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Thyroid autoantibody synthesis by cultures of thyroid and peripheral blood lymphocytes. II. Effect of thyroglobulin on thyroglobulin antibody synthesis

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SUMMARY

The influence of antigen (thyroglobulin, Tg) on Tg antibody synthesis has been investigated using cultures of Hashimoto thyroid and peripheral blood lymphocytes. In cultures of thyroid lymphocytes, Tg antibody synthesis was stimulated by a 24 h pulse of Tg (10–100 μg/ml) and similar results were obtained using spleen lymphocytes from BALB/c mice immunized with human Tg. In contrast, Tg antibody synthesis by Hashimoto peripheral blood lymphocytes was not affected by similar concentrations of Tg (1–240 μg/ml) in the presence or absence of pokeweed mitogen (PWM). However, peripheral blood lymphocytes from two out of nine patients produced increased levels of Tg antibody in the presence of very low concentrations of Tg (50 ng/ml). This increase in Tg antibody production was accompanied by a rise in total IgG synthesis indicating that the response to Tg was polyclonal. On the basis of other unusual features of the lymphocyte cultures from these two patients including a relatively small response to PWM and evidence of circulating plasma cells, it is suggested that sufficient numbers of lymphocytes responsive to Tg are only released into the circulation during active phases of the disease process.

INTRODUCTION

Lymphocytes from the peripheral blood of patients with Graves' disease synthesize detectable levels of TSH receptor antibodies when polyclonally activated with pokeweed mitogen (PWM) (McLachlan *et al.*, 1977, 1978). Similarly, Hashimoto peripheral blood lymphocytes produce antibodies to thyroid microsomes and thyroglobulin (Tg) when cultured with PWM or after infection with Epstein–Barr virus (McGregor *et al.*, 1979; McLachlan *et al.*, 1981). In the absence of stimulation by mitogen or virus, however, thyroid autoantibody synthesis is usually difficult to detect except in cultures of lymphocytes from patients with exceptionally high serum titres of such antibodies (McLachlan *et al.*, 1982). Attempts to stimulate TSH receptor antibody production by blood lymphocytes from Graves' patients with thyroid membranes as a source of TSH receptors have been unsuccessful using membranes alone or in combination with PWM (McLachlan *et al.*, 1978). However, Tg antibody synthesis is more suitable for investigations of this type and in this paper we describe a study of the influence of Tg on Tg antibody production by cultures of thyroid and peripheral blood lymphocytes.

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MATERIALS AND METHODS

Patients. Thyroid tissue was obtained from one female Hashimoto patient (DJ) aged 55 years and peripheral blood was taken from 15 Hashimoto patients (12 female and three male, age range 36–74 years, mean age 56 years). All Hashimoto patients were euthyroid on thyroxine and 14 showed serum microsomal (Mic) and thyroglobulin (Tg) antibody titres greater than 1:1,600 and 1:10,000 respectively. One patient (DJ) had a Tg antibody titre of 1:2,560 and a Mic antibody titre of 1:100.

Preparation of thyroglobulin . The procedure used for the isolation of Tg from Graves' thyroid tissue has been described in detail elsewhere (McLachlan *et al.*, 1982). Thyroglobulin was labelled with ^{125}I by a modification of the lactoperoxidase method of Marchalonis (1969) to a specific activity of about 10 μCi/μg, chromatographed on Sepharose 6B and stored at 4°C for up to 10 days.

Isolation and culture of lymphocyte suspensions. Peripheral blood lymphocytes were isolated from heparinized venous blood (60–120 ml) by centrifugation on 'Lymphoprep' (Böyum, 1976). Thyroid tissue was teased apart in balanced salt solution to release mononuclear cells and the suspensions purified on a 'Lymphoprep' gradient. The lymphocytes (greater than 90% viable for blood lymphocytes and 85% viable for thyroid lymphocytes) were resuspended in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin and 0·25 μg/ml fungizone) and incubated at $8–10 \times 10^6$ cells/ml in 1 ml aliquots in Marbrook flasks (Marbrook, 1967) or at $0·5 \times 10^6$ cells/ml in 1 ml aliquots in tubes (Falcon 2054).

Effect of thyroglobulin on thyroglobulin autoantibody production. Two series of experiments were carried out: (a) lymphocytes were incubated in Marbrook flasks (3–4 replicates per treatment) in the presence of increasing amounts of Tg (0–240 μg/ml). After 24 h, cell suspensions were removed from the flasks, washed three times in balanced salt solution and cultured in fresh medium in rinsed or new Marbrook flasks for a further 13 days. Experiments using peripheral blood lymphocytes were carried out with and without PWM (GIBCO, Grand Island, New York, USA 3 μl/ml); when the mitogen was used, it was present throughout the culture period. (b) Lymphocytes were cultured in Falcon tubes in the presence of Tg (5–200 ng/ml) which was left in the tubes for the duration of the culture period (14 days). Triplicate or quadruplicate cultures were set up for each Tg concentration as well as for cultures with and without PWM (3 μl/ml and 10 μl/ml).

Analysis of human immunoglobulins synthesized in culture. Total IgG synthesized in culture was measured in culture supernatants using a solid phase radioimmunoassay (McLachlan, Rees Smith & Hall, 1978). Thyroid autoantibodies were determined by ELISA (McLachlan *et al.*, 1982) or by tanned red cell haemagglutination (Bird & Stephenson, 1973; Cayzer, *et al.*, 1978) directly in culture supernatants or in culture concentrates prepared as described previously (McLachlan *et al.*, 1978a). ELISA measurements of Tg autoantibody were expressed in terms of an ELISA index as follows:

$$\text{ELISA index} = \frac{\text{Optical density of test sample}}{\text{Optical density of a standard serum dilution}}.$$

The standard was a sample of Hashimoto serum (tanned red cell haemagglutination titre 1:5,120) diluted 400 times.

Thyroglobulin antibody production by mouse spleen cells. Female BALB/c mice (6 weeks old) were immunized intraperitoneally with 100 μg human Tg in Freund's complete adjuvant. Four weeks later a second dose was administered (also in Freund's complete adjuvant) and mice were killed by cervical dislocation after 3–14 days to obtain spleen tissue and blood. Lymphocytes were extracted from the spleens by teasing them apart in balanced salt solution and lysing the erythrocytes using 0·87% buffered ammonium chloride. After washing three times, splenocytes were resuspended at 20×10^6 cells/ml in culture medium (containing 10% fetal bovine serum, 2 mM L-glutamine, antibiotics and 2×10^{-5} M mercaptoethanol) and 2 ml aliquots were incubated in Marbrook flasks in medium alone or with 1 or 100 μg/ml human Tg. After 24 h, the cells were washed three times, resuspended in fresh medium and incubated in 1 ml aliquots (10^7 cells/ml), in new flasks (four replicates per treatment) for 13 days. Culture supernatants were analysed for the presence of antibodies to human Tg using a radioimmunoassay as follows: 100 μl aliquots (diluted

1:2 and 1:10) were incubated at 4°C overnight with 100 μl ^{125}I-Tg; sheep anti-mouse immunoglobulin (100 μl, Seward Laboratories, diluted 1:50), was added to precipitate labelled Tg–antibody complexes and the percentage ^{125}I-Tg bound was determined. Controls included estimation of binding using second antibody alone (to determine non-specific binding to tubes) and the measurement of binding by culture medium. Analysis of serum in a similar manner demonstrated that mice immunized with Tg developed high titres of Tg antibodies (serum dilutions of $1:10^6$ able to precipitate 20% of ^{125}I-labelled Tg).

Statistical analysis of results. The significance of differences between the levels of total IgG or thyroid autoantibody synthesized by lymphocytes cultured in the presence or absence of PWM or autoantigen was determined by Student's *t*-test.

RESULTS

Initial studies were carried out using Hashimoto peripheral blood lymphocytes incubated for the first 24 h with PWM and increasing amounts of Tg; the lymphocytes were then washed and cultured

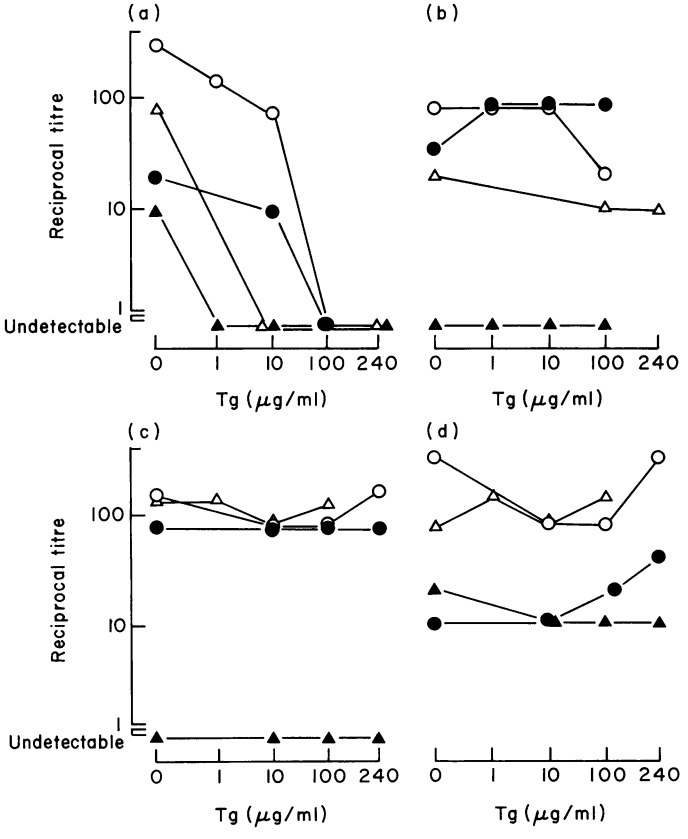

Fig. 1. Synthesis of antibodies to Tg and thyroid microsomes (measured by haemagglutination) in culture concentrates from Hashimoto peripheral blood lymphocytes pulsed for 24 h with PWM and Tg (0–240 μg/ml), washed and cultured in Marbrook flasks for 13 days. (a) Tg antibodies: final culture in rinsed flasks. (b) Microsomal antibodies: final culture in rinsed flasks. (c) Tg antibodies: final culture in new flasks. (d) Microsomal antibodies: final culture in new flasks. Results for lymphocytes obtained from individual patients are indicated by symbols; a & b: ○ MF, △ AM, ● JK ▲, LM; c & d: ○ CD, △ ES, ● MT, ▲ LM.

in rinsed flasks for a further 13 days with the mitogen only. In these cultures Tg antibody synthesis (measured by haemagglutination) appeared to be suppressed at Tg concentrations of 1–240 μg/ml (Fig. 1a). The inhibition was specific since microsomal antibody levels were unaffected (Fig. 1b) and the amount of total IgG synthesised by peripheral blood lymphocytes from individual patients was not significantly different whether Tg was present or absent (data not shown). Experiments using ^{125}I-Tg showed that although the lymphocytes could be washed free of Tg (less than 0·1% remaining) residual Tg could not be completely removed by rinsing the Marbrook flasks. Therefore a similar series of experiments was carried out transferring the washed lymphocytes to new Marbrook flasks. In these experiments the addition of increasing amounts of Tg had no effect on the levels of Tg antibody produced by PWM stimulated peripheral blood lymphocytes (Fig. 1c); nor was any change demonstrated in the levels of Mic antibody (Fig. 1d). The specific 'suppression' of antibody production by antigen seen in the previous set of experiments appeared to be an artefact due to incomplete removal of antigen.

Peripheral blood lymphocytes from three Hashimoto patients were exposed to a 24 h pulse of Tg (1–240 μg/ml) washed and cultured for 13 days in the absence of PWM. Under these conditions, Tg antibody synthesis (measured by haemagglutination) was undetectable.

In addition to studying the effect of a pulse of Tg in high density Marbrook cultures, peripheral blood lymphocytes from nine Hashimoto patients were cultured in tubes at low density ($0{\cdot}5 \times 10^6$ cells/ml) with Tg present throughout the culture period. The addition of Tg over the range 5–200 ng/ml had no significant effect on the level of Tg antibody synthesized *in vitro* by lymphocytes from seven of the nine patients, the amounts of Tg antibody (measured by ELISA) being low or undetectable in most cases except in lymphocyte cultures from patient CF (Table 1). However, the addition of PWM (3 μl/ml or 10 μl/ml) resulted in significant levels of Tg antibody production by peripheral blood lymphocytes from six of these individuals and the ratio of the Tg antibody ELISA Index in mitogen stimulated cultures to the values obtained in the absence of mitogen ranged from 5·0 to over 200 (Table 1).

A significant response to Tg was observed in peripheral blood lymphocytes from two Hashimoto patients (Fig. 2). In the presence of 50 ng/ml Tg, lymphocytes from both patients synthesized significantly more Tg antibody than cultures of lymphocytes incubated in medium only ($P < 0{\cdot}05$). Further, the response to Tg appeared to be polyclonal since the levels of total IgG production were also increased in the presence of Tg (Fig. 2d & f). Lymphocytes isolated from the blood of patient ED 6 months later showed no increase in Tg antibody synthesis in cultures containing Tg although there was a highly significant increase in the levels of Tg antibody and total IgG synthesized in the presence of PWM (Fig. 2b & e). The amount of Tg antibody produced in the absence of any stimulus was lower than on the previous occasion and this was reduced in the presence of 100 ng/ml Tg (Fig. 2b) presumably due to antigen–antibody complex formation.

A number of unusual features were associated with the lymphocyte cultures from patients ED and EB in which a response to Tg could be demonstrated: the ratio of Tg antibody synthesized by lymphocytes in the presence of PWM compared with cultures of lymphocytes in medium only was low, 2·8 for patient ED and 4·7 for patient EB; these values are similar to or lower than the values for the ratio of Tg antibody produced in the presence of 50 ng/ml Tg to Tg antibody synthesized by lymphocytes incubated in medium: 2·7 for patient ED and 8·0 for patient EB. This pattern of response is very different from that observed for lymphocytes which showed no detectable response to Tg in culture such as patient ED studied 6 months later (with ratios of 6·8 for Tg antibody synthesis +PWM: −PWM and 1·3 for Tg antibody synthesis in the presence of 50 ng/ml Tg: −PWM) and the seven patients in Table 1. Further, lymphocytes from patient ED (taken at the time she was responsive to Tg) produced, after irradiation (2,000 rad) highly significant levels of Tg antibody (ELISA index $1{\cdot}72 \pm 0{\cdot}04$ compared with $3{\cdot}01 \pm 0{\cdot}16$ for untreated lymphocytes cultured with PWM). Similarly, in the case of patient EB, low but still detectable levels of Tg antibody (ELISA index $0{\cdot}06 \pm 0{\cdot}01$) were present in tube cultures of her cells which had been frozen and thawed three times prior to culture. The release of specific antibody from cells which had been prevented from dividing or destroyed prior to culture strongly suggested that the antibody was derived from plasma cells present in the circulation. Evidence of circulating thyroid autoantibody secreting cells was not obtained for lymphocytes from patient ED on the second sampling occasion

Table 1. Synthesis of thyroglobulin antibody (measured by ELISA) in 14 day tube cultures of Hashimoto peripheral blood lymphocytes incubated in the presence of Tg (0–200 ng/ml) or PWM. The number of replicate cultures is given in parentheses

	Tg Antibody ELISA Index (Mean ± s.e. (mean))						
Culture conditions	Patient LM (4)	Patient ES (4)	Patient JW (3)	Patient EB (3)	Patient CD (4)	Patient CF (4)	Patient WJ (4)
Medium only	0·01 ± 0·01	0·01 ± 0·00	0·02 ± 0·01	0·03 ± 0·01	0·02 ± 0·01	0·09 ± 0·01	0·02 ± 0·01
5 ng/ml Tg	0·01 ± 0·01	0·04 ± 0·03	0·01 ± 0·01	0·02 ± 0·00	0·01 ± 0·01	0·13 ± 0·03	0·04 ± 0·01
10 ng/ml Tg	0·02 ± 0·01	0·06 ± 0·05	0·01 ± 0·01	0·03 ± 0·01	0·00 ± 0·00	0·18 ± 0·11	0·05 ± 0·01
50 ng/ml Tg	0·02 ± 0·01	0·02 ± 0·01	0·02 ± 0·01	0·03 ± 0·01	0·01 ± 0·01	0·17 ± 0·07	0·02 ± 0·02
100 ng/ml Tg	0·02 ± 0·01	0·04 ± 0·03	0·03 ± 0·01	0·02 ± 0·00	0·00 ± 0·00	0·10 ± 0·04	0·00 ± 0·00
200 ng/ml Tg	ND	ND	0·03 ± 0·01	ND	0·00 ± 0·00	ND	ND
PWM 3 μl/ml	0·74 ± 0·44†	2·01 ± 0·16†	0·11 ± 0·05	0·15 ± 0·06	0·60 ± 0·17*	1·06 ± 0·27*	1·77 ± 0·16*
PWM 10 μl/ml	1·08 ± 0·32*	1·87 ± 0·14*	0·12 ± 0·03†	0·14 ± 0·05	0·39 ± 0·06*	0·73 ± 0·09*	ND
Ratios:							
+PWM: medium only	74·0	201·0	6·0	5·0	30·0	11·8	88·5
+Tg: medium only	2·0	2·0	1·0	1·0	0·5	1·9	1·0

$$\text{Ratios: } +\text{PWM: Medium only} = \frac{\text{Tg Ab synthesized in the presence of PWM 3 } \mu\text{l/ml}}{\text{Tg Ab synthesized in medium only}}$$

$$+\text{Tg: Medium only} = \frac{\text{Tg Ab synthesized in the presence of Tg 50 ng/ml}}{\text{Tg Ab synthesized in medium only}}$$

Values significantly greater than those obtained for cultures without Tg or PWM: $*P < 0{\cdot}02$, $\dagger P < 0{\cdot}05$, ND: Not determined.

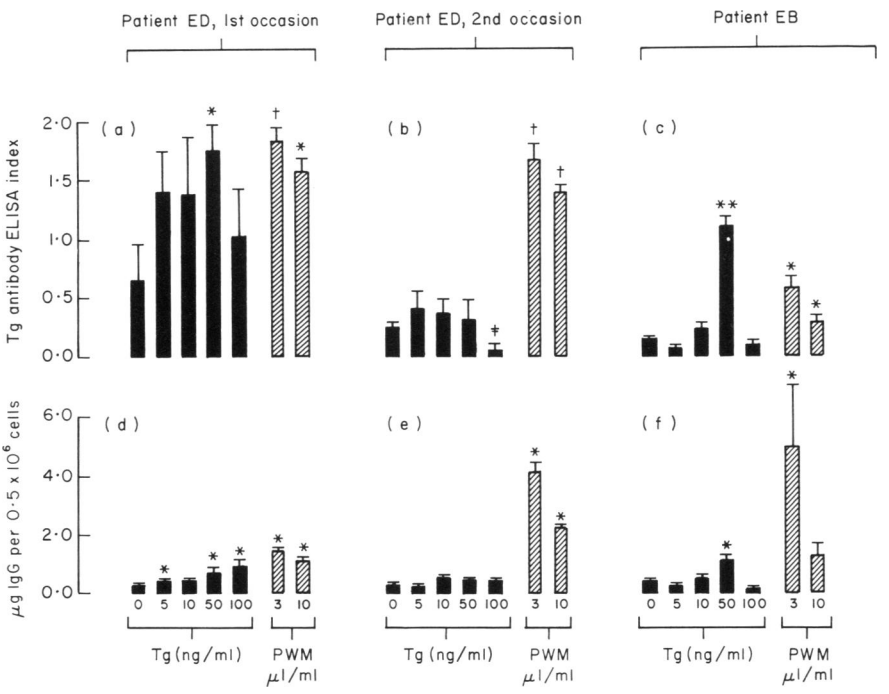

Fig. 2. Synthesis of Tg antibody and total IgG by Hashimoto lymphocytes (patients ED and EB) incubated in the presence of Tg (0–100 ng/ml) and PWM (3 and 10 μl/ml). Lymphocytes were obtained from patient ED on two separate occasions 6 months apart. Results are given for the mean ± s.e. (mean). Values significantly greater than those for lymphocytes incubated in culture medium only: $*P < 0{\cdot}05$, $\dagger P < 0{\cdot}002$; values significantly less than those for lymphocytes cultured in medium only: $\ddagger P < 0{\cdot}05$.

Table 2. Synthesis of Tg antibody (measured by haemagglutination) and total IgG by thyroid lymphocytes pulsed for 24 h with Tg (1–100 μg/ml) followed by incubation for 13 days in medium only in Marbrook flasks (three or four replicate cultures)

	Tg concentration (μg/ml)		
	1	10	100
μg IgG synthesized per 10^7 cells	147·7 ± 21·6	221·8 ± 54·9	159·4 ± 18·8
Tg antibody (Reciprocal titre):			
(a) culture supernatants	18·0 ± 3·0	27·0 ± 7·0	40·0 ± 0·0*
(b) culture concentrates	80	160	320
Specific activity (Reciprocal titre of Tg antibody per ug IgG in culture concentrates)	0·14	0·24	0·82

* Values significantly greater than results obtained for lymphocytes pulsed with 1 μg/ml Tg ($P < 0{\cdot}01$).

or in lymphocytes from patients CD and WJ whose cells did not respond to Tg (ELISA index $0{\cdot}02 \pm 0{\cdot}01$ in all three patients for cells frozen and thawed three times).

The effect of Tg was also studied in lymphocytes isolated from Hashimoto thyroid tissue. No significant change was observed in the level of total IgG synthesized by thyroid lymphocytes pulsed with 1–100 μg/ml Tg; however, Tg antibody levels measured by haemagglutination directly in culture supernatants were significantly higher in cultures pulsed with 100 μg/ml Tg compared with 1 μg/ml Tg ($P < 0{\cdot}01$) and the titres in culture concentrates showed this increase even more clearly (Table 2). Further, the specific thyroglobulin antibody activity was more than five times greater in lymphocyte cultures pulsed with 100 μg/ml Tg compared with cultures pulsed with 1 μg/ml Tg (Table 2).

Additional experiments on the effects of Tg on Tg antibody synthesis were carried out using spleen lymphocytes from mice. Splenocytes from two mice immunized with human Tg synthesized antibodies to Tg as shown by the ability of culture supernatants to precipitate significantly greater amounts of radiolabelled Tg than culture medium ($P < 0{\cdot}05$, Fig. 3). When these lymphocytes were pulsed for 24 h with 1 or 100 μg/ml Tg, higher levels of Tg antibody were detected ($P < 0{\cdot}02$, Fig. 3).

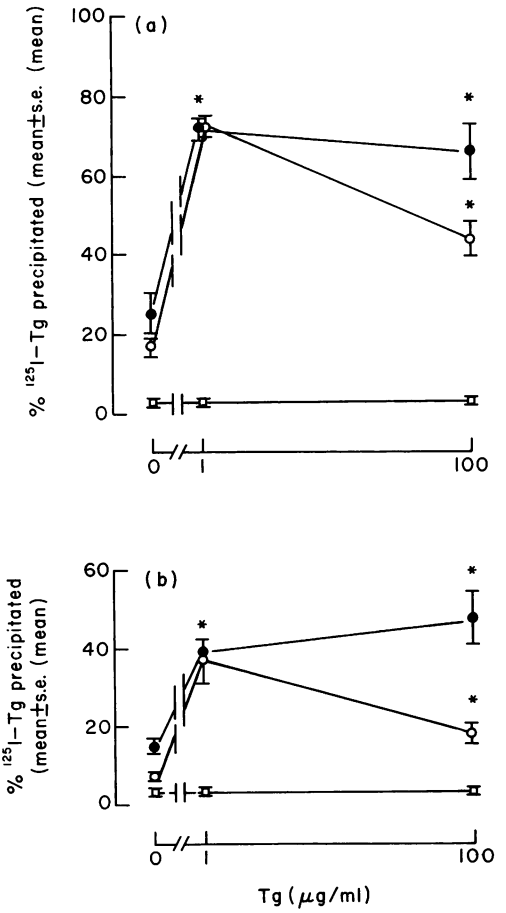

Fig. 3. Synthesis of Tg antibodies (measured by radioimmunoassay) by spleen lymphocytes from immunized mouse 1 (●) and mouse 2 (○) and from a non-immunized mouse (□). Lymphocytes were pulsed for 24 h with Tg (0–100 μg/ml), washed and cultured in Marbrook flasks for 13 days. Results are given for culture supernatants diluted (a) 1:2 and (b) 1:10. Values significantly greater than those obtained using lymphocytes cultured in medium only: $*P < 0{\cdot}02$.

In contrast, spleen lymphocytes from a control mouse did not produce detectable Tg antibody whether Tg was present or absent during the first 24 h of culture (Fig. 3).

DISCUSSION

Lymphocytes from Hashimoto tissue or the spleens of mice immunized with human Tg synthesized Tg antibody spontaneously in culture and responded to a pulse of Tg (1–100 μg/ml) by synthesizing increased amounts of Tg antibody. In contrast, Hashimoto peripheral blood lymphocytes did not respond specifically to a pulse of soluble Tg (1–240 μg/ml) whether PWM was present (four patients) or absent (three patients) from the culture system. Our results are therefore different from those obtained by Stevens (1981) for the tetanus toxoid antibody response in normal donors boosted with the toxoid; in this study, a pulse of tetanus toxoid (10 μg/ml) resulted in specific suppression of PWM-induced tetanus toxoid antibody synthesis.

Following the work of Volkman, Lane & Fauci (1981) on the stimulus of KLH antibody production (mainly IgM class) by 50 ng/ml KLH in peripheral blood lymphocytes from donors primed with KLH, we investigated the effect of Tg over the range 5–200 ng/ml in low density tube cultures of Hashimoto peripheral blood lymphocytes. No effect of Tg was observed on the amount of Tg antibody synthesized by peripheral blood lymphocytes from seven out of nine donors. However, peripheral blood lymphocytes from two patients synthesized increased levels of total IgG in the presence of Tg and the increase was associated with a significant rise in Tg antibody levels at 50 ng/ml. Lymphocyte cultures from these patients were further distinguished by showing a small response to PWM and evidence for the presence of circulating plasma cells. The response to Tg and the absence of a large increase in thyroid autoantibody synthesis when cultured with PWM are characteristics of thyroid lymphocytes (Table 2 & McLachlan *et al.*, 1983). If, as seems likely from previous studies (Rees Smith, 1981), the thyroid is a major site of thyroid autoantibody production, an increased immunologically related activity in the gland might be followed by the release into the peripheral circulation of lymphoblastoid cells secreting thyroid autoantibody. It is therefore suggested that the patients whose peripheral blood lymphocytes showed a response to Tg may have been undergoing an active phase of the disease process at the time of study.

The ability of thyroid lymphocytes to respond to Tg under conditions in which peripheral blood lymphocytes appear to be unaffected could be due to a number of factors. These include an increased B:T cell ratio (1:4 to 1:1 compared with 1:10 to 1:5 in blood lymphocytes, Totterman, 1978; McLachlan *et al.*, 1983) and the possibility that suitable antigen presenting cells occur in the thyroid. In this context it is of interest to note that Beal (1982) observed an increase in Tg antibody synthesis in cultures of peripheral blood lymphocytes containing suboptimal amounts of PWM and Tg insolubilized on beads. More recently, Noma *et al.*, (1982) demonstrated the development of Tg antibody plaque forming cells in response to Tg by Hashimoto B and T cells cultured at a ratio of 1:1. A major difference between thyroid and peripheral blood lymphocytes is the presence in the thyroid of significantly greater numbers of cells with a receptor for Tg (Totterman, 1978). Consequently, studies are in progress to specifically enrich for lymphocytes with a Tg receptor and to stimulate such populations with autoantigen.

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