Pediatric patients with acute lymphoblastic leukemia (ALL) are at an increased risk of thromboembolic events. Potential responsible mechanisms include the disease process itself, treatment with chemotherapeutic agents (particularly L-Asparaginase [ASP]), or a combination of the disease and treatment. We studied thrombin regulation in 26 consecutive children with ALL and 14 healthy age-matched controls by: (1) plasma concentrations of prothrombin; (2) plasma inhibition of \( ^{125} \text{I}-\alpha\)-thrombin; and (3) four biochemical markers of in vivo thrombin activation (thrombin complexed to its inhibitor antithrombin III [ATIII; TAT], prothrombin fragment 1.2 (F1.2), activated protein C complexed to the inhibitors \( \alpha \), antitrypsin [APCAT]), and protein C inhibitor [APC-PCI]). Measurements were made at presentation before treatment, after treatment with ASP alone, and during combination chemotherapy with and without ASP. At presentation, the capacity to generate thrombin (reflected by plasma prothrombin concentrations) and the capacity to inhibit thrombin \( ^{125} \text{I}-\alpha\)-thrombin—(inhibitor complex formation) were similar in children with ALL compared with that for healthy children. After ASP alone or as part of combination chemotherapy, prothrombin levels were preserved, whereas plasma inhibition of \( ^{125} \text{I}-\alpha\)-thrombin decreased significantly because of a decrease in plasma concentrations of inhibitors, most importantly ATIII. After combination chemotherapy without ASP, plasma concentrations of ATIII and the capacity to inhibit \( ^{125} \text{I}-\alpha\)-thrombin returned to normal values, whereas prothrombin levels increased above control values. Thrombin generation in vivo also differed from healthy controls. At presentation, plasma concentrations of three of four markers of in vivo thrombin activity (TAT, F1.2, APCAT, but not APC-PCI) were increased in children with ALL. Neither ASP alone nor combination chemotherapy with or without ASP significantly altered values of these three markers. In summary, although the in vitro capacity to generate thrombin was preserved, the in vivo capacity to inhibit \( ^{125} \text{I}-\alpha\)-thrombin decreased after ASP therapy. Evidence for increased endogenous thrombin generation was documented in children with ALL at presentation and throughout treatment. We speculate that poor regulation of this thrombin may contribute to thrombotic complications in children with ALL.

The treatment of childhood acute lymphoblastic leukemia (ALL) has advanced to the extent that 5-year event-free survival (EFS) rates of approximately 80% have been achieved. The chemotherapeutic protocols for childhood ALL developed at the Dana-Farber Cancer Institute (DFCI; Boston, MA) are currently among the most successful. Children with standard risk (SR) ALL, defined by age and white blood cell count, have an 86% chance of a 5-year EFS, and children with high risk (HR) disease, a 71% 5-year EFS.

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Increased Endogenous Thrombin Generation in Children With Acute Lymphoblastic Leukemia: Risk of Thrombotic Complications in L’Asparaginase-Induced Antithrombin III Deficiency

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MATERIALS AND METHODS

Patient population. The patient population was comprised of 26 consecutive children, newly diagnosed with ALL, admitted to the Children’s Hospital at Chedoke-McMaster between March 1989 and July 1991. Their ages ranged from 2 to 17 years with a median age of 5 years. All children were diagnosed with ALL as previously published

and is used aggressively in the consolidation phase of the DFCI ALL protocols. ASP interferes with protein synthesis, thereby impairing the production of some coagulation proteins.

The DFCI ALL protocols have an investigational window at diagnosis, facilitating evaluation of the effect of a single chemotherapeutic agent on untreated lymphoblasts in vivo. For the DFCI protocol 87-001, the single drug was ASP. This provided an novel opportunity to assess the effects of ASP on blood coagulation independent of previous or concurrent chemotherapy. In addition, blood coagulation was assessed at diagnosis before any intervention and after combination chemotherapy with and without ASP.

The generation and inhibition of thrombin constitute key steps in blood coagulation. Sensitive markers of endogenous generation of thrombin have been developed and include release of prothrombin fragment 1.2 (F1.2) by thrombinase; thrombin complexed to its main inhibitor antithrombin III (ATIII; TAT), and the generation of activated protein C as assessed by measuring protein-C complexes with its inhibitors \( \alpha \)-antitrypsin and protein-C inhibitor. The capacity of plasma to generate thrombin is reflected in the level of prothrombin.

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CHILDHOOD ALL: RISK OF ASP ATIII DEFICIENCY

The DFCl 87-001 protocols for ALL have an experimental window on day 0, at which time all patients receive 1 dose of E.coli ASP (25,000 U/m^2) only. The remainder of the induction therapy begins on day 5 for a total of 28 days. Induction is followed by a 20- to 40-week consolidation phase and a maintenance phase for the remaining time, up to 24 months. Details of these protocols have been published previously. In brief, all children received the same induction therapy after the experimental window with ASP (vincristine, adriamycin, methotrexate, prednisone). During the consolidation phase all children received ASP (25,000 IU/m^2, weekly), 6-mercaptopurine (daily for 2 of 3 weeks), prednisone (5 days every 3 weeks), and vincristine (week 1 of 3 weeks). SR children received 20 weeks of ASP, whereas children categorized as HR or very HR received 40 weeks of ASP and adriamycin (every 3 weeks) to a cumulative dose of 360 mg/m^2. Children categorized as very HR also received an additional 4-week intensification with the same drugs and cytosine arabinoside. The maintenance therapy was the same for all patients and consisted of 6-mercaptopurine, prednisone, vincristine, and methotrexate.

The children were studied at 4 time points in their therapy, and a minimum of 10 patient samples were assessed at each time point. Not all children were sampled at all times because of missed samples or because the central venous catheter did not bleed back necessitating a separate venipuncture. The times of sampling were day 0 (at diagnosis before treatment), day 5 (5 days after 1 dose of ASP), day 21 (21 days after combination chemotherapy with no ASP) and random (random time during the consolidation phase that included ASP). The final sample was drawn at week 14 from diagnosis, at which point they had undergone approximately 11 weeks of consolidation phase with ASP. The duration of ASP therapy at this time was 11.3 ± 3.3 weeks (mean ± 1 SD) with a median of 10.6 weeks. A 4-mL blood sample was collected into 3.8% buffered sodium citrate (1 part citrate to 9 parts blood) by venipuncture or from an indwelling catheter. Before sampling from the catheter, the catheter was flushed with 2 mL of saline, and 5 mL of blood was drawn and discarded.

Age-matched normal control values were obtained from children who were having minor elective surgery. Informed consent was obtained from the parents, and an additional 5-mL blood sample was drawn by venipuncture from these children at the time that preemptive blood work was drawn. Fourteen age-matched children served as concurrent controls.

Plasma concentrations of inhibitors of blood coagulation. α1-Antitrypsin (α1M) was measured by radial immunodiffusion technique using a commercially available antibody (Atlantic Antibodies, Toronto, Canada). Functional chromogenic assays were used to measure ATIII and heparin cofactor II (HCII; Stago, Wellmark Diagnostics, Guelph, Canada). Functionally available Protein Czymogen (PC) was measured by enzyme-linked immunosorbant assay (ELISA) as previously described. Protein S was measured as total protein S and free protein S by ELISA using protein S antibody from Affinity Biologicals (Hamilton, Canada). Free protein S was obtained by polyethylene glycol (PEG) precipitation according to the method of Comp et al. In vitro capacity to generate thrombin. Prothrombin levels were measured by clot-based assays in deficient plasma on an ACL 300R (IL Laboratories, Milan, Italy).

RESULTS

Patient population. Three of the 26 patients (11.5%) had thrombotic complications. All 3 children were classified as HR ALL, and the thrombotic complications occurred when they were receiving consolidation therapy that included ASP. The first patient (GW) had a right atrial thrombus (diagnosed by echocardiography) secondary to a central venous catheter. Because the mural thrombi increased in size, it was surgically removed, and in follow-up, the patient was treated with coumadin. Four months later, while the patient was on low dose coumadin (INR = 1.1), he had a pulmonary embolus diagnosed by a high probability ventilation/perfusion scan. The second patient (LA) presented with increasing pain and swelling in the left thigh. A thrombosis in the left common iliac vein was diagnosed by venography. She was treated with heparin followed by 3 months of coumadin therapy. The third patient (BS) was admitted with swelling and pain in the left leg. A thrombosis of the posterior tibial, peroneal, and common femoral veins was diagnosed by venography. He was also treated with heparin followed by 3 months of coumadin therapy. There were no remarkable differences in these patients’ results at presentation or during therapy (data not shown). ATIII levels were measured at the time of their events and were 0.46, 0.64, and 0.32 μg/mL, respectively.

In vitro capacity to generate thrombin. We have shown previously that the capacity of plasmas to generate thrombin in vitro is accurately reflected by plasma prothrombin concentrations. At presentation, prothrombin levels were similar in patients and controls (Table 1). Prothrombin levels did not significantly decrease after therapy with ASP. However, prothrombin levels significantly increased after combination chemotherapy both with and without ASP (Table 1).

Plasma concentrations of inhibitors of coagulation. Plasma concentrations of inhibitors of coagulation are also shown in Table 1. The same pattern of response was observed for all inhibitors, although the absolute values and degree of significance varied. At presentation (day 0), plasma concentrations of protein C, ATIII, α1M, and total protein S were similar to healthy age-matched control values, whereas levels of HCII were significantly increased. Free protein S was decreased. After ASP alone (day 5), plasma concentrations of all inhibitors decreased, and sig-
significant reductions were noted for ATIII (P < .0001), HCII (P = .008), protein C (P = .008), and free protein S (P < .0001). To compare the magnitude of the decrease of the four inhibitors, the plasma levels were standardized by the effect size (ES) calculation. The extent of the decrease was greatest for ATIII (ES = 2.56) as compared with free protein S (ES = 1.4), HCII (ES = 0.65), and α2M (ES = 0.46). The decrease for ATIII was statistically significantly greater than that for the other inhibitors measured. This agrees with our previous findings. After combination therapy without ASP (day 26), values rebounded to similar (ATIII, α2M, free protein S) or increased levels (HCII and total protein S) as compared with healthy controls. During consolidation therapy with combination chemotherapy that included ASP (random samples), only significant decreases in ATIII levels (P = .0014) were observed. A significant increase in total protein S was seen. α2M levels showed a tendency to be lower but remained within normal range for the entire study.

In vitro capacity to inhibit thrombin. The capacity of plasmas to inhibit thrombin was assessed by the addition of 125I-α-thrombin to plasma in vitro. A typical example of a patient’s plasma pattern of inhibition over therapy is shown in Fig 1. At presentation (day 0), and after combination chemotherapy with (consolidation) and without ASP (day 26), the inhibition of 125I-α-thrombin was similar to healthy age-matched controls (Table 2). However, after ASP alone (day 5), the inhibition of 125I-α-thrombin was significantly decreased (P = .0002; Table 2). 125I-α-thrombin–inhibitor—complex formation with ATIII, HCII, and α2M decreased; only the decrease in complexes with ATIII were statistically significant (P = .008; Table 2) and reflected plasma concentrations of inhibitors (Table 1). Combination chemotherapy with ASP did not significantly change thrombin inhibition.

In vivo generation of thrombin. Plasma concentrations of endogenous markers of thrombin generation are summarized in Table 3. There were no significant changes in the increased values over all 4 time points for TAT and APCAT. F1.2 levels were elevated on day-0 and day-5 time points (P = .009) but were decreased at the day-26 time point to values similar to normal controls.

DISCUSSION

The successful treatment of children with ALL permits clinicians to focus on the morbidity associated with the disease itself and on that which is a consequence of treatment. In this study, thrombin regulation was extensively evaluated both in vitro and in vivo using recently available sensitive measurements. The results show that the capacity of plasma to generate thrombin is preserved or increased throughout treatment for ALL and that the capacity to inhibit thrombin decreases intermittently, reflecting the plasma concentra-
Table 2. In Vitro Capacity of Plasma From Children With ALL to Inhibit Thrombin

<table>
<thead>
<tr>
<th>Complexes nmol/L (%) total</th>
<th>Pretreatment at Diagnosis (n = 15)</th>
<th>After Treatment With ASP Only (n = 15)</th>
<th>Combination Therapy Without ASP (n = 16)</th>
<th>Combination Therapy With ASP (n = 11)</th>
<th>Normal Controls (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATIII-lla</td>
<td>9.90 ± 0.53</td>
<td>8.21 ± 0.59*</td>
<td>10.98 ± 0.42</td>
<td>9.50 ± 0.87</td>
<td>10.16 ± 0.33</td>
</tr>
<tr>
<td>(64.7%)</td>
<td>(59.4%)</td>
<td>(72.1%)</td>
<td>(62.1%)</td>
<td>(63.4%)</td>
<td></td>
</tr>
<tr>
<td>hCII-lla</td>
<td>1.16 ± 0.1</td>
<td>0.81 ± 0.09</td>
<td>1.13 ± 0.14</td>
<td>0.81 ± 0.12</td>
<td>1.0 ± 0.07</td>
</tr>
<tr>
<td>(7.6%)</td>
<td>(5.9%)</td>
<td>(7.2%)</td>
<td>(5.2%)</td>
<td>(6.3%)</td>
<td></td>
</tr>
<tr>
<td>α2M-lla</td>
<td>4.08 ± 0.22</td>
<td>4.62 ± 0.39</td>
<td>3.12 ± 0.28</td>
<td>4.98 ± 0.63</td>
<td>4.82 ± 0.37</td>
</tr>
<tr>
<td>(27.1%)</td>
<td>(34.2%)</td>
<td>(20.3%)</td>
<td>(32.5%)</td>
<td>(30%)</td>
<td></td>
</tr>
<tr>
<td>Total Ila complexed</td>
<td>15.2 ± 0.52</td>
<td>13.7 ± 0.40*</td>
<td>15.3 ± 0.49</td>
<td>15.29 ± 0.78</td>
<td>16.0 ± 0.27</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± one standard error of the mean and as percentage of the total amount of thrombin inhibited.

Abbreviations: thrombin, Ila.

* Indicates a significant difference from healthy controls of at least P < .01.

Table 3. Evidence of In Vivo Generation of Thrombin in Children With ALL

<table>
<thead>
<tr>
<th>Test</th>
<th>Pretreatment at Diagnosis (n = 14)</th>
<th>After Treatment With ASP Only (n = 17)</th>
<th>Combination Therapy Without ASP (n = 19)</th>
<th>Combination Therapy With ASP (n = 16)</th>
<th>Normal Controls (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1.2 (nmol/L)</td>
<td>3.5 ± 0.92*</td>
<td>2.8 ± 0.68*</td>
<td>1.48 ± 0.34</td>
<td>1.67 ± 0.50</td>
<td>1.08 ± 0.099</td>
</tr>
<tr>
<td>TAT (pmol/L)</td>
<td>134 ± 50†</td>
<td>87 ± 14*</td>
<td>78 ± 18*</td>
<td>86.5 ± 16.5†</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>APCAT (nmol/L)</td>
<td>0.57 ± 0.073†</td>
<td>0.42 ± 0.57*</td>
<td>0.42 ± 0.11*</td>
<td>0.28 ± 0.18†</td>
<td>0.089 ± 0.017</td>
</tr>
<tr>
<td>APC-PC1 (nmol/L)</td>
<td>0.096 ± 0.03†</td>
<td>0.12 ± 0.035</td>
<td>0.083 ± 0.19</td>
<td>0.09 ± 0.04</td>
<td>0.047 ± 0.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± one standard error of the mean.

* Indicates a significant difference from healthy controls of at least P < .01.
† Indicates no significant change over the time points.
characterized by increased plasma concentrations of most coagulation proteins (XI, V, VIII, von Willebrand factor, II, ATIII, HCII, α2-antiplasmin, protein C, protein S) and with decreased levels of α2M and fibrinogen. Thus, the results at the end of induction and during consolidation may reflect the use of prednisone.

At presentation and during combination chemotherapy the capacity to inhibit thrombin was preserved in children with ALL compared with that for controls. As previously reported, α2M inhibited more thrombin in plasma from children compared with that from adults. However, ASP alone significantly decreased the capacity of plasma to inhibit thrombin because of decreases in plasma concentrations of inhibitors, most significantly ATIII. Previously, we reported that ASP caused a disproportional decrease in plasma concentrations of ATIII compared with several other coagulation factors and inhibitors. The current study supports the concept that low plasma concentrations of ATIII are fundamentally important to the development of thromboembolic complications. Plasma concentrations of ATIII in the 3 patients with thrombotic complications were 0.46, 0.64, and 0.32 μM/L at the time of their events. The mechanism for the prethrombotic state in children with ALL may be an acquired ATIII deficiency after treatment with ASP in the presence of a preserved capacity to generate thrombin. In our study, all three patients with thrombotic events presented during the consolidation phase. This is in keeping with the findings of others. The consolidation phase may be the period of greatest risk, because there is an increase in prothrombin levels concurrent with a decrease in ATIII levels.

In contrast to the ASP-related, acquired ATIII deficiency state in children with ALL, inherited ATIII deficiency is not characterized by thrombotic complications during childhood. Plasma from children with inherited ATIII deficiency inhibits thrombin effectively as age-matched controls and as well as effectively as healthy adults. This inhibition capacity is significantly better than that found in adults with inherited ATIII deficiency. Increased plasma concentrations of α2M during childhood, and to a lesser extent, the normal levels of HCII compensated for the inherited deficiency of ATIII. For children with ASP-induced ATIII deficiency, plasma concentrations of α2M and HCII are also decreased by ASP and can not compensate. The decreased plasma capacity of plasma to inhibit thrombin (13.7 ± 0.4 nmol/L) after treatment with ASP was comparable with that of ATIII deficient adults (12.8 ± 0.6 nmol/L).

At presentation and after administration of ASP alone, free protein S levels were decreased, and total protein S levels were normal. Combination chemotherapy with and without ASP caused an increase above normal levels for total protein S and to normal levels for free protein S. There was no correlation between the low levels of protein S, total or free, and the time of risk of thrombosis.

Decreased protein C levels have been associated with thrombosis in children with ALL. The ELISA used in this report is specific for unactivated or zymogen protein C but will recognize decarboxylated protein C. Because ASP does not effect the carboxylation of protein C and, therefore, does not cause qualitative abnormalities, the ELISA assay reflects functionally available protein C. After treatment with ASP alone, levels of protein C decreased, as did all other proteins. During combination chemotherapy without ASP, the level was significantly above normal, as were the two other vitamin K factors measured. During combination chemotherapy with ASP, the levels decreased but did not go below normal. Therefore, we found no evidence of a protein C deficiency at the time these patients were at risk for thrombosis.

At presentation, plasma concentrations of 3 of the 4 markers of endogenous thrombin generation (TAT, F1.2, and APCAT) were increased. APC-PCI was not increased at anytime, which may reflect the short half-life of this complex. The combination of consistent increased activation of the coagulation system with a normal capacity to generate thrombin and an acquired ASP-induced ATIII deficiency provides an explanation for the timing of thrombotic events during consolidation.

The predictability of the ASP-induced ATIII deficiency encourages trials testing prophylactic therapy. At least three approaches are possible. First, low dose coumadin is an effective form of prophylaxis, does not carry the risks of blood products, and is ATIII independent. The difficulty is maintaining an effective yet safe dose because there is a cycling of the coagulation protein levels during therapy. Second, prophylaxis with fresh frozen plasma (FFP) is used in some centres. However, FFP is a poor source for ATIII replacement and equally supplements the procoagulants. Finally, ATIII concentrates are available and may be effective. Although treated to remove viruses, ATIII concentrates are prepared from human plasma and, therefore, still carry potential risk of viral transmission.

We conclude that there is a consistent increase in endogenous thrombin generation in children with ALL. The acquired ATIII deficiency induced by ASP, in combination with concurrent decreases in α2M and HCII, impairs the capacity of the plasmas to inhibit thrombin. Concurrent increases in prothrombin levels sustain the capacity of these plasmas to generate thrombin. This combination of events may predispose children to thrombotic events. We speculate that prophylactic infusions of ATIII concentrates in high-risk patients (high ratio of prothrombin to ATIII) may correct this defect and result in fewer thrombotic complications with this therapy. However, this hypothesis must be tested in well-designed clinical trials.

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Increased endogenous thrombin generation in children with acute lymphoblastic leukemia: risk of thrombotic complications in L'Asparaginase-induced antithrombin III deficiency

L Mitchell, H Hoogendoorn, AR Giles, P Vegh and M Andrew