Thrombin Inhibition Is Impaired in Plasma of Sick Neonates¹

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ABSTRACT. The sick neonate may develop spontaneous or catheter-related thromboses, which must in part reflect poor regulation of the formation and activities of the coagulation enzyme, thrombin. We hypothesized that the balance between the generation and inhibition of thrombin may differ in sick neonates compared with healthy neonates. Fifty neonates with respiratory failure requiring mechanical ventilation and 40 healthy neonates were studied on d 1 of life. All neonates had normal coagulation screening tests and a platelet count greater than $150 \times 10^9/L$. Plasma pools from neonates with similar gestational age (GA), birth weight, and health status were prepared. Eight plasma pools from 40 healthy neonates of GA 30–38 wk were compared with six plasma pools from 30 sick neonates of GA 30–38 wk. An additional four plasma pools prepared from 20 sick neonates of GA <30 wk were studied. Thrombin generation was measured by amidolysis of a chromogenic substrate, S2238, after defibrination, contact activation, and recalcification of the test plasmas. The contributions of antithrombin III, heparin cofactor II, and α_2 -macroglobulin as inhibitors of ^{125}I -thrombin were quantitated by SDS-PAGE followed by autoradiography and densitometry. Thrombin generation was similar for both healthy and sick neonates of GA 30–38 wk. However, the inhibition of thrombin was impaired in plasma from sick neonates of GA 30–38 wk compared with plasma from healthy neonates of GA 30–38 wk (4.37 ± 0.22 versus 5.21 ± 0.21 nmol; $p < 0.05$). Plasma levels of antithrombin III and α_2 -macroglobulin were significantly lower in sick neonates of GA 30–38 wk compared with healthy neonates of GA 30–38 wk (0.30 ± 0.03 versus 0.41 ± 0.03 U/mL; $p < 0.05$ and 0.93 ± 0.07 versus 1.29 ± 0.10 U/mL; $p < 0.05$, respectively). The sick neonates of GA <30 wk had values similar to sick neonates of GA 30–38 wk for the generation and inhibition of thrombin and for plasma levels of inhibitors antithrombin III and α_2 -macroglobulin. Thus, sick neonates are able to generate thrombin as well as healthy age-matched neonates, but their ability to inhibit thrombin is significantly decreased. This acquired imbalance between the generation and inhibition of thrombin may place the sick neonate at risk for thrombotic complications in the immediate postnatal period. (*Pediatr Res* 31: 391–395, 1992)

Abbreviations

GA, gestational age
ATIII, antithrombin III
HCII, heparin cofactor II
 α_2 M, α_2 -macroglobulin
APTT, activated partial thromboplastin time
PT, prothrombin time
RDS, respiratory distress syndrome
AUC, area under the curve

The coagulation system in healthy preterm and full-term neonates is immature at birth and gradually evolves postnatally toward the mature adult system. The plasma concentrations of both procoagulants and inhibitors differ in healthy neonates compared with adults (1–3). The differences in the concentration of the procoagulants results in diminished ability to generate thrombin in neonates compared with adults (4, 5). Similarly, the concentrations of thrombin inhibitors are also altered, resulting in an impaired ability to inhibit thrombin in neonates compared with adults (6).

However, in healthy neonates, thrombin expression is controlled such that neither hemorrhagic nor thrombotic complications occur. Presumably, the plasma concentrations of coagulants and inhibitors, although significantly different from those of adults, are in a physiologic balance. In contrast, sick neonates with problems such as respiratory distress are prone to develop both hemorrhagic and thrombotic complications. The plasma concentrations of many procoagulants and inhibitors are decreased in sick premature neonates compared with healthy age-matched counterparts (7–15). The net effect of the altered concentrations of coagulants and inhibitors on thrombin regulation in sick neonates has not been studied previously with sensitive techniques that quantitate both the generation and inhibition of thrombin. We hypothesized that the regulation of thrombin was impaired in sick neonates, thereby contributing to the secondary hemostatic complications that these neonates may develop. By studying thrombin regulation in sick neonates, therapeutic strategies directed at reducing hemostasis-related complications could be devised and tested in clinical trials.

MATERIALS AND METHODS

Patient population. Neonates admitted on d 1 of life to McMaster University Medical Centre and St. Joseph's Hospital in Hamilton between March 1st, 1988 and September 30th, 1989 were eligible for the study if they fulfilled the following criteria: 1) GA greater than 24 wk, 2) birth weight greater than 500 g, 3) Apgar score ≥ 5 at 5 min, 4) either clinically normal or with respiratory failure requiring mechanical ventilation immediately after birth, and 5) normal coagulation screening tests [PT, APTT,

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thrombin clotting time (TCT), fibrinogen concentration, and platelet count].

Neonates were excluded if they did not meet the inclusion criteria and for the following reasons: 1) major congenital malformation, 2) culture-proven sepsis, and 3) heparin use before entry into the study. No infants had symptomatic thrombosis, and none were screened for asymptomatic thrombosis. GA was calculated from mother's expected date of confinement and antenatal ultrasound. All neonates received 1 mg of vitamin K intramuscularly before entry into the study. Informed parental consent was obtained, and the study was approved by the Research Advisory Group and Ethics Committee of both McMaster University Medical Centre and St. Joseph's Hospital.

Ninety neonates were studied on d 1 of life. Forty neonates of 30–38 wk GA were classified as healthy. These neonates were clinically normal, breathing room air, on i.v. glucose and electrolyte solutions, and/or establishing oral feeding without evidence of any complications. Thirty sick neonates of 30–38 wk GA and 20 sick neonates of 25–29 wk GA (GA < 30 wk) had respiratory failure requiring mechanical ventilation soon after birth. Of the 50 sick neonates, 45 had a diagnosis of hyaline membrane disease based on clinical and radiologic criteria (16), three had transient tachypnea of the newborn, *i.e.* type II RDS (17), and two had persistent pulmonary hypertension secondary to aspiration syndromes (18). Thus, three patient groups were identified and studied: 40 healthy neonates of GA 30–38 wk, 30 sick neonates of GA 30–38 wk, and 20 sick neonates of GA < 30 wk.

Materials. Arvin (Ancrod) was obtained from Connaught Laboratories, Toronto, Canada. The chromogenic substrate, H-D-Phe-Pip-Arg pNA (S2238), was purchased from Kabi Vitrum, Stockholm, Sweden. Activated partial thromboplastin time (APTT) reagent was purchased from Organon Teknika, Toronto, Canada. Prothrombin time reagent Thromborel S containing Human Thromboplastin was purchased from Behring, Montreal, Canada. Rabbit antibodies for measuring the concentration of ATIII, HCII, and α_2 M in human plasma were obtained from Atlantic Antibodies (NCS Diagnostics Inc., Mississauga, Canada). Fatty acid-free BSA was obtained from Sigma Chemical Co., St. Louis, MO. Human α -thrombin (sp act 2543 U/mg) was kindly provided by Dr. J. W. Fenton II, New York State Department of Health, Albany, NY.

Collection and preparation of plasma samples. Blood samples (1.8 mL) were obtained from all neonates within 12 h after birth. The blood sample was obtained from a dorsal hand vein of healthy neonates using a 1½-inch 21-gauge straight needle with the hub removed. The blood flowed directly into a premarked plastic tube containing 0.13 M sodium citrate (1 part citrate to 9 parts blood). The blood sample was obtained from umbilical arterial catheters of sick neonates into a polypropylene syringe containing 0.13 M sodium citrate. The latter samples were obtained before prophylactic heparin was added to the infusate to maintain umbilical arterial line patency. The blood was then transferred to a plastic tube. The platelet count was measured by a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Platelet-poor plasma was prepared, and the coagulation screening tests consisting of PT, APTT, thrombin clotting time, and fibrinogen were measured using previously described methods (19). The PT was expressed as an International Normalized Ratio (20).

The concentrations of ATIII, α_2 M, and HCII were measured immunologically in each neonate (19). Because of the small volume of individual blood samples, plasma was pooled to measure thrombin generation, prothrombin consumption, and inhibition of thrombin (subsequent section). Eight plasma pools ($n = 5$ per pool) from healthy neonates of GA 30–38 wk were compared with six plasma pools ($n = 5$ per pool) from sick neonates of GA 30–38 wk. Four plasma pools ($n = 5$ per pool) were prepared from the sick neonates of GA < 30 wk and are presented descriptively.

Thrombin generation and prothrombin consumption. Thrombin generation was quantitated using a previously described

technique (4). In brief, plasma was defibrinated by incubation with Arvin, activated by the addition of APTT reagent, and recalcified by the addition of a 0.04 M solution of calcium chloride in Michaelis buffer. The reaction was terminated at timed intervals by subsampling into cold EDTA. Aliquots of EDTA-plasma mixture were incubated in buffer containing the chromogenic substrate S2238. Amidolysis of S2238 was stopped at 10 min by addition of acetic acid. The amount of thrombin generated was quantitated by measuring the absorbance in OD at 405 nm in a spectrophotometer. Total amidolytic thrombin activity was measured for a total of 3 min and at least every 15 s when peak amidolytic activity appeared. Thrombin bound to α_2 M was quantitated by a previously described method and subtracted from the total thrombin activity (4). The amidolytic activities were plotted against time to produce thrombin generation curves for all plasma pools. Because the AUC is directly proportional to thrombin activity, the AUC was determined by use of the linear trapezoidal method (21).

Prothrombin levels in all plasma pools were measured by a standard one-stage prothrombin assay (19) and by ELISA (22). In addition, prothrombin levels were measured by ELISA in each sample to study prothrombin consumption in conjunction with thrombin generation. All assays were performed in duplicate.

Inhibition of thrombin and formation of thrombin inhibitor complexes. In brief, complex formation between 125 I- α -thrombin and plasma inhibitors was quantitated in polyacrylamide gels as previously described (6). Human α -thrombin was iodinated by the lactoperoxidase method using Enzymobead reagent (6). The activity was 4.9×10^5 cpm/U with over 85% of the radioactivity associated with the α -thrombin. Incubation of the iodinated thrombin with adult pool plasma for 5 min demonstrated 85% of total thrombin complexed to the inhibitors. Plasma was defibrinated with Arvin. 125 I-thrombin in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1% BSA was prewarmed to 37°C. This was added to an equal volume of prewarmed, defibrinated plasma (final concentration thrombin 10 mmol/mL). After incubation at 37°C for 90 s, one part of the reaction mixture was added to three parts of loading buffer (5% mercaptoethanol, 10% glycerol, 0.06 M Tris-HCl, 0.01% bromphenol blue, pH 6.8). The samples were boiled for 2 min, cooled on ice, and subjected to electrophoresis on 5–15% gradient polyacrylamide gels containing 0.01% SDS. Staining was performed using 0.1% Coomassie blue RC250 in a solution of 40% methanol and 10% acetic acid. Subsequently, the gels were washed in a solution of 45% methanol and 10% acetic acid and dried under vacuum at 80°C. After autoradiography, the covalent complexes of 125 I-thrombin and its plasma inhibitors were identified and quantitated by scanning densitometry. Previous time course studies done in our laboratory (6) have shown no differences in the relative percentage bound to the inhibitors in newborn or adult plasma.

Statistical analysis. Differences in mean values for prothrombin, thrombin generation and the inhibition of thrombin, and plasma inhibitors ATIII, α_2 M, and HCII between healthy and sick neonates of GA 30–38 wk were analyzed using an unpaired two-tailed *t* test. A *p* value of <0.05 was considered statistically significant. All results were expressed as mean $\pm$ SEM unless otherwise stated. Linear regression analysis with Pearson's coefficient of correlation (γ) was used where appropriate. The data for sick neonates of GA < 30 wk is presented descriptively because a healthy age-matched group of neonates could not be obtained for study.

RESULTS

The demographic characteristics and the coagulation tests for the study populations are given in Table 1. The results of the coagulation screening tests were within the normal range for gestational and postnatal age for all neonates. Healthy and sick

Table 1. *Demographic characteristics and coagulation parameters of study population**

	Healthy (n = 40)	Sick (n = 30)	Sick (n = 20)
GA	30–38 wk	30–38 wk	<30 wk
BW (g)	2306 ± 118	2244 ± 140	1098 ± 75
INR	1.3 ± 0.02	1.3 ± 0.04	1.3 ± 0.03
APTT (s)	48 ± 2.1	47 ± 2.2	49 ± 2.2
TCT (s)	24 ± 0.42	24 ± 0.56	24 ± 0.68
FBG (g/L)	2.8 ± 0.2	2.98 ± 0.17	2.66 ± 0.3
ATIII (U/mL)	0.41 ± 0.03	0.30 ± 0.03†	0.28 ± 0.03
α_2 M (U/mL)	1.29 ± 0.10	0.93 ± 0.07†	0.78 ± 0.05
HCII (U/mL)	0.18 ± 0.01	0.17 ± 0.01	0.17 ± 0.01

* Results are expressed as the mean ± SEM. BW, birth wt; TCT, thrombin clotting time; and FBG, fibrinogen. PT expressed as an international normalized ratio (INR).

† A *p* value less than 0.05 was considered statistically significant. Plasma concentrations of ATIII and α_2 M were significantly lower in sick neonates of GA 30–38 wk compared with healthy neonates of GA 30–38 wk. Plasma concentrations of all three inhibitors were similar for sick neonates of GA <30 wk and sick neonates of GA ≥30–38 wk.

neonates of GA 30–38 wk were similar for mean GA and birth weight.

Thrombin generation and prothrombin consumption. The total amount of thrombin generated, expressed as either peak amidolytic activity or as AUC, was similar in plasma from both healthy and sick neonates of GA 30–38 wk (Table 2). The initial prothrombin levels as measured by both functional and ELISA assays were significantly lower in plasma from sick neonates of GA 30–38 wk compared with healthy neonates of GA 30–38 wk (Table 2). By 75 s, mean prothrombin levels were <0.05 U/mL. The time point of maximum thrombin generation coincided with the time of maximum prothrombin consumption (data not shown). Plasma from sick neonates of GA <30 wk had values similar to those of sick neonates of GA 30–38 wk for peak thrombin generated, AUC, and prothrombin levels (Table 2).

Inhibition of thrombin and detection of thrombin-inhibition complexes. The total amount of thrombin inhibited was significantly less in plasma from sick neonates of GA 30–38 wk compared with healthy neonates of GA 30–38 wk (Table 2). Plasma from sick neonates of GA <30 wk inhibited thrombin in amounts similar to sick neonates of GA 30–38 wk. The relative proportions of thrombin complexed to the inhibitors ATIII, α_2 M, and HCII were similar in plasma from healthy and sick neonates of GA 30–38 wk. Unexpectedly, the amounts of thrombin complexed to HCII and ATIII in plasma from sick neonates of GA

<30 wk were increased over the values for healthy and sick neonates of GA 30–38 wk.

Figure 1 is a representative gel showing the pattern of thrombin inhibition and thrombin-inhibitor complex formation in plasma from all three groups of neonates. Thrombin was inhibited less effectively in plasma from both groups of sick neonates, and thus plasma from both groups of sick neonates had significantly more uncomplexed thrombin compared with healthy neonates. We next determined whether differences in the plasma concentration of inhibitors could provide an explanation for these observations.

Plasma levels of inhibitors ATIII, α_2 M, and HCII. Table 1 summarizes the levels of the three inhibitors measured on individual plasma samples from all healthy and sick neonates. Plasma levels of ATIII, HCII, and α_2 M were measured on adult and cord pooled plasma before and after Arvin, and no significant difference was observed (data not shown) as a result of Arvin treatment. Plasma from sick neonates of GA 30–38 wk had significantly lower levels of both ATIII and α_2 M compared with the healthy neonates of GA 30–38 wk. HCII levels were not significantly different between these two groups. The plasma levels of all three inhibitors were similar for sick neonates of GA <30 wk and sick neonates of GA 30–38 wk. Linear regression analysis showed a highly significant correlation between complexed thrombin and plasma inhibitor levels of ATIII ($\gamma = 0.75$, $p < 0.001$ and α_2 M $\gamma = 0.69$ $p < 0.01$, respectively) for both healthy and sick neonates of GA 30–38 wk. There was no correlation between complexed thrombin and plasma HCII levels in both groups.

DISCUSSION

In many disease states in neonates, including RDS, the circulating plasma concentrations of coagulation factors are decreased compared with healthy neonates (7–15). One of the most consistently reported alterations in RDS is low plasma levels of the inhibitor ATIII, probably secondary to consumption (23–25). There is also some evidence to show that plasma levels of prothrombin are lower in the presence of moderate to severe RDS (8, 26). The overall effect of the alterations in the hemostatic system on the ability to generate and inhibit thrombin has not been characterized previously with sensitive techniques. We chose to study the generation and inhibition of thrombin because thrombin is a key enzyme in the coagulation system. The current study assessed these parameters in neonates with respiratory distress and compared the results when possible with those for healthy age-matched counterparts.

The ability of plasma from sick neonates with respiratory distress to inhibit thrombin was significantly impaired compared

Table 2. *Thrombin generation, thrombin inhibition, and thrombin inhibitor complex formation**

	Healthy (GA 30–38 wk)	Sick (GA 30–38 wk)	Sick (GA <30 wk)
Thrombin generation			
Peak thrombin activity Abs (405 nm)	0.31 ± 0.01	0.30 ± 0.01	0.29 ± 0.01
AUC (Abs at 405 nm/s)	23.1 ± 0.9	23.0 ± 0.9	23.1 ± 1.6
Prothrombin ELISA (U/mL)			
Pre	0.50 ± 0.02†	0.39 ± 0.03†	0.38 ± 0.02
75 s	0.01 ± 0.006	0.04 ± 0.009	0.04 ± 0.004
Prothrombin functional (U/mL)	0.47 ± 0.02	0.37 ± 0.01†	0.36 ± 0.02
Thrombin inhibition			
IIa-inhibition (nmol)	5.21 ± 0.21	4.37 ± 0.22†	4.15 ± 0.25
IIa-ATIII (nmol) (%)	3.06 ± 0.11 (59)	2.69 ± 0.11 (62)	2.79 ± 0.12 (67)
IIa- α_2 M (nmol) (%)	1.76 ± 0.17 (34)	1.37 ± 0.21 (31)	0.85 ± 0.11 (21)
IIa-HCII (nmol) (%)	0.38 ± 0.06 (7)	0.31 ± 0.04 (7)	0.51 ± 0.06 (12)

* Results are expressed as the mean ± SEM. Abs, absorbance; IIa, thrombin. Healthy and sick neonates of GA 30–38 wk had similar peak thrombin activity and AUC for thrombin activity. Sick neonates of GA <30 wk had all levels similar to those of sick neonates of GA 30–38 wk. Plasma from sick neonates of GA 30–38 wk and sick neonates of GA <30 wk inhibited similar amounts of thrombin. Plasma from sick neonates of GA <30 wk had greater HCII-IIa complex formation compared with both healthy and sick neonates of GA 30–38 wk.

† A *p* value less than 0.05 was considered statistically significant.

Fig. 1. A SDS-PAGE of ^{125}I - α -thrombin (IIa) alone (lane 1) or added to pooled plasma from healthy neonates of GA 30–38 wk (lane 2), sick neonates of GA 30–38 wk (lane 3), and sick neonates of GA <30 wk (lane 4). In plasma, the thrombin complexed with three inhibitors, ATIII, HCII, and $\alpha_2\text{M}$. The smaller size band of free IIa in lane 2 (healthy neonates' sample) relative to lanes 3 and 4 (sick neonates' plasma) indicates that there is more free IIa in sick neonates' plasma. This demonstrates that plasma from healthy neonates inhibited thrombin more effectively than plasma from sick neonates. Sick neonates of GA <30 wk showed a high proportion of thrombin complexed to HCII.

with that from healthy counterparts. This was due, at least in part, to the decreased concentration of plasma inhibitors of thrombin. The highly significant correlation between complexed thrombin and plasma levels of the inhibitors ATIII and $\alpha_2\text{M}$ supports the concept that the plasma concentrations of these inhibitors are critical to the effective inhibition of thrombin. These conclusions are in agreement with previous studies of thrombin inhibition in which the concentrations of the inhibitors were raised (27).

In contrast to the inhibition of thrombin, the results of our studies show that the overall ability of plasma from sick neonates ventilated for respiratory distress to generate thrombin remains similar to that of healthy neonates. The latter result was unexpected because recent controlled experiments of exogenous prothrombin supplementation to cord plasma and to plasma from patients on Coumadin (Du Pont Pharmaceuticals, Wilmington, DE) have shown that thrombin generation is critically dependent on prothrombin levels (5, 28). The plasma prothrombin levels in sick neonates were lower than those in healthy neonates; therefore, we anticipated that the generation of thrombin would be decreased. However, plasma from sick neonates of all ages in our study generated similar amounts of thrombin compared with plasma from healthy neonates despite a lower level of prothrombin. The likely explanation is that the thrombin generation curve is the net effect of both prothrombin conversion to thrombin and subsequent neutralization of thrombin by the inhibitors ATIII, $\alpha_2\text{M}$, and HCII. Because the levels of ATIII and $\alpha_2\text{M}$ were significantly lower in plasma from sick neonates compared with healthy neonates, it seems likely that the lower concentration of inhibitors was reflected in the thrombin generation curve.

In this study, plasma from sick neonates of GA <30 wk had results similar to sick neonates of GA 30–38 wk with one exception. Sick neonates of GA <30 wk complexed greater amounts of thrombin to HCII than sick neonates of GA 30–38 wk despite similar plasma levels of HCII, $\alpha_2\text{M}$, and ATIII. The latter observation may be explained by the presence of a recently described glycosaminoglycan in cord blood, dermatan sulfate

(29). Dermatan sulfate potentiates the inhibition of thrombin by the natural inhibitor HCII. It is quite possible that in sick neonates the ability to clear the fetal glycosaminoglycan is impaired due to compromised renal function, leading to increased thrombin binding by HCII. Further studies are being conducted to support or refute this hypothesis.

Nearly all sick neonates in this study had RDS. RDS is a disorder characterized by surfactant deficiency and injury to alveolar epithelium and the capillary bed in the lungs (30). Although decreased surfactant is an important contributing feature of RDS, the replacement of surfactant improves only 50 to 70% of neonates (31). This suggests that other pathophysiologic features contribute to RDS. The pathologic features of both intravascular and intraalveolar fibrin deposition provide evidence for activation of the coagulation system in RDS (32–34). In the current study, we provide evidence that sick neonates with RDS maintain their ability to generate thrombin in amounts comparable to those generated by healthy counterparts. However, the ability to inhibit thrombin is significantly impaired in sick neonates, perhaps contributing to fibrin deposition in diseases such as RDS. In addition, this imbalance between the generation and inhibition of thrombin may predispose sick neonates to thrombotic events in the immediate postnatal period with its attendant complications. These observations provide a biologic rationale for exploring the prophylactic and therapeutic role of modalities such as low-dose anticoagulants and/or ATIII concentrates in sick neonates at risk for disorders characterized by abnormal fibrin deposition.

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