Autologous mixed lymphocyte reaction in Graves' disease: relationship to clinical status

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Abstract. The autologous mixed lymphocyte reaction (AMLR), i.e. the ability of T lymphocytes to proliferate when cultured with autologous non-T cell fractions, is an ‘in vitro’ phenomenon showing immunological memory and specificity, probably related to the cooperation ability of immunocompetent cells.

We have evaluated AMLR in 27 patients with Graves’ disease of varying clinical status (untreated and treated with antithyroid drugs, surgery or 131I). The results obtained show:

1) Impaired AMLR in untreated patients (as in other autoimmune diseases).
2) Significantly higher AMLR in cured patients, and that
3) AMLR in cured patients varies with the treatment (higher after surgery or radioiodine than after medical treatment).

These results are consistent with the hypothesis of a functional defect of T cells in Graves' disease, which improves when clinical remission is achieved.

Normal human T lymphocytes are able to proliferate in response to autologous non-T mononuclear cells. This phenomenon is known as the autologous mixed lymphocyte reaction (AMLR) (Opelz et al. 1975). The AMLR has typical immunologic features, in that it demonstrates both memory and specificity (Weksler & Kozak 1977).

Different functions have been attributed to the cells proliferating in AMLR (Vande Stouwe et al. 1977; Sakane & Green 1979; James et al. 1980). However, it is likely that this phenomenon reflects the ability of different immunocompetent cells to cooperate, although its exact in vivo equivalent has not been identified.

Impaired AMLR has been observed in neoplastic diseases (Weksler et al. 1981), autoimmune disorders (Weksler et al. 1981; James et al. 1980), atopy (Dirienzo et al. 1984), and in the elderly (Weksler et al. 1981).

Preliminary results obtained in our laboratory have demonstrated impaired AMLR in untreated Graves’ disease and Hashimoto’s thyroiditis (Canonica et al. 1984).

In the present work we have evaluated the AMLR in different groups of patients with Graves' disease, both untreated and treated with antithyroid drugs, surgery or radioiodine.

Materials and Methods

Patients

A total of 27 patients with Graves' disease (7 males, 20 females, mean age 45 (29–70) years) were studied. The diagnosis was established on the basis of usual clinical and laboratory parameters. Ten of the patients were hyperthyroid and untreated: 17 were in clinical remission after treatment with antithyroid drugs (ATD) (7), surgery (6) or radioiodine (4). ATDs had been discontinued from 4 to 2 years before this study; subtotal thyroidectomy performed from 5 years to 2 months before; 131I (1 or 2 doses, 80–100 μCi/g of thyroid tissue).
has been administered 3 to 9 years before. Clinical remission was defined using the following criteria: clinical euthyroidism, no evidence of relapse after treatment, normal circulating thyroid hormones and TSH, normal TSH response to TRH, no evidence of relapse in a 6–12 month follow-up period subsequent to this study. Five of the untreated patients had ophthalmopathy (class 1 to 3 according to A.T.A.) (Nos. 2, 4, 5, 6, 7). Mild 'inactive' ophthalmopathy was present also in 3 treated ones (Nos. 12, 14, 16). Clinical data are summarized in Table 1.

**Assay of thyroid hormones and TSH: evaluation of TSH response to TRH**

Serum T3, T4 and TSH were measured by radioimmunoassay using commercially available kits (Biodata, Rome, Italy). The normal values in our laboratory are 0.7–1.9 ng/ml for T3, 47–111 ng/ml for T4, 0.8–3.5 mU/l (WHO 68/38) for TSH. TSH response to TRH was evaluated after iv injection of 200 µg of TRH (Biodata, Rome, Italy) at 10, 20, 30, 45, 60, 90 min. An increase of 2 mU/l or more in blood TSH at 20–30 min is regarded as significant in our laboratory.

**Assay of thyroid autoantibodies**

Thyroid microsomal autoantibodies (TMA) were assayed by passive microhaemagglutination (Fujizoki, Tokyo, Japan). Antithyroglobulin autoantibodies (TGA) were measured by radioimmunooassay employing a commercially available kit (Biodata, Rome, Italy).

**Isolation and fractionation of mononuclear cells (MNC)**

Details of the methods employed have been reported elsewhere (Canonica et al. 1984). Briefly, MNC were isolated from heparinized blood samples by density gradient centrifugation. Following 1 h of incubation in an ice bath with neuroaminidase-treated sheep red blood cells, T-enriched and non-T-enriched cell fractions were separated by density gradients. Non-T fractions were recovered at the interphase: the pellet containing E-rosette forming cells (T fractions) was freed from erythrocytes by distilled water lysis. Both fractions were repeatedly washed with culture medium (RPMI 1640).

**Autologous mixed lymphocyte reaction**

AMLR was performed in triplicate using round-bottom

### Table 1.

Clinical data, thyroid function parameters, and autoantibody titres of the patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>Age</th>
<th>Therapy</th>
<th>T3</th>
<th>T4</th>
<th>TSH</th>
<th>TMA</th>
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<td>135</td>
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<tr>
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<td>1:20</td>
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<tr>
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</tr>
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microtitre plates (Sterilin), 1 x 10^6 T cells being mixed with 5 x 10^4 autologous non-T cells in a total volume of 0.2 ml. Separate control cultures were performed containing T or non-T cell fractions alone.

Culture medium was RPMI 1640 supplemented with t-glutamine, Gentamycin, 10 mM HEPES, and 10% foetal calf serum. Cultures were incubated for 7 days at 37°C in CO₂ in humidified air, and pulsed with 1 μCi of [³H] thymidine (Amersham Int., UK) for 18 h before harvesting using a Flow Titertek multiharvester. Radioactivity was counted in a Beckman automatic liquid scintillation counter.

T cell surface markers evaluation
The percentage of T cells, Fcγ-positive and Ia-positive (by means of D1/12 monoclonal antibody) was evaluated on T cells purified by subsequent density gradient centrifugations after adherent cell depletion, (purity 99%) strictly following previously described methods (Canonica et al. 1981, 1983a; Bagnasco et al. 1983).

Statistical analysis
The Mann-Whitney U-test was used.

Results
All the treated patients but one showed normal serum thyroid hormone and TSH levels, (as well as normal TSH response to exogenous TRH; data not shown). One patient (No. 20) had hypothyroidism subsequent to surgery and was undergoing

![Graph](https://via.placeholder.com/150)

**Fig. 1.**
AMLR results in patients and normal controls. Each point represents the mean [³H]TdR uptake value of triplicate cultures (coefficients of variation were constantly less than 18%).
Comparison between AMLRs of patients in clinical remission after ATD, surgery or radioiodine and of untreated patients.

I-thyroxine therapy (Table 1). The optimal culture period for AMLR was established on the basis of previous normal and pathological individuals: a 7-day culture was chosen. In some experiments, AMLR was performed using both non-irradiated and irradiated (3000 rads) non-T cells as stimulator: the entity of the response was unaffected by non-T cell irradiation (data not shown). Fig. 1 represents AMLR proliferation in untreated GD, clinical remission GD, and normal controls. A clear cut reduction of proliferative response is present in the first group ($P < 0.002$). On the other hand, $[^3H]$TdR incorporation does not significantly differ from normal subjects in the second group. The difference between untreated and cured patients is statistically significant ($P < 0.002$).

In order to exclude the possibility that AMLR reduction in untreated GD was due to thyroid hormone excess, we studied 4 patients with thyroid adenoma (whose serum total T$_4$ ranged from 132 to 261 µg/ml): AMLR response was normal (mean $[^3H]$TdR uptake ranging from 2550 to 7800 c.p.m.).

In the patients in clinical remission it was not possible to establish any clear correlation between AMLR, thyroid hormone levels, and other clinical and immunological (autoantibody titres) parameters.

Evaluation of T lymphocyte surface markers was performed in each patient: the number of T cells expressing Ia antigens was increased in most untreated GD patients (percentages on purified T
cells ranging from 8 to 32\%: mean + SEM 16 + 3) and constantly normal (1 to 9\%: mean + SEM 3.5 + 0.8) in cured patients. Moreover, T cells bearing Fcγ-receptors (TγC) were low in untreated (from 1 to 5\%: mean + SEM 2.5 + 0.7) and invariably normal in cured patients (from 9 to 15\%: mean + SEM 13 + 0.5) (Canonica et al. 1981, 1983a; Bagnasco et al. 1983). Within the group of cured patients there was no correlation between T subset percentages and AMLR.

On the other hand, a relationship was observed between AMLR and type of treatment: in fact, patients treated with ATD had reduced AMLR in comparison with patients treated with 131I or surgery. As compared with patients with active disease, the difference does not reach statistical significance for the ATD group, whereas it does for the other two groups (P < 0.02). Moreover, the difference between surgically and ATD-treated subjects was significant (P < 0.05) (see Fig. 2).

Discussion

A number of experimental data suggest that an abnormal regulation of immune response may play a crucial role in the pathogenesis of GD. Imbalances of T cell subsets have been found by several groups: specifically, as we previously described (Canonica et al. 1983a; Bagnasco et al. 1983), T lymphocytes expressing different ‘activation markers’ are increased during the active phase of the disease and T cells bearing Fcγ-receptors are reduced. Contrasting results have been obtained using the MAbs of the OKT series (Sridama et al. 1982; Iwatani et al. 1982). In some reports (Bonnyns et al. 1983; Canonica et al. 1983b), T cell subset imbalance has been observed also in MNC derived from thyroid tissue. In addition, function studies have demonstrated reduced ConA-induced suppression and defective suppressor activity in co-culture experiments (Aoki et al. 1979). A defect of the antigen-specific suppressor cells has been prospected on the basis of experiments concerning MIF production in response to thyroid antigen (Topliss et al. 1983). However, studies on mitogen-driven autoantibody production in vivo failed to demonstrate a major involvement of T suppressor cell defect (Beall et al. 1982; Mariotti et al. 1984). In this study we describe the presence in active GD of another functional defect involving T cells, namely AMLR reduction, which is in agreement with the recent data presented by Fournier et al. (1983) and our previous report (Canonica et al. 1984). A defect in AMLR may be due to alterations of both stimulator and responder cells, but the reported data do not render it possible to ascertain whether the former or the latter population is responsible. However, some findings may be consistent with responder T cell impairment: T cells bearing receptors for Fc fragment of IgG are markedly reduced in active GD (Bagnasco et al. 1983). In a previous work it was demonstrated that T cells able of proliferating in response to auto-logous stimulation are Fcγ-positive (Canonica et al. 1982). Moreover, they are not able to induce a marked proliferation. It is known that, in normal individuals, T cells bearing Ia antigens can stimulate other T lymphocyte subsets to proliferate (Mingari & Moretta 1982). Consequently, one might suppose that a stimulus other than non-T cells is present in patients with raised proportions of Ia-positive T cells.

It is unlikely, in our opinion, that thyroid hormone excess plays a role in the genesis of AMLR defect, although it is conceivable that thyroid hormones can somehow act on T cells (e.g. on subset distribution (Bonnyns et al. 1983). In fact, some patients with non-autoimmune hyperthyroidism proved to have normal AMLR in this study.

In addition to AMLR in untreated GD, we demonstrated that patients in clinical remission have a significantly increased AMLR. Using standard criteria for defining clinical remission, we selected 3 groups of patients with different therapy protocols. Autoantibody titres were similar in all groups. Moreover, no differences in T cell surface markers were observed: T cells were fully normal, and Ia-positive T lymphocyte percentages were constantly less than 7\% (as in normals) in all cured patients, whereas untreated patients had low T and high Ia-positive T cell proportions. Nevertheless, compared with the other two groups, AMLR was different in the ATD-treated group, in that proliferation was significantly reduced. Whatever the significance of different AMLR behaviour in patients treated according to different protocols, subjects with comparable T proportions have different AMLR responses. Evidence of discrepancies between phenotype and function is not unusual: the fact that the cells proliferating in AMLR bear Fcγ-receptor does not necessarily mean that all T
lymphocytes (whose heterogeneity is well known) are able to respond to autologous stimuli.

A possible explanation of the differences observed in cured GD might be researched in view of different effects on the immune system of ATD, surgery, and radiation. With regard to this point, two considerations should be made:

1) An ‘immunosuppressive’ effect of ATD has been described on the basis of in vitro experiments (McGregor et al. 1980). However, in the light of more recent studies (Wenzel & Lente 1984) the in vivo relevance of such an effect seems at least questionable.

2) Both surgery and radioiodine are ‘ablative’ treatments: there is no clear evidence of specific long-term effect on the immune system, and an important role of autoimmune mechanisms in the genesis of iatrogenic thyroid failure is unlikely (Doniach 1977).

Thus, it is possible that surgery or ¹³¹I therapy result in a more rapid normalization of AMLR. It is known that the thyroid gland is the primary site of immune organ-specific reactions in autoimmune thyroid disease (Canonica et al. 1983b). However, it remains to be explained how gland removal can lead to an important modification of AMLR of peripheral cells in a relatively short time. In this light, we think that it might be of interest to study GD patients cured with ATD many years previously. In conclusion, we have provided evidence of a functional T cell abnormality in GD, which can be reversed when clinical remission is achieved.

References


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