Immunoglobulin G Inhibitor of Thyroid-stimulating Antibody Is a Cause of Delay in the Onset of Neonatal Graves' Disease

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ABSTRACT Studies were carried out with the serum IgG from a mother and her two children who developed neonatal Graves' disease several weeks after birth. The maternal IgG: (*a*) stimulated the human thyroid in vitro, but maximal stimulation was found only with dilution of the IgG; (*b*) was very potent in the long-acting thyroid stimulator (LATS)-protector assay, but only when an inhibitor of the system was diluted out; (*c*) inhibited a standard preparation of LATS in the mouse bioassay; (*d*) was biphasic in the thyrotropin-binding inhibition (TBI) assay, i.e., enhanced binding at low concentrations of IgG and inhibited binding at high levels. Enhancement in the TBI assay was found only with particulate preparations of human thyroid membranes as receptor and not when that material was solubilized, nor with guinea pig fat cell membranes as receptor. Serial blood samples from the second child were obtained at birth and until 3 mo of age. In the thyroid slice (cyclic AMP) assay system there was a negative dose-response relationship in testing the IgG until age 45 d when it became positive, coinciding with the clinical recognition that hyperthyroidism had developed. The data are compatible with a concept that this mother's IgG contained thyroid-stimulating antibody (TSAb) and another moiety that inhibited TSAb through an action on the thyroid cell membrane, thus delaying the onset of hyperthyroidism in the neonate until the inhibiting IgG was metabolically cleared to an ineffective concentration.

INTRODUCTION

That neonatal Graves' disease is due to the transplacental passage of the thyroid-stimulating antibody (TSAb)[1] is complicated in part by the fact that different assay procedures have been used in the relevant studies (1). Since there is no universal agreement on the nature of the bioactivities measured in the various TSAb assays (2), data obtained with one procedure may or may not be relevant to data dependent upon another. Therefore, a recent description (3) of an example of the syndrome embodying apparently discordant data obtained by three different assay methods merits review. In this instance, the mother, whose thyroid diagnosis was Hashimoto's disease and who had never had clinical features of Graves' disease, gave birth to a child in whom thyrotoxicosis was not recognized until 4 mo of age. The exact time of onset of the neonate's hyperthyroidism was not clear. Assay of the mother's serum showed the IgG to be TSAb-positive, but maximal stimulation was found only on dilution of the IgG, and it was said to be both long-acting thyroid stimulator (LATS)- and LATS-protector (LATS-P)-negative. Studies we have carried out with sera from the family that was the subject of the earlier

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[1] *Abbreviations used in this paper:* C1 and C2, first and second children of M, respectively; LATS, long-acting thyroid stimulator; LATS-P, LATS-protector; M, mother; TBI, thyrotropin-binding inhibitor; TSAb, thyroid-stimulating antibody.

report (3) provide an entirely different perspective from that originally offered.

METHODS

Serial blood samples were obtained from the mother (M) between January 1981 (2 mo pregnant) and November 1981, including throughout the pregnancy that terminated uneventfully on August 8, 1981; this child (C2) is the sibling of the other (C1) who was the subject of the report by Hoffman et al. (3). C2 developed hyperthyroidism around 45 d.

Serial blood samples were also taken from C2 during the first three months of life. We are grateful to Dr. Hoffman who obtained the various bloods, separated the sera, and sent them to us either in Montreal or Miami; we are also most appreciative of the cooperation of the mother who traveled to Montreal on one occasion to undergo plasmapheresis.

TSAb measurement was by the human thyroid slice system, described previously in detail (4), in which an increase in the concentration of cyclic AMP in the tissue is the assay end point.

Assay of the LATS was carried out in mice (5) and the LATS-P assay was by the procedure reported in detail some years ago (6); samples were packed in dry ice and sent by air to Sheffield, for the latter measurement. Inhibition of the binding of ^{125}I-TSH to TSH-receptor (thyrotropin-binding inhibitor [TBI] assay) was studied by a standard procedure (7); the receptor preparations were human thyroid membranes, particulate or solubilized (7), and guinea pig fat cell membranes, particulate or solubilized (8).

All assays were carried out with IgG isolated from serum by precipitation with 1.64 M $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose (4). Protein concentration in membrane preparations was measured by the method of Lowry et al. (9) using bovine serum albumin as standard, with modification for solubilized preparations (10), or, for purified IgG, by spectrophotometry at 280 nm; assuming $E^{1\%}_{10\ mm} = 13.5$.

IgG containing only κ- or only λ-light chains was separated by affinity chromatography using antisera to κ- and λ-light chains (11).

RESULTS

TSAb Assays. Results of assays of IgG from M are shown in Table I. As quoted in the earlier report (3) initial assays carried out by our routine procedure (4) (i.e., 10 mg IgG/ml buffer) "gave uninterpretable results;" this statement reflected our surprise at the negative dose-response relationship now detailed in Table I, *a*. Assay of the separated IgGκ and IgGλ showed that all TSAb activity was associated with IgGκ and the maximum response was again significantly less than that produced by 5 mU TSH/ml (Table I, *b*). There was no significant qualitative or quantitative difference in the TSAb assay of M's IgG that was tested repeatedly with multiple samples of blood obtained over more than 2 yr; this point is exemplified by the fact that the data in Table I, *a* were obtained with IgG of serum taken in July 1978 and those in Table I, *b* with IgG from serum of January 1981.

Fig. 1 illustrates the pattern of TSAb-assay responses with serial samples of IgG from C2; the timing of the change from a negative to a positive dose-response relationship corresponded approximately to the date of the clinical diagnosis of thyrotoxicosis. It should be noted that with the inevitability of only small samples of blood being obtained from the neonate this series of assays could not be repeated; experience with assay of M's IgG indicates more pronounced differences in pairs of results would have been observed with testing of lesser concentrations of IgG, e.g., 0.1 and 2.0 mg/ml.

LATS assay. In Fig. 2 LATS bioassay data are shown indicating that there was no response to M's IgG and, in addition, it significantly inhibited the response to a known positive IgG (DeL).

LATS-P assay. These data are listed in Table II and indicate that although serum diluted twofold was

TABLE I
Assays of Mother's TSAb-IgG

	Test material	Concentration	Mean±SD	n
			pmol cAMP/ mg wet wt	
a	N IgG	0.20 mg/ml	0.20±0.04	(5)
	N IgG	10.0 mg/ml	0.22±0.03	(5)
	TSAb-IgG	0.02 mg/ml	0.96±0.27	(5)
	TSAb-IgG	0.06 mg/ml	1.84±0.10	(5)
	TSAb-IgG	0.10 mg/ml	2.33±0.83	(5)
	TSAb-IgG	0.20 mg/ml	2.86±0.50	(4)
	TSAb-IgG	2.0 mg/ml	1.88±1.48	(4)
	TSAb-IgG	5.0 mg/ml	0.87±0.23	(4)
	TSAb-IgG	10.0 mg/ml	0.54±0.20	(4)
	TSH	5 μU/ml	0.54±0.09	(4)
	TSH	5 mU/ml	5.95±0.68	(4)
b	N IgG	0.18 mg/ml	0.33±0.07	(5)
	TSAb-IgG	0.02 mg/ml	0.73±0.04	(3)
	TSAb-IgG	0.10 mg/ml	2.32±0.38	(4)
	TSAb-IgG	0.18 mg/ml	2.88±0.57	(4)
	TSAb-IgGk	0.02 mg/ml	1.01±0.05	(3)
	TSAb-IgGk	0.10 mg/ml	3.40±0.35	(4)
	TSAb-IgGk	0.18 mg/ml	3.43±0.15	(3)
	TSAb-IgGλ	0.10 mg/ml	0.35±0.06	(4)
	TSAb-IgGλ	0.18 mg/ml	0.37±0.05	(4)
	TSAb-IgGλ	0.37 mg/ml	0.43±0.08	(4)
	TSH	5 mU/ml	5.46±0.79	(4)

Assay of TSAb-IgG (*a*) and whole IgG, IgGk, and IgGλ (*b*) of M's serum; serum for section *a* was obtained July 1978 and for *b*, January 1981. In both *a* and *b*, the maximum response to TSAb-IgG is less than the maximum to TSH (i.e., response to 5 mU/ml). 5 and 10 mg TSAb-IgG/ml are less stimulatory than lower concentrations (section *a*). All activity is retained in TSAb-IgGk (*b*).

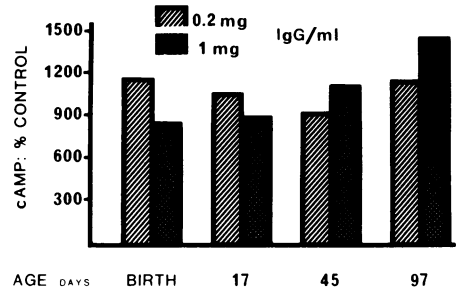

FIGURE 1 TSAb assay of C2's IgG. IgG of sera obtained at the ages shown were assayed at concentrations of 0.2 and 1.0 mg/ml. The responses are shown as the means of quadruplicate observations and are expressed as the percentage of control values obtained by assay of normal IgG at 1 mg/ml.

TABLE II
Assay of LATS-P in Mother's Serum

Serum dilution	Potency: Diluted serum	Potency: Whole serum
	U/ml serum	
1:2	10	20
1:20	17	348
1:60	13	814
1:200	6	1300

The definition of units based on a laboratory standard of LATS serum is given in reference 6. Serum was diluted as indicated, assayed, and the resulting observed potency was multiplied by the dilution factor to give the equivalent potency for whole serum.

moderately positive, the response was not diluted out up to a factor of 1:200. Thus, the final potency calculated was 1,300 U/ml.

TBI assays. The inhibition of ^{125}I-TSH binding to human thyroid membranes in particulate form by M's IgG, IgGκ, and IgGλ is illustrated in Fig. 3. ^{125}I-TSH binding was enhanced by the lowest concentrations tested with actual inhibition, compared with control, occurring only with 100 μg/ml for both IgG and IgGκ; IgGλ had no enhancing effect but was moderately inhibitory. The reproducibility of the TBI assay is affected by the inevitability of having to use different batches of thyroid membranes; repeated samples of M's IgG, concentrations being measured by spectrophotometry, no doubt also varied in the proportion of TSAb and other postulated activities in the total IgG.

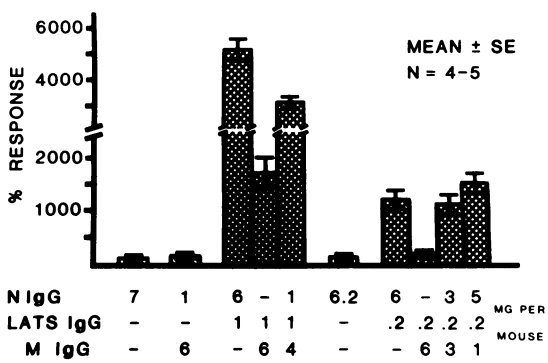

FIGURE 2 LATS assays. IgG from M's serum and from the serum of another Graves' disease patient (DeL, i.e., LATS IgG) were mixed in the concentrations indicated before injection into mice, all as 0.5 ml i.p. Data are shown as the increase in blood radioactivity (percentage increase over 0-h values) 24 h later. Inhibition of 1 mg DeL's LATS-IgG by 6 and 4 mg M's IgG, and of 0.2 mg by 6 mg, were all statistically significant ($P < 0.01$ by the t test).

Considering these facts, the many samples of M's IgG obtained over 2 yr were remarkably constant in both stimulating and inhibiting potencies in the TBI assay.

The pattern of inhibition of ^{125}I-TSH binding to thyroid membranes with serial samples of C2's IgG is shown in Fig. 4. The three preparations, compared with the effect of M's IgG, all enhanced binding but were progressively (corresponding to the child's age) less potent in inhibiting binding at the lower concentrations tested.

The binding-enhancing effect of M's IgG was affected by the TBI activity of DeL's IgG (a known potent preparation of TSAb) to a degree that was inversely proportional to the quantity of M's IgG used; 12.5, 25, and 50 μg of M's IgG per milliliter all enhanced thyrotropin binding and 100 and 500 μg of DeL's IgG per milliliter were inhibitory to binding. Mixing IgG as 12.5 μg M's plus 25 μg DeL's, or 25 μg M's plus 100 μg DeL's resulted in intermediate ef-

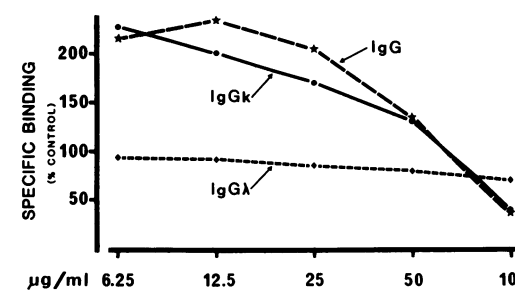

FIGURE 3 TBI assay. IgG from M's serum was isolated and separated into fractions containing only κ-light chains (IgGκ) and only λ-light chains (IgGλ) and tested at the concentrations shown. The data are shown as the means of closely agreeing triplicates and the specific binding is expressed as the percentage of that obtained (control) with buffer. There was no effect of normal IgG at these concentrations.

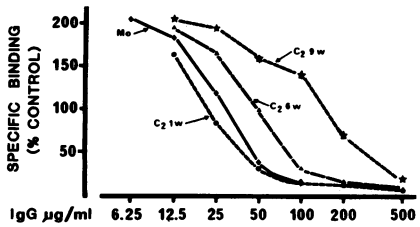

FIGURE 4 TBI assay. IgG was prepared from sera obtained from the child (C2) at 1, 6, and 9 wk (w) and from the mother at term (Mo) and assayed at the concentrations shown. Samples were diluted with normal IgG so that the total IgG concentration at each point was 500 µg/ml. The data are shown as the means of closely agreeing triplicates and the specific binding is expressed as the percentage of that obtained in the presence of buffer.

fects on the binding; the action of 500 µg DeL's IgG was only minimally influenced by admixture with M's IgG in the assay.

The binding-enhancing effect of M's IgG occurred only with human thyroid membranes in particulate form; there was only inhibition of binding with guinea pig fat cell membranes, particulate or solubilized, and with soluble thyroid membranes.

DISCUSSION

The initial report (3) of M and C1 indicated that neonatal Graves' disease had occurred because of the transplacental passage of TSAb but that the mother's serum contained neither LATS nor LATS-P activity; the conclusion was that TSAb was "distinct from LATS and LATS-P." Such a finding is in direct opposition to our belief that these activities, LATS and LATS-P, are intrinsic to TSAb and merely reflect a different method of assay (12, 13). We have shown that the LATS assay, which implies an interaction of the human antibody with a murine antigen, is more likely to be positive the more active the TSAb-IgG is in the human thyroid slice assay system (14). Therefore, a negative LATS assay with the extremely potent M's TSAb was most surprising. Regarding LATS-P, results of that assay have been shown to correlate closely with the development of neonatal Graves' disease (15), so that the negative LATS-P assay in the face of severe neonatal thyrotoxicosis was also difficult to accept. The results given in the present report, however, enable us to rationalize the apparent discrepancies and thus maintain the concept of the unity of TSAb, LATS, and LATS-P.

The biphasic effect of M's IgG in the TSAb assay, i.e., an initially positive dose-response relationship followed by a negative effect with higher concentrations of IgG, suggested to us that there was perhaps an inhibitor of TSAb in the IgG. The data obtained with the LATS assay support that view. The inhibition of DeL's LATS-IgG (Fig. 2) by M's IgG is compatible with the presence of an IgG that would inhibit M's own TSAb- (or LATS-) IgG when injected at the concentrations used.

The "uncovering" of LATS-P activity by dilution of M's serum is analogous to our experience with the thyroid slice system. The final value of 1,300 U/ml serum is amongst the highest in our experience (15). It should be emphasized that, in LATS-P-positive serum samples, dilution normally lessens the LATS-P activity. These data further support the concept of M's IgG containing an inhibitor to TSAb, as well as the stimulator itself. The LATS-P assay relies upon competition between TSAb in the test serum and a standard preparation of LATS for binding to human thyroid membranes; binding of the TSAb prevents, or "protects," the binding of the standard LATS (6). The concentration of standard LATS that remains unbound by the thyroid membranes, i.e., is in the supernatant solution when the membranes are sedimented, is assessed in the mouse bioassay. The postulated inhibitor of TSAb ought to "protect" the standard LATS, as well as TSAb, from being bound to thyroid membranes; however, it is safe to assume that significant quantities of the putative inhibitor also remain in the supernatant fluid and, when injected into the bioassay mouse, will inhibit LATS, as documented with the other LATS preparation described here (Fig. 2). Presumably, progressive dilution of M's serum not only diluted the inhibitor to permit binding of the very potent TSAb, and thus uncover LATS-P activity (Table II), but it also sequentially reduced the effect on the standard LATS that was assayed in the mouse.

The concept of the coexistence of TSAb-IgG and an inhibitor of that activity in M's IgG is further supported by C2's clinical course. This child did not become thyrotoxic until 45 d of age and our postulate is that the inhibiting IgG prevented TSAb from producing hyperthyroidism earlier. Assuming a critical minimum ratio of inhibitor to TSAb, the inhibitor may have been cleared more rapidly than the TSAb, thus explaining the delay in the onset of hyperthyroidism. Alternatively, if TSAb had a higher affinity than had the inhibitor for their relative antigens, the gradual clearance of both antibodies might result in TSAb's action eventually becoming dominant. Indeed, the data in Table I indicate that at certain concentrations the whole IgG becomes stimulatory, lending support to this postulate. The TSAb assay data we obtained with serial sera from C2 (Fig. 1) are also compatible with this concept in that the negative dose-response relationship was no longer seen with IgG obtained after the development of hyperthyroidism.

As previously reported (11), TSAb activity may be associated with IgG of restricted heterogeneity, characterized in those initial studies in seven of eight in-

stances by IgGλ. M's IgG was the eighth and was found to have TSAb restricted to IgGκ; judging from both TSAb and TBI assays, the inhibitory activity is similarly restricted.

Despite our emphasizing the apparent inhibitory nature, judging from TSAb, LATS, and LATS-P assay data, of the activity in M's IgG, the obvious effect in the TBI assays was enhancement of ^{125}I-TSH binding. Since TSAb, as studied with the IgG of the usual patient with Graves' disease, is an inhibitor of TSH-binding to its receptor, the inhibitory component of the dose-response slope of M's IgG in the TBI assay may be due to TSAb itself or to the putative inhibiting IgG. That is, we may view the factor that undoubtedly influences TSAb activity as akin to cholera toxin; this peptide also affects the action of TSAb (16) but in TBI assays it enhances TSH-binding at low concentrations and inhibits at high concentrations (17, 18). A hypothesis for these findings with cholera toxin, that may be equally applied to the relevant effects of M's IgG, is that at low concentrations there is perturbation of the thyroid plasma membrane to expose additional binding sites and at high concentrations a greater degree of perturbation results in binding sites being less available for the ligand (17, 18). Support for this view may also be seen in the fact that the TSH binding-enhancing activity of M's IgG occurred only with intact membranes and not with preparations of TSH receptor in which the integrity of the membrane had been disrupted by the action of detergent.

A further implication of the TBI assay data is that the other, i.e., non-TSAb, antibody in M's IgG is specific, in that binding to the TSH-receptor in guinea pig fat cell membranes was not enhanced, even in the particulate preparation. Acquiring proof for this and other postulates above would be greatly facilitated by separation of TSAb and its inhibitor. The prevalence and clinical significance of this inhibitor in the population of patients with autoimmune thyroid disease has still to be evaluated.

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