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Staphylococcus epidermidis biofilms induce lower complement activation in neonates as compared with adults

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BACKGROUND: *Staphylococcus epidermidis* (SE) is an important cause of late-onset sepsis in neonates. SE frequently produces a polysaccharide intercellular adhesin (PIA) biofilm, important in the pathogenesis of these infections. Little is known about how the neonatal innate immune system reacts to SE biofilm–associated infections. Our hypothesis was that SE biofilms induce a lower complement activation in neonates as compared with adults.

METHODS: Cord blood from term infants (n = 15) and blood from adults (n = 6) were studied in an *ex vivo* whole-blood sepsis model. A PIA biofilm–producing strain (SE1457) and its isogenic mutant (M10), producing a non-PIA biofilm, were used.

RESULTS: Both SE biofilms induced stronger complement activation in adult than in cord blood ($P \leq 0.033$). We found lower levels of antibodies toward both PIA ($P = 0.002$) and the whole bacterium ($P = 0.001$) in cord vs. adult blood. By contrast, the interleukin-8 (IL-8) and IL-6 secretion were higher in cord than in adult blood ($P \leq 0.002$). The PIA biofilm induced stronger complement activation than the non-PIA biofilm.

CONCLUSION: We conclude that the neonatal complement system exhibits a maturational deficiency. This may reduce the ability of neonates to combat biofilm-associated SE infections.

Staphylococcus epidermidis (SE) is the most prevalent pathogen causing late-onset sepsis in neonates (1). These infections are seldom lethal, but they cause significant morbidity, especially in preterm infants (1,2). SE infections are often associated with biofilm production on the surface of foreign-body implants (3,4). Biofilms are adherent multicellular bacterial aggregates embedded in a self-produced extracellular matrix. The best-described matrix compound in SE biofilms is a β-(1,6)-linked N-acetylglucosamine named polysaccharide intercellular adhesin (PIA) (5,6).

SE and SE biofilms interfere with the host immune response at different levels. PIA biofilms inhibit the action of antimicrobial peptides (7), decrease neonatal inflammatory response (8), and decrease phagocytosis and degranulation by neutrophils (7). Biofilms may "decoy" antibodies and complement on the bacterial surface and thereby decrease opsonization and phagocytosis (9–11). The ability of biofilm-producing bacteria to avoid immune clearance and the general immaturity of the neonatal immune system (12) are postulated to increase the risk of neonatal SE sepsis (13). As compared with adults, neonates have a lower quantitative and qualitative complement activation (14,15), reduced upregulation of cellular response (12), and an immature cytokine response pattern (16–19). Preterm infants demonstrate a markedly reduced capacity to upregulate oxidative burst in leukocytes in response to SE (20). However, some authors have reported that SE may induce a similar proinflammatory cytokine response in neonates and adults (19,21), indicating a differential maturation of various parts of the neonatal innate immune system. Differences in maturation have also been described when challenging the innate immune system with meconium, for which the cytokine/chemokine response was at least as potent in neonates as in adults, whereas the complement activation was markedly lower in the neonates (22).

The immunological response to SE biofilms is very complex. We have previously shown that SE biofilms induce a strong complement response in blood from healthy adults (23), indicating a putative important role of the complement cascade in SE infections. To our knowledge, no previous studies have analyzed the complement response to SE biofilms in neonates. Our main hypothesis was that SE biofilms induce a lower complement activation in neonates as compared with adults. We therefore performed experiments in an *ex vivo* whole-blood model comparing the complement activation upon exposure to SE biofilms in cord blood vs. blood from adults. We also compared the complement activation in blood after exposure to PIA biofilm vs. a non-PIA biofilm.

RESULTS

Complement Activation in Cord Blood as Compared With Adult Blood Upon Stimulation With PIA Biofilm and Non-PIA Biofilm

The PIA biofilm induced a higher activation of all complement activation products in adult (n = 6) blood as compared with cord (n = 15) blood; C1rs/C1 inhibitor complex, $P = 0.018$;

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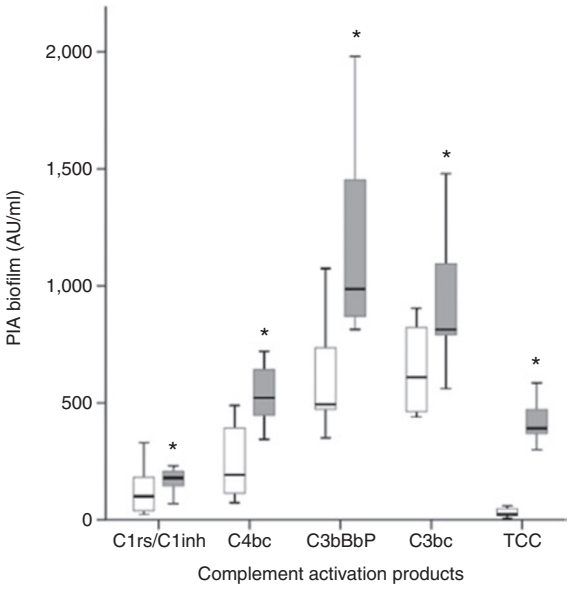

Figure 1. Comparison of the complement activation products in neonatal and adult blood after incubation with a PIA biofilm. The levels of the complement activation products C1rs/C1 inhibitor complex, C4bc, C3bBbP, C3bc, and terminal complement complex (TCC) were measured. White bars, neonate; gray bars, adult. *Statistically significant differences, $P < 0.05$. PIA, polysaccharide intercellular adhesin.

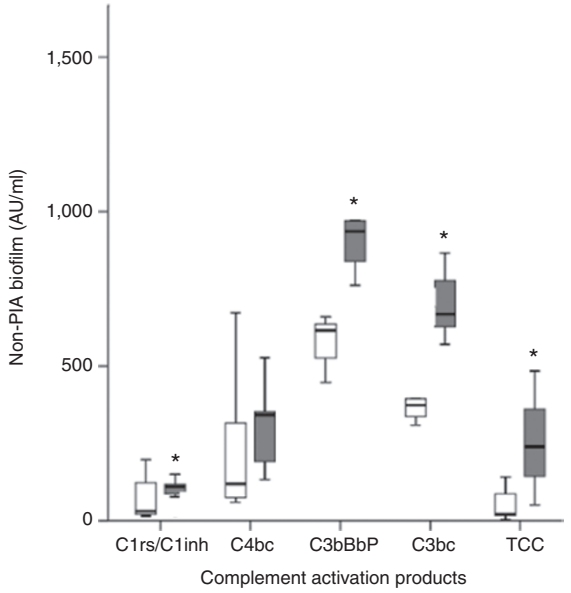

Figure 2. Comparison of the complement activation products in neonatal and adult blood after incubation with a non-PIA biofilm. The levels of the complement activation products C1rs/C1 inhibitor complex, C4bc, C3bBbP, C3bc, and terminal complement complex (TCC) were measured. White bars, neonate; gray bars, adult. *Statistically significant differences, $P < 0.05$. PIA, polysaccharide intercellular adhesin.

C4bc, $P = 0.008$; C3bBbP, $P = 0.003$; C3bc, $P = 0.012$; terminal complement complex, $P < 0.001$ (**Figure 1**).

The non-PIA biofilm induced a higher activation of all complement activation products in adult blood ($n = 6$) as compared with cord blood ($n = 15$); C1rs/C1 inhibitor complex, $P = 0.033$; C3bBbP, $P = 0.003$; C3bc, $P = 0.039$; terminal complement complex, $P = 0.001$ (**Figure 2**). As compared with data for the PIA biofilm (**Figure 1**), the overall complement activation was lower when challenging with the non-PIA biofilm (**Figure 2**).

Cytokine Secretion in Response to PIA and Non-PIA Biofilms

Both the PIA and the non-PIA biofilms induced a significant secretion of interleukin-6 (IL-6) and IL-8 in cord blood as compared with the nonstimulated control (**Figure 3a,b**). In adult blood, there were no differences in cytokine secretion between the nonstimulated control and the biofilms.

Secretion of IL-8 was significantly higher in cord as compared with adult blood when stimulating with both the PIA ($P < 0.001$) (**Figure 3a**) and the non-PIA biofilm ($P < 0.001$) (**Figure 3b**). A significantly higher IL-6 secretion was also observed in cord as compared with adult blood after stimulation with a PIA biofilm ($P = 0.002$), although in general the level of IL-6 secretion was low. No significant differences were found between PIA and non-PIA biofilms in the cord or adult blood.

Anti-PIA and Anti–*S. epidermidis* IgG Titers in Nonstimulated Cord Blood and Adult Blood

Significantly higher levels ($P = 0.002$) of anti-PIA IgG and anti–*S. epidermidis* IgG ($P = 0.001$) were found in the adult as compared with the cord blood (**Figure 4a,b**).

Complement Activation Induced by a PIA or a Non-PIA Biofilm in Cord Blood

Both biofilms induced a significant activation of all complement factors as compared with the nonstimulated control (**Figure 5**). Activation of the C1rs/C1 inhibitor complex, which is specific for the classic pathway, indicates initial activation of the complement cascade through the classic pathway. There was also an activation of C4bc, which is common for both the classical and lectin pathways. The increase in C3bBbP also indicates activation of the alternative pathway, probably through the alternative amplification loop.

The PIA biofilm (SE1457) induced a significantly higher activation of the C1rs/C1 inhibitor complexes ($P = 0.008$) and C3bc ($P = 0.001$) as compared with the non-PIA biofilm (M10) (**Figure 5**). The differences observed for the remaining activation product were all in the same direction (lower in the non-PIA biofilm) but did not reach statistical significance.

DISCUSSION

This is, to our knowledge, the first report on neonatal complement activation upon stimulation with SE biofilms. Previous studies in adults have suggested that the complement system is important in fighting biofilm-associated SE infections (10,23). We found lower complement activation in the cord blood as compared with adult blood but a more powerful secretion of early proinflammatory cytokines. Biofilm-associated SE infections are commonly encountered in modern neonatal intensive care (1,2). It is therefore important to understand the underlying pathogenic mechanisms in biofilm-associated SE infections in order to develop new treatment strategies.

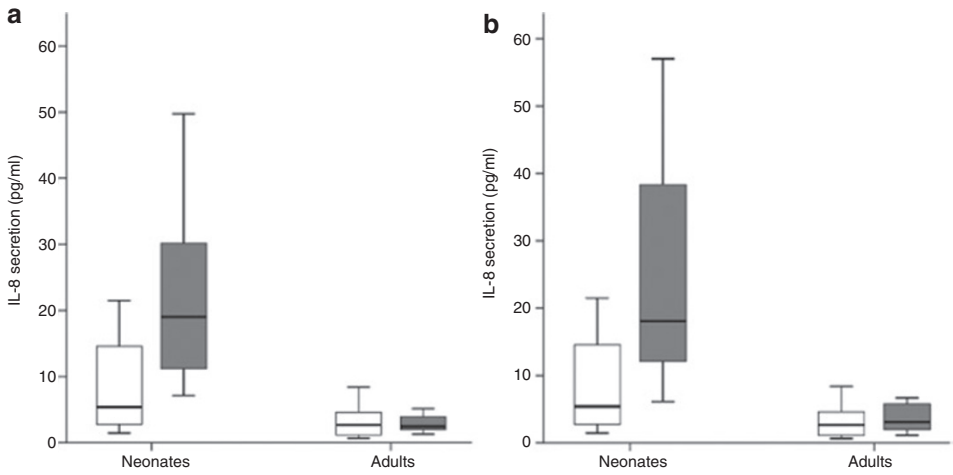

Figure 3. Comparison of the IL-8 secretion in umbilical cord and adult blood after incubation with (**a**) a PIA biofilm and (**b**) a non-PIA biofilm. The overall differences between neonate ($n = 15$) and adult ($n = 6$) secretion of IL-8 after incubation with (**a**) a PIA and (**b**) a non-PIA biofilm were statistically significant. Only the neonatal secretion was significantly higher than that of the nonstimulated control. White bars, nonstimulated sample; gray bars: (**a**) PIA-positive biofilm and (**b**) non-PIA biofilm. IL, interleukin; PIA, polysaccharide intercellular adhesin.

Both the PIA biofilm and the non-PIA biofilm induced higher complement activation in adult vs. cord blood. Our study indicates that the complement cascade was mainly activated through the classic pathway, although an increase in C4bc may also reflect activation of the lectin pathway. The substantial activation in the alternative pathway probably reflects an activation of the alternative amplification loop (24,25). In a previous study, we found the same pattern of complement activation in adults (23).

The classical pathway can be activated through different mechanisms: (i) direct binding of bacterial surface components to the first classic factor, C1q, (ii) binding through C-reactive protein, or (iii) opsonization by antibodies bound to antigens on the bacterial surface, such as PIA (26) or other antigens in the biofilm/on the bacterial surface. High PIA antibody titers have been reported both in patients with SE biofilm prosthesis infections and in healthy controls (26,27). Due to the short incubation time in our experiments, any antibody-dependent complement activation relies on preexisting antibodies. IgG antibodies are actively transported across the placenta in the last trimester of the pregnancy. Yet total IgG level is markedly lower in neonates (cord blood) than in adults (28). Furthermore, differences in transport kinetics between the IgG subclasses may cause qualitative differences in titers of IgG subclasses (29,30), e.g., IgG1, IgG3, and IgG4 are fairly efficiently transported across the placenta, whereas transport of IgG2 is less efficient (30). It has previously been reported that preterm neonates have considerably lower titers of anticoagulase-negative staphylococci IgG antibodies and antibody-mediated opsonic activity against coagulase-negative staphylococci as compared with adults (31,32). However, an association between level of anticoagulase-negative staphylococci antibodies and risk of neonatal coagulase-negative staphylococci sepsis has not been documented (32). PIA antibodies mainly belong to the IgG2 subclass (26,33), which are modest complement activators. Our study showed that there are significantly lower antibody titers both toward the biofilm matrix (PIA) and toward the whole bacterium (*S. epidermidis* SE1457) in cord blood of term infants as compared with adult blood. Lower levels of antibodies, in addition to an inefficient IgG2 complement activation, might be one reason for lower classic complement activation in neonates vs. adults.

Both PIA and non-PIA biofilms were capable of activating the complement cascade in both neonates and adults. However, they induced significantly higher activation of the alternative amplification loop in adults as compared with neonates. One purpose of this alternative amplification loop is to increase the amount of activated complement factors in the final common pathway and thus increase the total activity of the complement system (24,25). Neonates may have a deficient upregulation of the alternative pathway because of low levels of alternative pathway factors (15,34). A lower activity of the alternative amplification loop may also explain why we observed lower levels of activation products of the final common pathway in cord blood. We have previously shown that PIA biofilm and purified PIA induced a strong complement activation (23). Similar findings were observed in the current study. The PIA biofilm induced markedly stronger complement activation than the non-PIA biofilm, both in adult and cord blood. Collectively, these data support our hypothesis that PIA is a complement activator (10,23) in neonates as well.

We observed a higher secretion of both IL-6 and IL-8 in cord as compared with adult blood. Previous studies have indicated no difference in proinflammatory cytokine response between term neonates and adults after SE stimulation (19,21). However, differences in the study design regarding incubation times and their use of planktonic SE instead of the biofilm used in our study may explain these differences. Tatad *et al.* showed that after stimulating monocytes for 18 h with SE, there was a significantly higher secretion of IL-6, IL-8, and IL-12 in adult blood as compared with blood from term neonates (35). It is possible that the adult cytokine secretion would reach the same level as that of neonates if longer incubation times had been used in our

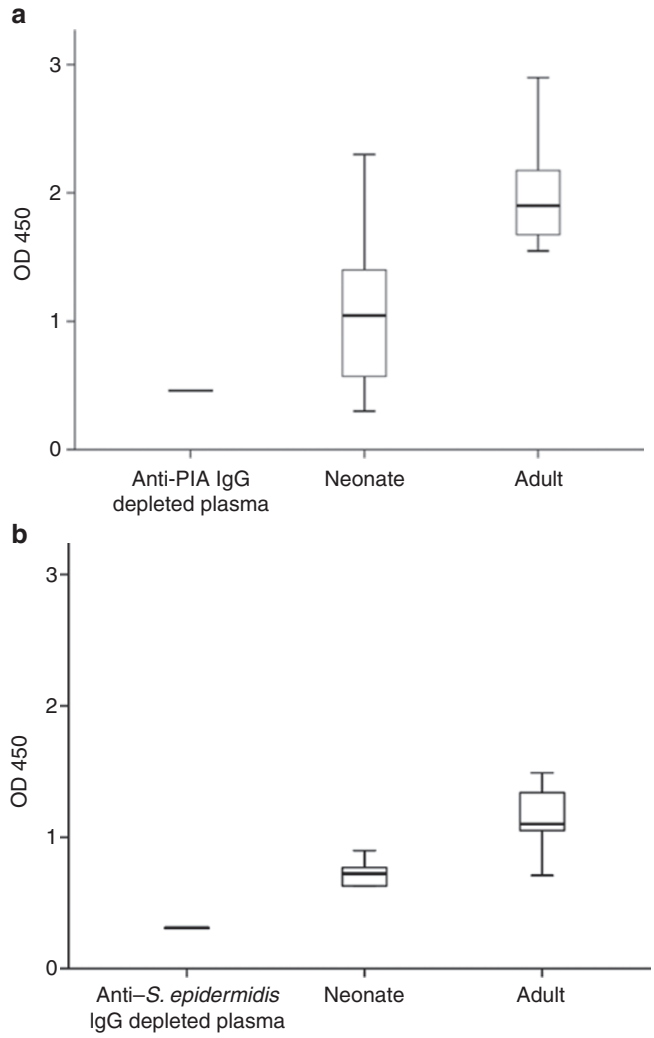

Figure 4. Comparison of (**a**) the anti-PIA IgG titers and (**b**) anti–*S. epidermidis* IgG titers (expressed as units of OD) in the nonstimulated umbilical cord and adult blood. (**a**) Anti-PIA IgG and (**b**) anti–*S. epidermidis* IgG-depleted blood were used to set the cutoff for nonspecific IgG binding in the assay. The overall difference between neonates and adults was statistically significant for both anti-PIA IgG ($P = 0.002$) and anti–*S. epidermidis* IgG ($P = 0.001$). OD, optical density; PIA, polysaccharide intercellular adhesin.

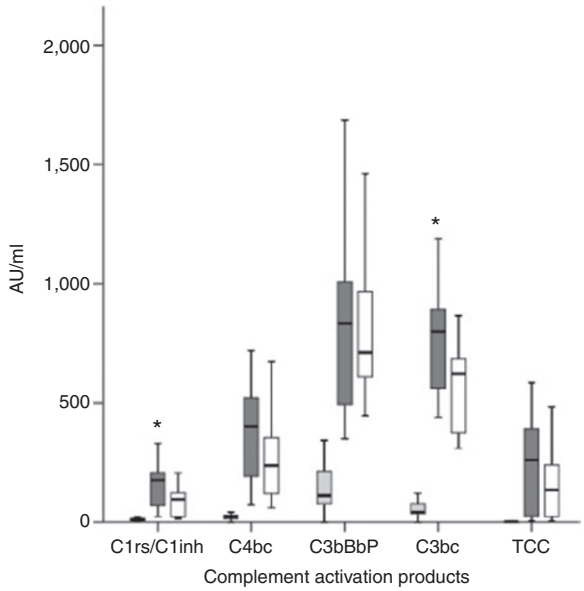

Figure 5. Comparison of the complement activation products in umbilical cord blood incubated with phosphate-buffered saline (nonstimulated), a PIA biofilm, and a non-PIA biofilm. The levels of the complement activation products C1rs/C1 inhibitor complex, C4bc, C3bBbP, C3bc, and terminal complement complex (TCC) were measured. Light gray bars, nonstimulated control; dark gray bars, PIA biofilm; white bars, non-PIA biofilm. *Statistically significant differences, $P < 0.05$. PIA, polysaccharide intercellular adhesin.

study. Consistent with our findings, the complement response was lower and the cytokine response was more pronounced in a study in which meconium was incubated with neonatal vs. adult blood (22). This underscores the notion that the innate immune system in neonates is not immature in all branches of the inflammatory network, as also confirmed in this study.

The current study shows contrary results for the complement activation and cytokine response when comparing neonates and adults. In a previous study, we also found a "delicate balance" between the complement activation and cytokine secretion wherein high complement activation seemed to be accompanied by low cytokine secretion and vice versa (23). The interplay between complement activation and cytokine secretion is complex, with some cytokines being complement dependent whereas others are complement independent (36,37). Cytokines may, however, indirectly contribute to complement activation, e.g., by increasing the expression of anaphylatoxin receptors (38). High levels of C5a may also inhibit neutrophils (39) and regulate the transcription of cytokines (40,41). The interplay between different parts of the innate immune response in neonates merits further investigation.

This study has limitations. First, the 30-min incubation time was a compromise to be able to analyze early activation of free circulating complement activation products (37). However, it may take hours before some of the proinflammatory cytokines are secreted in response to an infection. Thus, the current study describes only the initial cytokine response. Second, we included cord blood from term-born infants in this study. However, preterm infants represent the population "most at risk" for SE infections, and therefore future studies are needed to study this high-risk group. Third, we did not record the differential leukocyte counts for each experimental setup. We can therefore not totally rule out the possibility that quantitative differences in leukocyte counts may have influenced the inflammatory response. Fourth, the biofilm mass of the PIA biofilm was larger than that of the non-PIA biofilm (23). However, inflammatory response is not only positively correlated to biofilm mass. Indeed, a higher cytokine secretion and leukocyte activation was induced by a non-PIA biofilm (23). We therefore believe that our findings are not dependent on the biofilm mass but rather on differences in the activating potential of the components in the two biofilms. Finally, the *ex vivo* sepsis model enables us to study the host inflammatory response in whole blood, including detailed analysis

of complement and cytokine secretion and their interaction. However, this model is not able to inform on the cooperation with other body tissue compartments, e.g., the endothelium surface. Thus, *in vivo* experiments are needed to include all components of the inflammatory system when assessing the pathogenic mechanisms of biofilm-associated SE infections.

Conclusion

We found a significantly lower complement response in neonates as compared with adults, when stimulating with both a PIA and a non-PIA SE biofilm. Our findings indicate a maturational deficiency of the neonatal complement system. This may decrease the ability of neonates to combat biofilm-associated SE infections. Further studies are needed to assess the role of the complement system in neonatal SE infections in order to develop potential new therapeutic strategies.

METHODS

Bacterial Strains and Culture Conditions

The PIA-producing *S. epidermidis* strain SE1457 and its isogenic mutant M10 were used. SE1457 is a clinical strain from an infected central venous catheter (41). M10 was created by insertion of Tn917 into the *icaA* gene of the *ica*-operon, obliterating PIA production (41,42). We have previously shown that M10, when incubated in a glucose-rich medium, produces a biofilm consisting of proteins and DNA (23). Biofilm formation in polyvinyl chloride (PVC) tubing (length 30 cm, internal diameter 3 mm, Mediplast, Malmø, Sweden) was performed as described previously (23). Briefly, overnight cultures in tryptic soy broth were diluted 1:100 in tryptic soy broth with 1% glucose and transferred to the PVC tubing segments. Each segment was closed end to end to form small loops. The loops were rotated slowly in an incubator at 37 °C for 24 h, cultures were emptied from the loops, and the loops were then carefully washed once with sterile phosphate-buffered saline. Production of biofilm in the loops was verified by staining the biofilm with crystal violet as previously described, showing that M10 produced a biofilm with a lower biofilm mass as compared with that of SE1457 (23). The loops with preformed biofilms were then stored at −20 °C until they were thawed immediately before the final experiments were carried out. A pilot study showed that the complement activation generated by "fresh" and "frozen–thawed" biofilm was identical; therefore, the complement-activating properties of the biofilm were not affected by freezing.

Study Group

Pregnant women were recruited from a study investigating maternal, placental, and fetal hemodynamics in low-risk pregnancies. A convenience sample of cord blood from 20 term-born neonates was initially included in this study. The umbilical cord was clamped immediately after birth, and blood was obtained from the umbilical cord. Five samples were later discarded due to signs of perinatal infection, small sample volume, or a delay of >30 min from delivery until the experiment was started. Thus, the final study group constituted cord blood from 15 infants (9 girls) with a median (range) birth weight of 3,678 (2,898–4,360) g and median (range) Apgar scores of 9 (6–9) at 1 min and 10 (7–10) at 5 min after birth. The median (range) umbilical cord pH and base excess were 7.27 (7.14–7.40) and −3 (−7.2 to 7.2) mmol/l, respectively. Venous blood samples from a total of six healthy adults (four women) were used as controls in this study.

Ex Vivo Human Whole-Blood Model

Cord blood and blood from adult controls were collected in sterile polypropylene tubes (5 ml Nunc cryotubes; Nagle Nunc International, Naperville, IL) containing 50 µg/ml lepirudin (Refludan, CSL Behring, Marburg, Germany). Lepirudin, a recombinant hirudin analog with highly specific thrombin inhibitory activity, was used as an anticoagulant because it has no effect on complement activation (43). Induction of inflammatory response in fresh whole blood was carried out as described earlier (23,43), with the exception that only 900 µl whole blood (cord blood and whole blood from adults) was used for each sample. Each experiment commenced within 30 min after delivery to avoid nonspecific activation of the innate immune system. All experiments were run in parallel with neonatal and adult blood using (i) sterile PVC loops (i.e., nonstimulated control), (ii) PVC loops with preformed SE1457 biofilm, and (iii) PVC loops with preformed SE1457-M10 biofilm. After the incubation period of 30 min at 37 °C, EDTA was added to a final concentration of 10 mmol/l to stop further activation. Plasma was then separated and stored at −70 °C.

Table 1. Complement activation products

Complement activation products	Comment	Ref.
C1rs-C1 inhibitor complex	Specific for the classic pathway	45
C4bc	Common for the classic and lectin pathways	27
C3bBbP	Specific for the alternative pathway	37
C3bc	Final common pathway	46
Terminal complement complex (TCC)	C5b-9, terminal complement pathway	47

Enzyme Immunoassays for Complement Activation Products

Enzyme-linked immunosorbent assay (ELISA) was used to quantify central complement activation products (**Table 1**).

Cytokine Multiplex Assay

Multiplex assays for cytokines were used to study the secretion of four "early-response" proinflammatory cytokines (IL-6, IL-1β, IL-8, and tumor necrosis factor-α) in plasma. Analyses were performed by Bioplex cytokine assays (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The lower detection limits for the cytokines were 2.2 pg/ml for IL-1β, 1.8 pg/ml for IL-6, 1.4 pg/ml for IL-8, and 5.1 pg/ml for tumor necrosis factor-α.

Purification of PIA

PIA was prepared from a biofilm extract of a PIA-producing clinical strain, *S. epidermidis* CIP 109562, as previously described (23,44).

ELISA Determination of Anti-PIA IgG Titers in Nonstimulated Neonatal and Adult Blood

The ELISA was performed as previously described (26). Briefly, Microlon 600 plates (Greiner-Bio, Frickenhausen, Germany) were coated with 100 µl highly purified PIA at 1 µg/ml in 40 mmol/l sodium phosphate buffer, incubated overnight at room temperature, and then washed. The plates were blocked with 5% skim milk in Tris-buffered saline. Plasma was added at a concentration of 1:1,600 (diluted in Tris-buffered saline–0.05% Tween 20) and incubated for 1 h, washed with Tris-buffered saline–0.05% tween 20, and then incubated for 1 h with horseradish peroxidase-conjugated rabbit antihuman IgG (Sigma-Aldrich, St. Louis, MO), diluted 1:2,000 in Tris-buffered saline–0.05% tween 20. Color was developed with 100 µl of substrate solution (R&D Systems, Minneapolis, MN) for 15 min in the dark, and then 50 µl H_2SO_4 was added to stop the reaction. Optical density at 450 nm was measured. Background readings corresponding to the control wells incubated with blocking solution not containing sera were subtracted automatically. Anti-PIA IgG-depleted plasma was used as a cutoff marker for nonspecific IgG binding. Each experiment was conducted twice in triplicate. Antibody levels were expressed as units of optical density.

ELISA Determination of Anti–*S. epidermidis* IgG Titers in Nonstimulated Neonatal and Adult Blood

The ELISA was performed as previously described (32). Briefly, bacteria were killed by heat treatment (56° C, 1 h) and washed with Hanks

balanced salt solution (GIBCO Life Technologies, New York, NY). Polystyrene plates (Corning, New York, NY) were coated by adding 100 µl of bacterial suspension (1×10^7 colony-forming units/ml in phosphate-buffered saline) and incubated at 37 °C for 1 h and subsequently at 4 °C overnight. Pilot studies showed that a plasma concentration of 1:3,200 is adequate. Antihuman IgG conjugate goat antihuman antibody (Promega, Madison, WI) diluted 1:2,500 in phosphate-buffered saline was used as secondary antibody. Optical density at 450 nm was measured. Background readings corresponding to the control wells incubated with blocking solution not containing sera were subtracted automatically. Anti–*S. epidermidis* IgG-depleted plasma was used as a cutoff marker for nonspecific IgG binding. Each experiment was conducted twice in triplicate. Antibody levels were expressed as units of optical density.

Statistical Analyses

The Mann–Whitney *U* test was used to evaluate the differences in complement activation, cytokine secretion, and anti-PIA IgG and anti–*S. epidermidis* IgG titers between neonates and adults. The observed variations in adult complement activation and cytokine secretion were negligible when using blood from the same donor. To avoid problems with dependent observation among the adult controls, we used only the results from the first sample from each adult for the comparison with cord blood.

We performed parallel experiments with cord blood analyzing the responses upon exposure to the two biofilms. The immunological responses in each parallel experiment depend on individual properties of the blood, and we therefore used the nonparametric Wilcoxon's signed-ranks test to assess the level of significance. All tests were two-tailed, and statistical significance was defined as $P < 0.05$. All statistical analysis were performed using IBM-SPSS software, version 19.0 (IBM North America, New York, NY).

Ethics

The Regional Committee for Medical Research Ethics approved the collection of neonatal cord blood and blood from healthy adults for the immune response studies (REK number 2009/935-11). Informed written consent was obtained from the mothers of the neonates before delivery and from the adult control donors.

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