immunization a very low TRC antibody titer was apparent (Table I).

Gamma globulin (antibody)-binding of thyroxine. Two weeks after immunization paper electrophoretograms of thyroglobulin immune sera showed an average of about 5% retention of ^{125}I–T_4 radioactivity at the gamma globulin area which indicated the formation of a small population of T_4-binding antibodies. With increase in time there was a progressive elevation in T_4-binding antibody titer as shown by an increase in the proportion of radiothyroxine bound by gamma globulin. At 8 weeks after immunization, approximately half of ^{125}I–T_4 added to immune sera was bound by gamma globulin (Table I). After this interval only a slight increase in antibody-binding of ^{125}I–T_4 was noted. At 16 weeks after immunization, a slight decrease (over that noted at 12 weeks) in gamma globulin-binding of ^{125}I–T_4 was noted, followed by a rapid drop in antibody retention of ^{125}I–T_4 at 24 and 48 weeks. The amount of ^{125}I–T_4 bound by gamma globulin at 48 weeks after thyroglobulin immunization was approximately the same as at 5 weeks, indicating the persistence of a small population of T_4-binding antibodies.

Charcoal ^{125}I–T_3 Uptake. The variation of this parameter in animals at various intervals after thyroglobulin immunization essentially followed the same pattern as gamma globulin-bound radioactive thyroxine just described. Charcoal ^{125}I–T_3 uptake in sera was expressed as a ratio rather than in absolute values to overcome the possible inapparent inherent deficiencies in the technique: ^{125}I–T_3 uptake ratio (T_3 UR) = (^{125}I–T_3 charcoal uptake in immune or control serum)/(^{125}I–T_3 charcoal uptake in pooled normal rabbit serum). Prior to immunization no differences in T_3 UR between control and experimental animals were noted (Table I). In the sera of thyroglobulin-immune animals, however, antibodies provided additional thyroid hormone-binding sites so that more of the added ^{125}I–T_3 became bound resulting, therefore, in a decrease in charcoal radioactivity uptake; because of this decrease of the numerator, T_3 UR decreased. This decrease (over the preimmune values) already became significant ($p < .05$) 2 weeks after immunization as noted in comparisons either in the same group of animals or in relation to corresponding control values (Table I). The decrease in T_3 UR became more marked at 4 weeks after primary immunization. Subsequently T_3 UR decreased consistently up to 16 weeks after immunization when minimal values were noted. From this period onward, T_3 UR gradually increased and returned to preimmunization levels at 48 weeks after immuni-

TABLE I. Electrophoretic Gamma Globulin (Antibody) ^{125}I–T_4 Retention, Antibody Titer and *In vitro* Tests of Thyroid Function in Thyroglobulin Immunized and Control Rabbits.[a]

	Preimmunization or injection	Weeks after initial immunization or injection						
		2	4	8	12	16	24	48
Thyroglobulin immunized								
γ Globulin binding of ^{125}I–T_4 (% of total)	0.00 ± 0.00	5.59 ± 1.46	29.16 ± 4.27	55.26 ± 5.68	66.23 ± 6.50	59.22 ± 11.06	18.50 ± 2.60	4.88 ± 1.48
Total T_4 (μg/100 ml)	3.57 ± 0.21	4.25 ± 0.26	6.54 ± 0.68	10.33 ± 1.16	14.34 ± 1.24	14.14 ± 2.46	8.11 ± 0.68	3.89 ± 0.28
Total T_4 immunoassay (μg/100 ml)	2.93 ± 0.27	3.77 ± 0.29	—	8.93 ± 1.63	11.16 ± 1.24	—	7.23 ± 0.77	3.14 ± 0.23
T_3 uptake ratio	0.98 ± 0.01	0.88 ± 0.03	0.56 ± 0.06	0.22 ± 0.03	0.19 ± 0.03	0.16 ± 0.03	0.72 ± 0.06	0.98 ± 0.01
Median TRC antibody titer (reciprocal)	Negative	405	32,805	98,415	98,415	98,415	10,935	405
Albumin immunized								
Total T_4 (μg/100 ml)	3.67 ± 0.15	3.68 ± 0.22	3.16 ± 0.16	3.16 ± 0.17	4.05 ± 0.20	3.36 ± 0.23	3.12 ± 0.15	3.60 ± 0.28
T_3 uptake ratio	1.00 ± 0.01	1.02 ± 0.02	1.02 ± 0.02	1.04 ± 0.02	1.03 ± 0.02	0.99 ± 0.01	1.00 ± 0.01	0.99 ± 0.01
Median TRC antibody titer (reciprocal)	Negative	405	1215	10,935	32,805	3645	135	45
Adjuvant injected								
Total T_4 (μg/100 ml)	3.58 ± 0.15	3.64 ± 0.10	3.38 ± 0.23	3.42 ± 0.18	3.49 ± 0.15	3.41 ± 0.14	3.73 ± 0.23	3.92 ± 0.14
T_3 uptake ratio	0.97 ± 0.01	1.00 ± 0.01	0.98 ± 0.01	0.97 ± 0.01	0.99 ± 0.01	1.00 ± 0.02	0.98 ± 0.01	0.98 ± 0.14

[a] Excepting the TRC antibody titer, all values represent the mean ± SE. Each group represents 12 animals. No binding of ^{125}I–T_4 at γ globulin was noted in either albumin or in Freund's adjuvant treated groups.

Tests of significance

Analysis of variance between means of all groups

Total T_4	F
Before treatment	N.S.
2 Weeks after treatment	N.S.
48 Weeks after treatment	N.S.
T_3 UR	
Before treatment	N.S.
2 Weeks after treatment	$p < .01$
48 Weeks after treatment	N.S.

t Test of correlated means for the thyroglobulin immunized group

Total T_4	*t*
Preimmune vs. 2 week	$p < .05$
Preimmune vs. 48 week	N.S.
T_3 UR	
Preimmune vs. 2 week	$p < .05$
Preimmune vs. 48 week	N.S.

zation. T_3 UR at 48 weeks did not significantly differ from preimmune values as noted both in intra- and intercomparisons (see Table I).

Total thyroxine. As in the case of T_3 UR, the preimmunization total T_4 values in thyroglobulin immune animals did not significantly differ from that of controls immunized against albumin or those treated with CFA alone (see analysis of variance, Table I). The general pattern of changes in total T_4 in rabbits at various intervals after thyroglobulin immunization also was similar to that of gamma globulin-bound radiothyroxine and T_3 UR discussed above (Table I).

Although total T_4 at 2 weeks after thyroglobulin immunization (4.25 μg/100 ml) differed significantly from the preimmunization value of 3.57 μg/100 ml, it was still within the normal range of variation of T_4 in control animals (1.80–5.88 μg/100 ml). The 2-week postimmunization total T_4 values in thyroglobulin-immune animals did not differ significantly from the 2-week values in adjuvant-treated or albumin-immunized animals. The earliest time at which clear-cut changes in total T_4 were noted was at 4 weeks after immunization (Table I, 6.54 μg/100 ml at 4 weeks in comparison to the preimmunization value of 3.57 μg/100 ml or an increase of 83.2%); the 4-week postimmunization value was also significantly higher than the 4-week postalbumin immunization value (increase of slightly over 100%). The total T_4 increased until 12 weeks after immunization, followed by a plateau to 16 weeks, and then a decline. In the period of study total T_4 increased more than 300% over the preimmune value. Total T_4 at 48 weeks after thyroglobulin immunization (3.89 μg/100 ml) was not significantly different either from the preimmune value (3.57 μg/100 ml) or from that noted in controls at the corresponding interval (3.60 and 3.92 μg/100 ml in albumin-immunized and adjuvant-treated animals, respectively). Variation in total T_4 in thyroid-immune animals as determined by immunoassay was very similar to that just described above; the only difference was all immunoassay values were consistently lower than that obtained by Murphy's technique, and this is interpreted as a reflection of the specificity of the immunoassay (Table I). In thyroglobulin-immune sera, the seemingly contradictory observations of an increase in total T_4 in the context of a decrease in T_3 UR suggestive of a hypothyroid state, are readily resolved when one takes into consideration the additional thyroid hormone-binding sites provided by the antibody molecules.

Investigations with sera of albumin-immunized or adjuvant-injected animals. Albumin-immunized or adjuvant-treated animals did not give rise to T_4-binding antibodies. Therefore, only minor fluctuations were noted in total T_4 and T_3 UR tests carried out at various intervals corresponding to that of the thyroglobulin-immunized group (Table I). These fluctuations, still within the normal range of variation (total T_4, 1.80–5.88 μg/100 ml; T_3 UR, 0.85–1.08), were insignificant in comparison to the changes in total T_4 and T_3 UR noted at various intervals after thyroglobulin immunization. In the case of albumin-immunized animals, a low TRC antibody titer (1/405) was noted at 2 weeks after immunization which was followed by an increase up to 12 weeks (1/32,805), then a decline to extremely low levels (1/45) at 48 weeks after immunization.

Discussion. The investigations clearly indicate that in reflecting the presence of a low T_4-binding antibody titer electrophoretic techniques proved to be superior to *in vitro* thyroid function tests (total T_4 and T_3 UR) in providing consistently greater sensitivity (Fig. 1). Thus, at 2 and 48 weeks after thyroglobulin immunization electrophoretic techniques consistently detected a low grade serum antibody–T_4 interaction, whereas significant variations from preimmune values were noted with T_4 and T_3 UR only at the 2nd but not at the 48th week after immunization (Table I). Even the 2 week postimmunization T_4 values (while shown to be significantly different from the preimmune values in the correlated t test, Table I) were still within the range of variation of control values, and furthermore, did not differ from the control means at the corresponding time interval.

While it is true that hemagglutinating thy-

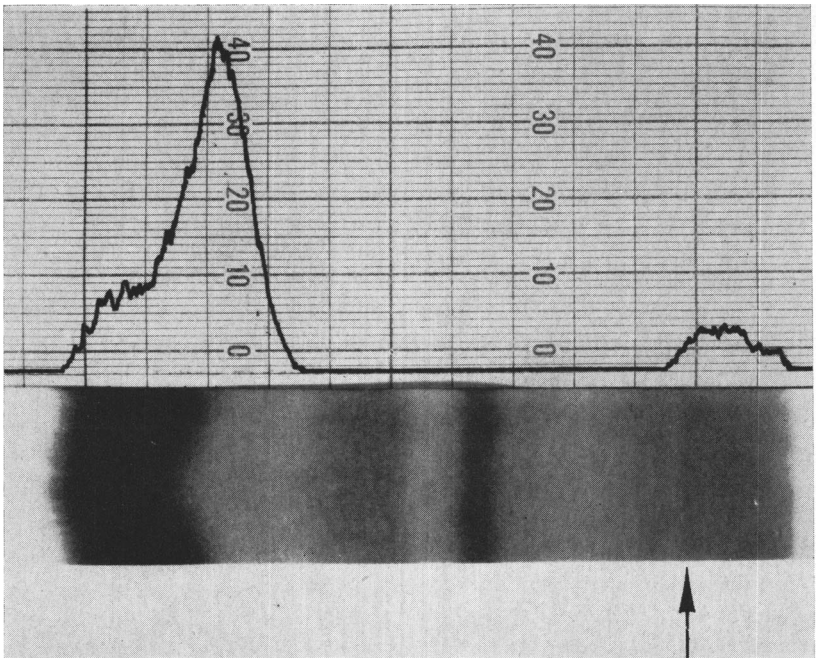

FIG. 1. Distribution of ^{215}I–T_4 in the serum paper electrophoretogram of a rabbit 48 weeks after thyroglobulin immunization. The arrow points the origin. Note the small radioactivity peak at the gamma globulin region (at and to the right of the arrow) which represents antibody binding of radiothyroxine. The *in vitro* tests (total T_4 and T_3 UR) failed to reflect T_4-binding antibodies in this serum as shown by the values which did not significantly differ from the respective preimmunization observations.

roglobulin antibody response was as sensitive as the electrophoretic technique in so far as detection of immune response at 2 and 48 weeks after sensitization (in contrast to the failure of T_4 and T_3 UR tests to consistently reflect immune-T_4 interaction at these time intervals), the presence of agglutinating thyroglobulin antibodies in circulation cannot, unfortunately, always be equated with T_4 antibody formation (14). Animals of various species differ substantially in their capacity to elicit antibodies to iodinated thyronine determinants despite the ease with which they form antibodies to thyroglobulin or the whole thyroid (15, 16). Furthermore, in obese strain chickens which develop thyroiditis spontaneously, antibodies to T_4 determinants form more slowly than antibodies to some of the larger antigenic fragments of thyroglobulin (17). That T_4-binding antibodies are long lasting was shown by a small but significant gamma globulin-binding of ^{125}I–T_4 even at 48 weeks after immunization.

Once a significant T_4-binding antibody population was generated in thyroglobulin-immune animals (gamma globulin-binding of at least 10%[5] of added ^{125}I–T_4), all the *in vitro* methods employed in the present studies did indicate significant changes in thyroid function in a direction consistent with what one would have anticipated in the context of increasing number of T_4-binding

[5] It should perhaps be stressed that the values denoting gamma globulin retention of ^{125}I–T_4 are only relative, and the value of 10% should not be considered as absolute in view of the varying serological characteristics of immune sera and the specific activity of radiothyroxine. It is entirely possible that in thyroglobulin immune sera as obtained from other laboratories, T_4 and T_3 UR tests may reflect immune-T_4 interaction, for instance, only when 20% or more of added ^{125}I–T_4 becomes bound by gamma globulin. The lack of a direct one-to-one relationship between gamma globulin-binding of ^{125}I–T_4 and total T_4 has also been made clear in the recent investigations of Chopra *et al.* (16) and Pogoriler *et al.* (6).

sites. It should be obvious, therefore, that an awareness of the probable antibody transport of thyroxine in active thyroid immunity is essential to avoid misinterpretation of seemingly paradoxical observations of various *in vitro* tests; this would also be true in human sera if they contain T_4-binding antibodies as has been described in certain pathological conditions (14, 18).

The inclusion of albumin-immunized animals in this study merits comment. We have routinely used such animals to serve as protein-administered controls in various investigations and it was of importance to note any changes in thyroid function in these animals. This suspicion was based on the following: (a) T_4 is generally believed to play a role during intense protein biosynthesis (19, 20), *e.g.*, in growth, and it was of interest, therefore, to determine whether or not intense antibody formation may have led to compensatory changes in total T_4. No outward signs of such a relationship seemed apparent in the present investigations, (b) it was also important to determine whether or not thyroxine would be bound by albumin–antialbumin complex, similar to the retention of thyroxine by prealbumin–antiprealbumin complex (21). The lack of marked and consistent changes in total T_4 or in other thyroid function tests in antialbumin sera suggests a lack of binding of T_4 by albumin–antialbumin complex; it may also indicate a lack of, or a very weak cross-reaction between antibovine albumin and rabbit albumin.

Summary. The sensitivity of *in vitro* thyroid function tests (charcoal ^{125}I–T_3 uptake ratio, total serum T_4) in reflecting antibody transport of thyroxine was evaluated in the sera of rabbits with varying amounts of thyroxine-binding antibodies as facilitated by bleeding rabbits at various intervals after bovine thyroglobulin immunization. Concurrent paper electrophoretic studies of distribution of ^{125}I–T_4 in thyroglobulin immune sera were also carried out. In sera with a very low titer of T_4-binding antibodies as revealed by a slight retention of ^{125}I–T_4 by gamma globulin in paper electrophoresis, *in vitro* tests did not provide consistent sensitivity in reflecting such abnormalities in thyroxine transport. In general when paper electrophoretograms of immune sera containing ^{125}I–T_4 showed gamma globulin retention of more than 10% of total radioactivity, *in vitro* thyroid function tests in such immune sera did reflect changes in a direction consistent with that expected in the context of increasing number of T_4-binding sites (as provided by thyroxine-binding antibody molecules). In contrast to the changes in ^{125}I–T_3 ratio and total T_4 noted in thyroglobulin-immunized animals, marked changes in these thyroid parameters were not noted in albumin-immunized or adjuvant-treated controls.

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