Neutrophil Adhesion Molecule Expression Is Comparable in Perinatal Rabbits and Humans

Chandra Ramamoorthy, M.B.B.S., F.R.C.A.(UK), W. Daniel Kovarik, M.D., Robert K. Winn, Ph.D., John M. Harlan, M.D., Sam R. Sharar, M.D.

Background: Human newborns, particularly those born before full term, are more susceptible to bacterial infections as a result of impaired host defense mechanisms. Compared with adults, circulating leukocytes from human newborns (preterm and full-term gestations) and newborn rabbits (full-term gestation) have low resting levels of CD62L (L-selectin) and do not significantly increase surface expression of CD18 after inflammatory stimulation. To determine the potential utility of preterm rabbits in investigations of perinatal human conditions, the authors compared the surface expression of the β2-integrin CD18 and CD62L (L-selectin) on polymorphonuclear leukocytes (PMNs) from perinatal rabbits and perinatal humans, both under resting conditions and after in vitro activation with inflammatory stimulants.

Methods: After erythrocyte lysis of whole-blood samples, leukocytes from 7-day-old, full-term (31-day gestation), and preterm (24-day gestation) rabbits, as well as full-term (37–42 week gestation) and preterm (27–36 week gestation) human newborns were prepared and stimulated in vitro at 37°C with either C5a or phorbol myristate acetate. After fluorescence labeling of CD18 and CD62L with monoclonal antibodies, PMN adhesion molecule expression was assessed by flow cytometry.

Results: Constitutive CD18 expression was not significantly different between perinatal and adult humans but was reduced in all perinatal rabbits compared with adults. Inflammatory stimulation caused significant increases in CD18 expression in adult human PMNs but not in full-term and preterm newborns. Changes in CD18 expression in adult and preterm rabbits after stimulation, although in the same direction as humans, were more variable. In both species, constitutive CD62L expression on PMNs from all perinates was significantly lower than in adults. However, CD62L was shed to similar degrees after inflammatory stimulation in all groups.

Conclusions: Preterm rabbits may provide a potentially useful experimental model to study PMN adhesion and host defense in the perinatal period, particularly preterm gestations. Specific advantages and limitations of rabbits in such studies are discussed. (Key words: Human and rabbit: adult; full-term newborn; preterm newborn; Leukocyte adhesion molecule expression: β2-integrins; L-selectin. Inflammatory stimuli.)

THE perioperative and intensive care of the septic newborn poses several challenges to both pediatric anesthesiologists and neonatal intensive care specialists. These challenges are encountered frequently because human newborns, particularly those born prematurely, are more susceptible to bacterial sepsis in the perinatal period compared with adults. This susceptibility is also associated with a high fatality rate despite aggressive antibiotic and supportive therapy. Because polymorphonuclear leukocytes (PMNs) provide the first line of host defense against bacterial infection, it is proposed that a maturity-dependent impairment in one or more basic PMN functions (e.g., mobility, chemotaxis, endothelial adherence, transendothelial migration, phagocytosis, or respiratory burst) may in part account for the increased risk of bacterial sepsis in neonates compared with adults. Furthermore, it is recently proposed that PMN-endothelial adhesion mechanisms play a significant role in perioperative inflammatory states,
ing pediatric surgical procedures. The focus of the current experiments is on molecular determinants of PMN-endothelial adherence that may be abnormal in the perinatal period.

To provide effective host defense against bacterial tissue invasion, circulating PMNs must first emigrate into the extravascular tissues at the infected site. This emigration process involves a multiple-step adhesion cascade with interaction between adhesion molecules expressed on PMNs and their cospecific ligands on vascular endothelium. Bacteria and their products, as well as local biochemical changes in vascular endothelium, induce changes in the surface expression of adhesion molecules on PMNs, resulting in an increased adhesive interaction between circulating PMNs and local vascular endothelium. This is followed by the transendothelial migration of adherent PMNs toward the bacterial source, where activated PMNs first phagocytize and then kill invading microorganisms.

The first step in the PMN-endothelial adhesion cascade results in low-affinity PMN adherence ("rolling") on endothelial cells and is mediated by the selectin family of adhesion molecules. The three selections in this family are structurally similar and found on circulating leukocytes (L-selectin) and activated endothelial cells (E- and P-selectin). L-selectin (designated CD62L) is constitutively expressed on resting, circulating PMNs and binds to various carbohydrate (e.g., sialyl Lewis X [SLeX]) and protein ligands on the endothelial cell. It is rapidly shed by proteolytic cleavage after PMN activation. Once the flow velocity of circulating PMNs is reduced by selectin-mediated rolling, firm PMN adherence to endothelial cells occurs and is mediated by members of the β2 integrin adhesion family. The β2 integrins share a common β subunit (CD18) associated with one of three distinct α subunits (CD11a, CD11b, or CD11c). CD11b/CD18 is the primary integrin mediating PMN binding to activated endothelium via its cospecific ligand intercellular adhesion molecule-1. CD11b/CD18 is constitutively expressed on resting, circulating PMNs but increases its surface expression after stimulation by bacteria or other chemokines. The recognition that congenital abnormalities in either β2 integrin- or selectin-dependent adhesion pathways (the leukocyte-adherence deficiency syndromes) can result in impaired bacterial host defense and sepsis suggests that similar abnormalities in the perinatal period may increase infectious risk in newborns.

Previous studies have described differences in the expression and regulation of adhesion molecules on human PMNs sampled during the perinatal period compared with those of adults. Polymorphonuclear cells from human full-term newborns show lower constitutive expression of CD62L, normal constitutive expression of CD11b/CD18, and attenuated upregulation of CD11b/CD18 in response to inflammatory stimuli. These observations correlate with impaired adherence of PMNs from full-term newborns to endothelial cells in vitro. Relevant to the issue of prematurity, findings of previous reports are more varied. Some studies show that preterm (24–32 weeks gestational age) and full-term (37–42 weeks) human newborn PMNs have similar patterns of CD11b/CD18 and CD62L expression at rest and with inflammatory stimulation, whereas others have shown gestational age-related differences in the stimulated expression of CD11b/CD18 and CD62L. Methodologic differences in the leukocyte isolation and preparation procedures used in these different studies may contribute to the various findings. One commonly used isolation technique (density gradient separation) can induce unpredictable changes in PMN surface molecule expression regardless of other stimuli. These artificial changes induced by cell preparation could then alter subsequent PMN responses to in vitro inflammatory stimulation.

Complementing these investigations of human newborn PMN adhesion mechanisms is a report of PMN adhesion and host defense in full-term newborn rabbits. Emigration of PMNs into the chemically inflamed peritoneum was reduced in 1-day-old rabbits compared with 14-day-old and adult rabbits and correlated with abnormal age-related expressions of CD11b/CD18 and CD62L on circulating PMNs that are similar to those reported from PMNs of full-term humans. These findings suggest the potential utility of rabbits in the study of perinatal PMN adhesion and host defense. However, resting and stimulated expression of PMN adhesion molecules in preterm rabbits, as well as a comparison with human preterm newborns under the same experimental conditions have not been reported.

We wanted to determine the potential biological and practical utility of the rabbit species for studies of leukocyte adhesion mechanisms in the perinatal period—including preterm gestation—by comparing observations of PMN adhesion molecule expression (CD18, CD62L) in rabbits with those of humans of similar gestational ages. In addition, to minimize potential artifactual changes induced by PMN isolation procedures, resting
and stimulated PMN surface adhesion molecule expression was determined on erythrocyte-lysed, whole-blood leukocytes rather than on physically isolated PMNs.

Materials and Methods

Human Samples
With approval from the institutional human subjects committee, blood was collected from the placental side of the umbilical cord after either cesarean section or vaginal delivery, because route of delivery is reported not to affect PMN adhesion molecule expression. Blood was obtained from 24–36-week gestation preterm (n = 9) and 37–42 week gestation full-term newborns (n = 12). The blood was anticoagulated with ethylene diaminetetraacetic acid and placed on ice immediately for transport to the laboratory for leukocyte preparation and labeling. Blood was also collected from healthy adult volunteers (n = 12) as controls.

Animal Samples
Institutional animal care and use committee approval was obtained, and National Institute of Health guidelines for experimental animals were followed for all experimental and euthanasia procedures. Seven-day-old neonatal rabbits (n = 8), full-term (31 days) rabbits less than 24 h old (n = 7), and 24–25-day pregnant doe rabbits (n = 5) were anesthetized with ketamine. Newborn and 7-day-old rabbits received intraperitoneal ketamine (10 mg/kg). After sternotomy, 3 ml blood was drawn by intracardiac aspiration. Pregnant rabbits received intravenous ketamine (1–2 mg/kg) and underwent cesarean section after local infiltration with 1% lidocaine. Umbilical vein blood was drawn from all intrauterine fetuses and pooled to a final volume of 1–4 ml for each doe. Blood was also drawn by peripheral arterial catheter from normal adult rabbits (n = 12) as controls. In all cases, blood was transferred to ethylene diaminetetraacetic acid-containing tubes, placed on ice, and prepared immediately for leukocyte labeling. After blood collection, the pregnant does and perinatal rabbits were killed with an overdose of intravenous (does) or intracardiac (newborns) pentobarbital.

Because ketamine was used to anesthetize 7-day-old, newborn, and pregnant rabbits, the effects of ketamine on PMN CD18 and CD62L expression were studied in a separate set of experiments. Blood (3 ml) was drawn by peripheral arterial catheter from adult rabbits (n = 4) into ethylene diaminetetraacetic acid-containing tubes. The animals were then anesthetized with 2 mg/kg intravenous ketamine and serial blood samples were drawn at 5, 15, 30, and 60 min, anticoagulated, and placed on ice for immediate leukocyte labeling.

Leukocyte Preparation
Rabbit and human leukocytes were prepared in a similar manner. All solutions and reagents were maintained and procedures conducted at 4°C to arrest cellular metabolism and to minimize artifactual changes in PMN adhesion molecule expression that occur with cellular metabolism or changes in temperature. Whole-blood erythrocytes were lysed with ammonium chloride/ethylene diaminetetraacetic acid, the sample was centrifuged, and the leukocytes were resuspended in cation-free phosphate-buffered saline. Leukocyte viability was confirmed by trypan blue exclusion.

Leukocyte Stimulation
After leukocytes were washed and resuspended in cation-free phosphate-buffered saline, aliquots (~5 × 10^6 cells) were stimulated for 15 min at 37°C with phorbol myristate acetate (PMA; 100 ng/ml) or human recombinant C5a (20 μg/ml). Two additional leukocyte aliquots, one at 4°C and the other at 37°C, did not receive inflammatory stimulation and served as controls for temperature-induced changes in CD18 and CD62L. After 15 min, stimulation was arrested by immersion in ice at 4°C. Leukocytes were centrifuged and resuspended in cation-free Hank’s balanced salt solution before immunostaining.

Immunostaining
Leukocyte samples were placed onto 96-well plates using a pipette, and surface F, receptors were blocked by incubation with heat-inactivated fetal calf serum. Stimulated leukocytes were labeled with 40 μg/ml murine IgG, monoclonal antibodies recognizing CD62L (LAM1.14 for humans and LAM1.3 for rabbits, gifts from T. Tedder, Ph.D., Duke University) or CD18 (mhm23, a gift from P. Jardeau, Ph.D., Genentech) or with an IgG1 isotype-matched, negative control monoclonal antibody (MOPC21; Organon Teknika-Cappel, Durham, NC). Cells were incubated for 30 min and then washed twice with cation-free Hank’s balanced salt solution with 2% heat-inactivated fetal calf serum. Fluorescein-conjugated goat anti-murine IgG, antibody (20 μg/ml; Southern Biotechnology, Birmingham, AL) was added, incu-
bated, and washed as before. Cells were fixed in 1% paraformaldehyde and stored in the dark at 4°C until flow cytometry was performed.

Flow Cytometric Analysis
The samples were analyzed on an Epics XL (Coulter, Hialeah, FL) flow cytometer. Granulocytes were gated based on their characteristic high forward and side light-scattering properties, with 525-nm (green) fluorescence displayed on a four-decade logarithmic scale. Mean fluorescence on the logarithmic scale was determined and reported as a ratio of the CD18 or CD62L fluorescence to that of the negative (isotype-matched IgG1) control. Sample sizes ranged from 5,000 to 20,000 cells, depending on sample yield.

Statistical Analysis
Data were treated as parametric and analyzed accordingly. For visual clarity, results depicted in figures are means ± SEM. Within each age group, stimulant-dependent differences in surface expression were analyzed by paired t tests. Constitutive adhesion molecule expression at 4°C was first compared with that at 37°C to note any effect of temperature change alone. The effects of either C5a or PMA stimulation at 37°C were then compared with the constitutive value at 37°C. Within each stimulation group, age-dependent differences were analyzed using analysis of variance and post hoc testing (Fisher’s protected least-significant difference test). Time-dependent variations in PMN surface expression after ketamine administration were analyzed by repeated-measures analysis of variance. All analyses were performed using Statview 4.1 (Abacus Concepts, Berkeley, CA), with significance set at P < 0.05.

Results

Expression of CD18 on Human Polymorphonuclear Cells
Constitutive PMN CD18 expression was not significantly different among adults, full-term newborns, and preterm newborns prepared completely at 4°C (fig. 1A). In vitro warming to 37°C without inflammatory stimulation caused a small but significant increase in PMN CD18 expression in all three age groups. After C5a stimulation, PMN CD18 expression increased by 66% in adults (P < 0.05 compared with that at 37°C without stimulation), but full-term and preterm newborns showed no increase in CD18 expression. After PMA stimulation, PMN CD18 expression increased in adults (by 53%; P < 0.05 compared with that at 37°C without stimulation) but did not change significantly in either full-term or preterm newborns.

Expression of CD62L on Human Polymorphonuclear Cells
Constitutive PMN CD62L expression was significantly lower (P < 0.05) in newborns, both full term and preterm, compared with adults (fig. 1B). In vitro warming to 37°C (without inflammatory stimulation) caused shedding of PMN CD62L in all three age groups (P < 0.05 only in full-term and preterm newborns). Stimulation with C5a resulted in additional CD62L shedding, whereas PMA stimulation resulted in nearly complete CD62L shedding in all three age groups (P < 0.05 compared with that at 37°C without stimulation).

Expression of CD18 on Rabbit Polymorphonuclear Cells
Constitutive PMN CD18 expression in the full-term rabbits was significantly lower compared with adult rabbits (P < 0.05; fig. 1C). Warming to 37°C without inflammatory stimulation (C5a or PMA) caused no change in CD18 expression in any of the groups. Adding C5a at 37°C did not significantly increase CD18 expression; however, addition of PMA at 37°C increased CD18 expression by 132% in adult and 158% in preterm rabbits (P < 0.05 compared to 37°C without stimulation). Polymorphonuclear cells from full-term and 7-day-old rabbits did not show such increased CD18 expression.

Expression of CD62L on Rabbit Polymorphonuclear Cells
Constitutive PMN CD62L expression was significantly higher (P < 0.05) in adults compared with each of the three newborn groups (fig. 1D). Warming to 37°C (without inflammatory stimulation) caused significant CD62L shedding in full-term rabbits. Stimulation with either C5a or PMA produced no further shedding of CD62L in premature rabbits but caused significant CD62L shedding in adult rabbits. CD62L expression on neonatal and full-term rabbit PMNs was not significantly lower after C5a or PMA stimulation, in part because their constitutive expression was already significantly attenuated.
Effect of Ketamine Administration on Rabbit PMN Adhesion Molecule Expression

Ketamine administration in vitro did not significantly affect the resting PMN expression of CD18 and CD62L in adult rabbits (Fig. 2).

Discussion

Previous studies in both full-term and preterm humans have reported abnormal expression and regulation of both CD62L and CD18 on circulating PMNs, as well as abnormal PMN-endothelial adhesion, compared with that seen in adult PMNs. However, the study of these phenomena in the perinatal period is limited because it can be difficult to obtain leukocyte or tissue samples from newborn humans, and even more so in obtaining samples in critically ill neonates with sepsis. Alternatively, systematic studies of these potential PMN-endothelial adhesion defects and their impact on host defense may be performed under controlled conditions in other species, such as rabbits, where molecular adhesion mechanisms in full-term newborns, as well as adults, are already preliminarily defined. The purpose of this study was to further evaluate the potential utility of rabbits for such studies, focusing on preterm PMN surface expression of CD18 and CD62L under resting and stimulated conditions in vitro compared with that of humans of similar gestational maturity in the perinatal period. Furthermore, a whole-blood leukocyte labeling technique was used to avoid artifactual changes in PMN surface molecule expression that can be induced by PMN isolation techniques.

Our finding that constitutive CD18 expression on human PMNs does not change with gestational maturity corresponds with previous studies of isolated PMNs. However, our finding of reduced constitutive expression of CD18 on perinatal rabbit PMNs differs from previous reports and may be the consequence of dif-

Fig. 1. CD18 (A) and CD62L (B) expression on circulating polymorphonuclear cells (PMNs) from human adult, full-term, and preterm newborns before and after inflammatory stimulation in vitro: CD18 (C) and CD62L expression (D) on circulating PMNs from rabbit adults, neonates, full-term, and preterm newborns before and after inflammatory stimulation in vitro. Within (†, ¥) and between (¥) age group comparison are shown. †P < 0.05, 37°C, no stimulation compared with 4°C, no stimulation. ¥P < 0.05, 37°C, C5a and phorbol myristate acetate stimulation compared with 37°C, no stimulation.

Fig. 2. Effect of intravenous ketamine on polymorphonuclear surface expression of CD18 and CD62L in adult rabbits.
ferent leukocyte preparation procedures. Briefly warming the cells to 37°C without other stimulation caused an increase in expression of CD18 on human cells from all age groups but not on rabbit PMNs. This finding in humans is consistent with reports that cooling leukocytes from body temperature to room temperature or 4°C has little effect on leukocyte integrin surface expression, whereas rewarming to 37°C after cooling to 4°C does increase integrin expression.29,31 This temperature dependence of surface expression underscores the importance of performing proper temperature control experiments (i.e., without inflammatory stimulants such as C5a or PMA) and referring changes induced by inflammatory stimulation to the appropriate temperature control. Why this same temperature-dependent change in constitutive CD18 expression was not demonstrated in rabbits is unclear.

In vitro stimulation of PMNs with C5a or PMA (substances that initiate PMN activation) resulted in increased CD18 expression in samples from human and rabbit adults but not from human full-term and preterm newborns or from full-term or 7-day-old rabbits. These observations are similar to those of previous reports using isolated PMNs, in which stimulation with C5a or PMAs resulted in either diminished or absent CD18 upregulation in the perinatal period.19,22,23 Impaired surface mobilization of preformed intracellular stores of CD11b/CD18 is the proposed mechanism of this abnormality.21 However, after stimulation of rabbit preterm PMNs in the current study with PMAs, we observed increased expression of CD18 to a level similar to that seen on both adult rabbit and adult human PMNs. One explanation for this finding is that in contrast to those reports using isolated PMNs, we studied unisolated, whole-blood leukocytes. Rebuck et al.24 reported similar findings in preterm humans using a whole-blood labeling technique, suggesting that leukocyte preparation techniques can significantly affect CD18 expression in this setting. However, these investigators also noted adult-like responses in stimulated CD18 expression in full-term human newborns. We did not find such CD18 upregulation on either human or rabbit full-term newborns, and thus it is unlikely that the upregulation we noted in preterm rabbits is entirely the consequence of the leukocyte isolation technique.

An alternative explanation for this unexpected upregulation of CD18 is our use of preterm fetal rather than preterm newborn PMNs in the rabbit studies. Smith et al.25 reported a correlation between gestational age and expression of PMN CD11b/CD18 in newborn humans, but they found no such correlation in human fetuses. Fetal PMNs (obtained by ultrasound-guided percutaneous umbilical cord sampling) showed greater CD18 expression with inflammatory stimulation in vitro than did preterm newborn PMNs, similar to our findings in preterm fetal rabbits. The mechanisms and implications of the differing patterns of CD18 regulation on preterm newborn and preterm fetal PMNs are unknown and worthy of future study.

Polymorphonuclear cell CD62L expression that we observed in perinatal humans and rabbits in our study is consistent with previously reported data in humans.20,32 Cooling cells to 4°C and rewarming to 37°C caused shedding of CD62L in full-term and preterm humans and in full-term rabbits. Further shedding occurred after either C5a or PMA stimulation in all the human age groups and in adult rabbits. Fetal rabbits were particularly resistant to the effects of inflammatory agents and appeared not to shed CD62L, although their resting expression was so low that additional shedding was difficult to assess. Although leukocyte isolation procedures can cause artifactual L-selectin shedding,27 our findings obtained from whole-blood leukocytes suggest that temperature-dependent CD62L shedding does occur in vitro, regardless of the cell preparation technique.

Our results for both resting and stimulated PMN CD18 and CD62L expression in perinatal rabbits were comparable to those found for perinatal humans. These data suggest that the rabbit may be potentially useful as an experimental model to study the effects of maternal disease, perinatal development, and neonatal sepsis on PMN-endothelial adhesion phenomena, including adhesion molecule expression, PMN adhesion, and trans-endothelial migration. Rabbits have short gestation periods to provide a convenient source of preterm newborns and have been used extensively to study leukocyte adhesion biology. Rabbits also provide advantages over humans for these types of studies in that (1) they can be studied under consistent and predictable delivery conditions in the laboratory, free from highly variable or unknown human maternal factors (e.g., substance abuse); (2) both blood and tissue can be easily sampled to permit simultaneous analysis of both leukocyte and endothelial adhesion factor expression and regulation; and (3) leukocyte–endothelial adhesive interactions can be viewed in situ by videomicroscopy.33 In addition, use of ketamine in these animals for anes-
thetic purposes has no significant effect on expression of the primary PMN adhesion molecules CD62L and CD18.

There are, however, some limitations to using perinatal rabbits in these types of studies. First, there are potential technical difficulties in obtaining large blood samplings from fetal rabbits because of their small size (∼50 g). Blood was pooled from several (up to eight) sibling fetuses to obtain sufficient quantity (1-4 ml) for a single study. In addition, monoclonal antibodies that recognize rabbit PMN adhesion molecules are not as readily available as they are for humans. In addition, we observed a greater variability in the surface expression of adhesion molecules on rabbits PMNs (particularly CD18) than on human PMNs. Finally, our observations of increased CD18 expression with inflammatory stimulation of preterm rabbit but not preterm human PMNs may be the result of fetal versus newborn blood sampling, as discussed. However, further explanation of this difference is necessary before results of similar studies in rabbits can be applied to humans.

Using a cell preparation and labeling technique that does not require density-gradient PMN isolation, we found that expression of the PMN adhesion molecules CD18 and CD62L in perinatal rabbits is similar to that in perinatal humans, both under resting and stimulated conditions. These data suggest that rabbits may provide a useful model to study PMN-endothelial adhesion and transmigration in the perinatal and perioperative periods.

References

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