Influence of Methimazole on Murine Thyroiditis

Evidence for Immunosuppression In Vivo

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Abstract. Thionamide drugs are immunosuppressives in vitro. To examine this action in vivo, A/J mice were immunized with human thyroglobulin (hTg) (0.5 mg intraperitoneal injections for 5 d) beginning on days 6, 24, and 43 with or without methimazole (M) (0.05%) and *l*-thyroxine (T4) (0.1 μg/ml to prevent thyroid hypertrophy) in their water supply. Groups (n = 8) were killed on days 37, 42, and 59. Spontaneous splenic IgG-secreting cells determined by Staphylococcus protein A-linked sheep erythrocytes (SRBC) via indirect plaque-forming cell (PFC) assay indicated polyclonal stimulation induced by the hTg exposure (controls = 2,285±599, hTg-only = 5,570±470 PFC per 10^6 spleen cells), but this was significantly reduced in the M plus T4-treated group (3,640±415 PFC, P = 0.05). hTg antibody was measured by specific PFC assay using hTg-linked SRBC. Anti-hTg PFC were absent in controls and were 147±41, 25±8, and 173±58 PFC per 10^6 spleen cells in the hTg-only groups on days 37, 42, and 59, respectively. Anti-hTg PFC results in the M plus T4-treated animals were significantly reduced to 0, 15±5, and 63±30 anti-hTg PFC. Histological examination revealed a marked thyroiditis in hTg-only animals and a significantly reduced degree of mononuclear cell infiltration and follicular destruction in the M plus T4-treated groups (graded 1.9 compared with 3.6 in hTg-only P = <0.01). Examination of IgG deposition using fluorescent anti-mouse IgG revealed a similar granular pattern and degree of staining in both immunized groups. Control animals that received concurrent T4 administration alone showed similar hTg-induced murine thyroiditis to non-T4-treated animals and could not explain the apparent immunosuppression observed.

In conclusion, these data demonstrated that M reduced both the splenic immune response and the degree of thyroiditis after heterologous Tg immunization, while a quantitative difference in the circulating and intrathyroidally deposited Tg antibody was not detected.

Introduction

There is accumulating evidence implicating an immunosuppressive action for the antithyroid drugs, which has been convincingly demonstrated in vitro at concentrations approximating intrathyroidal levels (1–3). Whether such an effect is of importance in autoimmune thyroid disease depends upon the influence of this immunosuppression on the course of the underlying dysfunction. So far, only changes in thyroid antibody (Ab)[1] levels have been observed in patients treated with such therapy and there is now general agreement that titers tend to decrease during therapy with thionamide drugs (2, 4–6). However, not all individuals demonstrate such falling titers and there also appears to be idiosyncrasy in the in vitro immunosuppressive action. Hence, we observed considerable variation amongst individuals in their immunosuppressive response to both propylthiouracil and methimazole (M) (3).

The present report describes a study undertaken to assess the influence of M on experimentally induced thyroiditis in order to determine the role of the immunosuppressive action on the progressive thyroid pathology. The model chosen was extrinsically induced thyroiditis in A/J mice using immunization with heterologous human thyroglobulin (hTg) (7).

Methods

Murine groups. Male A/J mice aged 3 mo (Jackson Laboratories, Bar Harbour, ME) were investigated as follows: group I (n = 24), controls;

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1. *Abbreviations used in this paper:* Ab, antibody; hTg, heterologous human Tg; M, methimazole; PFC, plaque-forming cell; SRBC, sheep erythrocytes; SPA, Staphylococcus protein A; Tg, thryoglobin; TSH, thyroid-stimulating hormone; T4, thyroxine.

group IIA (n = 24), hTg immunization; group IIB (n = 8), hTg immunization plus thyroxine (T4); group III (n = 24), hTg immunization plus M and T4; group IV (n = 8), M only. Groups II and III were immunized with 0.5 mg hTg by daily intraperitoneal injection in normal saline for 5-d-periods beginning on days 6, 24, and 43 after the initiation of the experiment. For group IIB and III water was supplemented with 0.05% methimazole (Sigma Chemical Co., St. Louis, MO) with or without 0.1 μg/ml T4 (Sigma Chemical Co.). Since M therapy alone results in thyroid stimulating hormone (TSH)-induced thyroid hypertrophy, we considered that such a difference would make histological changes uninterpretable when compared with animals immunized with hTg only. Hence, animals were maintained in a euthyroid state throughout the experiment.

Groups I, IIA, and III were killed in groups of eight on days 37 (8 d after second immunization), 42 (13 d after second immunization), and 59 (11 d after third immunization). Groups IIB and IV were killed on day 59. Mice were exsanguinated by retroorbital blood collection performed via Pasteur pipettes. Sera were pooled and stored in groups at −20°C. Thyroids were removed with trachea and esophagus on days 37 and 59 and half were preserved in Bouin's reagent overnight followed by 70% alcohol. The remaining thyroid tissue was immediately flash frozen in hexane cooled by a dry ice/alcohol mixture and stored at −70°C. Spleens were collected on each occasion and cells were obtained by needle perfusion with RPMI-1640 (Gibco Laboratories, Grand Island, NY). Purification of mononuclear cells was achieved using density gradient centrifugation of individual spleen cell preparations on a mixture of Ficoll and diatrozoate sodium (Pharmacia Fine Chemicals, Piscataway, NJ). All cells were prepared at 4°C.

Plaque-forming cell (PFC) assays. Murine spleen cells were assessed for spontaneous immunoglobulin secreting capacity by the use of indirect hemolytic PFC assays described in detail previously (3, 8). Essentially, these systems used sheep erythrocytes (SRBC) (Flow Laboratories, Inc., McClean, VA) linked to Staphylococcus protein A (SPA) (Pharmacia Fine Chemicals) or hTg using chromium chloride. hTg was prepared by a standard technique of column chromatography using supernatant from homogenized human thyroid and used Sephadex G-200 followed by Sepharose 6B (9).

Spleen cells, suspended in RPMI-1640 at a concentration of 10,000–100,000 for SPA-PFC or 100,000–500,000 for anti-hTg PFC, were layered with the coated SRBC in agarose on 60 × 15-mm plastic Petri dishes and incubated for 2 h at 37°C in 5% CO_2 and 95% humidity. Hemolytic plaques were developed with 1 ml amplifying rabbit antibody to murine IgG (Kallestad Laboratories, Inc., Austin, TX) at a predetermined optimal dilution (1/100). After a further 2 h this was replaced with guinea pig complement (Gibco Laboratories) at a dilution of 1/40 and plaques counted 60 min later. Results were expressed as PFC per 10^6 spleen cells. All experiments included control plates without spleen cells and spleen cells with unlabeled SRBC to detect any anti-SRBC activity that was negligible in this series of experiments. Data were analyzed by the t test.

Histological assessment of murine thyroids. Fixed thyroid specimens were prepared for hematoxylin and eosin staining by routine techniques. Histologic changes were assessed semiquantitatively by counting the number of mononuclear clear cell foci and damaged thyroid follicles per section. Counts were assigned blind. Frozen sections of thyroids were fixed in acetone and subjected to direct immunofluorescent staining using fluorescein isothiocyanate-labeled rabbit anti-murine IgG (Cappel Laboratories, Cochranville, PA). Fluorescein isothiocyanate-labeled rabbit anti-mouse albumin was used as control.

Thyroid functional assessment. Serum T4 was measured by solid-phase RIA (Corning Labs, Medfield, MA) and serum hTg Ab titers by tanned turkey erythrocyte hemagglutination (Burroughs Wellcome Co., Research Triangle Park, NC).

Results

Group characteristics. All groups, except T4-only (IIB), gained weight satisfactorily during the experiment and maintained similar water intake to the control animals (Table I). Limited T4 evaluation on grouped sera indicated that Group III animals remained euthyroid throughout the experiment and showed the effectiveness of the regimen. Serum hTg Ab titers were negative in the nonimmunized animals and were similar in groups II and III, whether M and T4 were administered or not (Table I).

Polyclonal activation. The indirect SPA-PFC detected spontaneous IgG-secreting splenic mononuclear cells. Similar numbers of IgG-secreting spleen cells were observed in the control animals (Group I) at each of the three time points investigated (Table II). Mice immunized with hTg alone showed an increased proportion of IgG-secreting cells (when expressed as a percentage of total spleen cells) rising from 0.23% in the controls to 0.56% (P = <0.05) on day 37, which followed the second 5-d immunization course. Data from days 42 and 59 were similar to the control animals (Table II). In contrast, the animals treated with M plus T4, in addition to the hTg immunization, (Group III) showed less spontaneous IgG-secreting cells than Group II at all times.

Hence, using the SPA-PFC a "non specific" early response was detectable after hTg immunization and represented almost

Table I. Group Characteristics

Group	Day	Mean weight gain	Water consumption	Serum* T4	hTg Ab‡ titer
		%	*ml/animal/w*	*μg/100 ml*	
I	37	13.0	6.6	5.4	–
	42	15.8	7.7	7.5	–
	59	19.4	9.7	7.2	–
IIA	37	22.8	6.5	7.8	1/80,000
	42	29.1	7.9	7.6	1/80,000
	59	26.6	8.7	7.7	1/640,000
IIB	59	15.0	10.0	11.4	1/640,000
III	37	21.6	5.6	7.0	1/80,000
	42	25.7	5.7	5.6	1/80,000
	59	32.4	8.0	8.2	1/640,000
IV§	59	23.5	4.9	3.9	–

* RIA of pooled sera.
‡ Hemagglutination assay on pooled sera for hTg Ab, not antibody to murine-Tg.
§ M only for last 30 d of experiment.

Table II. Spontaneous Plaque Forming Murine Spleen Cells

Group (n = 8)	Day 37	Day 43	Day 59
	group±SEM	*group±SEM*	*group±SEM*
SPA-PFC*			
I	P = <0.05 { 2,285±599	2,135±686	2,037±283
IIA	5,570±1470 } ‡	2,045±648	2,342±203
IIB			1,770±246
III	3,640±715	1,573±361	1,782±345
anti-hTg-PFC*			
I	0	0	0
IIA	147±41	25±8 } ‡	173±58 } P = <0.01
IIB			240±27
III	0	15±5	63±30

* PFC/10^6 spleen cells.
‡ P = NS.

a 100% increase in recruitment of spontaneous IgG-secreting cells. This increase was not seen during concomitant administration of M and T4.

Anti-hTg PFC data. Control animals had no demonstrable specific anti-hTg PFC at each of the time points assessed (Tables II and III). Group II animals demonstrated 2.6% of their IgG-secreting cells to be hTg Ab-specific on day 37 and this appeared to become less further from the immunizations as shown on day 42 (1.2%) (Table II). After a third immunization, there were 7.4% of IgG-secreting cells detected as anti-hTg PFC on day 59. Animals receiving T4 in addition to hTg immunizations developed an enhanced number of anti-hTg plaques. In contrast, mice receiving M and T4 (group III) had significantly less spontaneous anti-hTg PFC. There were no specific PFC detected on day 37 (Table II) and only 15 and 63 anti-hTg PFC per 10^6 spleen cells on days 42 and 59, respectively. Hence, there was evidence of significant immunosuppression in the murine spleen cells from animals treated with M plus T4 on the basis of both specific and nonspecific PFC assays. In addition, the percentage of IgG-secreting cells that were hTg specific was also reduced to 0.9 and 3.5%, suggesting excessive suppression of the antigenic response by the spleen. As stated earlier, this apparent suppression of anti-hTg PFC was not observed when hTg-immunized animals were treated with T4 only.

Thyroid pathology. In the thyroids of control animals, there were occasional foci of mononuclear cells that were small (Fig. 1 *A*), and not associated with follicular destruction. Similar observations were made in the animals treated with M only. In group IIA and B animals, there was a marked thyroiditis exhibited by multiple foci of interfollicular mononuclear cells associated with infiltration and destruction of thyroid follicles (Fig. 1 *B*, Table III). There was a distinctly reduced degree of thyroiditis in group III animals as reflected in the histometric scores on day 59 (Fig. 1 *C*). In contrast, the immunofluorescent detection of granular IgG deposits along the follicular basement membrane was equally intense in both groups II and III but absent from nonthyroid tissues and from control animals (Fig. 2, Table III). This was confirmed by dilution studies with the FITC-labeled anti-IgG, which gave similar intensity of staining to a dilution of 1:800 in both groups II and III. Fluorescein isothiocyanate-labeled antialbumin did not react with any of the tissues.

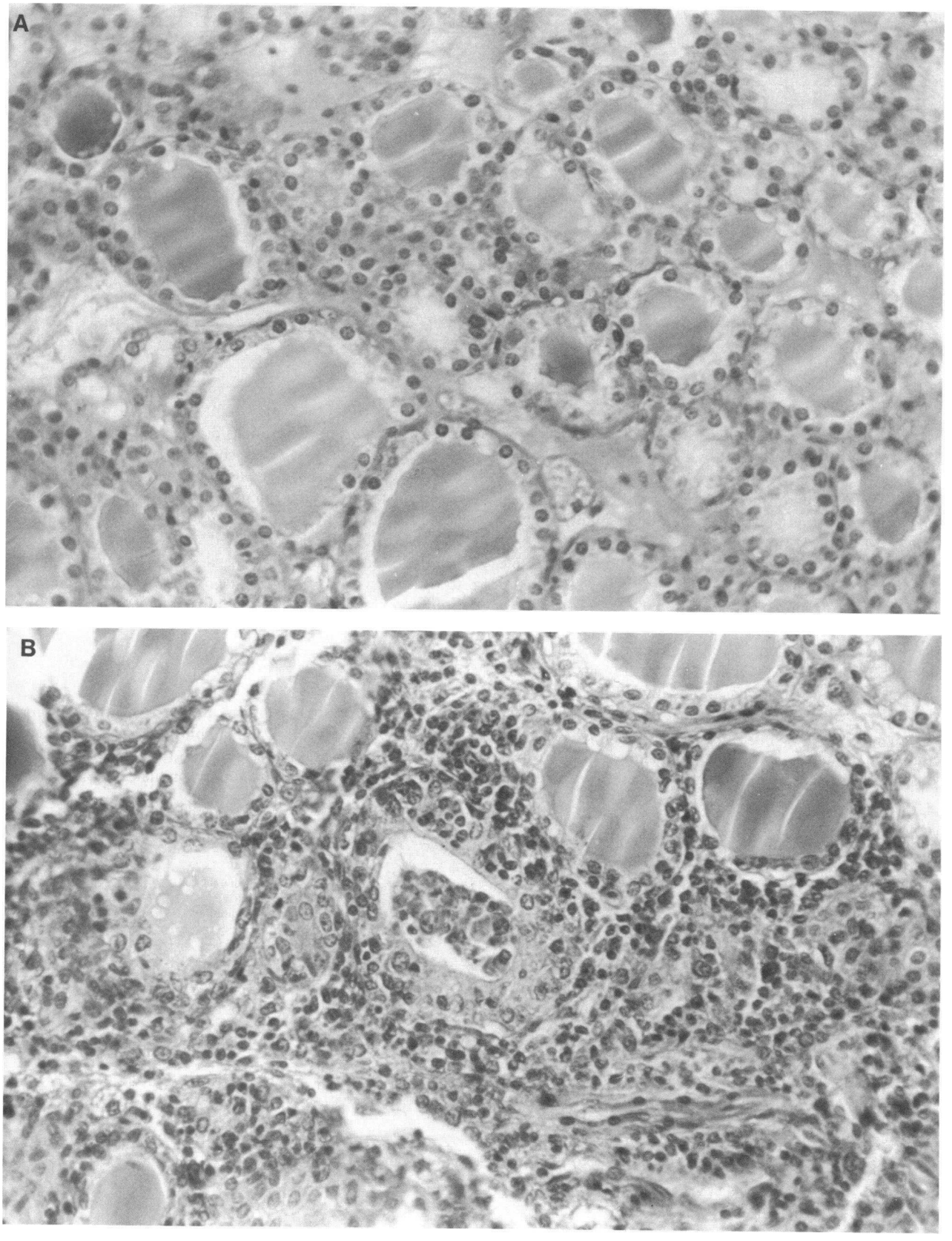
Discussion

Thyroiditis was initiated by immunization with heterologous thyroglobulin after a conventional intraperitoneal immunization regimen of daily injections for 5 d on up to three occasions. Using previously published studies for the onset of thyroiditis

Table III. Morphologic Analysis

Day	Group	Follicular destruction*	Mononuclear cell infiltration*	Mean rating	IgG flourescence
37	I	0.3	0.3	0.3	−
	IIA	3.75	1.5	2.63	+++
			P = NS		
	III	3.0	2.0	2.5	+++
59	I	0.0	0.17	0.08	−
	IIA	4.75	2.5	3.63	+++
	IIB	3.00	3.5 P = <0.01	3.25	−
	III	2.25	1.5	1.9	+++

* Mean number of foci per section.


A
B

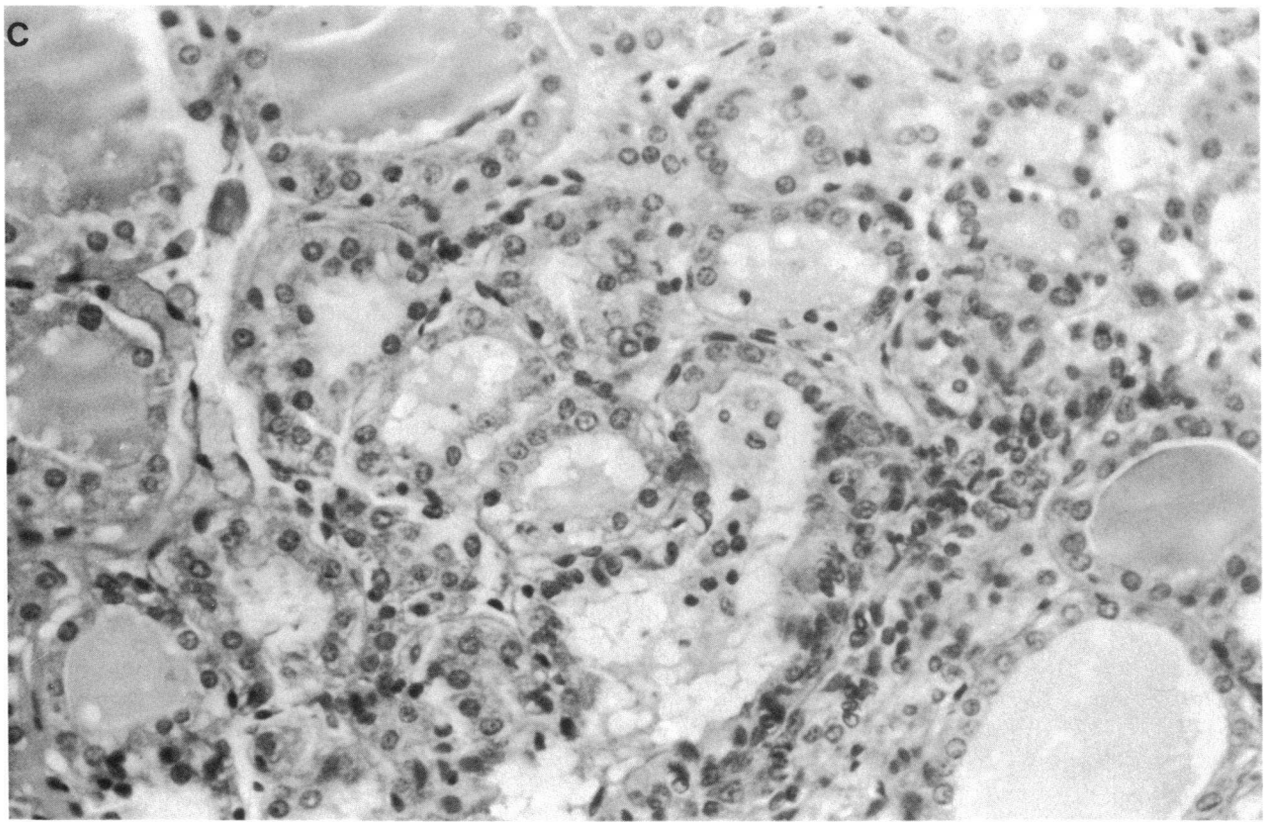
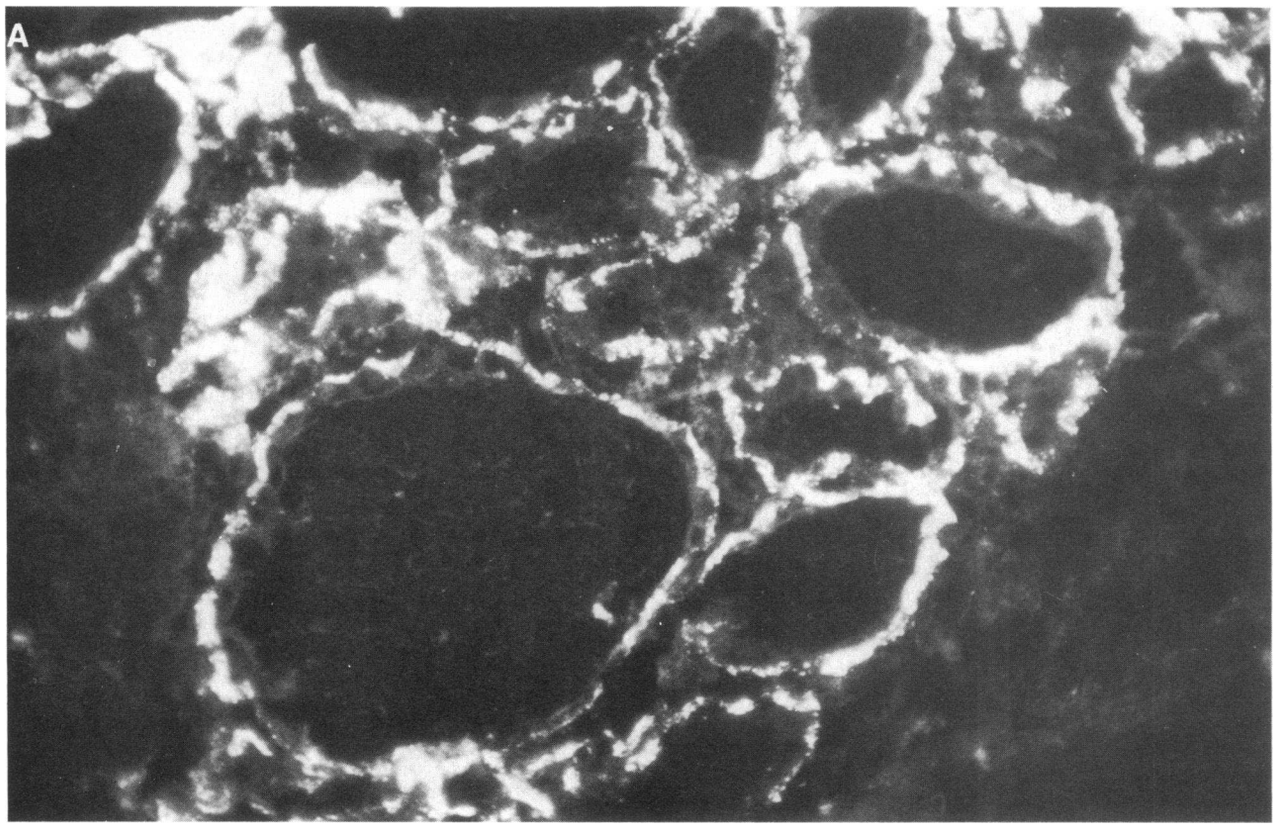


Figure 1. Histological presentation of sample thyroids from mice killed on day 59 from groups I (*A*), II (*B*), and III (*C*). Note the marked mononuclear infiltration in group II as reflected by morphological analysis in Table III and which is much reduced in group III (× 250).

in A/J mice under these conditions (7), immune function and thyroid pathology were investigated at intervals during and after the immunization regimen. The development of thyroiditis under these conditions may be dependent on both production of large quantities of antibody to heterologous thyroglobulin which exhibit cross-reactivity with autologous Tg, and the initiation of cell-mediated cytolysis (7, 10, 11). The mechanism by which antibodies reach the thyroid in order to damage tissue is uncertain. This may be by sequestration of circulating immune complexes, deposition of circulating Tg Ab onto the follicular cell, or deposition of locally secreted Tg Ab from within the gland itself. The fluorescence staining for murine IgG deposits within the thyroid glands suggested immune complex deposition at the base of the follicular cells presumably as Tg emerges from the cell itself (Fig. 2). This did not help us, however, define the source of the antibody deposited. It has been suggested that this type of interstitial immune complex-induced injury may be mediated primarily by the intrathyroidal mononuclear cell infiltration which was evident in all of the immunized animals (10, 11). Furthermore, there is accumulating evidence in human disease that thyroid antibodies are formed and released locally (12, 13). However, an animal model is not human disease. In the murine thyroiditis model there is an immune response to an exogenous antigen and thyroid antibodies will be made in large quantities outside the thyroid, which may then be able to damage the gland from without (7). Since hTg antibodies may not be complement fixing, the mononuclear cell infiltrate may represent a secondary response to the immune complex deposition and may be responsible for antibody-dependent cell-mediated injury to the thyroid follicles (11, 14). The balance between these mechanisms remains uncertain at present and, in particular, may vary at different periods after immunization (7).

This study presented two lines of evidence, which suggest that M may act as an immunosuppressive agent in vivo. Firstly, the plaque-forming spleen cell assay demonstrated evidence for inhibition of the polyclonal response to an external antigen (15) in addition to gross reduction in the specific PFC response to hTg. This was not seen in immunized mice that were treated concomitantly with T4. These data led us to suggest that oral M influenced the total immune response in the spleen of the murine model. Secondly, the interstitial thyroiditis elicited by



the heterologous Tg in the immunized animals was markedly reduced by administration of M plus T4 but not by T4 alone. This was reflected by the reduced mononuclear cell infiltration and the fewer follicles damaged. Nevertheless, certain factors need to be explained. Firstly, the fact that serum hTg Ab titers were similar in groups II and III indicated that any generalized immunosuppression was probably unimportant and that both groups of mice were able to mount an effective immune response as reflected by serum hTg Ab titers. The PFC data merely reflected the spontaneity of antibody secretion which appeared to be greater in the absence of M, indicating some reduction in antigen-induced B cell activation. The oral T4 may have diminished the effectiveness of the M-induced immunosuppression since it may stimulate the immune response (16) and appeared to enhance anti-hTg plaque formation, while other data have indicated that the influence of T4 on the immune system is likely to be only small (17, 18). However, the serum T4 levels in the M plus T4 animals were similar to the controls, again suggesting little possible influence.

The second disparity to be noted was that the immunofluorescence data did not support a significant effect for M. Since we know that the immune complexes deposited within the thyroid may be derived from circulating Tg Ab as well as intrathyroidally derived antibody (7), the similar levels of circulating Tg Ab and intrathyroidal immune complex deposition indicated that M was influencing another intrathyroidal mechanism for thyroiditis initiation and exacerbation. Furthermore, the fact that there was a marked reduction in mononuclear cell infiltration in the M-treated animals with preservation of immune complex deposition also suggested that the mononuclear cell infiltrate may not be the source of the deposited antibody and that the antibody itself may not have been responsible for the follicular destruction. Such logic suggests that the role of cell-mediated immunity in the follicular lesions may be significant and more important than generally recognized.

In vitro studies on the immunosuppressive action of the thionamide drugs have concentrated on inhibition of antibody secretion (2, 3). These studies showed marked inhibitory activity at concentrations $>10^{-4}$ M, compatible with levels thought to accumulate within the thyroid. Beck et al. (19) originally reported a significant decrease in lymphocytic infiltration of the thyroid in carbimazole-treated Graves' patients compared with that in a group of patients treated with propranolol only. Since that first observation in human disease, data has accumulated that confirms the in vitro evidence for antibody suppression. Since our studies were completed, similar findings have been described by Rennie et al. (20). These workers used an August rat model and included Freund's adjuvant in their immunization regimen.

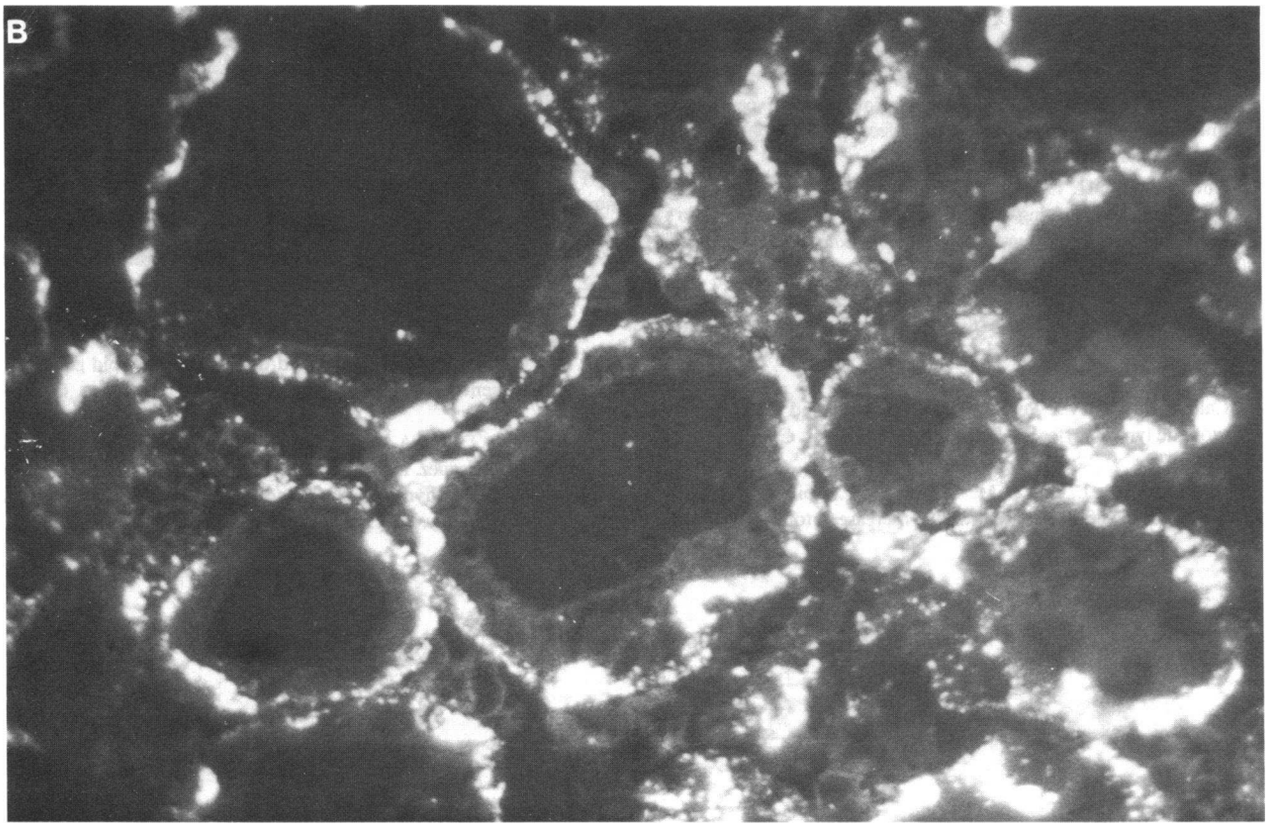


Figure 2. Immunofluorescent staining of murine IgG in frozen thyroid sections obtained at day 59 from groups II (*A*) and III (*B*). Note the similarity in the granular pattern and degree of staining (× 250).

Nevertheless, they clearly demonstrated a diminution in the thyroiditis when the animals were treated with M in the presence and absence of supplemental T4. However, no studies have pursued the effects of antithyroid drugs on cell-mediated immunity. The present experiments clearly lead us to suggest that such inquiries are long overdue.

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