A hormonally controlled serum factor stimulating the thymidine uptake into lectin-activated lymphocytes

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Abstract. The in vitro stimulation of [3H]thymidine uptake into lectin-activated lymphocytes in the presence of various sera was studied. The mean precision of the assay is 5%, and the study of the confidence intervals shows variations from 4% to 12%. Compared to a normal reference serum (fixed as 1 U/ml), the serum thymidine uptake stimulating activity (mean ± sem) was 1.04 ± 0.07 U/ml in normal adult males, 2.63 ± 0.48 U/ml in acromegalic patients, 1.51 ± 0.13 U/ml in constitutional dwarfism and 0.37 ± 0.04 U/ml in untreated hypopituitarism with a significant difference between the groups (P < 0.001). In patients with hypopituitarism a single im hGH dose (6 mg/m²) increased the thymidine uptake stimulating activity of serum within 24 to 48 h following injection. The effects of directly adding hGH, insulin and T₃ to the assay, have been studied: pharmacological concentrations are required to produce only a slight effect. Physiological concentration of a purified preparation of somatomedin A stimulated thymidine uptake and its effect is increased in the presence of serum. These data demonstrate that [3H]thymidine uptake into lectin-activated lymphocytes is stimulated by a GH-dependent serum factor. The data suggest that this method should be proposed for an accurate and sensitive biological evaluation of serum thymidine uptake stimulating activity.

Daughaday & Reeder (1966) were the first to observe that the stimulation by growth hormone of thymidine uptake into DNA of cartilage from hypophysectomized rats could be mediated by a serum thymidine factor which appeared similar to the sulphation factor described in 1957 (Salmon & Daughaday 1957). Thus the terms of sulphation and thymidine factor have been replaced by the general term of somatomedin activity (Daughaday et al. 1972). Since then all current bioassays for determining levels of somatomedin activity are based upon the stimulatory effect on the incorporation of [35S]sulphate into chondroitin and/or [3H]thymidine into the DNA of cartilage cells. They differ mainly by the origin of the tissue used and by the incubation schedule, but are all tedious, not very sensitive, and require important quantities of serum (Salmon & Daughaday 1957; Almqvist 1960; Hall 1970; Salmon & Du Vall 1970; Alford et al. 1972; Raben et al. 1972; Salmon 1977; Schimpff & Donnadieu 1973; Philips et al. 1974; Van den Brande & Du Caju 1974). To avoid these drawbacks, more specific radioimmunoassay (Furlanetto et al. 1977; Hall et al. 1979), radioreceptor assay (Hall et al. 1974) and binding protein assay (Zapf et al. 1975) of the somatomedins currently identified have recently been developed. However the effects of the somatomedin polypeptides on the biological system seem to represent only a part of the total serum somatomedin bioassayable activity, defined by Daughaday et al. (1972) as GH-dependent plasma factor(s) stimulating both incorporation of sulphate into the chondroitin sulphate, incorporation of thymidine into DNA, of proline into the

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hydroxyproline of collagen and of uridine into RNA. Thus the specific radioligand assays of the somatomedin peptides may not be sufficient to measure the total somatomedin activity of serum. Biological determinations, both as sulphation and as thymidine factor, in parallel with the radioligand methods, remain of interest.

We are reporting here the stimulation in vitro of thymidine uptake into lectin-activated lymphocytes by a hormonally controlled serum factor. This technique should be proposed for an accurate and sensitive evaluation of serum thymidine uptake stimulating activity (thymidine activity).

Material and Methods

Lymphocytes were purified using the Ficoll Telebrix technique (Sharif et al. 1977) from venous blood from normal male adults. Cells were then suspended in microtiter plates at a concentration of 2 × 10⁴ cells/200 μl of nutritive medium (RPMI 1640, Flow, Paris, France) containing phytohaemagglutinin (20 μg/ml), gentialine (0.2 mg/ml) and sera or different hormones at various concentrations (Fig. 1). After 48 h of incubation at 37°C, in a 5% CO₂ humidified atmosphere, 0.1 μCi of thymidine was added and the incubation time was prolonged for 18 h.

Cells were then rapidly harvested and rinsed in an automatic apparatus Mash II (Microbiological Associates, USA) which aspirates the content of microtiter plates, harvests lymphocytes on glassfiber membranes, and washes it three times. The glassfiber membranes supporting lymphocytes are dried, then put in 3 ml of scintillation liquid (Packard Filter Count, Packard Cy). Tritium radioactivity is counted in a spectrometer (Inter-technique, France). In routine assay, the [³H]thymidine uptake of the cells incubated with sera or hormones is expressed as the ratio of their radioactivity to the mean radioactivity of cells incubated without serum.

Reference serum is a pool of sera from 8 normal male adults. It has been studied at 8 concentrations from 0.1 to 20% in preliminary studies, which demonstrated that the relation dose/response was optimal between 0.5 and 5%. Thus routine assays were done using, for each serum, four concentrations from 0.5 to 5%.

Hormones added in vitro

Human growth hormone (Nanormon 4 IU = 1.8 mg – Nordisk Insulinlaboratorium, Copenhagen) was added at final concentration of 0.1 to 500 ng/ml (4.5 × 10⁻¹² to 2 × 10⁻⁸ M). Bovine insulin (Sigma 24 IU per mg) was added at final concentration of 0.25 μU/ml to 625 μU/ml (1.5 × 10⁻¹² to 4 × 10⁻⁹ M). L-triiodothyronine (ICN Pharmaceuticals) was added from 1 to 5000 ng/ml (1.25 × 10⁻¹² to 8 × 10⁻⁹ M). Finally, a partially purified preparation of somatomedin A (batch SPE 282-2 G-50-2) kindly supplied by Dr. L. Frykland (AB Kabi, Stockholm) was studied at final concentration of 10 and 100 ng/ml.

Patients

Seventeen children (8 males and 9 females) with idiopathic hypopituitary dwarfism had age ranging from 5 to 11 years and marked growth retardation of −2 SD to −4 SD with biological evidence of impaired secretion of all hormones from the anterior pituitary. The diagnosis of GH deficiency was established by the failure of plasma GH to respond to at least two stimulation tests (insulin-induced hypoglycaemia and arginine loading test). Somatomedin activity measured as sulphation factor by a previously described technique (Schimpff & Donnadieu 1973) remained in them below or equal to 0.5 U/ml. Eight of these patients were studied before and 24–48 h after a single injection of human Growth Hormone, 6 mg/m² (France-Hypophyse, Paris, France; 2 IU = 1 mg).

Three acromegalic male patients, 30, 43 and 45 years old, were studied. Assessment of clinical activity was based on the whole of historical, laboratory and X-ray evidence and on high levels of hGH (> 40 ng/ml) and sulphation factor (> 2 U/ml).

Seventeen children (12 males and 5 females) with constitutional dwarfism, age ranging from 6 to 12 years, were studied. The subjects showed growth delay (−2 SD to 13 SD) with normal endocrine and metabolic functions.

Nineteen normal male adults, age ranging from 20 to 45 years, regular blood donors, were compared to the reference serum.

Blood samples were collected from a peripheral vein after an overnight fast and 1 h of rest.

Somatomedin bioassay is measured as sulphation factor by a previously described technique (Schimpff & Donnadieu 1973).

Expression of results and statistical evaluation of assay

For the evaluation of thymidine activity, the [³H]thymidine uptake (absolute or relative) was plotted against the square root of the concentration of sera (Fig. 3). The dose response curve is a straight line, the slope of which depends upon the thymidine activity. Finney’s g test was used for comparison of different experimental curves, and the activity of unknown samples was calculated using Burn’s slope ratio assay (Burn et al. 1950), the reference serum being arbitrarily considered as activity of 1 U/ml. Statistical analysis was performed using Student’s t-test (Snedecor & Cochran 1967) for comparison of different groups, either on arithmetical or on logarithmic scale. The reproducibility was studied on three successive evaluations of thymidine activity performed on four sera, on different days spread over a period of 5 weeks.
Results

Effect of reference human normal serum and/or different hormones on thymidine uptake by lectin-activated lymphocytes

Human growth hormone, insulin and T3, added in vitro, even at pharmacological concentrations, only slightly stimulated the thymidine uptake in lymphocytes. The sensitivity of the system to the addition of normal serum is higher since 1 μl of serum is sufficient to significantly stimulate the thymidine uptake (Fig. 1). SMA added at the dose of 10 and 100 ng/ml in the medium provides significant stimulation of thymidine uptake which varies in relations to the dose of added SMA. This effect is increased in the presence of 2.5% of serum (Fig. 2).

Reference curve and assay

An example of thymidine activity assay is plotted in Fig. 3.

The mean precision is 5% and the study of the confidence intervals shows variations from 4% (high levels) to 12% (low levels). Variance analysis shows that interassay reproducibility is 0.06 U/ml (95% confidence intervals).

Thymidine activity in sera from human patients (normal and pathological groups)

Results are summarized in Table 1. Compared with the group of normal adults, serum activity was significantly increased in acromegalic patients ($t = 6.784, P < 0.001$) and in the group of children with constitutional growth delay ($t = 3.572, P < 0.001$). Thymidine activity was significantly decreased in pituitary dwarfs ($t = 7.020, P < 0.001$). Moreover, the thymidine activity was significantly less in hypopituitary dwarfs than in constitutional dwarfs of the same age ($t = 8.273, P < 0.001$).

Effect of hGH treatment on serum thymidine activity

Before hGH administration the mean ± SEM thymidine activity level in eight pituitary dwarfs was 0.39 ± 0.058 U/ml. In the 48 h following hGH injection, the mean ± SEM peak level rose to 1.58 ± 0.690 U/ml ($t = 3.207, P < 0.01$). When this mean value after hGH injection was compared to the mean value in all the untreated subjects under study, the difference was highly significant ($t = 3.857, P < 0.001$).
Correlation between [3H]thymidine activity measured by thymidine uptake into activated lymphocytes, and the somatomedin activity measured as sulphation factor

These two activities are in good correlation in various pathological circumstances dependent upon hGH levels, \( r = 0.937 \) n = 32 \( (P < 0.001) \). When the group of constitutional dwarfism was added, the correlation remained significant, \( r = 0.666 \) n = 49 \( (P < 0.01) \). In the group with constitutional dwarfism, in which hGH level was in the normal range, no correlation was found between thymidine activity and sulphation factor.

Discussion

The present method clearly discriminates normal subjects from acromegalic patients and pituitary dwarfs. Low values of thymidine activity were obtained in hypopituitary patients and returned to normal following i.m. hGH injection. In children with growth failure due to causes other than hGH deficiency, serum thymidine activity was found to be in the normal range in some of them or higher in some others. The mean value in this latter group is significantly higher than that of the normal adult group \( (P < 0.01) \) and than the reference serum value.

The stimulating effect of serum cannot be related to the GH, insulin or T3 content since physiological concentration of these hormones does not increase the thymidine uptake by activated lymphocytes. It may be attributed in part to the somatomedins. The relative lack of effect of the purified preparation of somatomedin in comparison with the effect exerted by serum which we observe, has been previously reported by several authors (Hayashi & Sato 1976; Cohen & Nislesly 1980) and probably reflects the necessity of serum co-factors which elicit the effect of somatomedin polypeptides (Stiles et al. 1979).

Serum somatomedin, as defined above, has multiple biological actions (Salmon & Du Vall 1970; Raben et al. 1972; Phillips & Vassilopoulos-Sellin 1980). The possibility of several mediators of GH action cannot be excluded at this stage though during purifications of somatomedin from human plasma it was not possible to separate the thymidine factor and sulphation factor (Van den Brande et al. 1971; Van Wyk et al. 1972). Therefore it may be

Table 1.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Age (years)</th>
<th>Serum thymidine activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean (SEM)</td>
<td>mean* (U/ml) SEM</td>
</tr>
<tr>
<td>Normal adults</td>
<td>19</td>
<td>42 (1.40)</td>
<td>1.04 (0.07)</td>
</tr>
<tr>
<td>Acromegalic patients</td>
<td>3</td>
<td>40 –</td>
<td>2.63 (0.48)</td>
</tr>
<tr>
<td>Constitutional dwarfism</td>
<td>17</td>
<td>7 (0.24)</td>
<td>1.51 (0.13)</td>
</tr>
<tr>
<td>Panhypopituitary dwarfs (untreated)</td>
<td>17</td>
<td>8 (0.40)</td>
<td>0.37 (0.04)</td>
</tr>
</tbody>
</table>

* Results are expressed as U/ml compared to the reference serum which is arbitrarily fixed as activity of 1 U/ml.
We are indebted to Dr. L. Fryklund (AB Kabi, Stockholm) for kindly supplying the purified preparation of SMA, and to Edgar Montana for his kind assistance in the preparation of the English text.

Work supported by INSERM, Contract 78.1.0374 and 81.40.37.

Technical assistance for somatomedin bioassay: B. Leduc and A. M. Repellin.

References


Acknowledgments

We would like to greatly thank Pr. J. C. Job for allowing us to study the patients of the Endocrine Department, and for helping during the discussion of results and preparation of this paper.


Received on December 4th, 1980.