Levels of Complement Anaphylatoxin C5a in Pulmonary Effluent Fluid of Infants at Risk for Chronic Lung Disease and Effects of Dexamethasone Treatment

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ABSTRACT. A number of studies have shown that increased numbers of neutrophils and macrophages are recruited into the airways during the development of chronic lung disease (CLD) in preterm infants. The objective of this study was to determine whether the anaphylatoxin C5a is detectable in tracheobronchial aspirate fluid of infants at risk for CLD and to evaluate the possible effects of dexamethasone (Dxm) treatment. C5a/C5a(des Arg) levels were determined by a sensitive ELISA based on a neoepitope-specific MAb. In a prospective study, 27 infants (birth weight 881 ± 169 g, mean ± SD) still on mechanical ventilation at d 10 postnatal age with fraction of inspired oxygen ≥ 0.3 and/or peak inspiratory pressure ≥ 16 cm H_2O were randomly assigned to Dxm treatment at d 10 ($n = 14$) or d 16 ($n = 13$). Ten mechanically ventilated infants with no respiratory disease or who had recovered from respiratory distress syndrome did not meet these criteria on d 10 and served as a control group (birth weight 928 ± 126 g). For the evaluation of Dxm therapy, the late treatment group was used as a control group for the early regimen. Compared with controls, C5a concentrations were higher in infants at risk for CLD on d 10 [median (25th–75th percentile): 2.40 (1.13–3.38) versus 0.82 (0.55–1.78) μg/L, $p < 0.05$]. After Dxm, C5a concentrations decreased significantly in the early treatment group compared with pretreatment values in the late treatment group [d 15, pre Dxm 2.22 (0.98–3.92), post Dxm 0.57 (0.18–1.02) μg/L, $p < 0.01$]. C5a levels in plasma of eight infants were not affected by Dxm treatment. Our results show that increased levels of complement anaphylatoxin C5a are present in lung effluent fluid of infants at risk for CLD, and that local but not systemic levels are affected by Dxm. These findings indicate a role of C5a in the recruitment of inflammatory cells into the airways of infants with CLD. (*Pediatr Res* 34: 586–590, 1993)

Abbreviations

RDS, respiratory distress syndrome
CLD, chronic lung disease
ARDS, adult respiratory distress syndrome
Dxm, dexamethasone
C5a, complement component C5 derived anaphylatoxin
C5a(des Arg), C5a without C-terminal argininyl residue
PBS-Tween, PBS containing 0.05% Tween 20

PIP, peak inspiratory pressure
L/S ratio, lecithin to sphingomyelin ratio

There is evidence that an inflammatory reaction is involved in the pathogenesis of CLD in preterm infants. This disorder is associated with an increased amount of neutrophils and macrophages in the pulmonary effluent fluid (1, 2). Additionally, considerable chemotactic activity of tracheal aspirate fluid was found in infants at high risk for CLD (3), which may account for the neutrophil influx into the airways. The neutrophil is supposed to play an important role in producing increased permeability pulmonary edema (4) and mediating a prolonged lung injury by releasing proteolytic enzymes (2, 5) and reactive oxygen radicals (6). During the acute phase of RDS, activation of the kallikrein-system may also contribute to the inflammatory process (7).

The anaphylatoxin C5a is generated during the activation of either the classic or the alternative pathway of complement. It has numerous biologic effects; one of the most important functions is the chemotactic activity toward neutrophils and monocytes (8). There is evidence that neutrophil recruitment into the lungs is regulated by C5a in ARDS (9), bacterial pneumonia (10), and cystic fibrosis (11). However, the possible role of C5a in neonates with CLD has not yet been defined.

Treatment with Dxm improves gas exchange and pulmonary mechanics in infants with CLD (12–17). These effects are apparently due to a decreased pulmonary inflammatory reaction (3, 18, 19), which affects pulmonary microvascular permeability (18, 20).

The objective of this study was to determine whether C5a could account for the increased chemotactic activity of lung effluent fluid in infants at risk for CLD by measuring C5a/C5a(des Arg) levels in tracheobronchial aspirate fluid and to evaluate possible effects of Dxm treatment. In human serum, the carboxyl-terminal arginine of C5a is rapidly cleaved by carboxypeptidase N. This leads to the formation of C5a(des Arg), which has reduced chemotactic activity compared with C5a. For the quantitation of both C5a and C5a(des Arg), a sensitive and highly specific enzyme immunoassay was applied (21).

MATERIALS AND METHODS

Patients and protocol. Sixty-seven preterm infants with birth weight < 1200 g who were consecutively admitted to the neonatal intensive care units of the Children's Hospital Cologne and the Perinatal Center at Women's Hospital, Cologne-Holweide, from June 18, 1991 to June 18, 1992, were enrolled into a prospective randomized study on the effects of early *versus* late Dxm treat-

ment on CLD. According to a clinical protocol, the criteria for randomization were: *1)* ventilator dependency at a postnatal age of 10 d, *2)* fraction of inspired oxygen ≥0.3 and/or PIP ≥ 16 cm H_2O, *3)* no radiologic evidence of pneumonia, no clinical or laboratory signs of local and systemic infection (immature/total neutrophil ratio < 0.2, serum C-reactive protein < 0.6 mg/dL, negative blood and tracheal cultures). Eight infants died during the first 10 d of life. Of the remaining 59 neonates, 24 met the criteria mentioned above and were randomly assigned to treatment with Dxm on d 10 (early treatment group) or d 16 (late treatment group). Because measurements of plasma C5a levels were done only in the last three infants, five additional patients entered the study after June 18, 1992. Thus, in eight infants, levels of C5a were determined in both plasma and lung effluent fluid. The results of the C5a concentrations in the respiratory fluids were nearly identical in these additional patients when compared with the original study group. Two infants of the total group were excluded from analysis because there was evidence of bacterial contamination of tracheobronchial aspirate fluid. Of the remaining 27 patients, 14 had been randomized into the early treatment group and 13 infants into the late treatment group. Ten of the 67 infants with birth weight <1200 g who were still intubated at d 10 postnatal age but did not meet the ventilatory criteria noted above served as a control group for the Dxm treatment groups. The effects of Dxm were investigated within each treatment group. Furthermore, both groups were compared at d 15 of postnatal age, when the late treatment group served as a control group for the effects of Dxm 5 d after initiation of therapy in the early treatment group. Dxm was given intravenously every 12 h in divided doses over a period of 28 d. The maximal dose was 0.5 mg/kg/d for the first 3 d and 0.3 mg/kg/d for the next 3 d. This dose was reduced by 10% until a dose of 0.1 mg was reached on d 24, which was given every second day until d 28. In 21 of 27 Dxm-treated infants, RDS was diagnosed clinically and confirmed by L/S ratio analysis. All infants with RDS were treated with natural porcine surfactant, as previously described (22). The study was approved by the hospital ethics committee. Informed consent of the parents was obtained.

Sampling of tracheobronchial aspirate fluid. Tracheobronchial aspiration was performed in a standardized manner at least three times daily, by instillation of 0.5 mL of 0.9% NaCl into the endotracheal tube. Suction catheters were inserted slightly beyond the distal tip of the tube, and lung and airway secretions were suctioned and collected in sterile specimen traps (Vygon, Ecouen, France). Tracheobronchial aspirate fluid was diluted with 0.9% saline to a total volume of 0.5 mL, centrifuged at 3000 rpm for 5 min, and cell-free supernatants were frozen at −30°C for later assay. During 24 h, one specimen was obtained in each 8-h period. Immediately before analysis, the three samples for 1 d were pooled. The lung effluent fluid of all infants was cultured for microbiologic analysis twice weekly; additionally, bacterioscopic examinations were performed.

Plasma samples. For determination of C5a/C5a(des Arg) in plasma, 500 μL of blood was obtained by venipuncture and was collected into a syringe containing dipotassium-EDTA. The plasma was separated from the blood cells by centrifugation (10 min/1000 × *g*) and was stored at −30°C within 1 h after puncture.

ELISA for C5a/C5a(des Arg) quantitation. The quantitation of C5a/C5a(des Arg) was performed as described previously (21). In this ELISA, an MAb is used with specificity for a neoantigenic determinant that is present on C5a/C5a(des Arg) but not on the native precursor protein C5. In brief, wells of microtiter plates were coated with 100 μL of anti-C5a MAb C17/5 (40 μg/mL) in carbonate buffer. After blocking of nonspecific binding sites, 100-μL samples were applied per well. Purified C5a or plasma samples that were diluted at least 2-fold in PBS-Tween–20 mM EDTA were allowed to bind to the solid phase for 2 h and were detected by adding, in sequence, the second biotinylated anti-C5a MAb G25/2 (1 μg/mL PBS-Tween) and a 1000-fold dilution

of streptavidin-peroxidase in PBS-Tween, both incubation steps lasting 1 h. Incubation periods were stopped by repeated wash cycles with PBS-Tween. C5a determinations were carried out by the colorimetric analysis of peroxidase-mediated hydrolysis of 2 mM 2,2′-azino-di-(3-ethylbenz-thiazoline sulfonate) in the presence of 2.5 mM H_2O_2 using a microplate photometer, read at 410 nm.

Albumin assay. Albumin in plasma and tracheobronchial aspirate fluid was measured by single radial immunodiffusion (NOR-, VLC- and LC-partigen, Behring, Marburg, Germany).

Concentrations of constituents in lung effluent fluid. To avoid errors due to the sampling procedure, concentrations of constituents in tracheobronchial aspirate fluid are usually related to albumin concentrations. However, this is only correct when albumin concentrations in respiratory fluids are constant over a certain period of time. Because albumin concentrations were markedly affected by Dxm treatment, we chose to express concentrations of all constituents of lung effluent as weight unit/mL of fluid. The suction technique was standardized as mentioned above. The recovered volume of pulmonary effluent by a single tracheal suction did not change in the week before and after Dxm treatment (155 ± 60 *versus* 158 ± 55 μL).

Analysis of L/S ratio. Amniotic fluid or tracheal aspirates were obtained immediately after birth and frozen at −30°C. Analysis of L/S ratio was done in modification as previously described (23).

Statistical analysis. Because of the skewed distribution of the results, data of C5a measurements are expressed as median (25th–75th percentile) and analyzed for statistical significance using the Mann-Whitney U test and the Wilcoxon signed-rank test, as appropriate. Clinical data are expressed as mean ± SD and analyzed for statistical significance by means of the paired and unpaired *t* test. A *p* value < 0.05 was considered statistically significant.

RESULTS

Patients. Birth weight and gestational age were slightly lower, but L/S ratios were significantly lower in infants with high risk for CLD compared with controls (birth weight 881 ± 169 *versus* 928 ± 126 g, NS; gestational age 26.9 ± 1.8 *versus* 28.1 ± 2.1 wk, NS; L/S ratio 1.7 ± 0.4 *versus* 2.6 ± 1.1, *p* < 0.01). There were no differences for birth weight, gestational age, or L/S ratio between the early and late Dxm-treatment group. Thirteen infants of the CLD-risk group, who had been treated with Dxm, but only one infant of the control group eventually developed CLD defined as radiologic abnormalities and oxygen requirement on d 28.

C5a concentrations in lung effluent fluid. C5a/C5a(des Arg) levels at d 10 were significantly higher in infants at risk for CLD compared with controls: 2.40 (1.13–3.38) *versus* 0.82 (0.55–1.78) μg/L, *p* < 0.05. After treatment with Dxm, C5a concentrations decreased both in the early treatment regimen initiated at d 10 postnatal age (*p* < 0.01) and in the late treatment regimen begun at d 16 (*p* < 0.01) (Table 1). On d 15, the late-treated group served as the control group for therapy effects of the early treatment group. After Dxm administration, C5a concentrations decreased in the early treatment group compared with pretreatment values of the late treatment group (*p* < 0.01).

Albumin concentrations in lung effluent fluid. Compared with control infants, albumin concentrations were higher in CLD-risk infants [0.25 (0.17–0.39) μg/L *versus* 0.15 (0.10–0.23) μg/L, *p* < 0.05]. After therapy with Dxm, albumin concentrations significantly decreased in both the early and late treatment regimen [early treatment group, before Dxm: 0.28 (0.10–0.39) g/L; after Dxm: 0.06 (0.03–0.11), *p* < 0.01; late treatment group, before Dxm: 0.26 (0.21–0.38) g/L; after Dxm: 0.09 (0.06–0.26) g/L, *p* < 0.05].

Severity of illness variables. Ventilator settings of fraction of inspired oxygen and PIP that were necessary to obtain blood

Table 1. *Concentration of C5a/C5a(des Arg) in pulmonary effluent and severity of illness variables of infants with low risk for CLD (controls) and infants with high risk for CLD who have been treated with Dxm on d 10 (early treatment group) or d 16 (late treatment group)**

| | | Dxm | | | | |
| | | Early treatment group ($n = 14$) | | Late treatment group ($n = 13$) | | |
	Controls ($n = 10$) (d 10)	Pre (d 10)	Post (d 15)	Pre (d 10)	Pre (d 15)	Post (d 20)
C5a/C5a(des Arg) concentration	0.82	2.73	0.57†‡	2.22	2.22	0.56†
(μg/L)	(0.55–1.78)	(1.22–3.38)	(0.18–1.02)	(1.09–3.66)	(0.98–3.92)	(0.15–0.91)
FiO$_2$	0.23 ± 0.03	0.51 ± 0.16	0.25 ± 0.04†‡	0.47 ± 0.13	0.50 ± 0.19	0.29 ± 0.09§
PIP (kPa)	1.19 ± 0.15	1.93 ± 0.54	1.34 ± 0.30†‖	1.81 ± 0.22	1.86 ± 0.35	1.42 ± 0.21§

* Values represent median (25th–75th percentile) for C5a measurements, and mean ± SD for clinical data. FiO$_2$, fraction of inspired oxygen.

† $p < 0.01$, pre- *vs* posttreatment within one group.

‡ $p < 0.01$, early group, posttreatment *vs* late group, pretreatment on d 15 between Dxm groups.

§ $p < 0.05$, pre- *vs* posttreatment within one group.

‖ $p < 0.05$, early group, posttreatment *vs* late group, pretreatment on d 15 between Dxm groups.

gases within normal ranges are shown in Table 1. According to the inclusion criteria, they were higher in infants at risk for CLD compared with controls and decreased after both early and late Dxm treatment.

C5a concentrations in plasma. In eight infants who had been treated with Dxm, quantitation of C5a in plasma was performed in parallel to determinations in lung effluent fluid. In contrast to levels in airway secretions [pre Dxm 1.97 (1.11–2.22), post Dxm 0.52 (0.17–0.86) μg/L, $p < 0.05$], C5a concentrations in plasma were not affected by Dxm treatment [pre Dxm 3.38 (2.04–4.41) μg/L, post Dxm 4.62 (1.65–5.80) μg/L, difference not significant].

Lung effluent fluid/plasma ratio. Lung effluent/plasma ratio of albumin on d 10 was 0.012 [albumin in lung effluent: 0.26 (0.12–0.35) g/L; albumin in plasma: 21.9 (16–24) g/L]. The lung effluent/plasma ratio of C5a was 0.59.

DISCUSSION

In this study, we provide evidence that the complement component C5a may be involved in the recruitment of inflammatory cells into the airways of preterm infants during the development of CLD. C5a concentrations in pulmonary effluent of infants at risk for CLD were higher compared with control infants. After treatment with Dxm, C5a concentrations significantly decreased. These results correspond well with the neutrophil count in respiratory fluids of ventilated preterm infants. Infants who later developed CLD had increased numbers of neutrophils in lung effluent fluid compared with controls (1, 2). After treatment with Dxm, neutrophil count in airway secretions was reduced (18). We have previously shown that infants at risk for CLD have high chemotactic activity of lung effluent fluid, and that this chemotactic activity was reduced after Dxm treatment (3). High chemotactic activity of airway fluids is a common feature of inflammatory lung diseases (24). Because C5a is a potent chemoattractant, it may considerably contribute to neutrophil influx into the airways. Additionally, C5a has marked effects on microvascular permeability (25). Whether C5a that is present in respiratory fluids alters lung permeability depends on where and how it is generated.

The source of C5a detectable in airway secretions is not clear. It may be of local or systemic origin. A systemic complement activation has been postulated for the pathogenesis of ARDS. In animal models, systemic activation can induce neutrophil accumulation in the lungs and an increased pulmonary epithelial permeability (26). However, only half of human patients with ARDS showed evidence for systemic complement activation, but nine of 10 patients had C5a in the bronchoalveolar lavage fluid (9). Systemic activation of the complement system does not seem

to be necessary for the observed pulmonary effects of C5a. Direct instillation of C5a into the lungs of rabbits is also associated with a massive influx of neutrophils into the airways (27). Neutrophil pulmonary recruitment mediated by C5a thus may occur via local activation of complement components. Although the liver is the major site of synthesis of complement proteins, components of both pathways are produced in lung tissue as well. C2, C4, and factor B have been found to be produced by macrophages (28, 29) and type II alveolar epithelial cells (29). Additionally, type II cells are capable of synthesizing of C3 and C5 (30). These factors secreted by cells of the alveolar wall are thought to contribute to local inflammatory responses in the lung. Neutrophils attracted by other chemotactic active material can cleave locally produced C5 to chemotactic C5a by elastase release (31) and thus amplify the inflammatory response. Free elastase activity has been detected in pulmonary effluent already in the early stages of RDS (1, 32).

The complement system may also be activated by trauma in the immature lung. As shown in our patients, C5a levels are closely related to barotrauma, indicated by PIP and high oxygen concentrations. In preterm infants with surfactant deficiency, positive pressure ventilation rapidly initiates epithelial disruption of the airways (33). Hyperoxia also damages the pulmonary tissue. Trauma can induce complement activation via the contact and the kinin systems (34). Furthermore, exposure of complement components present in interstitial or airway-lining fluid to subcellular membrane fragments can lead to an activation of the cascade (35). When activated locally, C5a may considerably contribute to increased lung permeability, which has been shown to be associated with CLD (20).

C5a is also detectable in plasma. In the blood of healthy individuals, there is a low-grade generation of C5a that is rapidly bound to white blood cells. C5a levels measured in plasma of our infants were comparable to those of healthy adults (21). Because lung microvascular permeability is increased in infants with CLD, high levels of C5a in lung effluent fluid thus may result from increased plasma leakage. This explanation would imply that C5a is not the primary agent that alters lung permeability. The cause of the increased pulmonary microvascular permeability in CLD is not known, but it is thought to be due to an inflammatory process that is associated with the development of the disease (36). Mediators other than C5a with marked effects on microvascular permeability have been detected in the airways of infants with CLD and could be responsible for the increased permeability observed (37).

Our data show that C5a concentrations in lung effluent fluid are significantly decreased after Dxm treatment. In contrast, C5a concentrations in plasma are not affected by Dxm. This finding may indicate that Dxm exerts different effects on the local

complement system and the systemic cascade. It is not clear whether complement metabolism and serum levels are affected by glucocorticosteroids (38–41). However, *in vitro* formation of C3a and C5a has been shown to be inhibited by glucocorticosteroids (41, 42). Furthermore, there is evidence that locally produced complement components are affected. The administration of glucocorticosteroids to guinea pigs inhibited C2 and C4 biosynthesis by bronchoalveolar macrophages (43).

Dxm may act not on local C5a generation but on factors that are necessary for the C5a-mediated mechanisms. The increase of vascular permeability induced by C5a is not due to a direct action on the endothelial cells but is caused by the interaction between endothelial cells and neutrophils. These effects are neutrophil dependent and are completely abolished by depletion of neutrophils (8). Dxm may act on agents that mediate this endothelial-neutrophil interaction. IL-1 induces endothelial expression of adhesion molecules, and Dxm effects on IL-1 production are well documented (44). Furthermore, vascular permeability is dependent on local blood flow (25). Effects of Dxm on local vasodilatatory prostaglandin synthesis (39) may also lead to a decreased C5a-mediated endothelial fluid leakage.

Dxm therapy has been shown to improve capillary integrity and thus to reduce pulmonary fluid leakage in infants with CLD (3, 18, 20). Similar results were obtained in our patients, inasmuch as albumin concentrations were significantly decreased after Dxm treatment. Diminished levels of C5a in lung effluent fluid after Dxm therapy thus may be due to a decreased leakage from plasma, without any effect on local or systemic C5a generation. Effects of Dxm on other mediators that increase lung permeability have been described. The generation of leukotrienes (45) and of platelet-activating factor (46) are both affected by Dxm therapy.

In conclusion, this study shows that increased levels of C5a are present in the airways during the early development of CLD. Regardless if it is produced locally or derives from increased leakage from plasma, C5a in pulmonary secretions may be capable of amplifying the inflammatory process by recruiting neutrophils and macrophages. However, currently we do not know whether increased concentrations of C5a in airway fluids induce an increased pulmonary microvascular permeability or whether the presence of C5a in respiratory secretions is a consequence of increased lung leakage. The inflammatory response is a complex reaction depending on a network of various humoral mediators and effector cells (25). Studies on the time-dependent generation and interaction of inflammatory mediators detectable in airway secretions of infants with CLD will further clarify the role of inflammation in the pathogenesis of this disease.

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