Serological Characteristics and Biological Effects of Antibodies Produced by Injection of Thyroid Materials[1] (35932)

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Antibodies heterogenous both as to immunoglobulin type and antigenic specificity have been found in the serum of animals immunized with thyroid materials.

Other evidences of alterations wrought by immunization have also been noted. Thyroid-stimulating activity in the serum has been found in rabbits immunized with human thyroid tissue by ourselves and others (1–3). Premachandra and co-workers described an increase in protein-bound iodine in guinea pigs immunized with thyroglobulin (4); and increases in total serum thyroxine (T_4) have been observed in rabbits immunized by several means (1, 3). We have recently described a double-isotopic modification of the McKenzie bioassay which has clarified the relationship of these phenomena and has demonstrated that activity which had been termed thyroid stimulating was actually the sum of separate thyroidal and extrathyroidal effects (5, 6). The thyroidal effect consists of a discharge of radioactive iodine (^{131}I) from the prelabeled thyroid gland. The extrathyroidal effect is, when most potent, a net mobilization of labeled thyroxine (^{125}I) from tissues to serum of the test animal; when less potent, the effect is simply a slowing of disappearance of ^{125}I from the serum. In the course of several investigations, during which we have immunized animals with thyroidal materials in different ways, we have noted some relationships between these and other phenomena. These relationships were further investigated and the results of these comparisons are presented below.

Methods. Double-isotopic McKenzie bioassay. A complete description has been published (6). To summarize: ^{125}I-labeled T_4 is injected 24 hr before the start of the standard McKenzie assay using mice whose thyroidal iodine pool has been prelabeled with ^{131}I. The test substance is injected after a 0 hr blood specimen is obtained. Blood specimens at 2 hr are compared with the 0 hr specimen for content of ^{131}I and ^{125}I. The results are expressed as response indices, that is, the percentage of the 0 hr value corrected for the response in control mice injected with 2% bovine serum albumin. If the mean ^{125}I-T_4 count rate in the 2 hr specimens from the test group is greater than from the control group, there is evidence for *slowing of disappearance of* T_4 from the serum. In the extreme case, the ^{125}I-T_4 counts actually increase over the 0 hr value, indicating a *net mobilization of* T_4 from extrathyroidal tissues. The ^{131}I response index is made up of both portions of this extrathyroidal phenomenon plus the effects of the test substance on the thyroid gland. Correction of the ^{131}I response index, which is the usual reported result of the McKenzie bioassay, by subtraction of the ^{125}I response index, to subtract the extrathyroidal effects, gives a value for the *thyroidal effect* of the test substance. We have expressed this $^{131}I - ^{125}I$ difference as a response index. Proof that thyroid stimulation is the source of this thyroidally derived radioactivity is as yet incomplete (6).

T_4-binding capacity and other methods. A radioelectrophoretic method was used to assay the T_4-binding capacity of gamma globulin (7). Serum total T_4 was measured by the 1965 modification of the method of Murphy and Pattee (8, 9) and serum free T_4 by a combination of the method of Schussler and

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Plager (10) with that of Sterling and Brenner (11). Hemagglutinating antibodies to human thyroglobulin (HTg) were measured by the method of Fulthorpe *et al.* (12).

Sera. Sera from 55 different animal sources were examined to obtain the results presented.

1. We immunized 5 Dutch rabbits with human thyroid microsomal fraction in complete Freund's adjuvant (HTM), 5 Dutch rabbits with rabbit thyroid microsomal fraction in complete Freund's adjuvant (RTM), 4 Dutch rabbits with human thyroglobulin without adjuvant (HTg), and 4 New Zealand rabbits with human thyroglobulin in complete Freund's adjuvant (HTg-A). Postimmunization sera from all 18 of the animals were assayed by all the means described above. Preparation of the materials and the immunization schedules have been described previously (13, 14).

2. We immunized 15 New Zealand rabbits with human thyroglobulin in Freund's adjuvant. Post-immunization sera from all these animals were assayed for hemagglutinating antibodies to human thyroglobulin, T_4-binding *in vitro* by gamma globulin, and the *in vivo* thyroidal and extrathyroidal effects of the sera in the double-isotopic McKenzie bioassay.

3. Twenty-two pools of sera from a larger number of animals immunized with HTM were subjected to the double-isotopic McKenzie bioassay. Eighteen of these pools were from guinea pigs and 4 from Dutch rabbits.

Effects of T_4 on HTg Hemagglutination. In one experiment, a solution of T_4 (100 μg/ml) was prepared in 0.4 *N* KOH containing 4% propylene glycol. The pH of the solution was reduced to 10.3 with dilute HCl. Duplicate specimens of serum to be tested were diluted in microtiter trays. A drop of the T_4-containing solution was added to each well in one series of dilutions; and a drop of the solvent without T_4 was added to the other. After incubation for 1 hr at room temperature, HTg-coated tanned red cells were added and the usual procedure was followed.

In another experiment, a solution of 0.001 *M* T_4 in normal rabbit serum was prepared by raising the pH of the serum to 8.5 with dilute NaOH. Duplicate portions of 29 sera from rabbits immunized with HTg, RTM, or HTM were incubated 1 hr at room temperature with an equal volume of either the normal rabbit serum containing T_4 or normal rabbit serum (pH 8.5) not containing T_4. Hemagglutination assays were then performed with the sera.

Absorption with HTg. The sera we studied were incubated with human HTg (4 or 12 mg/ml of serum) at 37° for 30 min and then overnight at 4°. They were centrifuged in the cold; and the supernatants were removed and assayed for T_4-binding gamma globulins, McKenzie bioassay activity, and HTg hemagglutination.

Statistical methods. Student's unpaired *t* test was used to compare sample means. The Spearman rank-order procedure was used to obtain the correlation coefficient (*r*). Statistical significance was ascribed to values of *t* or *r* for which *p* was .05 or less. Such values are denoted by footnote 2 throughout the text, and by a footnote to Tables I–V.

Results. The T_4-binding capacity of the gamma globulin fraction of the immunized rabbit sera varied quite considerably, from <0.3 to 106.5 μg/100 ml in the two series of rabbits in which it was measured. (Tables I and II). This capacity to bind T_4 correlated strongly with the extrathyroidal effects of the sera on ^{125}I-T_4 as detected by the bioassay in mice. Spearman rank-order correlation coefficients (4) were $+ 0.83$[2] and $+ 0.80$[2] for the two groups (Tables III and IV).

The T_4-binding capacity seemed to be related to the means by which the animals were immunized. Sera from 5 HTM-immunized Dutch rabbits and 4 HTg-A-immunized New Zealand rabbits had potent T_4-binding globulins (mean capacities were 26.5 and 55.0 μg/100 ml for the two groups, respectively). In contrast, sera from 5 RTM-immunized and 4 HTg-immunized Dutch rabbits bound only small amounts of T_4 (mean capacities 0.7 and 1.1 μg/100 ml, respectively).

There was no significant correlation ($r = +0.20$) of the *in vitro* T_4-binding capacity with the *thyoidal* effect of the serum, as re-

[2] *r* is statistically significant; $p = <.05$.

TABLE I.

Rabbit no.	Immunization[a]	γ-Globulin T_4-binding capacity (μg/100 ml)	McKenzie assay; 2 hr response indices ^{125}I	^{131}I	$^{131}I - ^{125}I + 100$	Anti-HTg hemagglutinin titer (reciprocal of serum dilution)	Total T_4 (μg/100 ml)
10-20	HTM	19.0	113[b]	108	95	10,240	3.8
10-47	HTM	53.0	130[b]	108	78	10,240	17.4
10-54	HTM	27.0	131[b]	105	74	20,480	18.8
10-16	HTM	26.0	127[b]	123	96	20,480	9.2
10-28	HTM	7.5	113[b]	127	114	10,240	6.6
10-19	RTM	1.6	104	73	69	640	2.8
10-45	RTM	0.8	104	89	85	2560	6.2
10-7	RTM	0.4	105	72	67	640	5.4
10-3	RTM	0.4	104	131	127	1280	5.4
10-53	RTM	0.3	103	73	70	640	5.8
10-49	HTg	<0.3	105	80	75	5120	5.6
10-51	HTg	0.3	104	76	72	5120	5.0
10-30	HTg	3.6	104	94	90	20,480	4.0
10-6	HTg	<0.3	104	121	117	40,960	3.2
RaTg	HTg-A	67.0	116[b]	129	113	20,480	—
11-16	HTg-A	36.0	114[b]	124	110	40,960	—
11-7	HTg-A	29.0	116[b]	144[b]	128	20,480	—
11-19	HTg-A	93.0	137[b]	152[b]	115	81,920	—

[a] HTM = human thyroid microsomes; RTM = rabbit thyroid microsomes; HTg = human thyroglobulin in saline; and HTg-A = human thyroglobulin in Freund's adjuvant.

[b] $p < .05$ (t test vs bovine serum albumin).

vealed by the $^{131}I - ^{125}I$ response index. The lack of correlation of T_4-binding with thyroidal effect was underlined by the results in the 15 animals of Group 2 immunized with HTg-A. Prominent extrathyroidal effects in the mouse bioassay and T_4-binding by gamma globulin *in vitro* were again demonstrated, but again none of the sera had significant thyroidal effect ($^{131}I - ^{125}I$ response index).

The capacity of the T_4-binding gamma globulin was inversely correlated with the fraction of T_4 which was dialyzable from the serum ($r = -0.88$[2]). However, the binding capacity did not correlate as well with the absolute free T_4 ($r = -0.56$[2]) nor with the total T_4 of the serum ($r = +0.49$).

Although antithyroglobulin hemagglutinating activity correlated with the T_4-binding activity of the gamma globulins ($r = +0.60$[2]) in group 1, the association may have been related only to interanimal variation in the degree of immunologic response. In group 2 animals immunized with HTg-A, the correla-

TABLE II. Animals Immunized with HTg-A Listed in Order of T_4-Binding Capacity.

γ-Globulin T_4-binding capacity (μg/100 ml)	2 hr double-isotope McKenzie assay ^{125}I response	Anti-HTg hemagglutinin titer (reciprocal of serum dilution)
106.5	129[a]	10240
102.0	140[a]	320
46.5	119[a]	5120
46.0	107	640
39.6	107	1280
38.5	108	10240
34.5	123[a]	640
32.2	118[a]	640
29.0	113[a]	10240
28.5	111[a]	1280
26.0	105	10240
13.0	102	10240
12.5	102	320
10.8	105	160
9.5	98	5120

[a] $p < .05$ (t test vs bovine serum albumin).

TABLE III. Group 1—18 Dutch Rabbits Immunized as Shown in Table 1.

	(^{131}I — ^{125}I)[a]	^{131}I[b]	^{125}I-T_4[c]	T_4-binding	Total serum T_4	HTg hemagglutinins
(^{131}I — ^{125}I)[a]	—	+0.93	+0.30	+0.20	+0.02	+0.64[d]
^{131}I[b]			+0.57[d]	+0.62[d]	+0.25[d]	+0.68[d]
^{125}I-T_4[c]				+0.83[d]	+0.61[d]	+0.53[d]
T_4-binding by gamma globulin					+0.49	+0.60[d]
Total serum T_4						+0.18

[a] Represents the effect of immune serum on the mouse thyroid.
[b] Represents the total effect of immune serum.
[c] Represents the effect of the immune serum on T_4 in serum and extrathyroidal tissues.
[d] r is statistically significant ($p < .05$).

tion coefficient between HTg hemagglutinins and T_4-binding capacity was +0.03 (Table IV). We have previously noted a poor correlation between thyronine-binding and HTg hemagglutination in antisera produced by HTg immunization (13).

Thyroid-iodine-releasing activity (the ^{131}I —^{125}I response index) correlated well with ^{131}I activity (total McKenzie assay response) ($r = +0.93^2$) but not significantly with the ^{125}I-T_4 activity (the extrathyroidal activity) ($r = +0.30$) in the double-isotopic McKenzie bioassay of sera from group 1. Since the range (67–128) of these response indices was rather small and none of the values were significant (Table I), we verified the poor correlation by carrying out a similar correlative study with all of the sera upon which we had performed a double-isotopic modification of the McKenzie assay. In this series of 40 sera (18 from group 1 plus 22 others) ^{131}I —^{125}I differences ranged from 67 to 316 and ^{125}I response indices from 103 to 185. The correlation coefficient for these pairs was only +0.45.

Thyroglobulin hemagglutination was unaffected by preincubation with T_4. When dilutions of a potent hemagglutinating serum were incubated with 100 μg/ml of T_4 before addition of the coated red cells, the resulting titer was 1:320,000. The same serum incubated with the KOH containing solvent without T_4 gave a titer of 1:160,000. Additionally, 29 undiluted rabbit sera were preincubated with 0.001 *M* T_4 and the hemagglutinating titers after dilution were compared to controls. Twenty were unchanged in titer, 5 increased, and 4 decreased in titer by one twofold dilution.

Thyroglobulin inhibited T_4-binding, antithyroglobulin, and ^{125}I extrathyroidal activity in sera possessing such activities (Table V). Thyroglobulin also decreased the ^{131}I–^{125}I activity in one experiment. Unfortunately, we have not had available enough serum with potent ^{131}I–^{125}I activity to repeat and clarify this finding.

Discussion. There are several lines of evidence indicating that a T_4-binding gamma globulin in the immunized rabbit serum was the cause of the slowing of disappearance of

TABLE IV. Group 2—15 New Zealand Rabbits Immunized with HTg-A.

	^{125}-T_4	T_4-binding	HTg hemagglutinins
^{125}I-T_4	—	+0.80[a]	+0.07
T_4-binding by gamma globulin		—	+0.03

[a] $p < .05$.

^{125}I-T_4 from the blood of the bioassay mouse. First, the two phenomena were closely related in the sera from each of two series of immunized rabbits ($r = +0.80^2$ and $+0.83^2$). Second, T_4-binding globulin (TBG) in human serum had a similar effect in the bioassay (5). Finally, absorption with thyroglobulin removed both the *in vitro* T_4-binding activity of the serum globulins and their ability to retard the disappearance of ^{125}I-T_4 from the blood of the bioassay mouse.

We have demonstrated that the ^{131}I–^{125}I bioactivity in the McKenzie assay involves thyroidal mechanisms (5–6). It is less certain whether this activity is independent of the gamma globulin–T_4-binding activity that causes the ^{125}I-extrathyroidal effects in the bioassay. Several of our findings suggest there is no relationship. T_4-binding did not correlate with the ^{131}I–^{125}I activity in the individual sera examined. Thyroxine-binding globulin also did not cause any increase in the thyroidal effects of the serum (5). Additionally, the thyroidal activity (^{131}I–^{125}I) seemed to be strongly related to immunization with microsomal fractions rather than with thyroglobulin, whereas production of T_4-binding gamma globulin was stimulated by both types of immunization. Addition of thyroglobulin to such a serum pool, however, resulted in the parallel loss of both bioactivities on the one occasion we were able to perform the experiment.

The T_4-binding globulin in these sera seemed to react specifically with thyroxine determinants. Thyroglobulin hemagglutination, on the other hand, must depend on some other determinants on the thyroglobulin molecule, since absorption with T_4 did not inhibit hemagglutination. Furthermore, there was a lack of correlation between T_4-binding and HTg hemagglutination when the two activities were compared.

Summary. We have studied the relationship of the serological and biological effects of sera from animals immunized with thyroid materials. Thyroglobulin hemagglutinating antibodies, thyroxine (T_4) binding by gamma globulin, the serum content of T_4 and the thyroid-stimulating (McKenzie bioassay)

TABLE V.

Serum	Immunization	γ-Globulin T_4-binding capacity (μg/100 ml)	Thyroid stimulation 2 hr response indices ^{125}I	^{131}I	^{131}I/^{125}I	Anti-HTg titer
Pool 3-70	HTM	226	170[a]	386[a]	316[a]	1:2560
Pool 3-70	+12 mg/ml of HTg	72	128[a c]	197[a c]	169[c]	1:80
LATS[b]	—	—	101[a]	501[a]	500[a]	<1:10
LATS	+12 mg/ml of HTg	—	99	444[a]	445[a]	<1:10
11-7 + 11-16	HTg-A	25.5	115[a]	103	88	1:20,480
11-7 + 11-16	+ 4 mg/ml of HTg	12.5	93[c]	97	104	1:20

[a] $p < .05$ (t test vs bovine serum albumin).

[b] LATS = serum from a human patient with Graves' disease containing long-acting thyroid stimulator. Data presented are 2 hr values; 8 hr values were higher.

[c] Significantly lower than preabsorption value ($p < .05$).

effects of the immune sera were compared.

Use of the double-isotopic McKenzie assay made it possible to distinguish extrathyroidal from thyroidal effects of the immune serum. The extrathyroidal effects of the bioassay correlated strongly with the T_4-binding capacity of gamma globulins detectable by electrophoresis of the immune serum. This, with other evidence, indicated that the same activity was being measured with both methods. Thyroidal effects (^{131}I–^{125}I) in the McKenzie bioassay appeared not to be due to T_4 binding, yet T_4 binding was always present in such sera.

Thyroglobulin (HTg) hemagglutination did not depend on thyroxine specific determinants since T_4 did not inhibit hemagglutination.

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