Virchows Arch. Abt. A Path. Anat. 361, 307—319 (1973)

Suppression of Experimental Allergic Thyroiditis in Guinea Pigs by Homologeous and Heterologeous Thyroglobulin*

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Received August 24, 1973

Summary. Guinea pigs with experimental allergic thyroiditis, induced by three intradermal injections of thyroglobulin in complete Freund's adjuvant, were treated by daily injections of thyroglobulin emulsified in incomplete Freund's adjuvant over a period of 12 days. Besides lightmicroscopical, ultrastructural and immunohistochemical studies the serum antibody titers were determined and skin tests for delayed hypersensitivity were performed in all animals. The following results were obtained.

1. Specific suppression of experimental thyroiditis by homologeous or heterologeous thyroglobulin resulted in almost complete absence of the inflammatory infiltrate in the thyroid glands.

2. The cellular hyperactivity and the toxic-degenerative changes of the thyroid cells were still present in the desensitized animals. These changes were regarded as residues of the former inflammation of the glands.

3. There was a good correlation between the presence of thyroiditis and delayed hypersensitivity in all animals. Such a correlation was not apparent between thyroiditis and antibody titers.

The data, presented in this study, further support the role of cellular immunity in the pathogenesis of experimental allergic thyroiditis. The mechanism by which the suppressive effect is mediated is discussed.

Zusammenfassung. Meerschweinchen mit einer experimentell induzierten Thyreoiditis wurde über einen Zeitraum von 12 Tagen täglich Thyreoglobulin, emulgiert in incomplettem Freundschem Adjuvans, injiziert. Neben licht- und elektronenmikroskopischen sowie immunhistochemischen Studien wurden Untersuchungen zur Überempfindlichkeit vom Spättyp an Hand von Hauttesten durchgeführt. Weiterhin wurde bei allen Tieren der Serum-Antikörpertiter gegen Thyreoglobulin bestimmt. Folgende Ergebnisse wurden erzielt.

1. Eine spezifische Desensibilisierung durch homologes oder heterologes Thyreoglobulin führte bei den Versuchstieren mit experimenteller Thyreoiditis zu einem fast vollständigen Rückgang der Entzündung innerhalb der Schilddrüse.

2. Das Fortbestehen stimulierter Follikelepithelien und toxisch-degenerativer Veränderungen in den Schilddrüsen desensibilisierter Tiere wurde als Hinweis für die vorausgegangene Entzündung gedeutet.

3. Zwischen dem Vorhandensein einer Thyreoiditis und der Überempfindlichkeit vom verzögerten Typ (Hauttest) besteht in allen Untersuchungsgruppen eine deutliche Korrelation. Eine solche Korrelation ließ sich nicht zwischen Thyreoiditis und Antikörpertiter nachweisen.

Die Befunde stützen die Hypothese, daß die celluläre Immunität für das Zustandekommen der experimentellen Thyreoiditis den wesentlicheren Faktor darstellt. Die Mechanismen, die zur Desensibilisierung führen, werden in der vorliegenden Arbeit diskutiert.

* This work was supported by the Deutsche Forschungsgemeinschaft.

Introduction

Morphological and immunological phenomena of experimental allergic thyroiditis (e.a.t.) have been extensively studied in various species (see reviews by Rose and Witebsky, 1971; Delespesse *et al.*, 1972). In guinea pigs inflammation of the thyroid gland has been described to occur 5 days to 26 months after a single injection of thyroid extract in complete Freund's adjuvant (Lerner *et al.*, 1963).

Suppression of e.a.t. has been achieved by the application of compounds like 6-mercaptopurine and aminopterin (Spiegelberg and Miescher, 1963). Eylar *et al.* (1972), on the other hand, were able to suppress experimental allergic encephalomyelitis by daily injections of the antigen by which they had induced the disease before.

As far as we know there have been no previous attemps to suppress e.a.t. by the application of the specific antigen. Our experiment demonstrates the effect of specific desensitization of e.a.t. The morphological alterations of the inflammatory infiltrate and the parenchymatous changes will be described and compared with results of delayed hypersensitivity skin reactivity and with humoral antibody responses.

Material and Methods

The experiment was performed on 30 female guinea pigs weighing 250–300 g. 15 animals were treated by homologeous thyroglobulin (see Table 1, groups I, II and V), 12 animals by heterologeous thyroglobulin (groups III and IV) and 3 animals were untreated controls (group VI). The experimental design is shown in Table 1. Sensitizing injections were given into the foot pads with a volume of 0.4 ml each. Suppression was initiated at the 28th day with a single intramuscular injection of 8 mg thyroglobulin in incomplete Freund's adjuvant and continued with daily subcutaneous injections of 3 mg thyroglobulin. 3 immunized animals were killed at the 28th day, all other animals at the 40th day of the experiment.

Antigen Preparation

Guinea pig thyroglobulin was prepared from saline guinea pig thyroid extract by $(NH_4)_2SO_4$ fractionation and further purified by gel filtration on Sephadex G 200 (Salvatore *et al.*, 1964). Bovine thyroglobulin was purchased from SIGMA and also purified on Sephadex G 200.

Immunological Methods

In all animals a skin test was performed at the 27th and 39th day of the experiment. 50 μg antigen solved in 0.1 ml saline were injected intradermally. A simultaneous injection of 50 μg gamma-globulin served as control. The skin test was considered positive when skin lesions of more than 5 mm in diameter developed at the site of antigen injection after 24 hours. The relative amount of precipitating serum antibody was studied by a radial immunodiffusion test of antigen in 90% serum-agar slides according to the method of Feinberg *et al.* (1969).

Immunohistochemistry

Thyroid tissue was fixed in cold 10% neutral formalin and embedded in low melting wax. Two indirect immunohistochemical techniques were used to study the distribution of thyroglobulin in the thyroid gland. FITC-labelled goat anti-rabbit IgG was used to demonstrate the specific binding of rabbit antibody to guinea pig thyroglobulin (for details of the method and specific controls see Böcker and Lietz, 1973). Additionally the method of Sternberger *et al.* (1970) was performed for the same purpose using a soluble peroxidase-anti-peroxidase (PAP) complex in the last step of the incubation process.

Table 1. Treatment of 30 guinea pigs

Group	Number of animals	Day	Immunisation			Suppression		
			1	20 . . .	27	28	29	40
I	6		2 mg gp thg + c.F.a.	2 mg gp thg + c.F.a.	1 mg gp thg + c.F.a.	saline + ic.F.a.	saline + ic.F.a.	*→†
II	6		2 mg gp thg + c.F.a.	2 mg gp thg + c.F.a.	1 mg gp thg + ic.F.a.	8 mg gp thg + ic.F.a.	3 mg pg thg + ic.F.a.	*→†
III	6		2 mg b thg + c.F.a.	2 mg b thg + c.F.a.	1 mg b thg + c.F.a.	saline + ic.F.a.	saline + ic.F.a.	*→†
IV	6		2 mg b thg + c.F.a.	2 mg b thg + c.F.a.	2 mg b thg + ic.F.a.	8 mg b thg + ic.F.a.	3 mg b thg + ic.F.a.	*→†
V	3		2 mg gp thg + c.F.a.	2 mg gp thg + c.F.a.	——————→	†		
VI	3		saline + c.F.a.	saline + c.F.a.	saline + c.F.a.	saline + ic.F.a.	saline + ic.F.a.	*→†

* Daily injections continued till sacrifice.
Abbreviations used in the tables: gp thg = guinea pig thyroglobulin, b thg = bovine thyroglobulin, c.F.a. = complete Freund's adjuvant, ic.F.a. = incomplete Freund's adjuvant.

Histological Investigations

Diagnosis and grading of e.a.t. were restricted to the presence and extent of actual inflammatory cellular reaction in the thyroid gland. Alterations in size or shape of the follicles were not considered to be specific for thyroiditis.

Specimens from lung, liver, kidney, spleen and lymph node from all animals were processed for routine histological investigations.

Electron Microscopy

Specimens of thyroid tissue of all animals of groups I and II were fixed by immersion in cold 2.5% glutar-aldehyde-cacodylate buffer for 2 hours and postfixed in buffered osmium-tetroxyde for 1 hour. The tissue was embedded in Epon 812. Thin sections were stained by uranyl acetate and lead citrate and examined in a Phillips EM 300 at 60 kV.

Results

The results of our experiment are summarized in Table 2. No histopathological changes of the thyroids were noted in control animals immunized with Freund's adjuvant. All but 3 out of 15 guinea pigs (groups I, III and V) which had previously been immunized with thyroglobulin showed inflamed thyroid glands with the characteristic interstitial and follicular infiltration by predominantly mononuclear cells (Fig. 1). Homologeous as well as heterologeous thyroglobulin resulted in identical thyroid lesions but the inflammation produced by guinea pig thyroglobulin was regularly far more intensive.

Table 2. Thyroiditis, circulating antibody and skin test in guinea pigs after sensitization and suppression with thyroglobulin

Group	Frequency of thyroiditis	Degree of thyroiditis					Precipitating antibody to		Positive skin test to	
		0	(+)	+	++	+++	gp thg	b thg	pg thg	b thg
I	5/6		1	1	2	2	1/6	—	5/6	4/6
II	1/6	4	1	1			1/6	—	0/6	0/6
III	4/6	1	1	4			0/6	6/6	0/6	6/6
IV	0/6	5	1				0/6	5/6	0/6	2/6
V	3/3				2	1	1/3	—	3/3	3/3
VI	0/3	—			—		0/3	0/3	0/3	0/3

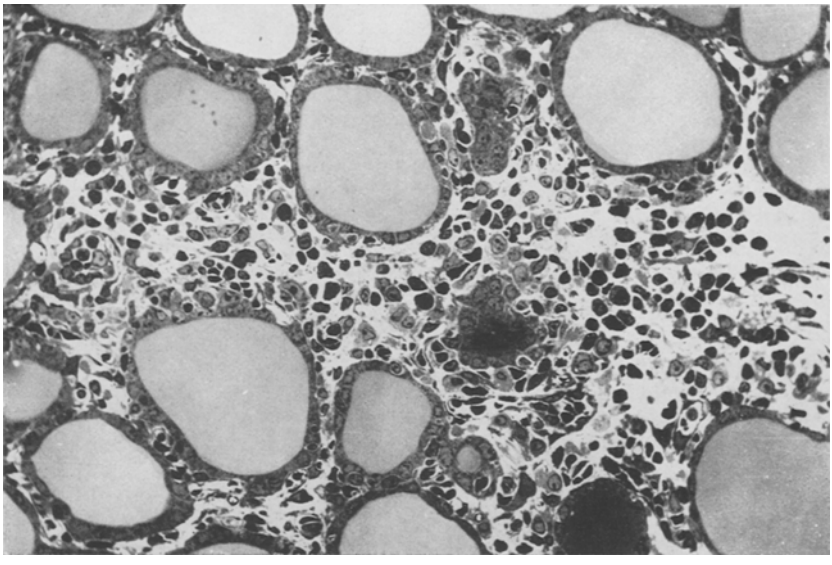

Fig. 1. E.a.t. (++, group I). Pleomorphic infiltrate consisting of small to medium-large lymphocytes, macrophages and plasma cells. Follicles partly invaded by inflammatory cells. Toluidin blue. ×250

Precipitating antibody to the antigen used for sensitization could consistently be demonstrated only in group III representing the animals which had received heterologeous thyroglobulin. There was no cross reaction to homologeous thyroglobulin in this group. All but one animal of groups I and III had positive delayed skin reactions after intradermal injection of the respective antigen. This delayed hypersensitivity reaction (Fig. 2) was positive with homologeous and heterologeous thyroglobulin in animals of group I but was restricted to heterologeous thyroglobulin in animals of group III.

The most striking result of this experiment seems to be the effect of specific suppression of e.a.t. (groups II and IV). Desensitization resulted in almost com-

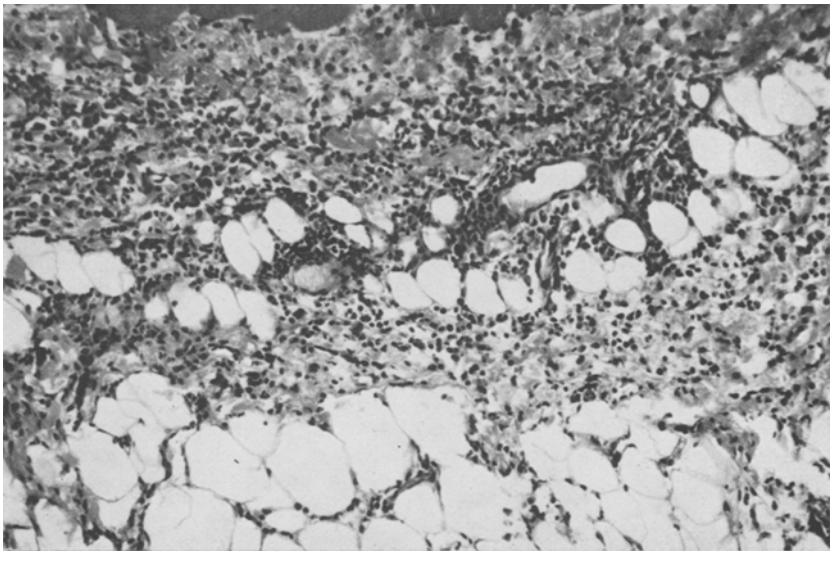

Fig. 2. 24 hour skin reaction to 50 μg guinea pig thyroglobulin. Same guinea pig as in Fig. 1. Mononuclear cells in deeper dermis and adipose tissue. H-E. ×250

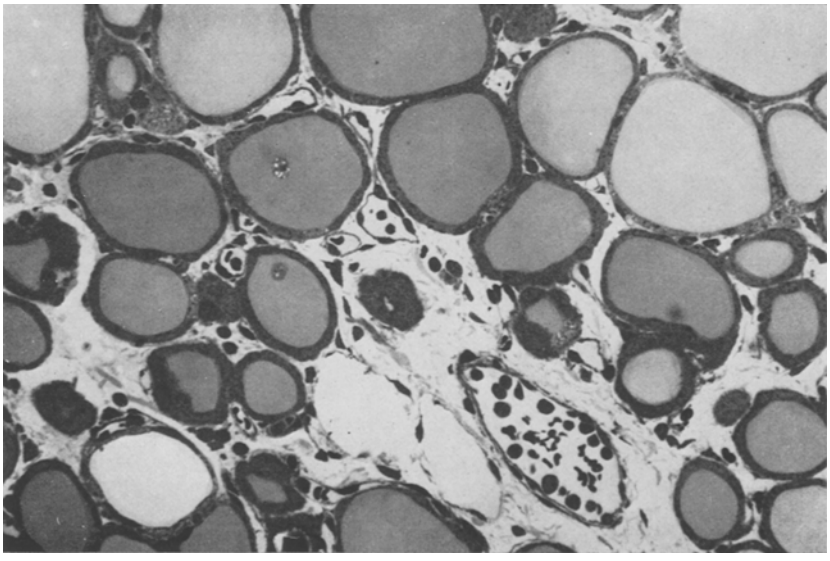

Fig. 3. Suppression of e.a.t. (group II). Note absence of cellular infiltration, interstitial edema and activation of follicle cells as only residues of the previous inflammation. H-E. ×250

plete absence of inflammatory infiltration of the thyroid glands at the end of the experiment (Fig. 3).

Comparision of the serum antibody titer of group I with II and III with IV did not show any significant effect of desensitization on humoral antibody

response when measured by the radial immunodiffusion method. On the other hand there was a significant effect of desensitization on cutaneous delayed hypersensitivity reaction. The skin test which had been clearly positive in all but one of the immunized animals at the 27th day turned out to become negative in all but 2 animals after specific suppression at the 39th day of the experiment.

Morphology of Thyroiditis

The cellular infiltrate of e.a.t. in guinea pigs has been described by Sobel and Geller (1965), Kosunen and Flax (1966) and Kåresen (1970). Our study confirms their descriptions (Fig. 1). Therefore no further detailed comment on the cellular infiltrate seems to be necessary.

The immunohistochemical studies on thyroglobulin (Fig. 4a and b) demonstrated the antigen in the follicular epithelium as well as in the interstitial space of the thyroid gland. The colloid of the follicles mostly gave negative results in the immunohistochemical reaction for thyroglobulin. This was a constant finding also in untreated guinea pigs (Böcker and Lietz, 1973). In the present study thyroglobulin could furthermore be localized within macrophages which were often located at the periphery of the inflamed follicles. C-cells which characteristically form large clusters in the guinea pig thyroid gland (Lietz, 1971) did not stain at all nor did they show any inflammatory infiltration or cellular alteration (Fig. 5a). Thyroglobulin staining of the follicular epithelium was irregular in inflamed glands when compared to normal control animals.

Results of electron microscopic investigation of all animals of groups I and II revealed characteristic parenchymatous lesions of the thyroid gland in addition to the inflammatory cellular infiltrate.

The ultrastructure of glands with e.a.t. clearly showed a hypertrophy of the follicular epithelium similar to the well known TSH-response. In the normal guinea pig thyroid the epithelium was flat while in glands with e.a.t. it became columnar and irregular with proliferation of the apical cytoplasm and formation of pseudopods. Nuclei and nucleoli increased in size. The microvilli became long, Golgi vesicles and lysosomal organelles of the apical cytoplasm became more prominent. A constant finding was the distension of the ergastoplasmic cisternae which were often cystic and occupied the basal as well as the apical cytoplasm (Fig. 6). Destruction of the thyroid follicles by invasion of mononuclear cells was a focal finding in 5 of 6 animals of group I. Necrobiosis of follicular cells began with cystic dilatation of the ergastoplasm, formation of large autophagic vacuoles and pyknosis of the nucleus. The characteristic degeneration of the parenchyma resulted in the so called "colloid cell" which is a constant feature of human autoimmune thyroiditis (Delespesse *et al.*, 1972) and has also been found in e.a.t. of monkeys (Themann *et al.*, 1968). Finally there was a detachement of the epithelial cells from the basement membrane, leakage of follicular content into the interstitium and phagocytosis of the necrobiotic cells by macrophages. Activated lymphocytes often penetrated the intercellular spaces (Fig. 5) which were constantly distended.

The endothelial cells of peri- and intrafollicular vessels were markedly swollen and often proliferation of the endothelium could be seen. The perifollicular fibro-

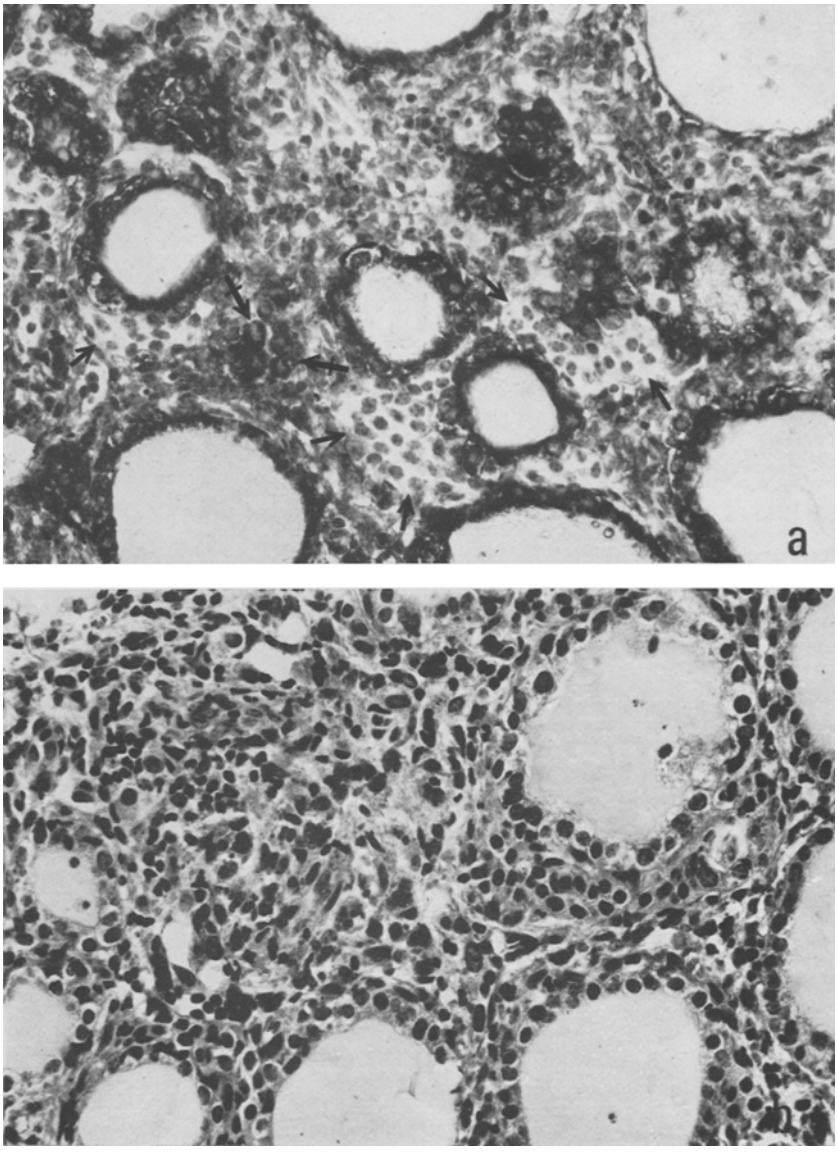

Fig. 4. a E.a.t. (group I). Immunohistochemical staining of thyroglobulin by the PAP-complex. Note the "untouched" C-cells (arrows). Intensely stained thyrocytes as well as some macrophages in the interstitium (cross-barred arrows). Haematoxylin. ×250. b Negative control. In the first step of the incubation process antibody freed serum has been used instead of rabbit anti thyroglobulin serum. Haematoxylin. ×250

cytes which exhibit characteristics of pericytes in the thyroid gland (Lietz, 1973) showed marked swelling of their cytoplasmic extensions and seemed to participate in the cellular proliferation within the interstitium.

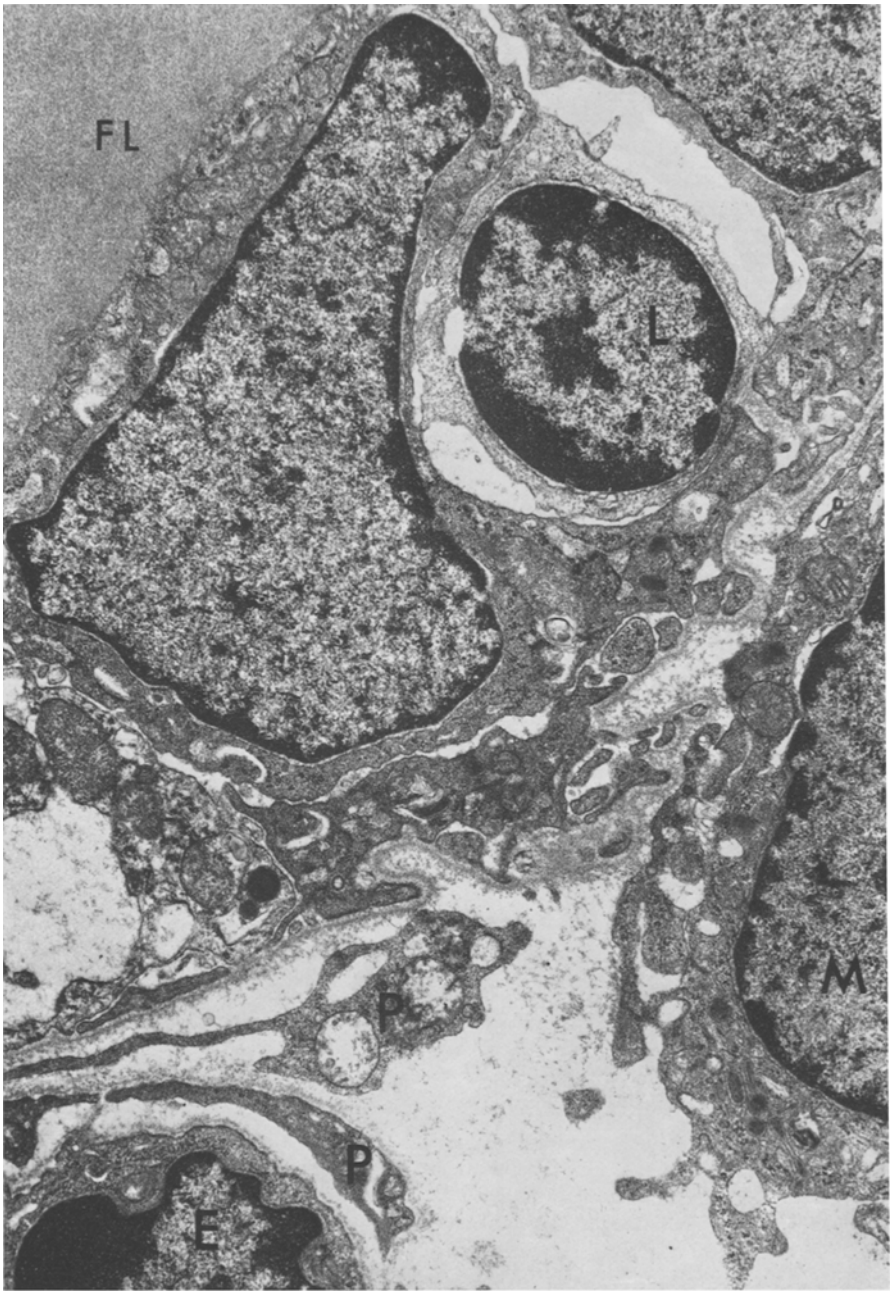

Fig. 5. E.a.t. (group I). Inflamed thyroid follicle with an interepithelial lymphocyte (*L*), swollen endothelial and pericytic cells (*E*+*P*) and a macrophage in the interstitium (*M*). *FL* follicular lumen. ×13500

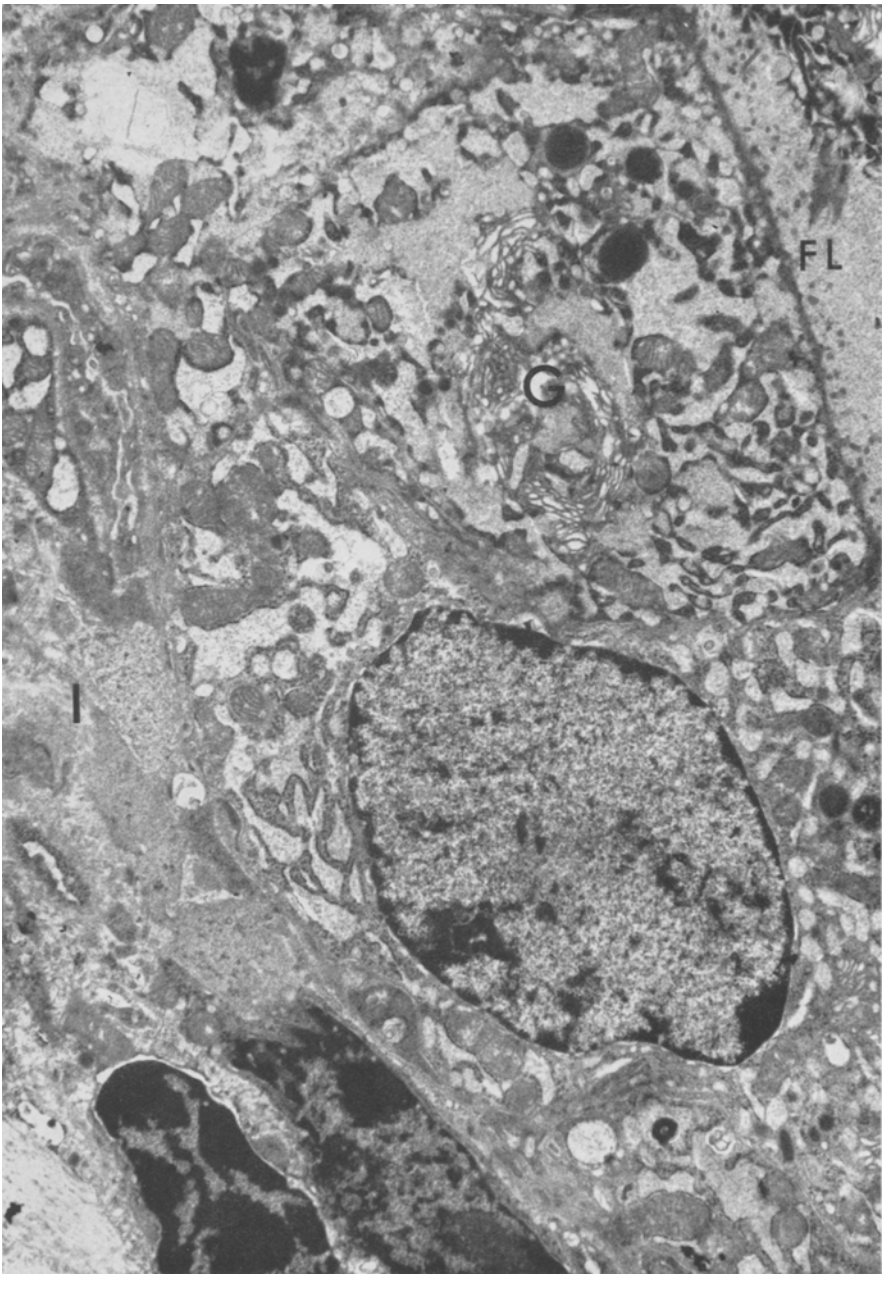

Fig. 6. E.a.t. (group I). Part of a follicle with extensive degenerative changes. Dilatation, hypertrophic golgi field (*G*) and lysosomes. *FL* follicular lumen, *I* interstitium. ×8000

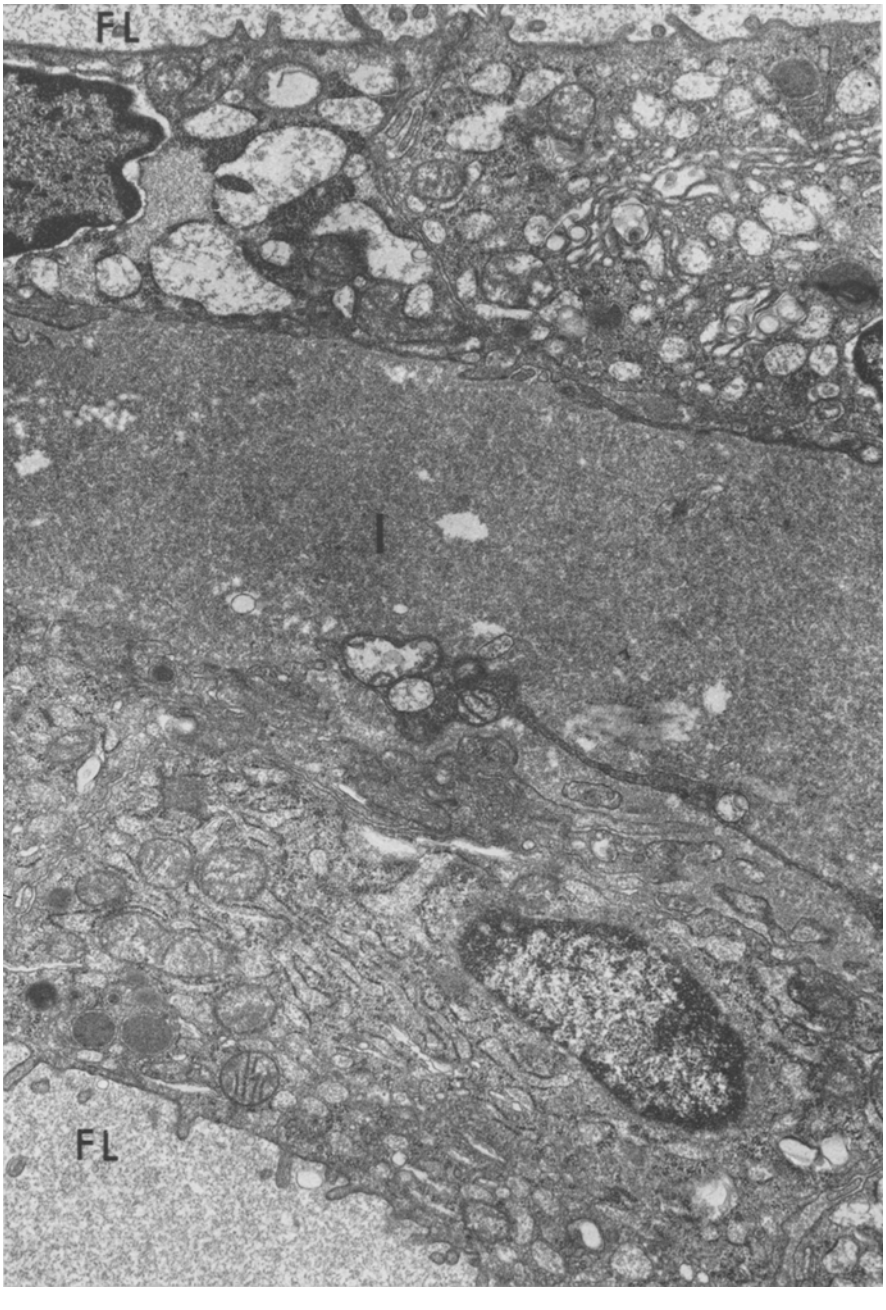

Fig. 7. Suppression of e.a.t. (group II). Widened interstitium (*I*) filled with granular colloid like material. Degenerative changes of thyrocytes. ×13500

Whereas the cellular infiltrate had rather constantly disappeared in all thyroids of desensitized animals (group II) the alteration of the parenchyma still persisted. The glands of group I animals regularly showed a marked distension of the interstitial spaces (Fig. 7) and a slight degree of interstitial fibrosis. Additionally the hypertrophy and the degeneration of the parenchymatous cells could still be shown.

Discussion

Our experiment has demonstrated that it is possible to suppress e.a.t. by daily injections of thyroglobulin incorporated in incomplete Freund's adjuvant. Our assumption that e.a.t. was present in all animals before specific desensitization started is based on several pilot studies on e.a.t. in guinea pigs, on literature data (McMaster *et al.*, 1960; Flax *et al.*, 1963; Lerner *et al.*, 1963; Koffler and Paronetto, 1964; Wasserman and Packalen, 1965; Kosunen and Flax, 1966) and on the presence of e.a.t. in 3 animals of group V which had been killed on the 28th day. Furthermore the positive delayed hypersensitivity skin test in all animals on the 27th day documented the positive immunological response in all sensitized animals.

Immune thyroiditis involves a rather uniform cellular inflammation during the first period of the disease. Later on the infiltrate becomes pleomorphic and includes macrophages as well as plasma cells (Kosunen and Flax, 1966). As could be shown immunohistochemically in our study there were scattered macrophages containing thyroglobulin. In connection with the plasma cells of the infiltrate it is tempting to hypothesize that the macrophages may act as mediators of processed antigen to lymphoid cells within the gland which under this instruction begin to synthesize antibody. Thus this process leads to a local antibody production (Kosunen and Flax, 1966).

The parenchymatous changes of the inflamed thyroid gland seems to be a characteristic result of the immune response as well in e.a.t. as in human chronic lymphocytic thyroiditis. This effect partly mimics the physiological TSH-effect on thyrocytes but in addition demonstrates a toxic-degenerative effect. Interestingly, this toxic-degenerative effect persisted even after the cellular infiltrate of the gland had completely disappeared in the desensitized animals. This might be due to the persistence of a humoral factor but cannot be explained as yet. The stimulatory effect is believed to be mediated by a humoral factor comparable to Lats (Ochi and de Groot, 1969; Néve, 1971). Our data on hypersensitivity skin reactivity and on precipitating serum antibody further support the role of cellular immunity in the pathogenesis of e.a.t. (McMaster *et al.*, 1960; Flax *et al.*, 1963; Lerner *et al.*, 1963; Spiegelberg and Miescher, 1963; Salvin and Liauw, 1966; Warnatz *et al.*, 1970). Thus there was a good correlation between the presence of thyroiditis and positive delayed skin reaction in sensitized and in desensitized animals. Such a correlation was not apparent between precipitating serum antibody and thyroiditis. In this connection it is worth to note that it was not possible to show delayed skin reactivity to guinea pig thyroglobulin in animals sensitized to bovine thyroglobulin although a mild degree of thyroiditis suggested cross reactivity (see also Flax *et al.*, 1963).

The present experiment does not allow any conclusion as to the mechanism by which the suppressive effect of desensitization of e.a.t. is mediated. In experimental autoimmune disease it is generally believed that sensitized lymphocytes initiate the inflammation in the target organ (Werdelin, 1972). Thus one possibility could be the inactivation of sensitized lymphocytes or blast cells by thyroglobulin which is released continuously from the injection site. Such an effect on sensitized cells would be consistent with the present interpretation of immune paralysis (Diener, 1970; Vasalli and McCluskey, 1971).

On the other hand, Eylar *et al.* (1972) noted an increase in the number of animals which produced antibody in the course of suppressive treatment of experimental allergic encephalomyelitis. Although precipitating antibodies to thyroglobulin in our series did not show significant differences between sensitized and desensitized animals, the presence of blocking antibodies could not be excluded.

Our presented results give evidence that experimental autoimmune thyroiditis elicited by thyroglobulin can be specifically suppressed by this antigen. The fact that the suppressive treatment is still effective once the disease has developed may become of practical importance in human thyroid disease.

The authors wish to thank Miss U. Zeiger and Miss M. Fischer for skilled technical assistance.

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