

THE ROLE OF SELF-ANTIGEN IN THE DEVELOPMENT OF  
AUTOIMMUNITY IN OBESE STRAIN CHICKENS  
WITH SPONTANEOUS AUTOALLERGIC THYROIDITIS\*

BY L. C. PONTES DE CARVALHO, JANE TEMPLEMAN, G. WICK, AND  
I. M. ROITT

*From the Department of Immunology, Middlesex Hospital Medical School, London W1, and the Institute for  
General and Experimental Pathology, University of Innsbruck Medical School, Innsbruck A6020*

It has been taken for granted that spontaneous autoimmunity is both triggered and maintained by antigen. However, the existence of antigen-independent mechanisms for the stimulation of immune responses implies that this assumption should be questioned. For example, there is evidence that B cells from New Zealand black (NZB) mice, which develop spontaneous autoimmune hemolytic anemia, are in a stimulated state 4 mo before the establishment of the autoimmune process (1). This does not appear to be due to abnormal T cell function, because it is also observed in athymic nude mice with an NZB background (1). The mechanism is presumably antigen independent, because spleens from 4-wk-old NZB mice contain an increased number of spontaneous plaque-forming cells directed against haptens such as trinitrophenyl (1) and fluorescein (2). Furthermore, the demonstration of anti-idiotypemediated helper or amplifier mechanisms (3, 4) and the evidence for direct stimulation of B cells by anti-idiotypic antibodies (3) raise the possibility of a perturbation in the immunological network actively leading to autoimmunity through the proliferation of autoreactive clones.

We therefore decided to examine the role of autoantigen in spontaneous autoimmunity and chose the Obese strain (OS)<sup>1</sup> of White Leghorn chickens as the model for study. These animals regularly develop an autoallergic thyroiditis, which closely mimics Hashimoto's disease in the human with respect to its histological and serological features (5). In particular, a high proportion of OS chickens have circulating autoantibodies to the thyroid-specific protein, thyroglobulin (Tg), and it is possible to investigate the dependence of these antibodies on the presence of autoantigen by surgical removal of the thyroid at hatching, the animals being maintained on a thyroid hormone dietary supplement. This model also enabled us to study the possibility that the production of Tg antibodies (Tg-Ab), whether or not initiated by the thyroid gland autoantigen, might nevertheless be maintained by an antigen-independent mechanism, involving, for example, idiotypic interactions, because we could investigate the effect of adult thyroidectomy on Tg-Ab levels in birds with an already established autoimmune response.

An additional aspect of the role of antigen in the development of spontaneous

\* Supported by the Austrian Scientific Research Fund, project 4114.

<sup>1</sup> *Abbreviations used in this paper:* OS, Obese strain; PBS, phosphate-buffered saline; SAT, spontaneous autoimmune thyroiditis; Tdx, thyroidectomized; Tg, thyroglobulin; Tg-Ab, thyroglobulin antibodies.

autoallergic thyroiditis is also dealt with in this paper. Embryonic OS chicken thyroids display an increased iodine uptake when compared with normal White Leghorn (NWL) controls (6), which suggests some primary abnormality of the thyroid gland. Conceivably, this could result in the release of an abnormal Tg molecule, which would be one of the factors involved in the pathogenesis of spontaneous autoimmune-thyroiditis (SAT). To see whether Tg of the OS was an absolute requirement for the formation of Tg-Ab, thyroidectomized (Tdx) OS chickens, negative for serum Tg-Ab, were immunized with soluble normal chicken Tg. Preparations of Tg from OS chickens and their normal counterparts were also compared using conventional immunochemical procedures. Our results show that spontaneous thyroid autoimmunity in OS chickens is dependent upon exposure to normal thyroglobulin.

### Materials and Methods

**Animals.** OS and NWL chickens were raised under standardized conditions (7) in the Institute for General and Experimental Pathology, Innsbruck, Austria. Experimental and control OS groups were matched for major histocompatibility antigens (B locus).

**Thyroidectomy.** Neonatal thyroidectomy was performed within 1–2 d after hatching. Adult thyroidectomy was performed on 3-mo-old birds. The thyroid lobes were carefully detached from the jugular veins with two fine forceps under a dissecting microscope. The perijugular and common carotid regions were then aspirated by a vacuum pump with a blunt-edged pasteur pipette (2.5-mm Diam) bent through 60°, until no thyroid remnants could be seen. Some control chickens were hemithyroidectomized (only one of the two thyroid lobes removed) or sham-thyroidectomized (by exposing the thyroid, and then closing the wound without removing the gland). All animals were given 4.5 µg thyroxine/kg of feed throughout the experiment, and tetracycline, 2 g/liter of drinking water during the first 2 d and 1 g/liter for the next 8 d. The effectiveness of thyroidectomy was evaluated in NWL chickens thyroidectomized 2–10 d after hatching and not treated with thyroxine. These chickens showed: (a) severe impairment of body development, and (b) decreased iodine uptake (Table I). Some of them died with signs of myxedema before appropriate thyroid hormone replacement therapy could be initiated. All OS chickens that had undergone total thyroidectomy were carefully checked for thyroid remnants by histological examination of the tissue juxtaposed to the jugular vein near the angle formed by the divergence of the subclavian and common carotid arteries after fixation in 4% buffered formaldehyde pH 7.4, sectioning in 8–10 planes, and staining with hematoxylin-eosin.

**Thyroglobulin.** Crude thyroid extracts were prepared in 0.15 M phosphate-buffered saline (PBS), pH 7.2 from pools of four 1-d-old NWL or OS chick thyroids (not yet infiltrated by leukocytes at this time) or from adult NWL chicken thyroids (Pel-Freeze Biologicals, Rogers, AR). Thyroglobulin was purified by precipitation between 37% and 45% saturation with

TABLE I  
*Impairment of Growth and Iodine Uptake in Tdx NWL Chickens*

Experiment	Treatment	Number of animals	Body weight	Iodine uptake
1*	Tdx	5	136 ± 48‡	0.49 ± 0.21§
	Sham-Tdx	3	224 ± 39	1.94 ± 0.42
2	Tdx	5	178 ± 31	0.49 ± 0.08
	Sham-Tdx	7	315 ± 66	2.62 ± 0.89

\* Measurements performed 4.5 wk after hatching.

‡ Mean ± SD in grams.

§ Ratio between radioactivity measured over thyroid region (neck and upper thorax) and radioactivity over abdomen 16 h after intraperitoneal injection of 1 µCi (<sup>131</sup>I)NaI in 0.2 ml isotonic saline.

|| Measurements performed 7 wk after hatching.

ammonium sulphate and gel filtration on Sepharose 6B as described previously (8). The protein was radiolabeled with  $^{125}\text{I}$  (NaI, Radiochemical Centre, Amersham, England) by the method of Fraker and Speck (9), which uses 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (iodogen) as an oxidizing agent. 20 nmol of iodogen dissolved in 50  $\mu\text{l}$  dichloromethane was allowed to evaporate in each plastic iodination vial with occasional stirring to increase the area of plastic coming into contact with the reagent. 50  $\mu\text{g}$  of normal or OS Tg in 200  $\mu\text{l}$  PBS and 500  $\mu\text{Ci}$  of  $^{125}\text{I}$  were added to each iodination vial and incubated at room temperature for 10 min with mixing every minute. This was followed by Sephadex G-25 chromatography, in an 8-ml column pre-equilibrated with PBS containing 0.4% bovine serum albumin, to remove the free iodine. The specific activities were 5.8 and 3.8  $\text{Ci}\cdot\text{g}^{-1}$  for normal and OS Tg, respectively, which corresponded to 1.3 and 0.9  $^{125}\text{I}$  atoms per Tg molecule.

**Serology.** Serum levels of Tg-Ab were determined either by hemagglutination of Tg-coated sheep erythrocytes (results expressed as  $\log_2$  of highest serum dilution giving a positive reaction) (10) or by an immunoradiometric assay using Tg bound to plastic tubes as solid phase (8). Sera were centrifuged (1,500  $g$  for 15 min) before assay.

**Antisera.** Sera from 2-6-mo-old OS chickens with high titers of antibodies as determined by hemagglutination were selected as a source of autoantibodies for the immunochemical comparison of normal and OS Tg. Rabbit antiserum to OS thyroid extract was raised by 2 intramuscular injections of 0.4 mg Tg in complete Freund's adjuvant (1.5 mg *Mycobacterium tuberculosis* per ml emulsion) separated by a 12-d interval. These were followed 10 wk later by a booster consisting of 0.3 mg Tg in alum subcutaneously. The animals were bled 12-26 d after the last injection. Anti-chicken immunoglobulin (Ig) was produced in rabbits by immunization with 1 mg chicken serum fraction II (Miles Laboratories Ltd., Slough, England) in complete Freund's adjuvant intramuscularly, followed by two boosters consisting of 0.2 mg alum-precipitated fraction II injected subcutaneously at 2-wk intervals.

**Investigation of the Proportion of Iodinated Thyroid Protein with Antigenic Activity by Double Antibody Immunoradiometric Assay.** All immunoradiometric assays were carried out using PBS containing 0.4% bovine serum albumin, 0.05% Tween 20, and 0.05% sodium azide. Different volumes of OS (OS 4441) or NWL serum (0.04, 0.08, 0.16, 0.32, 0.64, and 1.28  $\mu\text{l}$ ) were incubated with fixed amounts of  $^{125}\text{I}$ -labeled normal Tg (25 ng) or labeled OS Tg (25, 44, or 48.5 ng) in LP3 (Luckham Ltd., Sussex, England) tubes for 1 h at 37°C, followed by 1 h at 4°C. The total reaction volume at this point was 0.4 ml. To this, 0.1 ml of a 1:4 dilution of rabbit anti-chicken Ig was added (final dilution was 1:20), and the reagents further incubated for 16 h at 4°C under constant mixing. After centrifugation at 700  $g$  for 20 min, the precipitate was washed once with  $\sim 2$  ml cold PBS, and the radioactivity bound determined in a gamma counter (LKD Instruments Ltd., Surrey, England). 5 ng of  $^{131}\text{I}$ -labeled normal chicken Ig was added to the assay as an internal control to determine the efficacy of the precipitation. In another experiment, various pooled thyroid extracts were labeled and allowed to react with pooled sera from eight OS chickens, using the conditions described above to estimate the proportion binding to autoantibody.

**Immunoabsorption of Sera.** A solid-phase immunosorbent of normal chicken Tg was prepared by incubating 28.6 mg of the antigen in 2.75 ml PBS with 4 ml of Sepharose 6B preactivated with cyanogen bromide, by the method of Axen et al. (11). The suspension was mixed on a magnetic stirrer for 16 h at 4°C, and the solid phase was washed on a glass scinter with 100 ml PBS. Approximately 3.5 mg of Tg was coupled to each ml of Sepharose 6B. 1 ml of OS serum or 0.1 ml of rabbit anti-OS thyroid extract together with 0.4 ml of normal rabbit serum were applied to the solid phase Tg in a 5-ml plastic syringe and allowed to pass through under gravity. This was repeated once or twice, after elution of the bound protein with 0.1 M HCl-glycine buffer, pH 2.8. The solid-phase Tg was found to retain its full antigenic characteristics after incubation with this buffer for 16 h. All absorbed sera were concentrated to their original concentrations with Aquacide II-A (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA). The antigen-binding ability of the absorbed OS sera was determined by incubating 0.1  $\mu\text{l}$  of serum with 25 ng  $^{125}\text{I}$ -labeled normal Tg or with 48.5 ng labeled OS Tg, and carrying out the precipitation with rabbit anti-chicken Ig as described above in the immunoradiometric assay. The absorbed rabbit anti-OS Tg (diluted 1:1000) was tested in a solid-phase immuno-

radiometric assay using Tg-coated plastic tubes and a purified goat anti-rabbit Ig antibody labeled with  $^{125}\text{I}$  (8).

*Competition between OS and Normal Tg for Homologous Antibody.* 0.1- $\mu\text{l}$  samples of OS serum were incubated with varying amounts of unlabeled normal Tg (0, 0.05, 0.1, 0.2, 0.4, 4, 40, or 80  $\mu\text{g}$ ) for 1 h at 37°C, followed by 1 h at 4°C in a total volume of 0.3 ml. 0.1 ml of buffer containing 25 ng  $^{125}\text{I}$ -labeled normal Tg or 48.5 ng  $^{125}\text{I}$ -labeled OS Tg (giving equivalent amounts of antigenically active labeled protein; see Results) was added to each tube, and the reagents were incubated for 3 h at 4°C, followed by 16 h at 4°C in the presence of 0.1 ml of rabbit anti-chicken Ig (final dilution: 1:20). The precipitation of the chicken Ig and assessment of bound radioactive Tg were performed as described above.

## Results

*Tg Autoantibody Response in Neonatally Tdx Chickens.* OS chicks were surgically thyroidectomized at hatching to see whether the presence of antigen was necessary for the development of Tg autoantibodies. Only 4 out of a total of 33 OS chickens thyroidectomized in three different experiments had detectable Tg-Ab in the circulation, as measured by hemagglutination (experiments 1, 2, and 3) and by immunoradiometric assay (experiment 1) 7–10 wk after hatching (Table II). This incidence was significantly lower ( $P = 0.00018$  by Fisher's exact probability test) than the incidence in control groups (47 Tg-Ab positive animals out of 95). Two of the thyroidectomized groups with Tg-Ab were found to have thyroid remnants. Neither sham-thyroidectomy, hemithyroidectomy, nor sham-hemithyroidectomy affected the incidence or levels of circulating Tg-Ab. In early bleedings (5 wk after hatching in experiment 2, and 4 wk after hatching in experiment 3), however, Tg-Ab were detected in some Tdx chickens in amounts which, although apparently lower, were not statistically different from those of control sham-Tdx animals (Figs. 1 and 2). All but 4 of the thyroidectomized animals and, surprisingly, 5 of the 27 positives in the

TABLE II  
Effect of Neonatal Tdx on Autoantibody Formation in OS Chickens

Experiment	Treatment	Tg autoantibodies	
		Incidence	Titer ( $\log_2$ ) of positive sera (mean $\pm$ SD)
1*	Tdx	0/5 $\ddagger$	<1
	None	18/25	3.8 $\pm$ 2.9
2 $\parallel$	Tdx	1/15	3
	Sham-Tdx	11/24	5.0 $\pm$ 2.4
	Hemi-Tdx	6/9	5.8 $\pm$ 2.6
	Sham-hemi-Tdx	7/9	4.3 $\pm$ 2.7
	None	5/12	4.6 $\pm$ 1.9
3 $\nabla$	Tdx	3 $\ast\ast$ /13	2.0 $\pm$ 1.0
	Sham-Tdx	11/16	3.7 $\pm$ 2.8

\* Tg-Ab measured 10 wk after thyroidectomy.

$\ddagger$  Number of animals with Tg-Ab (hemagglutination)/total number in group.

$\S$   $P$  values calculated by Fisher's exact probability test.

$\parallel$  Tg-Ab measured 9 wk after thyroidectomy.

$\nabla$  Tg-Ab measured 7 wk after thyroidectomy.

$\ast\ast$  Two of these animals had thyroid remnants.

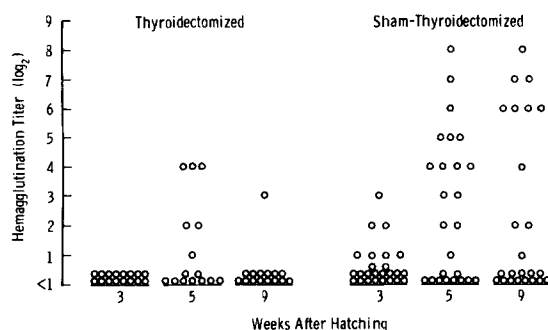


FIG. 1. Levels of autoantibodies to Tg in neonatally Tdx and sham-Tdx OS chickens. Each circle represents the Tg-Ab level in one individual chicken. The figures at 9 wk after surgical treatment correspond to the data presented in Table II, experiment 2.

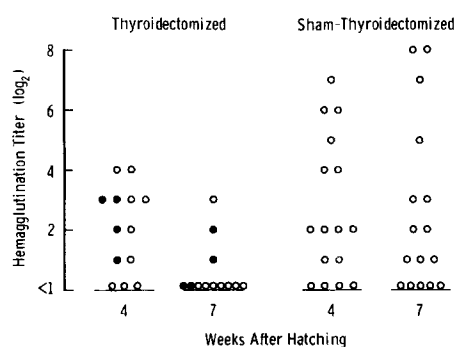


FIG. 2. Levels of autoantibodies to Tg in neonatally Tdx and sham-Tdx OS chickens. The figures at 7 wk after surgical treatment correspond to the data presented in Table II, experiment 3. ●, animals with thyroid remnants.

sham-thyroidectomized group, became negative at a later bleeding.

*Tg Autoantibody Response in Adult Tdx Chickens.* To investigate the possibility that once established, autoantibody production could be maintained by some antigen-independent mechanism such as anti-idiotypic stimulation, 3-mo-old birds were surgically thyroidectomized, and serum antibody titers were measured 100 d after the operation. The levels of Tg-Ab in Tdx chickens were significantly lower than in control sham-Tdx animals ( $P < 0.05$  by paired Student's  $t$  test;  $P < 0.05$  by Wilcoxon's rank sum test of the changes of hemagglutination titers in individual animals, i.e., titer before minus titer after surgery). Five out of seven Tdx birds had no detectable Tg-Ab, whereas only two out of eight control animals were negative for Tg-Ab (Fig. 3).

*Immunization of Neonatally Tdx OS Chickens with Normal Chicken Tg.* Presumably, autoantibody production in the OS is a consequence of an unusually responsive immune system, or an abnormal antigen, or both. The neonatally Tdx chicks that failed to produce Tg-Ab provided an ideal system for the elucidation of this problem. Accordingly, Tdx and control animals received 1 mg Tg in saline/kg body weight intravenously followed by identical boosters at 1–2-wk intervals. Four out of five Tdx OS chickens that received two intravenous injections of soluble normal chicken Tg produced circulating Tg-Ab 3 wk after the first injection (Fig. 4, experiment 1). Treatment of nine NWL chickens with the same immunization schedule did not lead to Tg-Ab formation.

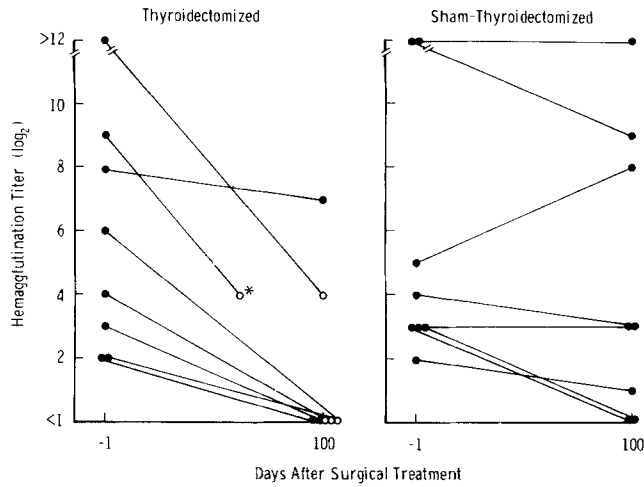


FIG. 3. Levels of autoantibodies to Tg in adult Tdx or sham-Tdx OS chickens. ●, animals with thyroid remnants; \*, animal died before completion of experiment.

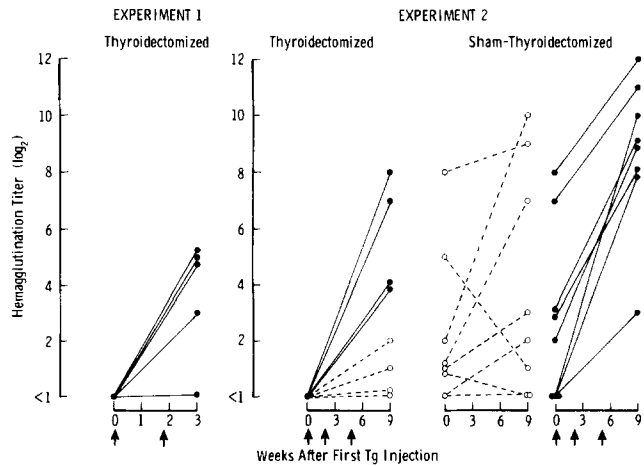


FIG. 4. Immunization of neonatally Tdx OS chickens with normal chicken Tg. Arrows on horizontal axis indicate intravenous injections with soluble Tg (1 mg Tg/kg body weight). First injection in experiment 1 given 10 wk after Tdx; in experiment 2, 7 wk after Tdx or sham-Tdx. ●, immunized animals; ○, nonimmunized controls.

In a second experiment, three injections of soluble normal Tg significantly increased the levels of Tg-Ab in four Tdx chickens when compared with control nonimmunized Tdx chickens ( $P < 0.01$ , Student's  $t$  test;  $P < 0.05$ , Wilcoxon's rank sum test of the changes of hemagglutination titers in individual animals; Fig. 4, experiment 2). Immunization of sham-Tdx chickens similarly increased the serum levels of Tg-Ab when compared with nonimmunized sham-Tdx animals ( $P < 0.02$ , paired Student's  $t$  test;  $P < 0.05$ , Wilcoxon's rank sum test of the changes of hemagglutination titers in individual animals; Fig. 4, experiment 2).

*Immunochemical Analyses of OS and Normal Tg.* These studies show that normal Tg is capable of provoking autoantibodies in OS birds, and it seems unlikely that the Tg in these birds is structurally abnormal. We carried out a further series of experiments to

see whether OS Tg can be differentiated from the normal by immunochemical means. Thyroid extracts from normal and OS thyroids were iodinated and allowed to react with a high titer OS serum. The proportion of labeled protein binding to autoantibody was higher in the extracts prepared from normal birds (Table III). However, in each case the binding was reduced to background levels if an excess of unlabeled normal Tg was added. The same relationship held when purified Tg preparations were labeled, the OS serum precipitating a higher proportion of radioactivity in the normal as compared with OS Tg. On the other hand, when the amount of OS Tg added to each tube was increased so that comparable total amounts of active (precipitable) Tg were present in the OS and in the normal preparations, the precipitation curves obtained with both preparations on diluting the antiserum could be superimposed (Fig. 5); thus, although a smaller fraction of the OS Tg is antigenic, the two preparations are qualitatively indistinguishable.

That the Tg autoantibodies in OS serum do not appear to recognize extra determinants on OS as compared with normal Tg is evident from the data presented in Table III and by the finding that five OS sera, with initial binding capacities ranging from 1.1 to 4.7  $\mu\text{g}$  Tg/ml, after passage through normal Tg immunosorbents, gave values of  $0.5 \pm 0.1$   $\mu\text{g}$  Tg/ml (mean  $\pm$  SD); this compares with the binding capacity of six control NWL sera of  $0.6 \pm 0.3$   $\mu\text{g}$  Tg/ml. Furthermore, in a more discriminating study, it was found that preincubation of six different OS sera with

TABLE III  
*Binding of OS and Normal Chicken Thyroid Extras to OS Serum and its Blocking by an Excess of Normal Chicken Tg*

Experiment	Thyroid extract		Percent bound to OS serum*		Percent bound to OS serum in presence of normal Tg	Percent bound to NWL serum
	Strain of chicken	Number of thyroids in pool	Individual values	Mean $\pm$ SD		
1	NWL outbred	4	44.5		2.6	2.8
2	NWL outbred	4	39.5		2.8	2.4
3	NWL outbred	4	39.4	$34.7 \pm 8.3\ddagger$	1.4	1.5
4	NWL outbred	12	29.5		2.9	2.3
5	NWL B21B21	4	21.4		1.6	2.1
6	NWL B21B21	4	34.0		2.7	2.7
7	OS B3B3	4	24.5		2.6	3.8
8	OS B3B3	4	25.2		2.4	2.4
9	OS B3B3	4	24.1		3.4	4.0
10	OS B1B3	4	29.3		2.4	2.0
11	OS B1B1	4	26.5	$25.6 \pm 3.6\ddagger$	2.6	2.7
12	OS B1B4	4	23.1		2.9	2.9
13	OS B4B4	3	19.2		3.5	3.1
14	OS random	22	27.2		4.1	3.8
15	OS random	11	31.6		3.4	3.5

\* 25 ng of each thyroid extract preparation was incubated with 0.16  $\mu\text{l}$  of a pool of sera from eight 2-3-month old OS chickens.

‡ Values differ significantly ( $P < 0.02$  by Student's  $t$  test).

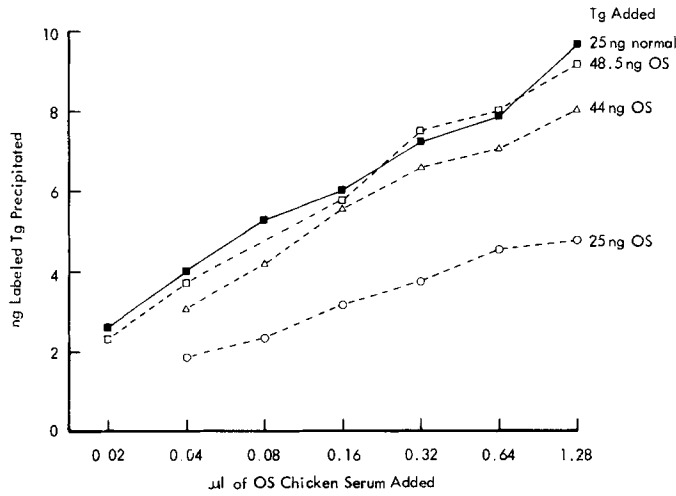


FIG. 5. Binding of  $^{125}\text{I}$ -labeled normal and OS Tg to increasing volumes of OS 4441 serum. Values are corrected for the amount precipitated in control tubes in which normal is substituted for OS serum. The amount of Tg added to each tube is shown at the right of the curves. Note superimposition of curves obtained with 25 ng of normal Tg and with 48.5 ng OS Tg.

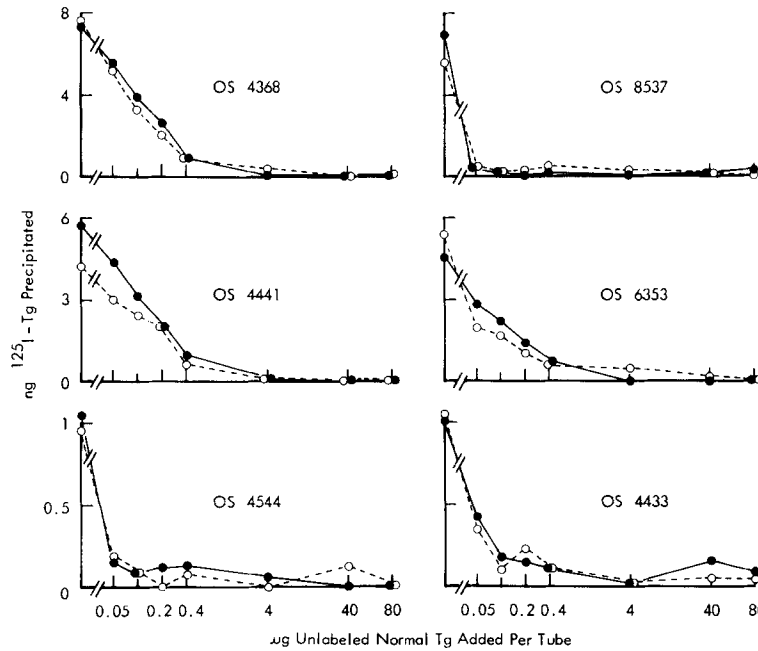


FIG. 6. Competition between unlabeled normal Tg and  $^{125}\text{I}$ -labeled normal Tg (●) or  $^{125}\text{I}$ -labeled OS Tg (○) for OS autoantibodies. Fixed volumes ( $0.1 \mu\text{l}$ ) of six different OS sera were preincubated with varying amounts of unlabeled normal Tg before being allowed to react with the  $^{125}\text{I}$ -labeled proteins.

increasing amounts of unlabeled normal Tg gave rise to closely similar patterns of inhibition of binding to labeled normal and OS Tg preparations (Fig. 6).

After passage of rabbit anti-OS thyroid extract over a normal Tg immunosorbent



column, the binding capacity for OS thyroid extract, as measured by a solid phase immunoradiometric assay, fell from 354  $\mu\text{g}$   $^{125}\text{I}$ -goat anti-rabbit Ig/ml to a value of 7.6  $\mu\text{g}$ ; likewise, the binding capacity for normal thyroid extract was reduced from 286 to 10.9  $\mu\text{g}$   $^{125}\text{I}$ -goat anti-rabbit Ig/ml. This compares with binding capacities for OS and normal thyroid extracts of 11.6 and 6.9  $\mu\text{g}$ , respectively, obtained with a normal rabbit serum. Because rabbit antisera are generally considered to be directed against more epitopes than autoantisera (12), this provided further evidence that neither protein is deficient in immunogenic determinants relative to the other. As found with the homologous Tg-Ab, this rabbit antiserum also precipitated a larger proportion of NWL thyroid extracts than of OS thyroid extracts.

### Discussion

A useful feature of the spontaneous thyroiditis model provided by the OS chicken is the relative ease with which the role of autoantigen can be investigated. Using the same experimental procedure, i.e., thyroidectomy, three different aspects of the immune response to Tg in OS chickens have been approached in this study: (a) the requirement of the tissue autoantigen for triggering autoimmunity, (b) the necessity for the presence of autoantigen in the maintenance of autoimmunity, and (c) the autoimmunogenicity of normal Tg in OS birds.

We feel that our results deal with the first point very convincingly: in the absence of the thyroid gland, no Tg-Ab could be detected in 29 out of a total of 33 Tdx birds 7–10 wk after hatching. Some Tdx animals, however, did show Tg-Ab in the circulation 4 or 5 wk after hatching, which disappeared after a further 3–4-wk interval. This initial, transient autoantibody response could be explained by stimulation of self-reactive lymphocytes by the Tg that would have been released during the process of surgical removal of the thyroid glands. In the absence of further antigenic stimulation, the autoantibody response would appear to subside. Alternatively, thyroid remnants could maintain the autoantigenic stimuli until they were destroyed by the host's immune system: imperfect thyroidectomy would be completed *in vivo*. Indeed, although the thyroidectomies seemed in many cases to be complete, judging by the severe impairment of development of Tdx normal chickens and by their inability to survive without thyroid hormone-replacement therapy, in a few animals thyroid remnants were found. A maternal origin for the Tg-Ab in the 5-wk-old Tdx chicks of experiment 2 can be excluded, because those animals had no detectable Tg-Ab 3 wk after hatching (Fig. 1).

The fact that the group of chickens subjected to neonatal hemithyroidectomy had a normal incidence of Tg-Ab argues against the possibility that an abnormal release of Tg into the circulation during the surgical procedure could have tolerized the Tdx OS animals. Neither could the failure to make Tg-Ab be attributed to a nonspecific immunological defect caused by loss of the thyroid, because these animals were maintained on thyroid hormone and in any case, were shown to make these antibodies when injected with Tg.

Removal of the thyroid gland in adult OS chickens with established disease produced a sharp fall in Tg-Ab, in most cases to undetectable levels, indicating that Tg is of importance not only for triggering but also for maintaining the autoantibody response. Thus, our findings argue against the hypotheses that (a) nonspecific stimulation of self-reactive lymphocytes accounts for the development of the autoimmune

response to Tg, and (b) stimulatory anti-idiotypes, perhaps of maternal origin, or generated spontaneously within the immune network, would cause or contribute to that autoimmune response.

The fact that neonatally Tdx OS chickens produced Tg-Ab on immunization with soluble Tg derived from normal birds would suggest that normal and OS Tg molecules share the antigenic determinants involved in the development of OS autoimmunity, and that no abnormality in the structure of OS Tg need be postulated to account for Tg-Ab production. However, OS chickens might respond to normal Tg for two reasons quite unrelated to their autoimmune condition: (a) the response could be due to allotypic antigenic determinants, i.e., the response would not be autoimmune, and (b) the response could be due to a loss of tolerance resulting from the complete absence of Tg from the body fluids for a relatively prolonged interval before immunization, because tolerance to Tg has been ascribed to the constant presence of low concentrations of the protein in the circulation ("low dose" tolerance; 13, 14). This last point could be settled by immunization of neonatally thyroidectomized normal chickens with normal Tg but so far, in a preliminary experiment, we have failed to detect Tg-Ab under such circumstances.

For these reasons it seemed worthwhile to investigate possible differences between OS and normal Tg by other means. Thus, preabsorption of five OS sera with normal chicken Tg completely abolished the specific binding to OS Tg, whereas the reactions of nine different OS thyroid extracts with one OS chicken serum were completely blocked by the addition of an excess of normal Tg. It would therefore appear that normal chicken Tg had autoantigenic determinants similar or identical to all those recognized on the OS Tg molecule. Additional support for this view comes from the finding that OS and normal Tg gave identical precipitation curves with varying dilutions of OS serum (Fig. 5) and virtually identical patterns of inhibition of binding in blocking studies with increasing amounts of unlabeled normal Tg (Fig. 6), implying that there are neither quantitative autoantigenic differences, because OS and normal Tg molecules display the same number of epitope specificities, nor qualitative differences, in that the avidity characteristics of all six OS chicken sera tested appeared to be the same for both OS and normal Tg. Thus, insofar as the autoantigenicity of Tg is reflected in the autoantibody response, i.e., by the different B cells stimulated by it, normal and OS Tg would seem to be identical. In addition, our results with the heterologous antiserum to an OS Tg preparation, which failed to detect any private antigenic specificities, make it unlikely that there are differences involving determinants recognized only by helper or cytotoxic T cells but not by B cells. Therefore, putative abnormalities of OS Tg do not appear to contribute to the development of thyroiditis.

The finding that a smaller proportion of the protein in the OS chicken Tg preparations was antigenic when compared with normal Tg (Table III) could reflect real quantitative or qualitative differences in the proteins present in normal or OS thyroids: alternatively, it could be accounted for by different susceptibilities to damage during *in vitro* preparation. Although the evidence overwhelmingly suggests that there are no abnormal features of OS Tg itself that contribute to the development of thyroiditis, other circumstances concerning the antigen, such as the concentration at which it circulates and its mode of presentation by accessory cells, could affect the state of responsiveness of the immune system.

### Summary

Neonatal thyroidectomy of Obese strain (OS) chickens showed that the spontaneous development of thyroid autoimmunity in these animals was fully dependent upon the presence of autoantigen, and could not be ascribed essentially to antigen-independent mechanisms such as polyclonal lymphocyte activation or innate distortions within the idiotypic network. Similarly, removal of the gland in animals with established thyroiditis demonstrated the need for antigen to maintain the autoimmune response. Thyroglobulin from normal chickens induced autoantibodies in neonatally thyroidectomized OS birds, suggesting that an abnormality in the structure of this protein is not a prerequisite for the development of autoimmunity. This contention is supported by the finding that OS and normal thyroglobulin were immunochemically indistinguishable, whether compared using OS autoantibodies or rabbit anti-chicken thyroglobulin sera.

We gratefully acknowledge the technical assistance of Mrs. Pia Ulrike-Müller.

*Received for publication 30 November 1981.*

### References

1. Chused, M. T., H. M. Moutsopoulos, S. O. Sharrow, C. T. Hansen, and H. C. Morse. 1978. Mechanism of autoimmune disease in New Zealand black mice. *In Genetic Control of Autoimmune Disease*. N. R. Rose, P. E. Bigazzi, and N. L. Warner, editors. Elsevier/North-Holland, New York. 177-190.
2. Izui, S., and F. J. Dixon. 1978. Spontaneous B cell activation and autoantibody formation in autoimmune mice. *Fed. Proc.* **37**:1373.
3. Eichmann, K. 1978. Expression and function of idiotypes on lymphocytes. *Adv. Immunol.* **26**:195.
4. Cooke, A., and P. M. Lydyard. 1981. The role of T cells in autotimmune diseases. *Pathol. Res. Pract.* **171**:173.
5. Wick, G., R. Boyd, K. Hala, L. de Carvalho, R. Kofler, P. U. Müller, and R. K. Cole. 1981. The Obese strain (OS) of chickens with spontaneous autoimmune thyroiditis: review of recent data. *Cur. Top. Microbiol. Immunol.* **91**:109.
6. Sundick, R. S., and G. Wick. 1974. Increased iodine uptake by Obese strain thyroid glands transplanted to normal chick embryos. *J. Immunol.* **116**:1319.
7. Kite, Jr. J. H., G. Wick, B. Twarog, and E. Witebsky. 1969. Spontaneous thyroiditis in an Obese strain of chickens. II. Investigations on the development of the disease. *J. Immunol.* **103**:1334.
8. de Carvalho, L. C. P., I. M. Roitt, and G. Wick. 1980. A three-layer immunoradiometric assay for antibodies in different immunoglobulin classes and its application to the detection of chicken thyroglobulin autoantibodies and of antibodies to sheep erythrocytes. *J. Immunol. Methods.* **39**:15.
9. Fraker, P. J., and J. C. Speck. 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenyl-glycoluril. *Biochem. Biophys. Res. Commun.* **80**:849.
10. Kofler, R., and G. Wick. 1977. Some methodological aspects of the chromium chloride method for coupling antigen to erythrocytes. *J. Immunol. Methods.* **16**:201.
11. Axen, R., J. Porath, and S. Ernback. 1967. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature (Lond.)*. **214**:1302.

12. Nye, Lynn, L. C. P. de Carvalho, and I. M. Roitt. 1980. Restrictions in the response to autologous thyroglobulin in the human. *Clin. Exp. Immunol.* **41**:252.
13. Roitt, I. M., and G. Torrigiani. 1967. Identification and estimation of undegraded thyroglobulin in human serum. *Endocrinology.* **81**:421.
14. Allison, A. C. 1971. Unresponsiveness to self-antigens. *Lancet.* **II**:1401.