Cold reactive lymphocytotoxic activity in autoimmune thyroid disease

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Abstract. An increased incidence of cold-reactive lymphocytotoxic activity (LCTA) has been demonstrated in the sera of patients with autoimmune thyroid disease. Twenty-six of 79 (33%) patients with Graves' disease and 9 of 21 (43%) patients with Hashimoto's thyroiditis had cold-reactive LCTA detected by microcytotoxicity assay compared to 6 of 42 (14%) normal controls. There was no correlation between LCTA and age, sex, MCHA titre or TGHA titre. A positive correlation with FTI and LCTA in Hashimoto's patients was demonstrated, but no such correlation was demonstrable in Graves' patients. The lymphocytotoxic activity was directed preferentially against B cells. There was no preferential lysis of T-cell subsets as defined by monoclonal antibodies, and the lymphocytotoxins were equally reactive with normal lymphocytes and toxic Graves' lymphocytes. The significance of cold-reactive lymphocytotoxic activity in the pathogenesis of autoimmune thyroid disease remains to be determined.

Cold-reactive lymphocytotoxicity has been demonstrated in the sera of patients with autoimmune thyroid disease (Ozturk & Terasaki 1979), as well as in the sera of patients with other diseases including systemic lupus erythematosus (Ozturk & Terasaki 1979), multiple sclerosis, viral and parasitic infections (Ozturk & Terasaki 1979; Gilbreath et al. 1983), insulin-dependent diabetes mellitus (Serjeantson et al. 1981), mycosis fungoides (Schocket et al. 1982), and Hodgkin's disease (Ozturk & Terasaki 1979). These lymphocytotoxins have been most extensively investigated in systemic lupus erythematosus where they have been shown to be predominantly IgM antibodies and to be optimally reactive at 15°C (Winfield et al. 1975). Their specificity has been in dispute, and they have variously been reported to react with B and/or T lymphocytes, as well as T lymphocyte suppressor-cytotoxic subsets (Ozturk & Terasaki 1979; De Horatius et al. 1980; Williams et al. 1981; Koike et al. 1979). The antibodies have been demonstrated to be autotoxic. They are thought not to have HLA specificity, since they react with lymphocytes from a wide variety of donors (Ozturk & Terasaki 1979).

Less information concerning the nature, specificity, and function of these lymphocytotoxins is available in autoimmune thyroid disease. Since the lymphocytotoxins may function to modulate the immune response in patients with autoimmune disease, we have further characterized the cold-reactive lymphocytotoxic activity in the sera of patients with Hashimoto's thyroiditis and Graves' disease.

Materials and Methods

Patients

Seventy-nine patients with Graves' disease and 21 patients with Hashimoto's thyroiditis were studied. The patients were all outpatients seen in the Endocrinology Clinic at the University of Chicago Hospitals and Clinics. Their average age was 38 years (range 15 to 70 years). Diagnosis was based on standard clinical and/or laboratory criteria. Forty-two control subjects were selected from hospital personnel and included clinical, clerical, and laboratory staff lacking a history of significant medical illness and without known thyroid disease. The control subjects were of the same age and sex distribution as the patients.

Supported in part by United States Public Health Service Grants AM13377, AM27384, and AM07011 (Training).
Sera were obtained from patients and controls and immediately frozen at −20°C. Prior to the preparation of microtitre plates (Histoplate, Cooke Laboratory Products, Alexandria, VA), complement was inactivated by heating each serum at 56°C for 30 min. One µl of serum was then added to each microtitre well and the plates were stored at −70°C.

Lymphocytes

Lymphocytes were obtained from at least 5 members of a panel of 15 healthy donors. Lymphocytes were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) density gradient centrifugation. B lymphocytes and T lymphocytes were separated by the nylon wool column method (Handwerger & Schwartz 1974). Cell viability was determined by trypan blue dye exclusion. Background levels of cell death were usually <5% for the T lymphocyte fraction, and <8% for the B lymphocyte fraction.

Preparation of T cell subsets using monoclonal antibodies

T cell subsets were obtained by the negative immunoselection method of Reinherz et al. (1981). Briefly, un fractionated T cells were reacted with one of two hybridoma antibodies, termed anti-T₄ (OKT₄) and anti-T₈ (OKT₈) (both from Ortho Pharmaceutical Co., Raritan, NJ). The anti-T₄ reactive (OKT₄+) subset was previously shown to define the human helper/inducer T-cell subset, whereas the OKT₈+ subset contains cytotoxic/suppressor T cells. The monoclonal antibody-coated T cells were separated into adherent and non-adherent fractions based on binding to plastic dishes coated with affinity-purified goat antimouse immunoglobulin. The non-adherent fraction was used as the T₄-enriched or T₈-enriched cell population in the lymphocytotoxicity assay. Re-analysis by indirect immunofluorescence indicated that the populations were at least 80% pure.

Determination of lymphocytotoxic activity

The lymphocytotoxic activity of sera was measured using the microcytotoxicity method of Terasaki & McClelland (1964). One µl of the subject's serum was incubated with 3 × 10⁶ target cells (either B, T, or T cell subsets as described above) at 15°C for 30 min in a well of a microtitre plate. Five µl of diluted normal rabbit serum was added to each well, and the mixture was incubated for 3 h at 15°C. At the end of this incubation, 2 µl of eosin dye was added, followed, in 2 min, by 3 µl of formaldehyde. The viability of the target cells in each well was determined by eosin dye exclusion with phase contrast microscopy, and the per cent killed cells was recorded. Cytotoxicity for each serum was expressed as the mean per cent of lymphocytes killed in separate concurrent assays with 5 different normal donor lymphocytes. A serum was considered to demonstrate abnormal cytotoxicity when the mean per cent of lymphocytes killed was 15% or more. Reproducibility of the assay was determined by obtaining essentially identical results when the same serum was directed against the same targets at different times. Serum from a patient with Graves’ disease which consistently killed >50% of target B cells was used as the positive control in all experiments; a negative control of target cells and complement without serum was included in each assay.

Other determinations

Total thyroxine (TT₄), total triiodothyronine (TT₃), and TSH were measured by radioimmunoassay. Normal ranges were as follows: TT₄, 5.0–12.0 µg/dl; TT₃, 80–175 ng/ml; and TSH < 5.0 µU/ml. The free thyroxine index (FTI) was calculated from the TT₄ and the resin T₄ uptake ratio as previously described (Robin et al. 1971); normal range 6.0–10.5. Antimicrosomal and antithyro globulin antibodies were measured by the tanned red cell haemagglutination technique.

Statistics

Chi-square tests for proportions were done when appropriate. Linear correlations were calculated by the least squares method.

Results

Incidence of lymphocytotoxic activity

Twenty-six of 79 (33%) patients with Graves’ disease and 9 of 21 (43%) patients with Hashimoto’s thyroiditis had cold-reactive lymphocytotoxic activity (LCTA) as detected by the microcytotoxicity assay (Table 1). These incidences were significantly higher than the control group (P < 0.05). Six of 42 (14%) normal subjects demonstrated lymphocytotoxic activity. When the Graves’ disease patients were separated into euthyroid treated and toxic

<table>
<thead>
<tr>
<th>Table 1. Incidence of lymphocytotoxic activity (LCTA)</th>
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<tr>
<td>No. of subjects</td>
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<td>Graves' disease (total)</td>
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*P < 0.05 compared to normal controls.
untreated groups, there was no significant difference in the incidence of lymphocytotoxic activity (Table 1). Twenty-one of 62 (34%) euthyroid treated Graves’ disease patients had positive cytotoxic reactions compared to 5 of 17 (29%) untreated hyperthyroid patients. Furthermore, when the group of treated Graves’ patients was subdivided by type of treatment (RAI, surgery, propylthiouracil), there was no significant difference detected in incidence of lymphocytotoxic activity between the groups: 11 or 30 euthyroid patients previously treated with RAI, 5 of 19 euthyroid patients previously treated with PTU, and 5 of 13 euthyroid patients who had undergone subtotal thyroidectomy for Graves’ disease were found to have serum lymphocytotoxic activity.

**Correlation of cytotoxicity with clinical parameters**

A significant correlation ($P < 0.001$) between lymphocytotoxicity and serum FTI levels was found in patients with Hashimoto’s thyroiditis (Fig. 1). There was no significant correlation between lymphocytotoxicity and FTI levels in Graves’ patients, however, nor were there significant correlations between lymphocytotoxicity and age, MCHA titre or TGHA titre in either group. The female to male ratio was 4:1 in the patients, and this female preponderance was unchanged in the LCTA positive and LCTA negative group.

**Cellular specificity of lymphocytotoxins**

As can be seen in Table 2 and Fig. 2, lymphocytotoxic activity was preferentially directed against B cells. Of the 26 lymphocytotoxic Graves’ sera, 18 (69%) were reactive with B cells alone, 7 (27%) were reactive with both B and T cells, and one was reactive with T cells alone. Similarly, in the 9 lymphocytotoxic Hashimoto’s sera, 5 (56%) were...

**Table 1.** Percentage B cell lysis is plotted against the free thyroxine index. Normal range for free thyroxine index is indicated by shaded area.

**Table 2.** Cell types against which lymphocytotoxic sera are directed in autoimmune thyroid disease.
Fig. 2.

Lymphocytotoxicity assay performed with sera from normal controls (N), patients with Graves’ disease (G), or patients with Hashimoto’s thyroiditis (H). Target cells obtained from normal donors were (a) total peripheral blood lymphocytes, (b) B lymphocyte, or (c) T lymphocytes. Figures in parentheses denote number of sera assayed in each category. Sera with mean cytotoxicity < 15% (as indicated by ———) are considered positive for lymphocytotoxic activity.

reactive with B cells alone, 4 (44%) were reactive with both B and T cells, and none were reactive with T cells alone. Both suppressor-enriched and helper-enriched T cell subsets were targets of lymphocytotoxic activity in sera with T-directed lymphocytotoxicity (Fig. 3), and there was no preferential lysis of a particular T cell subset.

Five selected sera which demonstrated lymphocytotoxicity with normal control peripheral blood lymphocytes (PBL) were tested against PBL from 3 patients with untreated Graves’ disease. There was no difference in mean percentage of lymphocytotoxicity depending upon the source of the lymphocytes.

Discussion

Cold-reactive lymphocytotoxicity was first described in patients with infectious mononucleosis, rubella, and measles (Mottironi & Terasaki 1970). Cold-reactive lymphocytotoxicity has subsequently been found in patients with systemic lupus erythematosus, multiple sclerosis, insulin dependent diabetes, rheumatoid arthritis, inflammatory bowel diseases, and malignancies, particularly those with a possible viral etiology such as Hodgkin’s disease, cervical carcinoma, and nasopharyngeal carcinoma (Ozturk & Terasaki 1979; Lamelin et al. 1977; Vos et al. 1975). Cold-reactive lymphocytotoxins have
also been detected in up to 16% of normal control subjects, and their incidence appears to increase with advancing age (Dawkins et al. 1978).

Although most investigation has been done in patients with SLE, these lymphocytotoxins have also been described in patients with autoimmune thyroid disease. Naito et al. (1971) found an incidence of lymphocytotoxic activity of 62% in 26 patients with Graves’ disease and 50% in 8 patients with Hashimoto’s thyroiditis. Eguchi et al. (1982) and Pruzanski et al. (1984) found similar incidences in patients with Hashimoto’s thyroiditis (51 and 48%, respectively). Pruzanski et al. (1984) found a somewhat lower incidence of 25% in Graves’ disease patients, and Eguchi et al. (1982) found a difference in incidence of LCTA in untreated and treated Graves’ patients (54 and 16%, respectively). Our incidences are similar to those of previous investigators: 33% of our Graves’ patients and 43% of our Hashimoto’s patients had LCTA. Similar to previous studies, 14% of our normal controls had cold-reactive lymphocytotoxic activity. Unlike Eguchi et al. (1982), however, we could detect no significant difference in incidence of LCTA between treated and untreated Graves’ patients.

As in SLE, the cell target of lymphocytotoxic activity in autoimmune thyroid disease is unclear. Ozturk & Terasaki (1979) and Pruzanski et al. (1984) have found these lymphocytotoxins to be predominantly directed against B cells in both Graves’ disease and Hashimoto’s thyroiditis patients. Eguchi et al. (1982), in contrast, find LCTA to be predominantly directed against the suppressor cell (OKT\(_8^+\)) subset of T lymphocytes. Our data support the findings of Ozturk & Terasaki (1979) and Pruzanski et al. (1984) that B cells were the predominant, although not the exclusive, target cells in all groups studied including the normal controls with demonstrable LCTA. Additionally, preferential lysis of T cell subsets, either helper-enriched or suppressor enriched populations (as defined by OKT\(_4^+\) negative and OKT\(_8^+\) negative populations), was not detected.

We could demonstrate no correlation of LCTA with age, sex, TGHA, or MCHA titres. Interestingly, there was a significant correlation between FTI and LCTA in Hashimoto’s patients, but no correlation was demonstrable in the Graves’ group. All our patients with Hashimoto’s thyroiditis were on replacement thyroxine therapy. If the metabolic status of the patient affects immunoregulation and expression of lymphocytotoxic activity, as suggested by this correlation, a similar result should be seen in hyperthyroid Graves’ patients. In actuality, no such correlation between FTI and LCTA is present in these patients, and therefore, it cannot be concluded that the metabolic status per se is correlated with lymphocytotoxic activity.

Pruzanski et al. (1984) have demonstrated that length of disease is correlated with the presence or absence of lymphocytotoxic activity in Graves’ patients. They found a mean duration of disease of 15 months in LCTA positive Graves’ patients and 55 months in LCTA negative patients. We were unable to detect a difference in incidence of LCTA between patients with a short duration of disease (newly diagnosed untreated Graves’ patients) and those who had undergone therapy and were essentially euthyroid at the time of the investigation. Interestingly, LCTA has similarly been associated with duration of disease in insulin-dependent diabetes mellitus. With increasing duration of disease,
the lymphocytotoxic activity diminishes in patients with insulin-dependent diabetes mellitus (Serjeantson et al. 1981).

The lymphocytes from patients with toxic untreated Graves' disease were as susceptible to lysis by LCTA positive sera as were normal control lymphocytes. A study of patients with inflammatory bowel disease demonstrated reduced cytotoxicity of patient's serum for allogeneic lymphocytes, if the target lymphocytes were from patients with active inflammatory bowel disease (Brown & Jewell 1982). Lymphocytes from patients in remission were as susceptible to lysis as normal donor lymphocytes. The exact mechanism whereby the lymphocytes from patients with active Crohn's disease were protected from the effects of homologous lymphocytotoxins is unknown. However, our data do not demonstrate such a protective effect in Graves' disease patients.

The potential significance of these lymphocytotoxins in autoimmune thyroid disease is still undetermined. Certainly their reactivity with large numbers of peripheral B cells in vitro would suggest that their effect is non-specific; however, it is possible that the in vivo effect of these lymphocytotoxins is specific. Clearly cell lysis is not an in vivo effect, since it is a cold temperature dependent phenomenon, and has not been demonstrated to occur at physiologic temperatures. Effects of LCTA on lymphocyte function at 37°C have been demonstrated; inhibition of the mixed lymphocyte culture reaction at 37°C by cold-reactive cytotoxic sera has suggested a role for LCTA in the modulation of the immune response in vivo (Matsumoto et al. 1983).

In conclusion, there was an increased incidence of cold reactive lymphocytotoxic activity in patients with Graves' disease and Hashimoto's thyroiditis. The lymphocytotoxic activity was directed preferentially against B cells; a possible correlation with FTI and LCTA in Hashimoto's patients was demonstrated but no such correlation was demonstrable in Graves' patients. The significance of these lymphocytotoxins remains unclear; they may represent physiologically unimportant byproducts of an immune response, or they may play a significant role in immunoregulation. Additional studies are indicated to further define the in vivo specificity and functional significance of these cold-reactive lymphocytotoxins.

References


Received on February 20th, 1985.