In vitro lymphocyte proliferation in response to polyclonal activators and microbial antigens, and production of immunoglobulins stimulating thyroid adenylate cyclase in Graves' disease

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Abstract. Cultures of blood lymphocytes from 16 patients with Graves' disease (GD) and 14 matched controls were studied. Incorporation of \(^{14}C\)thymidine was significantly increased in unstimulated cultures of GD lymphocytes, while the incorporation after stimulation with polyclonal activators (concanavalin A, pokeweed mitogen, phytohaemagglutinin), microbial antigens (E. coli, Candida albicans extract, purified protein derivative of tuberculin, Yersinia enterocolitica serotype 3) and subcellular fractions of human thyroid antigens did not differ from the controls. Due to the increased incorporation in unstimulated cultures, stimulation index is not suitable as an indicator of lymphocyte sensitization. After polyclonal activation or stimulation with thyroid antigens the lymphocytes were cultured for up to 21 days, and the supernatants were investigated for thyroid adenylate cyclase stimulating immunoglobulins (TACSI). No TACSI were demonstrated in supernatants of the lymphocyte cultures neither after polyclonal activation nor after specific stimulation with several thyroid antigens.

Several autoantibodies against components in cytoplasm membranes of the thyroid cell have been demonstrated in Graves' disease (GD). Particularly, the thyroid stimulating immunoglobulins (Ig) have evoked interest, because they are assumed to be involved in the pathogenesis of the hyperthyroid state, and it has been reported that GD lymphocytes produce thyroid stimulating Ig in vitro after specific stimulation with thyroid extract and after non-specific polyclonal activation (McKenzie & Gordon 1965; Miyai et al. 1967; Wall et al. 1973; Knox et al. 1976a,b; McLachlan et al. 1978).

The purpose of the present investigation was to study the ability of various polyclonal activators, microbial and thyroid antigens to stimulate cultures of blood lymphocytes from patients with GD, and to investigate the ability of the lymphocytes to produce thyroid adenylate cyclase stimulating Ig (TACSI) in vitro when cultured for up to 21 days.

Materials and Methods

Patients
GD was defined as hyperthyroidism with diffuse goitre. Sixteen unselected outpatients (13 women and 3 men) with untreated GD (age 49 ± 14 years (mean ± sd)) were studied. Serum total thyroxine was 16.9 ± 3.0 \(\mu g\)/100 ml, serum total triodothyronine was 547 ± 140 mg/100 ml and serum resin T\(_3\) uptake was 1.58 ± 0.2. Blood lymphocyte concentrations were 2.1 ± 0.6 \(\times\) 10\(^8\) cells/l. Four patients were TACSI negative, while the levels in the others ranged from 110—130% per cent (mean 249 ± 299 per cent).

Controls
Fourteen healthy persons (11 women and 3 men) without clinical or laboratory evidence of thyroid disease and without family history of thyroid disorders were studied in parallel (age 50 ± 16 years). Serum thyroxine was...
6.7 ± 1.5 μg/100 ml, serum triiodothyronine 138 ± 47 ng/100 ml and resin T₃ uptake 0.89 ± 0.13. All were TACSI negative.

Antibodies against Yersinia enterocolitica serotype 3 were determined by an agglutination technique, and none had significantly elevated titers (< 80).

**Lymphocyte cultures**

Lymphocytes were isolated by Isopaque-Ficoll, and after washing 10⁵ or 10⁶ cells per vial were cultured in triplicates in medium RPMI 1640 with heparin 10.000 IU per 400 ml, 15 per cent pooled human serum or 10 per cent foetal calf serum (Andersen et al. 1975). As polyclonal activators phytohaemagglutinin (PHA, Difco, 0.5 μg per 500 μl), concanaaval A (Con A, Pharmacia, 20 μg per 500 μl), pokeweed mitogen (PWM, Gibco, 2 μg per 500 μl) were employed; these cultures were incubated for 72 h. The antigens were suspensions of heat-killed Yersinia enterocolitica serotype 3 (10⁷ to 10² per 500 μl), E. coli (5 × 10⁴ per 500 μl), an extract of Candida albicans (100 μg per 500 μl), purified protein derivative (PPD, 5 μg per 500 μl), and subcellular fractions of human thyroid homogenate obtained by differential centrifugation (crude fraction, 1000 g, 5000 g, 10,000 g, 37,000 g pellets and 37,000 g supernatant (Bech & Madsen 1978)) were employed at two-fold dilutions up to 1:1000. These cultures were incubated for 120 h. In all cases [¹⁴C]thymidine was added 24 h before harvest.

To detect production of TACSI, 10⁵ or 10⁶ lymphocytes per vial were cultured in triplicates for 6, 12 and 21 days with or without stimulation with polyclonal activators or thyroid antigens as described above. The medium was changed twice weekly, and the 10⁶ cells were cultured in Falcon flasks.

**Preparation of Ig**

Ig were prepared from serum or the supernatant of the cultures by ammonium sulphate precipitation at a final concentration of 1.6 mol/l followed by dialysis (Bech & Madsen 1979).

**The adenylate cyclase (AC) assay**

Ig derived from the supernatants of the lymphocyte cultures, ATP, and ATP regenerating system, a phosphodiesterase inhibitor and the 5000 g fraction of human thyroid homogenate (0.04 mg protein/incubate) were incubated at 37°C for 60 min. The reaction was stopped by boiling for 5 min (Bech & Madsen 1978). The cyclic AMP in the incubates was measured by a competitive protein binding assay. AC activity above the 95th percentile of the controls indicated presence of TACSI.

**Results**

Lymphocyte proliferation as determined by thymidine uptake was studied in 9 patients and 9 controls (Table 1 and Fig. 1). Unstimulated cultures from patients with GD showed significantly higher [¹⁴C]thymidine incorporation than controls, while uptake after stimulation with polyclonal activators showed no difference.

After stimulation with microbial antigens all responses were lower in GD, but this was significant only in case of Candida albicans (P < 0.025, Mann-Whitney U-test). The response to Yersinia was also lower in patients and paralleled the responses to E. coli.

Stimulation with subcellular thyroid antigens was studied using a wide range of concentrations, but no significant difference between GD and controls was observed. In 1 patient and 1 control stimulation occurred, defined as an increase above 2.5 times the incorporation of the corresponding unstimulated culture.

Presence of TACSI was studied in supernatants from lymphocytes (10⁵ or 10⁶ cells per vial) cultured for 6, 12 and 21 days with and without stimulation with PHA, PWM or the subcellular fractions of human thyroid homogenate. Thirteen patients were studied using 10⁵ cells per vial and 3 patients using 10⁶ cells per vial. No difference between AC activity by supernatants of GD and

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<td><strong>Patients</strong></td>
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<tr>
<td>No stimulation</td>
</tr>
<tr>
<td>PHA</td>
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<td>PWM</td>
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| No stimulation | 289 | 209 | n.s.
| E. coli | 1598 | 2514 | |
| Candida albicans | 369 | 2071 | < 0.025 |
| PPD | 1645 | 5451 | |
| Yersinia | 1660 | 3390 | n.s. |
| Thyroid | 182 | 293 | |

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control lymphocyte cultures was demonstrated, and no correlation between serum TACSI and AG activity was found.

Discussion

In the present study of blood mononuclear cells obtained from patients with GD, we were not able to demonstrate lymphocyte proliferation after stimulation with thyroid antigens. This is in contrast to observations of Mäkinen et al. (1978) using the 10 000 g pellet and Wall et al. (1980) using the 6500 g pellet and a cytosol fraction of human thyroid homogenate.

By cytochemical and radioreceptor assays McLachlan et al. (1978) have demonstrated production of Ig with thyroid stimulating activity in 3 of 7 patients with GD; the maximal amounts were produced after 20 days incubation. Sugenooya et al. (1978) used a slice technique to detect these Ig and observed detectable levels after 6 days incubations. In contrast Wall et al. (1979) by radioreceptor assay, like we, were unable to demonstrate production of thyroid stimulating Ig.

The lack of response to thyroid antigens and production of TACSI in the present study could be due to the increased [14C]thymidine incorporation in unstimulated cultures of GD lymphocytes, reflecting an increase of spontaneously activated cells as previously suggested by Folb & Bank (1976) and Maciel et al. (1976). Beall & Kruger (1979) have demonstrated that antithyroglobulin production in vitro can be suppressed by a large number of T-cells in the cultures, but Aoki et al. (1979) found evidence of impaired suppressor cell function in GD by measurement of enhanced activity; this has recently been supported by Okita et al. (1981) and Pacini (1981) who demonstrated that T cells in GD were unable to suppress the Ig synthesis by normal B cells in vitro.

Fig. 1.

[14C]thymidine uptake in counts per min (cpm) by lymphocytes from 9 patients with Graves' disease (●) and 9 controls (○) after stimulation with polyclonal activators (PHA, PWM and Con A) or with microbial antigens (E. coli, Candida albicans, PPD, Yersinia) and the plasma membrane fraction of human thyroid homogenate (5000 g). [14C]thymidine was added 24 h before harvest.
It has recently been shown that target organ lymphocytes have a more pronounced capacity of antibody synthesis as compared to blood lymphocytes (McLachlan et al. 1979). Further studies comparing different subsets of lymphocytes isolated from the thyroid gland and from the blood might therefore elucidate the regulatory role of lymphocytes in the synthesis of thyroid stimulating Ig in GD.

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References


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