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SUPPRESSION OF THYROID LESIONS IN RABBITS BY TREATMENT WITH CYCLOPHOSPHAMIDE AFTER THE INDUCTION OF THYROIDITIS

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SUMMARY

An acquired immunologic unresponsive state to bovine thyroglobulin has been induced in adult rabbits following simultaneous injections of cyclophosphamide and bovine thyroglobulin. The acquired unresponsive state in some rabbits lasted at least 3 months after the last injection of bovine thyroglobulin. After induction of autoimmune thyroiditis by soluble injections of altered homologous arsanil-sulphanil thyroglobulin or bovine thyroglobulin, a decrease in the incidence of thyroid lesions and the level of antithyroglobulin antibody production was observed following simultaneous injections of cyclophosphamide and native thyroglobulin.

INTRODUCTION

Under selected conditions immunologic unresponsiveness to protein antigens can be induced in adult animals (Weigle, 1967; Dresser & Mitchison, 1968). The condition selected must be such that an effective concentration of an antigen persists for a critical period of time in the absence of antibody production (Weigle & Golub, 1968). It has previously been shown that injection of certain antigens into animals in which the immune response has been temporarily blocked with irradiation (Linscott & Weigle, 1965) or immunosuppressive drugs (Gabrielsen & Good, 1967) results in a specific unresponsive state following recovery. Cyclophosphamide has been used effectively to induce unresponsiveness in adult animals to sheep red blood cells (Aisenberg, 1967; Dietrich & Dukor, 1968), serum proteins (Salvin & Smith, 1964), and to fortify the natural unresponsive state to homologous thyroglobulin (Salvin & Liauw, 1967). In the present study, cyclophosphamide was used to induce immunological unresponsiveness in adult rabbits to heterologous thyroglobulin. In addition, experiments were designed to suppress the production of thyroid lesions and autoantibody

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after induction of autoimmune thyroiditis. Rabbits possess an unresponsive state to their own thyroglobulin (Nakamura & Weigle, 1967) which is readily terminated following injection of aqueous preparations of either heterologous or altered homologous thyroglobulins resulting in both thyroiditis and circulating antibody to rabbit thyroglobulin (Weigle, 1965a; Nakamura & Weigle, 1967). Following a latent period after induction of autoimmune thyroiditis, these same animals responded to an injection of native thyroglobulin with an increase in both circulating autoantibody and severity of thyroid lesions (Weigle, 1965b). However, upon repeated injections of native thyroglobulin, most of the rabbits appeared to slowly lose their ability to respond to native thyroglobulin (Weigle, 1965b). Injections of native homologous thyroglobulin in normal rabbits do not result in a detectable immune response. In the present study simultaneous injections of cyclophosphamide and native thyroglobulin after induction of thyroiditis were given in an attempt to re-establish the natural unresponsive state to native thyroglobulin.

MATERIALS AND METHODS

Animals

New Zealand white adult rabbits (2·0–3·0 kg) were used in the experiments. Animals were given water containing KI to block reincorporation of radioactive iodine during isotope tracer studies.

Isolation and purification of thyroglobulin

Fresh bovine thyroids were obtained from a local slaughter house. Rabbit thyroids were obtained fresh and unfrozen from Pel-Freez, Rogers, Arkansas. Pooled bovine or rabbit thryoglobulin was purified according to a modified ultracentrifugation method previously described (Weigle, 1965a). Protein nitrogen determinations were performed by a modification of the micro-Kjeldahl technique using the Technicon auto-analyser (Ferrari, 1960).

Preparation of arsanil-sulphanil thyroglobulin

The thyroglobulin was coupled to the diazonium derivatives according to a procedure described in detail elsewhere (Weigle, 1965a). The arsanil-sulphanil thyroglobulin contained about 50 azo linkages/molecule of thyroglobulin.

Elimination of thyroglobulin from the circulation

Five mg samples were labelled with ^{131}I in phosphate buffer, pH 7·0, according to the method described by McConahey and Dixon (1966). Serum elimination studies with ^{131}I-labelled thyroglobulin were performed as previously described (Nakamura & Weigle, 1967).

Antibody analysis

The levels of precipitating antibody to thyroglobulin were measured by a quantitative precipitation technique (Talmage & Maurer, 1953) using ^{131}I-labelled thyroglobulin. At a point near equivalence where 80% of the antigen added is precipitated, the antibody to antigen weight ratio is approximately one for precipitating antibodies to the various thyroglobulins. Haemagglutinating antibodies were measured by a modification of the technique described by Boyden (1951) with use of the spiral dilution technique as previously described (Nakamura & Weigle, 1967).

Induction of unresponsiveness to bovine thyroglobulin in adult rabbits with cyclophosphamide

Two kg rabbits were given daily intravenous injections of 25 mg/kg cyclophosphamide (kindly donated by Mead-Johnson Research Center, Evansville, Indiana) for 14 days. On day 4, they were injected intravenously with 10 mg ^{131}I bovine thyroglobulin. Animals showing an elimination of the labelled thyroglobulin were bled 3 days after the elimination was complete and the sera analysed for antibody to thyroglobulin. The rabbits demonstrating a non-immune elimination of ^{131}I bovine thyroglobulin were rechallenged intravenously 1 month after the last injection of cyclophosphamide with 10 mg ^{131}I bovine thyroglobulin. Again, the responsive animals were bled 3 days post immune elimination. The animals which failed to show an immune elimination of the second injection of bovine thyroglobulin were tested for unresponsiveness with a third injection given 3 months later.

Five control animals were injected with cyclophosphamide alone according to the above schedule. After a rest period of 1 month, the animals were injected with 10 mg ^{131}I bovine thyroglobulin intravenously and bled 3 days post immune elimination. Nine normal rabbits were injected intravenously with 10 mg ^{131}I bovine thyroglobulin without cyclophosphamide. The rabbits were bled 3 days post immune elimination for antibody analyses.

Injection of cyclophosphamide and native thyroglobulin in animals previously immunized with homologous arsanil-sulphanil thyroglobulin and bovine thyroglobulin

Rabbits were injected subcutaneously each day for 4 days with 15 mg rabbit arsanil-sulphanil thyroglobulin. On the 5th day, they received 15 mg intravenously. Two weeks later the series of injections was repeated. The rabbits were bled 7 days after the last injection and the sera were analysed for haemagglutinating antibody to rabbit thyroglobulin. The animals were divided into three groups containing similar proportions of high, low and medium antibody producers.

Group I: Fifty-six days after the last injection of arsanil-sulphanil thyroglobulin the animals were bled and injected intravenously with 15 mg ^{131}I native rabbit thyroglobulin and bled 3 days post immune elimination. Forty-two days after the first injection of rabbit thyroglobulin, the rabbits were bled and again injected with 15 mg ^{131}I rabbit thyroglobulin and followed for elimination of thyroglobulin from the blood. The rabbits were bled 3 days after complete elimination.

Group II: These animals were injected with cyclophosphamide alone. Cyclophosphamide in doses of 25 mg/kg body weight was injected intravenously each day for 14 days. The first injection of native thyroglobulin was not given. An injection of ^{131}I native rabbit thyroglobulin was given to Group II animals at the same time the second injection of native thyroglobulin was given to Groups I and III. The animals were bled on the 3rd day after complete elimination.

Group III: These animals were injected with native thyroglobulin similar to Group I. In addition, intravenous injections of 25 mg cyclophosphamide/kg body weight were given daily for 14 days starting 4 days prior to the injection of rabbit thyroglobulin. The elimination of the ^{131}I rabbit thyroglobulin was followed and the animals were bled 3 days after complete elimination. All rabbits of Groups I, II and III were killed and the thyroid glands were removed for histologic studies 15 days after the last injection of rabbit thyroglobulin. The sera were analysed for haemagglutinating antibody to rabbit thyroglobulin.

The above experiments were repeated with injections of aqueous bovine thyroglobulin instead of arsanil-sulphanil rabbit thyroglobulin. Rabbits were injected each day for 4 days

with 20 mg of bovine thyroglobulin. On the 5th day, they received 20 mg of bovine thyroglobulin intravenously. The series of injections was repeated 2 weeks later, but the fifth injection was given subcutaneously. The rabbits were bled 7 days after the last injection and the sera were analysed for haemagglutinating antibody. The rabbits were divided into Groups I, II and III containing similar proportions of high, medium and low antibody producers. The groups were injected with either native thyroglobulin alone, cyclophosphamide alone, or both native thyroglobulin and cyclophosphamide as described above. The animals in Groups I and III were injected with 15 mg ^{131}I native rabbit thyroglobulin 60 days after the last injection of bovine thyroglobulin; the second injection of rabbit thyroglobulin was given 60 days after the first injection. The elimination of the ^{131}I rabbit thyroglobulin was followed and the animals were bled 3 days after complete elimination. All rabbits were sacrificed and the thyroid glands were removed for histologic studies 15 days after the last injection of rabbit thyroglobulin. The sera were analysed for haemagglutinating antibody to rabbit thyroglobulin.

Histology

The thyroid tissue was fixed in Bouin's solution. The tissue was embedded in paraffin, sectioned and stained with haematoxylin and eosin. The thyroid lesions were graded as 1+ if at least 5 foci the size of one follicle were present in a longitudinal section of one thyroid lobe. Lesions were graded as 2+ if 10 to 20 foci were observed which occupied an area the size of one or more follicles.

RESULTS

Immune response of rabbits given injections of bovine thyroglobulin with and without cyclophosphamide

Following intravenous injection of 10 mg ^{131}I bovine thyroglobulin, all nine adult rabbits showed an immune elimination of the bovine thyroglobulin between days 7 and 8. The serum precipitating antibody level to bovine thyroglobulin on the 3rd day post immune elimination varied from 5·6 to 34·1 μg N/ml with an average of 23·2 μg N/ml (Table 1).

Ten of thirteen animals given injections of cyclophosphamide and 10 mg ^{131}I bovine thyroglobulin did not show an immune elimination. Three animals showed a delayed immune elimination pattern and the precipitating antibody levels to bovine thyroglobulin were 0·2, 0·5 and 6·0 μg N/ml respectively. These levels were much lower than the average level observed in normal animals given bovine thyroglobulin alone.

The immune response of rabbits previously injected with cyclophosphamide and bovine thyroglobulin and after a latent period given a second injection of bovine thyroglobulin alone was studied. This experiment was performed to test whether the initial injections of cyclophosphamide and bovine thyroglobulin induced an acquired unresponsive state in the ten animals which showed a non-immune elimination pattern on the first injection of bovine thyroglobulin. Four of ten rabbits were still unresponsive, in that they failed to show an immune elimination of a second injection of 10 mg ^{131}I bovine thyroglobulin. Six of the rabbits showed an immune elimination and the precipitating antibody levels to bovine thyroglobulin measured on the 3rd day after complete elimination varied from 0·6 to 21·8 μg N/ml with an average level of 4·8 μg N/ml. This average of 4·8 μg N/ml is less than that

observed in the average primary response in a group of nine normal animals which was 23·2 μg N/ml. The four animals which did not respond were still unresponsive to bovine thyroglobulin when tested 3 months after the second injection of bovine thyroglobulin.

The following control experiment was performed to determine if the immune system of the animals would recover 1 month after injections of cyclophosphamide. Following the intravenous injection of 10 mg ^{131}I bovine thyroglobulin, all five animals previously treated with cyclophosphamide demonstrated an immune elimination on day 7 or 8. The average precipitating antibody level on the 3rd day post immune elimination was 22·9 μg N/ml and is very similar to the average antibody response of 23·2 μg N/ml observed in a group of normal rabbits.

The effect of injections of cyclophosphamide and native thyroglobulin on production of anti-thyroglobulin and lesions in rabbits previously immunized with arsanil-sulphanil thyroglobulin

All animals in each group were initially immunized with arsanil-sulphanil thyroglobulin and showed evidence of haemagglutinating antibody to rabbit thyroglobulin (Table 2). The

TABLE 1. Immune response of rabbits given injections of bovine thyroglobulin with and without cyclophosphamide

	Bov Tg* alone	First injection Bov Tg and cyclophosphamide	Second injection Bov Tg
Number of animals	9	13	10§
Fraction showing antibody production	9/9	3/13	6/10
Antibody†	23·2	0·51	4·8

* Bovine thyroglobulin. † Precipitating antibody to Bov Tg (μg nitrogen/ml). § All animals showed a nonimmune elimination of the first injection of ^{131}I Bov Tg injected simultaneously with cyclophosphamide.

haemagglutinating antibody (H.A.) titre in all three groups of animals had decreased to zero or very low levels prior to the subsequent injections of native thyroglobulin. Log_2 H.A. titre to rabbit thyroglobulin varied from 0 to 3. Similar to previous studies (Weigle, 1965b), the antibody levels increased and reappeared in the majority of animals in Group I following the first and second injections of native thyroglobulin. Two of the animals in Group I showed no evidence of haemagglutinating antibody production following injections of native thyroglobulin. In Group I, one to two plus thyroid lesions were observed in nine of ten animals following a subsequent injection of native thyroglobulin without cyclophosphamide.

In Group II the animals were given injections of cyclophosphamide and the first injection of native thyroglobulin was omitted. However, the Group II animals were given an injection of native rabbit thyroglobulin at the same time the second injection of native thyroglobulin was given to Groups I and III. The average log_2 H.A. titre to rabbit thyroglobulin increased from 2 to 6 following injection of the native thyroglobulin in a group of four rabbits. One

animal did not show any evidence of haemagglutinating antibody production following the injection of the native thyroglobulin. Three of the four animals showed a 1+ to 2+ thyroiditis.

TABLE 2. The effect of injection of cyclophosphamide and native thyroglobulin (Tg) on production of antithyroglobulin and lesions in rabbits previously immunized with arsanil-sulphanil (A-S) Tg

Group: Treatment*	Rabbit No.	Antibody†			
		After injection of A-S Tg	After injecting native Tg		
			First§	Second‖	Lesions‡
I: Native Tg	1	5	5	3	++
	2	10	10	7	++
	3	10	9	7	++
	4	5	9	6	++
	5	11	10	10	+
	6	4	0	0	−
	7	11	9	10	++
	8	9	7	3	++
	9	11	10	10	++
	10	4	0	0	++
II: Cyclophosphamide	1	11		7	++
	2	9	not	9	+
	3	11	tested	10	++
	4	5		0	−
III: Cyclophosphamide + native Tg	1	6	4	0	−
	2	8	0	0	−
	3	11	11	11	++
	4	5	0	0	−
	5	8	1	0	−
	6	9	1	0	−

* Rabbits given ^{131}I native Tg were injected intravenously with 15 mg 60 days after the last injection of A-S Tg. Cyclophosphamide was given intravenously for 14 days starting 56 days after the last injection of A-S Tg.

† Log_2 of the reciprocal of the highest serum dilution showing complete agglutination of sheep red blood cells coated with native rabbit Tg.

§ First injection of native Tg given to Groups I and III only.

‖ 15 mg ^{131}I native Tg injected 42 days after first injection of native Tg.

‡ Tissue removed 15 days after second injection of native Tg.

In Group III, where cyclophosphamide and native thyroglobulin were both injected, only one of six rabbits showed evidence of thyroiditis and haemagglutinating antibody to rabbit thyroglobulin after the second injection of native thyroglobulin. Five of the six animals showed no thyroid lesions and no haemagglutinating antibody to rabbit thyroglobulin after the second injection. Several of these animals demonstrated a non-immune elimination of the second injection of ^{131}I native rabbit thyroglobulin.

Effect of injections of cyclophosphamide and native thyroglobulin on production of antithyroglobulin and lesions in rabbits previously immunized with bovine thyroglobulin

All the animals in this experiment were initially immunized with soluble bovine thyroglobulin and their sera contained haemagglutinating antibody (H.A.) to rabbit thyroglobulin. The average $\log_2$ H.A. titre in all of the animals had decreased to values between 0 and 3 when tested prior to subsequent injections of native thyroglobulin (Table 3). Similar

TABLE 3. The effect of injection of cyclophosphamide and native rabbit thyroglobulin (Tg) on production of antithyroglobulin and lesions in rabbits previously immunized with bovine (Bov) Tg

Group: Treatment*	Rabbit No.	Antibody†			
		After injection of Bov Tg	After injection of native Tg		
			First§	Second‖	Lesions‡
I: Rabbit Tg	1	6	1	0	+
	2	9	7	8	+
	3	5	1	1	−
	4	7	10	10	++
	5	8	11	7	++
	6	9	6	6	−
	7	10	7	8	+
II: Cyclophosphamide	1	6	2	7	+
	2	9	5	6	++
	3	8	1	9	+
	4	7	3	1	−
	5	5	0	6	+
III: Cyclophosphamide + rabbit Tg	1	7	2	1	−
	2	6	1	2	−
	3	10	11	5	+
	4	6	2	0	−
	5	9	1	0	−

* Rabbits given ^{131}I rabbit Tg werei njected intravenously with 15 mg 60 days after the last injection of bovine Tg. Cyclophosphamide was given intravenously each day for 14 days starting 56 days after the last injection of bovine Tg.

† $\log_2$ of the reciprocal of the highest serum dilution showing complete agglutination of sheep red blood cells coated with native Tg.

§ First injection of rabbit Tg given to Groups I and III.

‖ 15 mg ^{131}I rabbit Tg injected 60 days after first injection of rabbit Tg.

‡ Tissue removed 15 days after second injection of native Tg.

to the previous experiment, the haemagglutinating antibody was increased and reappeared following subsequent injections of native thyroglobulin in practically all of the animals in Group I. The average $\log_2$ H.A. titre was 6·1 and 5·7 following the first and second injections of native thyroglobulin respectively. In Group I the animals were injected with native thyroglobulin alone and five of seven animals demonstrated a 1+ to 2+ thyroiditis. Definite correlation between severity of thyroid lesions and haemagglutinating antibody titre was not observed.

In Group II the animals were injected with cyclophosphamide alone after immunization with bovine thyroglobulin. Cyclophosphamide was given intravenously each day for 14 days starting 56 days after the last injection of bovine thyroglobulin. The initial average $\log_2$ H.A. titre was 7·0 which decreased to an average titre of 2·2 by the time injections of cyclophosphamide were completed. An injection of ^{131}I rabbit thyroglobulin was given 50 days after the last injection of cyclophosphamide. The average $\log_2$ H.A. titre increased to 5·6 following this injection. Four of five rabbits showed a 1+ to 2+ thyroiditis.

In Group III, after the initial injections of cyclophosphamide and native thyroglobulin, four out of five animals showed a $\log_2$ H.A. titre of 1 to 2 with no significant increase when compared to the pre-injection titre. After the second injection of native thyroglobulin, the same four animals in Group III showed no evidence of thyroid lesions and the $\log_2$ H.A. titre varied from 0 to 2. These four rabbits were followed for 14 days and did not demonstrate evidence of a sharp immune elimination pattern of the ^{131}I rabbit thyroglobulin. Animal number 3 showed an immune elimination of the second injection of ^{131}I rabbit thyroglobulin and a 1+ thyroiditis with a $\log_2$ H.A. titre of 5 following the second injection of native thyroglobulin.

DISCUSSION

The present experiments have demonstrated that specific immunologic unresponsiveness can be induced in adult rabbits to a heterologous thyroglobulin by simultaneous injections with cyclophosphamide. Ten of thirteen animals given injections of cyclophosphamide with 10 mg ^{131}I bovine thyroglobulin did not show an immune elimination. The ten animals were rechallenged 1 month later and four animals were still unresponsive to bovine thyroglobulin. The remaining six animals were hyporesponsive to bovine thyroglobulin and the average precipitating antibody response was one-third of that observed in the primary response of normal animals. The unresponsive state to bovine thyroglobulin was still maintained in the four animals when tested 3 months later. Thus, it appears that an acquired unresponsive state can be induced in adult rabbits if an effective concentration of the thyroglobulin persists in the absence of antibody production. The success of the induction of unresponsiveness in this case apparently lies in both the immunosuppressive property of cyclophosphamide and the ability of thyroglobulin to persist in the circulation and equilibrate between the intra- and extravascular fluid compartments (Nakamura *et al.*, 1968) permitting it to reach all potential antibody-producing units.

In addition, the present studies have demonstrated that after the induction of autoimmune thyroiditis, simultaneous injections of cyclophosphamide and native thyroglobulin decreased the incidence of thyroid lesions and production of antithyroglobulin antibody. However, two of eleven animals had very high levels of haemagglutinating antibody following injections of altered or heterologous thyroglobulin and still demonstrated thyroid lesions after simultaneous injections of cyclophosphamide and native thyroglobulin.

The simultaneous injections of cyclophosphamide and native thyroglobulin following immunization with altered homologous or heterologous thyroglobulin may help to restore a natural unresponsive state. It has been previously shown that many of the rabbits which made circulating antibody to arsanil-sulphanil thyroglobulin made an antibody response to subsequent injections of native thyroglobulin with an increase in severity and frequency of thyroid lesions and reappearance of antibody to native thyroglobulin (Weigle, 1965b).

However, upon repeated injections, most of the rabbits slowly lose their ability to respond to native thyroglobulin (Weigle, 1965b). It may be that the natural unresponsive state is reinduced as a result of injections of native antigen. The latter concept is supported by Humphrey's observation that rabbits unresponsive to human serum albumin which spontaneously lost their unresponsive state could again be rendered unresponsive to injections of appropriate doses of soluble human serum albumin (Humphrey, 1964). Furthermore, the acquired unresponsive state to bovine serum albumin in rabbits slowly returns following its termination by immunization with cross-reacting albumins (Weigle, 1964).

The present data and the successful treatment of experimental allergic encephalomyelitis with cyclophosphamide (Paterson & Drobish, 1969) suggest that simultaneous administration of an immunosuppressive agent with native antigen to reduce the incidence of lesions may provide another approach to treatment of autoimmune diseases. On the other hand, the present experiments have shown that when high levels of autoantibodies were seen in experimental thyroiditis, significant thyroid lesions were still observed after simultaneous injections of cyclophosphamide and native antigen. This is in agreement with the findings that immunosuppressive agents are not very effective when given after establishment of the immune response (Gabrielsen & Good, 1967).

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