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Antithrombin III levels in term and preterm infants measured by rate nephelometry

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The plasma antithrombin III level was measured in term and premature newborns within the first 24 h of life; in addition to the authors', own immunological method utilizing rate nephelometry the assays of Laurell and Kabi Coatest were used. Antithrombin III concentration showed a linear correlation with the degree of maturity, in accordance with previous data. The lowest values were found in premature babies affected by idiopathic respiratory distress syndrome who later developed pulmonary haemorrhage. At all gestational ages the antithrombin III functional index was lower than 1.0, indicating antithrombin III consumption. Since both concentration and activity of antithrombin III are low in prematures, they are at an increased risk for coagulopathies, hence substitution therapy is indicated. Measurement of antithrombin III by rate nephelometry can also be used for following changes in inhibitor concentration.

Haemorrhage, local or generalized, is a significant factor in morbidity and mortality in the neonatal period. In the majority of cases affected by bleeding tendency some kind of deficiency in haemostasis can be demonstrated. Its clinical manifestations are manyfold: diffuse purpura, melaena, haematuria, massive pulmonary haemorrhage (MPH) and intracranial haemorrhage (ICH). The low level of the vitamin K dependent II, VII, IX, X and contact factors in preterm and term newborns is well known. It contributes to the proneness of pathological newborns to disseminated intravascular coagulation (DIC). Due to their vulnerable metabolism and underdeveloped reticuloendothelial system, adequate circulation in the microvasculature is difficult to be maintained in newborns afflicted by any severe disorder. During the newborn period the hepatic functions are immature, and this may lead to impaired compensatory synthesis of circulating anticoagulants and their inhibitors [11, 21]. MPH and ICH are not necessarily caused by a haematological disorder but DIC can be demonstrated in all infants affected by them. Intraventricular haemorrhage (IVH) is ultimately due to the weakness of the periventricular capillaries of the germinal matrix and to the damaged autoregulation of cerebral circulation of the newborn [13, 22]. The aetiological factors precipitating MPH are also the risk factors capable of deepening the severity of IVH: respiratory distress syndrome, asphyxia, acidosis,

infection, hypoglycaemia, hypercapnia, jaundice, oxygen toxicity, congenital malformations of the heart, fetal distress and adverse birth conditions [12, 20]. DIC is the result of all these circumstances [16].

DIC plays a central part in perinatal pathophysiology. The basic processes in its background are accelerated procoagulant activity and pathological generation of thrombin and other serine proteases. Antithrombin III (AT III), the inhibitor of plasma serine proteases, plays an important role in the pathogenesis of DIC and other neonatal conditions with bleeding tendency. AT III is a linear glycoprotein having a molecular weight of about 65,000 [15]. It has an inhibitory action not only on thrombin but also on other serine proteases formed during clotting, like factors X*a*, IX*a*, XI*a*, XII*a*, plasmin and callicrein [9, 19, 24]. The serine protease receptor site on the AT III molecule is a structure containing arginine, which can be linked to the active serine of the coagulants. This reaction is slow but can be speeded up 2000-fold by heparin; the latter is attached to the lysine of the AT III molecule, causing thereby configurational changes in it [3]. In addition to this main anticoagulant effect heparin also exerts a minor anticoagulant action by disrupting the complex of factor X and prothrombin activator [17]. In spite of this the central target of heparin is AT III. Serial determination of AT III in term and preterm newborns gains importance by the fact that the equilibrium between serine proteases and AT III is established at a low level.

AT III measurement methods belong to either of two groups: functional and immunological assays. The functional methods are based either on coagulation or on synthetic substrate assays. The coagulation assay works on the following principle: the sample to be tested is incubated with a known quantity of thrombin in excess, so that an AT III-thrombin complex is formed. After incubation the residual thrombin is retitrated by human plasma clotting and the initial AT III concentration is calculated. The synthetic substrate assays result in either chromogenic or fluorogenic compounds. The basic reactions of this technique are as follows [6, 7]:

AT III + heparin → (AT III–heparin)

(AT III–heparin) + thrombin → → (AT III–heparin–thrombin) + + thrombin residue

thrombin residue + R-pNA → R-OH + pNA,

where R-pNA is a synthetic chromogenic substrate.

The more exact immunological assays measure both the functioning and non-functioning forms of AT III. In contrast to the rapid synthetic substrate assays they are rather slow and cumbersome. Therefore we have elaborated an immunological rate nephelometry method which is rapid. The reaction time in this is one minute, a useful feature in AT III determinations in immature babies with bleeding tendency.

In the present study the level and course of AT III of term and preterm newborns were investigated. AT III was measured by our own rapid rate nephelometry method and by the immunological assay of Laurell and, in addition, by a functional method, the Kabi Coatest, based on the use of a synthetic chromogenic substrate.

AT III levels were also measured in infants affected by IRDS and MPH.

Materials and Methods

Samples

Citrated plasma was used. The blood was mixed with 3.8% trisodium citrate (9 volume blood with 1 volume citrate), the plasma was separated by centrifugation (3000 *g*, 15 min, 4°C), aspirated and kept at −38°C.

Pooled normal human plasma obtained from 20 healthy adults was used as a standard. Calibration was performed with the standard plasma, and the obtained AT III level was taken as 100%.

Patients

Plasma AT III was measured in 47 newborns admitted to our department. Blood was taken from a peripheral vein within 24 h after birth. The newborns were grouped according to gestational age and disease, the data of patients affected by IRDS and MPH were treated separately.

There were 12 healthy term newborns. Their mean gestational age was 39.2 ± ± 1.1 weeks, their mean birthweight, 3203 ± 395 g. Thirteen premature infants without IRDS had a mean gestational age of 32.1 ± 3.6 weeks and a mean birthweight of 1415 ± 541 g. Twenty-two premature babies were affected by IRDS, their mean gestational age was 30.3 ± 2.5 weeks and the mean birthweight was 1450 ± ± 381 g. Eight of them had also MPH; all of these infants died and necropsy revealed alveolar haemorrhage in all of them. The mean gestational age of these eight patients was 29.5 ± 2.4 weeks, their mean birthweight was 1356 ± 331 g.

Methods

Rate nephelometry

Our immunological method for AT III determination has been described in detail elsewhere [1]. In summary, the measurements were performed in a Beckman rate nephelometer (Beckman Immunochemistry System, Fullerton, Ca—ICS) by manual mode, utilizing M—CAL and M-22 Manual Mode programme cards.

A Behring antiserum (Behring OTPV 04/05) was used as anti-AT III antibody. The calculated antibody dilution was 1:2.5. First a calibration curve was constructed using pooled normal human plasma. The results obtained from the patients' samples were calculated by help of the calibration curve. The optimum sample dilution was 1:12. 20 µl plasma was needed for double determination, the nephelometric value measured in the pooled normal adult plasma was taken as 100% of AT III concentration. The results obtained in the patients' samples were expressed as percentages. According to the literature 100% corresponds to 0.30 g AT III/liter plasma [7, 8].

Functional AT III assay

For the measurement of heparin cofactor activity the Kabi Coatest kit based on the amidolytic method was used.

Laurell's immunological method

This one-dimensional immunoelectrophoretic technique was carried out as described in the literature [26]. The results were expressed in percentage of the pooled adult plasma content.

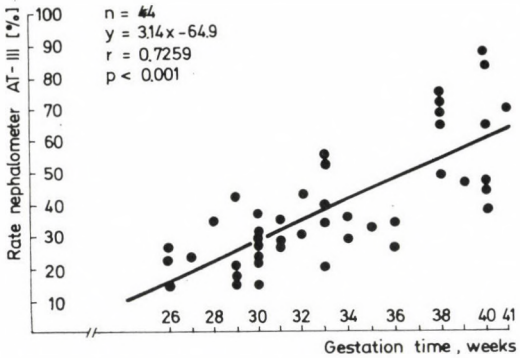

FIG. 1. Plasma AT III, measured by rate nephelometry, plotted against gestational age

Statistics

Student's t test was used for significance calculations between means; the relationship between two variables was characterized by the correlation coefficient.

RESULTS

Plasma AT III levels measured by rate nephelometry in 44 newborns were correlated to gestational age (Fig. 1). There was a fairly close correlation between the two variables ($r = 0.72$). In the most immature babies about one fifth of the adult AT III level was found and even the healthy term babies did not attain the adult mean. Patients affected by IRDS and MPH had a significantly lower level (Fig. 2). The mean AT III level of term babies was about 2/3, that of all preterms about 1/3 of the adult level. The lowest levels (mean: 23.4%) were encountered in prematures affected by MPH. In 35 newborns AT III was measured also by

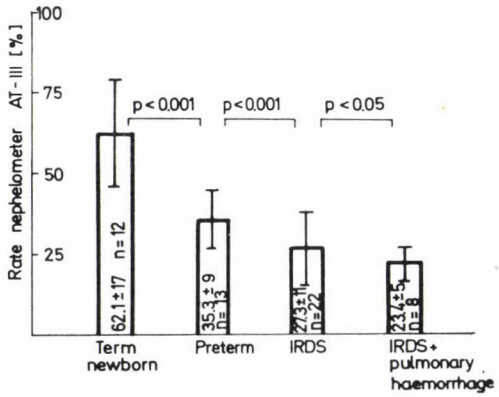

FIG. 2. Plasma AT III, measured by rate nephelometry, in healthy term newborns, in unaffected preterm babies and in preterm babies affected by idiopathic respiratory distress syndrome alone or complicated by massive pulmonary haemorrhage

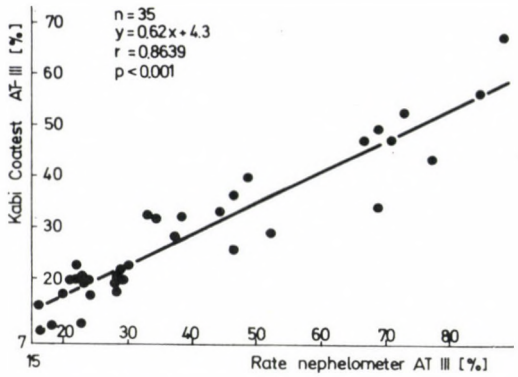

FIG. 3. Relationship between AT III values obtained by rate nephelometry (immunological assay) and those yielded by Kabi Coatest (functional assay)

the functional Kabi Coatest assay. The correlation between the two methods was satisfactory (r = 0.86, see also Fig. 3).

All heparin-cofactor activity values were lower than the corresponding individual nephelometric values in all gestational age groups. If both non-functional and functional methods are available, specific activity or the functional index (quotient of the percentual values obtained by the functional resp. immunological assay) can be calculated [6]. The quotient of the Coatest result per the result obtained by nephelometry (Fig. 4) termed functional index, was invariably less than 1.0, the mean value being 0.77. A very close correlation was found between the results obtained by the two immunological methods (r = = 0.91, see Fig. 5).

DISCUSSION

Several compounds with antithrombin activity have been described but only three are of importance: AT III, fibrin, and fibrinogen-fibrin degradation products. AT III is mainly pro-

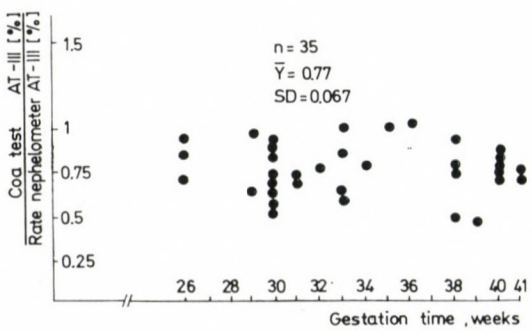

FIG. 4. Relationship between the AT III functional index and gestational age

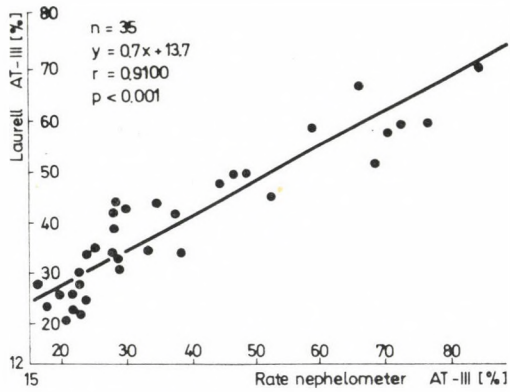

FIG. 5. Correlation between the AT III levels obtained by Laurell's and the rate nephelometric immunological methods

duced by the liver, endothelial cells and perhaps by megakaryocytes [5]. A low level may induce pathological clotting in the case of hereditary deficiency, deep vein thrombosis, pulmonary embolism, coronary disease renal and hepatic diseases, and DIC [2]. There are "physiological" low levels in newborns. Our data obtained by rate nephelometry agreed well with results published in the literature, i. e. 28 % in prematures, 40–54–87 % in term newborns [4, 14, 23]. The lowest AT III levels were encountered in very immature babies, the inhibitor is thus lacking just in the population at highest risk of severe bleeding disorders. This was confirmed by our own finding of very low levels in the group subsequently developing massive pulmonary haemorrhage.

AT III proteins of various forms and different activity may be present in the plasma [18, 25]. The depressed functional activity is, however, mainly due to partial denaturation of AT III and to slow release of the already inactive AT III from the thrombin — AT III complex [10]. This fact was probably the cause of our finding lower Coatest values than the nephelometric ones; the overall low mean value of the functional index (0.77) supports this assumption. Even in healthy term newborns AT III consumption was higher than in adults, and the inactive AT III released from the complexes maintained a higher level measurable with immunological methods than the values obtained with the functional Coatest.

In addition, deficient hepatic production of AT III may aggravate the situation, especially in premature infants. Increased protein catabolism may be an aspecific cause of AT III losses.

Low levels of AT III may be an important risk factor in immature babies, in whom one-fourth of the immunologically measurable level is inactive. Administration of AT III concentrates may be of great help in all newborns at risk of DIC, or where

protein synthesis is impaired or protein breakdown augmented.

Our new immunological method performed by rate nephelometry seems to be useful in measuring or monitoring AT III levels parallel with functional determinations.

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