THE PRODUCTION OF THYROIDITIS AND ANTIBODY FOLLOWING INJECTION OF UNALTERED THYROGLOBULIN WITHOUT ADJUVANT INTO RABBITS PREVIOUSLY STIMULATED WITH ALTERED THYROGLOBULIN*

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Acquired tolerance induced in rabbits to serum protein antigens by neonatal injection of the antigens can be readily terminated by injecting either certain antigens which cross-react with the tolerated antigen (1) or certain altered preparations of the tolerated antigens (2). Similarly, antibody to thyroglobulin and, in some cases, thyroiditis can be produced in rabbits by injecting them with altered preparations of homologous thyroglobulin incorporated into incomplete (without mycobacteria) Freund's adjuvant, while similar injections of native thyroglobulin did not result in appreciable production of antibody to thyroglobulin or thyroiditis (3). The injection of rabbits with thyroglobulin altered by coupling to diazonium derivatives without adjuvant also was shown to be an effective way to produce both thyroiditis and circulating antithyroglobulin (3). The use of altered thyroglobulin without adjuvant to produce autoimmune thyroiditis permits the investigations of the effect of booster injections of native thyroglobulin, since the altered thyroglobulin is rapidly catabolized and does not persist as a continuous stimulus, as is the case when Freund's adjuvant is employed. The present experiments were designed to study the production of autoimmune thyroiditis following a course of injections of altered thyroglobulin without adjuvant and the subsequent injections of native thyroglobulin.

Materials and Methods

Isolation and Purification of Thyroglobulin.—Rabbit thyroglobulin was isolated and purified as previously described (3) using a modification of the method described by Edelhoch (4).

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Fresh unfrozen tracheae of New Zealand white rabbits were obtained from Pel-Freeze, Rogers, Arkansas. The lobes of the thyroids were removed and stripped relatively free of fat. The glands were minced with scissors, suspended in 0.15 M NaCl (100 gm of tissue/150 ml) and passed through a tissue press. Tissue debris was removed by filtration through stainless steel mesh and centrifugation at 20,000 *g* for 30 minutes, in a Spinco model L preparatory centrifuge. The thyroglobulin was isolated by centrifugation at 105,000 *g*. Following centrifugation the thyroglobulin was present in the lower one-third of the tube. The upper two-thirds of the fluid was aspirated and the lower portion decanted. A small amount of heavy material forming a button at the bottom of the tube was discarded. The thyroglobulin was diluted to one-half the original concentration and purified further by repeated centrifugation at 105,000 *g*.

Preparation of Diazo Thyroglobulin.—Rabbit thyroglobulin was coupled to the diazonium derivatives of arsanilic and sulfanilic acid by a slight modification of the method described by Baker *et al.* (5). The diazonium derivatives were prepared by dissolving 0.069 gm of sulfanilic acid or 0.078 gm of arsanilic acid in a mixture of 0.9 ml of 1 N HCl and 0.72 ml of 0.5 N $NaNO_2$. The solutions were brought to 5 ml with distilled water and added dropwise, at 0°C with constant stirring, to 1.0 gm of thyroglobulin in 60 ml of phosphate buffer, pH 7.5 and μ = 0.1. The pH was maintained at 7.5 to 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. The non-coupled derivatives were removed from the modified thyroglobulin by passage through sephadex G-25. The number of azo linkages was estimated spectrophotometrically at 335 mμ employing an extinction coefficient of 26,000 (6). The arsanil-sulfanil-thyroglobulin preparations employed in this study contained approximately 50 azo linkages/molecule of thyroglobulin.

Injection and Bleeding of Rabbits.—Rabbits were injected subcutaneously each day for 4 days with 15 mg of altered thyroglobulin. On the 5th day they received 15 mg intravenously. Two weeks later this series of injections was repeated. The rabbits were bled 7 days after the last (tenth) injection. Subsequent injections of native thyroglobulin were given intravenously and the animals bled 7 days later.

Antibody Analysis.—The levels of circulating antibody to native thyroglobulin were measured by both a quantitative precipitin (7) and the hemagglutination (8) techniques. The quantitative precipitin test employed involved the use of I^{131}-labeled antigen, *i.e.*, in this case I^{131} thyroglobulin. This test measures the amount of I^{131} thyroglobulin precipitated at equivalence by 1 ml of serum. At equivalence the antibody/antigen ratio is approximately 1 with the thyroglobulin-rabbit antithyroglobulin system. In the hemagglutination technique, a 2.5 per cent suspension of tannic acid–treated sheep erythrocytes was sensitized with 0.5 mg of native thyroglobulin per ml. Before use, sera were absorbed with an equal volume of sheep erythrocytes and heated at 56°C for 20 minutes. Qualitative tests for the presence of precipitating antibody were performed by Preer's double diffusion in agar technique (9).

In one experiment, tests for hemagglutinating antibody to native thyroglobulin were performed both prior to and after treatment of the sera with 2-mercaptoethanol. The sera were diluted twofold with 0.15 M NaCl and dialyzed overnight at room temperature against 0.1 M 2-mercaptoethanol in 0.15 M NaCl. The sera were then dialyzed for 24 hours at 0–3°C against 0.01 M iodoacetamide in 0.15 M NaCl. The excess iodoacetamide was removed by dialysis against 0.15 M NaCl.

Histology.—Thyroid tissue was usually taken both by hemithyroidectomy during the experiment and when the rabbits were sacrificed after the terminal bleeding. The tissue was fixed in Bouin's solution, embedded in paraffin, and sections cut through the long axis. The sections were mounted and stained with hematoxylin and eosin.

The grading of thyroiditis was determined by the degree of inflammatory, cellular infiltration. The lesions were graded + if at least 5 foci (the size of one follicle or less) of infiltrating cells were present in the longitudinal section of one lobe. Lesions were graded ++ if 10 to 20

foci were present each of which occupied the area of several follicles. Lesions were graded +++ if either numerous foci were present in each section each of which occupied areas the size of a number of follicles or the entire lobe was involved with a more diffuse infiltration.

RESULTS

Injection of Arsanil-Sulfanil-Thyroglobulin and One Subsequent Injection of Native Thyroglobulin—Seven days after the last of a series of injections of arsanil-sulfanil-thyroglobulin without adjuvant the sera of most rabbits contained hemagglutinating antibody to native thyroglobulin (Table I). After 1 month, the titers had decreased appreciably; however, thyroid lesions were observed in some tissue taken at this time by hemithyroidectomy. Following a subsequent injection of 15 mg of native (unaltered) thyroglobulin the level of hemagglutinating antibody to native thyroglobulin in the sera of many of these rabbits greatly increased. In some cases the titers were higher after the injection of native thyroglobulin than after the initial injections of arsanil-sulfanil-thyroglobulin. The lesions observed in thyroid tissue increased after the injec-

TABLE I

Production of Hemagglutinating Antibody in Rabbits Injected with Arsanil-Sulfanil-Thyroglobulin (Tg) and Subsequently Injected with Native Tg*

Rabbit No.	After injection of arsanil-sulfanil-Tg	Injection of native Tg	
		Pre	Post
1	16	0	0
2	128	2	0
3	2,048	64‡	4,096§
4	256	32§	1,024§
5	8	0	0‡
6	128	2‡	64‡
7	4	0‡	0‡
8	1,024	128	16,384
9	16	0	0‡
10	64	64	4
11	512	16	32
12	2,048	16	1,024‡
13	256	16‡	512§
14	8	0	0
15	32	0‡	0‡
16	512	Died	
17	4,096	1,024‡	524,288‡

* Reciprocal of the highest serum dilution completely agglutinating sheep red blood cells coated with native thyroglobulin.

‡ Denotes presence of thyroid lesions (+ lesion).

§ Indicates more severe lesions (++ lesions).

tion of native thyroglobulin. However, there was no correlation between levels of antibody and extent of lesions.

Similar results were obtained when precipitating antibody to native thyroglobulin was measured. Fourteen of the 17 rabbits produced precipitating antibody to arsanil-sulfanil-thyroglobulin following the injections of arsanil-sulfanil-thyroglobulin. Eight of these 14 rabbits also produced antibody which precipitated with native thyroglobulin (Table II). One month after the injec-

TABLE II

Production of Precipitating Antibody (μg N) in Rabbits Injected with Arsanil-Sulfanil-Tg and Subsequently Injected with Native Tg

Rabbit No.	After injection of arsanil-sulfanil-Tg	Injection of native Tg	
		Pre	Post
1	0	0	0
2	0	0	0
3	2.2	0*	4.5‡
4	1.4	0‡	0.9‡
5	0	0	0*
6	0	0*	0*
7	0	0*	0*
8	16.0	1.2	27.0
9	0	0	0*
10	1.8	0	0
11	0	0	0
12	6.5	0	5.4*
13	2.0	0*	1.0‡
14	0	0	0
15	0	0*	0*
16	2.3	—	—
17	35.7	6.2*	76.0

* Denotes presence of thyroid lesions (+ lesions).

‡ Indicates more severe lesions (++ lesions).

tions of arsanil-sulfanil-thyroglobulin the level of precipitating antibody decreased substantially. Six of the 8 rabbits responded to a subsequent injection of 15 mg of native thyroglobulin. In several rabbits the level of precipitating antibody to native thyroglobulin was higher after injection of the native thyroglobulin than after the prior injections of arsanil-sulfanil-thyroglobulin.

Ten rabbits initially given a series of injections of native thyroglobulin followed by a single injection of 15 mg of native thyroglobulin at no time had antibody to thyroglobulin nor thyroid lesions.

Injection of Arsanil-Sulfanil-Thyroglobulin and Three Subsequent Injections of Native Thyroglobulin.—As in the above experiment, most of the rabbits given

two series of injections of arsanil-sulfanil-thyroglobulin contained hemagglutinating antibody to native thyroglobulin in the sera obtained 7 days after the last injection (Table III). Lesions (+ to ++) were observed in thyroid tissue taken at this time from 4 of the 12 rabbits. Again, there was no correlation between the presence or absence of lesions and the level of hemagglutinating antibody. The rabbits were then given 3 monthly injections of native thyroglobulin and the sera analyzed for hemagglutinating antibody to native thyroglobulin, both before and 7 days after each injection. Following the first

TABLE III

Production of Thyroiditis and Hemagglutinating Antibody to Native Thyroglobulin Following Injection of Arsanil-Sulfanil-Tg and Three Subsequent Injections of Native Tg into Rabbits*

Rabbit No.	After injection of arsanil-sulfanil-Tg		Antibody after monthly injections of native Tg						Lesions after the last injection of native Tg
			1		2		3		
	Lesions	Antibody	Pre	Post	Pre	Post	Pre	Post	
1	−	128	8	64	16	256	64	4,096	+++
2	+	64	4	0	0	0	0	0	+
3	+	16	64	512	2,048	256	256	4,096	++
4	−	2,048	2,048	8,192	2,048	16,384	2,048	2,048	+
5	−	0	0	0	0	0	0	0	−
6	−	512	256	256	64	32	16	32	+
7	−	0	0	0	Died				
8	+	16	4	0	0	0	0	0	−
9	−	2,048	128	2,048	64	256	128	64	+
10	++	2,048	1,024	65,536	262,144	2,097,152	16,384	16,384	++
11	−	64	32	64	32	64	32	32	−
12	−	512	512	16,384	524,288	8,192	1,024	4,096	++

* Reciprocal of the highest serum dilution completely agglutinating sheep red blood cells coated with native thyroglobulin.

injection, the sera of 6 of the 10 rabbits which responded after injection of arsanil-sulfanil-thyroglobulin contained increased levels of hemagglutinating antibody to native thyroglobulin, the sera of 2 showed no change in levels of hemagglutinating antibody and the sera of 2 others contained no hemagglutinating antibody. The 2 rabbits which did not respond to injections of arsanil-sulfanil-thyroglobulin did not respond to any of the subsequent injections of native thyroglobulin. During the next 30 days, 2 rabbits showed an increase in the level of antibody, although no additional injection was given. After the second monthly injection of native thyroglobulin, the sera of 5 of the 8 rabbits which showed a response to the first injection showed an increase in the level of antibody to native thyroglobulin, 2 showed no change, and 1 showed a de-

crease in level. Following the third injection, most of the rabbits appeared to be losing their ability to make hemagglutinating antibody to native thyroglobulin. The sera of only 2 rabbits contained a level of hemagglutinating antibody as high or higher than the previous peak titer.

The precipitating antibody in the sera of the rabbits also was measured. Eight of 12 rabbits produced precipitating antibody to arsanil-sulfanil-thyroglobulin, following the 10 injections of arsanil-sulfanil-thyroglobulin. Four of these 8 rabbits also produced antibody which precipitated with native thyro-

TABLE IV

Production of Thyroiditis and Precipitating Antibody (μg N/ml serum) to Native Thyroglobulin Following Injection of Arsanil-Sulfanil-Tg and Three Subsequent Injections of Native Tg Into Rabbits

Rabbit No.	After injection of arsanil-sulfanil-Tg		Antibody after monthly injections of native Tg						Lesions after the last injection of native Tg
			1		2		3		
	Lesions	Antibody	pre	Post	Pre	Post	Pre	Post	
1	−	0	0	0	0	Trace*	Trace	0.2	+++
2	+	0	0	0	0	0	0	0	+
3	+	0	0	0	0	0.3	0.2	0.3	++
4	−	10.1	2.9	5.6	1.9	4.6	1.8	0.2	+
5	−	0	0	0	0	0	0	0	−
6	−	0	0	0	0	0	0	0	+
7	−	0	0	0	Died				
8	+	0	0	0	0	0	0	0	−
9	−	0.4	0	0.8	0	0.2	0	0	+
10	++	24.8	6.1	51.0	17.9	29.4	16.3	32.6	++
11	−	0	0	0	0	0	0	0	−
12	−	6.1	1.3	20.8	6.8	5.4	2.6	6.2	++

* Detected by double diffusion in agar but the amount was too small to quantitate.

globulin (Table IV). During the next month the levels decreased considerably. The sera of all 4 rabbits showed a subsequent rise in titer following an injection of native thyroglobulin and in the sera of 3 of the rabbits the level of antibody was higher than after the injection of arsanil-sulfanil-thyroglobulin. Again, the levels of precipitating antibody decreased considerably over the next 30 days. Following a second injection of 15 mg of native thyroglobulin, the sera of 3 of these 4 rabbits showed an increase in precipitating antibodies, however, the levels were lower than after the first injection of native thyroglobulin. The sera of two additional rabbits which previously contained no precipitating antibody contained small amounts of precipitating antibody after the second injection. Of the 4 rabbits which contained precipitating antibody to native thyroglobu-

lin after the injection of arsanil-sulfanil-thyroglobulin, 2 failed to produce precipitating antibody to a third injection of native thyroglobulin and 2 showed an increase in the level of precipitating antibody, but the level was less than that produced after the first injection of native thyroglobulin. The 2 rabbits which produced small amounts of precipitating antibody after the second injection also produced small amounts after the third injection.

With some of the sera there was no correlation between the rise and drop in the levels of precipitating and hemagglutinating antibodies to native thyro-

TABLE V

Comparison of Precipitating and Hemagglutinating Antibody to Native Thyroglobulin Following Injection of Arsanil-Sulfanil-Tg and Three Subsequent Injections of Native Tg into Rabbits

Rabbit No.	Serological test	Antibody after injection of arsanil-sulfanil-Tg	Antibody after monthly injections of native Tg					
			1		2		3	
			Pre	Post	Pre	Post	Pre	Post
4	P*	10.1	2.9	5.6	1.9	4.6	1.8	0.2
	H*	2,048	2,048	8,192	2,048	16,384	2,046	2,048
	H-M*	—	—	—	1,024	4,096	4,096	128
10	P	24.8	6.1	51.0	17.9	29.4	16.2	32.6
	H	2,048	1,024	65,136	262,144	2,097,152	16,384	16,384
	H-M	2,048	1,048	8,196	2,048	8,192	4,096	16,384
12	P	6.1	1.3	20.8	6.8	5.4	2.6	6.2
	H	512	512	16,384	524,288	8,192	1,024	4,096
	H-M	1,024	128	8,196	4,096	4,096	2,048	8,192

* P, precipitating antibody, μg antibody N/ml serum; H, hemagglutinating antibody. Reciprocal of the highest serum dilution completely agglutinating sheep red blood cells coated with native thyroglobulin; H-M, hemagglutinating antibody after treatment with 2-mercaptoethanol.

globulin (Table V). This lack of correlation was especially noticeable when extremely high levels of hemagglutinating antibody were present. In order to determine if the high levels of hemagglutinating antibody were the result of The presence of 19S antibody, the sera were analyzed after treatment with 2-mercaptoethanol. After treatment of the sera, the rise and drop in the levels of hemagglutinating antibody correlated more closely with the rise and fall in the levels of precipitating antibody (Table V). The titers in sera from 2 rabbits (Nos. 3 and 9) which produced precipitating antibody (Table IV), but not extremely high levels of hemagglutinating antibody (Table III) were not affected by treatment with 2-mercaptoethanol and the results are given in Table V. As shown previously (3), the antibody produced following injection

of arsanil-sulfanil-thyroglobulin is apparently 7S antibody and not affected by 2-mercaptoethanol. However, following subsequent injections of native thyroglobulin, some rabbits began to produce some mercaptoethanol sensitive (19S) as well as mercaptoethanol insensitive (7S) antibody. With 2 of the rabbits (Nos. 10 and 12), the 19S antibody was most abundant in sera taken preceding and/or just following the second monthly injection of native thyroglobulin. During the next month the 19S antibody virtually disappeared from these sera and a subsequent injection of native thyroglobulin resulted in production of only 7S antibody. Another rabbit (No. 4) which was losing its ability to make precipitating and 7S hemagglutinating antibody did produce some 19S after the third injection of native thyroglobulin. In terms of absolute weight, the amount of 19S antibody was probably quite small, since 19S antibody is much more efficient in hemagglutinating (10, 11) and the high levels of 19S antibody did not result in high levels of precipitating antibody. All of the 19S antibody apparently precipitates with the 7S antibody since absorption of sera, containing high titers of hemagglutinating antibody, at equivalence with thyroglobulin removed all hemagglutinating antibody.

Lesions observed in thyroid tissues taken after the third monthly injection of native thyroglobulin were both more frequent and severe than those observed after the injections of arsanil-sulfanil-thyroglobulin. Two different histological pictures were observed in the tissue taken at this later time. The lesions in tissue taken from some rabbits were characterized by foci of dense infiltration of mononuclear cells (Figs. 1 *a* and 1 *b*). This type of lesion was usually observed in tissue taken from rabbits which contained no precipitating antibody (rabbits 2, 3, 6, and 9) (Table IV). The same type of lesions was observed in tissue taken after injections of only arsanil-sulfanil-thyroglobulin. With one exception (rabbit 3) these lesions were the least severe lesions. This rabbit also contained a small amount of antibody in its serum. The second type of lesion was characterized by diffuse infiltration of mononuclear cells and a marked increase in connective tissue (Figs. 2 *a* and 2 *b*), although occasional foci of dense infiltration of mononuclear cells were observed. These lesions were usually more severe and seen in tissue taken from rabbits which contained precipitating antibody in their sera (rabbits 1, 4, 10, and 12). It is likely that the later lesion is a progressive phase of the former lesion.

Rabbits initially given two series of injections of native instead of arsanil-sulfanil-thyroglobulin and then injected with 3 subsequent injections of native thyroglobulin produced no circulating antibody and contained no significant thyroid lesions.

Injection of Arsanil-Sulfanil-Thyroglobulin and Subsequent Autotransplantation of Thyroid Tissue.—As with the previous 2 experiments, the sera of most rabbits given two series of injections of soluble arsanil-sulfanil-thyroglobulin contained hemagglutinating antibody to native thyroglobulin and the titer of

this antibody markedly decreased during the next 30 days (Table VI). At this time one lobe of the thyroid gland was removed and transplanted to the exterior surface of the splenius capitis muscle of the neck. Under these conditions, the lobe of the thyroid undergoes central necrosis (Figs. 3 *a* and 3 *b*), presumably with release of thyroglobulin into the tissue but the peripheral follicles are preserved. With approximately 60 per cent of these rabbits there was no significant change in the titer of sera taken before and 10 days after autotrans-

TABLE VI

Production of Hemagglutinating Antibody in Rabbits Injected with Arsanil-Sulfanil-Tg and Received an Autotransplant of Thyroid Tissue

Rabbit No.	After injection of arsanil-sulfanil-Tg	Autotransplant		
		Antibody*		Lesions
		Pre	Post (10 days)	
1	256	16	64	+
2	256	32	4	+
3	256	32	512	++
4	8	0	0	−
5	128	16	32	+
6	256	64	32	+
7	1,024	128	8,192	+
8	4,096	16,384	1,049,376	+++
9	32	4	0	−
10	4,096	1,024	131,172	−
11	256	16	32	++
12	512	512	1,024	−
13	256	64	64	+

* Reciprocal of the highest serum dilution completely agglutinating sheep red blood cells coated with native thyroglobulin.

plantation of thyroid tissue. However, with some of the rabbits (Nos. 1, 3, 7, 8, and 10) there was a marked increase in hemagglutinating antibody to native thyroglobulin after autotransplantation. Four of the above 5 rabbits also produced precipitating antibody to native thyroglobulin (Table VII). Another rabbit which produced a small amount of precipitating antibody after injection of arsanil-sulfanil-thyroglobulin made no precipitating antibody following autotransplantation.

Sera taken from 6 normal rabbits (not injected with arsanil-sulfanil-thyroglobulin) 10 days after autotransplantation of thyroid tissue contained no antibody to thyroglobulin and thyroid tissue taken at this time showed no lesions.

DISCUSSION

It has become possible to study autoimmunity to thyroglobulin and experimental thyroiditis without use of adjuvant. As shown previously (3) circulating antibody to native thyroglobulin and thyroiditis readily can be produced in rabbits by injecting homologous thyroglobulin without adjuvant which is first altered by coupling to diazonium derivatives of arsanilic and sulfanilic acids. Following a latent period, many of the rabbits then will respond to injections of native (unaltered) thyroglobulin by producing hemagglutinating antibody and, in some cases, precipitating antibody to native thyroglobulin. Some of the rabbits respond to as many as 3 subsequent injections of native

TABLE VII

Production of Precipitating Antibody (μg N/ml serum) in Rabbits Injected with Arsanil-Sulfanil-Tg and Subsequently Received an Autotransplant of Thyroid Tissue

Rabbit No.	After injection of arsanil-sulfanil-Tg	Autotransplant	
		Pre	Post (10 days)
1	3.1	0	0.1*
6	0.5	0	0*
7	1.2	0	1.0*
8	27.2	13.8	46.4‡
10	65.6	3.6	32.8

* Denotes thyroid lesions (+ lesions).

‡ Indicates more severe lesions (++ lesions).

thyroglobulin. The subsequent injections of native thyroglobulin also appear to result in an increase in both severity and frequency of thyroid lesions. The injections of native thyroglobulin without adjuvant into rabbits not previously immunized with arsanil-sulfanil-thyroglobulin induces neither circulating antibody nor thyroiditis.

The present results suggest the possibility that at least some autoimmune diseases may be induced following an immune response to an altered body constituent. Furthermore, these results indicate that after the initial insult, which results in antigenic alteration of host constituents, is corrected the disease may be perpetuated by stimulation with the unaltered body constituent. Following the induction of an immune state to thyroglobulin by injecting rabbits with homologous arsanil-sulfanil-thyroglobulin without adjuvant, the level of circulating antibody steadily declined over the next month. When a lobe of the thyroid gland was then autotransplanted to a distal area, some of the animals again began to produce precipitating and hemagglutinating antibody to native thyroglobulin. Also, lesions observed at this time in the *in situ* lobe appeared to be more frequent and severe than in thyroid tissue taken

from rabbits injected only with arsanil-sulfanil-thyroglobulin. When a thyroid lobe is transplanted to some distal area in the same rabbit it undergoes a central necrosis with loss of structure of the colloid follicle and presumably release of thyroglobulin into the tissue. The thyroglobulin which is released apparently is capable of further stimulating the immune mechanisms of the previously sensitized rabbit.

The mechanisms involved in the induction of autoimmunity to thyroglobulin by injecting altered thyroglobulin may be similar to those involved in the termination of acquired immunological tolerance (1). Similar to the production of antibody to native thyroglobulin following injection of rabbits with arsanil-sulfanil-thyroglobulin, acquired tolerance to bovine serum albumin (BSA), in the rabbit is lost and antibody to native BSA appears in the sera following injection of certain altered preparations of BSA, including arsanil-sulfanil-BSA (2). Also, these rabbits now will make an antibody response to subsequent injections of native BSA given at monthly intervals. However, they slowly lose their ability to respond to native BSA and by the seventh monthly injection most of them return to the tolerant state (12). Likewise, many of the rabbits which made circulating antibody to arsanil-sulfanil-thyroglobulin made an antibody response to subsequent injections of native thyroglobulin. However, by the third monthly injection most of the rabbits appeared to be losing their ability to respond to native thyroglobulin. In the case of both acquired tolerance to BSA or natural "tolerance" (unresponsiveness) to thyroglobulin, it is not certain whether there is a spontaneous return to the tolerant state or rather that the tolerant state is reinduced as the result of injections of antigen. That this later possibility is correct is supported by the experiments of Humphrey (13) who observed that rabbits tolerant to human serum albumin (HSA) which spontaneously lost their tolerant state can again be rendered tolerant to injection of appropriate doses of soluble HSA.

Some rabbits in the present study produced some 19S antibody (mercaptoethanol sensitive) which reacted with native thyroglobulin. As observed previously (3), the sera obtained from rabbits after injection of arsanil-sulfanil-thyroglobulin contained only 7S antibody to native thyroglobulin. However, after a subsequent injection of native thyroglobulin 19S antibody appeared in the sera of some rabbits. In 2 rabbits the 19S antibody appeared after the first injection of native thyroglobulin. Although no additional injection was given during the next 30 days, the titer of hemagglutinating 19S antibody increased dramatically. This antibody may have resulted from stimulation by the rabbit's own thyroglobulin which could have leaked from the thyroid had it been damaged at that time. By the third injection of native thyroglobulin both rabbits lost their ability to produce 19S antithyroglobulin and made only 7S antibody. Another rabbit in the same experiment made a small amount of 19S after the third injection, but did not make any after the first injection. As has been shown

by others (10, 11), the 19S antibody was much more efficient in hemagglutinating than the 7S antibody. The present results are in agreement with studies in humans. The sera of most patients with Hashimoto's thyroiditis contained only 7S antibody to thyroglobulin (14–17), however, some cases have been reported in which the sera contained 19S antibody (16–18). In the latter cases it was not shown whether the production of 19S was transient or persisted during the entire course of the disease.

There was no correlation between the level of either hemagglutinating or precipitating antibody to native thyroglobulin and the presence or absence of thyroid lesions in rabbits which received only injections of arsanil-sulfanil-thyroglobulin or those which received both the injections of arsanil-sulfanil-thyroglobulin and a single injection of native thyroglobulin. However, with rabbits given multiple monthly injections of native thyroglobulin after the injections of arsanil-sulfanil-thyroglobulin there was a correlation between the histological appearance of the lesions and the presence or absence of precipitating antibody. The lesions observed in rabbits which contained little or no precipitating antibody in their sera were characterized by discrete foci of dense infiltration of mononuclear cells, while the lesions observed in rabbits which contained precipitating antibody were characterized by a marked increase in connective tissue and a diffuse infiltration of mononuclear cells. The latter type of lesion may represent only a progression of the former type. It is possible that the lesions are initiated by factors other than circulating antibody and once the lesions are present, prolonged interaction between the exposed thyroglobulin and circulating antibody may result in additional tissue damage.

SUMMARY

Injection of rabbits with arsanil-sulfanil-thyroglobulin without adjuvant resulted in the production of hemagglutinating and precipitating antibody to native thyroglobulin and thyroiditis. Following a latent period of 1 month, these same rabbits responded to an injection of native thyroglobulin with an increase in both circulating antibody to native thyroglobulin and severity and frequency of thyroid lesions. Some rabbits initially immunized with arsanil-sulfanil-thyroglobulin also responded to a second and third monthly injection of native thyroglobulin, but the response to the third injection was usually not as good as the response to the first injection. A few of the rabbits showed a transient production of 19S antibody after the injection of native thyroglobulin was initiated. Neither circulating antibody nor thyroiditis were ever observed in rabbits injected with only native thyroglobulin without adjuvant. The correlation of the thyroiditis with the presence or absence of circulating antibody was discussed. Following a latent period after injections of aqueous arsanil-sulfanil-thyroglobulin some rabbits made a response to their own thyroglobulin released from an autotransplant of thyroid tissue.

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BIBLIOGRAPHY

1. Weigle, W. O., The immune response of rabbits tolerant to bovine serum albumin to the injection of other heterologous serum albumins, *J. Exp. Med.*, 1961, **114,** 111.
2. Weigle, W. O., Termination of acquired immunological tolerance to protein antigens following immunization with altered protein antigens, *J. Exp. Med.*, 1962, **116,** 913.
3. Weigle, W. O., The induction of autoimmunity in rabbits following injection of heterologous or altered homologous thyroglobulin, *J. Exp. Med.*, 1965, **121,** 289.
4. Edelhoch, H., The properties of thyroglobulin. I. The effects of alkali, *J. Biol. Chem.*, 1960, **235,** 1326.
5. Baker, M. C., Campbell, D. H., Epstein, S. I., and Singer, S. J., Physical chemical studies of soluble antigen-antibody complexes. VII. Thermodynamics of the reaction between benzenearsonic acid-azo-bovine serum albumin and rabbit antibodies to benzenearsonic acid, *J. Am. Chem. Soc.*, 1956, **78,** 312.
6. Gelewitz, E. W., Riedemann, W. L., and Klotz, I. M., Some quantitative aspects of the reaction of diazonium compounds with serum albumins, *Arch. Biochem. and Biophysics*, 1954, **53,** 411.
7. Talmage, D. W., and Maurer, P. H., I^{131}-labeled antigen precipitation as a measure of quantity and quality of antibody, *J. Infect. Dis.*, 1953, **92,** 288.
8. Boyden, S. V., Adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera, *J. Exp. Med.*, 1951, **93,** 107.
9. Preer, J. R., A quantitative study of a technique of double diffusion in agar, *J. Immunol.*, 1956, **77,** 52.
10. Greenburg, C. L., Moore, D. H., and Nunn, L. A. C., Reaction of 7S and 19S components of immune rabbit antisera with human group A and AB red cells, *Immunology*, 1963, **6,** 421.
11. Grey, H. M., Studies on changes in the quality of rabbit bovine serum albumin following immunization, *Immunology*, 1964, **7,** 82.
12. Weigle, W. O., The immune response of BSA tolerant rabbits to injections of BSA following the termination of the tolerant state, *J. Immunol.*, 1964, **92,** 791.
13. Humphrey, J. H., Immunological unresponsiveness to protein antigens in rabbits. I. The duration of unresponsiveness following a single injection at birth, *Immunology*, 1964, **7,** 449.
14. Forrigiani, G., and Roitt, I. M., Sedimentation characteristics of human thyroid autoantibodies, *Immunology*, 1963, **6,** 73.
15. Shulman, S., and Witebsky, E., Studies on organ specificity, *J. Immunol.*, 1960, **85,** 559.
16. Korngold, L., van Leeuwen, B. A., and Brener, J. L., The determination of the

molecular weight of different autoprecipitins in Hashimoto's disease by a new immunologic method, *J. Lab. and Clin. Med.*, 1959, **53**, 517.

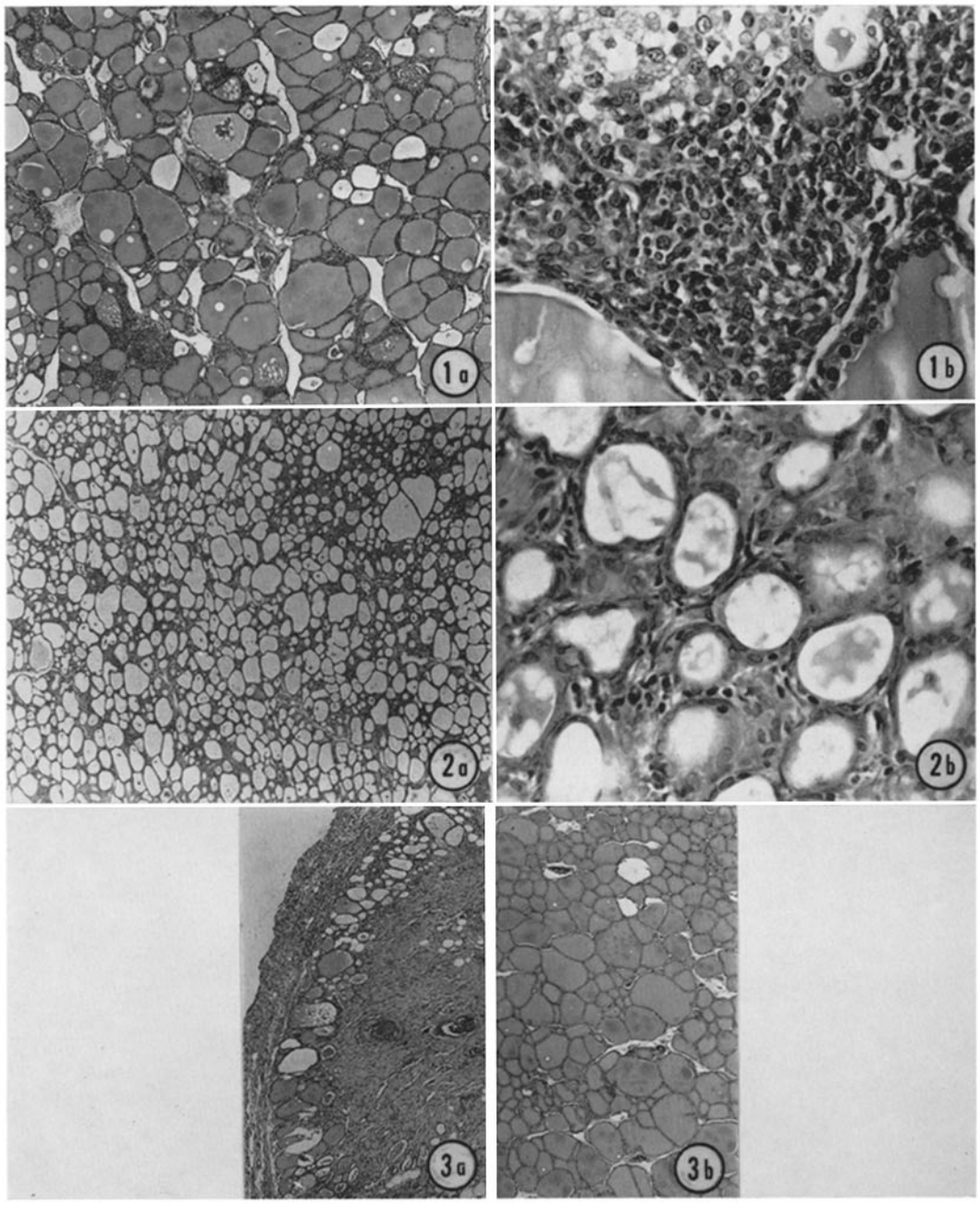
17. Fahey, J. L., and Goodman, H. C., Characterization of anti-thyroglobulin factors in human serum, *J. Clin. Invest.*, 1960, **39**, 1259.
18. Pressman, D., James, A. W., Yagi, Y., Hiramoto, R., Woernley, D., and Maxwell, W. T., *Proc. Soc. Exp. Biol. and Med.*, 1957, **96**, 773.

EXPLANATION OF PLATE 64

FIGS. 1 *a* and 1 *b*. Section of thyroid taken from a rabbit after a series of injections of arsanil-sulfanil-thyroglobulin and 3 monthly injections of native thyroglobulin. Lesion characterized by foci of dense infiltration of mononuclear cells. Hematoxylin and Eosin. Fig. 1 *a*, × 30; Fig. 1 *b*, × 250.

FIGS. 2 *a* and 2 *b*. Section of thyroid taken from a rabbit after a series of injections of arsanil-sulfanil-thyroglobulin. Lesion characterized by increase in connective tissue and diffuse infiltration of mononuclear cells. Hematoxylin and Eosin. Fig. 2 *a*, × 30; Fig. 2 *b*, × 250.

FIGS. 3 *a* and 3 *b*. Sections of thyroid taken 10 days after autotransplantation of one lobe. Hematoxylin and Eosin. Fig. 3 *a*, transplanted lobe, × 30; Fig. 3 *b*, intact lobe, × 30.



(Weigle: Production of thyroiditis and antibody)