INTERSTITIAL IMMUNE COMPLEX THYROIDITIS IN MICE

The Role of Autoantibody to Thyroglobulin*

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Immunization of mice with cross-reacting heterologous thyroglobulin (Tg)[1] leads to termination of unresponsiveness to autologous Tg and results in production of autoantibody to Tg and thyroiditis (1). The obligatory role of antibody in the induction of thyroiditis can be shown in antigen suicide experiments; ^{125}I-labeled syngeneic Tg affects only bone marrow (B) cells and diminishes the incidence and severity of lesions (1). Furthermore, passive transfer of Tg-specific antibody produces thyroiditis in rabbits (2), guinea pig (3, 4), and mice (5). These observations are compatible with the cellular events that have been suggested to result in thyroiditis in rabbits (6) and mice (1). It has been postulated that T cells are unresponsive to autologous Tg, but B cells are fully competent and that autoantibody and thyroiditis results from stimulation of competent B cells by bypassing the need for specific T cells (7).

The mechanism by which anti-Tg autoantibody produces thyroiditis remains unclear. Therefore, the present investigations were undertaken to study the role of anti-Tg autoantibody in the production of the thyroiditis in A/J mice immunized with cross-reacting soluble heterologous Tgs. The immunologic response of the immunized mice was compared with the course of histologic, immunohistochemical, and electron microscopic thyroid alterations.

Materials and Methods

General Protocol. 6- to 8-wk old female A/J mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally (i.p.) with 0.5 mg of a mixture of soluble human, equine, and bovine

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[1] *Abbreviations used in this paper:* ABC, antigen-binding capacity; FRA, fibrinogen-related antigens; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte, Tg, thyroglobulin.

Tg daily for 5 days. A second and third series of injections were given i.p. and subcutaneously with a 2-wk interval between each course of injections. Groups of 6–10 immunized mice were sacrificed by exsanguination under ether anesthesia at appropriate times during the experiment. Tissues were removed for immunofluorescence and histological analyses. Serum from each animal was stored at −20°C. Control, sex-, and age-matched nonimmunized A/J mice were sacrificed at corresponding times.

Isolation of Thyroglobulins. The isolation and purification of Tg from bovine, equine, human, and murine thyroids have been described previously (1).

Iodination of Proteins. All proteins were iodinated by the chloramine-T method of McConahey and Dixon (8).

Histology. Portions of kidney, heart, lung, liver, spleen, pancreas, uterus, intestine, and gluteus maximus were excised and fixed in Bouin's solution for 8–16 h. Hematoxylin-eosin sections were examined for increased cellularity, particularly about vessels. Thyroid sections were scored from 0 to 4+ (1); 0 being normal and 4+ when greater than 40% of the thyroid was replaced by inflammatory cells.

Quantitation of Antibody to Mouse Tg (1). Briefly, 20 μl of antibody, serially diluted in 1:2 pooled normal mouse sera was added to 20 μl of ^{131}I-labeled murine Tg (3.85 μg N/ml) at 0.1–0.5 μCi/μg protein; 1 μg (5 μl) of ^{125}I-labeled normal mouse IgG or ^{125}I-labeled PC 5 myeloma protein (γ2aK) was added to each tube as an internal control in order to determine the amount of mouse immunoglobulin (Ig) precipitated by the coprecipitating antiserum. The tubes were held at 37°C for 1 h and overnight at 4°C; next, an appropriate volume of goat antimouse Fab (IgG) or antimouse IgG antiserum was added to each tube in order to precipitate the mouse Ig. The mixture was centrifuged and the pellet washed and counted in a dual channel gamma scintillation counter. For each dilution of antiserum, the percentages of Tg and Ig precipitated were calculated. The dilution at which 33% of the Tg was bound was ascertained from a semilogarithmic graph, and was used to calculate the antigen-binding capacity (ABC) value for each antiserum. Normal mouse serum served as a background control for each experiment with less than 2% of the Tg added being entrapped. Between 85–100% of the mouse Ig in each antiserum dilution was precipitated by 200 μl of goat antimouse Fab (IgG) or antimouse IgG. Quantitative studies on the inhibition of ABC by excess cold antigen have been presented previously (1).

Immunofluorescence. Samples of kidney and thyroid from each animal were embedded in Tissue Tek (Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.) and snap-frozen in liquid nitrogen. Frozen sections 4 μm thick were air dried, washed in phosphate-buffered saline (PBS), fixed in ether-ethyl alcohol (1:1) and ethyl alcohol (95%), and washed again in PBS. The fixed sections were stained for immunofluorescence microscopy with fluoresceinated goat antimouse Fab (IgG), rabbit antimouse albumin, and rabbit antimouse C3. Rabbit antimouse immunoglobulin heavy-chain and antimouse fibrinogen antisera (reactive with fibrinogen-related antigens [FRA], including fibrin) were used for indirect immunofluorescence with a fluoresceinated goat antirabbit IgG antisera. All antisera were demonstrated to be specific for their respective antigens by immunoelectrophoretic and double-diffusion analyses. The specificity of the mouse heavy-chain antisera was shown (titer greater than 1:40) by indirect immunofluorescence on ethanol-fixed mouse myeloma cells (S121, W33, MOPC 315, MOPC 104E, PC-5, and 183) (1). Immunofluorescence controls included the use of known negative and positive kidney sections, adsorption of the antisera with specific antigen, and prior blocking with the specific reagent unconjugated (except anti-C3). The sections were examined with a Zeiss fluorescence microscope (9) (Carl Zeiss, Inc., New York). The amount of specific immunofluorescence in the thyroid was graded 0 when no fluorescence was demonstrable, 1+ when small granular or lumpy deposits were located near the base of the follicular cells in 10–40% of the follicles, 2+ when the bases of 50–90% of follicular cells stained, and 3+ when the deposits were nearly confluent around the bases of 100% of follicular cells.

Elution of Immunofluorescent Thyroid Deposits. The effect of 0.02 M (pH 3.2) citrate buffer and 2 M NaSCN (pH 4.5) on unfixed cryostat serial sections of thyroids exhibiting Ig deposits was investigated. Some sections were incubated with elution buffers for 15–120 min, washed in PBS for 5–10 min, and stained with fluoresceinated antimouse Fab or with fluoresceinated mouse antiheterologous Tg antiserum. As controls, other sections were incubated with PBS for corresponding amounts of time and stained.

Relationship between Thyroid Follicular Basement Membrane and Immunofluorescent Deposits. The relationship between the immunofluorescence-positive deposits and the follicular basement membrane was investigated. Thyroid sections with known immune deposits were stained with a concentrated rhodamine-conjugated rabbit antimouse IgG (Cappel Laboratories, Downingtown, Pa.). The follicular basement membrane was then outlined by indirect immunofluorescence using horse antirabbit renal basement membrane antiserum and fluoresceinated rabbit antihorse IgG. These sections were examined by fluorescence microscopy using KP-546 and KP-500 excitation filters (Carl Zeiss, Inc.), coupled with appropriate barrier filters, to detect the fluorescence of rhodamine and fluorescein, respectively. The anatomical position of rhodamine-stained deposits of mouse Ig and the fluorescein-stained basement membrane could be compared and photographed.

Quantitation of Available Tg in Thyroids of Immunized and Control Mice. Guinea pig antibodies to heterologous Tg were purified by precipitation at equivalence. The complexes were washed seven times with cold PBS and redissolved in cold 0.025 M citrate buffer, pH 3.2. This solution was layered onto a G-200 Sephadex column (2.5 $\times$ 30 cm) equilibrated with the citrate buffer; the chromatographic separation was carried out at 6°C. The protein content was measured as the OD at 280 nm. Before use, the column was calibrated by chromatographing ^{131}I-labeled bovine Tg and ^{125}I-labeled normal guinea pig IgG. In order to determine the effect, if any, of the pH 3.2 citrate buffer on the IgG molecules, normal guinea pig IgG was equilibrated in the citrate buffer for the same amount of time as guinea pig anti-Tg IgG. The guinea pig anti-Tg IgG preparation recovered from the G-200 column and the normal guinea pig IgG were dialyzed against normal saline and then against PBS. Any precipitated material was removed by centrifugation (3,000 *g*). Both preparations were radioiodinated (sp act usually 0.2–1.0 μCi/μg protein) and deaggregated by ultracentrifugation at 100,000 *g* for 150 min (10). Immunized A/J mice whose thyroids were known to contain Ig deposits and nonimmunized age- and sex-matched A/J control mice had their drinking water supplemented with 0.1% KI 5–10 days before and during the experimental period. Each immunized and control mouse was injected intravenously with 30 μg of ^{125}I-labeled guinea pig anti-Tg IgG plus 30 μg ^{131}I-labeled normal guinea pig IgG. The mice were exsanguinated after 14–18 h, and the left ventricle of each animal was slowly infused with 20–30 ml of heparinized saline. The thyroids were removed, weighed, and counted in a dual channel gamma scintillation counter. In addition, a sample of serum from each animal was counted after precipitation with an equal volume of 10% TCA. The specific antibody content in the thyroid was calculated as follows:

$$\text{specific thyroid } [^{125}\text{I}]\text{IgG} = \text{thyroid } [^{125}\text{I}]\text{IgG} - \left[\frac{\text{thyroid } [^{131}\text{I}]\text{IgG} \times \text{serum } [^{125}\text{I}]\text{IgG}}{\text{serum } [^{131}\text{I}]\text{IgG}}\right].$$

In Vivo Localization of [^{125}I]Anti-Tg Antibodies in Thyroid. Mice previously immunized with heterologous Tgs were injected with 60–70 μg protein of either ^{125}I-labeled guinea pig anti-Tg IgG or ^{125}I-labeled normal guinea pig IgG. Nonimmunized control mice were injected only with 60–70 μg protein of ^{125}I-labeled guinea pig anti-Tg IgG. The drinking water of all groups of mice was supplemented with 0.1% KI 5 days before and during the 72 h experimental period. The mice were exsanguinated and their thyroids were sectioned in a cryostat. Sections (4-μm thick) were washed in PBS, stained for Ig with fluoresceinated anti-Fab (IgG) antiserum, and photographed; the sections were then washed (three times) in PBS, fixed in formalin, and emulsed in NTB-2 (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.). After 7–14 days, the autoradiographs were developed, stained with hematoxylin-eosin, and rephotographed. The fluorescense and the autoradiographic photomicrographs were compared to determine the coincidence of fluorescent deposits and silver grain distribution. Sections were also processed for autoradiography alone to determine the effect, if any, of the fluorescence procedures on the development of silver grains.

Electron Microscopic Examination of Thyroids. Control and immunized A/J mice were exsanguinated under ether anesthesia. Their thoracic cavities were opened and the hearts exposed; 10 ml of normal saline containing 10 U heparin/ml were infused into the left ventricles followed by 6–10 ml of buffered 2% glutaraldehyde. The thyroids were dissected free of the trachea, cut into 1-mm fragments, immersed in glutaraldehyde for 2–3 h, washed, and postfixed in buffered OsO_4 for 1 h. The fragments were dehydrated in a graded series of alcohols, propylene oxide, and propylene oxide plus activated Vestopal W (Henley & Co., Inc., New York) (1:1) for 24 h. The fragments were then em-

bedded in gelatin capsules with pure activated Vestopal W. After 3 days, the blocks were cut into 1-μm thick sections on an LKB Ultrotome (LKB, Stockholm, Sweden) microtome fitted with glass knives and the thick sections were stained with toluidine blue. Thin sections (300–400 Å) were cut using a diamond knife; the sections were stained with 2% uranyl acetate for 20–30 min followed by a 3 min stain with lead citrate. The tissue specimens were examined in a Hitachi Hu 11A electron microscope (Hitachi Ltd., Tokyo, Japan)

Results

Immunologic, Histologic, and Immunohistochemical Analysis. The sequential development of heterologous Tg-induced murine thyroiditis was correlated with the levels of antibody to mouse Tg and the deposits of mouse Ig, C3 and inflammatory cells in the thyroid (Table I). There was no detectable serum antibody (<2 μg AbN/ml serum) at 8 and 15 days after the first series (days 1–5) of Tg injections. At these times, the thyroids had minimal Ig and C3 deposits

TABLE I

Comparison of Immunofluorescence and Serum Antibody with Histological State of Thyroids of A/J Mice

Day of experimentation	ABC value*	Immunofluorescent staining			Histological state of thyroid‖	
		Fab(IgG)	C3	IgG	Mononuclear	PMN
8	<2	0/4¶ (0)**	ND‡	ND	0/6§ (0)	0/6 (0)
15	<2	4/4 (1+)	4/4 (1+)	ND	0/4 (0)	0/4 (0)
25	<2	0/3 (0)	ND	ND	0/3 (0)	0/3 (0)
28	56	5/6 (1+)	5/6 (1+)	ND	0/3 (0)	1/3 (1+)
32	153	4/4 (1+)	4/4 (1+)	ND	2/4 (1+)	1/4 (1+)
35	172	3/6 (1+)	3/6 (1+)	ND	2/6 (2+)	2/6 (2+)
43	23	6/6 (1+)	6/6 (1+)	ND	0/6 (0)	0/6 (0)
45	39	4/6 (1+)	ND	ND	4/5 (0)	1/5 (0)
48	371	6/6 (3+)	6/6 (3+)	6/6 (3+)	2/6 (1+)	3/6 (2+)
50	1097	4/4 (3+)	4/4 (3+)	4/4 (3+)	4/4 (2+)	4/4 (1+)
51	165	4/4 (3+)	4/4 (3+)	4/4 (2+)	4/5 (3+)	1/5 (1+)
52	208	10/10 (3+)	10/10 (3+)	10/10 (3+)	5/6 (2+)	5/6 (3+)
53	121	9/9 (3+)	9/9 (3+)	9/9 (2+)	2/6 (1+)	1/6 (1+)
54	385	6/6 (3+)	6/6 (3+)	6/6 (2+)	1/5 (3+)	2/5 (2+)
55	185	3/3 (3+)	3/3 (3+)	3/3 (3+)	2/6 (3+)	0/6 (0)
65	33	6/6 (3+)	6/6 (3+)	6/6 (3+)	5/5 (1+)	0/5 (0)
72	ND	4/4 (3+)	4/4 (3+)	4/4 (3+)	3/4 (3+)	3/4 (2+)
75	65	3/3 (3+)	3/3 (3+)	3/3 (3+)	3/6 (3+)	1/6 (1+)

* ABC of serum antibody expressed as micrograms of antibody nitrogen per milliliter.
‡ ND, not done.
§ Denominator equals total number of mice examined and numerator equals number of mice exhibiting inflammation.
‖ Number in parenthesis is the average inflammatory score (1) for those mice exhibiting lesions.
¶ Denominator equals total number of mice examined and numerator equals number of mice with positive immunofluorescence.
** Number in parenthesis is the average immunofluorescence score for those mice exhibiting positive immunofluorescence.

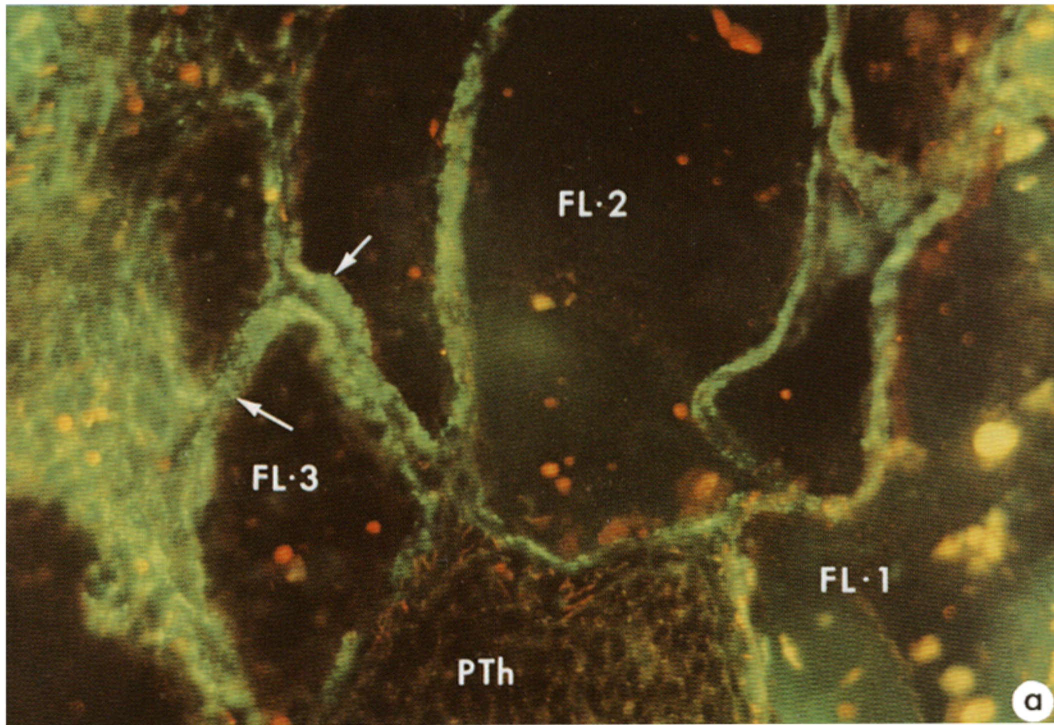
(1+) and contained no inflammatory cells. The second series of daily Tg injections was given from days 20 through 24. 4 days later, on day 28, detectable amounts of antibody appeared in the serum, and Ig and C3 were deposited in the thyroids. Even though the antibody level had increased (days 32 and 35), the incidence of thyroid Ig and C3 deposits and of inflammatory cells remained low. The third and final series of Tg injections lasted from days 40 through 44. On days 43 and 45 serum antibody levels had decreased (88%) when compared to those of day 35. This decrease probably resulted from absorption of a portion of the antibody reactive with murine Tg by the immunizing cross-reacting heterologous Tgs. As the Tgs were removed from the circulation, the serum antibody levels increased dramatically at days 48 and 50. There was a simultaneous increase in the deposition of Ig and C3 in the thyroids (Fig. 1 *a*). At day 51 the serum antibody level dropped strikingly and remained low during the rest of the experiment. The incidence and severity of the thyroid lesions reached a maximum at day 52. At this time, the most striking feature of the inflammatory lesions was the extensive number of neutrophils with scattered mononuclear elements (Fig. 2 *a*). The quality and quantity of Ig and C3 deposits remained unchanged during the remainder of the experiment. However, the incidence of histologic lesions declined rapidly. In those thyroids with residual inflammatory involvement, the noteworthy histopathological feature was the disappearance of neutrophils with only mononuclear cells remaining (Fig. 2 *b*).

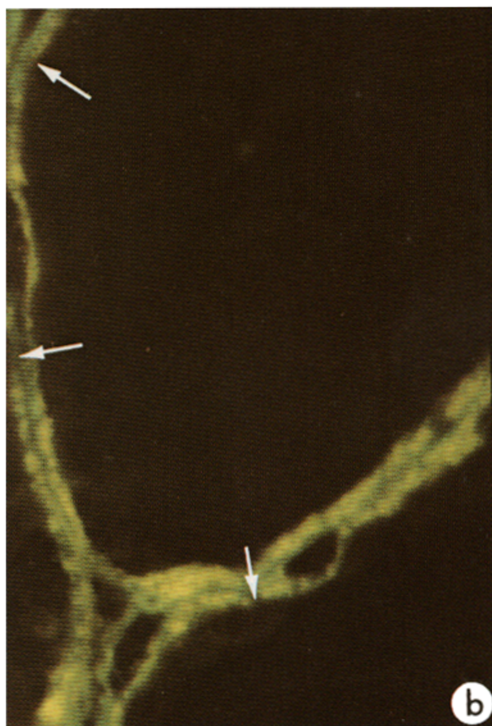
Thyroids from days 48 and 75 were examined for the presence of IgM and IgA in addition to IgG; however, only IgG was found in the deposits formed near the basal portion of the follicular cells. The IgG deposits were usually granular to lumpy in nature; however, when heavy they became almost confluent. In no case did the thyroid from experimental or control animals show positive immunofluorescence for albumin, although some glands did show intravascular FRA by immunofluorescence.

To determine whether there was any spontaneous thyroiditis in A/J mice, 40 age- and sex-matched nonimmunized mice were sacrificed throughout the experiment. None of these control mice had detectable lesions, deposits of Ig or C3 in the thyroid, or antibody to murine Tg in the serum.

Mild glomerular alterations were present with equal frequency in the immunized and control mice. There was no detectable increase in the incidence or quantity of glomerular Ig or C3 in immunized mice compared to control mice. There was also no histologic evidence of vasculitis in the kidney, lung, liver, spleen, pancreas, intestine, uterus, voluntary muscle, or heart of the immunized or control mice.

Elution of Immunofluorescent Thyroid Deposits. This experiment was designed to answer two questions: Are the deposits in the thyroid an antigen-antibody complex and is Tg one of the antigens in the deposit? It has been reported that antigen-antibody complexes in situ may be partially or totally solubilized by acid conditions or by chaotrope-containing buffers, such as NaSCN, but not by PBS (11). The data in Table II show that the deposits of Ig in the thyroids were removed after short (15 min) exposures to 2 M NaSCN and within 60 min after exposure to 0.02 M citrate buffer (pH 3.2). There was no decrease in intensity of staining for Ig when the sections were placed in PBS, even for 24 h. Fluorescein-


FL·2
FL·3
FL·1
PTh
a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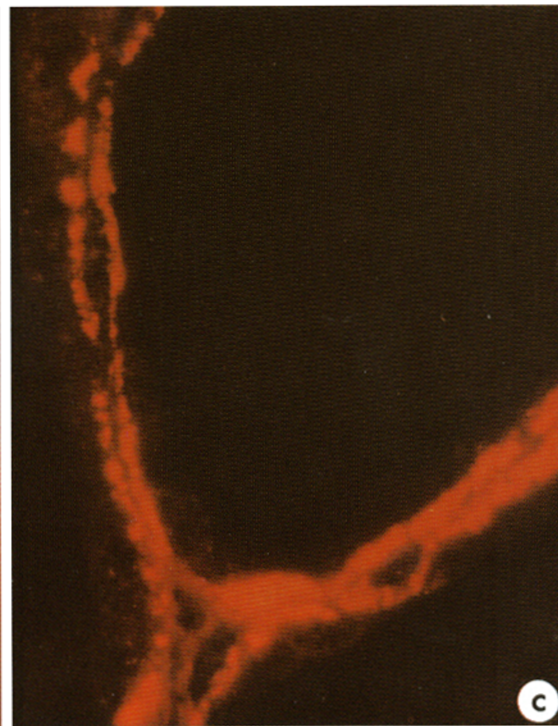
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ated mouse antiheterologous Tg detected small granular deposits near the basal area of follicular epithelial cells after 15- to 30-min elutions of Ig with the acid citrate buffer (Fig. 3). These Ig deposits corresponded in location to the IgG and C3 deposits which suggested that Tg was involved in the presumed immune complex formation.

Determination of Available Tg in the Thyroids of Immunized and Control Mice. Because of the relative difficulty in demonstrating (by immunofluorescence) Tg as an integral constituent of the deposits found in diseased thyroids, the in vivo paired label technique, a more sensitive and quantitative technique, was employed. The premise of this experiment was that the presumed immune complexes found in the diseased thyroid contained Tg as one of the antigens and some Tg antigenic determinants would be available to bind with [^{125}I]anti-Tg IgG, thus causing accumulation of ^{125}I above that attributable to blood equilibration. The data in Table III show that immunized mice whose thyroids were positive for immunofluorescent Ig deposits bound approximately 270-fold more [^{125}I]anti-Tg IgG than did nonimmunized, age- and sex-matched controls. Further, the specific binding of [^{125}I]anti-Tg IgG bound in the thyroid was a function of the quantity of the antibody injected. When the level of [^{125}I]anti-Tg diminished from 30 to 3 μg protein, there was a 6.5-fold decrease in the quantity of [^{125}I]anti-Tg IgG bound. Although not shown in Table III, the spleens, lungs, kidneys, and hearts of both nonimmunized and immunized mice were examined for specific binding of [^{125}I]anti-Tg IgG. There was no statistical difference between these tissues from nonimmunized and immunized mice.

In Vivo Localization of [^{125}I]Anti-Tg Antibodies in Immunized and Normal Mice. The in vivo paired label experiment revealed that Tg (presumably in immune complexes) was available to antibody in the thyroids of mice immunized with heterologous Tgs. A combination of fluorescence microscopy and autoradiography was used to determine the anatomical location of the Tg and the coincidence of mouse Ig and Tg was confirmed as shown in Figs. 1 *a* and 4 *a*. Results of immunofluorescence microscopy (Fig. 1 *a*) and the corresponding autoradiograph (Fig. 4 *a*) are presented with particular attention directed to area b. Fig. 4 *b* is a higher magnification of a portion of area b where silver grains are located or associated with the same anatomical loci as the fluorescent deposits. In

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FIG. 1. (*a*) Granular immunofluorescent deposits (arrows) of mouse Ig in presumed immune complexes were present in thyroid glands of immunized mice from day 48 throughout the experimental period. Heavy granular Ig deposits in a follicle disrupted by inflammatory cells are apparent adjacent to follicle 3 (FL-3). No Ig deposits are observed in the parathyroid (PTh) gland. (Stained with fluorescein-conjugated goat antimouse Fab (IgG) serum, original magnification × 100.) (*b*) This photomicrograph depicts the relationship between the follicular basement membrane and the IgG deposits in the thyroid of an immunized mouse. Ig deposits stained with rhodamine-conjugated rabbit antimouse IgG (yellow deposits) are shown in intimate relationship to the follicular basement membrane (arrows) outlined in green by indirect immunofluorescence using horse antirabbit renal basement membrane antiserum. (Original magnification × 440, KP 500 exciter filter.) (*c*) The thyroid follicle in Fig. 1 *b* was photographed with an exciter filter which allows viewing of the rhodamine-labeled antimouse IgG antibody alone. Note the cherry-red coloration of the granular Ig deposits which follow the outline of the follicle. (Original magnification × 440, KP-546, exciter filter.)

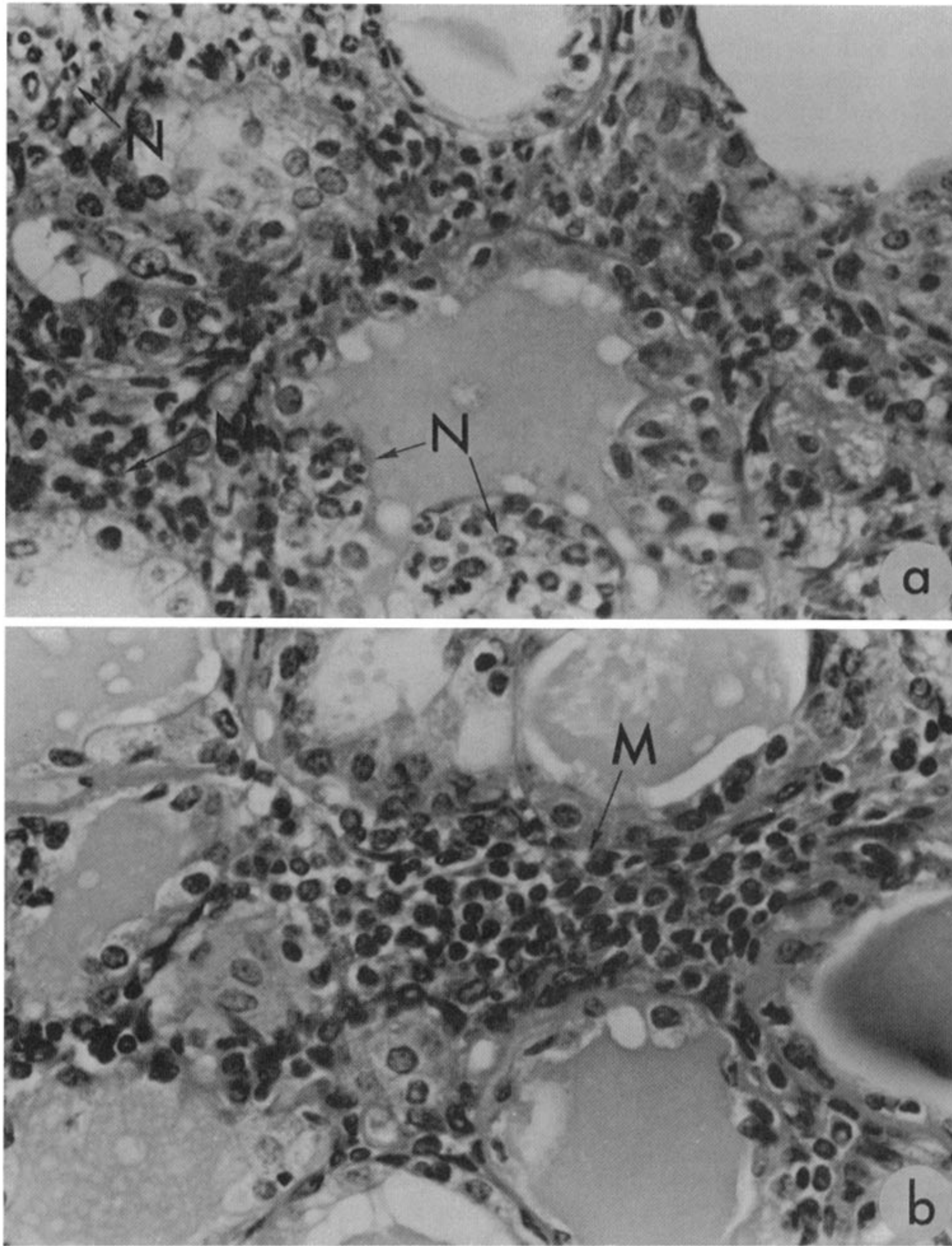


FIG. 2. (*a*) The histologic changes representative of the immunized mice at day 52 of the experimental period consisted of collections of neutrophils (N) surrounding and penetrating the thyroid follicles. (Original magnification × 250.) (*b*) From day 60 to the conclusion of the experiment (day 365), the thryoid alterations were characterized by mononuclear (M) inflammatory cell infiltrates surrounding and invading the follicles. (Original magnification × 250.)

TABLE II

Effect of Various Elution Buffers on Fluorescent Thyroid Deposits

Minutes of treatment*	Elution buffers		
	0.02 M Citrate‡	2 M NaSCN	PBS§
15	2+ ‖	—	3+
30	±	—	3+
60	—	—	3+ ¶

* All sections were unfixed, washed once in PBS, immersed in specified elution buffers, washed once in PBS, and stained with fluoresceinated goat antimouse Fab (IgG).

‡ 0.02 M citrate buffer, pH 3.2.

§ PBS, pH 7.2.

‖ Amount of immunofluorescent Ig remaining after treatment. Before elution 3+ amounts of Ig were present.

¶ Specimens exposed to PBS for up to 24 h remained 3+.

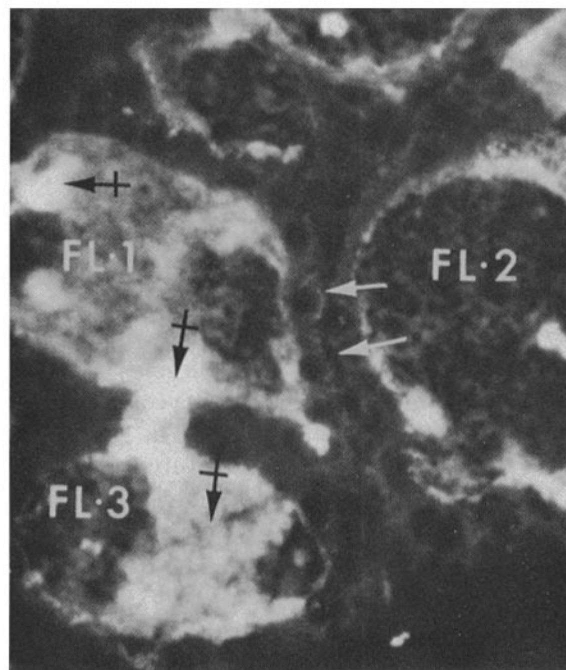


FIG. 3. Tg deposits in the thyroid of an immunized mouse are shown within the follicular lumens (FL) (black arrows) of FL-1 and FL-3, and to a lesser extent in FL-2. Fine granular deposits of Tg (white arrows) are also seen at the bases of the follicular cells in the same area in which Ig was identified. (Section stained with fluoresceinated mouse anti-Tg antiserum after 30-min elution in 0.02 M citrate buffer, pH 3.2, original magnification × 250.)

TABLE III

Quantitative Determination of Available Thyroglobulin in Thyroids of Immunized and Normal Mice

Amount of *I proteins injected*	No. of animals	Nanograms of specific Ab protein per thyroid	Status of thyroids
μg			
30	8	6.986 (2.20–12.63)‡	Ig deposits present
3	4	1.092 (0.63–1.79)	Ig deposits present
30	6	0.026§	No deposits

* Equal quantities of ^{125}I-labeled anti-Tg IgG and ^{131}I-labeled normal IgG injected intravenously into each mouse.

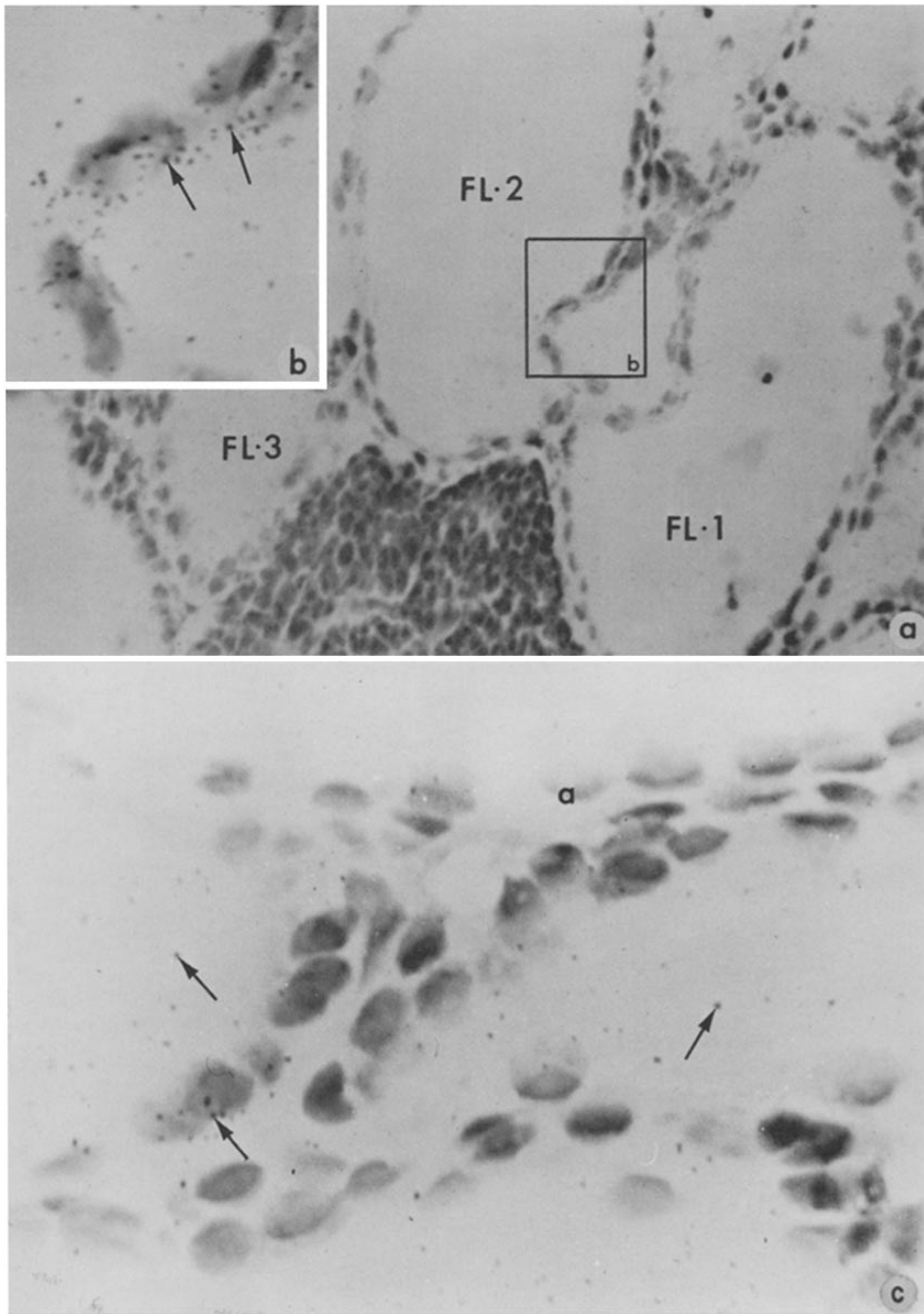
‡ Numbers found in parenthesis are the range of values observed.

§ 5/6 with "zero" specific Ab protein per thyroid.

an autoradiograph from a control mouse (Fig. 4 *c*) a few silver grains are randomly distributed in the basal area of the follicles and in the follicular lumen as well as interstitially. As a further control, immunized mice injected with 60–70 μg of ^{125}I-labeled normal IgG were processed similarly. Large granular deposits of mouse Ig were observed at the basal area of all of the follicle cells; however, the autoradiographs revealed a paucity of silver grains which appeared to be equally distributed in the follicular lumen and in the interstitial spaces with no selective accumulation at the bases of follicular cells (the area of IgG deposition). Autoradiographs which had not been processed for fluorescence microscopy also demonstrated the specific accumulation of [^{125}I]anti-Tg IgG in thyroids from immunized mice (Fig. 5 *a*). While similarly immunized mice, injected with ^{125}I-labeled normal IgG had very little randomly distributed label in their thyroids (Fig. 5 *b*).

Relationship between the Thyroid Follicular Basement Membrane and Immunofluorescent Ig Deposits. By using two fluorochromes the mouse IgG deposits at the bases of the follicular cells of immunized mice could be shown to be in close proximity to the follicular basement membrane. The fluorescein-labeled basement membrane appeared as a green linear structure forming the outline of the follicle (Fig. 1 *b*). When viewed with the fluorescein excitation filter, the yellowish rhodamine-labeled Ig deposits were seen in close proximity to the

FIG. 4. (*a*) A 7-day autoradiograph of the section shown in Fig. 1 *a* from an immunized mouse injected with ^{125}I-labeled guinea pig anti-Tg antibody is used for comparison of the Ig and Tg deposits. The follicular lumens are marked (FL-1, 2, and 3) for reference. (Original magnification × 100.) (*b*) The insert is an enlargement of area b and depicts the distribution of silver grains (^{125}I-labeled guinea pig anti-Tg antibody) about the basal area and interstitial spaces of the follicular epithelial cells. Note the coincidence of silver grains to the Ig deposits shown in Fig. 1 *a*. (Original magnification × 960.) (*c*) A 7-day autoradiograph of the thyroid of a nonimmunized control mouse shows the scant and random distribution of silver grains (arrows) within follicular lumen and interstitial spaces. The mouse was injected with ^{125}I-labeled guinea pig anti-Tg antibody. (Original magnification × 440.)



FL·2
FL·3
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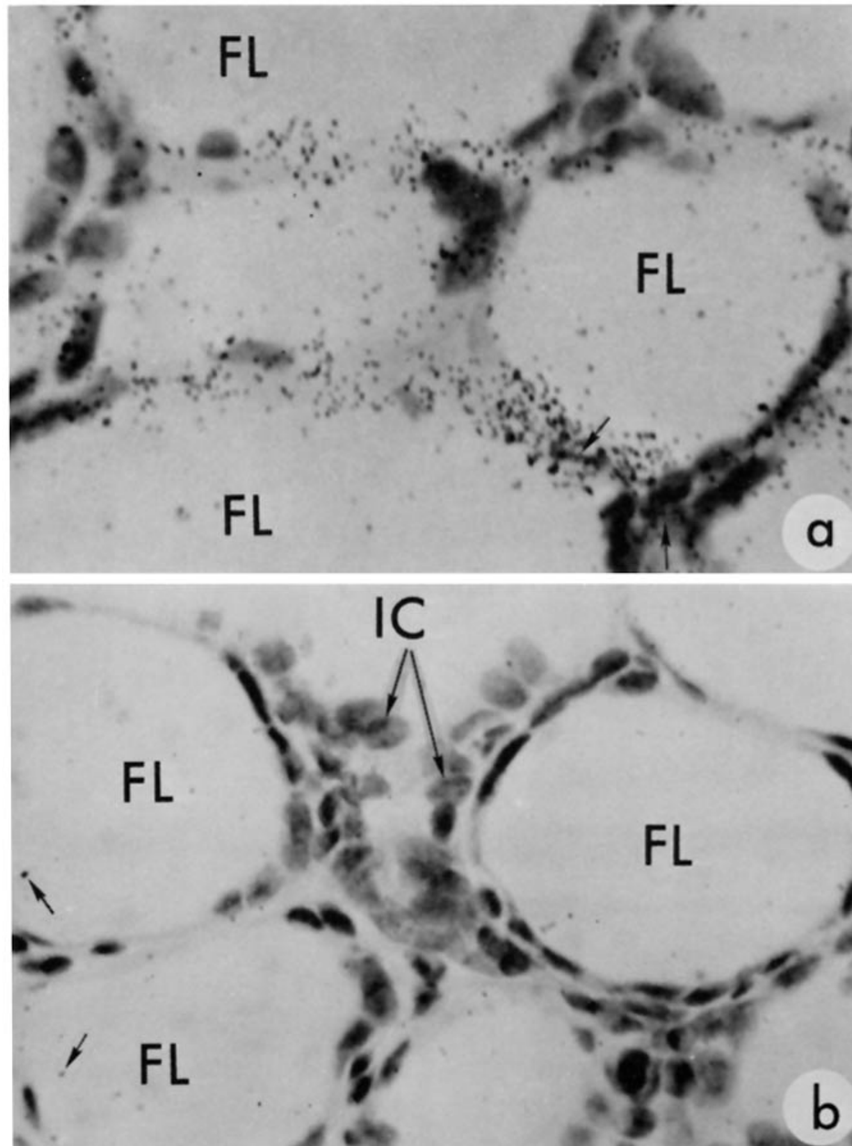

FIG. 5. (*a*) A 14-day autoradiograph of the thyroid from an immunized mouse injected with ^{125}I-labeled guinea pig anti-Tg antibody is shown. Note the heavy accumulation of silver grains (arrows) which suggest binding of the [^{125}I]anti-Tg antibody to Tg in interstitial immune complexes. (Original magnification × 96.) (*b*) A control 14-day autoradiograph of the thyroid from an immunized mouse injected with ^{125}I-labeled normal guinea pig antibody is shown. Note the paucity and random distribution of silver grains (arrows). (Original magnification × 96.)

basement membrane toward the follicular side. When the rhodamine excitation filter was used, the Ig deposits were more easily visualized with the lumpy aggregated appearance emphasized (Fig. 1 *c*). When thyroids from nonimmunized mice were treated in a similar way, no Ig deposits were observed, and the

basement membrane formed an uninterrupted linear structure around each follicle.

Electron Microscope Examination of Thyroids. In order to pinpoint the precise ultrastructural location of the presumed immune complexes observed by fluorescence microscopy, election microscopy of thyroids from immunized mice was performed. Moderate electron dense masses, assumed to represent the Ig, C3, and Tg deposits, were observed near the bases of the follicular cells (Fig. 6 *a*). At a higher magnification (Fig. 6 *b*), the electron dense deposits were observed to be between the follicular basement membrane and plasma membrane of the follicle cell. When thyroids from age- and sex-matched nonimmunized mice were examined in a corresponding manner, no electron dense deposits were observed.

Discussion

The development of autoimmune thyroiditis in A/J mice immunized with heterologous Tg, is dependent in part, if not totally, on the production of large quantities of autoantibody to autologous Tg. Circulating autoantibody appears to react with autologous Tg as it emerges from the follicular cell, leading to the

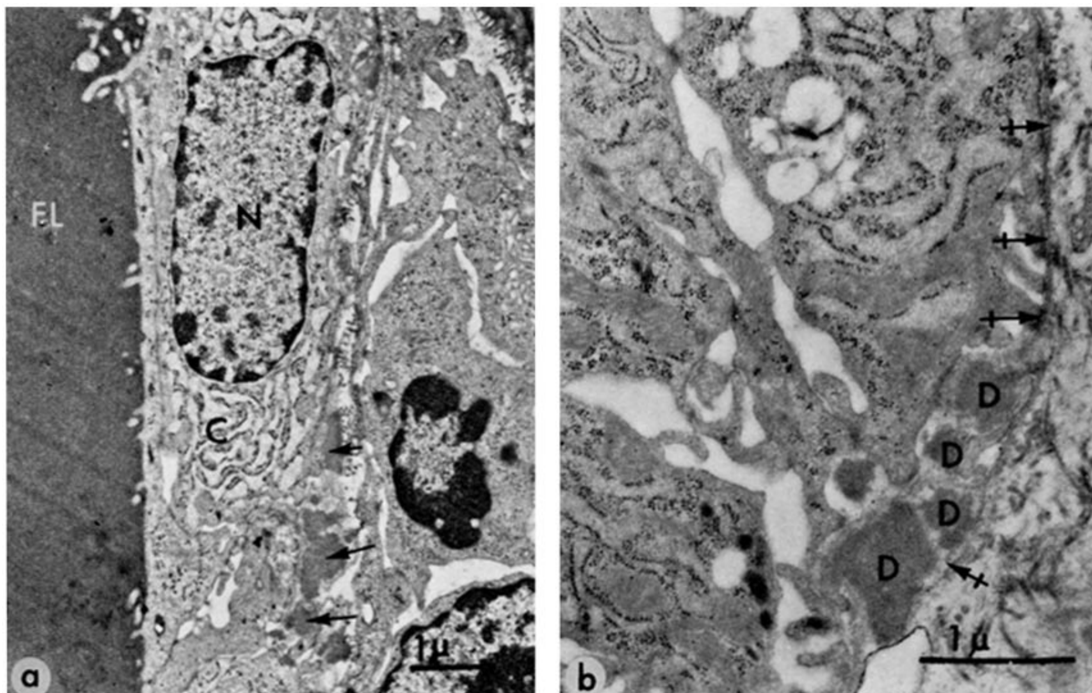

FIG. 6. (*a*) An electron photomicrograph of the thyroid of an immunized mouse is shown. The moderately electron dense colloid is seen in the follicular lumen (FL). The nucleus (N) and cytoplasm (C) of a follicular cell can be seen with irregular electron dense deposits (arrows) adjacent to the basal area of the cell. (Magnification × 11,200.) (*b*) At higher magnification the irregular dense deposits (D) appear to be interposed between the follicular cell plasma membrane and the follicular basement membrane (arrows). (Magnification × 20,480.)

formation of immune complexes in the potential interstitial space between the follicular basement membrane and the plasma membrane of the follicular cell. The moderate electron dense electron microscopic deposits observed in this location in the thyroids of the immunized mice probably correspond to the granular Ig and C3 deposits detected by immunofluorescence. Simultaneous, fluorochrome labeling of the immune deposits and the follicular basement membrane demonstrated the intimate physical proximity of these two materials. Recently, Käresen (12) has reported that administration of passive antibody with specificity for guinea pig Tg formed electron dense deposits in the intracellular spaces and between the follicular basement membrane and the follicular cell plasma membrane in guinea pig thyroids.

There was a direct correlation between the circulating levels of autoantibody to murine Tg and the quantity of Ig deposits (presumed in situ formed interstitial immune complexes) in the thryoid. Any artifactual origin of the Ig deposits appears to have been excluded by the following considerations: (*a*) the selective accumulation of only IgG; of 55 thyroids examined from days 45 to 75, all were positive for IgG and negative for IgM and IgA. On a limited number of thyroids obtained from mice immunized 1 yr before sacrifice, this class restriction was still evident; (*b*) the absence of albumin in the thyroids; (*c*) the coincidence of C3 with the IgG; (*d*) the ability to elute or dissociate the IgG and C3 from the tissue with acid or chaotropic ion-containing buffers with the subsequent detection of Tg in a similar location; and (*e*) the direct correlation of serum autoantibody specific for murine Tg, the Ig deposits in the thyroid, and the appearance of pathologic lesions.

As the level of circulating autoantibody increased concomitant with each series of immunization, the incidence and quantity of Ig deposits in the thyroid increased. There was a dramatic drop in serum antibody at day 50 concomitant with the appearance of the intense Ig and C3 deposits within the thyroid. The events from the present study were in parallel to those reported by Clinton and Weigle (6) for a rabbit model of thyroiditis induced by repeated injections of a single soluble heterologous Tg (bovine). They found an excellent correlation between the appearance of plaque-forming cells for rabbit Tg in the thyroid gland and the appearance of thyroid lesions. Further, they noted a drop in the levels of serum antibody to rabbit Tg which preceded the appearance of the lesions. It is possible that the presumed immune complex formation in the interstitium of the thyroid thought to be responsible for the histologic lesion may be fostered by an "early" antibody similar to that postulated for tissue deposition of circulating immune complexes (13). Subtle injury induced by such an antibody between days 45 and 48 with changes in vascular permeability and other tissue alterations may make the thyroid more susceptible to damage by the increasing levels of IgG autoantibody (day 50) from the serum and by some produced locally by lymphoid cells in the thyroid. Passive transfer studies have shown that thyroiditis in rabbits can be transferred only with the sequential administration of antibody collected at different times after immunization (2).

The interstitial immune complex-induced injury may be mediated by polymorphonuclear leukocyte (PMN) infiltration (14, 15) which was prominent during the initial stages of the histologic lesion. Compatible with this hypothesis,

C3 was associated with IgG deposits and as the quantity of IgG deposits increased, so did the quantity of C3 (judged by immunofluorescence). C components (C3a, C5a, and C$\dot{5}$67 complex [16]) are known to be chemotactic for PMN. No formal proof has been presented which demonstrates that the PMN exudation was C dependent. The possibility exists that the neutrophil influx might possibly be unrelated to C as has been reported in experimental allergic encephalomyelitis (17); however, this would seem unlikely. It is also of interest that as with other antibody-associated lesions (18, 19) typified late in the course by mononuclear infiltrates, an early PMN infiltration was evident. The finding of large numbers of mononuclear elements has led to the suggestion that such lesions are "cell mediated" when in reality they were induced by Ig and presumably mediated by C and PMN with the mononuclear elements occurring only late in the course.

Immunofluorescent deposits of Tg corresponding to the IgG and C3 deposits suggest that Tg is an important, if not the sole, antigen involved in this interstitial immune complex induced thyroiditis. This observation is strengthened by the autoradiograph studies. When a specifically purified and iodinated heterologous antibody with specificity for murine Tg, through cross-reacting determinants, was injected intravenously into immunized mice, the antibody was demonstrated to be specifically localized near the basal portion of the follicular cells. Of most importance, the distribution of radiolabeled antibody was coincidental to that of Ig deposits as determined by immunofluorescence. In the control mice no Ig deposits were observed which correlated with the absence of localization of heterologous anti-Tg antibodies by autoradiography. The in vivo paired label experiments support in a quantitative manner the above concept. Only thyroids from immunized mice had Tg which was accessible for specific radiolabeled antibody and therefore demonstrated a specific accumulation of isotope. Käresen and Godal (3, 4), and Sharp et al. (20) have been able to demonstrate that Tg plays a role in the formation of interstitial "immune complexes" in the basal area of the follicular cells of the guinea pig thyroid. These authors passively transferred anti-Tg antibody to guinea pigs and observed the transient accumulation of donor Ig in the recipient thyroid followed by a mild thyroiditis. Koffler and Paronetto (21) also reported that Tg accumulated in the interstitial areas of guinea pig thyroid with heavy inflammatory involvement in areas with Ig extravasates.

The interstitial immune complex formation postulated in this murine thyroiditis would seem similar to that seen in the Arthus reaction in which either antigen or its corresponding precipitating antibody present in the tissue combines with its counterpart from the circulation to form immune complexes in the walls of small vessels. The immune complexes lead to vascular injury mediated by C and PMN (14). Similar, presumed in situ formed immune complex deposits have been shown by electron microscope and immunofluorescence techniques in rabbits after immunization with homologous kidney in adjuvant (22). In these rabbits Ig deposits were found between the tubular basement membrane and the renal tubular plasma membrane, a potential anatomic space similar to that in the thyroids in this study. The interstitial type of immune complex injury postulated in this study is contrasted to that caused by tissue deposition of

immune complexes from the circulation, such as those responsible for serum sickness. The glomerulus is a frequent site of injury produced by deposition of circulating immune complexes (23). Such a deposition can be identified as irregular, granular deposits of Ig, usually accompanied by C3 (24). An ever increasing number of circulating exogenous (drugs, inoculations, and infectious agents) and endogenous (nuclear, renal tubular, and erythrocyte) antigens (24), including Tg (25, 26) have been identified in glomerular immune complex deposits. Circulating Tg-anti-Tg immune complexes sufficient to produce glomerular deposition and injury have usually been associated with manipulations that increase the amount of Tg available for soluble immune complex formation (27). Manipulation of antibody levels to allow a condition of antigen excess might also result in the formation of soluble and potentially nephritogenic immune complexes. The lack of extrathyroidal (glomerular or vascular) deposition of immune complexes in the mice in the current study suggests that conditions favoring formation of soluble pathogenic (circulating) immune complexes did not occur and that injury was apparently confined to the in situ formation of immune complexes in the interstitium of the thyroid.

Werner et al. (28) have reported that thyroids from over 70% of patients with Graves' disease contained IgM, IgE, IgG, C1q, and C3 distributed focally as granular deposits often associated with the follicular basement membrane. By contrast, in Hashimoto's thyroiditis, Kalderon et al. (29) found only one of eight thyroids with demonstrable immunofluorescent deposits of IgG, IgA, and C3 in a heavy granular pattern in the area of the follicular basement membrane. All eight cases, however, had electron dense deposits at the interface of the follicular basement membrane and the basal plasma membrane. They also observed that three thyroids diagnosed as Graves' disease were positive for immune deposits by immunofluorescence but contained no electron dense deposits. In general, these observations in humans would correlate well with the observations made in murine thyroiditis reported in this paper, and suggest that formation of immune complexes in the area of the follicular basement membrane by circulating antibody and Tg may be the inciting event responsible for thyroiditis in man.

Summary

Mice immunized with soluble heterologous thyroglobulins developed autoantibody that cross-reacted with autologous thyroglobulin. There was a direct correlation between the temporal appearance and quantity of serum autoantibody and the presumed in situ formation of immune complexes in the interstitium of the thyroid glands. Immediately after the formation of interstitial immune complexes containing antibody of the IgG complement-fixing type, the thyroids were invaded by a transient but intense neutrophil infiltrate which within 1 wk was replaced by chronic mononuclear elements. By the combination of fluorescence microscopy and autoradiography, thyroglobulin was demonstrated to be one, if not the sole, antigen in the interstitial immune complexes. The interstitial immune complexes were granular to lumpy in appearance and formed at the basal area of the follicular cells in intimate association with the follicular basement membrane. Electron microscopy revealed electron dense

deposits, presumably immune complexes, between the follicular basement membrane and the plasma membrane. The presumed in situ formation of immune complexes in this model is similar to that which occurs in the Arthus reaction and is a different mechanism of immune complex injury than that caused by tissue deposition of circulating immune complexes as occurs in serum sickness.

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