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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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 thrombin; (2) plasma inhibition of 125I-α-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 α1 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 (125I-α-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 125I-α-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 125I-α-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 125I-α-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.

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The DFCI 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/m²) 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.1 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 U/m², weekly), 6-mercaptopyrurine (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/m². 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 26 (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 preparative blood work was drawn. Fourteen age-matched children served as concurrent controls.

Plasma concentrations of inhibitors of blood coagulation. α2-Macroglobulin (α2M) was measured by radial immunodiffusion technique13 using a commercially available antibody (Atlantic Antibodies, Toronto, Canada). Functionally chromogenic assays were used to measure ATIII and heparin cofactor II (HCII; Stago, Wellmark Diagnostics, Guelph, Canada). Functionally available Protein C zymogen (PC) was measured by enzyme-linked immunosorbent assay (ELISA) as previously described.12 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.13

In vitro capacity to generate thrombin. Prothrombin F1.2 and TAT complexes were measured by ELISA technique (Behring Diagnostics, Hoescht, Montreal, Canada). Activated protein C-1 antitrypsin (APCAT) and activated protein C-protein C inhibitor (APC-PC1) were quantitated by ELISA.12

Statistical analysis. For comparison of F1.2, TAT, APCAT, and APC-PC1 values, data were log-transformed. A one-way analysis of variance was performed, and if a difference in the population was detected, an unpaired Student's t-test was performed at each time point. Significance level was adjusted by a Bonferroni correction factor for multiple comparisons. Values for inhibitors were compared using an unpaired Student's t-test.

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 μ/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.8 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, α2M, 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.\(^1\) The extent of the decrease was greatest for ATIII \(\text{ES} = 2.56\) as compared with free protein S \(\text{ES} = 1.4\), HCII \(\text{ES} = 0.65\), and \(\alpha_2\)M \(\text{ES} = 0.46\). The decrease for ATIII was statistically significantly greater than that for the other inhibitors measured. This agrees with our previous findings.\(^1\) After combination therapy without ASP (day 26), values rebounded to similar (ATIII, \(\alpha_2\)M, 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. \(\alpha_2\)M 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 \(^{125}\)I-\(\alpha_2\)-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 \(^{125}\)I-\(\alpha_2\)-thrombin was similar to healthy age-matched controls (Table 2). However, after ASP alone (day 5), the inhibition of \(^{125}\)I-\(\alpha_2\)-thrombin was significantly decreased \((P = .0002; \text{Table 2})\). \(^{125}\)I-\(\alpha_2\)-thrombin–inhibitor—complex formation with ATIII, HCII, and \(\alpha_2\)M decreased; only the decrease in complexes with ATIII were statistically significant \((P = .008; \text{Table 2})\) and reflected plasma concentrations of inhibitors (Table 1). Combination chemotherapy with ASP did not significantly change thrombin inhibition.

*Fig 1*. An SDS-PAGE of thrombin inhibition of a patient’s plasma over therapy \(^{125}\)I-\(\alpha_2\)-thrombin (IIA) alone (lane I), at diagnosis before treatment (lane II), 5 days after one dose of L-asparaginase (ASP) only (lane III), at the end of induction with combination chemotherapy without ASP (lane IV), during combination chemotherapy with ASP (lane V), and an age-matched, normal control (lane VI).
Children with ALL are at risk for thrombotic complications for at least two reasons. First, several forms of cancer including myeloproliferative disorders are associated by activation of the coagulation system.16,17 Mechanisms such as tissue factor (TF) activity,18 TF-VII complexes,19 and cancer procoagulant (CP)20 have been described. To date, only the presence of the CP has been described21 in ALL. A second risk factor may be iatrogenic. The chemotherapeutic agent ASP has been repeatedly linked to thrombosis in children and adult patients.2,3,14,22,24 Usually the thrombotic events occur during consolidation therapy when ASP is administered as part of combination chemotherapy. This means it is impossible to isolate ASP as the causative agent because of the multiple confounders of concurrent therapy and disease. In the largest study, the location for the thrombotic episodes was the central nervous system.22 In addition, there are case reports of peripheral events.23,22 The true incidence and main location of thrombosis remains to be determined. Three (11%) of the children in our study had documented peripheral thrombotic complications. In keeping with the literature, these events occurred during consolidation therapy consisting of combination chemotherapy with weekly ASP.

ASP interferes with protein synthesis by catalyzing the hydrolysis of L-asparagine to L-aspartic acid and ammonia. Most cells produce the enzyme asparagine synthetase that replaces asparagine. However, leukemic lymphoblasts lack this enzyme and, therefore, rely solely on exogenous asparagine.26 ASP has a number of well-documented side effects related to interference with protein synthesis.27 After administration of ASP, plasma concentrations of many coagulation proteins are decreased.3,7,28,33 The latter has been speculated to be the mechanism for the thrombotic episodes. Previous studies reported values for selected coagulants and/or inhibitor proteins with inconsistent findings that most likely reflect variation in sampling times and concurrent use of other chemotherapeutic agents.

The DFCI 87-001 ALL protocol offered a unique opportunity to study the effect of the disease itself, of ASP alone, and of combination chemotherapy with and without ASP on thrombin regulation. The in vitro capacity of plasma to generate thrombin is reflected in the plasma concentration of prothrombin.4,9 Age-specific, healthy controls were necessary because the capacity of plasma from healthy children to generate plasma is decreased by approximately 20% compared with that for adults.34 In this report, the capacity to generate thrombin was preserved at presentation and throughout treatment. In fact, prothrombin levels significantly increased after combination chemotherapy both with and without ASP. These results agree with our previous study that used a chromogenic substrate to assess the capacity of plasma from children with ALL to generate thrombin.15 The mechanism(s) responsible for the significant increase in prothrombin concentration after combination chemotherapy is not known because of the multiple drugs administered. However, prednisone administration has been associated with changes in the hemostatic system.
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. In our study, all three patients with thrombotic events presented during the consolidation phase. This is in keeping with the findings of 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 μ/mL 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 cannot 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 when 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.

REFERENCES

4. Homans AC, Ryback ME, Baglin RL, Tiarks C, Steiner ME,
CHILDHOOD ALL: RISK OF ASP ARINASE DEFICIENCY


34. Andrew MA, Vegh PA, Mitchell LG: Thrombin regulation in children differs from adults both in the absence and presence of heparin. Thromb Haemost (submitted)


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