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Thyroid antibodies are produced by thyroid-derived lymphocytes

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SUMMARY

The significance of the characteristic lymphocytic infiltrate in the target organ in organ-specific autoimmune disease is unknown. We have demonstrated the production of thyroglobulin antibodies and immunoglobulins (IgG, IgM and IgA) by thyroid-derived lymphocytes in Graves' disease and Hashimoto's thyroiditis using two plaque forming cell (PFC) assays. The thyroid appears to be an important site of thyroglobulin antibody production but the thyroid lymphocytes also contain many IgG PFCs of non-thyroglobulin specificity. Short-term culture and direct thyroglobulin antibody assay on micro-ELISA plates confirmed the results of the PFC assay. Therapies such as carbimazole may therefore be acting on a localized source of autoantibody production.

INTRODUCTION

There is little direct information regarding the site of autoantibody production in man, yet such data would be valuable not only with respect to pathogenic mechanisms but also with regard to therapy. Several indirect lines of evidence suggest that the target organ itself may be the main source of antibody in organ-specific autoimmune disease. In untreated Graves' disease and Hashimoto's thyroiditis there is lymphocytic and plasma cell infiltration of the thyroid, and in the latter condition the extent of this correlates with circulating autoantibody levels (Yoshida *et al.*, 1978). In Graves' disease, thyroidectomy usually leads to a fall in thyroid-specific autoantibodies (Mukhtar *et al.*, 1975) and lymphocytes derived from the thyroids of two patients with Hashimoto's thyroiditis produced autoantibodies following culture *in vitro* (McLachlan *et al.*, 1979). Fine needle aspiration of the thyroid has yielded sufficient cells for an increase in the B to T cell ratio to be demonstrated in these conditions (Totterman, 1978).

We have employed two sensitive plaque assays to detect cells spontaneously secreting immunoglobulins and thyroglobulin specific antibody—these assays permit investigation of lymphocytes without further *in vitro* manipulation to allow direct demonstration of their function. Independent confirmation of the importance of these target organ lymphocytes in the synthesis of autoantibody was obtained using brief (18 hr) *in vitro* cultures.

MATERIALS AND METHODS

Patients. Thyroidectomy specimens were obtained from six women with Graves' disease (mean age 32, range 23–46). Four of these patients had thyroglobulin antibodies in their serum (titre > 1 : 40), as assessed by standard haemagglutination tests (Wellcome). Fine needle aspiration

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(2–3 passes) of thyroid tissue was performed on six women with Hashimoto's thyroiditis (mean age 55, range 30–69) and two women with simple diffuse goitre (age 59–70). All the patients with Hashimoto's thyroiditis and neither with simple goitre had thyroglobulin antibodies.

Six healthy women without family history of autoimmunity (mean age 52·9, range 40–58) donated peripheral blood lymphocytes (PBL) as controls for PBL obtained from the patients.

Preparation of lymphocytes. Thyroid-derived lymphocytes (TL) were obtained by teasing thyroidectomy specimens with blunt forceps to release cells into RPMI 1640 medium (Flow, Irvine, UK) or by fine needle (21 gauge) aspiration of goitres. Purification was achieved by Ficoll-Hypaque (Nyegaard, Oslo, Norway) sedimentation. PBL from each patient were simultaneously obtained on Ficoll-Hypaque. Cells were washed three times in RPMI 1640 before use. Cell concentration of the final suspension was estimated by haemocytometer and viability was always greater than 95%, as assessed by trypan blue exclusion. Over 85% of cells from the thyroid preparations were mononuclear after Giemsa staining. The yield from thyroidectomy specimens was always greater than 10×10^6 and from aspiration $0{\cdot}6–3{\cdot}0 \times 10^6$.

Plaque assays: methods and control experiments. The protein A haemolytic plaque assay adapted for human use and thyroglobulin specific antibody–plaque assay were employed as previously described (Gronowicz, Coutinho & Melchers, 1976; McLachlan *et al.*, 1981). Briefly, 1 part protein A (Pharmacia, Uppsala, Sweden), 0·5 mg/ml, or human thyroglobulin (prepared by Dr A.B. Parkes, Cardiff), 0·5 mg/ml, was mixed with 1 part phosphate-free sheep red blood cells (SRBC, Tissue Culture Services, Slough, UK) and 10 parts chromic chloride solution ($2{\cdot}5 \times 10^{-4}$ M in normal saline). After incubation at 30°C for 30 min the SRBC were washed three times and resuspended in 5 parts Hanks balanced salt solution (HBSS, Flow, Irvine, UK) for use.

Tubes containing 800 μl 0·5% agar (DIFCO, Detroit, USA) in HBSS supplemented with 0·45 mg/ml DEAE-dextran (Pharmacia, Uppsala, Sweden) were kept at 46°C in a water bath and to these in turn were added 25 μl developing antiserum [rabbit anti-human γ chain for thyroglobulin plaques; γ, α and μ for protein A plaques (Dako, Denmark) diluted 1:30 in HBSS], 50 μl coated SRBC, 100 μl TL or PBL suspension and 25 μl SRBC absorbed guinea-pig complement (Flow, Irvine, UK) diluted 1:4 in HBSS. The tubes were vortexed and 3×100 μl drops placed on a 90×15 mm petri dish and covered with 22×22 mm coverslips. This produced a thin agar film and a monolayer of SRBC. The plates were incubated for 4 hr in a humid atmosphere at 37°C and the plaques were enumerated using indirect light. Results were expressed as mean PFC/10^6 mononuclear cells added.

In each assay the presence of a central lymphocyte in random plaques was verified microscopically. Omission of complement or antiserum abolished PFC in both assays and no plaques were obtained using uncoated SRBC. Both PFC responses were also abolished by preincubation of the lymphocyte suspension with cycloheximide (Sigma, St. Louis, USA), 100 μl/ml, for 60 min at 37°C, and increasing cell washes to six instead of three before assay had no effect on PFC numbers, demonstrating that plaques were due to synthesized rather than adherent antibody. This was confirmed by the inability of trypsin pretreatment of the lymphocytes to alter PFC numbers. Since cytotoxic cells can produce PFC in certain circumstances (Wahlin, Perlmann & Perlmann, 1976) the possibility that thyroglobulin plaques were due to such cells was excluded by assays using lymphocytes and coated SRBC with or without 45 min preincubation of the SRBC with sera containing 1:640 or 1:160 antithyroglobulin antibodies. No direct or antibody dependent cell cytotoxicity could be detected by plaque formation in PBL or TL from two Graves' or two Hashimoto patients in three of whom TL produced thyroglobulin plaques when rabbit anti-human IgG and complement were added in the conventional plaque assay. The specific nature of the thyroglobulin PFC response was demonstrated for each subject by inhibition of PFC with 25 μg/ml free thyroglobulin added to the agar before assay—bovine serum albumin had no effect. This inhibitory effect of thyroglobulin has previously been shown to be dose–dependent (McLachlan *et al.*, 1981).

Culture and antibody assay. Lymphocytes from three patients were cultured on plates which allowed direct estimation of antibody using an enzyme linked immunosorbent assay (ELISA). Ninety-six well micro-ELISA plates (M129B; Dynatech, Billinghurst, UK) were coated with human thyroglobulin 10 μg/ml in carbonate buffer pH 9·6 for 12 hr at 4°C, washed four times with

phosphate buffered saline −0·05% Tween 20 (PBS-T) and 200 μl aliquots of cells ($1{\cdot}0 \times 10^6$/ml in RPMI 1640 with 10% fetal calf serum; Flow, Irvine, UK) added to the wells. Standard positive and negative sera and medium alone in wells on the same plate were added as controls. The plates were incubated for 18 hr at 37°C in 5% CO_2, 95% air, 100% humidity and then washed four times with PBS-T. The plates were incubated for 2 hr at room temperature with anti-human IgG-alkaline phosphatase conjugate (Sigma, St Louis, USA) diluted 1:1000 in PBS-T, and after further PBS-T washes, the substrate pNPP 0·1% (Sigma, St Louis, USA) in diethanolamine buffer pH 9·8 was added. The optical density was read at 15 min on a Flow Titertek Multiskanner. At least four wells were used for each specimen and the results expressed as mean ± s.d.

RESULTS

In all the Graves' patients and all but one of the Hashimoto patients who had detectable serum thyroglobulin antibodies, thyroglobulin PFC were demonstrated in the thyroid derived lymphocytes (Table 1). Such PFC were never found in the peripheral blood or in the thyroid lymphocytes from the four patients (two Graves' disease, two simple diffuse goitre) without serum thyroglobulin antibodies. There was no correlation between the PFC response and serum antibody titre.

There were large numbers of IgG and to a lesser extent IgM PFC present in the lymphocyte population from the Graves' and Hashimoto glands detected by the protein A plaque assay. Previous studies have shown bone marrow aspirates obtained during routine surgery in otherwise healthy individuals yield PFC numbers of similar magnitude—in seven age-matched subjects the PFC/10^6 (± 1 s.d.) were IgG 1650 ± 1395; IgM 261 ± 212 and IgA 778 ± 691 (unpublished data). The thyroid IgG and IgM response for each patient with autoimmune thyroid disease was always much greater than the peripheral blood PFC response. There was no significant difference between the peripheral blood PFC of the patient and control population for all Ig classes ($P > 0{\cdot}1$, Student's *t*-test).

The results obtained from short term culture on precoated micro-ELISA plates are shown in Table 2. The mean optical density of culture medium alone for each culture was regarded as background optical density and any change greater than 2 s.d. above this was regarded as significant. There was no change in optical density with control or patient PBL, or with TL derived from a patient without serum thyroglobulin antibodies (Table 2). This latter patient did not have thyroglobulin PFC in the TL. However, in two other patients with Graves' disease, both of whom had serum antibodies and thyroglobulin specific PFC, there was a significant increase in optical density with TL (but not PBL)—indicating thyroglobulin antibody production by the TL added to

Table 1. PF< responses/10^6 mononuclear cells (± 1 s.d.) in patients with antoimmune thyroid disease and control subjects

	PFC response (mean ± 1 s.d.)/10^6 mononuclear cells			
	IgG	IgM	IgA	Tg
Thyroid lymphocytes				
Graves' disease ($n=6$)	1,473 ± 1,044	279 ± 356	215 ± 212	169 ± 138*
Hashimoto's thyroiditis ($n=6$)	2,394 ± 2,124	191 ± 130	225 ± 275	385 ± 509
Simple diffuse goitre ($n=2$)	0	0	20	0
Peripheral blood lymphocytes				
Graves' disease ($n=6$)	12 ± 10	53 ± 50	201 ± 208	0
Hashimoto's thyroiditis ($n=6$)	52 ± 60	20 ± 12	128 ± 174	0
Control subjects ($n=6$)	14 ± 23	37 ± 84	103 ± 138	0

* Excluding two patients with no PFC response and no serum thyroglobulin antibodies.

Table 2. Change in optical density following culture of lymphocytes in micro-ELISA plates

	Optical density	Thyroglobulin PFC/10^6 cells	Serum antithyroglobulin titre
Peripheral blood lymphocytes			
Controls ($n=2$)	n.s.	0	0
Graves' disease ($n=3$)	n.s.	0	1:80–1:240
Hashimoto's thyroiditis ($n=1$)	n.s.	0	1:5120
Thyroid lymphocytes			
Graves' disease			
Patient 1	n.s.	0	0
Patient 2	$0{\cdot}229 \pm 0{\cdot}032$	150	1:160
Patient 3	$0{\cdot}182 \pm 0{\cdot}011$	133	1:80

n.s. = not significant, regarded as a change in optical density < 2 s.d. above 'background optical density' produced by culture medium alone.

Background optical density (±1 s.d.) for patient $2 = 0{\cdot}128 \pm 0{\cdot}011$ and patient $3 = 0{\cdot}106 \pm 0{\cdot}008$.

the plate (background optical density mean ± 1 s.d. for patient $2 = 0{\cdot}128 \pm 0{\cdot}011$ and patient $3 = 0{\cdot}106 \pm 0{\cdot}008$). There was no difference between background OD for these two cultures and those negative for antibody ($P > 0{\cdot}1$, Student's *t*-test).

DISCUSSION

We have shown that spontaneous thyroglobulin PFC occur in the TL population and have confirmed that simultaneously obtained PBL do not display such activation (McLachlan *et al.*, 1981). In this previous study thyroglobulin PFC were only obtained from PBL by the use of polyclonal activators such as pokeweed mitogen which indicates that further differentiation of this B cell population is required. The use of such agents has an uncertain relationship to the situation *in vivo*. By contrast the TL form plaques without need for mitogens and are therefore an activated site of autoantibody synthesis.

The assay for thyroglobulin PFC has been shown to be specific and sensitive (McLachlan *et al.*, 1981) and using pokeweed mitogen stimulated PBL from Hashimoto patients we have found an intra-assay variation of less than 10% for thyroglobulin (and protein A) PFC (unpublished data). The control studies which we have performed confirm the specific nature of the thyroglobulin PFC response and demonstrate its reliance on *de novo* antibody synthesis. In addition they extend the previous observations by precluding any role for cytotoxic cells in the production of PFC.

Although it is apparent that large numbers of TL PFC are specific for thyroglobulin, these formed at the most 24% of the number of IgG PFC detected by the protein A PFC assay and in one subject less than 1% of IgG PFC were thyroglobulin specific. Differences in the antibody affinity requirements of the two PFC assays and between patients make such comparisons only approximate at best but it seems likely that TL contain cells secreting IgG of many specificities other than thyroglobulin. No thyroglobulin PFC could be found in the absence of rabbit antiserum, which precludes any IgM class thyroglobulin antibodies being produced by TL and similarly no PFC could be found when anti-human IgA was used instead of anti-IgG in the thyroglobulin PFC assay. However, large numbers of IgM and IgA PFC were detected in TL by the protein A assay and therefore these antibodies must also have specificities other than thyroglobulin. Some of the IgA which were obtained in TL may be due to contamination of the specimen by PBL since the corresponding responses obtained from the PBL show IgA PFC predominance. This has been documented previously but the exact origin and nature of these cells is uncertain (Freijd & Kunori,

1980). However blood contamination cannot account for all of these PFC since there is such disparity between the IgG, IgM and thyroglobulin PFC responses of the two populations. One possibility is that other thyroid autoantibodies are also being synthesized by these lymphocytes in the target organ. However, in view of the magnitude of the protein A PFC response, which is equivalent to or greater than the response of marrow, a second possibility could be that the target organ cells are polyclonally activated, since the protein A PFC assay has been used as an estimate of polyclonal activation (Ringden *et al.*, 1979; Hammarström *et al.*, 1980). If the TL are polyclonally activated this may be analogous to the secondary polyclonal activation which can be shown to occur following primary specific stimulation (Fauci, 1980). The specificities of these TL is at present being investigated.

The results of short-term culture on micro-ELISA plates correspond with those obtained from PFC responses. Although TL obtained from two Hashimoto patients have been cultured *in vitro* to demonstrate autoantibody synthesis these were long-term (14 day) cultures which made no allowance for contamination by PBL or mitogenic induction by fetal calf serum (McLachlan *et al.*, 1979). The advantages of both the PFC assay and the short-term culture system are that they avoid the use of conventional mitogens and that the duration of the assays minimises the effect of *in vitro* factors. They also allow for simultaneous assessment of PBL under exactly similar circumstances to allow for contamination of the population by the PBL. Combined with the rapidity of the assays and their requirement for small numbers of cells, there is no doubt that these techniques are more suitable for the study of TL than the previously described *in vitro* system (McLachlan *et al.*, 1979).

Although we have shown that TL are actively synthesizing thyroglobulin antibody the relative contribution of other sites such as bone marrow remains to be determined. Additional sites of synthesis would account for the absence of thyroglobulin PFC in the TL of one of the patients described above with thyroglobulin antibodies in the serum. If indeed the target organ is a major site of autoantibody synthesis, this would have potential clinical significance. For instance it is known that carbimazole, which is concentrated by the thyroid, leads to a fall in serum autoantibody levels in Graves' disease and can inhibit antibody production *in vitro* (McGregor *et al.*, 1980). One possibility is that it is acting as a local immunosuppressant on the main source of antibody and this may indicate a method of controlling the autoimmune process without systemic effects.

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REFERENCES

FAUCI, A.S. (1980) Immunoregulation in autoimmunity. *J. Allergy clin. Immunol.* **66,** 5.

FREIJD, A. & KUNORI, T. (1980) Spontaneous plaque-forming human lymphocytes detected with the protein-A plaque assay. *Scand. J. Immunol.* **11,** 283.

GRONOWICZ, E., COUTINHO, A. & MELCHERS, F. (1976) A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol.* **6,** 588.

HAMMARSTRÖM, L., SMITH, C.I.E., PETTERSON, D., MELLSTEDT, H. & HOLM, G. (1980) The protein-A plaque assay: a new system for the detection of cells secreting a given idiotype. *J. Immunol.* **124,** 140.

MCGREGOR, A.M., PETERSEN, M.M., MCLACHLAN, S.M., ROOKE, P., REES SMITH, B. & HALL, R. (1980) Carbimazole and the autoimmune response in Graves' disease. *New Engl. J. Med.* **303,** 302.

MCLACHLAN, S.M., BIRD, G., WEETMAN, A.P., SMITH, B.R. & HALL, R. (1982) Use of plaque assays to study thyroglobulin autoantibody synthesis. *Scand. J. Immunol.* **14,** 233.

MCLACHLAN S.M., MCGREGOR, A.M., REES SMITH, B. & HALL, R. (1979) Thyroid-autoantibody synthesis by Hashimoto thyroid lymphocytes. *Lancet*, **i,** 162.

MUKHTAR, E.D., SMITH, B.R., PYLE, G.A., HALL, R. & VICE, P. (1975) Relation of thyroid stimulating immunoglobulins to thyroid function and effects of surgery, radioiodine and antithyroid drugs. *Lancet*, **i,** 713.

RINGDEN, O., RYNNEL-DAGÖÖ, B., KUNON, T., SMITH, D.I.E., HAMMARSTRÖM, L., FREIJD, A. & MÖLLER, E. (1979) Induction of antibody synthesis in human B lymphocytes by different polyclonal B cell activators: evaluation by direct and indirect PFC assays. *Immunol. Rev.* **45,** 195.

TOTTERMAN, T.H. (1978) Distribution of T-, B- and thyroglobulin-binding lymphocytes infiltrating the gland in Graves' disease, Hashimoto's thyroiditis and de Quervain's thyroiditis. *Clin. Immunol. Immunopath.* **10,** 270.

WAHLIN, B., PERLMANN, H. & PERLMANN, P. (1976) Analysis by a plaque assay of IgG- or IgM-dependent cytolytic lymphocytes in human blood. *J. exp. Med.*, **144,** 1375.

YOSHIDA, H., AMINO, N., YAGAWA, K., UEMURA, K., SATOH, M., MIYAI, K. & KUMAHARA, Y. (1978) Association of serum antithyroid antibodies with lymphocytic infiltration of the thyroid gland: studies of seventy autopsied cases. *J. clin. End. Metab.* **46,** 859.