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Biochemical Abnormalities of the Third Component of Complement in Neonates[1]

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ABSTRACT. The third component of complement, C3, is of central importance as an opsonin in the nonimmune host. Although gestational deficiencies in C3 levels are well recognized in neonates, defects in complement-mediated functions have not in every case correlated with low levels of complement proteins. Because opsonic functions of C3 are mediated through a reactive thiolester bond, we hypothesized that a biochemical dysfunction at this active site could explain the newborn's predisposition to infection, even with relatively normal C3 levels. We therefore examined the biochemical integrity of the C3 thiolester in an assay independent of all other complement proteins. As measured by ELISA, mean C3 levels from 44 neonates (24–43 wk) were significantly lower in infants < 30 wk gestational age (0.79 ± 0.13 mg/mL) than in full-term newborns (1.19 ± 0.27 mg/ml, $p < 0.05$). Furthermore, biochemical reactivity of the thiolester bond, as measured by incorporation of the radiolabeled nucleophile, methylamine, correlated significantly with gestational age ($r = 0.45$, $p < 0.01$). Functional C3 was defined as the product of thiolester reactivity and C3 level; 9/11 premature and 2/17 full-term infants had levels of functional C3 which were less than 50% of the adult norm. Structural analysis of neonatal C3 revealed the two-chain structure in all neonates; four neonates had an additional band at 205 kD which may represent an impairment in posttranslational processing of a precursor molecule. We conclude that defects in thiolester reactivity may constitute a newly identified mechanism for the newborn's susceptibility to infection. (*Pediatr Res* 26: 116–120, 1989)

Abbreviations

PAS, pooled adult serum
CH_3NH_2, methylamine

Sepsis is a leading cause of morbidity and mortality in neonates, with premature infants affected more frequently than full-term newborns (1). This predisposition to infection is ascribable, at least in part, to age-related defects in host defense (2, 3). Primary among humoral defenses are the complement proteins, which serve as opsonins for common neonatal pathogens such as the group B Streptococcus or the K1 capsular serotype of *Escherichia coli* (4, 5).

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The third component of complement, C3, is the pivotal protein in the opsonization of bacteria by either the classical or the alternative complement pathway. Binding of C3b to the bacterial surface constitutes the central mechanism of antibody-independent opsonization and initiates complement-dependent lysis or phagocytosis. The opsonic functions of C3 are mediated through an internal thiolester bond, which upon activation binds covalently to hydroxyl or amino groups on the microbial surface (6). The presence of the reactive thiolester bond is essential for the opsonic and covalent binding of C3b to pathogenic bacteria such as the type III group B Streptococcus (7).

Previous studies have demonstrated quantitative deficiencies in C3 and other complement proteins in the neonate (8–12). In addition, function of the newborn's complement system is also impaired and may increase the risk of infection (9–13). For example, Cairo *et al.* (14) have recently shown that a low CH50, a measure of the hemolytic activity of complement, is the most significant determinant of mortality in septic newborns, regardless of gestational age.

Because of the central role of C3 in opsonization, we have examined C3 functional activity per milligram of protein in a manner independent of all other complement proteins. This method permits the ready identification of C3 dysfunction per se and facilitates the analysis of structural aberrancies as well.

MATERIALS AND METHODS

Serum. Serum was obtained from cord blood or venous access in 50 neonates, aged 24–43 wk gestation, within the first 3 d of life. Review of each infant's hospital chart indicated no clinical or microbiologic evidence of infection. PAS obtained from six nonpregnant adults served as control. Serum was separated by centrifugation and frozen in aliquots at −70°C until testing. The study was approved by the University of Minnesota's Committee on the Use of Human Subjects in Experimentation.

ELISA assay for C3. Purified human C3 was isolated from fresh pooled plasma by the method of Tack *et al.* (15). Sheep antihuman C3 was a kind gift from Dr. B. F. Tack (La Jolla, CA). Goat anti-human C3 (Atlantic Antibodies, Scarborough, ME) was conjugated to horseradish peroxidase by the periodate method (16). Methods for the ELISA assay, a modification of that developed by Voller *et al.* (17), have been published previously (18). Briefly, wells of a microtiter plate were coated with a 1:400 dilution of sheep antihuman C3 in carbonate/bicarbonate buffer (pH 9.6), incubated overnight at 4°C, and washed three times with 0.05% Tween 20 in PBS. Serum was serially diluted 1:5000 to 1:20,000 in 1% BSA/1% Tween 20/PBS, added to duplicate wells in a volume of 0.1 mL, and incubated for 1 h at 37°C. After three washes, 0.1 mL of a 1:800 dilution of peroxidase-conjugated goat antihuman C3 was added to each well, and the incubation was continued for 1 h at 37°C. The developing reagent consisted of 0.4 mg/mL of *o*-phenylenediamine in 0.2 M Na_2HPO_4 (pH 5.0 with 0.1 M citric acid), containing 0.4 μL

35% H_2O_2/mL. Color was developed for approximately 25 min. Absorbance was measured at 405 nm in a Titertek Multiskan ELISA Reader (Flow Laboratories, McLean, VA). The standard curve was calibrated with known concentrations of purified human C3. As additional verification, concentrations of C3 in PAS and five neonatal serum samples were measured by radial immunodiffusion in the clinical laboratories of the University of Minnesota Hospital (19). As shown in Table 1, there was no significant difference between mean C3 levels in neonates as determined by ELISA (1.20 ± 0.38) and RID (1.18 ± 0.30, p = 0.67).

Biochemical assay for reactivity of C3 thiolester bond. Nucleophilic amines, such as CH_3NH_2, are inherently reactive with thiolester bonds; binding of the nucleophile is stoichiometric, with 1 mol of the nucleophile covalently bound for each mol of thiolester disrupted (20). Titration of thiolester reactivity was performed by a modification of the procedure of Tack *et al.* (20) and Hostetter *et al.* (21). Briefly, 0.45 mL of serum from 34 neonates was incubated with 0.01 mL of 1 M CH_3NH_2 (Sigma Chemical Co., St. Louis, MO) in 0.1 M TRIS/0.01 M EDTA/ pH 8.0; to this was added 0.01 mL of $^{14}CH_3NH_2$ (Amersham, Arlington Heights, IL) which had been diluted with unlabeled CH_3NH_2 to a specific activity of 2.6 mCi/mmol. A 20-fold molar excess of 0.44 M iodoacetamide (Sigma) in TRIS/EDTA, pH 8.0, was added to prevent disulfide bond formation. The final volume of the reaction mixture was 0.522 mL. For each day's experiments, 0.45 mL of PAS, identically treated, served as a control. After 2-h incubation in a water bath at 37°C, C3 was then separated from other serum proteins by affinity chromatography on cyanogen-bromide activated Sepharose-4B (Pharmacia, Piscataway, NJ) which had been coupled to affinity purified goat antihuman C3 (Atlantic Antibodies), according to manufacturer's instructions. Bound C3 was eluted with 0.02 M HCl, and the concentration of C3 in each fraction was determined spectrophotometrically at 280 nm. Eluates containing C3 were then pooled and exhaustively dialyzed against 0.1% SDS in PBS to remove noncovalently bound CH_3NH_2. Incorporation of $^{14}CH_3NH_2$ was quantitated with a Beckman LS scintillation counter (Beckman Industries, Fullerton, CA). Reactivity of the C3 thiolester bond was expressed as mol of $^{14}CH_3NH_2$ incorporated per mol C3 eluted and was thus independent of the serum level of C3. Comparative analysis of the C3 thiolester reactivity of serum obtained from cord blood and venous sampling from four premature neonates showed no differences attributable to source of the serum (88 ± 1.9 *versus* 92 ± 16.9%, p = 0.69).

C3 hemolytic assay. Neonatal C3 dysfunction as measured by C3 level and thiolester bond reactivity was confirmed in six premature neonates by a C3 hemolytic assay (Cordis Laboratories, Miami, FL). In brief, serial serum dilutions were incubated in the presence of preformed EAC14 and excess C2 and C5–9. Hemolysis was measured spectrophotometrically at 415 nm, and the C3 hemolytic titer was determined.

Structural analysis of C3. C3 purified from serum by affinity chromatography was analyzed by SDS-PAGE in a 6% polyacrylamide gel under reducing conditions according to the procedure of Laemmli (22) and stained with a commercial silver stain (Bio-Rad, Richmond, CA). In other experiments, SDS-PAGE gels were transferred to nitrocellulose paper (Sigma) in a 40-min transfer, according to the conditions of Towbin *et al.* (23). The transferred proteins were visualized by immunodetection using a biotin-avidin-peroxidase system as previously described (6). Nonspecific binding sites were blocked by incubation with 1% BSA. The nitrocellulose was then incubated sequentially at 23°C with 1:250 (v/v) goat antihuman C3 for 1 h, 1:500 (v/v) biotin conjugated rabbit antigoat IgG (Cappel Laboratories, Malvern, PA) for 40 min, and 1:500 (v/v) peroxidase-conjugated avidin (Cappel) for 30 min. Three 5-min washes with PBS/0.05% Tween 20 were performed after each incubation, and the blot was then developed with 1.1 mM 3,3′-diaminobenzidine (Sigma) in 50 mM TRIS/0.3% H_2O_2 (pH 7.4).

Statistical analysis. The two-tailed Student's t test, ANOVA, and linear regression analysis for determination of the coefficient of correlation were used for statistical analysis. Results are expressed as mean ± SD and $p < 0.05$ was considered statistically significant.

Table 1. *C3 concentration by ELISA and radial immunodiffusion (RID)*

Subject	ELISA (mg/mL)	RID (mg/mL)
Neonate 1	0.86	0.92
Neonate 2	0.83	0.75
Neonate 3	1.57	1.34
Neonate 4	1.13	1.08
Neonate 5	1.63	1.50
PAS	1.68	1.81

RESULTS

Concentrations of C3 in neonatal and adult sera. Levels of C3 in serum from 44 neonates ranged from 0.49 to 1.74 mg/mL. Concentration of C3 in PAS was 1.68 mg/mL. As shown in Figure 1, mean C3 levels were significantly lower in infants <30 wk gestation (0.79 ± 0.13 mg/mL) than in full-term newborns (1.19 ± 0.27 mg/mL, $p < 0.05$). C3 concentrations at 30–37 wk gestation (1.02 ± 0.37 mg/mL) were not significantly different from either group.

Reactivity of C3 thiolester. Reactivity of the C3 thiolester was quantitated by incorporation of the radiolabeled nucleophile $^{14}CH_3NH_2$. CH_3NH_2 incorporation was measured daily in PAS and ranged from 0.88–0.96 mol of methylamine/mol of C3.

Incorporation of CH_3NH_2 in neonatal C3 is shown in Figure 2. Thiolester reactivity correlated significantly with gestational age (r = 0.45, $p < 0.01$). All of the premature neonates exhibited reduced thiolester reactivity when compared to PAS (mean =

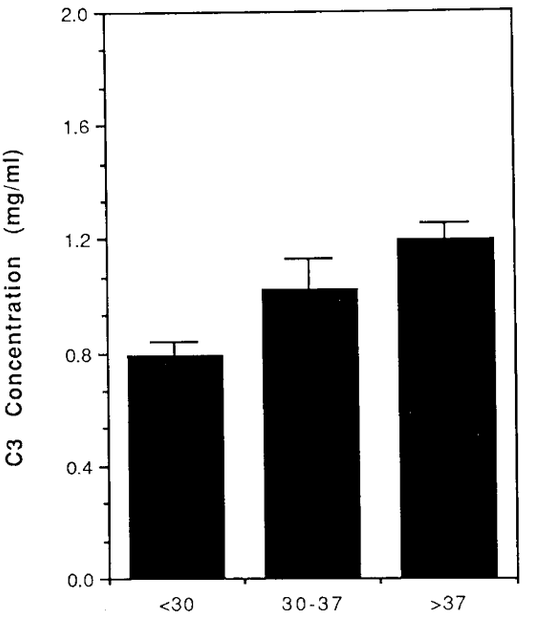

Fig. 1. Mean C3 concentration by ELISA assay in neonates <30 wk (n = 8) was significantly lower than neonates >37 wk gestation (n = 23, $p < 0.05$). Neonates 30–37 wk gestation (n = 13) were not significantly different from either group. C3 concentration in PAS was 1.68 mg/mL. Values are expressed as mean ± SE.

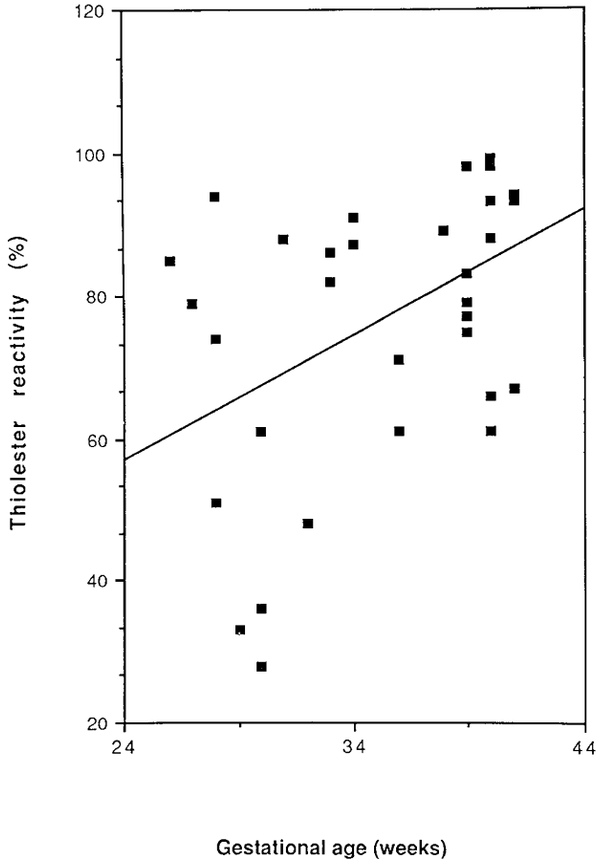

Fig. 2. Reactivity of the C3 thiolester bond in neonates quantified by incorporation of $^{14}CH_3NH_2$. Reactivity of the C3 thiolester correlates with gestational age ($r = 0.45$, $n = 34$, $p < 0.01$).

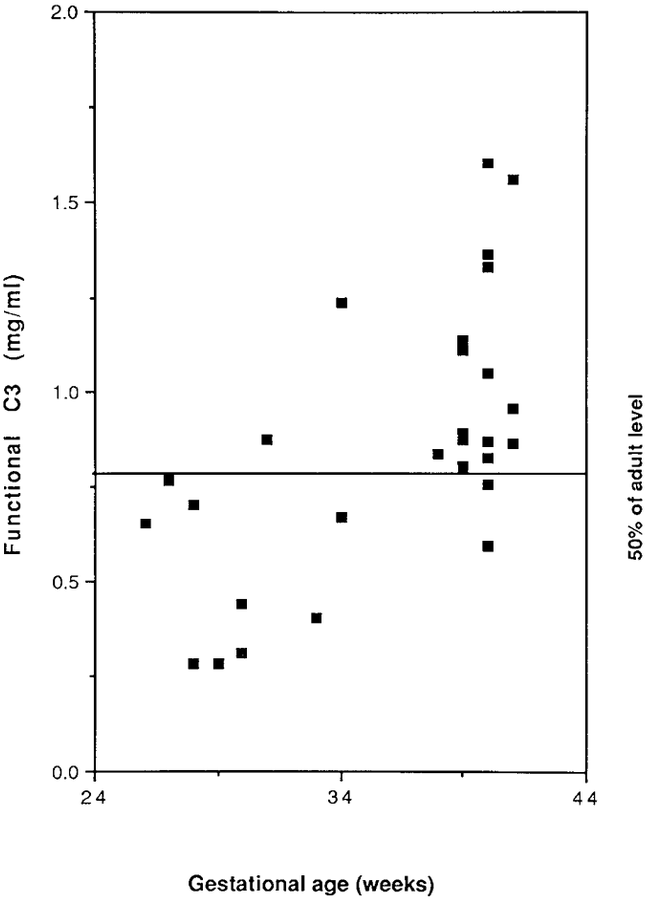

Fig. 3. Correlation of functional C3 with gestational age ($r = 0.63$, $n = 28$, $p < 0.001$). The *horizontal line* indicates 50% of the adult norm.

69% ± 22%). In full-term newborns, methylamine incorporation ranged from 61–99% (mean = 85% ± 12%).

Functional C3. We defined functional C3 as the product of C3 concentration and thiolester reactivity. Therefore, for a given concentration of C3, this value represents the amount that is functionally active. Functional C3 also correlated well with increasing gestational age ($r = 0.63$, $p < 0.001$) (Fig. 3). Moreover, nine of 11 premature infants and two of 17 term newborns had concentrations of functional C3 which were less than 50% of the adult norm. A C3 hemolytic assay provided verification of neonatal C3 dysfunction in six premature neonates. The C3 hemolytic titers expressed as the neonatal/adult ratio did not differ from the neonatal/adult ratio of calculated functional C3 (respectively, mean ± SE = 0.67 ± 0.22 *versus* 0.62 ± 0.06, $p = 0.78$).

Structural analysis of neonatal C3. The structure of neonatal C3 isolated from 31 newborns was examined by 6% SDS-PAGE under reducing conditions. All patients demonstrated the two-chain structure of mature C3 with an α-chain at 115 kD and a β-chain at 75 kD. Four of the premature infants exhibited a single band at 205 kD which corresponds to the reported structure of pro-C3 (24). By densitometric scanning, all bands other than the α-chain and β-chain constituted < 10% of the total C3 (Fig. 4). Bands at 115 kD (α-chain) and 75 kD (β-chain) were shown to be of C3 origin by Western blotting and immunodetection with goat antihuman C3 (Fig. 5).

DISCUSSION

The propensity of the newborn to life-threatening infection is without parallel in later life (1). A variety of deficiencies in host defenses has been described in the neonate, with the premature infant affected more severely than the term neonate (2, 3). In the absence of transplacentally acquired antibodies, the neonate must rely on the complement system for opsonization of invading bacterial pathogens. The importance of an intact complement system in septic neonates has been recently emphasized; in the report by Cairo *et al.* (14), impaired complement function, as measured by an abnormally low CH50, was the single most important predictor of mortality in infected newborns.

In the complement cascade, C3 sits at the junction of the classical and the alternative pathways of activation. A two-chain protein with an α-chain of 115 kD and a β-chain of 75 kD, C3 possesses an internal thiolester bond which links a cysteinyl and a glutamyl residue within the C3d subdomain of the α-chain (15, 25). Activation of either the classical or the alternative pathway results in the enzymatic cleavage of the C3a fragment from the amino terminus of the α-chain and the formation of C3b. Concomitant with the formation of C3b there is a disruption of the internal thiolester bond with exposure of the reactive glutamyl residue. This residue is the site of covalent attachment of C3b to amino or hydroxyl groups on nearby acceptor surfaces (26). The covalent linkage between the reactive glutamyl residue and carbohydrate or amino groups on the bacterial surface is the central mechanism of C3b-mediated opsonization (6).

Using radial immunodiffusion assays, investigators have previously shown that serum concentrations of C3 are reduced in full-term and premature neonates in comparison to adults. Some have found lower levels in the premature infant in comparison to the term newborn (8, 10–12). We used a sensitive ELISA assay for measurement of C3 concentrations and calibrated it with known concentrations of purified human C3 as an internal

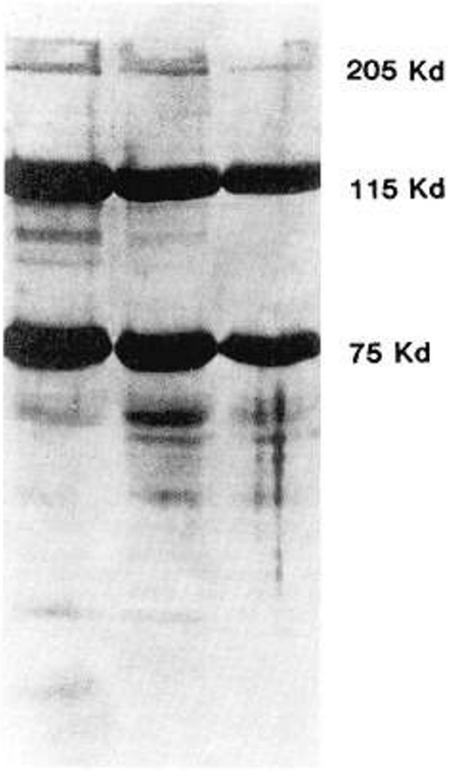

Fig. 4. Structure of C3 isolated by affinity chromatography from three premature neonates and examined by 6% SDS-PAGE under reducing conditions. The two-chain structure of mature C3 is demonstrated with an α-chain at 115 kD and a β-chain at 75 kD. A single band at 205 kD corresponding to the structure of pro-C3 is also present.

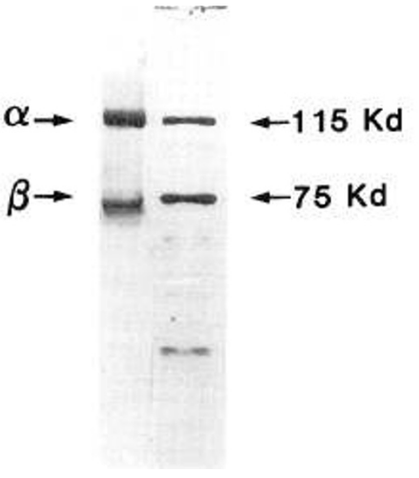

Fig. 5. Two-chain structure of C3 isolated by affinity chromatography from a premature neonate (*right lane*) and examined by Western blotting and immunodetection with goat anti-human C3. C3 standard shown in the *left lane.*

control, as well as with radial immunodiffusion. In comparison to full term infants, only extremely premature infants (<30 wk gestation) had significantly lower C3 levels (Fig. 1).

Previous studies of complement function in neonates have demonstrated impairments in both the classical and the alternative pathways (9–13). In several studies, decreases in complement-mediated opsonization or bacterial killing in neonatal serum have correlated with reduced serum levels of various complement components including factor B (10, 27), C3 (28), C1q, C4, and factor H (12). However, in many cases, defects in neonatal complement function have been reported despite relatively normal serum concentrations of C3 (10, 12). These latter reports give rise to the hypothesis that the function of C3 may be aberrant in the neonate, even though the C3 concentration is essentially normal.

Our experiments test this hypothesis by assessing the function of the third component of complement in a system independent of other complement proteins. Furthermore, because nucleophilic amines bind stoichiometrically to reactive thiolester groups regardless of protein concentration, our assay is not influenced by differences in C3 concentration, or by the presence or absence of other complement proteins such as factors B, H, or I (20, 21). When we examined the reactivity of the C3 thiolester bond under these conditions we found a significant correlation with gestational age.

The critical level of functional C3 is unknown. Patients homozygous for C3 deficiency are markedly susceptible to severe infections with pyogenic organisms while their heterozygous parents, whose serum C3 concentrations are 50% of the norm, display no increased risk of infection (29, 30). Therefore, half-normal serum concentrations of C3 are still protective, provided that each molecule is functionally active.

We defined functional C3 as the product of thiolester reactivity and C3 concentration. Nine of 11 premature infants and two of the 17 term newborns had concentrations of functional C3 which were less than 50% of the adult norm (Fig. 3). Although the results in prematures are not surprising, given their generally low serum concentrations, the results in term newborns may help to explain why a high proportion of early onset group B streptococcal disease arises in full-term infants despite adequate serum concentrations of complement proteins (31).

The aberrant structure of C3 in some newborns (Fig. 4) provides at best a partial explanation for the functional defects which we observed. Although local production of C3 by monocytes/macrophages (32), type II alveolar epithelial cells (33), and endothelial cells (34) is undoubtedly important at sites of inflammation which are sequestered from the bloodstream, 90% of total C3 is synthesized in the hepatocyte as a single-chain precursor molecule, pro-C3 (24). Posttranslational modification of pro-C3 includes proteolytic separation of α- and β-chains with formation of the disulfide bridge, deamination of a glutamine residue to the glutamyl of the intact thiolester, and glycosylation of the native two-chain structure (35–37).

The majority of posttranslational modification occurs intracellularly; however, the exact site of the reaction and nature of the responsible enzymes are unknown (37). Structural analysis of neonatal C3 revealed the two-chain structure in all neonates; four neonates had an additional band at 205 kD which may represent unmodified pro-C3. In none of the four, however, was the amount of the 205-kD band sufficient to account for the full degree of functional abnormality. An alternative explanation for this band is dimerization of α-chains; however, this possibility is deemed unlikely because formation of disulfide bonds was blocked in our assay by alkylation with iodoacetamide. Moreover, the mol wt of α-chain dimers (230 kD) exceeds that which we observed (205 kD).

It is unlikely that these observations are due to increased degradation of C3 because densitometric scanning quantified all bands other than those at 115 and 75 kD as constituting <10% of the total C3.

A possible explanation for decreased thiolester reactivity in neonatal C3 may be derived from the experiments of Iijima *et al.* (38). These investigators used rabbit liver mRNA to characterize an intermediate C3 translational product which failed to react with methylamine and was thus biochemically inactive; this defect was corrected by liver homogenate (38). It is well known that the activity of other fetal hepatic enzymes, such as UDP-glucuronyl transferase, is deficient in early gestation and

matures with increasing gestational age (39). Other enzymatic or biochemical deficiencies in newborn liver may affect posttranslational modification of neonatal pro-C3.

In conclusion, these studies are the first to characterize and to quantitate a biochemical dysfunction of neonatal C3 which is independent of all other complement proteins. Because the functional integrity of the thiolester is required for opsonization of bacterial pathogens (6, 7), aberrant function of the thiolester bond in neonates may constitute a newly identified mechanism for the newborn's susceptibility to infection.

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