Reduced expression of C5a receptors on neutrophils from cord blood

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Abstract

Aim—To describe further functional deficiencies of neonatal neutrophils by measuring the expression of C5a receptors.

Methods—C5a uptake was measured using flow cytometry with fluorescein isothiocynate labelled recombinant C5a. The response of neutrophils to stimulation with C5a and fMLP was tested by measuring migration and exocytosis of myeloperoxidase and lactoferrin.

Results—C5a mean fluorescence on neutrophils from neonates was significantly lower (22.4 (SD 3.5)) than in adult controls (31.5 (3.1)). Neutrophils from neonates migrated poorly towards both C5a and fMLP compared with those from adult controls. Exocytosis of myeloperoxidase, but not lactoferrin from neonatal neutrophils stimulated with C5a, was significantly lower than in adult controls. fMLP stimulation, on the other hand, resulted in significantly higher exocytosis in neonates.

Conclusion—The lower expression of C5a receptors on neutrophils from neonates could be related to reduced C5a mediated exocytosis of myeloperoxidase.

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The immunological host defences of neonates are not fully developed, and this may contribute to the high incidence of bacterial infections and associated morbidity and mortality in term neonates.[1–3] This immaturity seems to be multifactorial and different defects have been described, including quantitative and functional deficiencies in immunoglobulins, the complement system, and phagocytosis.[3–5] However, the most consistent defect described is impaired function of polymorphonuclear neutrophils (PMN).[6–8] Defective chemotaxis is thought to have a major role.[1 7 8] The precise mechanisms underlying decreased motility of PMN are still unclear, but may involve both cellular and humoral deficiencies.[1 9]

These deficiencies include abnormal membrane fluidity,[10 11] abnormalities in the microfilamentous cytoskeletal organisation,[12] and variation in both membrane potential and free intracellular ionised calcium.[13] Furthermore, a decrease in stimulus induced adhesion and migration has been shown in vivo[14] and in vitro.[8 15] Impaired upregulation of CD11b/CD18 (Mac-1),[4, 15 16] diminished Mac-1 mobilisation from gelatinase granules,[17] and decreased total cell content of Mac-1[18] may be related to this decreased ability to adhere. The importance of these findings should be put in the context of observed differences between adults and neonates that may be due to leucocyte isolation procedures.[19] However, Falconer *et al*, using a whole blood assay,[20] have shown that PMN from preterm and stressed neonates have significantly fewer receptors for complement factors C3b (CR1, CD35) and iC3b (Mac-1, CR3) than term neonates and adults. Term neonates, on the other hand, had higher PMN Mac-1 than adults, supporting the findings of Rebuck *et al*.[19]

Human complement component 5a (C5a) is cleaved from C5 during activation of the complement system[21] and is an important mediator of the inflammatory response. Inter alia, C5a promotes PMN chemotaxis and activation[22] by binding to a specific receptor (C5aR).[23 24] C5aR has been cloned and sequenced,[25], and is characterised as a 7 transmembrane (7TM) G protein linked receptor. Furthermore, when C5aR is occupied, it becomes phosphorylated and is subsequently internalised.[26] Thereafter, the receptor is believed to be dephosphorylated in intracellular compartments before being recycled to the cell surface. The receptor for N-formyl-methionyl-leucyl-phenylalanine (fMLP) is also a 7TM G protein linked receptor that is internalised when activated. However, its re-expression rate is much faster than that for C5aR,[27] because replacement fMLP receptors are translocated from pre-existing intracellular pools in the secretory vesicles,[28] and not directly recycled. We decided to investigate the expression of C5aR on neonatal PMN and the responses of neonatal PMN to C5a and fMLP. As others have shown that neonatal PMN express normal numbers of fully functional fMLP receptors,[8 15 29] fMLP was used as a comparative standard.

Methods

Twenty healthy term neonates, with normal weight for gestational age (average weight 3540 g (SD=492)), born at Odense University Hospital, entered the study. K₃-EDTA stabilised cord blood (10 ml) was obtained immediately after birth. The study was approved by the ethical committee. Informed consent was obtained from the parents. Donor blood was obtained from healthy laboratory staff aged 25–35.

Polymorphonuclear neutrophils (PMN) were isolated from cord blood within 2 hours of birth, using gradient centrifugation. Whole blood was centrifuged with Polymorfprep (Nycomed Inc.) at $400 \times g$ for 35 minutes, and PMN resuspended in Hanks's Buffered Salt Solution (HBSS) (Gibco), washed three times

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and centrifuged at $900 \times g$ for 7 minutes. PMN were then resuspended (2.0×10^{-6} cells/ml) in HBSS with 0.2% human serum albumin (HSA) (Behring). This suspension was used in the following assays unless otherwise stated. From half of the neonates, 2 ml of plasma were taken to determine the level of complement activation by measuring C3d, as described by Brandslund *et al.*[30]

Migration was assessed using a modified Boyden technique,[31] with a 48 well microchemotaxis chamber (NeuroProbe Inc., USA) with 3 μm millipore filter, type SS. fMLP (10^{-8} M) (Sigma, USA) and recombinant C5a (rC5a) (10^{-9} M) (Sigma, USA) were used as chemoattractants. The incubation time was 30 minutes at 37°C. Directed migration was measured by the leading front technique[32] with three cells in one high power field.

PMN were kept on ice for 20 minutes and then pre-incubated for 5 minutes at 37°C, followed by addition of the stimulus rC5a (5×10^{-7} M) or fMLP (5×10^{-6} M). Incubation was terminated after 10 minutes by the addition of 2 volumes of ice cold HBSS and sedimentation of the PMN by centrifugation at 4°C for 10 minutes. The supernatant fluid (1 ml) was aspirated and the concentrations of myeloperoxidase (MPO) and lactoferrin (LF) were measured by ELISA, as described before.[33][34] To assess the total amount of MPO and LF in the PMN, cell pellets from unstimulated PMN suspensions from the exocytosis assay were sonicated after addition of 10 μl 5% Triton X-100. Cell suspensions were sonicated for 15 seconds at full amplitude with Soniprep 150 (Radiometer, Denmark). Exocytosis of granule markers (MPO and LF) was calculated as amount of enzyme released following stimulation minus the spontaneous enzyme release, and also expressed as per cent of total cell content. All assays were done with eight replicates. The interassay variation was 13.4% and 9.6% for the MPO assay (for C5a and fMLP, respectively), and 9.7% and 9.0% for the LF assay.

FLOW CYTOMETRY

Forty nM rC5a in 200 μl 0.1 M sodium phosphate, pH 7.0, was incubated with 120 nM of fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO, USA) at room temperature for 45 minutes.[35] The reaction was terminated by removing free fluorescein using gel filtration of the reaction mixture on a 5 ml column of Bio-Gel (BioRad, Richmond, CA) equilibrated with 30% acetic acid. The labelled derivatives were collected and lyophilised to remove acetic acid. rC5a concentration was measured using amino acid analysis. FITC concentration was measured by spectrophotometric analysis at 493 nm, pH 7.0. The extent of fluorescein incorporation into rC5a was 0.45 mol of fluorescein per mol of peptide.

PMN were incubated with 3.33×10^{-10} M Fl-rC5a at room temperature for 45 minutes. The background fluorescence of activated PMN was measured after incubation with 3.33×10^{-10} M Fl-rC5a and 10^{-7} M rC5a. PMN were then washed twice with buffer (PBS, pH 7.4), resuspended in buffer with 0.5% parafor-
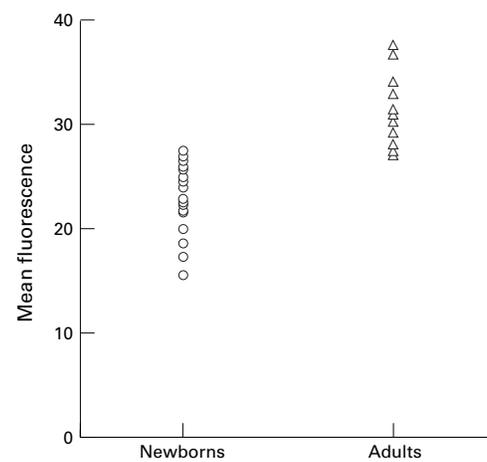

Figure 1   Relative fluorescence intensities of newborn and adult PMN. The PMN were isolated and incubated with 3.33×10^{-10} Fl-C5a. The difference in the mean FI for the two populations (Fl = 22.4 ± 3.5 and 31.5 ± 3.1) indicates that neonatal PMN express significantly fewer C5aR than adult PMN (p<0.001). (N=19 for neonatal and N=16 for adults).

maldehyde, and stored in the dark at 4°C until analysis within 24 hours by flow cytometry using a FACScan (Becton Dickinson, USA), with a 488 nm argon laser and 530 nm band pass filter. PMN were gated on the basis of their forward scatter/side characteristics and the arithmetic means of their fluorescence intensities were measured.

Statistical significance of differences between groups was determined using a two sample *t* test. A value of p<0.05 was considered significant.

Results

The relative fluorescence intensities of newborn and adult PMN incubated with a saturating concentration of FITC labelled rC5a are shown in fig 1. The significant difference (p<0.001) in the mean (SD) fluorescence intensity for the two populations (22.4 (3.5) and 31.5 (3.1)), indicates that neonatal PMN bear fewer C5aR than adult cells. To confirm low levels of complement activation, a potential confounding factor, we measured C3d plasma concentration and found a C3d mean value of 21 arbitrary units (range 15–40 AU), which is well under the limit indicating activation (52 AU).

The release of MPO after stimulation of newborn PMN with C5a was significantly lower than from adult PMN (p<0.05) (table 1), while exocytosis of LF showed no significant difference (p=0.75). On the other hand, following stimulation with fMLP, newborn PMN released about three times more MPO than adult PMN (p<0.001), while the release of LF from newborn PMN was also significantly higher (p<0.01). The total PMN MPO was not significantly different in adults and neonates, while the total PMN LF was significantly lower in neonates (p<0.001). The percentage of total MPO and LF released by neonatal PMN was much higher following fMLP than C5a stimulation: this difference was much less obvious in adults.

Table 1 C5a and fMLP mediated exocytosis of azurophil and specific granules from newborn and adult PMN

	Newborns (n=20)		Adults (n=20)	
	ng/10^{-7} cells (SD)	Release percentage (SD)	ng/10^{-7} cells (SD)	Release percentage (SD)
C5a triggered exocytosis of MPO	438 (191)★	1.2 (0.7)	602 (258)†	1.5 (0.5)
fMLP triggered exocytosis of MPO	1861 (937)	5.2 (3.4)	632 (258)	1.6 (0.5)
C5a triggered exocytosis of LF	657 (475)‡	7.1 (5.7)	694 (225)★★	4.0 (1.5)
fMLP triggered exocytosis of LF	1979 (1085)	22.0 (13.6)	1039 (409)	5.8 (2.2)

★ Total MPO = 40.1 µg/10^{-7} cells; † total MPO = 39.8 µg/10^{-7} cells; ‡ total LF = 10.7 µg/10^{-7} cells; total LF = 20.1 µg/10^{-7} cells.
The exocytosis was calculated as amount of enzyme released after subtraction of the spontaneous enzyme release and expressed in ng/10^{-7} cells.
The percentage of total cell content is calculated by dividing the enzyme release with the total cell content measured after sonication of the PMN.
n=8 for all experiments.

With both fMLP and C5a as stimuli, less migration of neonatal PMN was observed than in adult PMN. With C5a, neonatal PMN migrated 29.1 µm/30 minutes (SD 8.2) compared with 58.4 µm/30 minutes (SD 7.5) for adult PMN. The fMLP stimulated migration of PMN from newborns was 14.9 µm/30 minutes (SD 5.6) compared with 37.8 µm/30 minutes (SD 5.7) for adult PMN.

Discussion

As C5aR, in contrast to fMLP receptors, are not found as a substantial intracellular pool that can be rapidly translocated to the cell surface, the significantly lower neonatal expression of C5aR could possibly lead to a reduction of signalling by phosphorylated C5aR. This reduced expression does not seem to arise from receptor downregulation, due to complement activation (and thereby C5a generation) in the neonate, in that C3d concentrations measured in cord blood were, as in adult blood, not increased. Furthermore, earlier studies of priming of PMN have shown no alteration in C5aR expression.[36] Recently, Rebuck *et al*[19] reported lower responses to fMLP stimulation in isolated neonatal PMN than adult PMN, a difference not seen in whole blood PMN. This indicates that newborn PMN are more susceptible to isolation procedures than adult PMN. However, in our study, exocytosis after fMLP stimulation of isolated PMN from neonates was higher than in adult PMN.

It has been reported that neonatal PMN may be primed[37–39] and this could explain why we find a significantly higher release of MPO and LF from newborn PMN, when stimulated with fMLP, than from adult PMN, as newborn PMN are said to express normal numbers of fMLP-R. The amount of LF being released by neonatal PMN was higher, in spite of the fact that we and others have found a significantly lower total LF in newborn PMN.[15 37 39] It has recently been shown that a higher proportion of total intracellular LF in newborn PMN is associated with the lighter and more readily mobilisable gelatinase granules than in adult PMN,[39] and this, together with priming, could account for the high levels of LF exocytosis from newborn PMN. Priming of newborn PMN might also be expected to lead to increased exocytosis, in response to C5a. However, we found a similar release of LF in neonates and adults (in the context of lower total LF contents in neonates) and modestly lower MPO release in neonates (in the context of similar total MPO contents in both groups). These relatively low neonatal responses could be partly explained by low C5aR expression.

The significant difference we observe between the migration of adult and newborn PMN towards both C5a and fMLP is fully consistent with other reports.[1 7 40] Furthermore, the fact that the proportional reduction, in comparing neonatal and adult PMN migration, is essentially the same for both stimuli, suggests that the level of receptor expression for C5a and fMLP on PMN is not critical for their migratory activity. This conclusion is strengthened by the observation that we could show no correlation between PMN migration and C5aR expression either in neonates or adults. Further studies will be necessary to investigate whether there is any functional relation between levels of C5aR expression and exocytosis in infants and whether our observations have any clinical importance.

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