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PERPETUATION OF AUTOIMMUNE THYROIDITIS AND PRODUCTION OF SECONDARY RENAL LESIONS FOLLOWING PERIODIC INJECTIONS OF AQUEOUS PREPARATIONS OF ALTERED THYROGLOBULIN

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SUMMARY

Periodic injections of aqueous preparations of thyroglobulin coupled to the diazonium derivatives of arsanilic and sulphanilic acids (arsanil–sulphanil thyroglobulin) resulted in perpetuation of both the synthesis of circulating antibody to native thyroglobulin and thyroiditis. Two months after the last injection, the rabbits made a strong immune response to a subsequent injection of native thyroglobulin. Without the periodic injections both circulating antibody and thyroid lesions disappeared and the ability to respond to native thyroglobulin was lost. The level of circulating antibody and the incidence and severity of lesions were considerably greater in rabbits receiving periodic injections over a 6-month period of time than in rabbits given a series of injections during a period of 1 month. The relation of these findings to the progressive nature of some autoimmune diseases is discussed. Complex-induced renal injury, secondary to the immune response to arsanil–sulphanil thyroglobulin, was observed in the rabbits given the periodic injections over a 6-month period of time. Chronic glomerulonephritis apparently resulted from deposition of thyroglobulin–anti-thyroglobulin complexes and complement along the glomerular basement membranes.

INTRODUCTION

Rabbits given a series of injections of aqueous preparations of either homologous thyroglobulin coupled to the diazonium derivatives of arsanilic and sulphanilic acids (arsanil–sulphanil thyroglobulin) (Weigle, 1965) or certain heterologous thyroglobulins (Weigle & Nakamura, 1967) develop both thyroiditis and circulating antibody to thyroglobulin. The thyroid lesions appear to be similar to those observed in rabbits injected with homologous thyroglobulin incorporated into complete Freund's adjuvant (Rose & Witebsky, 1956). The disease produced following injections of the aqueous preparations is not progressive

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and after 2 months both the thyroid lesions and circulating antibody disappear (Nakamura & Weigle, 1967). At this time the rabbits will make a secondary response to an injection of either native homologous (Weigle, 1965) or autologous thyroglobulin (Weigle & High, 1967). However, the rabbits can respond to the native thyroglobulin for only a limited time and after 5 months most of them return to the unresponsive state (Weigle & Nakamura, 1967). The present experiments were designed to determine if a progressive disease of long duration could be produced by periodic injections of aqueous preparations of arsanil–sulphanil thyroglobulin over a prolonged period of time and what effect such injection had on the immune response to a subsequent injection of native thyroglobulin. In addition, the ability of these periodic injections of altered thyroglobulin to cause secondary kidney lesions was investigated.

MATERIALS AND METHODS

Isolation and purification of thyroglobulin

Rabbit thyroglobulin was isolated and purified as previously described (Weigle, 1965). Fresh, unfrozen tracheae of New Zealand White rabbits were purchased from Pel-Freez, Rogers, Arkansas. Following removal of the glands they were stripped relatively free of fat, minced with scissors, suspended in 0·15 M-NaCl (100 g tissue/150 ml) and passed through a tissue press. Debris was removed by filtration through stainless steel mesh and centrifugation at 20,000 *g* for 30 min in a Spinco model L centrifuge. The thyroglobulin was isolated by repeated centrifugation at 105,000 *g* (Edelhoch, 1960). Following centrifugation the thyroglobulin was present in the lower third of the tubes. The upper two-thirds of the fluid was aspirated and the lower portion decanted and retained.

Preparation of diazo thyroglobulin

Thyroglobulin was coupled to the diazonium derivatives of arsanilic and sulphanilic acids by a slight modification of the method described by Baker et *al.* (1956). The diazonium derivatives were prepared by dissolving 0·69 g of sulphanilic acid or 0·78 g of arsanilic acid in a freshly prepared mixture of 9·0 ml 1 N-HCl and 7·2 ml of 0·5 N-$NaNO_2$. The solutions were brought to 50 ml with distilled water and 5 ml of each preparation was added dropwise at 0°C with constant stirring to 1·0 g of thyroglobulin in 60 ml of phosphate buffer, pH 7·5 and $\mu = 0{\cdot}1$. The pH was maintained at 7·5–7·8 by addition of 0·2 N-NaOH. The final pH was adjusted to 7·8 and the conjugate stored overnight at 0–3°C. Non-coupled derivatives were removed by passage through Sephadex G-25. The thyroglobulin contained 50 azo linkages/molecule as determined spectrophotometrically at 335 mμ employing an extinction coefficient of 26,000.

Injection and bleeding of rabbits

Four groups of rabbits were injected subcutaneously each day for 4 days with 15 mg of arsanil–sulphanil thyroglobulin in an aqueous preparation. On the 5th day they received 15 mg intravenously. Two weeks later this series of injections was repeated and the rabbits were bled 7 days after the last injection. Rabbits in Groups I, II and III received a single injection of 15 mg native thyroglobulin 1, 2 and 4 months, respectively, after the last injection of arsanil–sulphanil thyroglobulin. The fourth group of rabbits received six

additional series of injections (five daily injections) given at monthly intervals. The rabbits in this group were bled 7 days following the fifth injection of each series. These rabbits received an injection of 15 mg of native thyroglobulin 2 months after the last injection of arsanil–sulphanil thyroglobulin. The rabbits in all groups were bled 7 days after the injection of native thyroglobulin and thyroid tissue was taken for histology.

Antibody analysis

The levels of circulating antibody to native thyroglobulin were measured by both haemagglutination (Boyden, 1951) and quantitative precipitation (Talmage & Maurer, 1953). In the haemagglutination technique, a 2·5% suspension of tannic acid-treated sheep cells was sensitized with 0·5 mg native thyroglobulin/ml. Before use, sera were absorbed with an equal volume of sheep erythrocytes and heated at 56°C for 20 min. The quantitative precipitin test employed involved the use of ^{131}I-labelled thyroglobulin. This test measures the amount of ^{131}I-labelled thyroglobulin precipitated at equivalence by 1 ml of serum. At equivalence the antibody–antigen ratio is approximately one with the thyroglobulin–rabbit antithyroglobulin system. The thyroglobulin was labelled with ^{131}I by the method described by McConahey & Dixon (1966).

Preparation of fluorescent antiserum reagents

Sheep anti-rabbit γ-globulin (RGG) was obtained from sheep injected periodically with RGG incorporated into complete Freund's adjuvant. The sheep γ-globulin was isolated by DEAE-cellulose chromatography using 0·0175 M-phosphate buffer at pH 6·5. Anti-rabbit thyroglobulin was obtained from guinea-pigs immunized with rabbit thyroglobulin incorporated in incomplete Freund's adjuvant and anti-β_{1C} was prepared by immunizing guinea-pigs with zymosan–β_{1C} complexes incorporated in Freund's adjuvant (Mardinay & Müller-Eberhard, 1965). Guinea-pig γ-globulin was isolated by DEAE-cellulose chromatography using 0·02 M-phosphate buffer at pH 8·0. The γ-globulin preparations were conjugated to fluorescein isothiocyanate by the method of Clark & Shepard (1963).

Histology

Thyroid tissue was taken when the rabbits were killed after the terminal bleeding. In addition, tissue was taken from two groups of rabbits by hemithyroidectomy during the experiment. The lobes were fixed in Bouin's solution, embedded in paraffin and stained with haematoxylin and eosin. The grading of thyroiditis was determined by the degree of inflammatory infiltration. The lesions were graded + if at least five foci (the size of one follicle) of infiltrating cells were present in the longitudinal section of one lobe. Lesions were graded ++ if ten to twenty foci were present, each of which occupied the area of several follicles. Lesions were graded +++ if either the entire lobe was involved with a diffuse infiltration of cells or numerous foci were present in each section which occupied relatively large areas.

When the rabbits were killed, the kidneys were removed, fixed in formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Fixed sections also were stained with periodic acid–Schiff (PAS) reagent. Glomerular lesions were graded according to the degree of both cellular proliferation and thickening of basement membrane.

Fluorescent antibody studies were performed by conventional methods previously

described (Unanue & Dixon, 1964). The degree of localization of anti-RGG, antithyroglobulin and anti-β_{1C} was determined by the intensity of fluorescence along the glomerular basement membrane and graded as +, ++ or +++.

Elution of γ-globulin from glomerular basement membrane

γ-Globulin was eluted from whole kidneys from three rabbits and sections of kidneys from five other rabbits as previously described by Lambert & Dixon (1968). All of the kidneys showed localization of fluorescent anti-RGG along the glomerular basement membrane. Homogenized kidney tissues were extensively washed with 0·15 M-NaCl and eluted for 90 min at 37°C with 0·02 M-citrate buffer, pH 3·2. The eluate was adjusted to pH 7·0 and NaCl molarity of 0·15 and concentrated by pressure dialysis, then conjugated to fluorescein isothiocyanate.

RESULTS

One series of injections of arsanil–sulphanil thyroglobulin

Approximately 75% of rabbits given a series of ten injections of arsanil–sulphanil thyroglobulin over a period of 1 month produced antibody to native thyroglobulin averaging

TABLE 1. The production of antibody to thyroglobulin (Tg) and thyroiditis in rabbits receiving injections of arsanil–sulphanil thyroglobulin (A–S Tg) and a subsequent injection of native thyroglobulin

Group	No. of rabbits	Antibody*			Lesions†							
		After A–S Tg	After native Tg		After A–S Tg‡				After native Tg			
			Pre§	Post	0	+	++	+++	0	+	++	+++
I	11	3·4 (9/11)	0·6	3·8 (8/11)	5	5	1	0	4	5	2	0
II	10	4·1 (8/10)	0·2	2·2 (5/10)					4	3	2	0
III	14	3·2 (10/14)	0	0·7 (4/13)					9	5	0	0
IV	18	53·8 (18/18)	4·7	52·9 (14/14)	0	6	9	4	0	4	4	6

Group I received one course of A–S Tg and 15 mg native Tg 1 month later.
Group II received one course of A–S Tg and 15 mg native Tg 2 months later.
Group III received one course of A–S Tg and 15 mg native Tg 4 months later.
Group IV received 6-month courses of A–S Tg and 15 mg native Tg 2 months later.

* μg precipitating antibody N/ml serum.
† Number of rabbits showing lesions of varying severity.
‡ Tissue taken by biopsy 7 days after last injection of A–S Tg.
§ Blood obtained immediately before injection of native Tg.

3·5 μg N/ml of serum (Table I, Groups I, II and III). Most of the responding rabbits produced antibody of approximately the same level following a subsequent injection of 15 mg of native thyroglobulin given 1 month later (Group I). However, when there was a longer interval between the last injection of arsanil–sulphanil thyroglobulin and injection of native thyroglobulin fewer of the rabbits responded to the injection of native thyroglobulin. Most of the rabbits injected with native thyroglobulin 4 months after the last

injection of arsanil–sulphanil thyroglobulin failed to produce antibody and the incidence of thyroiditis appeared to be less with the 4-month interval than with shorter intervals between the injections (Group III, Table 1).

In contrast, rabbits which continued to be injected monthly with arsanil–sulphanil thyroglobulin showed a progressive autoimmune disease. The level of precipitating antibody to thyroglobulin increased with each monthly injection (Fig. 1) and after the sixth series of injections the level was approximately fifteen times that observed after the two series of injections given during the 1st month (Table 1). The failure of the level of haemagglutinating antibody to significantly increase after several of the monthly injections may be the result of minor fluctuations in a highly efficient haemagglutinating antibody (19S). Thyroid tissue of rabbits taken by biopsy after the last series of injections (6th month) contained lesions and these were usually more severe than those observed in the thyroids of rabbits

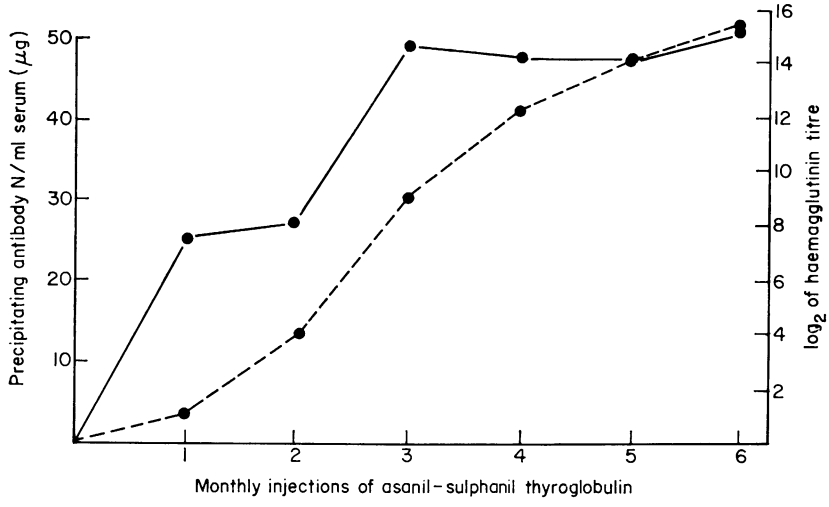

FIG. 1. Level of precipitating (– – –) and haemagglutinating (——) antibody following monthly injections of arsanil–sulphanil thyroglobulin.

given only the first series of injections (Table 1). With some rabbits almost the entire lobe was involved (Fig. 2). Two months after the sixth series of injections the level of precipitating antibody decreased dramatically. However, an injection of 15 mg of native thyroglobulin resulted in an increase in antibody in all the rabbits (Table 2). The average level of antibody was similar to that observed after the sixth series of injections of arsanil–sulphanil thyroglobulin (Table 1). Furthermore, the incidence of thyroid lesions was as frequent and at least as severe after the injection of native thyroglobulin as it was after the last injection of arsanil–sulphanil thyroglobulin (Tables 1 and 2).

Histological changes in the kidney

Kidneys of about 50% of the rabbits given six series (monthly) of injections of arsanil–sulphanil thyroglobulin contained chronic glomerular lesions. In most cases the kidneys were removed 7 days after a subsequent injection of native thyroglobulin given 2 months

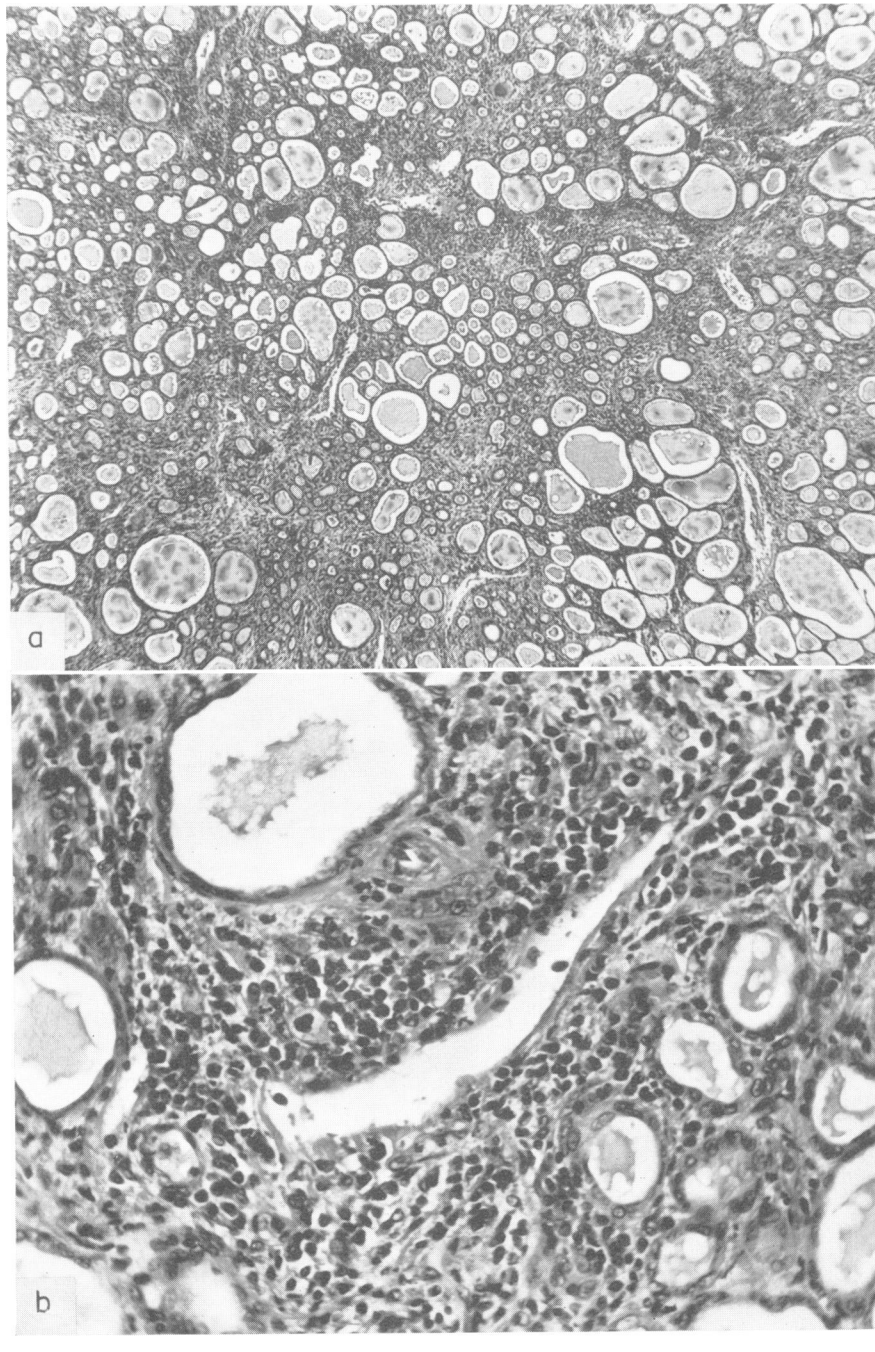

FIG. 2. Section of thyroid gland removed 7 days after last injection of arsanil–sulphanil thyroglobulin. H & E, (a) × 23, (b) × 200.

after the sixth series. The lesions in most kidneys were characterized by proliferation of endothelial cells and thickening of the glomerular basement membrane. The kidney of one rabbit (No. 10, Table 2) which died of apparent anaphylaxis following an intravenous injection of arsanil–sulphanil thyroglobulin during the 6th month contained quite severe glomerular lesions (Fig. 3) with marked cellular proliferation, thickening of basement membrane, scarring and crescent formation. Rabbit γ-globulin was localized along the

TABLE 2. Changes in the thyroid and kidneys resulting from both injections of arsanil–sulphanil thyroglobulin (A–S Tg) over prolonged periods of time and a subsequent injection of native thyroglobulin*

Rabbit No.	Thyroid lesions		Kidney				Antibody§	
	After injection of A–S Tg‡	After injection of native Tg	Lesions	Localization of: Anti-RGG	Anti-Tg	Anti-β_{1C}	Before injection of native Tg	After injection of native Tg
1	+	+	0	0		0	0	4·6
2	+	+	++	+++		++	9·4	34·6
3	++	+	+	+		+	0	10·1
4	++	+	0	+		0	0	10·1
5	++	+++	0	0		0	7·2	62·4
6	+	++	+++	+++	+	++	6·1	47·2
7	+	+++	++	++	+	++	0	5·9
8	+	++	+	+		0	6·6	110·2
9	+	Died	0	0		0		
10†	+++	Died	++++					
11	++	++	0	0		0	0	1·9
12	++	Died						
13	+++	+++	+	++		+	2·6	36·0
14†	++	Died	0	+		+		
15	+++	+++	++	++	+	++	16·5	88·2
16	++	++	0	0		0	0	3·7
17	++	+++	0	0		0	10·9	77·8
18†	+++	Died	0	+		0		
19	++	+++	+	++	+	+	6·4	52·9

* Given six monthly courses of A–S Tg and injected with natural (unaltered) Tg 2 months after the last course.

† Rabbits died before injection of native Tg and kidneys removed shortly following death.

† One lobe removed by biopsy 7 days after injection.

§ μg precipitating antibody N/ml serum.

basement membrane of the glomeruli of all rabbits having obvious glomerular lesions and several having no apparent lesion (Table 2, Fig. 4). Because of the density of fluorescent staining, the pattern of localization in a large part of the glomeruli was difficult to determine. However, close observation of less dense stain areas reveals the granular pattern of the localization. A higher magnification of a less dense area is shown in Fig. 5. β_{1C} also was localized along the basement membrane of the glomeruli of most of the rabbits showing RGG localization. Four of the kidneys showing glomerular lesions and localization of both RGG and β_{1C} were tested for localization of rabbit thyroglobulin by the indirect

fluorescent technique. The basement membrane of these animals showed both definite and specific localization, but the staining was not very strong. There was no correlation of histology of the kidneys with degree of thyroiditis. Likewise, there was no relationship between the level of circulating antigen and thyroiditis. However, there was a correlation between the peak level of precipitating antibody to native thyroglobulin and kidney histology, in that the rabbits that showed localization of RGG along the basement membrane usually had the higher levels of antibody. None of three rabbits with less than 10 μg antibody N/ml of serum and only one of seven rabbits with less than 20 μg antibody N/ml of serum showed localization of RGG. In contrast, nine of eleven rabbits having 45 μg antibody

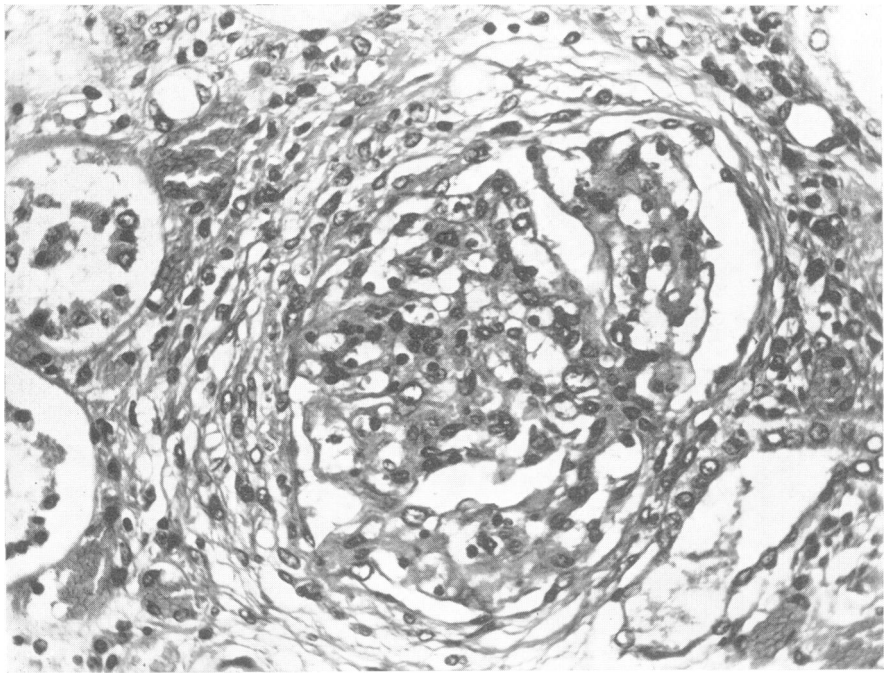

FIG. 3. Glomerulus of kidney from rabbit No. 10 (Table 2). Figure shows marked cellular proliferation, thickening of basement membrane, scarring and cresent formation. H & E, $\times$ 250.

N/ml of serum or greater showed localization. The serum of the rabbit which had the most severe glomerulonephritis (No. 10, Table 2) and died of anaphylaxis contained 240 μg antibody N/ml following the last series of injections of arsanil–sulphanil thyroglobulin.

Fluorescent labelled γ-globulin eluted from the kidneys of rabbits showing both glomerulonephritis and localization of RGG along the basement membrane failed to fix to basement membrane in a kidney section obtained from a normal rabbit.

DISCUSSION

In contrast to persisting autoimmune thyroiditis induced in rabbits by injecting thyroglobulin incorporated into complete adjuvant, thyroiditis resulting from injections of

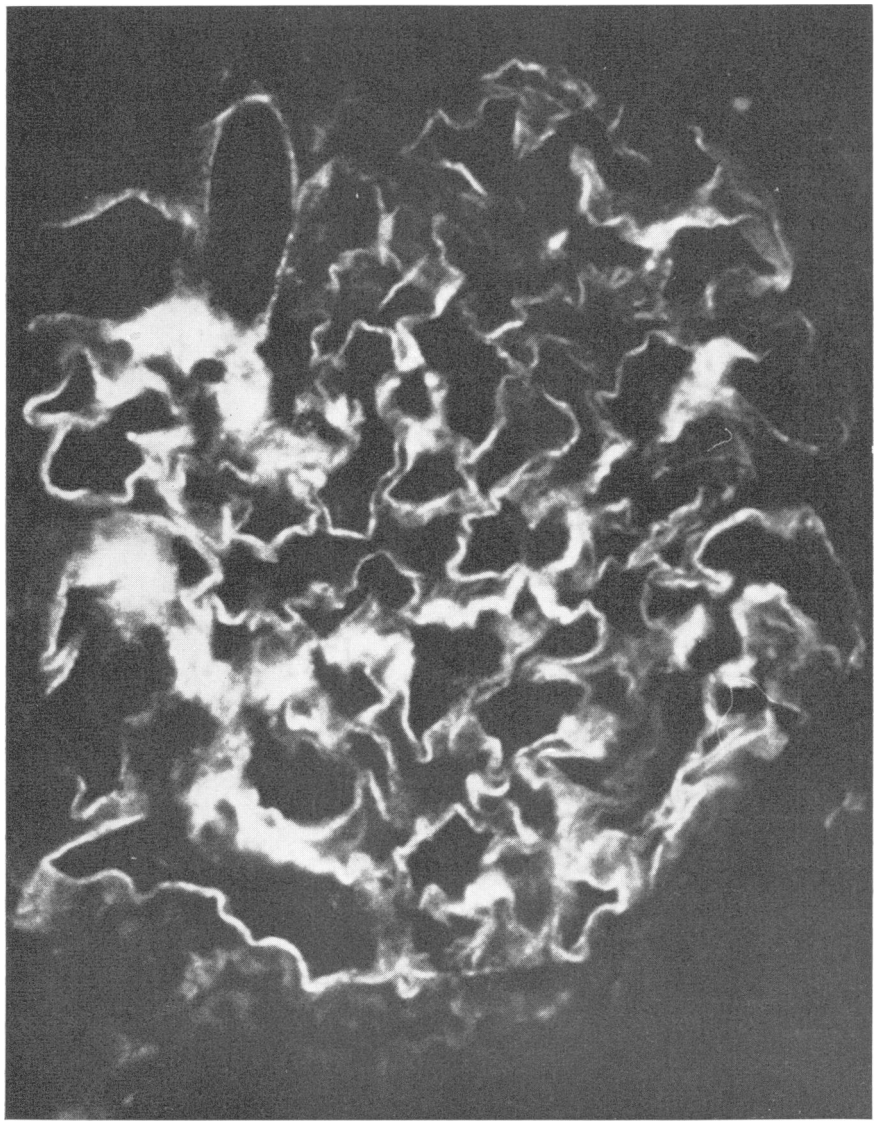

FIG. 4. Localization of rabbit γ-globulin along basement membrane of glomerulus from kidney removed 7 days after last injection of arsanil–sulphanil thyroglobulin. Stained with fluorescein labelled anti-rabbit γ-globulin. $\times$ 1025.

aqueous preparations of thyroglobulin coupled to the diazonium derivatives of arsanilic and sulphanilic acids is only transient. Both the thyroid lesions and the circulating antibody to native thyroglobulin begin to disappear shortly after the last of a series of injections of the arsanil-sulphanil thyroglobulin and after 2 months thyroiditis and antibody cannot be detected (Nakamura & Weigle, 1967). The rabbits do respond to subsequent injections of native thyroglobulin, but their ability to do so rapidly declines. Likewise, most rabbits injected with an aqueous preparation of heterologous thyroglobulin respond with both

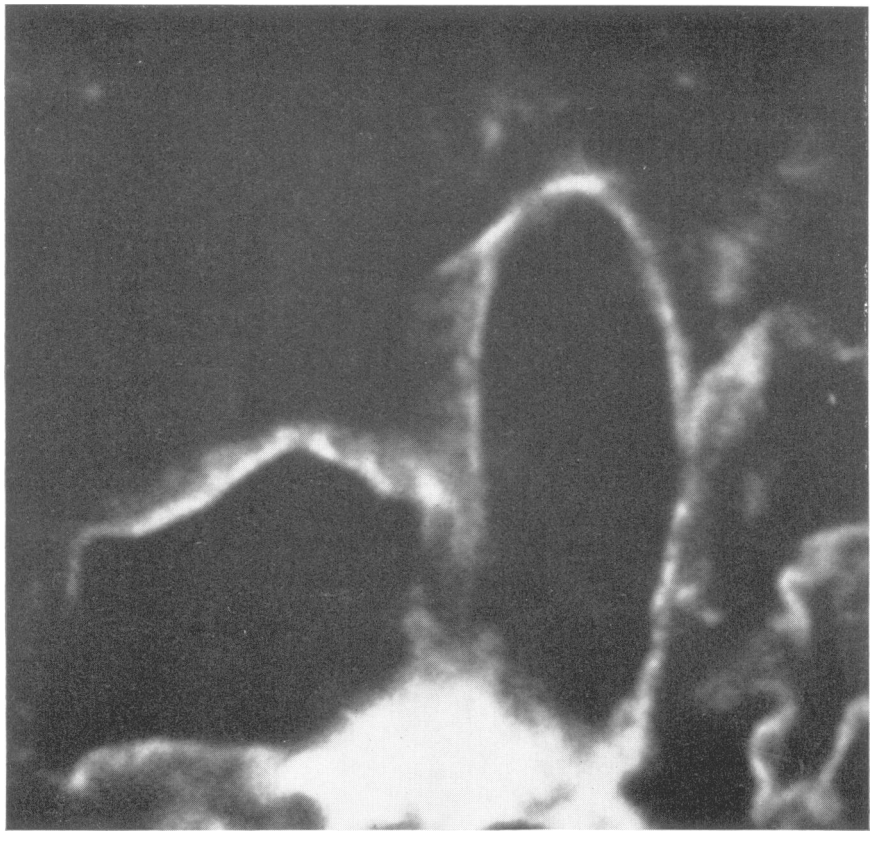

FIG. 5. Enlarged (× 2375) area of Fig. 4 showing the granular nature of the localization.

increased thyroiditis and antibody following a subsequent injection of rabbit thyroglobulin 2 months later, but only a few respond to a similar injection given at 5 months (Weigle & Nakamura, 1967). Thyroiditis and the immune response to thyroglobulin can both be perpetuated and markedly enhanced by periodic injections of aqueous preparations of arsanil-sulphanil thyroglobulin. Rabbits given a series of injections of arsanil-sulphanil thyroglobulin each month for 6 months continue to show significant increases in the level of precipitating antibody, even after the 6th month. The incidence and severity of the lesions at this time were much greater than following a series of injections given over a period of 1 month. The ability of rabbits to respond to native thyroglobulin is also much

more pronounced in rabbits receiving periodic injections over 6 months than in rabbits receiving periodic injections during 1 month.

If some autoimmune diseases are the result of an immune response to altered self antigens, as has been suggested, then progressive autoimmune diseases like Hashimoto's thyroiditis may require altered antigens to persist throughout the life of the individual. Transient trauma or infection may be expected to result only in a transient disease which may be perpetuated by unaltered tissue antigens for only a limited period of time (Weigle, 1967), but shortly after the insult that caused the alteration was corrected the autoimmune state would be alleviated. Such may be the case in acute transient forms of acquired haemolytic anaemia and idiopathic thrombocytopenic purpura. If altered tissue components are responsible for autoimmunity, it appears from the present studies that the alteration would have to be permanent in the progressive autoimmune diseases. It seems that a genetic alteration possibly involving a viral transformation would most likely result in permanent structural changes. In any event, structural studies of subunits of thyroglobulin isolated from both normal and Hashimoto's patients might be helpful in determining if such alterations take place.

Chronic glomerular lesions occur in the kidneys of rabbits injected with six monthly courses of aqueous arsanil–sulphanil thyroglobulin which are secondary to the immune response to the arsanil–sulphanil thyroglobulin and possibly, in part, to the thyroiditis. The lesions probably result from localization of complexes formed in the circulation between circulating antibody and the injected arsanil–sulphanil thyroglobulin and/or native thyroglobulin released from the injured thyroid gland. The experimental data overwhelmingly support the contention that the glomerular lesions are induced by complexes rather than antibody direct to the basement membrane.

(1) There is a correlation between the presence of lesions and the level of antibody to thyroglobulin.

(2) The deposition of rabbit γ-globulin along the basement membrane is granular in nature. It has previously been shown that granular or lumpy deposition of RGG along the basement membrane is characteristic of a complex induced lesion, while a linear deposition is characteristic of a disease resulting from antibody direct to basement membrane (Unanue & Dixon, 1967).

(3) Thyroglobulin also is found deposited in a granular pattern along the basement membrane.

(4) γ-Globulin extracted from basement membrane of rabbits with both lesions and deposition of RGG did not fix to glomerular basement membrane of normal kidneys. The mechanism involved is probably similar to that resulting in chronic glomerulonephritis following daily injections of heterologous serum proteins (Dixon, Feldman & Vazquez, 1961). Similar lesions were observed in rabbits given a series of injections of arsanil–sulphanil thyroglobulin and at a later time autologous thyroglobulin being released into the circulation following destruction of the thyroid gland (Weigle & High, 1967). Complexes formed between several self antigens and their respective antibody have been reported by others to be responsible for glomerular lesions. Edgington, Glassock & Dixon (1967) have reported that a renal tubular epithelial component is deposited in association with γ-globulin (antibody) and complement in glomeruli from rats with glomerulonephritis induced by immunization with renal tubular components incorporated into complete Freund's adju-

vant. Kunkel *et al.* (1961) detected γ-globulin complexes in the sera of patients with rheumatoid arthritis which apparently represented complexes between γG and anti-γG. Tan, Schur & Kunkel (1966) and Krishman & Kaplan (1966) implicated complexes formed between DNA and anti-DNA as the causative agent for renal injury in patients with systemic lupus erythematosus. It should be noted that there is no evidence at the present time for complex-induced injury in Hashimoto's thyroiditis.

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