# Composition and efficacy of iodothyronine immunogens and host responsiveness in radioimmunoassay (RIA) for serum thyroxine (T4)

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*Summary.* An immunogen was prepared consisting of iodinated and thyroxylated bovine thyroglobulin which on theoretical and practical grounds was highly specific for thyroxine; a rabbit antiserum derived from it fell within limits for RIA specification under conditions wherein a T4-BSA immunogen product was inactive.

Thyroglobulin (Tg) and iodothyronine-artifical protein carrier immunogens[1-3] have been employed for antisera production intended for thyroxine (T4) or triiodothyronine (T3) RIA. The advantages of the former type of immunogen include large molecular size and the native carrier property of its nondeterminant moieties; a disadvantage is the degree of cross-reactivity of the derived antisera with other iodothyronines. Although the problem has been circumvented by selection of high T4 - low cross-reactant sera[1], it is never completely abrogated. A T3/BSA (bovine serum albumin) product was introduced by Gharib[2] (methodology essentially identical for T4/BSA conjugate preparation), but in this case the molar ratios were low, and a synthesis was required. That cross-reactivity would be a problem with Tg was shown previously[4]; thus bovine Tg-induced antibodies contained a mixed set of specificities elicited by the various iodinated forms of l-thyronine.

A method of utilizing bovine Tg resulting in antisera of high specificity, high avidity, and early elevated serum titers is reported below. In the method the Tg is first iodinated by the chloramine-T method[5], then thyroxylated with free-acid T4 using a water-soluble carbodiimide. Proof of enhanced efficacy is shown by quantitative comparison with T4-BSA conjugates.

In the hands of the author, molar T4/BSA ratios consistently fell within the range of 9-12. With such preparations the total quantity of conjugate required to reach effective titer and avidity in the complete Freund adjuvant was 20-28 mg. Several months were necessary to reach the required properties for T4 RIA. In these studies, a) molar ratios were determined by incorporating 'tracer' quantities of $T4^{125}$ I into the reaction mixtures followed by exhaustive dialysis; b) titers were determined in an RIA system without standard T4 in which labelled T4 (0.04-0.06 µCi), T4 depleted serum (10 µl), an inhibitor of TBG binding (magnesium 8-anilino-l-naphthalene sulfonate, 600 µg) and antiserum dilution (100 µl), in barbital buffer (0.075 M, pH 8.8) to 1 ml, were reacted statically at 37 °C. The bound and free label were then effectively separated by a resin strip consisting of Amberlite anion exchanger. The dilution giving a bound/total count ratio of 70% constituted the end-point; c) cross-reactivity with T3 was determined by the radiometric titer system above and the % effect was given by:

$$\frac{\text{titer at 70\% with T3-}^{125}\text{I}}{\text{titer at 70\% with T4-}^{125}\text{I}} \times 100,$$

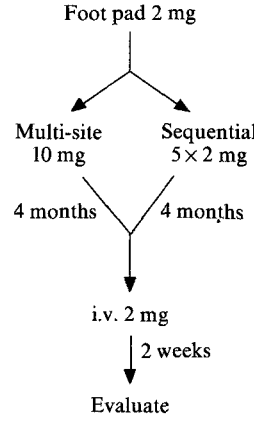
d) avidity was expressed as the decrease in B/T effected by increasing quantities of standard T4; thus a $\Delta$ 0-40 µg% value of 40% describes the steepness of the linear regression plot produced; this method, unlike the standard Scatchard plot, provides for a statistical control; thus at $\Delta$ values below 40% small variation in B/T measurement effect larger than desired variations in serum T4 as read by extrapolation.

Preliminary efforts centered around establishing baseline values with T4-BSA in donkey and rabbit (table 1). From the data it was apparent that rabbit peak titers persisted while donkey titers declined early, apparently irreversibly, although avidity and cross-reactivity values remained within acceptable limits. For this reason the rabbit was the animal of choice.

The iodination of bovine Tg was accomplished as follows: To 100 mg bovine Tg (Sigma Chemical) dissolved in 15 ml 0.05 M phosphate buffer, pH 7.2 (PB) were added 300 mg KI in 1 ml PB and 422 mg chloramine T in 3 ml PB; the mixture was stirred for 4 h at room temperature and the reaction stopped by adding 870 mg $Na_2S_2O_5$ in 3 ml PB. The resultant yellow precipitate after washing was taken up in 30 ml PB. The product was thyroxylated according to the T3 method of Gharib[2] with BSA carrier protein except that quantities were adjusted to accomodate the larger Tg.

With T4-BSA conjugates in the rabbit, 3 injections of 2 mg each produce antibody which is only detectible by comparison of count data with the undiluted pre-immune sample

Scheme for comparing methods of T4 antiserum preparation with bovine albumin carrier.




All injections except last given in complete Freund adjuvant.

Table 1. Titer comparison for donkey and rabbit antisera: T4-BSA immunogen

| Donkey Date | Titer | Rabbit Date | Titer |
|---|---|---|---|
| 20 Jan 77 | 200 | 28 Sept 78 | 10,000 |
| 11 Mar 77 | 13,000 | 15 June 79 | 14,750 |
| 30 Mar 77 | 7,600 | 20 July 79 | 12,000 |
| 4 Jan 78 | 450 | 19 Dec 79 | 11,000 |

Donkey titers showed relatively early decline to a level of 59% of peak at less than 3 weeks and to 3.4% of peak at about 1 year; rabbit titers remained rather constant for over 1 year. The immunosuppressive effect could not be reversed with T4/poly-l-lysine (7 injections of 2 mg each, molar ratio - 8). Slope values and cross-reactivities remained within specification limits with each at time of peak titers. Injections were given i.v. after titer maxima were reached. Prior to reaching maxima cell injections were given s.c. in complete Freund adjuvant.

and there is no titer per se. By comparison, the iodinated Tg-T4 preparation for the same quantity produced a Δ 0–40 μg% of 40.6% and a titer of 750×. With this latter antiserum 35 pg of T4 could be detected. Cross-reactivity with T3 was only 7.1% (table 2), not limiting when considering the ratio of T3/T4 in human serum ($^1/_{50}$ to $^1/_{100}$). This early and intense response provided an antiserum with the required properties for direct use.

In the iodination 308 moles of I were bound per mole Tg. This figure is obtained by multiplying the % radiolabel bound × KI added (gravimetric correction for I in KI), converting to g and dividing by 126.9. The calculation of moles Tg reacted (mol.wt 669,000) then yields the ratio. Drawing an analogy with rat Tg for which published mole% amino acid composition data are available, one can estimate 100 residues of tyrosine per molecule (calculation based on prosthetic carbohydrate - less Tg) each taking up 2 atoms of I; thus over 100 moles I were available for the saturation iodination of lower-substituted thyronines; even with iodohystidyl formation (approximately 50 residues), the fact that some of the tyrosines would be present in iodinated form would compensate for the histidine participation, at least in part. The iodotyrosines are not immunocross-reactive with T4, that event requiring the aromatic ether linkage and B-ring iodination[4].

A discussion of the magnitude of thyroxylation is in order. A ratio of T4/ITg of 266 was obtained. There are an estimated 850–950 groups potentially reactive with thyroxine under these conditions; these figures are obtained from mole% composition data for aspartic and glutamic acids (include asparagine and glutamine) and lysine. However, there must certainly be some T4-T4 reactivity as well. One could possibly abrogate this inter-thyroxine effect by use of the N-acyl or alkyl ester derivatives or by using tetraiodothyroacetate or similar analogues.

A change in immunization procedure which could improve the T4-BSA method is worth discussing. When most of the inoculum mass is given simultaneously, followed by a single i.v. injection 4 months later, an improvement in titer with equivalent cross-reactivity is observed (table 3). It may also be possible to retain the improvements with shorter times before the i.v. challenge.

Table 2. Performance characteristics of antisera prepared with different types of T4 immunogens: early responses

| Immunogen | Total dose (mg) | Titer | Δ0–40 μg** (%) | T3 cross-reactivity*** (%) |
|---|---|---|---|---|
| Iodinated Tg-T4 | 6.0 | 750 | 40.6 | 7.1 |
| T4-BSA | 6.0 | nil* | | |

* Represents all findings from several determinations. ** Represents decrease in standard slope effected by 40 μg%. *** Represents the % reactivity of the antiserum with T3-$^{125}$I. Only minor changes in T4-$^{125}$I uptake were noted with the T4-BSA immunogen, while a significant titer and standard curve slope steepness were noted with the Iodinated Tg-T4 preparation; cross-reactivity with this immunogen was within acceptable limits. Injections of 2 mg were given on alternate weeks for 3 weeks; the preparations were given s.c. in complete Freund adjuvant.

Table 3. Simultaneous T4-BSA immunogen administration compared with sequential T4-BSA administration

| Method | x̄ T4 titer (n = 4) | Range T3 cross-reactivity (%) | Δ0–40 μg (%) |
|---|---|---|---|
| a) Simultaneous | 17,650 | 1.8–7.5 | 46.5 |
| b) Sequential | 7,000 | 1.9–6.3 | >40.0 |

A gain of titer was noted with method (a) without sacrifice of specificity or avidity.


1 I.J. Chopra, J.C. Nelson, D.H. Solomon and G.N. Beall, J. clin. Endocr. Metab. *32*, 299 (1971).
2 H. Gharib, R.J. Ryan and W.E. Mayberry, J. clin. Endocr. Metab. *31*, 709 (1970).
3 J.M. Lieblich and R.D. Utiger, J. clin. Invest. abstr. No. 199, 600 (1971).
4 S. Margherita and B.N. Premachandra, J. Immun. *102*, 1511 (1969).
5 F.C. Greenwood and W.M. Hunter, Nature *194*, 495 (1962).


## Suppressed collagenolytic activity in polymorphonuclear leucocytes from diabetic humans[1]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*Summary.* Extracts of polymorphonuclear leucocytes (PMNL) from diabetic humans exhibited less collagenolytic activity than extracts from normoglycemic control subjects. Partially purified control extracts produced $\alpha^A$ and $\alpha^B$ collagen breakdown products of the type generated by mammalian collagenase; the diabetic preparation produced decreased amounts of the same products. The diabetic PMNLs may synthesize abnormally low levels of collagenase or contain inactive forms of this enzyme.


Experimental diabetes in rats has been shown to increase the collagenolytic activity of gingival tissue in culture[3,4] and to result in collagen loss in skin[5] and bone[6]. In contrast, however, Golub et al.[7] found that diabetes suppressed the loss of collagen normally seen in an inflammatory gingival lesion. To explain this unexpected effect, the following were proposed: (a) that diabetes increased collagen cross-linking rendering the fibrils less susceptible to degradation by collagenase; experimental evidence now exists for this proposal,[8,9] and/or (b) that diabetes inhibited the activity of leucocytes which mediate collagen degradation during inflammation. Although several studies have demonstrated that diabetes inhibits various aspects of the inflammatory response[10,11], including leucocyte chemotaxis[12–14], the present study is the first to demonstrate an effect of diabetes on leucocyte collagenolytic activity.