Bioassay determinations of thymopoietin and thymic hormone levels in human plasma

(Thy 1.2 antigen induction/ubiquitin)

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ABSTRACT Thymopoietin is a thymic hormone that induces differentiation of thymocytes from precursor cells which arise in hemopoietic tissues. This paper describes a sensitive in vitro assay for the induction of Thy 1.2 antigen on null lymphocytes from germ-free athymic (nu/nu) mice. The sensitivity and specificity of the bioassay were increased by adding high concentrations of ubiquitin (a nonspecific inducer) to the induction incubations. The bioassay was sufficiently sensitive to detect thymopoietin at <0.25 ng/ml. A dose-response relationship was shown between thymopoietin concentration and the percentage of cells induced to express Thy 1.2 antigen. When normal human plasma was assayed, induction was registered with activity corresponding to thymopoietin at >1 ng/ml in plasma from infants or young adults. Activities in the thymopoietin range of 0.25 ng/ml were registered with plasma from healthy subjects over 50 years of age. Thymectomy was followed by loss of this inductive activity from the plasma. This bioassay permits clinical studies on T (thymus-derived) cell inducers released by the human thymus into the circulation.

The function of the thymus is to promote differentiation of lymphocytes (T cells) that participate in effecting and regulating immune responses. Abnormalities of the thymus have been detected with certain human diseases such as myasthenia gravis (1), systemic lupus erythematosus (2), and immune deficiency states (3–5). Thymic dysfunction may be implicated in a broader range of human disorders and also with aging (1). The lack of sensitive and precise tests for thymic function has hampered attempts to clarify the clinical significance of this immunologic organ.

Thymopoietin (molecular weight 5562) is a polypeptide hormone of defined amino acid sequence (6) that induces differentiation of precursor lymphoid cells to thymocytes (7) and also has secondary effects upon neuromuscular transmission (8). Thymopoietin is secreted by epithelial cells of the thymus (9). Lymphocytes that differentiate under the influence of thymopoietin subsequently give rise to distinct subclasses of T cells with potential to develop discrete effector, helper, or suppressor functions after encounter with antigen (10). The relationship of thymopoietin to the later stages of the functional differentiation of T cells remains to be elucidated.

Thymopoietin can be measured in vitro by its ability to differentiate precursor cells from bone marrow or spleen to cells that express characteristic surface antigens of T cells (7, 11, 12). Induction of T cell differentiation by thymopoietin is accomplished by the generation of cyclic AMP (13), which accounts for the ability of other agents that elevate intracellular cyclic AMP to induce T cell differentiation in a relatively nonspecific fashion (14). Of these other agents, ubiquitin (molecular weight 8451), whose amino acid sequence has been determined (15), is noteworthy because of its presence in all body tissues that have been tested (16). Although present in high concentrations in the thymus, ubiquitin is not a thymic hormone.

Induction by thymopoietin and ubiquitin can be distinguished as follows. (a) Thymopoietin induces selective T cell differentiation in vitro, whereas ubiquitin induces both T and B (thymus-independent) cell differentiation (16). (b) Ubiquitin, at high concentrations (>100 μg/ml), does not cause induction (17). (c) The β-adrenergic inhibitor propranolol ablates induction by ubiquitin but does not affect induction by thymopoietin (16). This suggests that ubiquitin, but not thymopoietin, acts via adrenergic receptors. (d) High concentrations of ubiquitin do not inhibit induction with thymopoietin (17); this may be due to null lymphocyte membranes having distinct receptor sites for thymopoietin and ubiquitin.

We have utilized these differences in induction with thymopoietin and ubiquitin to develop a sensitive bioassay for thymopoietin. This test provides the potential to measure T cell induction activity released from the thymus into the circulation. This biologic activity requires the presence of thymic tissue and declines with age.

MATERIAL AND METHODS

Substances Tested. Thymopoietin II and ubiquitin were isolated as described previously (8, 16). Mouse serum was obtained from 12- to 24-week-old nu/nu mice and 12- to 18-week-old thymus-intact C3H/He mice. Human plasma from heparinized blood was tested after filtration through PM30 membranes (Amicon Corp., Lexington, Mass.), which have a nominal retention of 30,000 molecular weight; >95% of the plasma volume was recovered. Human plasma was obtained from cord blood, from infants 2–4 months of age, and from healthy adults in the third and sixth age decades. Plasma was also obtained from five patients with myasthenia before and 1 week after thymectomy.

Induction Assay. Spleen cell suspensions were prepared from athymic (nu/nu) mice outbred on a C3H/He background, whose thymocytes bear Thy 1.2 antigen. The nu/nu mice were maintained in a germ-free colony. The spleen cell suspensions were incubated for 45 min in equilibrated nylon wool columns (18). The 4–7% of these cells that were eluted from the columns demonstrated excellent viability by trypan blue exclusion. Induction incubations included 0.5 × 10^6 indicator cells suspended in 0.1 ml of medium 199 that contained bovine serum albumin at 50 mg/ml plus 0.1 ml of (a) mouse serum, (b) filtered human plasma, or (c) thymopoietin or ubiquitin suspended in medium. Induction incubations were for 18 hr at 37°C, at the end of which cells were washed with 0.87% ammonium chloride and then with medium. Slightly lower and more
variable values were recorded when induction was terminated after 2 hr of incubation. Display of Thy 1.2 antigen on indicator cells was detected using an enzymatic cytotoxicity test (19, 20), which detects lesser degrees of cell injury than dye exclusion or the release of membrane-bound isotope. To $0.25 \times 10^{6}$ cells in 0.05 ml of medium was added 0.025 ml of antiserum to Thy 1.2 antigen (AKR anti-C3H thymocytes), 0.025 ml of guinea pig complement (Gibco, Grand Island, NY), and 0.025 ml of normal saline. Optimal dilutions of antiserum and complement, which had been adsorbed with mouse liver powder, were determined from prior testing on healthy C3H thymocytes. Paired incubations were routinely performed with complement and heat-inactivated complement as a control. After incubating for 1 hr at 37°C, 0.125 ml of protease in physiologic saline (5 mg/ml, Pronase Grade B, Calbiochem, San Diego, CA) was added to each tube. Hemocytometer cell counts were performed after an additional 30 min of incubation and the induction index was calculated according to the formula $[(a - b)/a] \times 100$, where $a =$ cell count with heat-inactivated complement and $b =$ cell count in the presence of complement and the same inducing agent. Incubated cells were kept on wet ice until cell counts were performed. The specificity of this cytotoxicity test was established with C3H/He (Thy 1.2+) and AKR (Thy 1.2−) thymocytes: Antiserum to Thy 1.2 antigen plus complement injured $>90\%$ thymus lymphocytes from the Thy 1.2+ mice and $<2\%$ thymus lymphocytes from Thy 1.2− mice as measured by the protease test.

RESULTS

Induction by Thymopoietin and Ubiquitin. Fig. 1 shows the percentages of indicator null cells induced to express Thy 1.2 by various concentrations of these substances. Thymopoietin induced appreciable T cell differentiation at $<1$ ng/ml; maximal induction occurred at $1 \mu g/ml$, which was the highest concentration of thymopoietin tested. Ubiquitin induced null cells to express Thy 1.2 at 0.01 ng/ml, induced maximally at 1 ng/ml, but induced little Thy 1.2 antigen at 100 ng/ml.

Induction by Thymopoietin in the Presence of High Ubiquitin Concentrations. Induction by 0.1 to 1.0 ng thymopoietin/0.2 ml was measured in incubations that also included 125 ng of ubiquitin. Induction by 125 ng of ubiquitin without thymopoietin (mean ± SEM, 3.9 ± 0.6% Thy 1.2+ cells) was subtracted in each experiment. In each experiment, correction was made for variations in the percent of inducible cells in individual indicator cell preparations by expressing the data as percentages of optimal induction that was achieved with 1 ng thymopoietin plus 125 ng ubiquitin/0.2-ml incubation. As shown in Fig. 2, an excellent relationship was demonstrated between thymopoietin concentration added to the incubations and Thy 1.2 induction (correlation coefficient >0.9). Appreciable induction was obtained with as little as 0.2 ng thymopoietin/0.2 ml.

Induction by Plasma and Serum. T cell differentiation was induced by serum from thymus-intact C3H mice but not by serum from nu/nu athymic mice (Table 1). Plasma from human infants induced brisk T cell differentiation. Although the molecular size of thymopoietin is sufficiently small to permit diffusion across the placental barrier, comparable Thy 1.2+ induction was recorded with plasma from cord blood and with plasma from 2- to 4-month-old infants. Plasma from young adults also induced brisk, but somewhat lower, levels of Thy 1.2 induction, while plasma from subjects aged 50-55 years, when used alone, showed little inducing activity.

Induction by Human Plasma in the Presence of High Ubiquitin Concentrations. The high levels of induction obtained with neonatal or young adult plasmas without added ubiquitin were not significantly enhanced by adding 125 ng ubiquitin to 0.2 ml incubations (Table 1). However, induction by plasma from 50- to 55-year-old subjects rose from negligible to significant levels when 125 ng ubiquitin was added to the incubations ($P < 0.01$, Student t test). Likewise, induction with 0.5 ng thymopoietin (which is a suboptimal concentration) rose significantly from 12.2 ± 2.0% to 21.6 ± 1.4% Thy 1.2+ cells when 125 ng ubiquitin was added to the induction incubations ($P < 0.01$).

The percentages of Thy 1.2+ cells induced after 18 hr of incubation with serial dilutions of plasma from a healthy 27-year-old male, without and with the added presence of 125 ng ubiquitin, are shown on Fig. 3. The dose–response curve demonstrated a satisfactory relationship between plasma dilution and Thy 1.2+ induction. The sensitivity of the assay for the more dilute plasma preparations was greatly increased by adding 125 ng ubiquitin per 0.2 ml induction incubation.
Loss of Plasma Inductive Activity after Thymectomy. Plasma obtained from five patients with myasthenia gravis was tested before and 1 week after thymectomy. The prethymectomy specimens from these patients caused regular Thy 1.2 induction. Indeed, the concentrations observed were significantly higher than expected for individuals in their age range (Table 2). Plasma collected 1 week following thymectomy induced little Thy 1.2+ on indicator cells in any of the five thymectomized patients.

Reproducibility of the Assay. Indicator cells tested immediately after preparation or after 18 hr of incubation with nu/nu mouse serum always contained ≤% Thy 1.2+ cells. In triplicate assays with potent induction using 1 μg thymopoietin/0.2 ml, variation between replicate assays was ≤4%.

Indicator Cells. About 80–90% of cells in indicator preparations were morphologically mature lymphocytes by light and electron microscopic examination (21). Less than half of these lymphocytes could be induced to express detectable Thy 1.2 under optimal conditions. At most, 7% bore membrane Ig by immunofluorescence test or had complement receptors.

DISCUSSION

The bioassay for T cell differentiation used in this study represents a modification of the Komuro-Boyse system (11). The modification employed herein greatly increases the sensitivity of their test. Our procedure proved capable of registering T cell induction with <1.0 ng/ml of thymopoietin or 0.01 ng/ml of ubiquitin. It was also possible to detect T cell-inducing activity in plasma or serum, which suggests that this bioassay is suitable for clinical studies. The use of filtered plasma excluded antibodies that might interact with indicator cells and interfere with the reaction (22).

On a weight by volume basis, ubiquitin is more active in inducing precursors to T cells than is thymopoietin. Because ubiquitin is not unique to thymic tissue, it is possible that biologically active concentrations of this peptide could be released from nonthymic tissues into the circulation with tissue injury or with other disease states. This would introduce an element of uncertainty regarding the source of inductive activity when human plasma is bioassayed. The present bioassay distinguishes induction due to ubiquitin from induction due to thymopoietin by utilizing a crucial dose–response relationship of ubiquitin in the induction assay (17). At concentrations at or above 100 μg/ml, ubiquitin by itself causes minimal induction. These self-inhibitory concentrations of ubiquitin do not interfere with induction by thymopoietin.

There is a second advantage to including high concentrations of ubiquitin when testing for Thy 1.2 antigen induction with low concentrations of thymopoietin. The assay for thymopoietin is considerably more sensitive in the added presence of ubiquitin at 625 μg/ml. The sensitivity of the test for diluted plasma from healthy young adults or undiluted plasma from subjects in the sixth decade is appreciably increased by the added presence of high concentrations of ubiquitin.

The mechanisms whereby ubiquitin enhances sensitivity to induction of Thy 1.2 antigen by thymopoietin is not known, but may be related to interactions between ubiquitin and adren-

Table 2. Induction of Thy 1.2+ cells with plasma from patients with myasthenia gravis obtained before and 1 week after thymectomy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, years</th>
<th>% Thy 1.2+ cells induced Before thymectomy</th>
<th>% Thy 1.2+ cells induced After thymectomy</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>58</td>
<td>32</td>
<td>1</td>
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</tr>
<tr>
<td>5</td>
<td>59</td>
<td>16</td>
<td>0</td>
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</tbody>
</table>

Undiluted plasma was tested in the presence of ubiquitin at 125 μg per 0.2-ml incubation mixture.

FIG. 3. Thy 1.2 antigen induction by serial dilutions of normal human plasma without (○) and with (■) the added presence of 125 μg of ubiquitin per 0.2-ml incubation mixture.
ergic receptors. Pharmacologic studies suggest that interaction with β-adrenergic receptors is necessary for the induction of T cell differentiation by ubiquitin (16). Phenotamine, which blocks α-adrenergic receptors, enhances T cell induction with thymopoietin (M. P. Scheid, G. Goldstein, and E. A. Boyse, unpublished observation). Perhaps ubiquitin, in high concentrations, interacts with both α- and β-adrenergic receptors, and thereby increases the inductive sensitivity of null cells to agents that act independently of adrenergic receptors while, at the same time, inhibiting induction mediated via β-adrenergic receptors.

The present study confirms that normal serum or plasma can induce T cell antigens on xenogeneic null cells. The fact that serum or plasma from athymic nu/nu mice or from thymectomized patients was inactive in our assay is forceful evidence that the activity being measured in the circulation is, in fact, thymic hormone. The active material has a molecular weight of <30,000, as do both thymopoietin (6) and ubiquitin (15). These findings are in keeping with earlier studies based on the neuromuscular effects of thymopoietin, which established that thymopoietin is a thymic hormone (20). The present study does not exclude the possibility that the thymus also releases into the circulation other substances that are capable of or are involved normally in T cell induction.

A direct relationship was demonstrated between Thy 1.2 induction and thymopoietin concentrations with 125 μg ubiquitin in 0.2 ml incubations. A similar dose–response relationship was observed with dilutions of human plasma. Thus, it is possible in our test system to express β-adrenergic-receptor-independent Thy 1.2 induction by plasma as being comparable to that of a known amount of thymopoietin. This activity exceeds the activity of 1 ng thymopoietin/ml in human infant and young adult plasma and declines to that comparable to 0.25 ng/ml by the sixth age decade. The fact that T cell induction is detectable through the sixth decade may be related to the fact that epithelial cell remnants persist even in severely invovled thymus tissue (1).

Bach et al. (24) have described a bioassay for T cell induction by human serum that is based upon increasing the percentage of mouse bone marrow lymphocytes that form azathioprine-sensitive rosettes. This bioassay has proved technically difficult and usually fails to detect induction after the fourth decade. The present bioassay appears to have a higher level of sensitivity. Recently, Astaldi et al. (25) reported an indirect bioassay that is based upon the stimulation of cyclic AMP synthesis by incubated thymocytes (13). The relative specificity and sensitivity of this assay remain to be determined.

It is apparent that the thymus releases into the circulation biologically active molecules that induce T cell differentiation. This suggests that thymopoietin (and perhaps other thymic products) may have peripheral endocrine function in addition to intrathymic actions on thymocyte differentiation. Biologically active products of the thymus may serve an important role in maintaining the T cell series throughout life as well as orchestrating T cell differentiation during immunologic maturation. This possibility is supported by the decline of T cell function with age (26) and following adult thymectomy (27, 28). Measurement of plasma thymic hormone activity may serve as a useful test with which to monitor age-related involution of the immune system and for assessment of T cell deficiencies in immunodeficient patients.

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