Clin. exp. Immunol. (1983) **53**, 397–405.

Enrichment and depletion of thyroglobulin autoantibody synthesizing lymphocytes

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(Accepted for publication 7 March 1983)

SUMMARY

Lymphocyte populations enriched for (or depleted of) a receptor for thyroglobulin (Tg) have been prepared from Hashimoto peripheral blood mononuclear cells (PBM) by rosetting with Tg coated erythrocytes. Removal of Tg binding cells from PBM or B cell preparations resulted in $>85\%$ reduction in their ability to synthesize Tg antibody when stimulated with pokeweed mitogen (PWM) or EB virus (EBV); the depletion was specific since the ability of Tg receptor negative cells to secrete microsomal antibody and total IgG was unimpaired. Hashimoto lymphocytes (PBM or B cells) enriched for Tg binding cells produced only small amounts of Tg antibody when cultured with PWM even in the presence of irradiated T cells and monocytes; exposure to autoantigen followed by mitogen appeared to be inhibitory. However, the Tg receptor positive fraction was readily activated by EBV to synthesize Tg antibody with a specific activity 4–10 times higher than that secreted by unfractionated lymphocytes. The ability to isolate Tg specific B cells from peripheral blood will facilitate the development of EBV transformed cell lines secreting monoclonal Tg antibody and such antibodies will provide invaluable probes in the investigation of autoimmune thyroid disease.

Keywords thyroglobulin autoantibody Hashimoto's disease autoantibody synthesis Tg antibody synthesizing cells

INTRODUCTION

The presence of autoantibodies to thyroid antigens in the serum of patients with Hashimoto's disease (Roitt *et al.*, 1956; Belyavin & Trotter, 1959) is associated with the ability of lymphocytes from such patients to synthesize antibodies to thyroglobulin (Tg) and thyroid microsomes (Mic) when cultured with pokeweed mitogen (PWM) (McGregor *et al.*, 1979) or after infection with Epstein–Barr virus (EBV) (McLachlan *et al.*, 1981). Since the immunoglobulin on the membrane of an individual B cell has the same antigenic specificity as that of the antibody the cell is programmed to secrete, it would be anticipated that cells capable of binding thyroid antigens would exist in patients with autoimmune thyroid disease. A receptor for Tg has been demonstrated on Hashimoto lymphocytes using a variety of methods including rosette formation with Tg coated erythrocytes (Perrudet-Badoux & Frei, 1969) and binding of radiolabelled Tg (Roberts, Whittingham & Mackay, 1973; Totterman, 1978). We have made use of this receptor to prepare cell populations enriched for, or depleted of, the ability to bind Tg and we have studied the capacity of these cell fractions to synthesize thyroid autoantibodies in culture.

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MATERIALS AND METHODS

Patients. Lymphocytes were obtained from seven Hashimoto patients, five female and two male (age range 47–67 years, mean age 54 years). The patients were euthyroid on thyroxine at the time of study and all except one had microsomal (Mic) and Tg antibody titres of $> 1:1,600$ and $> 1:10,000$ by haemagglutination tests. Patient PP had a Tg antibody titre $> 1:10,000$ but Mic antibodies were undetectable.

Preparation of thyroglobulin and thyroglobulin coated erythrocytes. Thyroglobulin was prepared from Graves' thyroid tissue obtained at operation by a method described previously (McLachlan *et al.*, 1982). Sheep or ox erythrocytes (SRBC or OxRBC, Tissue Culture Services) were coated with Tg (4 μg/ml) or bovine serum albumin (BSA) using the chromic chloride method (Dresser, 1978). The presence of Tg on the surface of the red blood cells was established by haemagglutination tests using Hashimoto sera positive for Tg antibodies.

Isolation of lymphocytes and fractionation into Tg receptor$^+$ and Tg receptor$^-$ cells. Hashimoto peripheral blood mononuclear cells (PBM) were isolated from 120 ml heparinized venous blood by density gradient centrifugation on 'Lymphoprep' (Böyum, 1976), and washed extensively. To enrich for Tg binding lymphocytes, PBM (10^7/ml) were mixed with Tg coated erythrocytes (0·5%) and fetal calf serum (absorbed against red blood cells) in the ratio 1:2:1 respectively. After centrifugation at 150*g*, the mixture was allowed to stand at 4°C for 3 h or overnight. The pellet was then carefully resuspended using a Pasteur pipette and centrifuged at 800*g* for 15 min on 'Lymphoprep' to give an interface population depleted of Tg binding cells (Tg receptor negative, Tg-R$^-$) and a pellet enriched in cells binding to Tg (Tg receptor positive, Tg-R$^+$). In some experiments, the Tg-R$^+$ cells were depleted of T cells using neuraminidase treated SRBC (Weiner, Bianco & Nussenzweig, 1973). An alternative approach involved the separation of PBM into T and non-T cells before enriching the non-T fraction (referred to as 'B cells') for Tg binding lymphocytes as described above. To control for non-specific binding to erythrocytes, Hashimoto PBM were allowed to interact with BSA coated erythrocytes and rosette forming cells separated to give a BSA-R$^-$ fraction at the interface and BSA-R$^+$ fraction in the pellet. After lysis of the red cells using buffered NH_4Cl at 4°C, cell suspensions were washed twice and resuspended in culture medium before counting. The viability (assessed by Trypan blue exclusion) of all cell fractions was $> 90\%$.

Lymphocyte cultures. PBM or cell fractions were cultured in 1 ml aliquots at $6–8 \times 10^6$ cells/ml in Marbrook flasks (Marbrook, 1967) or at 5×10^5 cells/ml in tubes (Falcon 2054). The culture medium was 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. Replicate cultures (3–4) were incubated in the presence of PWM (GIBCO, Europe, 3 μl/ml) or following infection with EBV using B95-8 supernatants (1 ml/5×10^6 cells for 1 h at 37°C); cyclosporin A (Sandoz) was usually added to EBV infected cultures to give a final concentration of 0·2 μg/ml in order to prevent the development of cytotoxic T cells (Bird, McLachlan & Britton, 1981). When non-T cell fractions were stimulated with PWM, equal numbers of autologous T cells which had been irradiated (2,000 rad) were added to the cultures. After 14 days at 37°C in the presence of 5% CO_2 in air, cultures were harvested by centrifugation at 400*g* and the supernatants removed and stored at −70°C.

Characterization of cell fractions. The numbers of total T cells were determined by indirect immunofluorescence using the monoclonal antiserum OKT3 and goat anti-mouse immunoglobulin conjugated to fluorescein (Ortho Pharmaceuticals). Cell suspensions were assayed for surface immunoglobulin positive cells (B lymphocytes) using rabbit anti-human immunoglobulin and fluorescein conjugated goat anti-rabbit immunoglobulin (Wellcome Laboratories). Differential staining was used to assess the proportion of monocytes and the results have been expressed as a percentage of the total number of lymphocytes.

Analysis of immunoglobulin synthesized in culture. Total IgG was analysed using a solid phase radioimmunoassay (McLachlan, Rees Smith & Hall, 1978) and Mic and Tg antibodies of IgG class by ELISA techniques described in detail elsewhere (Schardt *et al.*, 1982; McLachlan *et al.*, 1982); 100 μl aliquots of culture supernatant were assayed in duplicate and the results expressed as an ELISA index as follows:

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

In Tg antibody assays the standard was a Hashimoto serum with a haemagglutination titre of 1:5,120 diluted 400 times; when Mic antibody was being measured the reference sample (negative for Tg antibody) had a Mic antibody titre of 1:204,800 and was diluted 4,000 times. In all assays background values were assessed using culture medium and normal serum (diluted 1:100).

RESULTS

Depletion of Tg binding lymphocytes

In PWM stimulated cultures, a significant reduction in Tg antibody synthesis ($P<0{\cdot}05$) was consistently demonstrated in PBM depleted of cells binding to Tg compared with unfractionated PBM or PBM depleted of BSA-R$^+$ cells (nine cultures from five Hashimoto patients, Fig. 1a).

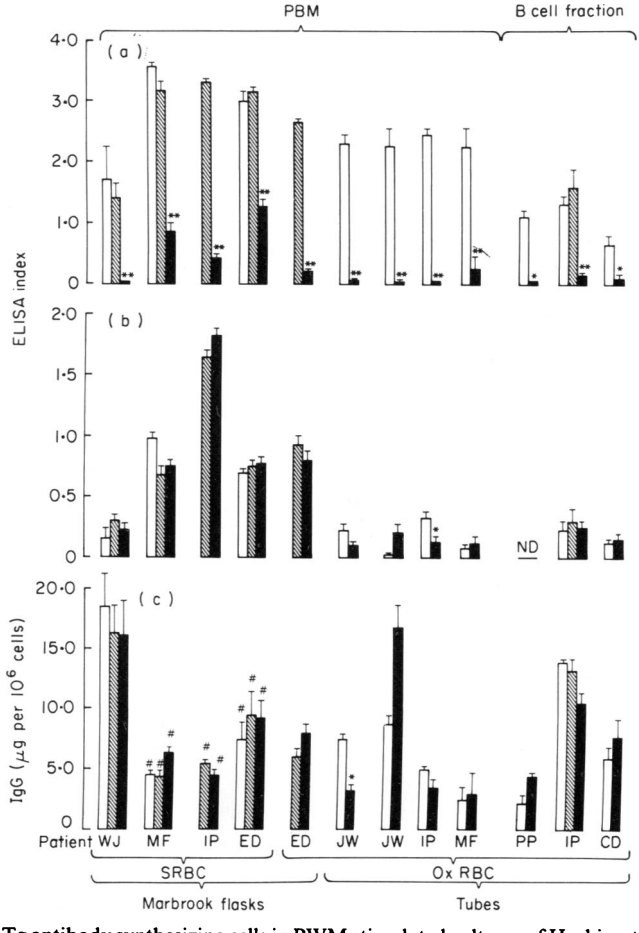

Fig. 1. Depletion of Tg antibody synthesizing cells in PWM stimulated cultures of Hashimoto PBM or the B cell fraction (plus irradiated T cells) from which Tg binding cells had been removed. Values are given as Mean + s.e. for three or four replicate cultures. (a) Tg antibody; (b) Mic antibody and (c) total IgG. □ = Unfractionated PBM or B cells; ▨ = Tg-R$^-$ PBM or B cells; ■ = BSA-R$^-$ PBM or B cells. Significantly lower than values obtained for unfractionated PBM or B cells, $*P<0{\cdot}05$, $**P<0{\cdot}002$. #Values divided by 10. ND = not determined.

Similarly, the Tg-R$^-$ fraction of B cells (non-T fraction) from three Hashimoto patients also produced significantly lower amounts of Tg antibody ($P<0{\cdot}01$) when cultured with PWM in the presence of irradiated T cells (Fig. 1a). In contrast, the amounts of Mic antibody and total IgG synthesized were generally similar in unfractionated cells, BSA-R$^-$ and Tg-R$^-$ cells (Fig. 1b & c). Specific depletion of cells with the ability to synthesize Tg was achieved using cells cultured in Marbrook flasks or in tubes; depletion was similarly effective with Tg coupled to sheep erythrocytes or to ox red cells (Fig. 1a).

Using EBV as a polyclonal activator, the ability to synthesize Tg antibody was also reduced in Hashimoto PBM or B cells from which Tg-R$^+$ lymphocytes had been removed (Fig. 2a). This decrease in the amount of Tg antibody synthesized by Tg-R$^-$ cells could not be attributed to a reduction in total immunoglobulin synthesis in lymphocyte cultures from patients ED, MF, IP, CD and JW′ in which the levels of IgG or Mic antibody synthesized were not significantly different from the amounts produced by the unfractionated cells (Fig. 2b & c). However, a decrease in total IgG production was observed in Tg-R$^-$ cultures from patient JW (Fig. 2c). To correct for the problems

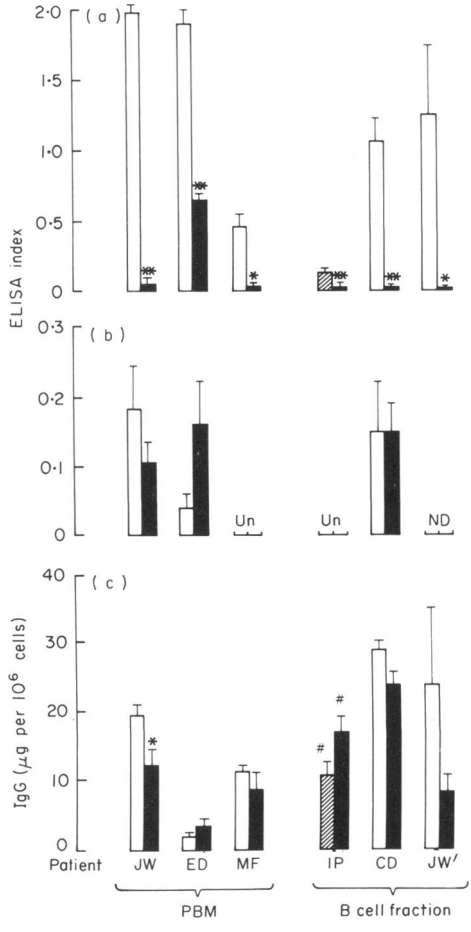

Fig. 2. Depletion of Tg antibody synthesizing cells in EB virus stimulated cultures of Hashimoto PBM or the B cell fraction from which Tg binding cells had been removed. Values are given as the Mean + s.e. for three or four replicate cultures. (a) Tg antibody; (b) Mic antibody and (c) total IgG. □ = Unfractionated PBM or B cells; ■ = Tg-R$^-$ PBM or B cells; ▨ = BSA-R$^-$ PBM or B cells. Significantly lower than values obtained for unfractionated PBM, $*P<0{\cdot}05$, $**P<0{\cdot}002$. #Values multiplied 10 times. Un = undetectable; ND = not determined.

involved in assessing changes in Tg antibody production against differences in total immunoglobulin synthesis, the specific antibody activity was calculated as follows:

$$\text{Specific thyroid antibody activity} = \frac{\text{ELISA index in 100 } \mu\text{l supernatant}}{\text{Total IgG present in 100 } \mu\text{l supernatant}}.$$

When the results are expressed in this way, it can be seen that the decrease in Tg antibody synthesis by Tg-R$^-$ cells of patient JW stimulated with EBV (Fig. 2a) was not the result of a decrease in total IgG production (Fig. 2c) since the specific activity for Mic antibody synthesized by these cells was virtually the same as the value obtained for unfractionated lymphocytes (Table 1). In contrast the Tg antibody specific activity was reduced by one-twentieth (Table 1). The specific activities for Tg antibody and in some cases for Mic antibody in lymphocyte cultures from three other Hashimoto patients are also given in Table 1; the results show that the Tg antibody specific activities produced by unfractionated PBM stimulated with PWM or EBV were similar and that the extent of Tg antibody depletion by the rosetting procedure was comparable using these two B cell activators.

Enrichment of Tg binding lymphocytes

PBM enriched for Tg binding lymphocytes cultured with PWM either produced Tg antibody with a specific activity similar to that synthesized by untreated PBM or produced virtually no Tg antibody (Table 2). The rosetting procedure used to obtain such cells was not responsible for this inability to secrete Tg antibody since BSA-R$^+$ PBM secreted Tg antibody in four out of five lymphocyte cultures and in three of these experiments the specific activity was greater than that of unfractionated PBM (patient MF, ED and IP) (Table 2). Preliminary studies suggest that this increase (which was also seen in Mic antibody activity) may have resulted from the sedimentation of plasma cells during separation of rosetting cells.

In order to investigate the lack of thyroid autoantibody synthesis by Tg-R$^+$ cells, lymphocyte markers were compared in unfractionated and Tg-R$^+$ and Tg-R$^-$ populations (Fig. 3). Tg-R$^-$ cells contained similar proportions of total T cells (OKT3$^+$), B cells and monocytes compared with PBM; however, the Tg-R$^+$ fraction was characterised by the presence of significantly fewer B lymphocytes ($P < 0{\cdot}05$, paired t-test) and in general more T cells than the unfractionated PBM. As the increased T : B cell ratio could have accounted for the lack of response to PWM (De la Concha *et*

Table 1. Specific thyroid autoantibody activities in unfractionated cells and Tg-R$^-$ populations cultured with PWM or after infection with EBV. Values are given as the mean ± s.e. of four replicate cultures

		Tg antibody (specific activity*)		Mic antibody (specific activity*)	
Patient	Cell fraction	+PWM	+EBV	+PWM	+EBV
JW	PBM	3·1 ± 0·3	2·0 ± 0·1	0·029 ± 0·007	0·019 ± 0·006
	Tg-R$^-$ PBM	0·1 ± 0·0§	0·1 ± 0·0§	0·025 ± 0·009	0·019 ± 0·008
JW′	B cells†	—	2·8 ± 0·8	—	ND
	Tg-R$^-$B cells†	—	0·0 ± 0·0‡	—	ND
IP	BSA-R$^-$ B cells†	1·2 ± 0·1	2·5 ± 0·6	0·20 ± 0·06	undetectable
	Tg-R$^-$ B cells†	0·2 ± 0·0‡	0·5 ± 0·2‡	0·22 ± 0·03	undetectable
CD	B cells†	1·3 ± 0·5	0·7 ± 0·1	2·10 ± 0·29	0·99 ± 0·45
	Tg-R$^-$ B cells†	0·1 ± 0·1‡	0·0 ± 0·0§	2·15 ± 0·50	0·87 ± 0·26
MF	PBM	1·7 ± 0·2	0·7 ± 0·2	0·06 ± 0·02	undetectable
	Tg-R$^-$ PBM	0·2 ± 0·0§	0·1 ± 0·1‡	0·07 ± 0·03	undetectable

* See text for definition. † Cultured with irradiated T cells in the presence of PWM. ND = not determined.

Values significantly higher than in cultures of Tg-R$^-$ PBM or Tg-R$^-$ B cells ‡ $P < 0{\cdot}05$; § $P < 0{\cdot}002$.

Table 2. Specific activity of thyroid autoantibodies synthesized in PWM stimulated cultures of Hashimoto lymphocytes before and after enrichment for cells with a receptor for Tg (Tg-R$^+$) or BSA (BSA-R$^+$) cells

		Tg antibody (mean ± s.e.)			Mic antibody (mean ± s.e.)		
Donor	*n*	untreated	Tg-R$^+$	BSA-R$^+$	untreated	Tg-R$^+$	BSA-R$^+$
PBM							
WJ	2–3	0·1 ± 0	0 ± 0	0 ± 0	un	un	un
MF	1–4	0·1 ± 0	0·3 ± 0·2	0·8	0·03 ± 0	0·13 ± 0·07	0·08
ED	1–3	0·6 ± 0·1	1·2 ± 0·3	2·2	0·14 ± 0·03	0·65 ± 0·11	0·31
CD	4	5·2 ± 1·6	0 ± 0	1·4 ± 0·4	ND	ND	ND
JW	4	3·1 ± 0·3	0·2 ± 0	—	0·29 ± 0·07	0·31 ± 0·31	—
*B cell fraction untreated or enriched for TgR$^+$ (or BSA-R$^+$) cells**							
IP	3–4	1·6 ± 0·1	0·9 ± 0·4	3·5 ± 0†	0·45 ± 0·02	0·48 ± 0·15	0·75 ± 0·05†
CD	4	1·3 ± 0·5	0 ± 0	—	0·21 ± 0·03	0·59 ± 0·21	—
*Tg-R$^+$ cells untreated or depleted of T cells**							
PP	4	5·0 ± 0·6	0 ± 0	—	ND	ND	—
IP	4	4·6 ± 0·3	0 ± 0	—	0·65 ± 0·10	1·58 ± 0·48	—

n = number of replicate cultures; ND = not determined; un = undetectable; *cultured with irradiated T cells.

† Significantly greater than values obtained for untreated PBM or B cells, $P < 0·01$.

al., 1977), Tg-R$^+$ PBM were further fractionated into T and non-T cells. The Tg-R$^+$ B cell fraction prepared by this method contained approximately equal proportions of B cells and monocytes (Fig. 3). In an alternative approach, the non-T cell fraction of PBM (B cells) was enriched for Tg binding cells; however, this method yielded very small numbers of lymphocytes and sufficient cells were only available in one out of three separations for the determination of markers as well as functional studies (Fig. 3). Tg-R$^+$ B cells (obtained by either of the above procedures) were cultured with PWM in the presence of irradiated T cells; however, Tg antibody synthesis was absent or present at very low levels (Table 2).

In contrast to the inability of Tg binding cells to respond to PWM, Tg-R$^+$ PBM or Tg-R$^+$ B cells synthesized Tg antibody when activated with EBV (Fig. 4a) and the specific activity of this antibody was higher than that of unfractionated PBM, usually significantly ($P < 0·05$, five out of seven cultures). Using the Wilcoxon matched pairs rank sign test (Siegel, 1956) for the data from all seven

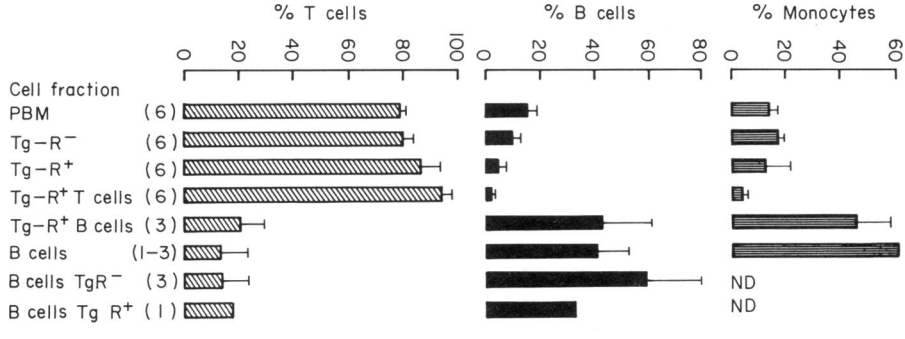

Fig. 3. Changes in proportion of T cells (OKT3$^+$), B cells and monocytes at different stages in the fractionation of Tg-R$^-$ and Tg-R$^+$ cells. Values for T and B cells are expressed as a percentage of the total number of lymphocytes and monocytes as a percentage of the total number of mononuclear cells. Error bars indicate the s.e. (mean); the number of experiments is given in parenthesis. ND = not determined.

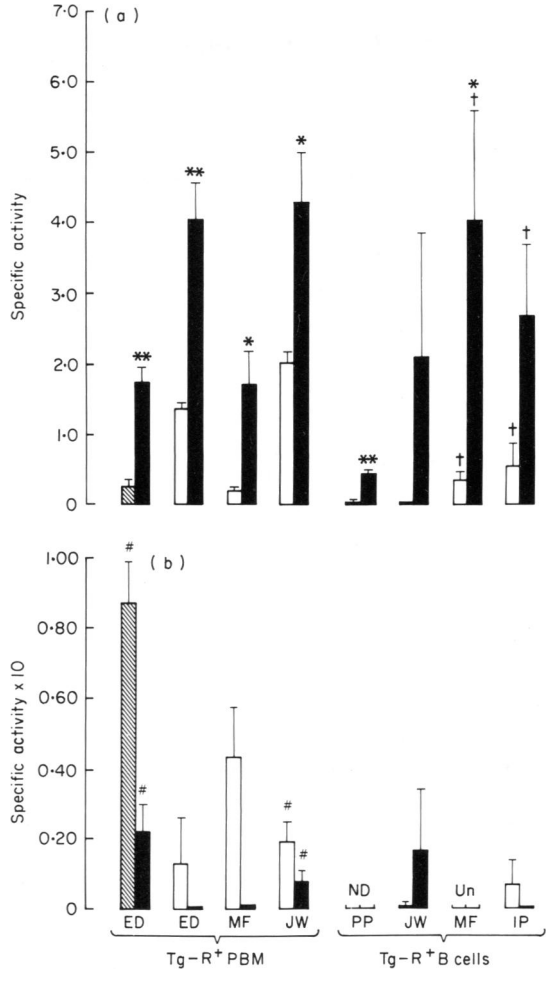

Fig. 4. Enrichment of Tg antibody synthesizing cells in EBV stimulated cultures of Hashimoto PBM or the B cell fraction from which lymphocytes with the ability to bind Tg coated erythrocytes were selected. Results are given as the Mean + s.e. for the specific activity (defined in the text) for thyroid autoantibodies (three to four replicates). (a) Specific activity for Tg antibody and (b) specific activity for Mic antibody. □ = Unfractionated PBM; ■ = Tg-R$^+$ PBM or B cells; ▩ = Tg-R$^-$ PBM. Significantly greater than values obtained for untreated PBM or Tg-R$^-$ PBM, $*P < 0{\cdot}05$, $**P < 0{\cdot}01$. †Values divided by 2. #Values divided by 10. Un = undetectable. ND = not determined.

patients, it was shown that the specific activity of Tg antibody synthesized by the Tg-R$^+$ fraction was significantly higher than that produced by unfractionated cells ($P < 0{\cdot}02$). Fig. 4a also shows that Tg-R$^+$ cells from patient ED synthesized Tg antibody with a higher specific activity than that secreted by the Tg-R$^-$ fraction. Tg-R$^+$ cells secreted little if any Mic antibody and the specific Mic antibody activity was either lower than, or not significantly different from, that of the unfractionated population (Fig. 4b).

DISCUSSION

Hashimoto peripheral blood lymphocytes from which Tg binding cells have been removed have a reduced capacity to synthesize Tg antibody without affecting the ability of these cells to produce Mic antibody. This specific deletion of Tg antibody synthesizing cells was shown using the

T-dependent activator, PWM (Keightley, Cooper & Lawton, 1976) as well as by stimulation with EBV which activates human B cells without the requirement for T cell help (Bird & Britton, 1979). The results of these experiments indicate that the Tg receptor positive population consists mainly of B lymphocytes and this is in accordance with the findings of Totterman (1978) in studies of Tg binding by lymphocytes from patients with autoimmune thyroid disease. The lower density of antigen receptors on T cells compared with B lymphocytes (Hammerling & MacDevitt, 1974) also suggests that B cells rather than T lymphocytes would be selected by the rosetting procedure.

The process of enriching peripheral blood lymphocytes for Tg binding cells simultaneously increases the proportion of total T cells and the small percentage of B lymphocytes in such fractions synthesized negligible or small amounts of Tg antibody when stimulated with PWM. B cells prepared from Tg receptor positive PBM were also unable to produce Tg antibody in the presence of irradiated T cells and PWM. The reason for this lack of response is not known but it is possible that the interaction with antigen coupled to erythrocytes combined with mitogen, provided a suppressor signal. Comparable inhibition using tetanus toxoid and PWM has been shown by Stevens & Saxon (1978) in their study of tetanus toxoid antibody synthesis by cultures of human peripheral blood lymphocytes.

Although it was not possible to stimulate Tg receptor positive lymphocytes to secrete Tg antibody using PWM, this cell fraction could be activated by EBV to synthesize Tg antibody with a higher specific activity than unfractionated PBM. No comparable increase was observed in microsomal antibody production and the enrichment was therefore specific. The extent of enrichment ranged from four to 10 times, the maximum value being similar to the upper limit observed by Wolf, Gattringer & Wick (1979) for the isolation of BSA rosette forming cells in chickens immunized with albumin. However, the degree of enrichment of Tg antibody synthesizing cells was much lower than the figure obtained by Nossal, Pike & Battye (1978) for enrichment of hapten positive mouse spleen cells from unimmunized mice using two cycles of gelatin adherence followed by selection on the fluorescence activated cell sorter (600-fold enrichment). Recently a number of improved techniques have been described for enrichment of antigen specific cells including magnetic separation of rosette forming cells and the use of polyacrylamide beads coupled to fluoroscein labelled antibody (Owen *et al.*, 1982; Baran *et al.*, 1982) but the small number of cells obtained has so far precluded extensive studies of the functional capacity of the enriched populations. However, the use of such techniques alone or in combination with the rosetting method described above might further increase the extent of enrichment obtained in these studies for Tg antibody synthesizing cells.

The proportion of circulating lymphocytes with a receptor for Tg in Hashimoto patients is small, of the order of 0·1% (Roberts *et al.*, 1973; Totterman, 1978). Consequently, the ability to isolate functional Tg receptor positive B lymphocytes from Hashimoto patients will substantially increase the possibility of developing Tg antibody secreting cell lines using the EBV transformation technique developed by Steinitz & Klein (1977). Such studies are currently in progress.

We are most grateful to Mrs Marian Atherton, Miss Sharron George, Mr B. Phail and Miss Shirley Middleton for technical assistance, to Miss Kathleen Hughes for secretarial help and Dr T. Bell for providing B95-8 supernatants. The work was supported by grants from: The Medical Research Council, the Wellcome Trust, the North of England Campaign for Cancer Research, the League of Friends of the Royal Victoria Infirmary, the Clement Lawson Fund and the Scientific and Research Committee of the Newcastle Area Health Authority.

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