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Effects of Various Isolation Methods for Human Peripheral Lymphocytes on T Cell Subsets Determined in a Fluorescence Activated Cell Sorter (FACS), and Demonstration of a Sex Difference of Suppressor/Cytotoxic T Cells

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The effects of various methods for removal of monocytes and residual erythrocytes from human peripheral blood mononuclear cells (PBMC) on T cell subsets were examined. T cell subsets were determined in a fluorescence activated cell sorter (FACS) using fluorescein-conjugated monoclonal antibodies (anti-Leu-1, anti-Leu-2a and anti-Leu-3a antibodies). Removal of monocytes by methods based on adherence or phagocytosis decreased yields of lymphocytes and caused changes in the percentage and/or the fluorescence intensity of T cell subsets. Exclusion of monocytes from the FACS analysis by setting of the scatter gates was incomplete (about 80%). Removal of residual erythrocytes after Ficoll separation by ammonium chloride treatment also changed the percentage of T cell subsets. A method using PBMC without removal of monocytes and erythrocytes was chosen as best and simplest for routine use in FACS analysis of lymphocytes. Erythrocytes could be excluded from the FACS analysis by setting the scatter gates and the percentage of T cell subsets was corrected after measurement of monocytes, identified by peroxidase staining.

The reproducibility of measurements of T cell subsets made at different times was examined using PBMC obtained from the same healthy man during 12 weeks. For standardization of the assay, the peak positions of scatter and fluorescence intensity of each PBMC labeled with anti-Leu-3a were adjusted to standard values in each FACS analysis. Under these conditions, variations of other parameters of this standard PBMC were very small in 12 different assays. Using this standard PBMC, satisfactory, reproducible results were also observed on PBMC obtained from another normal subject. Therefore, this standard PBMC labeled with anti-Leu-3a was used as a standard in FACS analysis. Under these accurately standardized conditions, it was demonstrated that the peak position of fluorescence intensity of Leu-2a (suppressor/cytotoxic T) cells was significantly lower ($P < 0.01$) in women than in men.

Key words: *FACS — monoclonal antibody — helper T cell — suppressor T cell — sex difference — fluorescence intensity*

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Introduction

Human T cell subsets may be identified with antibodies that detect stable glycoprotein antigens on their surface. The importance of a balance between helper and suppressor T cells in maintaining immune homeostasis in various diseases has been illustrated using these antibodies (Reinherz and Schlossman, 1981).

Various methods for isolation of human peripheral lymphocytes have been used in fluorescence activated cell sorter (FACS) analysis, but few data are available on the relative merits of different separation methods, especially those designed to remove monocytes and erythrocytes. Peripheral blood mononuclear cells of normal subjects isolated by density gradient centrifugation contain 10–20% of contaminating monocytes and those of pregnant women and patients with infections contain 20–40% of contaminating monocytes. Cells from pregnant women and patients with anemia are also heavily contaminated with erythrocytes. These contaminating cells may influence the analysis of T cell subsets, but the effects of various methods of removal of monocytes and erythrocytes on the determination of T cell subsets by FACS analysis are unknown.

In FACS analysis the percentage of fluorescence-positive cells and the peak position or pattern of the fluorescence histogram are analyzed. The peak position of fluorescence intensity is generally thought to be proportional to the antigen density on the cell surface (Herzenberg and Herzenberg, 1978; Ledbetter et al., 1980). However, no data are available on the position of the peak of fluorescence intensity of human lymphocytes. Therefore, we studied the effects of various methods of isolation of lymphocytes for FACS analysis and the most suitable method for routine use in FACS analysis of human peripheral lymphocytes. We also evaluated the significance of the peak position of fluorescence intensity.

Materials and Methods

Preparation of peripheral blood mononuclear cells (PBMC)

Samples of 10 ml of heparinized venous blood (15 U preservative-free heparin/ml) were obtained from healthy volunteers. Samples were always drawn at 10:00–11:00 a.m. Standard PBMC for standardization of the FACS analysis were obtained from one of us (Y.I.) at 10:30–11:00 a.m. once a week.

The heparinized venous blood was mixed with an equal volume of Dulbecco's phosphate-buffered saline (PBS), and 20 ml of this diluted blood was layed on 15 ml of Ficoll-sodium metrizoate solution (density 1.077 g/ml), and centrifuged at $400 \times g$ for 30 min. The mononuclear cells at the interface were washed 3 times and resuspended in PBS at a concentration of 1×10^6 cells/ml. For routine analyses, 6 ml samples of heparinized venous blood were used.

Removal of monocytes from PBMC

Adherence method. PBMC were incubated in plastic tissue culture dishes (No. 3002, Falcon Plastics) at 37°C under 5% CO_2 for 1 h in 4 ml of medium 199 (with

Hanks' solution; Flow Laboratories, McLean, VA) containing 10% fetal calf serum (FCS) (Flow Laboratories, Stanmore, NSW). Nonadherent cells were then obtained by gently rocking the plastic dish for 30 sec and aspirating the supernatant. These cells were washed twice and resuspended in PBS at a concentration of 1×10^6 cells/ml.

Phagocytosis method. Ten milliliters of heparinized peripheral venous blood were incubated with 1 ml of 5% suspension of silica (silicon deoxide; Japan Immunoresearch Laboratories, Tokyo) in PBS at 37°C for 1 h in a glass tube with gentle mixing every 10 min, and then centrifuged on Ficoll-sodium metrizoate solution at $1040 \times g$ for 30 min. Lymphocytes from the interface were washed 3 times and resuspended in PBS at a concentration of 1×10^6 cells/ml.

Removal of erythrocytes from PBMC

For complete removal of residual erythrocytes after Ficoll separation, PBMC were incubated with Tris-buffered 0.83% ammonium chloride for 10 min, washed twice with PBS and resuspended in PBS at a concentration of 1×10^6 cells/ml.

Analysis of lymphocyte populations by FACS

Fluorescein conjugated monoclonal anti-Leu-1, anti-Leu-2a and anti-Leu-3a antibodies (Becton Dickinson, Mountain View, CA) were used. Anti-Leu-1 reacts with human T cell antigen (Leu-1), anti-Leu-2a with human T suppressor/cytotoxic cell antigen (Leu-2a), and anti-Leu-3a with human T helper/inducer cell antigen (Leu-3a) (Engleman et al., 1981; Evans et al., 1981; Ledbetter et al., 1981).

A sample of 1×10^6 cells was incubated for 45 min at 4°C with 1 μg of fluorescein conjugated monoclonal antibody in a total volume of 100 μl PBS containing 2% of inactivated FCS and 0.1% of sodium azide. During incubation, tubes were covered with aluminum foil to exclude light. The cells were then washed twice and resuspended at approximately 10^6 cells/ml in PBS containing 0.1% sodium azide at 4°C. Before FACS analysis, the final cell fraction was checked for monocyte contamination by peroxidase staining. The viability of cells in each experiment was more than 95% as judged by trypan blue exclusion.

Quantitative fluorescence measurements were made in a FACS IV (Becton-Dickinson FACS systems, Mountain View, CA), which showed the distribution of fluorescence intensity over a range of 1–255. First the FACS was finely tuned to obtain the sharpest peaks for fluorescence and scatter with standard chicken erythrocytes (CRBC) rendered fluorescent by glutaraldehyde fixation and standard human PBMC labeled with fluorescein-conjugated anti-Leu-3a. The peak positions for scatter and fluorescence intensity of the standard human PBMC were then adjusted to correspond to standard values (channel numbers) on the pulse height analyzer (150 for scatter and 45 for fluorescence). The initial conditions were maintained by re-running the standard human PBMC after every 5–10 samples and the FACS was readjusted if necessary to give the original peak channel and sharpness. In FACS analysis of each sample, a scatter histogram was obtained first and the scatter gate was set to exclude erythrocytes and/or dead cells from the fluorescence histogram. Then a fluorescence histogram was obtained with 2×10^4

live PBMC, and the threshold (gate) position for positivity, the count of positive cells and the peak position of fluorescence intensity were recorded. The total count of lymphocytes in the fluorescence histogram was determined by the following calculation:

$$\left(1 - \frac{\text{peroxidase positive percentage}}{100}\right) 2 \times 10^4$$

E and EAC rosette tests

Peripheral T and B lymphocytes were also measured quantitatively by a micromethod for detection of sheep erythrocyte (E) rosettes and ox erythrocyte-antibody-complement (EAC) rosettes, with a commercially available kit (Japan Immunoresearch Laboratories, Tokyo), as described previously (Mori et al., 1980). Monocyte contamination was checked in another microplate by peroxidase staining. Finally the percentage of T and B lymphocytes were calculated by the following formulas based on the percentages of E rosettes (E%), EAC rosettes (EAC%) and peroxidase positive cells (P%):

$$\text{T (corrected E)\%} = \text{E} \times \frac{\text{E} + \text{EAC}}{\text{E} + \text{EAC} - \text{P}}$$

$$\text{B (corrected EAC)\%} = (\text{EAC} - \text{P}) \times \frac{\text{E} + \text{EAC}}{\text{E} + \text{EAC} - \text{P}}$$

Statistical analyses were carried out by Student's *t*-test for sex differences of T cell subsets and the paired *t*-test for other values.

Results

Effect of removal of monocytes

Table I shows the effects of 2 different treatments for removal of monocytes on the lymphocyte subpopulations detected by E and EAC rosette formation in 6 normal blood specimens. With the adherence method, the recovery of lymphocytes based on the yield without treatment was very low. Furthermore the percentage of T (corrected E) cells was increased and that of B (corrected EAC) cells was decreased. With the phagocytosis method the yield of lymphocytes was slightly reduced and the percentage of B (corrected EAC) cells was decreased.

Table II shows the effect of these treatments on T cell subsets detected by FACS analysis in the same 6 samples. With the adherence method the percentages of Leu-1 cells and Leu-3a cells were increased and so the ratio of Leu-3a to Leu-2a cells was increased. With the phagocytosis method the percentage of Leu-1 cells was increased and the positions of the peaks of fluorescence intensity for Leu-1, Leu-2a or Leu-3a cells were increased. We examined the effect of incubation alone by incubating peripheral blood from 6 normal donors for 1 h at 37°C without addition of silica

TABLE I

EFFECTS OF TREATMENTS FOR REMOVAL OF MONOCYTES ON THE DETERMINATION OF LYMPHOCYTE SUBPOPULATIONS BY MEASURING ROSETTE FORMATION

Values are means±S.D. for 6 samples of normal blood.

	Yield of lymphocytes %	E %	EAC %	Peroxidase positive %	T cell (%) (corrected E)	B cell (%) (corrected EAC)
No treatment	100	65.7±4.9	31.9±3.7	16.1±3.4	78.7±4.3	18.9±3.9
Treatment						
adherence	33.4± 5.3	86.0±2.7 [a]	14.4±5.1 [a]	0.3±0.1 [a]	86.0±2.7 [b]	14.2±5.1 [c]
phagocytosis	75.0±14.2	78.8±3.2 [b]	14.3±4.8 [a]	0.4±0.2 [a]	79.1±3.3	14.0±4.8 [c]

[a] Significant difference from value of untreatment group at $P<0.001$.
[b] Significant difference from value of untreatment group at $P<0.01$.
[c] Significant difference from value of untreatment group at $P<0.05$.

TABLE II

EFFECTS OF TREATMENTS FOR REMOVAL OF MONOCYTES ON DETERMINATION OF T CELL SUBSETS BY FACS ANALYSIS

Values are means±S.D. for 6 samples of normal blood.

	Percentage of positive cells			Peak channel of fluorescence intensity			Ratio of Leu-3a/ Leu-2a
	Leu-1	Leu-2a	Leu-3a	Leu-1	Leu-2a	Leu-3a	
No treatment	62.4±5.0	27.3±4.0	39.6±5.3	53.3±4.8	135.7±2.6	45.2±0.8	1.47±0.27
Treatment							
adherence	77.4±6.3 [a]	28.6±4.8	45.5±6.5 [b]	57.0±5.0 [b]	140.3±6.5	45.5±1.4	1.63±0.37 [c]
phagocytosis	71.7±8.0 [b]	28.3±4.0	38.8±6.9	57.0±5.9 [c]	141.8±4.3 [c]	47.3±1.5 [b]	1.38±0.31

[a] Significant difference from value of untreatment group at $P<0.001$.
[b] Significant difference from value of untreatment group at $P<0.01$.
[c] Significant difference from value of untreatment group at $P<0.05$.

TABLE III

EFFECT OF INCUBATION OF PERIPHERAL BLOOD FOR 1 H AT 37°C ON DETERMINATION OF T CELLS SUBSETS

Values are means ± S.D. for 6 samples of normal blood. None of the values for the two groups are significantly different.

	Percentage of positive cells			Peak channel of fluorescence intensity			Ratio of Leu-3a/ Leu-2a
	Leu-1	Leu-2a	Leu-3a	Leu-1	Leu-2a	Leu-3a	
No incubation	67.0 ± 6.8	24.7 ± 5.4	42.0 ± 8.4	54.7 ± 4.9	156.1 ± 12.6	46.3 ± 3.0	1.82 ± 0.68
Incubation	65.7 ± 6.2	25.0 ± 5.4	42.5 ± 6.7	54.7 ± 6.6	145.9 ± 28.7	45.7 ± 2.5	1.79 ± 0.53

TABLE IV

EFFECT OF TREATMENT WITH AMMONIUM CHLORIDE ON DETERMINATION OF T CELL SUBSETS BY FACS ANALYSIS

Values are means ± S.D. for 5 samples of normal blood.

	Percentage of positive cells			Peak channel of fluorescence intensity			Ratio of Leu-3a/ Leu-2a
	Leu-1	Leu-2a	Leu-3a	Leu-1	Leu-2a	Leu-3a	
No treatment	64.7 ± 5.0	22.1 ± 7.0	43.2 ± 6.4	53.8 ± 4.6	132.0 ± 16.1	45.8 ± 3.1	2.16 ± 0.75
Treatment	63.5 ± 5.5 [a]	21.6 ± 6.9 [b]	44.6 ± 5.5 [a]	54.4 ± 2.9	128.6 ± 15.3	44.8 ± 2.0	2.26 ± 0.80 [a]

[a] Significant difference from value of untreatment group at $P < 0.05$.

[b] Significant difference from value of untreatment group at $P < 0.02$.

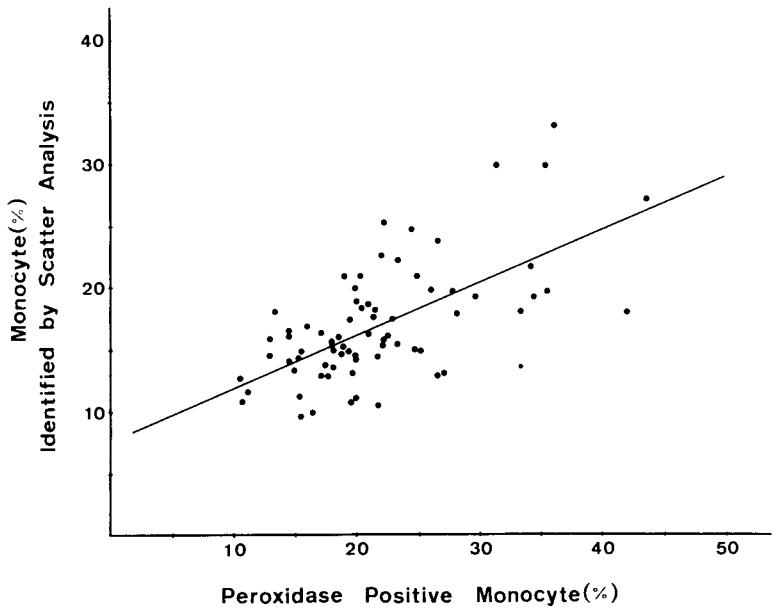

Fig. 1. Relationship between the percentage of monocytes determined by setting the scatter gates of the FACS analysis and the percentage measured by peroxidase staining; n=73, $r=0.635$, $P<0.001$, $y=0.430x+7.54$.

(Table III). Neither the percentage nor the peak fluorescence intensity of Leu-1, Leu-2a or Leu-3a cells was significantly changed by this treatment. Therefore, treatment with silica seemed to cause a significant increase in fluorescence intensity of T cell subsets. Thus these treatments for removal of monocytes seemed to be unsuitable for routine use in FACS analysis because they decreased yields of lymphocytes and changed the percentage and/or fluorescence intensity of each T cell subset.

Next, we examined the possibility of excluding monocytes by FACS analysis. The percentages of monocytes were calculated by setting the scatter gates for larger populations. The percentages of monocytes obtained correlated well with the percentages of monocytes determined by peroxidase staining of the same samples of PBMC from 73 subjects (11 normal individuals, 25 pregnant women and 37 patients with autoimmune thyroid disease) (Fig. 1). However the values estimated by FACS analysis were usually lower (about 80%) than those determined by peroxidase staining. Therefore, this method for exclusion of monocytes was not adequate, particularly when contamination was great.

Effect of removal of erythrocytes

Treatment of PBMC with Tris-buffered 0.83% ammonium chloride for hemolysis of erythrocytes influenced the percentages of T cell subsets determined by FACS analysis and increased the ratio of Leu-3a to Leu-2a cells (Table IV).

TABLE V

STABILITY OF FLUORESCENCE INTENSITY ON LYMPHOCYTES AFTER LABELING

	Percentage of positive cells			Peak channel of fluorescence intensity			Ratio of Leu-3a/ Leu-2a
	Leu-1	Leu-2a	Leu-3a	Leu-1	Leu-2a	Leu-3a	
Control	64.6±6.0	23.4±7.5	45.9±4.3	56.8±5.3	145.0±23.5	49.2±5.1	2.16±0.70
Storage for 3 h [a]	64.8±5.5	23.3±8.0	45.4±4.4	55.7±6.0	144.0±25.6	48.7±4.9	2.19±0.79

[a] PBMC labeled with fluorescein conjugated antibodies were stored at 4°C in PBS containing 0.1% sodium azide in the dark. None of the values for the two groups were significantly different.

TABLE VI

REPRODUCIBILITY OF MEASUREMENT OF T CELL SUBSETS BY FACS ANALYSIS USING STANDARD PBMC

Values are means ± S.D. for 12 different analyses of standard PBMC and 6 of the other PBMC during the observation period of 12 weeks.

	Leu-1	Leu-2a	Leu-3a
A. Standard PBMC			
Percentage of positive cells	57.8 ± 3.9	29.6 ± 1.5	32.7 ± 3.5
Peak channel of fluorescence intensity	50.8 ± 3.7	144.3 ± 3.1	45 [a]
Threshold channel for positivity	31.3 ± 2.1	71.3 ± 4.2	21.5 ± 1.4
B. Another sample of normal PBMC			
Percentage of positive cells	60.7 ± 2.5	22.7 ± 1.4	39.5 ± 1.6
Peak channel of fluorescence intensity	57.5 ± 1.9	131.0 ± 5.8	43.7 ± 0.5
Threshold channel for positivity	30.5 ± 1.4	68.2 ± 5.3	23.7 ± 1.2

[a] The peak position for fluorescence intensity of standard PBMC labeled with anti-Leu-3a was adjusted to channel 45 as a standard channel number and other data were measured.

Stability of labeled PBMC

PBMC labeled with fluorescein conjugated monoclonal antibodies were stored at 4°C in PBS containing 0.1% sodium azide. The percentages and the peak fluorescence intensities of Leu-1, Leu-2a and Leu-3a cells in 6 normal blood donors did not significantly change during storage for at least 3 h (Table V).

Standardization and reproducibility of measurement of T cell subsets using standard PBMC

Standard PBMC were obtained from the same healthy man once a week, and analyzed in the FACS. For standardization of the assay, the peak positions of scatter and fluorescence intensity of each standard PBMC labeled with anti-Leu-3a were adjusted to standard channel numbers: 150 for scatter and 45 for fluorescence. Under these conditions, variations of other parameters of this standard PBMC were very small in 12 different assays during the observation period of 12 weeks (Table VI.A). The ratio of Leu-3a to Leu-2a cells was 1.11 ± 0.14 (mean ± S.D., $n = 12$). Satisfactory, reproducible results were also obtained on another sample of normal PBMC (Table VI.B). The ratio of Leu-3a to Leu-2a cells was 1.74 ± 0.07 (mean ± S.D., $n = 6$). These data indicate that standard PBMC obtained on different days give constant values on FACS analysis. Therefore, this standard PBMC could be used for standardization of the FACS analysis.

Sex difference in T cell subsets

The T cell subsets of 17 healthy men aged 23–32 years and 26 healthy women aged 21–33 years were analysed in the FACS with the standard CRBC and standard PBMC described above. There was no difference in the average ages of the 2 groups. The peak position of fluorescence intensity of Leu-2a cells in the women was significantly ($P < 0.01$) lower than that of the men (Table VII).

TABLE VII

SEX DIFFERENCE IN T CELL SUBSETS DETERMINED BY FACS ANALYSIS

Values are means ± S.D.

Subjects	Number examined	Percentage of positive cells			Peak channel of fluorescence histogram			Ratio of Leu-3a/ Leu-2a
		Leu-1	Leu-2a	Leu-3a	Leu-1	Leu-2a	Leu-3a	
Males	17	66.6 ± 7.6	22.7 ± 5.0	43.9 ± 7.7	56.2 ± 4.5	139.1 ± 12.5	47.1 ± 2.7	2.06 ± 0.67
Females	26	66.8 ± 9.1	23.1 ± 6.1	44.6 ± 7.0	55.4 ± 4.6	129.0 ± 8.7 [a]	46.4 ± 2.2	2.10 ± 0.76

[a] Difference from males significant at $P < 0.01$.

Discussion

Flow cytometer cell sorters are playing an important role in research in immunology and several methods for operating procedures have been reported (Herzenberg and Herzenberg, 1978; Hoffman et al., 1980; Miller et al., 1981). The most important requirements of a cell sample for flow cytometry are that it be monodisperse and free of damaged cells and a large excess of irrelevant cells (Miller et al., 1981), although some laboratories reported a method using whole blood for analyzing T cell subsets (Hoffman et al., 1980). We studied the effect of removing monocytes and residual red blood cells from Ficoll separated peripheral blood mononuclear cell fraction on FACS analysis.

Removal of monocytes on the basis of their capacity for adherence or phagocytosis seems to be unsuitable for routine FACS analysis, since these treatments decreased yields of lymphocytes and caused variations in the percentages and/or fluorescence intensities of T cell subsets (Tables I, II). On removal of monocytes by the adherence method, B cells seemed to be more adherent to the plastic dish than T cells, and Leu-2a cells more than Leu-3a cells. On removal of monocytes by the phagocytosis method, treatment with silica seemed to cause significant increase in the fluorescence intensity of T cell subsets. Furthermore there was a difference between Leu-1 cells and corrected E cells, though both cells are defined as T cells. The percentage of Leu-1 cells was increased but that of corrected E cells was not changed after incubation with silica. However, incubation without silica (Table III) and exclusion of most monocytes from the FACS analysis by setting the scatter gates (unpublished data) did not influence the percentage and fluorescence intensity, respectively, of T cell subsets. Thus these data indicate that expression of Leu-1 antigen, which is different from E receptor, is increased by incubation with silica. This finding is consistent with the report that Leu-1 antigen apparently differs from E receptor (Engleman et al., 1981). In the phagocytosis method, silica treatment also caused a decrease of B cells. A similar decrease of B cells has been reported on carbonyl iron treatment to remove monocytes (Rumpold et al., 1979). Exclusion of monocytes from the FACS analysis by setting the scatter gates was incomplete; only about 80% of the monocytes were excluded in this way. Removal of erythrocytes by ammonium chloride also changes the percentage of T cell subsets and may damage lymphocytes to some extent. Thus use of PBMC without removal of monocytes and erythrocytes was finally chosen as best and simplest for routine use in FACS analysis. Erythrocytes were excluded from the FACS analysis by setting the scatter gates, and the percentages of T cell subsets were corrected for monocyte contamination, determined by peroxidase staining.

In many laboratories, CRBC rendered fluorescent by glutaraldehyde fixation are used for standardization of scatter and fluorescence in FACS analysis (Herzenberg and Herzenberg, 1978). However, with this method it is difficult to detect minor changes of labeling conditions and the properties of fluorescein conjugated antibodies in different assays. Therefore, we used standard PBMC, which were obtained from the same man, in each experiment in addition to fixed CRBC. The standard PBMC were sufficiently constant to use as a standard in FACS analysis (Table VI).

Under these conditions of standardization of the FACS, we obtained a significantly lower value for the peak position of fluorescence intensity on Leu-2a (suppressor/cytotoxic T) cells in normal women than in normal men. This probably indicates a lower Leu-2a antigen density on the cell surface in women. It has been reported that women are, in general, more resistant to infection than men (Washburn et al., 1965). The incidence of autoimmune disease is known to be higher in women. Quantitative immunological studies have shown that girls and women also tend to have higher IgM levels than boys and men (Butterworth et al., 1967). Moreover in older children the antibody response to rubella vaccine was found to be significantly higher in girls than boys (Michaels and Rogers, 1971). The reasons for these sex differences are unknown, but the decreased fluorescence intensity on Leu-2a (suppressor/cytotoxic T) cells observed in this study may be related to these phenomena. Further studies are necessary on the relation between the function and fluorescence intensity, that is the antigen density, of Leu-2a cells.

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T Lymphocyte Subsets in Autoimmune Thyroid Diseases and Subacute Thyroiditis Detected with Monoclonal Antibodies*

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ABSTRACT. Peripheral T lymphocyte subsets were analysed with monoclonal antibodies, by highly standardized fluorescence-activated cell sorter analysis instead of manual counting by the indirect immunofluorescence method, in autoimmune thyroid diseases and subacute thyroiditis. Total lymphocyte counts were increased in patients with thyrotoxic Graves' disease and subacute thyroiditis. The percentage of total T (Leu 1) cells was significantly lower in patients with thyrotoxic Graves' disease and Hashimoto's disease with destructive thyrotoxicosis than in normal subjects. No significant changes were observed in the percentages of suppressor-cytotoxic T (Leu 2a) cells or helper-inducer T (Leu 3a) cells or in the Leu 3a-Leu 2a ratio in different groups of patients. There were no correlations between the percentages of E rosette-forming cells and Leu 1 cells and between the percentages of Tγ cells and Leu 2a cells in normal subjects and patients. The peak position of fluorescence intensity of Leu 2a cells showed a significant sex difference even in normal controls. The most important finding was a significant decrease in the peak position of Leu 2a cells in patients with thyrotoxic Graves' disease and with hypothyroid or thyrotoxic Hashimoto's disease. These findings indicate the significant association of qualitative, but not quantitative, abnormality of suppressor-cytotoxic T (Leu 2a) cells with thyroid dysfunction in autoimmune thyroid diseases. (*J Clin Endocrinol Metab* 56: 251, 1982)

HASHIMOTO'S DISEASE and Graves' disease are considered to be typical organ-specific autoimmune diseases (1–3). It has been postulated that a lack of suppressor function of T lymphocytes might underlie the break in immunological tolerance that leads to autoimmune diseases. However, results on suppressor function of T lymphocytes in autoimmune thyroid diseases are conflicting. Aoki *et al.* (4) showed a decrease in suppressor T lymphocyte function in thyrotoxic Graves' disease but not in Hashimoto's disease by functional assay of Concanavalin A-induced suppressor activity. However, MacLean *et al.* (5) reported normal suppression by Concanavalin A-stimulated lymphocytes in Graves' disease and Hashimoto's disease of polyclonal immunoglobulin G and antimicrosomal antibody synthesis. Beall and Kruger (6) also reported normal helper or suppressor function of T lymphocytes on immunoglobulin G and antithyroglobulin synthesis induced by stimulation by pokeweed mitogen in patients with autoimmune thyroid diseases. Furthermore, Beall (7) showed normal T lymphocyte regulation of antithyroglobulin synthesis induced by thyroid antigen and suboptimal amount of pokeweed mitogen in autoimmune thyroid diseases. Okita *et al.* (8) observed a decrease of thyroid-specific suppressor function of T lymphocytes in thyrotoxic Graves' disease and Hashimoto's disease by assay of thyroid antigen-induced inhibition of T lymphocyte migration.

Recently, peripheral T lymphocyte subsets were enumerated by indirect immunofluorescence microscopy with monoclonal antibodies. Thielemans *et al.* (9) reported a decrease in the percentage of suppressor-cytotoxic T (OKT8) cells in both Hashimoto's and Graves' disease, whether thyrotoxic or euthyroid. Very recently, Sridama *et al.* (10) reported a decrease in the percentage of suppressor-cytotoxic T (OKT8) cells in thyrotoxic Graves' disease and Hashimoto's disease, but not in treated Graves' disease. In this study we examined peripheral T cell subsets in autoimmune thyroid diseases and subacute thyroiditis using monoclonal antibodies (Leu 1, Leu 2a, Leu 3a) by fluorescence-activated cell

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sorter (FACS) analysis, which was accurately standardized (11), instead of by manual counting by the indirect immunofluorescence method.

Subjects and Methods

Subjects

The normal controls consisted of 22 women and 18 men. The patients with Graves' disease consisted of patients with thyrotoxic Graves' disease (19 women and 3 men) and Graves' disease in remission (7 women). The patients with Hashimoto's disease consisted of euthyroid (11 women), hypothyroid (7 women), and destruction-induced thyrotoxic (see Ref. 12) (5 women) patients. Five women with subacute thyroiditis were also examined. None of the patients were receiving medication. Cases with other associated autoimmune diseases or infections were excluded from this study to avoid effects other than those of thyroid autoimmune abnormality. The patients with Graves' disease in remission had been euthyroid for at least 6 months without antithyroid drugs before examination.

Analysis of lymphocyte populations by FACS

Fluorescein-conjugated monoclonal anti-Leu 1, anti-Leu 2a, and anti-Leu 3a antibodies (Becton Dickinson Co., Mountain View, CA) were used. Anti-Leu 1 reacts with human T cell antigen (Leu 1), anti-Leu 2a with human T suppressor-cytotoxic cell antigen (Leu 2a), and anti-Leu 3a with human T helper-inducer cell antigen (Leu 3a).

T cell subsets were analyzed as described previously (11). Briefly, a sample of 1×10^6 peripheral blood mononuclear cells (PBMC), which were obtained by density gradient centrifugation, was incubated for 45 min at 4 C with 1 μg fluorescein-conjugated monoclonal antibody, and was then washed twice. Before FACS analysis, the final cell fraction was checked for monocyte contamination by peroxidase staining. Quantitative fluorescence measurements were made in a FACS IV (Becton-Dickinson Co.). The peak positions for scatter and fluorescence intensity of the standard human PBMC labeled with anti-Leu 3a were adjusted to correspond to standard values (channel numbers) on the pulse height analyser (150 for scatter and 45 for fluorescence). Then a fluorescence histogram was obtained with 2×10^4 live PBMC, and the count of positive cells and the peak position of fluorescence intensity were recorded. The total count of lymphocytes in the fluorescence histogram was determined by the following calculation: $2 \times 10^4 \times$ (1-peroxidase positive percentage/100).

The value for the absolute number of peripheral lymphocytes was calculated as described previously (13). The absolute count of T cell subsets was calculated as the product of the absolute lymphocyte count and the percentage of each T cell subset.

Sheep erythrocyte (E), ox erythrocyte-antibody complement, and ox erythrocyte-antibody rosette tests

Peripheral T, B, and Tγ lymphocytes were measured quantitatively by a micromethod for detection of E rosettes, ox erythrocyte-antibody-complement rosettes, and ox erythrocyte-antibody rosettes, using a commercially available kit (Japan Immunoresearch Laboratories, Tokyo, Japan), as described previously (14, 15).

Serology and thyroid function tests

Antithyroglobulin antibodies and antithyroid microsomal antibodies were measured by the tanned red cell haemagglutination technique as previously reported (16). Serum T_4, T_3, and resin T_3 uptake were measured, and the free T_4 index and free T_3 index were calculated as described previously (13). The size of goiter was expressed as the transverse width of the thyroid gland.

Statistical analysis of data was carried out by Student's *t* test. The analysis of variance was performed by the F test, and Mann-Whitney U test was used when it was unequal.

Results

The percentages of peripheral T lymphocyte subsets in normal subjects and patients with autoimmune thyroid diseases and subacute thyroiditis are shown in Table 1. Total lymphocyte counts were increased in untreated patients with thyrotoxic Graves' disease and subacute thyroiditis. The percentages of Leu 1 cells in untreated patients with thyrotoxic Graves' disease and Hashimoto's disease with destructive thyrotoxicosis were significantly lower than those of controls. No significant change was observed in the percentages of Leu 2a cells and Leu 3a cells, or in the Leu 3a-Leu 2a ratio in different groups. The absolute counts of Leu 1, Leu 2a, and Leu 3a cells in all group of patients were also not significantly different from those of controls. The percentage of peroxidase-positive cells in the mononuclear cell fraction was higher in untreated patients with thyrotoxic Graves' disease ($23.2 \pm 6.2\%$; $n = 19$; $P < 0.001$) and subacute thyroiditis (24.3 ± 14.5; $n = 5$; $P < 0.05$) than in controls ($16.9 \pm 3.8\%$ $n = 40$).

The peak position of fluorescence intensity of Leu 2a cells showed a sex difference ($P < 0.01$) even in normal controls (men, 137.9 ± 12.7 channel, $n = 18$; women, 127.5 ± 10.9 channel, $n = 22$). Therefore, the peak positions of fluorescence intensity of peripheral T lymphocyte subsets in normal subjects and patients with autoimmune thyroid diseases and subacute thyroiditis were examined in women (Table 2). The most important finding was a decrease in the peak position of Leu 2a cells in untreated patients with thyrotoxic Graves' disease and hypothyroid or thyrotoxic Hashimoto's disease. Interestingly, the peak position of Leu 1 cells was shifted to the right in subacute thyroiditis.

The percentages, absolute counts, and peak positions of fluorescence intensity of Leu 1, Leu 2a, and Leu 3a cells were not significantly correlated with the serum level of T_4, free T_4 index, T_3, or free T_3 index, the titer of antithyroid antibodies (either antithyroglobulin antibodies or antithyroid microsomal antibodies), the size of

TABLE 1. Peripheral T lymphocyte subsets in normal subjects and patients with Graves' disease, Hashimoto's disease, and subacute thyroiditis

	No. examined	Age (yr)	Total lymphocytes (count/mm^3)	Percentage of positive cells			Leu 3a-Leu 2a ratio
				Leu 1	Leu 2a	Leu 3a	
Normal controls	40	28.0 ± 5.8	1950 ± 370	67.6 ± 8.1	23.3 ± 5.2	44.6 ± 7.1	2.03 ± 0.63
Graves' disease							
Thyrotoxic	22	31.3 ± 11.6	2360 ± 760[a]	62.3 ± 11.1[b]	21.1 ± 6.9	41.2 ± 8.2	2.16 ± 0.86
Remission	7	29.3 ± 4.5	2060 ± 260	67.9 ± 4.0	25.7 ± 7.2	44.9 ± 6.0	1.89 ± 0.72
Hashimoto's disease							
Euthyroid	11	31.6 ± 7.4	1960 ± 700	69.5 ± 11.7	22.1 ± 5.7	45.8 ± 8.7	2.18 ± 0.59
Hypothyroid	7	29.4 ± 9.1	1900 ± 570	63.6 ± 11.2	23.2 ± 8.1	43.8 ± 7.2	2.14 ± 0.90
Thyrotoxic	5	29.6 ± 4.8	2170 ± 480	57.9 ± 14.6[b]	23.5 ± 5.1	39.3 ± 15.2	1.83 ± 0.98
Subacute thyroiditis	5	36.2 ± 4.1[a]	2410 ± 630[b]	65.1 ± 11.0	21.0 ± 5.0	43.7 ± 9.9	2.15 ± 0.94

Data are expressed as means ± SD.
[a] $P < 0.01$ *vs.* normal controls
[b] $P < 0.05$ *vs.* normal controls.

TABLE 2. Peak position of fluorescence intensity of peripheral T lymphocyte subsets in normal subjects and patients with Graves' disease, Hashimoto's disease, and subacute thyroiditis

	No. examined	Age (yr)	Peak position of fluorescence intensity		
			Leu 1	Leu 2a	Leu 3a
Normal controls	22	27.9 ± 5.5	56.6 ± 4.1	127.5 ± 10.9	46.6 ± 3.8
Graves' disease					
Thyrotoxic	19	30.8 ± 12.3	59.1 ± 6.3	111.9 ± 17.4[a]	44.9 ± 4.2
Remission	7	29.3 ± 4.5	57.1 ± 5.9	120.0 ± 14.4	46.3 ± 2.7
Hashimoto's disease					
Euthyroid	11	31.6 ± 7.4	58.9 ± 3.4	121.5 ± 15.1	47.8 ± 2.0
Hypothyroid	7	29.4 ± 9.1	55.9 ± 8.6	107.7 ± 14.2[a]	44.9 ± 2.7
Thyrotoxic	5	29.6 ± 4.8	59.4 ± 5.9	107.8 ± 14.9[b]	48.0 ± 4.2
Subacute thyroiditis	5	36.2 ± 4.1[b]	61.4 ± 3.9[c]	122.0 ± 27.8	47.4 ± 4.6

All subjects examined were females. Data are expressed as the means ± SD.
[a] $P < 0.001$ *vs.* normal controls.
[b] $P < 0.01$ *vs.* normal controls.
[c] $P < 0.05$ *vs.* normal controls.

goiter, or the degree of proptosis. Furthermore, there were no correlations between the percentages of E rosette-forming cells and Leu 1 cells ($n = 97$, $r = 0.083$) or between the percentages of Tγ cells and Leu 2a cells ($n = 97$, $r = -0.148$) in normal subjects and patients.

Discussion

A decrease in the percentage of total T lymphocytes detected with monoclonal antibody (Leu 1) was confirmed in untreated patients with thyrotoxic Graves' disease. Furthermore, a decrease of Leu 1 cells was found in destruction-induced thyrotoxic Hashimoto's disease. However, there was no correlation between the percentages of E rosette-forming cells and Leu 1 cells in normal subjects or patients with autoimmune thyroid diseases. This result is compatible with the report that E receptor apparently differs from Leu 1 antigen (17).

With regard to the percentages of suppressor T cells, we found no difference in the percentage or absolute count of Leu 2a cells in normal controls and patients with autoimmune thyroid diseases. These results are incompatible with previous reports by Thielemans *et al.* (9) and Sridama *et al.* (10). At least three explanations of these discrepancies should be considered. First, in the previous two studies monoclonal antibodies of the OKT series were used, whereas we used those of the Leu series. Second, the methods used for analysis of T cell subsets were different: the manual counting method with indirect immunofluorescence was used in the previous two studies, whereas we used accurately standardized FACS analysis (11). We found a decrease in the peak position of fluorescence intensity of Leu 2a cells in untreated patients with thyrotoxic Graves' disease and hypothyroid or thyrotoxic Hashimoto's disease. This decrease in the peak position of fluorescence intensity indicates less intense fluorescence of the cells. Weak immunofluorescence may be missed and recorded as a negative reaction on microscopic manual counting. If this is so, the previous reports of decrease in OKT 8-positive cells can be explained by our finding of a decreased peak position. Third, monocyte contamination was not clearly excluded

in previous studies. We observed an increase in the percentage of peroxidase-positive cells in untreated patients with thyrotoxic Graves' disease and subacute thyroiditis, indicating a relative increase of monocytes and decrease in the lymphocyte population. This might misleadingly suggest a lower percentage of T cell subsets in untreated patients with Graves' disease.

We know of no previous clinical reports on the peak position of fluorescence intensity of each T cell subset. In this work we examined the peak position and found a decrease in that of suppressor-cytotoxic T (Leu 2a) cells in thyrotoxic Graves' disease and hypothyroid or thyrotoxic Hashimoto's disease, but normal values in Graves' disease in remission, euthyroid Hashimoto's disease, and subacute thyroiditis. The peak position of fluorescence intensity represents the antigen density specific for each T cell subset. If the antigen density of Leu 2a is correlated with suppressor T cell function, our findings indicate that generalized suppressor T cell function is decreased in thyrotoxic Graves' disease and hypothyroid or thyrotoxic Hashimoto's disease. From all these results, we suggest that suppressor T cell function is decreased only in active autoimmune thyroid diseases. The qualitative abnormality of suppressor-cytotoxic T (Leu 2a) cells in autoimmune thyroid diseases may induce thyroid dysfunction, or be induced by thyroid dysfunction. However, the latter possibility seems unlikely, since the fluorescence intensity was decreased similarly in both thyrotoxic and hypothyroid states.

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A MICROCYTOTOXICITY ASSAY FOR THYROID-SPECIFIC CYTOTOXIC ANTIBODY, ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY AND DIRECT LYMPHOCYTE CYTOTOXICITY USING HUMAN THYROID CELLS

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A microcytotoxicity assay for detection of thyroid-specific complement-dependent cytotoxic antibody (cytotoxic antibody), antibody-dependent cell-mediated cytotoxicity (ADCC) and direct lymphocyte cytotoxicity was developed using human thyroid epithelial cells as targets. Thyroid tissue was obtained from patient with Graves' disease and was treated with collagenase and then trypsin. The red blood cells, interfollicular fibroblasts and infiltrating lymphoid cells in thyroid tissue from patients with Graves' disease could be removed by this procedure. To obtain entirely single cells as target cells, the suspension of dispersed thyroid epithelial cells was allowed to stand for 1 h at 4°C in culture medium to allow cell clumps to settle. For assay of cytotoxic antibody, a mixture of the single target cells, patient's serum and human complement was incubated in microwells for 18 h. After removing the detached cells, remaining target cells in the wells were fixed, stained and counted to assess cytotoxicity. For assay of ADCC and of direct lymphocyte cytotoxicity, target thyroid cells were precultured in the microwells for 18 h. Then effector cells with or without patient's serum were added and cultured further for 24—72 h. Cytotoxicity was assessed as described above. Adherence of effector cells to target thyroid cells sometimes disturbed the enumeration of target cells when the effector/target cell ratio was high. With this microassay system 3 different cytotoxic immune reactions against human thyroid cells could be measured quantitatively at the same time on 2—3 ml blood samples.

Keywords: *autoimmune thyroid disease — cytotoxicity assay — cytotoxic antibody — ADCC — lymphoid cytotoxicity*

INTRODUCTION

Cytotoxic immune reactions have been examined extensively in vitro (Wells, 1978). In Hashimoto's disease, a typical organ-specific autoimmune

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disease, complement-dependent cytotoxic antibody (Pulvertaft et al., 1961; Forbes et al., 1962; Irivine, 1962), antibody-dependent cell-mediated cytotoxicity (ADCC) (Calder et al., 1973b; Wasserman et al., 1974), direct lymphocyte cytotoxicity (Podleski, 1972; Calder et al., 1973a) and lymphotoxin (Amino and DeGroot, 1974, 1975) have been demonstrated in vitro. However, it is unknown which is the most important destructive factor in vivo. Most assays reported were semiquantitative and needed large blood samples and many target cells. Moreover, we do not know of any reports on the quantitative assay of ADCC or of direct lymphocyte cytotoxicity on human thyroid epithelial cells. Since these factors should be examined simultaneously in patients, we developed a quantitative microassay for simultaneous assays of thyroid-specific cytotoxic antibody, ADCC and direct lymphocyte cytotoxicity, by the microplate method (Takasugi and Klein, 1970; Amino and DeGroot, 1973) using single thyroid epithelial cells.

MATERIALS AND METHODS

Preparation of target human thyroid epithelial cells

Human thyroid tissues (5—10 g) obtained from patients with Graves' disease were separated from adipose and connective tissues and minced with scissors. The strawberry juice-like preparation obtained was washed 3—5 times with Dulbecco's phosphate-buffered saline free of divalent cations [PBS(—)] until the supernatant became clear. The sediment containing fine tissue fragments was then transferred to 50 ml of 0.3% collagenase (CLS II, Worthington Biochemical Co., Freehold, NJ) in culture medium, consisting of medium 199 (with Hank's solution, Flow Labs., McLean, VA) supplemented with 20% heat-inactivated fetal calf serum (FCS; Flow Labs., Stanmore, N.S.W.), 100 μg/ml streptomycin and 100 U/ml penicillin (Flow Labs., McLean, VA), and gently mixed with a magnetic stirrer for 60 min at 37°C. The suspension was then allowed to stand for 5 min and the supernatant containing red blood cells, endothelial cells, fibroblasts and infiltrating lymphoid cells, was discarded. The sediment of thyroid tissue fragments was resuspended in 50 ml of 0.3% collagenase solution, and the treatment was repeated. The final suspension of tissue fragments was again allowed to stand for 15 min. The sediment, containing fine thyroid follicles, was treated with 0.25% trypsin (Chiba-ken Kessei Labs., Chiba, Japan) for 20 min at 37°C. In some experiments, dispase (II, Godo-Shusei Co., Chiba, Japan) was used instead of trypsin. The trypsinized cell suspension was allowed to stand for 15 min and then the supernatant, containing single cells, was centrifuged at $400 \times g$ for 10 min to obtain a pellet of single thyroid cells. The sedimented cell clumps were trypsinized again and the supernatant centrifuged as described above to obtain further single cells. These cell pellets were combined and suspended in culture medium. The effectiveness of the procedure was improved by pipetting the tissue fragments 10 times at the beginning and end of the trypsinization procedure. Several methods,

such as filtration (steel mesh, Buchner filter or lens paper), low speed centrifugation and spontaneous sedimentation, were used to remove small cell clumps. In the filtration method a 20—30 μm or a 40—50 μm fritted-ware funnel (17 G2 or 17 G3, Funnel, Buchner type, with a fritted disc, Iwaki Glass Co., Tokyo), lens paper (Duster K3, Ozu Co., Tokyo) and stainless steel meshes (SUS 304 No. 42 100 mesh, SUS 316 No. 46 150 mesh, Mesh Kogyo Co., Osaka) were used. The final preparation of single cells was gently resuspended in 2 ml of culture medium, mixed with 8 ml of 0.8% NH_4Cl and kept for 5 min at room temperature to hemolyze the few contaminating red blood cells present. Then the single cells were washed 3 times with 40 ml of PBS(−). Finally the single thyroid epithelial cells were suspended in culture medium at a concentration of 1.2×10^6 cells/ml.

Loss of thyroid cells by adhesion to the walls was prevented by siliconizing all glassware with 3% Dow-Corning 360 Medical Fluid (350 cs viscosity) in xylene and heating it in dry air at 200°C for 90 min before use.

Preparation of effector cells

Lymphocytes. Heparinized human venous blood (10 U heparin/ml blood) was mixed with an equal volume of PBS(−) and the mixture was carefully layered over sodium metrizoate-Ficoll solution of specific gravity 1.077. The mononuclear cells obtained by centrifugation at $400 \times g$ for 30 min were washed 3 times with PBS(−) and then suspended in medium 199.

Leukocytes. Heparinized human venous blood was mixed with one-half volume of 6% dextran in 0.9% saline in a plastic syringe. After standing at 37°C for 30 min to allow sedimentation of erythrocytes, the leukocyte-rich plasma was centrifuged at $160 \times g$ for 10 min. The cell pellet was resuspended in 2 ml of culture medium and mixed with 8 ml of 0.8% NH_4Cl for 5 min at room temperature to hemolyze contaminating red blood cells. Then the leukocytes were washed 3 times with PBS(−) and suspended in medium 199.

Inactivation of serum

All test sera were inactivated before assay by heating at 53°C for 90 min and were stored at −20°C. This treatment completely inactivated complement and reduced anticomplementary activity which developed during heating and interfered with many immunological assays (Soltis et al., 1979). Normal human serum from a healthy subject of blood type AB was used as a source of complement, since this serum was less toxic than guinea-pig complement. Aliquots of serum were stored at −70°C.

Microcytotoxicity assay

The technique used was an adaptation of the microassay described by Takasugi and Klein (1970) and Amino and DeGroot (1973).

A mixture of 10 μl of solution containing equal volume of complement and inactivated patient's or control serum, and 10 μl of target cell suspension were placed in each well of a plastic microtest tissue culture plate (No.

3034, Falcon Plastics, Oxnard, CA) with a microsyringe (No. 750, Hamilton Co. Whittia, CA) with a dispenser attachment. Six replicates of each sample were tested. The plates were incubated for 18 h at 37°C in an incubator with a water jacket in an atmosphere of 5% CO_2 in air. The microplates were then inverted for 1 h and shaken briskly to remove medium, dead cells and debris from the bottom of the wells. The wells were then washed 3 times with PBS containing divalent cations [PBS(+)] and living cells attached to the bottom of the well were fixed in absolute methanol for 30 min and stained with Giemsa. The number of remaining thyroid epithelial cells was then counted under an inverted light microscope with a 40 X objective lens.

For assays of ADCC and direct lymphocyte cytotoxicity, 10 μl samples of target thyroid cell suspension were placed in the wells of a microtest plate and preincubated for 18 h at 37°C. Then 10 μl of a suspension of effector cells and inactivated serum were added to each well and the plates were incubated for 24—72 h. The test was scored as described above.

The significance of cytotoxic activity was determined by Student's *t* test.

RESULTS

Preparation of complete single thyroid cells

Even after treatment with 0.3% collagenase and 0.25% trypsin, the thyroid epithelial cell suspension contained many cell clumps. Several methods were tested for removal of these cell clumps. In the filtration method, entirely single cells were obtained when a 20—30 μm fritted ware filter was used, but the recovery of cells was very low. In the centrifugation method, thyroid epithelial cell suspension was centrifuged for 30 sec at up to 500 rev/min and then centrifugation was stopped rapidly. However, the supernatant obtained still contained clumps of thyroid cells. Contamination of the supernatant with thyroid cell clumps was examined after standing for 5—120 min. Standing for 1 h at 4°C in culture medium was found to be the best way to obtained single thyroid cells. Fig. 1 shows the human thyroid epithelial cells obtained by filtration through stainless steel meshes (A) and by spontaneous sedimentation (B).

Cytotoxic antibody assay

In the presence of complement, inactivated sera obtained from patients with Hashimoto's disease had significant cytotoxic activity. However, in some cases, serum diluted with inactivated FCS had higher cytotoxic activity than the original undiluted serum or the control (inactivated FCS alone). Results with such a serum are shown in Fig. 2. Freeze-thawing for 5—10 times destroyed the cytotoxic activity of Hashimoto's serum (Fig. 3). The susceptibility of thyroid cells to cytotoxic antibody disappeared on preincubation of thyroid cells for more than 18—36 h. Thyroid epithelial cells dispersed with 1000 PU/ml of dispase, instead of trypsin, had similar susceptibility to cytotoxic antibody.

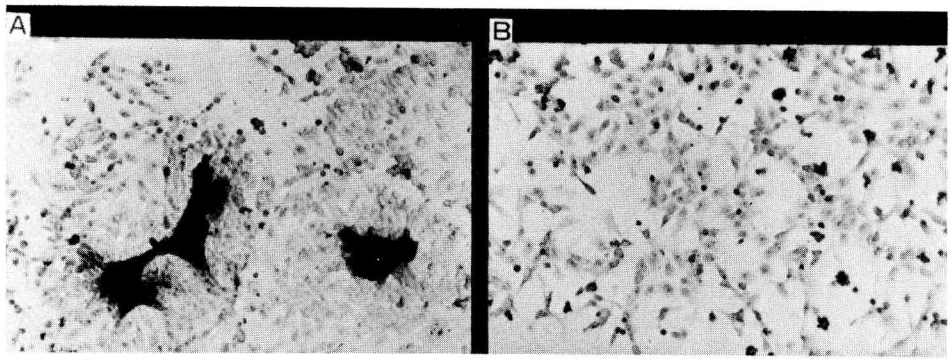

Fig. 1. Human thyroid epithelial cells obtained by filtration through stainless steel mesh (A) and by spontaneous sedimentation (see Materials and Methods) (B) were dissolved for 18 h in microplate wells.

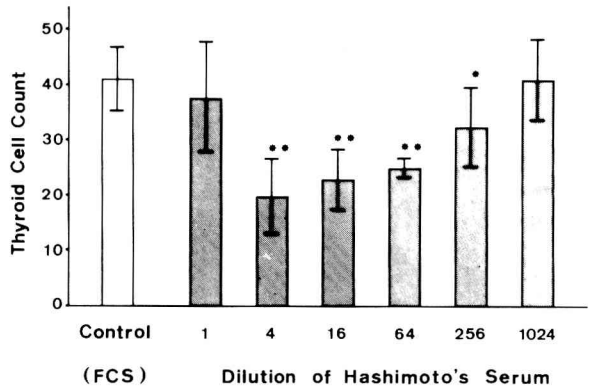

Fig. 2. Dose-response relationship in assay for cytotoxic antibody. The undiluted original serum showed less activity. Cell counts were significantly different from those of the control at $P < 0.05$ (*) and $P < 0.001$ (**).

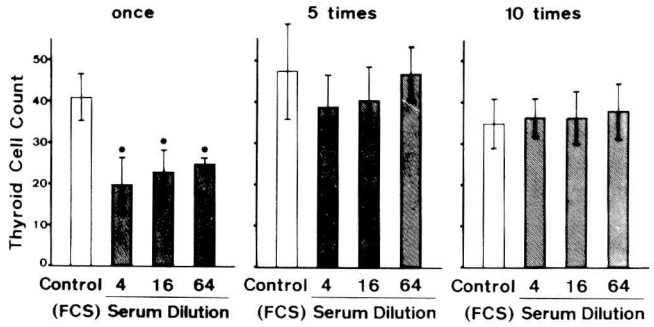

Fig. 3. Effect of freeze-thawing on cytotoxic activity of serum of a case of Hashimoto's disease. Cytotoxicity was lost by freeze-thawing more than 5 times. Cell counts were significantly different from those of the control at $P < 0.001$ (*).

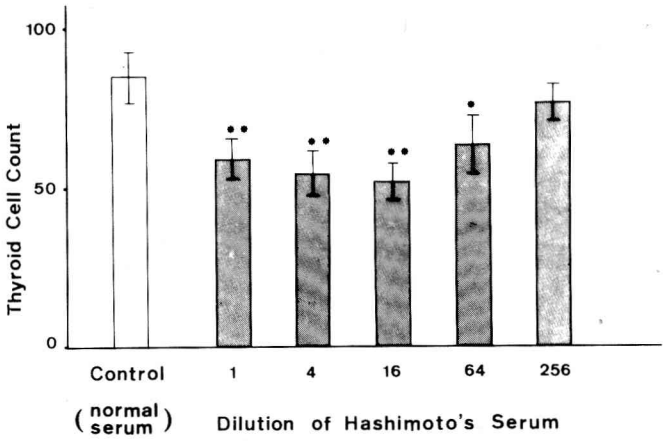

Fig. 4. Dose-response relationship in lymphocyte-mediated ADCC assay. After preincubation of target thyroid epithelial cells for 18 h, normal human lymphocytes mixed with various dilutions of serum of a case of Hashimoto's disease were added to the targets. The effector/target cell ratio was 15/1. Remaining target thyroid cells were counted after incubation for 48 h. Cell counts were significantly different from those of the control at $P < 0.01$ (*) and $P < 0.001$ (**).

ADCC assay

In the presence of normal human lymphocytes, inactivated Hashimoto's serum had significantly more cytotoxic activity than inactivated normal

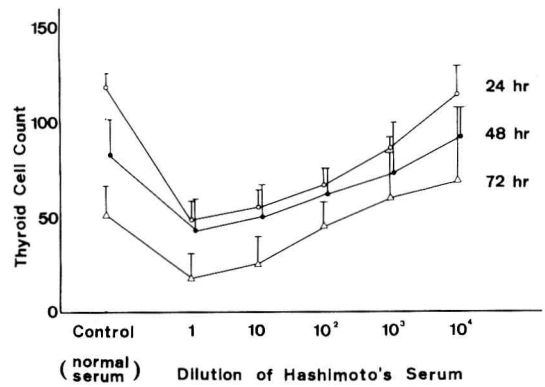

Fig. 5. Dose-response relationship and time-response relationship in leukocyte-mediated ADCC assay. After preincubation of target thyroid epithelial cells for 18 h, normal human leukocytes mixed with various dilutions of serum of a case of Hashimoto's disease were added to target cells and cultured from 24 to 72 h. The effector/target cell ratio was 12/1.

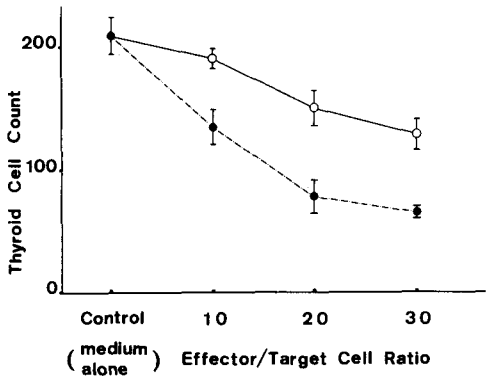

Fig. 6. Effect of various effector/target cell ratios on direct lymphocyte cytotoxicity. After preincubation of target thyroid epithelial cells, normal lymphocytes (○———○) or lymphocytes from a patient with Hashimoto's disease (●- - - -●) were added to the target cells at the indicated E/T ratios and cultured for further 48 h.

serum (Fig. 4). As in the case of cytotoxic antibody, some sera showed higher ADCC activity after dilution. Normal human leukocytes, instead of lymphocytes, with inactivated Hashimoto's serum also showed significant ADCC activity. Fig. 5 shows the dose- and time-response relationships in ADCC assay using leukocytes. Cytotoxic activity increased with the incubation time both in control wells and test wells. Incubation for 24 h was sufficient for detection of ADCC.

Direct lymphocyte cytotoxicity assay

Lymphocytes obtained from the patient with Hashimoto's disease had significantly higher cytotoxic activity than normal human lymphocytes. The cytotoxic activity increased with increase in the effector/target cell ratio (Fig. 6).

Adherence of effector cells to target cells

In assays of ADCC and direct lymphocyte cytotoxicity, considerable adherence of effector cells to target cells was observed when the effector/target cell ratio (E/T ratio) was more then 10/1. This phenomenon often interfered with the counting of target cells. Even shaking the microplates under various conditions, as reported by Huber et al. (1978), did not achieve complete removal of adherent effector cells.

DISCUSSION

In studies on the pathogenesis of immune-related tissue destruction, it is necessary to have a simple method for simultaneous determination of complement-dependent cytotoxic antibody, ADCC and direct lymphocyte cyto-

toxicity using small blood samples. Therefore, we adapted our previous microassay system for lymphocyte-mediated cytotoxicity (Amino and DeGroot, 1973) for testing thyroid-specific cytotoxicity. In this method it is essential to obtain a completely single thyroid cell suspension to allow counting of cells. Usually it is difficult to obtain human target cells from specific organs, but thyroid tissue from Graves' disease can be obtained at operation. In this study, we established a method of obtaining a preparation consisting of single thyroid epithelial cells after removal of red blood cells, fibroblasts, infiltrating lymphoid cells and small thyroid epithelial cell clumps. Recently, Shimazaki and Mitsuhashi (1977) also developed a microcytotoxicity assay for cytotoxic antibody using the microtest plate, but they used serum dilution for quantitation of cytotoxicity rather than counts on target cells.

From cytotoxic antibody assays, Irvine (1962) reported that sera from most cases of Hashimoto's disease have cytotoxic activity at over 10^4-fold dilution, but in our study no cytotoxic activity was found at dilutions above 10^3-fold. This difference may be due partly to the different method and different serum samples used. In some cases of Hashimoto's disease, diluted sera had stronger cytotoxic activities than the original sera. This may be related to the anticomplementary activity of immune complexes present in the original sera. In any event, it is necessary to use not only the original undiluted serum but also diluted serum for cytotoxic antibody assay. It has been reported that thyroid cells prepared by collagenase treatment only are not susceptible to cytotoxic antibody, and that trypsinization is essential for susceptibility (Pulvertaft et al., 1961; Forbes et al., 1962; Irvine, 1962). In this study, treatment with dispase was also found to be effective. Proteolytic enzymes such as trypsin and dispase may be essential to obtain thyroid cells susceptible to cytotoxic antibody. We confirmed the finding that preincubation of thyroid cells for more than 36 h resulted in loss of susceptibility to cytotoxic antibody (Pulvertaft et al., 1961; Forbes et al., 1962). This suggests that digestion of the surface of thyroid cells with proteolytic enzymes may uncover antigenic determinants essential for the cytotoxic reaction, and that these may be restored gradually during preincubation. The activity of cytotoxic antibody disappeared on freeze-thawing. Freeze-thawing may produce aggregated immunoglobulins that interfere with the cytotoxic reaction, or directly inactivate the biological activity of cytotoxic antibody. Thus stored samples after frequent freeze-thawing may not be suitable for assay. Forbes et al. (1962) reported that the activities of cytotoxic antibody correlated well with the titers of microsomal antibody demonstrated by immunofluorescence using fluorescein-coupled anti-immunoglobulin (Holborow et al., 1959). However, the titers of microsomal antibodies (MCHA) demonstrated in the hemagglutination test (Amino et al., 1976) did not seem to correlate with the activity of cytotoxic antibodies (unpublished). Further study is necessary to clarify the nature of cytotoxic antibody.

Calder et al. (1973b) and Wasserman et al. (1974) examined ADCC

assays using human thyroglobulin-coated chicken erythrocytes as targets by measuring ^{51}Cr release. However, no assay for ADCC against human thyroid epithelial cells has been reported. In our method using human thyroid epithelial cells, lymphocyte- and leukocyte-mediated ADCC could be measured, and significant cytotoxic activities were observed in Hashimoto's disease.

Ling et al. (1965) first examined the assay of direct lymphocyte cytotoxity on human thyroid cell monolayers as targets by estimating morphological change and/or release of ^{32}P. However, no significant cytotoxic activity was found on incubation for 2 or 24 h, even at E/T ratios of 10^2—10^3/l. Later, Podleski (1972) and Calder et al. (1973a) reported significant cytotoxicity using thyroid antigen-coated mastocytoma cells or chicken erythrocytes and measuring ^{51}Cr release. On the other hand, Laryea et al. (1973) found significant cytotoxic activity in leukocytes from patients with Hashimoto's disease in an assay using human thyroid cell monolayers and measuring uptake of ^{131}I. In our assay using a human thyroid cell monolayer formed by single thyroid epithelial cells, significant direct lymphocyte cytotoxicity was demonstrated in Hashimoto's disease at an E/T ratio of about 10/1.

Ling et al. (1965) observed that not only lymphocytes from cases of Hashimoto's disease but also normal lymphocytes tended to clump in areas of cultured thyroid cells and that this was more common at higher lymphocyte concentrations. Using our assay system, we also observed that effector cells tended to clump and become attached to target thyroid cells regardless of the presence or absence of serum from cases of Hashimoto's disease in assays of ADCC and direct lymphocyte cytotoxicity, particularly when the E/T ratio was more than 10. This adherence of effector cells to target thyroid cells sometimes interfered with counting of remaining thyroid cells. It is necessary to develop a simple and effective method for removal of nonspecific adherent effector cells and to clarify the mechanism of this adherence.

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HIGH PREVALENCE OF TRANSIENT POST-PARTUM THYROTOXICOSIS AND HYPOTHYROIDISM

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THE frequent occurrence of post-partum hypothyroidism associated with endemic goiter was described by Roberton in 1948.[1] In general, however, post-partum hypothyroidism is thought to result from hypopituitarism.[2] We have found that in Japan transient thyrotoxicosis or hypothyroidism or transient thyrotoxicosis followed by hypothyroidism often occurs after delivery in patients with autoimmune thyroid diseases.[3,4] Similar post-partum syndromes have been observed in Canada,[5] England,[6] and the United States.[7] We suggested that the physiologic and immunologic changes associated with gestation might induce post-partum aggravation of autoimmune thyroid diseases, and speculated that a subclinical form of the disease might develop into overt disease after delivery.[8,9] Subclinical autoimmune thyroiditis has been found in 8.5 per cent of women in the general population of Japan.[10] Therefore, we made a population survey of post-partum thyroid dysfunction and found that 5.5 per cent of post-partum women had transient thyrotoxicosis or hypothyroidism. The prevalence of thyroid dysfunction was higher in women giving birth to a girl. Interestingly, the condition of some of these patients mimicked so-called post-partum psychosis.[11,12]

METHODS

All 507 mothers who had delivered babies at Aizenbashi Hospital between September 1979 and September 1980 were examined three months post partum. The neck was palpated by one of us (N.A.), and serum thyroxine (T_4) (normal, 4.6 to 11.0 μg per deciliter [59 to 142 nmol per liter]), triiodothyronine (T_3) (normal, 110 to 300 ng per deciliter [1.7 to 3.1 nmol per liter]), thyrotropin (TSH) (normal, <8 mU per liter), antithyroglobulin antibody, antithyroid microsomal hemagglutination antibody, complete blood counts, and 25 biochemical constituents were measured in all subjects. Any subject in whom the transverse width of the thyroid gland was more than 4.0 cm was considered to have a goiter.

All 22 subjects who had abnormal thyroid function three months post partum, 25 of 43 euthyroid subjects with positive tests for microsomal hemagglutination antibodies, and 16 of 21 euthyroid subjects with goiter and negative tests for antibodies were followed. These 63 subjects were examined monthly from the third through the sixth month after delivery. Any subject in whom thyroid function continued to be abnormal was followed longer. In subjects with high or low serum levels of thyroid hormones, free T_4 (normal, 1.0 to 2.1 nanograms per deciliter [13 to 27 pmol per liter]) was measured. In some subjects, the serum concentration of T_4-binding globulin (normal, 13.9 to 31.4 μg per milliliter) was measured to confirm a suspected abnormality. Thyrotoxicosis was diagnosed on the basis of clinical and laboratory findings indicating hyperactive thyroid function (serum T_4>11.0 μg per deciliter [>142 nmol per liter], T_3>200 ng per deciliter [>3.1 nmol per liter], and free T_4>2.1 ng per deciliter [>27 pmol per liter]). Primary hypothyroidism was diagnosed on the basis of hypometabolic signs and symptoms of reduced thyroid function (T_4<4.6 μg per deciliter [<59 nmol per liter], free T_4<1.0 ng per deciliter [<13 pmol per liter], and TSH>10 mU per liter). The uptake of radioactive iodine by the thyroid gland was measured with the standard technique in some patients with thyrotoxicosis. Serum levels of T_4, T_3, TSH, T_4-binding globulin, free T_4, antibody to thyroglobulin, and microsomal hemagglutination antibody were measured as described previously.[13-15]

Symptoms at three months post partum were investigated in all subjects with a questionnaire containing 47 items on thyroid dysfunction and mental disorders. Statistical analyses were performed with Student's t-test, the Mann–Whitney U test, and the chi-square test.

RESULTS

Table 1 shows the abnormalities in thyroid function present three to eight months after delivery. Two of the 507 subjects were diagnosed as having "simple goiter" before pregnancy, but the other 505 had no previous history of a thyroid abnormality. Most patients with post-partum thyroid dysfunction had positive tests for antithyroid microsomal hemagglutination antibodies. Half the patients with post-partum thyrotoxicosis had goiter. Only one patient, who first had transient thyrotoxicosis and then had transient hypothyroidism, had neither thyroid enlargement nor

Table 1. Abnormal Thyroid Function Three to Eight Months Post partum among Subjects.

TYPE OF ABNORMALITY	NO. OF SUBJECTS (PER CENT)	NO. WITH ABNORMAL FINDINGS *		
		GOITER	TGHA	MCHA
Transient thyrotoxicosis alone	13 (2.6)	6	5	11
Transient thyrotoxicosis followed by transient hypothyroidism	7 (1.4)	4	3	6
Persistent thyrotoxicosis	0	0	0	0
Transient hypothyroidism alone	7 (1.4)	7	3	7
Persistent hypothyroidism	1 (0.2)	1	1	1
Total	28 (5.5)	18	12	25

*TGHA denotes antithyroglobulin antibodies, and MCHA antithyroid microsomal hemagglutination antibodies.

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antithyroid antibodies during the observation period. All the other patients had antithyroid antibodies or thyroid enlargement or both.

Three months after delivery, 19 subjects had high levels of both T_4 and T_3. Of these, 17 subjects had increased levels of free T_4 and were diagnosed as having thyrotoxicosis. In all patients the thyrotoxicosis was transient, and in five patients transient hypothyroidism subsequently developed at four to six months post partum. Two subjects with normal levels of free T_4 had high levels of T_4-binding globulin (32.0 and 36.3 μg per milliliter, respectively); the results of their liver-function tests were normal, and they were considered to have only a slight increase in T_4-binding globulin. Primary hypothyroidism was found three months post partum in five subjects, in four of whom it was transient. Forty-one euthyroid subjects who had antithyroid antibodies or goiter or both three months post partum were available for follow-up: transient thyrotoxicosis followed by transient hypothyroidism developed in two, transient thyrotoxicosis in one, and transient hypothyroidism in three. In all, 28 of the 507 subjects (5.5 per cent) were found to have thyrotoxicosis or hypothyroidism. Pituitary hypothyroidism was not observed in this study. Two subjects had a small nodular goiter suggestive of thyroid adenoma.

In seven of eight patients with post-partum hypothyroidism, thyroid function spontaneously returned to normal four to six months post partum, but one patient with hypothyroidism (T_4<1.0 μg per deciliter [<13 nmol per liter]; T_3, 70 ng per deciliter [1.1 nmol per liter]; free T_4, 0.3 ng per deciliter [3.9 pmol per liter]; and TSH, 364 mU per liter) did not recover. Replacement therapy with T_3 was started eight months after delivery because of severe symptoms of hypometabolism. Replacement therapy was stopped 13 months after delivery; when the same degree of hypothyroidism recurred, the patient was finally diagnosed as having persistent hypothyroidism.

At three months post partum, antithyroid antibodies were found in 62 subjects (12.2 per cent). This prevalence was significantly higher than that in the nonpregnant controls of similar age (37 of 477, or 7.8 per cent) ($P<0.02$).

The thyrotoxic state usually persisted for one to two months, but in three cases it persisted for nearly three months. None of the patients with post-partum thyrotoxicosis had neck pain or fever. All cases of thyrotoxicosis except one were considered to have been induced by thyroid destruction,[13] because of the later development of hypothyroidism, a low T_3/T_4 (nanograms/micrograms) ratio[13] (<20), or low radioactive-iodine uptake. One patient with thyrotoxicosis of three months' duration and an exceptionally high T_3/T_4 ratio (26.4) seemed to have stimulation-induced thyrotoxicosis; however, her radioactive-iodine uptake was not measured because she wished to continue breast-feeding. The levels of free T_4 were 3.5±0.9 ng per deciliter (45±12 pmol per liter) (mean ±S.D.) in 20 patients with post-partum thyrotoxicosis — significantly higher than those in 33 normal controls (1.6±0.2 ng per deciliter [21±2.6 pmol per liter]) ($P<0.001$) and significantly lower than those in 15 patients with untreated Graves' disease (5.4±1.4 ng per deciliter [70±18 pmol per liter]) ($P<0.01$). The levels of free T_4 and TSH in 15 patients with post-partum hypothyroidism were 0.5±0.2 ng per deciliter (6.5±2.6 pmol per liter) and 99±96 mU per liter, respectively.

Table 2 compares the symptoms of patients with post-partum thyrotoxicosis with those of post-partum controls. Mild anemia and abnormal liver function were found in 3.6 per cent and 8.1 per cent, respectively, of the post-partum subjects. Control subjects had many symptoms. Therefore, only fatigue and palpitation were significantly different in prevalence when the patients were compared with the controls. However, the severity of these symptoms was more marked in the patients in whom thyrotoxicosis continued for more than two months. One patient was admitted to the hospital about one month post partum because of psychoneurotic reaction and was found to have thyrotoxicosis. Another patient with thyrotoxicosis had symptoms similar to those of "transient attacks of excitement and confusion" [16]; the value for T_4 was 17.9 μg per deciliter (231 nmol per liter), T_3 was 474 ng per deciliter (7.3 nmol per liter), and free T_4 was 4.3 ng per deciliter (55 pmol per liter). One patient with hypothyroidism (T_4<1.0 μg per deciliter [<13 nmol per liter]; T_3, 93 ng per deciliter [1.4 nmol per liter]; free T_4, 0.4 ng per deciliter [5.2 pmol per liter]; and TSH, 280 mU per liter) had a depressive reaction characterized by physical and mental lethargy, retardation of thought and action, and emotional depression. The psychotic symptoms of these three patients disappeared when they became euthyroid again.

The development of post-partum thyroid dysfunction was not related to age, parity, breast-feeding, or the economic situation of the mother. Interestingly, 22 of 28 patients (79 per cent) with post-partum thyroid dysfunction gave birth to girls, in contrast to 208 of

Table 2. Symptoms in 20 Patients with Post-partum Thyrotoxicosis and 254 Post-partum Controls.

SYMPTOM	PATIENTS	CONTROLS
	no. (%)	*no. (%)*
Shoulder stiffness	13 (65)	119 (47)
Fatigue	11 (55) *	75 (30)
Increased appetite	8 (40)	72 (28)
Increased sweating	4 (20)	23 (9)
Palpitation	4 (20) †	13 (5)
Nervousness	3 (15)	39 (15)
Hypersensitivity to heat	2 (10)	22 (9)
Weight loss	1 (5)	8 (3)

*Significantly different from control value ($P<0.02$ by chi-square test).
†Significantly different from control value ($P<0.05$ by chi-square test).

442 controls (47 per cent) ($P<0.01$). In all, 30 subjects had antithyroid-antibody titers of 10×2^7 or higher three months post partum, and of these, 18 had girls and 12 had boys. In 13 of the former 18 subjects (72 per cent) and in three of the latter 12 subjects (25 per cent) post-partum thyroid dysfunction occurred ($P<0.05$).

Discussion

It is known that post-partum hypothyroidism occurs as a result of hypopituitarism.[2] The incidence of post-partum hypopituitarism has been estimated to be 0.003 per cent (four of 124,752 deliveries).[17] This study, however, revealed a higher rate (3.0 per cent) of primary hypothyroidism in the post-partum period, although in most cases the disorder was transient. The prevalence of persistent primary hypothyroidism was only 0.2 per cent, but this figure was still far higher than that for post-partum pituitary hypothyroidism ($P<0.001$). The prevalence of post-partum thyroid dysfunction increased to 5.5 per cent when thyrotoxicosis was included. It is likely that some transient abnormalities had disappeared by three months post partum. Some subjects with antithyroid microsomal hemagglutination antibodies could not be followed in this study. Therefore, the real incidence of post-partum thyroid dysfunction may be even higher.

In autopsy studies we found an association between the presence of antithyroid antibodies and lymphocytic infiltration in the thyroid gland in subjects without overt thyroid disease.[18] Therefore it has been suggested that subjects with these antibodies have autoimmune thyroiditis irrespective of whether they have thyroid enlargement.[10] The high prevalence of post-partum thyroid dysfunction associated with antithyroid antibodies in this study strongly supports our hypothesis that these post-partum abnormalities are induced by aggravation of subclinical autoimmune thyroid disease.[8,9] Women with autoimmune thyroiditis and preexisting Graves' disease often have a dramatic fall in antithyroid antibody titers during gestation, and more than half these patients have a transient increase in these titers three to four months post partum to levels greater than those present before pregnancy.[9] In some cases, antibody became detectable transiently after delivery.[9] Such changes may be related to the high incidence of antithyroid antibodies three months post partum in this study.

Sheehan reported that 79 per cent of patients with post-partum hypopituitarism had round-cell infiltration and lymphoid follicles in the thyroid gland.[2] This finding suggests the coexistence of post-partum hypopituitarism and autoimmune thyroiditis. Thus, it may be necessary to investigate primary thyroid dysfunction in patients with Sheehan's syndrome.

It should be emphasized that half the patients with post-partum thyrotoxicosis in our study did not have thyroid enlargement. Therefore, even if post-partum women have no palpable goiter, their thyroid function should be examined if they have hypermetabolic symptoms. It is unlikely that the occurrence of post-partum thyrotoxicosis is related to the decrease in T_4-binding globulin that occurs after delivery, since free T_4 levels were normal even one month post partum.[15] All except one of our cases of post-partum thyrotoxicosis were considered to be due to thyroid destruction. Measurement of radioactive-iodine uptake is more reliable for differentiation of destruction-induced thyrotoxicosis from Graves' disease[13] but is contraindicated in women who wish to breast-feed. The T_3/T_4 ratio is also useful for differentiation of two types of thyrotoxicosis,[13] although this point is not accepted by all workers.[19]

All cases of post-partum thyrotoxicosis were self-limited, and thyrotoxic symptoms were transient. The control post-partum women also had various symptoms (also reported by Jacobson et al.[20]), which may have obscured the existence of post-partum thyroid dysfunction. Only the frequency of thyrotoxic symptoms of fatigue and palpitation differed in our patients and controls, although the severity of symptoms was greater in some of the patients. The three patients with post-partum thyroid dysfunction had symptoms similar to those of patients with so-called post-partum psychosis.[11,12,16] It is interesting to speculate that an acute, dramatic change in serum levels of thyroid hormones may induce overt psychosis in subjects with a predisposition to psychosis. Further studies are necessary to clarify the relation between post-partum psychosis and thyroid dysfunction. When patients report moderate or severe symptoms of thyrotoxicosis, a mild tranquilizer or β-blocker may be used temporarily. Replacement therapy with a submaximal dose of T_3 may be useful for post-partum hypothyroidism when patients report severe hypometabolic symptoms, since spontaneous recovery of thyroid function can be predicted.[21]

Surprisingly, thyroid dysfunction was more frequent in women giving birth to girls (female births: male births, 4:1). Moreover, among subjects with antithyroid microsomal hemagglutination antibody titers of 10×2^7 or higher, mothers of girls had a higher rate of post-partum thyroid dysfunction. It is possible that the gestational and post-partum state of immunologic surveillance in women who conceive females differs from that in women who conceive males.

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