Plasma Zinc Level and Thymic Hormone Activity in Young Cancer Patients

By Eugenio Mocchegiani, Paolo Paolucci, Donatella Granchi, Laura Cavallazzi, Lory Santarelli, and Nicola Fabris

It has been reported that in many neoplastic diseases, including leukemia, alterations in plasma zinc levels may frequently occur, although the causes for such alterations have yet to be clearly defined. Since zinc is required to induce biological activity to thymulin (Zn-FTS), a biochemical defined thymic hormone, and marginal zinc deficiencies may prevent its peripheral biological activation, we investigated the plasma level of zinc and of both active thymulin (Zn-FTS) and total zinc saturable thymulin (Zn-FTS + FTS) in 91 young patients affected by acute lymphoblastic leukemia (ALL) at various stages of the disease. It was discovered that the plasma zinc level was reduced at the onset and relapse, whereas in complete remission and in off-therapy it was in the normal range. Total zinc-saturable thymulin concentration did not change during the disease, whereas the active fraction was reduced at the onset and in relapse when compared with values observed in the other stages of the disease or in healthy controls. These data suggest that zinc plasma deficiency is present in ALL patients at the onset and during relapse, and that such a deficiency causes a decrease in the activity of thymulin despite a nearly normal production by the thymus. An impairment of peripheral immune efficiency in ALL patients is commonly found. The existence of positive correlations between zinc or active thymulin and peripheral immunological parameters (phytohemagglutinin [PHA] and concanavalin A [ConA]) at various stages of the disease suggests a link between derangement of peripheral immune function, thymic hormone activity, and zinc failure. These findings, considered together, suggest the possibility of a carefully controlled clinical trial with zinc in ALL patients at the onset and in relapse even in the light of in vitro ineffectiveness of physiological zinc or thymulin concentrations on the duplicative index of human lymphoblastic cells.

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therapy when all therapy was finished, according to the individual protocol. All tests were performed with the written consent of the parents after the approval of the institutional human subjects' review committee. ALL subjects were hospitalized patients of "Sant'Orsola" Hospital, II Pediatric Clinic, University of Bologna, Italy.

Plasma samples for both thymic hormone and zinc determination were separated from heparinized blood at 4°C and frozen at −70°C until used.

The analysis of peripheral immune function (T-cell subsets and mitogen responsiveness) was performed at random in ALL patients (5 subjects for each stage of the disease). The same patients were also assayed for thymulin (active and total) and zinc at various stages of the disease (Table 1). These patients came from the original selected population.

**Normal subjects.** Eighteen plasma samples from normal subjects, selected at random, from 2 years to 15 years old were used as controls. Normal subjects were chosen from patients admitted to the hospital for minor surgery.

**Thymulin determination.** Spleen cells from young mice include T and B lymphocytes carrying receptors for sheep erythrocytes (SRBCs) and, therefore, capable of forming rosettes when mixed with SRBCs. T and B rosettes can be distinguished by adding 10 µg/mL azathioprine, which selectively inhibits T-rosette formation.

The azathioprine sensitivity of T cells is strictly dependent on the thymic endocrine function. Removal of the thymus in mice induces the complete disappearance of the azathioprine sensitivity, although thymic hormone is removed completely by passing plasma samples through an antithymulin immunoabsorbent. The sensitivity of this bioassay allows for the detection of 1 pg/mL of synthetic thymulin (obtained from Serva, Heidelberg, Germany). Since in two consecutive blind assays no difference of more than one log₂ was found in all samples, the assay is considered reliable.

**Determination of inactive plasma thymulin.** The determination of inactive thymulin was based on the property of the inactive hormone to inhibit the biological effect of synthetic zinc-bound active thymulin (Serva). The method, originally developed by Bach and Beaurain to detect any plasma inhibitory activity on thymulin, was later modified to induce specificity for the inactive thymulin, by concomitantly measuring thymulin activity and inhibitory effect after adding zinc sulfate to the plasma samples. If an inactive hormone is present (inhibitory effect), the addition of zinc ions induces the disappearance of the inhibitory effect and increases in the active thymulin titer. Briefly, 60 µL of plasma diluted 1:50 with Hanks’

<table>
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<th>Immunological Parameters</th>
<th>At Diagnosis</th>
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<tr>
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<td>6-11</td>
<td>5-10</td>
<td>7-12</td>
<td>3-15</td>
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<tr>
<td>PHA response (cpm/culture × 10⁻²)</td>
<td>45.3 ± 5.9*</td>
<td>67.7 ± 8.6</td>
<td>30.7 ± 3.3*</td>
<td>86.7 ± 3.9</td>
<td>95.3 ± 7.5</td>
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<tr>
<td>Con A response (cpm/culture × 10⁻³)</td>
<td>60.9 ± 3.9*</td>
<td>81.5 ± 5.4</td>
<td>51.7 ± 4.7*</td>
<td>95.7 ± 5.3</td>
<td>110.8 ± 5.1</td>
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<tr>
<td>MNC (absolute no./µL)</td>
<td>2,818 ± 1851</td>
<td>2,560 ± 2151</td>
<td>ND</td>
<td>4,380 ± 170</td>
<td>4,665 ± 175</td>
</tr>
<tr>
<td>CD2 (absolute no./µL)</td>
<td>1,437 ± 771</td>
<td>1,475 ± 871</td>
<td>ND</td>
<td>2,830 ± 83</td>
<td>3,010 ± 81</td>
</tr>
<tr>
<td>CD3 (absolute no./µL)</td>
<td>1,319 ± 91</td>
<td>1,420 ± 801</td>
<td>ND</td>
<td>2,700 ± 73</td>
<td>2,625 ± 90</td>
</tr>
<tr>
<td>CD4 (absolute no./µL)</td>
<td>903 ± 15</td>
<td>913 ± 20</td>
<td>ND</td>
<td>1,985 ± 83</td>
<td>2,130 ± 53</td>
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<td>CD8 (absolute no./µL)</td>
<td>987 ± 22</td>
<td>936 ± 36</td>
<td>ND</td>
<td>1,010 ± 22</td>
<td>1,075 ± 39</td>
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<tr>
<td>Active thymulin (log₂)</td>
<td>0.9 ± 0.66*</td>
<td>3.4 ± 0.55</td>
<td>1.7 ± 0.45*</td>
<td>4.1 ± 0.45</td>
<td>5.0 ± 0.62</td>
</tr>
<tr>
<td>Total thymulin (log₂)</td>
<td>4.2 ± 0.45</td>
<td>4.8 ± 0.84</td>
<td>4.4 ± 0.55</td>
<td>6.4 ± 0.65</td>
<td>5.5 ± 0.53</td>
</tr>
<tr>
<td>Inhibitory substances (pg/mL)</td>
<td>87.1 ± 2.70*</td>
<td>3.6 ± 1.191</td>
<td>37.7 ± 9.70*</td>
<td>1.8 ± 0.90</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Zinc (µg/dL)</td>
<td>59.3 ± 9.6*</td>
<td>90.8 ± 6.4</td>
<td>72.8 ± 5.9*</td>
<td>107.5 ± 6.2</td>
<td>112.2 ± 2.7</td>
</tr>
<tr>
<td>Range</td>
<td>(49-70)</td>
<td>(83-97)</td>
<td>(68-80)</td>
<td>(101-115)</td>
<td>(107-115)</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD. Number of patients studied: five, chosen at-random, for each group, belonging to the original selected population.

Abbreviation: ND, not determined.

* P < .01 when compared with values in complete remission, off-therapy, and healthy controls.

† P < .05 when compared with values of healthy controls.

‡ P < .01 when compared with values in off-treatment and healthy controls.
solution was mixed with 60 μL of different amounts of synthetic thymulin corresponding to a final concentration of 0.1, 0.5, 1.0, 10, 50, and 100 pg/mL. The 1:50 dilution of plasma was used to avoid interference by endogenous thymulin. The mixture was immediately filtered through a CF50 Amicon membrane (cone size, 50,000 d) for 30 minutes at 4°C. The filtrates were tested for thymulin activity in the rosette assay. The thymulin inhibitory activity was expressed as the highest concentration of synthetic thymulin inhibited by the plasma sample.

In the modified test, zinc sulfate was added to Hanks’ solution at a final concentration of 200 nmol/L. This zinc concentration was chosen on the basis of previous experiments performed with graded concentrations ranging from 1 pmol/L to 10 μmol/L, which showed that the 200-nmol/L concentration was optimal for unmasking inactive thymic hormone molecules.20

Zinc determination. Plasma zinc was measured using the method reported by Fernandez and Kahn.21 Blood samples were collected into fluorinated tubes to avoid contamination (no. 115317, L.P. Milan, Italy) and centrifuged for 20 minutes, then at 300 g for 10 minutes. Plasma samples were then frozen at −70°C and stored until use. Zinc was determined by atomic absorption spectrophotometry against a standard reference as suggested by Evenson and Warren.22

Lymphocyte subsets analysis. Mononuclear cells (MNCs) from 20 ALL patients at various stages of disease were obtained from peripheral blood by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient sedimentation. The MNCs were washed and resuspended in phosphate-buffered saline (PBS) containing 5% heat-inactivated fetal calf serum (FCS) in the amount of 1×10⁶/mL. Fifty microliters of MNCs, isolated as described above, were incubated at 4°C for 30 minutes with 50 μL of monoclonal antibodies for CD antigens, and conjugated with goat antimouse fluorescein isothiocyanate (FITC) at a final dilution of 1:100. After washing with PBS-FCS, the cells were analysed in a fluorescence-activated cell sorter (Epics V; Coulter, Hialeah, FL). The positively stained cells were those with a greater fluorescence intensity than cells labeled with the second antibody alone (FITC) and were expressed as absolute number per microliter. Approximately 10⁵ randomly accumulated viable cells were analyzed. The monoclonal antibodies used were CD2 (E-rosette T cells), CD3 (total T cells), CD4 (t-helper cells) and CD8 (T suppressor/cytotoxic cells; Becton Dickinson, San Jose, CA).

Lymphocyte proliferative response. The lymphocyte proliferative response was assessed in 20 young ALL patients at various stages of the disease (5 at onset, five in complete remission, five at relapse, and five off-therapy). Ficoll-Hypaque–enriched peripheral blood MNCs, at a concentration of 1×10⁶/mL in RPMI 1640 (GIBCO, Gaithersburg, MD) containing 10% FCS (GIBCO), penicillin (100 U/mL) and streptomycin (100 μg/mL). Their vitality was determined by trypan blue exclusion. Aliquots of 0.1 mL were distributed in microwells.

Zinc sulfate was added in the amount of 10 μL/well to reach a final concentration of zinc ions of 10⁻⁴, 10⁻³, and 10⁻² mol/L.23

Synthetic active thymulin (Zn-FTS) (Serva) was added in the amount of 10 μL/well to reach a final concentration of 10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/L H3-Td was added in the amount of 1 μCi/well.

Statistical analysis. Differences between the groups and controls were assessed by Student’s t-test. The correlation was determined by linear regression analysis and the least-squares methods. Differences were considered significant at P < .05. Variations during the various stages of the disease were calculated using analysis of variance (ANOVA). Furthermore, ANOVA (one-way and two-way) was used for variations from in vitro data of blast cell cultures.

RESULTS

Zinc-dependency of low thymulin levels in ALL patients. Figure 1 shows that ALL patients at the onset of the disease and during relapse have low plasma levels of active thymulin (Zn-FTS) when compared with values of age-matched control subjects (P < .01), whereas the concentrations of inhibitory substances (inactive thymulin) are extremely high (P < .01). The in vitro addition of zinc sulfate to the ALL plasma samples at the onset and during relapse induces a strong reduction of inhibitory activities and the reappearance of thymulin activity (total thymulin = Zn-FTS + FTS) to levels comparable to those present in plasma samples from healthy control subjects. By contrast, the plasma active thymulin levels show, in complete remission and in off-therapy stages, only slight and not significantly reduced values when compared with those observed in young healthy controls. However, a small but statistically significant increase of the plasma concentration of inhibitory substances is present (P < .05), and the in vitro zinc addition induces a nearly complete disappearance of the inhibitory activity.

A statistically significant reduction in plasma zinc levels is present at the onset and during relapse of the disease when compared with the values of healthy controls (P < .01) (Fig 1), whereas no significant differences are observed in complete remission and in off-therapy stages. If the plasma zinc levels, regardless of stage of disease, are plotted against the corresponding values of in vitro thymic hormone saturable fraction (Zn-FTS + FTS/Zn-FTS), a significant positive correlation is present (r = .87; P < .01) (Fig 2).
Lymphocyte subsets and mitogen responsiveness in ALL patients. Table 1 shows that both at the time of diagnosis and in complete remission, ALL patients show reduced MNC counts, as well as reduced absolute numbers of CD2+, CD3+, and CD4+ cells, when compared with the values of age-matched healthy controls (P < .01). However, off-therapy patients with ALL showed normal counts of MNCs and lymphocyte subsets (CD2+, CD3+, and CD4+). CD8+ cells did not indicate significant differences among the patient groups and the controls.

The proliferative responses of peripheral blood lymphocytes to PHA and ConA are significantly reduced (P < .01) in ALL patients at the time of diagnosis and during relapse of the disease as compared with the values observed in the other stages of the disease or in normal controls.

Table 1 also shows the plasma thymulin (active and total) levels, plasma inhibitory substance (inactive thymulin) levels, and plasma zinc concentrations at various stages of the disease in the same ALL patients who were tested for the peripheral immune efficiency. With regard to thymulin levels and zinc concentrations, the data reported in Table 1 show a strong reduction of both active thymulin and zinc plasma levels, whereas the concentration of inactive thymulin is extremely high at the onset and during relapse of the disease when compared with controls (P < .01). However, in complete remission and in off-therapy patients, small but significant variations of inactive thymulin are present when compared with the values of age-matched healthy subjects (P < .05). Nevertheless, when the values of PHA and ConA mitogen responsiveness were plotted against plasma zinc concentrations or active thymulin plasma levels at various stages of the disease, significant positive correlations were found (Fig 3A, C, D, and F) and negative ones against inhibitory substances (inactive thymulin) (Fig 3B and E).

Effect of in vitro addition of zinc and active thymulin (Zn-FTS) on duplicative index of lymphoblastoid cells. The possible effect of zinc or of active thymulin (Zn-FTS) on the proliferative activity of lymphoblastoid cells was studied on cell lines established from bone marrow of ALL patients and on laboratory lymphoblastoid cell lines. The phenotypic characteristics of ALL patients, from which cell cultures were derived, are reported in Table 2.

Table 3 shows that zinc does not increase the proliferative rate of human lymphoblastoid cells at 10^-6 or 10^-5 mol/L concentrations when compared with values of control cultures, while a modest and statistically significant increment is observed at the concentration of 10^-4 mol/L (P < .05). On the contrary, the proliferation rate of human blast cell cultures is significantly reduced when active thymulin is added at the concentration of 10^-5 mol/L (P < .05), whereas lower thymulin concentrations, slight or no variation is observed. Such a decrement of proliferative rate is observable both in ALL-T and ALL-C patients. The effect of zinc and active thymulin on the proliferative rate of laboratory lymphoblastoid cell lines is also reported in Table 3. The in vitro addition of active thymulin is also able to induce a slight but significant decrement of the proliferative rate in these cells (P < .05) when compared with untreated control cultures. Zinc, by contrast, does not induce a significant increment of the proliferative rate. Only at the concentration of 10^-4 mol/L in the JM cell line is zinc able to induce a significant increment of the duplicative index (P < .05). Furthermore, it is relevant to note that, although a reduction is present in the proliferative rate when zinc, at the dose of 10^-4 mol/L, is added in vitro to blast cell cultures, a statistically significant variation of the duplicative index is observed only for active thymulin (10^-5 mol/L). (P < .05).

DISCUSSION

The present report shows that both plasma zinc level and thymulin activity are strongly reduced in leukemic patients at the onset and during relapse of the disease when compared with values of age-matched controls, whereas concomitantly high levels of inhibitory substances on thymic peptide activity are observed. At other stages of the disease, plasma concentrations of thymulin, inhibitory substances, and zinc are close to those observed in age-matched young healthy subjects. The findings on plasma zinc levels are in accordance with previous studies. They show a reduction of plasma zinc level at the onset of the disease in young ALL patients and a recovery of zinc values during complete remission and in off-therapy patients whereas they are in disagreement with previous reports that show normal plasma zinc values at the onset of the disease. However, it is interesting to note that in these last studies the range of zinc values in healthy subjects is extremely wide (from 60 to
![Graph showing significant correlations between plasma zinc level and fraction of zinc-saturable thymic hormone (Zn-FTS + FTS/Zn-FTS) in 91 ALL patients during various stages of disease. Normal controls (○), at disease onset (●), at relapse (△), in complete remission (□), off-treatment (□).](image)

Zinc and Thymulin in Young All Patients

300 µg/dL and the number of patients is quite limited, whereas in our measurements, as reported by other investigators, the zinc range is much more restricted and the number of patients is large.

The causes for the reduction of zinc plasma levels in ALL patients are not yet established. It has been suggested recently that the low zinc values observed in many neoplastic diseases, including leukemia, may result from an inadequate intake of zinc due to malnourishment or, especially during the relapse stage of the disease, it may be a consequence of the administration of antimitabolite drugs, which have been shown to enhance urinary secretion of bivalent cations. Although a definite interpretation of the low zinc levels in ALL patients is still not available, these findings suggest the presence of a low bioavailability of zinc ions at the onset and relapse of the disease.

The relevance of zinc in maintaining immune system efficiency is, at present, well documented. In particular, zinc plays a key role in inducing biological activity to one of the best known thymic hormones, the facteur timique sérique (FTS), more recently called thymulin in its zinc-bound active form. Thymulin is a zinc conjugate of a nonapeptide FTS synthesized by the epithelial cells of the thymus; its biological activity resides in the conjugate form, whereas the zinc-unbound form is inactive and can inhibit the active form. The mechanism of such an inhibition is unknown; it can be suggested that the inactive zinc-unbound form may compete with the biologically active form (thymulin) for FTS receptors, as do synthetic FTS analogs.

It has been shown that in marginal zinc deficiencies, dependent on mild reduction of zinc intake or associated with sickle cell anemia, acrdematite enteropathica, Down’s syndrome, and Duchenne’s syndrome, acquired immunodeficiency syndrome, and juvenile type 1 diabetes, the thymic peptide FTS may be synthesized and secreted in normal amounts, but only a fraction of it becomes bound to zinc ions and is therefore active. The inactive zinc-unbound form present in these conditions may be revealed by the fact that it exerts an inhibitory action on the active form and that in vitro addition of zinc ions causes the disappearance of this inhibitory activity with a concomitant increase of the active form.

Our findings clearly show that in the leukemic condition, at the onset and during relapse of the disease, low thymulin levels are not due to a failure of the endocrine thymus, but to a reduced peripheral saturation of the thymic peptide by zinc ions. Such an assumption is supported by the fact that in vitro addition of zinc to the ALL plasma samples at the onset and during relapse was able to induce a complete saturation of thymic hormone molecules present, whereas in complete remission and in off-therapy patients, only a slight effect of in vitro addition of zinc to plasma samples on thymulin activity was observed. This interpretation is further supported by the strict correlation existing between plasma zinc level and the degree of in vitro zinc saturation of thymic hormone regardless of the stage of the disease.

A major criticism of this interpretation is whether a 20% to 30% reduction of plasma level of zinc may be of practical significance. However, note that thymulin (kd, 10⁻⁷ mol/L) is not first in the affinity scale of available zinc; being preceded by nerve growth factor (kd, 10⁻¹¹ mol/L) and metallothioneins (kd, 10⁻⁸ mol/L), it is nearly equal to metalloproteins (kd, 10⁻⁷ mol/L) and followed by many other zinc-
dependent enzymes such as alkaline phosphatase (kd, 10^{-6} mol/L). Note that the much lower plasma concentrations of thymulin (3 mg/dL) in respect to the concentrations of other zinc-bound proteins, even with lower kd, can further reduce zinc availability for the hormone. Zinc supplementation experiments may confirm the role played by the 20% to 30% to decrease in plasma zinc observed in ALL patients at the onset and during relapse of the disease. In other human marginal zinc deficiencies, such as in Down's syndrome and uremic conditions, both of which are characterized by a ≤20% reduction of plasma zinc level and low thymulin titers, zinc supplementation had a beneficial effect in recovering plasma zinc levels, thymulin titers, and some crippled immune functions.

However, it must be noted that the in vitro addition of zinc to plasma of ALL patients does not totally eliminate the presence of inhibitory substances, as inhibiting activity remains, particularly at the onset and during relapse. Such a finding is difficult to explain. The existence of low-molecular weight substances, other than zinc-unbound FTS, capable of inhibiting active thymulin, may be suggested. Some experimental findings support this, but their nature is, at present, unknown.

Profound disturbance of the immune system is frequently found in patients suffering from neoplastic diseases, including leukemia, either at diagnosis or after treatment with radiation or chemotherapy.

Cell-mediated immunity, assessed by the measurement of both the absolute number of T-cell subsets and mitogen responsiveness, is impaired in leukemic patients even after
therapy has been discontinued. Our data are in agreement with these findings. A significant decrement both in T-cell subsets and in mitogen responsiveness was present at the onset and during relapse of the disease in our ALL patients. These parameters are normal in the off-therapy stage, but are in disagreement with other reports. The existence of positive correlations among PHA and ConA mitogenesis and active thymulin or zinc during the various stages of the disease clearly suggest a causative relationship between derangement of peripheral immune function at the onset and relapse of the disease and thymic hormone or zinc failure.

A clinical trial with zinc in these patients at the onset and during relapse of the disease might, therefore, have a beneficial effect in order to increase the biological activity of the thymic hormones, as well as to recover the crippled immune functions, as demonstrated in other human marginal zinc deficiencies, in some neoplastic diseases, and in physiological aging, all of which are characterized by reduction of zinc plasma levels and by low thymulin titers.

However, the recent discovery that zinc induces mitogenesis, raises the question of its possible effect on neoplastic cells. An analysis of the effect of zinc on lymphoblastoid cells has shown that zinc is mitogenic only when used at pharmacologic doses, whereas at physiologic doses it has no effect on neoplastic cellular duplication.

By contrast, thymulin in its zinc-bound active form (Zn-FTS), at the dose of $10^{-5}$ mol/L corresponding to the physiologic amount of thymulin in the blood circulation of young healthy individuals, induced a slight but significant decrement of the mitotic index both in human lymphoblastoid cells and in some laboratory blast cell lines. The mechanism by which active thymulin affects the mitotic rate of blast cells remains unknown.

According to these last findings, the proposed clinical trial with physiologic doses of zinc, in particular at the onset and during relapse of the disease, in order to induce a complete saturation of thymic hormone and possibly an improvement of peripheral immune efficiency, may find a further rationale.

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