Eur J Pediatr (2001) 160: 478–482

ORIGINAL PAPER

George D. Georgeson · Barnabás J. Szőny · Károly Streitman · Attila Kovács
László Kovács · Aranka László

Natural killer cell cytotoxicity is deficient in newborns with sepsis and recurrent infections

Received: 30 August 2000 and in revised form: 9 January 2001 / Accepted: 22 March 2001

Abstract We investigated natural killer (NK) cell cytotoxicity in healthy preterm and full-term newborns in comparison to adults, to elucidate the possible role of delivery mode in influencing the NK activity and to evaluate the NK activity in severe neonatal pathological conditions such as bacterial sepsis and recurrent infections. NK cell cytotoxicity was investigated using a 4 h ^{51}Cr release assay with K562 cells as targets expressed as percentage kill in the following study groups: full-term normal spontaneous vaginal delivery ($n = 55$), full-term caesarean section ($n = 51$), preterm normal spontaneous vaginal delivery ($n = 34$), preterm caesarean section ($n = 28$), bacterial sepsis ($n = 15$), recurrent neonatal infections ($n = 8$) and healthy adults aged between 22–42 years ($n = 89$). NK activity for the normal newborns was determined in paired cord and 2–4 day-old neonate blood. The NK cell cytotoxicity in healthy newborns was significantly lower than in adults ($P < 0.01$). Prematurity was associated with a significant decrease in NK cell activity compared to full-term neonates ($P < 0.05$). The mode of delivery did not influence the NK cytotoxicity. In sepsis and recurrent infections, a dramatic decrease in NK cell cytotoxicity was seen related to healthy newborns ($P < 0.01$).

Conclusion Natural killer cell cytotoxicity is deficient in both neonatal sepsis and recurrent infections.

Key words Chromium release assay · Natural immunity · Neonatal pathology · Recurrent infection · Sepsis

Abbreviations *FT-CS* full-term caesarean section · *FT-NSVD* full-term normal spontaneous vaginal delivery · *IFN* interferon · *IL* interleukin · *NK* natural killer · *PT-CS* preterm caesarean section · *PT-NSVD* preterm normal spontaneous vaginal delivery

Introduction

Natural killer (NK) cells are a distinct subpopulation of lymphocytes [12, 16] which in contrast with cytotoxic T lymphocytes (T killer cells) do not rearrange the T lymphocyte antigen receptor genes, do not have an antigen specific receptor and do not require previous antigenic activation [23]. NK cells can kill a variety of tumour cells and virally infected cells [5, 15, 17, 20, 21,

G. D. Georgeson
Mullikin Medical Centers Artesia, CA 90701, USA

B. J. Szőny (✉) · L. Kovács
Department of Obstetrics and Gynaecology,
Albert Szent-Györgyi Medical University,
Semmelweis u. 1, 6725 Szeged, Hungary
Tel.: +36-62-545497; Fax: +36-62-545711
e-mail: szony@obgyn.szote.u-szeged.hu

K. Streitman · A. László
Department of Paediatrics,
Albert Szent-Györgyi Medical University,
Szeged, Hungary

A. Kovács
Blood Transfusion Centre,
Albert Szent-Györgyi Medical University,
Szeged, Hungary

24, 48]. Apparently they have a role in haematopoiesis, suppressing the development of haematopoietic progenitors [8, 14, 41, 43]. NK cell activity also seems to be the first line mechanism in transplanted organ rejection and graft survival [25, 27, 33, 45, 47].

The mechanism of lysis is not well understood. Osmotic lysis mediated by perforins [3, 29, 30], necrosis and apoptosis are the most important mechanisms of target cell killing [49].

NK cytotoxicity is subject to a complex regulation. Pro-inflammatory mediators such as interleukin-2 (IL-2), and interferon (IFN) γ were shown to be NK activity augmenting cytokines, both in adults and neonates [4, 19] Incubation of cord cells with IL-15 and IL-12 results in an increase in NK cell cytotoxicity. IL-15 increases the number of NK cells, suggesting that it might be clinically useful in treating immunodeficient patients [34, 35]. In contrast, IL-4, a known Th2-type cytokine, is a potent suppressor of NK cell mediated necrotic and apoptotic cytotoxicity [11]. Other bioactive molecules such as hormones and several drugs might also modulate NK cell function. The NK activity depressing effect of theophyline, epinephrine, corticosteroids and members of the arachidonic acid cascade such as prostaglandin E_2 has been well documented [6, 22, 26, 32, 36, 42].

Fetal and neonatal NK cell activity is significantly decreased compared to adults [13, 28] which explains the increased neonatal susceptibility to infections. Until now, no neonatal normal values of NK activity were established. Data concerning the NK activity in health threatening conditions such as neonatal sepsis and recurrent infections are also missing. The endocrine-metabolic and immune variations observed after different degrees of delivery-related stress and the anaesthetic drugs given to the mother during caesarean section might influence cord blood NK activity. In the present work we investigated the role played by the NK cell cytotoxicity in recurrent infections and neonatal sepsis contrasted to healthy neonatal and adult NK activity as well as the possible influence exerted by the mode of delivery.

Patients and methods

The study was previously approved by the Human Investigation Review Board and blood samples were collected after informed consent had been obtained. According to the mode of delivery and gestational age four study groups were established: full-term normal spontaneous vaginal delivery (FT-NSVD, $n = 55$), full-term caesarian section (FT-CS, $n = 51$), preterm normal spontaneous vaginal delivery (PT-NSVD, $n = 34$) and preterm caesarean section (PT-CS, $n = 28$). In each case venous blood was obtained at the time of delivery from the cord vein and after 2–4 days by venopuncture of a peripheral vein of the newborn. Two additional categories were established for the study of NK cell cytotoxicity related to neonatal infection. The first group consisted of 15 newborns, who were diagnosed with sepsis by positive blood cultures. The second pathological population consisted of eight different newborns who presented a primitive, early onset infection at birth or within the first days of life and during hospitalisation developed a secondary infection such as bacterial sepsis, pneumonia and/or fungal infection occurring consequently within 28 days of life. The septic newborns from the first group were infected with group B streptococci ($n = 6$), *Listeria monocytogenes* ($n = 3$) or *Escherichia coli* ($n = 6$). Newborns having recurrent infections ($n = 8$), in addition to *Escherichia coli*, *Listeria monocytogenes* or group B streptococci positive blood cultures, later developed pneumonia and/or *Candida albicans* generalised infections.

Both full-term and preterm healthy neonates irrespective of the mode of delivery were appropriate for gestational age, born to healthy mothers with negative medical and obstetrical history having a 5 min Apgar score ≥7, at 38–42 and 31–37 weeks of gestation, respectively (mean gestational age ± SD was 39.5 ± 1.1 weeks for FT-NSVD, 39.6 ± 1.5 weeks for FT-CS, 34.4 ± 1.8 weeks for PT-NSVD and 34 ± 1.7 weeks for PT-CS). Septic newborns and those having had recurrent infections were appropriate for gestational age, born at 31–42 weeks of gestation with 5 min Apgar scores of 6–8 and with a maternal history of fever (temperature > 38 °C) and/or premature rupture of membranes greater than 24 h (mean gestational age ± SD for septic newborns and those having had recurrent infections were 35.7 ± 3.3 and 32.6 ± 1.4 weeks, respectively). In these cases, mothers were treated with antibiotics. Neonatal NK activity was compared to healthy adults aged between 22–42 years ($n = 89$).

Natural killer cell cytotoxicity assay

The NK cell cytotoxicity was measured using the standard 4 h ^{51}Cr release cytotoxicity assay as previously described [37]. Briefly, peripheral blood mononuclear cells were isolated from heparinised venous blood samples on Fycoll-Hypaque gradient (Pharmacia, Piscataway, N.J.) Blood samples were processed within 12 h from the time obtained. After washing, the cell count was adjusted to 2×10^6 viable cells/ml in RPMI 1640 containing 10 mM HEPES, 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Sigma, St. Louis, MO.) As targets we used ^{51}Cr-labelled K562 human erythroleukaemic cells. Prior to the experiments, K562 cells were maintained in complete medium without HEPES but with 5 mM extra L-glutamine and 60 μg/ml Tylocine. For labelling, 10^6 target cells in 100 μl of medium were incubated with 100 μCi of ^{51}Cr (sodium chromate) in physiological saline solution. The labelled cells were washed three times in medium and resuspended at the appropriate concentration, usually 3×10^4 cells/ml in order to give 3,000 cells/well. Prior to each experiment the viability of both target and effector cells was tested using Trypan blue exclusion. Effector and target cells were than co-cultured in 96 well V-bottomed microplates at various effector-target cell ratios ranging from 100:1 to 3:1, achieved by making serial doubling dilutions of the effector cell suspension. The six effector-target ratios used were set up in triplicate. The plates were then centrifuged at $150 \times g$ for 1 min and placed in a 37 °C humidified 5% CO_2 incubator for 4 h. Thereafter the plates were centrifuged again for 10 min and 100 μl aliquots were removed for counting in a gamma counter. The cpm of the total incorporated label was determined by resuspending the target cells and harvesting 100 μl. The percentage cell mediated lysis (%CML) was calculated as follows:

$$\%CML = \{[cpm(test) - cpm(medium)] / [cpm(max) - cpm(medium)]\} \times 100\%$$

The Student s *t*-test was used to determine differences in NK cell cytotoxicity at the effector-target cell ratio at 50:1 and differences in slopes.

Results

NK cytotoxicity of healthy neonates proved to be significantly lower than in adults as indicated by our ^{51}Cr

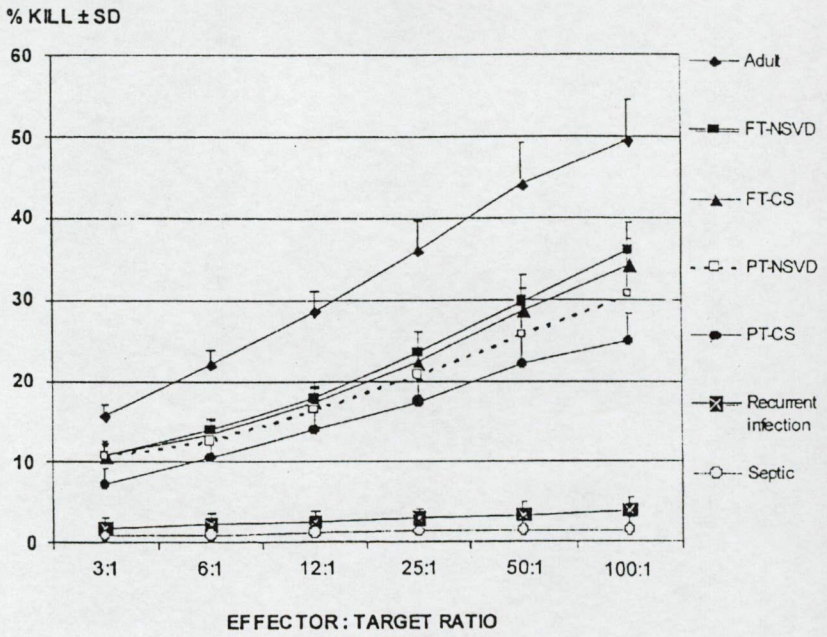


Fig. 1 NK cell cytotoxicity of FT-NSVD ($n = 55$), FT-CS ($n = 51$), PT-NSVD ($n = 34$), PT-CS ($n = 28$) and newborns diagnosed with recurrent infections ($n = 8$) or sepsis ($n = 15$) compared to healthy adults ($n = 89$), expressed as percentage kill values at different effector-target ratios

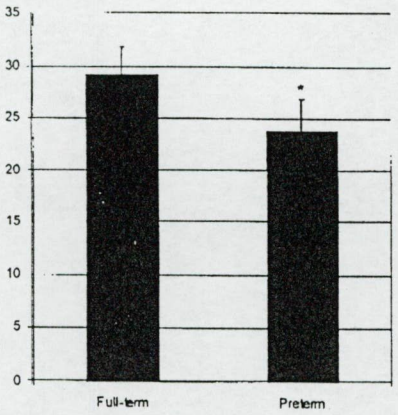


Fig. 2 Comparison between average percentage kill ± 1 SD values for full-term ($n = 106$) and preterm ($n = 62$) healthy neonates at 50:1 effector-target ratio showing a significant ($P < 0.05$) decrease in NK cell cytotoxicity of preterm neonates

cytotoxicity assays (44.2 ± 5% kill for adults and 26.6 ± 3.21% kill for the healthy neonatal population; $P < 0.01$) (Fig. 1). The possible role of the prematurity in influencing the cytotoxic activity of NK cells was also investigated. On the basis of the percentage kill values obtained, the preterm neonatal population compared to full-term healthy neonates showed a defective NK cell cytotoxicity, probably related to the immaturity of the immune system ($P < 0.05$) (Fig. 2). The influence of the delivery mode on neonatal NK cell response within the two populations (i.e. preterm and full-term healthy newborns) showed no significant differences at none of the effector-target ratios tested. Although the slopes of NK cytotoxicity versus effector to target ratios for FT-NSVD and FT-CS were decreased, they were not statistically significant.

The pathological cases (i.e. sepsis and recurrent infections) demonstrated a marked failure of NK response in comparison to both adults and healthy neonates ($P < 0.01$). The average value of the percentage kill for septic neonates was never higher then 1.5%. Neonates having recurrent infections showed a slight but statistically not significant elevation of the NK cell cytotoxicity (3.5% kill at 50:1 effector-target ratio; Fig.1). At lower concentrations of the effector cells, the difference between percentage kill values of neonates diagnosed with recurrent infections and those having neonatal sepsis was only faintly evident. Similarly, when paired cord and neonate blood samples were analysed, no statistically significant difference was found.

Discussion

The role of NK cells in normal immunity is not clearly established. These cells were attributed to antiviral and anti-neoplastic defence, however, neither viral infection associated nor tumour associated inflammatory infiltrates show significant numbers of NK cells. The one setting in which large numbers of NK cells predominate in the lesions is the graft-versus-host disease in recipients of bone marrow transplants. During fetal development, NK cell activity can be first identified in the fetal liver at 9 weeks of gestation. At 19 weeks, NK cell-like activity can be detected both in fetal liver and spleen and by the 28th week it appears in the peripheral blood as well [44, 46]. Previous studies have shown that at birth, healthy newborns have significantly lower NK activity than adults [7, 18]. Similarly the decreased NK

cytotoxicity of preterm versus full-term newborns has also been demonstrated [31, 40]. Our results are in perfect accordance with previously reported data. One possible explanation of the diminished NK activity seen in preterm newborns in comparison to that of full-term babies could rely on the insufficient maturation of the immune system. The concordance of our and previously reported data clearly demonstrates the reliability of our cytotoxicity assay.

The decreased NK activity in bacterial sepsis and in recurrent infections was somewhat surprising because as we have already discussed, the physiological role of NK cells in natural immunity has been assigned mainly for antiviral, anti-tumoural and anti-graft defense. Recently considerable attention has been paid to the elucidation of the role that NK cells might play in antibacterial and antifungal defence. Previous studies revealed that human NK cells exhibit potent bactericidal activity against both gram-negative and gram-positive bacteria at least in vitro. Ultrastructural studies revealed close contact between NK membranes and bacteria without any evidence of phagocytosis [9]. Other studies proposed that NK cell-bacteria contact is not necessary for efficient killing [10]. In accordance with the later findings, other investigators have demonstrated that the antibacterial activity derived from human T and NK cells may be partly mediated by antibacterial peptides LL-37 and alpha defensins (HNP 1–3). These peptides were also shown to possess chemotactic activity for other immune effector cells such as polymorphonuclear leucocytes and CD4-positive T-lymphocytes [2]. In mice lacking functional T cells, NK cell-derived IFNγ was able to activate macrophages to kill infectious organisms such as *Listeria monocytogenes* [1]. Taken together all these results indicate that NK cells are involved in antibacterial protection both through direct NK cell-bacteria contact, through antibacterial peptides and through a cross-talking between NK cells and other effector cells of the immune system via cytokines. In vitro studies of NK cell cytotoxic activity in adults with septic shock gave significantly lower results than in normal adults and this functional deficiency was paralleled by a severe decrease in NK cell number [38, 39]. This finding gave us the impetus to investigate the NK cell cytotoxicity in such an important clinical entity as neonatal sepsis and recurrent infection. Our results demonstrate that in these conditions the NK cell activity is dramatically decreased in comparison to normal preterm and full-term newborns. The lack of the diagnosis of an immunodeficiency syndrome further supports the significance of these results showing a decrease of NK cytotoxicity in severe bacterial infections. It is debatable if this insufficiency of NK cell function is a cause of the developing bacterial sepsis or it is rather an effect of this condition. This issue could be clarified by comparing NK cell cytotoxicity before and after sepsis and/or recurrent infection. However, the elucidation of this question was beyond the scope of the present study and awaits further investigation. To the best of our knowledge, this is the first publication showing a defective NK cell cytotoxicity in bacterial sepsis and recurrent infections of the neonate.

References

1. Abbas AK, Lichtman AH, Pober JS (1994) Cellular and molecular immunology. 2nd edn. WB Saunders. Philadelphia. pp 274–276
2. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jornvall H, Wigzell H, Gudmundsson GH (2000) The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 96: 3086–3093
3. Bonavida B (1987) Molecular mechanism of natural killer cell mediated cytotoxicity. Adv Exp Med Biol 213: 299–307
4. Braakman E, Van Tunen A, Meager A, Lucas CJ (1986) IL-2 and IFN gamma enhanced natural cytotoxic activity: analysis of the role of different lymphoid subsets and implications for activation routes. Cell Immunol 99: 476–488
5. Chin TW, Plaeger-Marshall S, Ank BJ, Pressmann SR, Stiehm ER (1991) Effects of herpes simplex virus on induced cell-mediated cytotoxicity in neonates and adults. Nat Immun Cell Growth Reg 10: 237–246
6. Ching CY, Ching N, Seto DS, Hokama Y (1984) Relationships of prostaglandin levels and natural killer (NK) cell cytotoxicity of mononuclear cells in cord blood. J Med 15: 233–236
7. Dominguez E, Madrigal JA, Layrisse Z, Cohen SB (1998) Fetal natural killer cell function is suppressed. Immunology 94: 109–114
8. Fujimori Y, Hara H, Nagai K (1988) Effect of lymphokine-activated killer cell fraction on the development of human hematopoietic progenitor cells. Cancer Res 48: 534–538
9. Garcia-Penarrubia P, Koster FT, Kelley RO, McDowell TD, Bankhurst AD (1989) Antibacterial activity of human natural killer cells. J Exp Med 169: 99–113
10. Garcia-Penarrubia P, Bankhurst AD, Koster FT (1989) Experimental and theoretical kinetics study of antibacterial killing mediated by human natural killer cells. J Immunol 142: 1310–1317
11. Gardiner CM, Renn DJ (1998) Differential cytokine regulation of natural killer cell-mediated necrotic and apoptotic cytotoxicity. Immunology 93: 511–517
12. Grossi CE, Ferrarini M (1982) Morphology and cytochemistry of human large granular lymphocytes. In: Herberman RB (ed) NK cell and other natural effector cells. Academic Press. New York, pp 1–8
13. Hallberg A, Malmstrom P (1982) Natural killer cell activity and antibody-dependent cellular cytotoxicity in newborn infants. Acta Paediatr Scand 71: 431–436
14. Hamood M, Corazza F, Bujan-Boza W, Sariban E, Fondu P (1995) Natural killer (NK) cells inhibit human umbilical cord blood erythropoiesis. Exp Hematol 23: 1187–1191
15. Hayward A, Laszlo M, Vafai A (1989) Human newborn natural killer cells responses to activation by monoclonal antibodies. Effects of culture with herpes simplex virus. J Immunol 142: 1139–1143
16. Herberman RB (1986) Natural killer cells. Ann Rev Med 37: 347–352
17. Herberman RB, Ortaldo JR (1981) Natural killer cells: their roles in defense against disease. Science 214: 24–30
18. Hoshina T, Kida K, Ito M (1999) Difference in response of NK cell activity in newborns and adults to IL-2, IL-12 and IL-15. Microbiol Immunol 43: 161–166
19. Ibayashi Y, Tokuda Y, Saks ER, Sarna GP, Golub SH (1987) In vivo and in vitro activation of NK cytotoxicity with IL-2. Prog Clin Biol Res 244: 275–285
20. Jenkins M, Mills J, Kohl S (1993) Natural killer cytotoxicity and antibody dependent cellular cytotoxicity of human immunodeficiency virus infected cells by leucocytes from human neonates and adults. Pediatr Res 33: 469–474

21. Kaminsky P, Skopinska-Rozewska E, Marianowski L, Majewski S, Radomska M (1994) Relationship between decreased activity of NK cells in umbilical cord blood and infection incidence in children. Wiad Lek 47: 103–106
22. Kotiranta-Ainamo A, Apajasalo M, Pohjavuori M, Rautonen N, Rautonen J (1999) Mononuclear cell subpopulations in preterm and full-term neonates: independent effects of gestational age, neonatal infection, maternal pre-eclampsia, maternal betamethasone therapy and mode of delivery. Clin Exp Immunol 115: 309–314
23. Lanier LL, Phillips JH (1988) What are natural killer cells? Immunology 1: 15–19
24. London L, Perussia B, Trinchieri G (1985) Induction of proliferation in vitro of resting human natural killer cells: expression of surface antigens. J Immunol 134: 718–726
25. Lotzova E, Savary CA (1977) Possible involvement of natural killer cells in bone marrow graft rejection. Biomedicine 27: 341–344
26. Lotzova E, Savary CA (1981) Parallelism between the effects of cortisone acetate on hybrid resistance and natural killing. Exp Hematol 9: 766–774
27. Lotzova E, Savary CA, Pollack SB (1983) Prevention of rejection of allogeneic bone marrow transplants by NK1.1 antiserum. Transplantation 35: 490–494
28. Lubens RG, Gard SE, Soderberg-Warner M, Stiehm ER (1982) Lectin dependent T-lymphocytes and natural killer cytotoxic deficiencies in human newborns. Cell Immunol 74: 40–53
29. Marx JL (1986) How killer cells kill their targets? Science 231: 1367–1369
30. Masson D, Tschopp J (1985) Isolation of a lytic, pore-forming protein (perforin) from cytotoxic T lymphocytes. J Biol Chem 260: 9069–9072
31. McDonald T, Sneed J, Valenski WR, Dockter M, Cooke R, Herrod HG (1992) Natural killer cell activity in very low birth weight infants. Pediatr Res 31: 376–380
32. Milch PO, Salvatore W, Luft B, Baker DA (1988) Suppression of newborn natural killer cell activity by prostaglandin E2. Am J Obstet Gynecol 159: 895–896
33. Nemlander A, Saksela E, Hayry P (1983) Are "natural killer" cells involved in allograft rejection? Eur J Immunol 13: 348–350
34. Nguyen QH, Roberts RL, Ank BJ, Lin SJ, Lau CK, Stiehm ER (1998) Enhancement of antibody-dependent cellular cytotoxicity of neonatal cells by interleukin-2 (IL-2) and IL-12. Clin Diagn Lab Immunol 5: 98–104
35. Nguyen QH, Roberts RL, Ank BJ, Lin SJ, Thomas EK, Stiehm ER (1998) Interleukin (IL)-15 enhances antibody-dependent cellular cytotoxicity and natural killer activity in neonatal cells. Cell Immunol 185: 83–92
36. Okumura Y, Kudo J, Ikuta T, Kurokawa S, Ishibashi H, Okubo H (1985) Influence of acute-phase proteins on the activity of natural killer cells. Inflammation 9: 211–219
37. Pross HF, Maroun JA (1984) The standardization of NK cell assays for use in studies of biological response modifiers. J Immunol Methods 68: 235–249
38. Puente J, Miranda D, Gaggero A, Maturana P, Godoy G, Salazar R, Sepulveda C (1991) Immunological defects in septic shock. Deficiency of natural killer cells and T-lymphocytes. Rev Med Chil 119: 142–146
39. Puente J, Carvajal T, Parra S, Miranda D, Sepulveda C, Wolf ME, Mosnaim AD (1993) In vitro studies of natural killer cell activity in septic shock patients. Response to a challenge with alpha-interferon and interleukin-2. Int J Clin Pharmacol Ther Toxicol 31: 271–275
40. Sancho L, de-la-Hera A, Casas J, Vaquer S, Martinez C, Alvarez-Mon M (1991) Two different maturational stages of natural killer lymphocytes in human newborn infants. J Pediatr 119: 446–454
41. Scala G, Allavena P, Ortaldo JR, Herberman RB, Oppenheim JJ (1985) Subsets of human large granular lymphocytes (LGL) exhibit accessory cell functions. J Immunol 134: 3049–3055
42. Screpanti I, Santoni A, Gulino A, Herberman RB, Frati L (1987) Estrogen and anti-estrogen modulation of the levels of mouse natural killer activity and large granular lymphocytes. Cell Immunol 106: 191–202
43. Shau H, Golub SH (1985) Signals for activation of natural killer-like activity. Nat Immun Cell Growth Reg 4: 113–119
44. Toivanen P, Uksila J, Leino A, Lassila O, Hirvonen T, Ruuskanen O (1981) Development of mitogen responding T cells and natural killer cells in the human fetus. Immunol Rev 57: 89–105
45. Uhteg LC, Kupiec-Weglinsky JW, Rocher LL, Salomon DR, Tilney NL, Carpenter CB (1986) Systemic natural killer activity following cardiac engraftment in the rat: lack of correlation with graft survival. Cell Immunol 100: 274–279
46. Uksila J, Lassila O, Hirvonen T, Toivanen P (1983) Development of natural killer cell function in the human fetus. J Immunol 130: 153–156
47. Weber B, Welte M, Hammer C, Stadler J, Koller C, Caspo C (1984) Increase of natural killer cells in rejecting kidney grafts. Transplant Proc 16: 1177–1178
48. Zhuo AS, Luo LL, Sun M, Han YK (1993) Effect of immune RNA on NK activity of infants born to mothers with cytomegalovirus infection. Chin Med J (Engl) 106: 361–365
49. Zychlinsky A, Zheng LM, Liu CC, Young JD (1991) Cytotoxic lymphocytes induce both apoptosis and necrosis in target cells. J Immunol 146: 393–400

Antioxidant enzyme activities are decreased in preterm infants and in neonates born via caesarean section

George D. Georgeson[1], Barnabás J. Szőny[2,3], Károly Streitman[4], Ilona Sz. Varga[5], Attila Kovács[6], László Kovács[3], Aranka László[4]

[1]Mullikin Medical Centers Artesia, CA. 90701 USA; [2]Department of Pathophysiology; [3]Department of Obstetrics and Gynecology, WHO Collaborating Center for Research in Human Reproduction, [4]Department of Pediatrics, [5]Biological Isotope Laboratory; [6]Blood Transfusion Center; University of Szeged, Hungary

Corresponding Author: László Kovács, M.D., D.Sc., Department of Obstetrics and Gynaecology, WHO Collaborating Center for Research in Human Reproduction, Albert-Szent-Györgyi Medical and Pharmaceutical Center, University of Szeged, H-6725, Semmelweis u. 1., Szeged, Hungary.

Phone: +36-62-545 498, Fax:+36-62-545 711

E-mail:kovacs@obgyn.szote.u-szeged.hu

Abstract

OBJECTIVES: To investigate the antioxidant defence potential of human neonates according to gestational age and mode of delivery.

STUDY DESIGN: Four study groups were established: full-term normal spontaneous vaginal delivery (FT-NSVD, n=24), full-term caesarean section (FT-CS, n=19), preterm normal spontaneous vaginal delivery (PT-NSVD, n=15) preterm caesarean section (PT-CS, n=21). The activity of catalase (CAT), glutathion peroxidase (GPX), Cu/Zn superoxide dismutase (Cu/Zn-SOD) were determined from cord blood. Statistical analysis was made by ANOVA.

RESULTS: CAT activity was significantly higher in full-term than in preterm newborns. In both categories neonates born via caesarean section had significantly lower CAT activities. GPX activity was significantly higher in the FT-NSVD group than in any other group. Cu/Zn-SOD activity was significantly higher in full-term neonates than in preterms and no difference was found related to the mode of delivery.

CONCLUSIONS: Prematurity and caesarean section may cause a deficiency of antioxidant defence in human newborn.

Keywords: neonate, caesarean section, antioxidant enzymes, preterm newborn, full-term neonate

Introduction

Aerobic organisms possess antioxidant defense systems that deal with reactive oxygen species (ROS) as a result of aerobic respiration and substrate oxidation. ROS, including hydroxil radicals (•OH), superoxid anions (O2•—) and hydrogen peroxide (H2O2), are generated in response to external and internal stimuli [1-3]. Low levels of ROS are necessary for several biological processes including intracellular differentiation and cell progression or arrest of growth, apoptosis, immunity and defense against microorganisms [4]. In contrast, high doses and/or inadequate removal of ROS result in oxidative stress, which may cause damages to biological macromolecules. The naturally occurring antioxidants in low-density lipoproteins (LDLs) and plasma protect cells from oxidation. The prevention of lipid peroxidation is an essential process because lipid peroxidation products can cause DNA damage and directly inhibit proteins such as Na+/K+-ATPases and glutamate transporters [5].

The enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Superoxide dismutase (EC1.15.1.1) is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to O2 and H2O2. In humans there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD and extracellular SOD. Cu/Zn-SOD is believed to play a major role in the first line of antioxidant defense and high SOD activities were correlated with high immune competence [6]. CAT and GPX play an important role in the detoxification of H2O2. CAT (EC1.11.1.6) reacts very efficiently with H2O2 to form water and molecular oxygen and with hydrogen donors with peroxidase activity thus protecting cells against the H2O2 generated within them. GPX (EC1.11.1.19) catalyses the reaction of hydroperoxides using GSH, protecting mammalian cells against oxidative damage. In fact glutathione metabolism is one of the most essential antioxidative defense mechanisms [7].

The onset of labor is associated with an increased production of proinflammatory mediators, which might induce an increase in the production of free radicals. In addition, oxygenation of both mother

and child tissues oscillate frequently during labour, leading to an overproduction of free radicals as a consequence of tissue reoxigenation, which suggests that both maternal and fetal antioxidant systems might be overloaded during vaginal delivery [8]. This hypothesis is supported by several findings, including those which indicate that xanthine oxidase activity in placentae of labouring women was higher than in placentae obtained from pregnancies terminated by caesarean section. These data further indicate that labour might enhance free radical production [9]. However, the competence of enzymatic antioxidant systems through which the newborn might protect himself against an increased load of free radicals during labor depends on the gestational age, since antioxidant enzymes display lower activities during intrauterine life and in preterm than in full-term neonates [10, 11, 12]. Therefore, in the present study we proposed to elucidate the antioxidative enzyme activity in full-term and preterm neonates and the correlation between the way of delivery (per vias naturales vs. caesarean section) and the antioxidant defense systems of the newborn.

Materials and methods

Patients

The study was previously approved by the Human Investigation Review Board, and blood samples were collected after informed consent had been obtained. According to the mode of delivery and gestational age four study groups were established: full-term normal spontaneous vaginal delivery (FT-NSVD, n=24), full-term caesarean section (FT-CS, n=19), preterm normal sponatneous vaginal delivery (PT-NSVD, n=15) and preterm caesarean section (PT-CS, n=21). In both the full-term and preterm category only elective caesarean section cases have been included. The indication for caesarean section was initiation of preterm labor and/or previous caesarean section associated or not with cephalopelvic disproportion. In each case venous blood was obtained at the time of delivery from the cord vein.

Both full-term and preterm healthy neonates irrespective of the mode of delivery were appropriate for gestational age, born to healthy mothers with negative medical and obstetrical history having a five-minute Apgar score ≥7, at 38-42 and 31-37 weeks of gestation, respectively (mean gestational age ± SD was 39.22 ± 1.15 for full-term infants, 34.3 ± 1.6 weeks for preterm neonates, 39.1 ± 1.1 weeks for FT-NSVD, 39.3 ± 1.3 weeks for FT-CS, 34.5 ± 1.8 weeks for PT-NSVD and 34 ± 1.8 weeks for PT-CS).

Determination of antioxidant enzyme activities

Cu/Zn-SOD activity was determined via inhibition of the epinephrine-adrenochrome autooxidation at 480 nm [13]. CAT activity was measured spectrophotometrically at 240 nm in aliquots of hemolysates. Enzyme activities were expressed in Bergmeyer units (BU). One BU is the amount of CAT that decomposes 1000 mg H2O2/min. GPX activity was determined in supernatant aliquots. As substrates reduced glutathione (GSH) and cumene hydroperoxide were used. GSH degradation was measured using Ellman's reagent [14, 15].

Other measurements

Lipid peroxidation was determined with the thiobarbituric acid (TBA) method [15, 16] which determines the level of the total TBA-reactive substance. Plasma GSH was determined using Ellman's reagent. Protein content was determined using the Folin reagent [17].

Statistical analysis

Statistical analysis of the data was made by ANOVA. For significant ANOVA values groups were compared by Tukey's test for multiple comparisons with unequal cell size. A probability level of 0.05 was accepted as indicating significant differences.

Results

CAT activity in healthy neonates proved to be significantly higher in the FT-NSVD category in comparison to PT-NSVD (average ± SE was 2.10 ± 0.16 mBU/mg protein for full-term healthy neonates and 1.86 ± 0.16 mBU/mg protein for the preterm healthy neonatal population). We found that the neonates born via cesarean section had a significantly lower CAT activity than the vaginally delivered babies in both the full-term and the preterm category. (Figure 1.A.).

GPX activity showed to be significantly higher in the FT-NSVD category than in the other study groups (average ± SE for the FT-NSVD category was 1.25 ± 0.07 mU/mg protein whereas the values for the other study groups were 0.71 ± 0.14 mU/mg protein for FT-CS, 0.91 ± 0.08 mU/mg protein for PT-NSVD and 0.79 ± 0.19 mU/mg protein for PTCS). The comparison of the PT-NSVD group to the two cesarean section categories showed that the average value of the GPX activity was higher in this study population, however no statistical significance could be demonstrated (Figure 1.B.)

Cu/Zn-SOD activities were significantly elevated in the full-term neonates in comparison to the preterm population, irrespective to the way of delivery (Figure 1.C.).

As for lipid peroxidation and GSH levels we could not find any significant difference between the four study populations neither in the plasma nor in red blood cells (data not shown).

Discussion

Oxygen species are important participants in damage caused by virus infections, progression to cancer and neurodegenerative processes [18]. They can be toxic at the molecular level and they are important effectors in ageing and lifespan determination. Our study raised the question of possible correlation between the antioxidant defense of the neonate and the way of delivery. This has been hypothesized by several recent findings, especially those which are indicating an increase in free radical production during vaginal delivery in contrast with ceasarean section, in which a diminished

free radical load seems to be likely. Free radicals were reported to play an important role in the pathogenesis of several pathological conditions such as haemolytic disease of the newborn, bronchopulmonary dysplasia, and retinopathy of prematurity [19]. Indeed, neonates born by caesarean section have an increased incidence of these conditions. The involvement of oxidative damage in pathological pregnancies is another important issue with clinical relevance since in pregnancy induced hypertension an increased lipid peroxidation was reported, which may also impact on the vascular function and antioxidant status of the fetus [20]. However, the elucidation of this question was beyond the scope of our present work. Our results clearly show that the antioxidant defense mechanisms of the neonate are profoundly modulated by both the gestational age and the way of delivery, specifically the CAT activity is significantly higher in full-term than in preterm neonates and similarly, it is higher in neonates born by spontaneous vaginal delivery, than in those which were born by caesarean section. The other antioxidant enzyme activities were less affected by the way of delivery, but the gestational age proved to be a determinant factor of the activity levels. This later finding is in perfect accordance with previous data published by this laboratory and by other investigators, showing that the antioxidant status of preterm newborns is lower compared to full-term neonates [10], which emphasizes the reliability of our measurements. The significantly lower enzyme activities which were seen in premature newborns could partly be explained by the immature enzyme systems of these infants. However, recent data showing that the biosynthesis of glutathione was active in leukocytes from preterm infants seem to suggest that enzyme maturity is not always the limiting step in determining the competence of cellular antioxidant systems [11]. The significance of our present findings is further emphasized by the fact that the study was conducted on purified study populations, excluding any pathological condition which might contaminate the results.

Increased free radical production during labour suggests another interesting possibility which is that the oxidative burden of infants born via caesarean section might be lower, thus probably resulting

in lower antioxidant enzyme expression, and lower activity levels, as well. This might explain our findings and could be addressed by investigating the expression of antioxidant enzymes at molecular level.

Despite different antioxidant enzyme activities, the lipid peroxidation and GSH levels were not significantly different in the four study populations neither in plasma nor in red blood cells. This might indicate that in a population with negative internal medical and perinatal history the prematurity and/or caesarean section *per se* do not affect significantly lipid peroxidation. However, these data draw our attention to the fact that any concomitant disease condition which orchestrates the mechanisms of oxidative stress may result in a more rapidly developing and more severe oxidative damage in premature neonates and in those born via caesarean section.

Based on our measurements we conclude that preterm babies and those born via caesarean section might be predisposed to pathological conditions in which reactive oxygen species may play a pathogenic role, due to deficient antioxidant defence.

References

1. Hurst R, Bao Y, Jemth P, Mannervik B, Williamson G. Phospholipid hydroperoxide glutathione peroxidase activity of rat class Theta glutathione transferase T2-2. Biochem Soc Trans 1997;25:S559.

2. Jornot L, Petersen H, Junod AF. Hydrogen peroxide induced DNA damage is independent of nuclear calcium but dependent on redox-active ions. Biochem J 1998;335:85-94.

3. Mills EM, Takeda K, Yu ZX et al. Nerve growth factor treatment prevents the increase in superoxide produced by epidermal growth factor in PC12 cells. J Biol Chem 1998;273:22165-22168.

4. Lee YJ, Galoforo SS, Berns CM et al. Glucose deprivation-induced cytotoxicity and alterations in mitogen-activated protein kinase activation are mediated by oxidative stress in multidrug-resistant human breast carcinoma cells. J Biol Chem 1998;273:5294-5299.

5. Patterson RA, Leake DS. Human serum, cysteine and histidine inhibit the oxidation of low density lipoprotein less at acidic pH. FEBS Lett 1998;434:317-321.

6. Prasad T, Kundu MS. Serum IgG and IgM responses to sheep red blood cells (SRBC) in weaned calves fed milk supplemented with Zn and Cu. Nutrition 1995;11:712-715.

7. Sigalov AB, Stern LJ. Enzymatic repair of oxidative damage to human apolipoprotein A-I. FEBS Lett 1998;433:196-200.

8. Stipek S, Mechurova A, Crkovska J, Zima T, Platenik J. Lipid peroxidation and superoxide dismutase activity in umbilical and maternal blood. Biochem Mol Biol Int 1995;35:705-711.

9. Many A, Roberts JM. Increased xanthine oxidase during labour-implications for oxidative stress. Placenta 1997;18:725-726.

10. Novak Z, Varga SI, Kovács L, Pál A, Pataki L, Matkovics B. The effects of Oradexone and Ambroxol pretreatment of the oxidative sensitivity of the red blood cells in preterm infants. Clin Chim Acta 1989;182:241-245.

11. Lavoie JC, Chessex P. Development of glutathione synthesis and gamma-glutamyl transpeptidase activities in tissues from newborn infants. Free Radic Biol Med 1998;24:994-1001.

12. Lindeman JH, Lentjes EG, van Zoeren-Grobben D, Berger HM. Postnatal changes in plasma ceruloplasmin and transferrin antioxidant activities in preterm babies. Biol Neonate 2000;78:73-76

13. Misra HP, Fridovich I. The role of superoxide anion in the antioxidation of epinephrine and a simple measurement of superoxide dismutase. J Biol Chem 1977;247:3170-3175.

14. Chiu DTY, Stults FH, Tappel AL. Purification and properties of rat lung soluble glutathione peroxidase. Biochem Biophys Acta 1976;445:558-566.

15. Sedlak I, Lindsay RH. Estimation of total protein-bound and non-protein sulfhydryl groups in tissue with Ellman`s reagent. Anal Biochem 1968;25:192-205.

16. Placer ZA, Cushman L, Johnson BC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical system. Anal Biochem 1966;16:359-364.

17. Lowry OH, Rosebrough NJ, Farr AL et al. Protein measurement with the Folin reagent. J Biol Chem 1951;193:265-275.

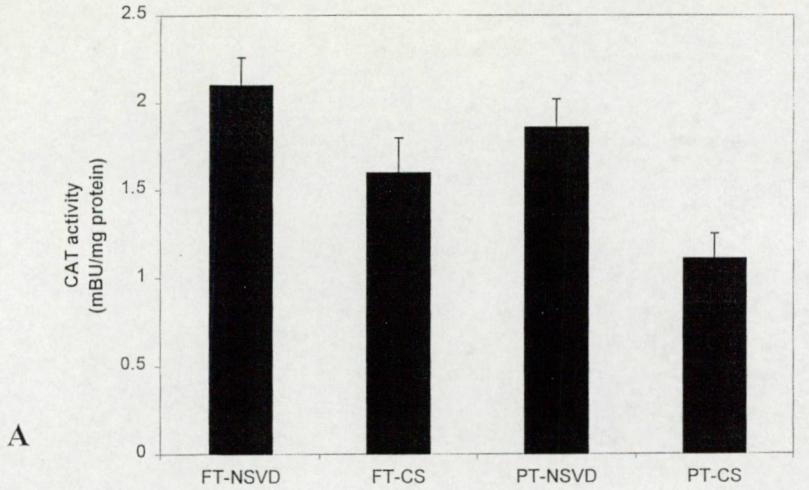
18. Facchinetti F, Dawson VL, Dawson TM. Free radicals as mediators of neuronal injury. Cell Mol Neurobiol 1998;18:667-682.

19. Halliwell B, Gutteridge JMC. Free radicals in Biology and Medicine, New York: Oxford University Press, 1999.

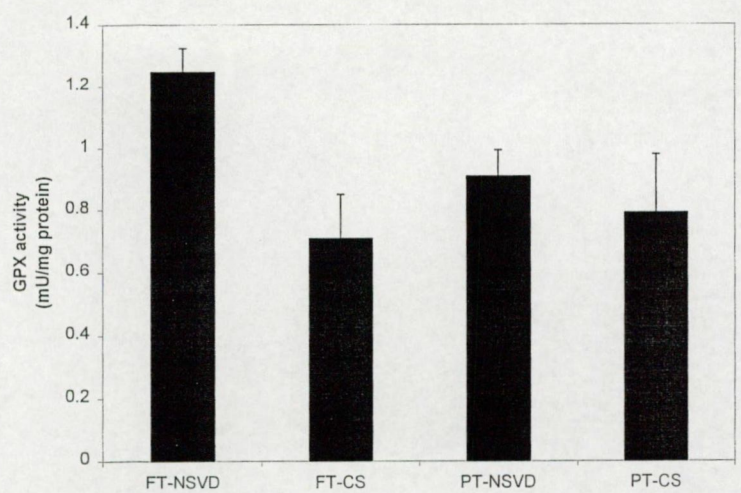
20. Uotila J, Tuimala R, Pyykko K, Ahotupa M. Pregnancy-induced hypertension is associated with changes in maternal and umbilical blood antioxidants. Gynecol Obstet Invest 1993;36:153-157.

Legend to figures

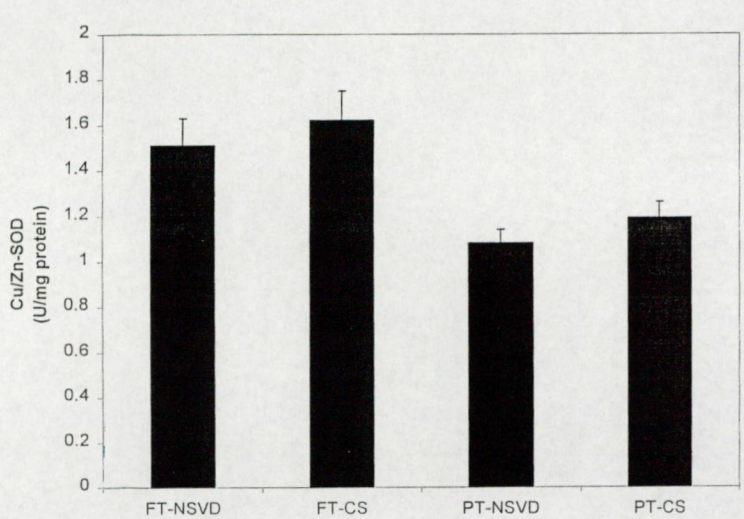
Figure 1. Antioxidant enzyme activities in full-term normal spontaneous vaginal delivery (FT-NSVD, n=24), full-term caesarean section (FT-CS, n=19), preterm normal spontaneous vaginal delivery (PT-NSVD, n=15) and preterm caesarean section (PT-CS, n=21) newborns. Enzyme activities were measured in cord blood hemolysates. Staistical analysis was made by ANOVA. For significant ANOVA values groups were compared by Tukey's test for multiple comparisons with unequal cell size. A probability level of 0.05 was accepted as indicating significant differences. Groups marked in a similar way are not statistically different. A. Catalase (CAT) activity±SE in mBU/mg protein. B. Glutathione peroxidase (GPX) activity±SE in mU/mg protein. C. Copper-zinc superoxide dismutase (Cu/Zn-SOD) activity±SE in U/mg protein.


2.5
2
1.5
1
0.5
0
CAT activity
(mBU/mg protein)
FT-NSVD
FT-CS
PT-NSVD
PT-CS

A


1.4
1.2
1
0.8
0.6
0.4
0.2
0
GPX activity
(mU/mg protein)
FT-NSVD
FT-CS
PT-NSVD
PT-CS

B


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cu/Zn-SOD
(U/mg protein)
FT-NSVD
FT-CS
PT-NSVD
PT-CS

C

Arylsulfatase-A in umbilical cord blood: gestational age and way of delivery do not influence enzyme activity

George D. Georgeson[1], Barnabás J. Szőny[2], Károly Streitman[4], Éva Sallay[4], Attila Kovács[5], László Kovács[3], Aranka László[4]

[1]Mullikin Medical Centers Artesia, CA. 90701 USA; [2]Department of Pathophysiology; [3]Department of Obstetrics and Gynecology, WHO Collaborating Center for Research in Human Reproduction, [4]Department of Pediatrics; [5]Blood Transfusion Center; University of Szeged, Hungary

Corresponding Author: László Aranka, M.D., D.Sc., Department of Pediatrics, Albert-Szent-Györgyi Medical and Pharmaceutical Center, University of Szeged, H-6720, Korányi fasor 14-15, Szeged, Hungary.

Phone: +36-62-545 330, Fax:+36-62-545 329

E-mail:laszloar@pedia.szote.u-szeged.hu

Summary:

The possibility to use umbilical cord blood for transplantation in several enzyme deficiencies has received increasing attention because of the availability of cord blood, the reduced incidence of post-transplantation complications, such as graft-versus-host disease and the possible accomplishment of good corrective results following transplantation, even in case of greater HLA disparity. The use of hematopoietic stem cells from unrelated donors is even more recommendable for the treatment of inherited enzyme deficiencies, because it might reduce the risk of originating the transplanted cells from a carrier of the defect, which might have an inadequate corrective ability. Our study was designed to elucidate whether the gestational age and way of delivery influences the arylsulfatase-A activity in the umbilical cord blood. Enzyme activities proved to be similar in the four populations studied (full-term normal spontaneous vaginal delivery, full-term caesarean section, preterm normal spontaneous vaginal delivery and preterm caesarean section). Therefore, umbilical cord blood samples seem to be suitable for transplantation in metachromatic leukodystrophy, regardless of gestational age and way of delivery. Moreover, our results are the first published data on normative values for arylsulfatase-A activity in human umbilical cord blood.

Keywords: cord blood, arylsulfatase-A activity, gestational age, way of delivery

Running Head: Arylsulfatase-A in human umbilical cord blood

Introduction

Human lysosomal arylsulfatase-A (ASA) is a member of the highly conserved sulfatase gene family. It is synthetized as a 507 amino acid precursor and is processed in the endoplasmic reticulum to yield a mature 489 amino acid protein. Each sulfatase is characterized by high substrate specificity. From ten of the different human sulfatases which are known until today, six are located in the lysosomes where they are responsible for the degradation of glycosaminoglycanes and sulfolipids. Besides their physiological substrates arylsulfatases also degrade synthetic chromogens and fluorogens.[1] ASA`s major natural substrate is cerebroside 3-sulfate which will accumulate if there is a deficiency in ASA, resulting in a lysosomal storage disorder, known as metachromatic leukodystrophy.[2]

Recently, umbilical cord blood has received increasing attention as a source of unrelated hematopoietic stem cells for transplantation. Engraftment using umbilical cord blood has proven effectiveness in treating lysosomal diseases, as indicated by normalization of the defective enzymatic activity in such important clinical entities like globoid cell leukodystrophy, metachromatic leukodystrophy, mannosidosis, fucosidosis, aspartylglucosaminuria, Hurler, Maroteaux-Lamy, Sly syndromes and Gaucher disease Type III.[3] The use of umbilical cord blood has several advantages above the well-known bone-marrow transplantation from unrelated donors, such as the ready availability of donor cells and the lower incidence of graft-versus host disease. The latter provides the ability to use umbilical cord blood even if there is a greater HLA disparity.[4-6]

Pulkkinen et al.[7] were the first who determined normative values for ASA and some steroid-sulfatases during intrauterine development. Previous results concerning the

activity of α-glucosidase, mannosidase, fucosidase and ASA in chorionic villi did not show any correlation of enzyme activity with gestational age, except for α-glucosidase.[8] Recent clinical data indicate that an incomplete reconstitution of the enzyme activity following the engraftment of bone-marrow cells from related donors who may carry the genetic defect (heterozygotes) results in a lessened improvement of the central nervous system status for diseases so treated, probably due to the inadequate corrective ability of the donor cells.[9] Therefore, the correct choice of the donor is of particular importance. The use of umbilical cord blood for treatment of enzymatic defects seems to be a reliable alternative for bone-marrow transplantation. However, our present knowledge regarding the levels of activity of lysosomal enzymes in cord blood is related only to one pioneering study showing that α-L-iduronidase, galactocerebrosidase and ASA levels in cord blood do not differ from adult levels.[10] Since no previous data exist regarding the levels of activity of ASA in umbilical cord blood related to gestational age and way of delivery, we cannot know if any cord blood sample, regardless the gestational age and way of delivery are equally effective in treating metachromatic leukodystrophy by transplantation. Therefore, the decision was made to investigate ASA activity in umbilical cord blood samples from preterm and full-term newborns, born by vaginal delivery and cesarean section.

Materials and methods

Patients

The study was previously approved by the Human Investigation Review Board, and blood samples were collected after informed consent had been obtained. According to the mode of delivery and gestational age four study groups were established: full-term normal spontaneous vaginal delivery (FT-NSVD, n=38), full-term caesarean section (FT-CS, n=22), preterm normal spontaneous vaginal delivery (PT-NSVD, n=26) and preterm caesarean section (PT-CS, n=21). In both the full-term and preterm category only elective caesarean section cases have been included. In each case venous blood was obtained at the time of delivery from the cord vein.

Both full-term and preterm healthy neonates irrespective of the mode of delivery were appropriate for gestational age, born to healthy mothers with negative medical and obstetrical history having a five-minute Apgar score ≥7, at 38-42 and 34-37 weeks of gestation, respectively (mean gestational age ± SD was 39.1 ± 1.1 weeks for FT-NSVD, 39.3 ± 1.3 weeks for FT-CS, 35.5 ± 1.8 weeks for PT-NSVD and 35.2 ± 1.6 weeks for PT-CS). The entire patient population included in this study had negative anamnestic history in siblings, parents and grandparents for any inherited metabolic diseases.

Determination of ASA activity in cord blood samples

ASA activity was measured in leukocyte homogenates prepared from cord blood samples obtained at the time of delivery. Briefly, umbilical cord blood (10 ml) transported to the laboratory within 1 hour from the time obtained, was subjected to Ficoll-Hypaque

gradient centrifugation (Pharmacia, Piscataway, N.J.). After washing, the cell count was adjusted to 30×10^6 cells/ml in physiologic saline solution. The obtained cell suspension was then subjected to 5 freezing-thawing cycles in order to lyse the cells. The cellular debries were removed from the lysed leukocyte suspension by centrifugation at 8000×g for 10 min. The precleared leukocyte homogenate was further used for the determination of protein content by the method of Lowry et al.[11] and for the direct measurement of ASA activity.

ASA was assayed by the method of Singh et al.[12] All the reagents used in the assay were purchased from Sigma (Budapest, Hungary). The assay was performed using 20 mM nitrocatechol sulfate as substrate in 0.2 ml M-sodium acetate buffer (pH 4.9), also containing 0.5 M $Na_4P_2O_7$ and 1.7 M NaCl. To this, 200 µl of leukocyte homogenate was added and the mixture was incubated at 37 °C for 4 hours. The reaction was terminated by the addition of 100 µl 2.5 M NaOH and 100 µl 0.15 M EDTA. Liberated nitrocatechol was measured at 515 nm with nitrocatechol (20 µM) as standard, and ASA activity was expressed as nmoles nitrocatechol/h/mg protein. In the control samples leukocyte homogenate and substrate were incubated separately and mixed immediately prior to the addition of NaOH and EDTA.

Statistical analysis

Statistical analysis of the data was made by ANOVA. For significant ANOVA values groups were compared by Tukey's test for multiple comparisons with unequal cell size. A probability level of 0.05 was accepted as indicating significant differences.

Results

The mean values of ASA activity in cord blood leukocytes are indicated in Figure 1. No significant difference in ASA activity was detected in preterm versus full-term newborns (61.6 ± 41.4 for preterms and 67.6 ± 40.4 for full-terms; mean ± 1 SD) (Fig. 1A) In FT-NSVD newborns the enzyme activity was 61.3 ± 35.2 (range 12.1-143). The mean ASA activity was slightly elevated in both the FT-CS and PT-CS category, whithout reaching statistical significance (87.5 ± 50.3, range 38-193 for FT-CS and 81.8 ± 39.6, range 43-159 for PT-CS). In contrast, PT-NSVD infants had a lower ASA activity (31.4 ± 22.2 range 9.6-68), which was not significantly different from the previous categories either, as shown by ANOVA (Fig. 1B). The percentage of values below 30, which were considered as being indicative of a possible pseudo-deficiency was 18.4% for FT-NSVD, 0% for FT-CS, 15.4% for PT-NSVD and 4.7% for PT-CS, respectively. The frequency of possible pseudo-deficiency on the basis of enzyme activity level below 30 for the full-term population was 11.7% as well as for preterm newborns 10.6% (11.2% for the entire population).

Discussion

This is the first study determining the values of ASA in four different newborn categories. Prior to this study it has been shown that ASA activity of umbilical cord blood is equivalent to the activity of normal adults.[10] Metachromatic leukodystrophy is an important pediatric disease which is inherited in an autosomal recessive manner. Its incidence is particularly high in the North American and Eastern European population and represents an important burden for both the patient's parents and the health care

providers. Until the advent of the very promising new modality of treatment which is represented by the transplantation of bone-marrow derived cells thought to be able to overcome the genetic defect, the traditional cure for this disease had very poor results. However, even this new treatment modality was shadowed by the important difficulties because of availability of suitable donors, which is significantly restricted due to HLA disparity. Whilst in other cases the most recommendable donor is a close relative of the patient, in the case of genetic disorders such as metachromatic leukodistrophy the use of cells obtained from related donors is not recommendable because even if the donor is clinically healthy it cannot be ruled out that the related donor is a heterozygote carrier of the genetic defect. This incovenience became evident after incomplete restauration of the enzyme activity resulting in a lessened improvement of the central nervous system status in patients so treated has been reported.[9] Therefore, one possible alternative for bone-marrow transplantation would be the engraftment of umbilical cord blood. In favor of this treatment modality pleedes the fact that umbilical cord blood is one of the most available sources of hematopoietic stem cells. Moreover, after umbilical cord blood transplantation, even in the case of a greater HLA disparity, the incidence of graft-versus-host disease is lower. Thus the only limitation to the use of umbilical cord blood for the treatment of metachromatic leukodystrophy seems to be related to the possibility that the newborn from whom the cord blood had been obtained might be a carrier of the genetic defect as well. Another possible limitation to qualify as a donor is the high incidence of pseudodeficiency of the enzyme in the general population, which could be as high as 10-15 %.[13] The very recent data reported by de Gasperi and co-workers[10] concerning the ASA activity in umbilical cord blood refer to a random neonate population, which

certainly increase the value of the reported data as reference values for the enzyme activity. However, our knowledge about the ASA activity in different newborn categories remains insufficient. The variation of enzyme activities with gestational age is not an uncommon phenomenon, raising the possibility that ASA activity could be lower in prematures than in full-term newborns. In the today`s HMO oriented cost effective health care system the use of easy to obtain donor cells with maximal therapeutic potential and with minimal costs is highly recommended. Therefore the study of the ASA activity in different newborn categories in order to verify the potential corrective ability of the cells to be engrafted and to establish the reference values of ASA activiy as a function of gestational age and way of delivery is of paramount importance. Our data clearly demonstrate that the activity of ASA is not different in any of the categories studied. Moreover, our data are comparable to those reported by de Gasperi et al.[10] As a result, every normal newborn`s umbilical cord blood could qualify equally effective as donor cell source for the treatment of metachromatic leukodystrophy. However, before engrafting umbilical cord cells, the determination of the enzyme activity by a qualified laboratory in order to exclude any possible pseudodeficiencies would be recommendable.

References

1. Von Figura K, Schmidt B, Selmer T, Dierks T. A novel protein modification generating an aldehyde group in sulfatases: its role in catalysis and disease. *BioEssays* 1998; **20**: 505-510.
2. Parenti G, Meroni G, Ballabio A. The sulfatase gene family. *Curr Opin Genet Dev* 1997; **7**: 386-391.
3. Krivit W, Peters C, Shapiro EG. Bone-marrow transplantation as effective treatment of central nervous system disease in globoid cell leukodystrophy, metachromatic leukodystrophy, adrenoleukodystrophy, mannosidosis, fucosidosis, aspartylglucosaminuria, Hurler, Maroteaux-Lamy, Sly syndromes and Gaucher disease Type III. *Curr Opin Neurol* 1999; **12**: 167-176.
4. Kurtzberg J, Laughlin M, Graham ML *et al.* Placental blood as a source of hematopoietic stem cells for transplantation into unrelated patients. *New Engl J Med* 1996; **335**: 157-166.
5. Wagner JE, Rosenthal J, Sweetman R *et al.* Successful transplantation of HLA-matched and HLA mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. *Blood* 1996; **88**: 795-802.
6. Wagner JE, Kurtzberg J. Cord blood stem cells. *Curr Opin Hematol* 1997; **4**: 413-418.
7. Pulkkinen M. (1961) Arylsulfatase and the hydrolysis of some steroid sulphatases in developing organism and placenta. *Acta Physiol Scand* 1961; **32** (Suppl. 180)

8. Németh M, László A, Kovács A, Falkay G. Lysosomal enzyme activities in frozen, non-cultured chorionic villi for prenatal diagnosis of enzymopathies. *Acta Med Hung* 1992-93; **49**: 143-148.

9. Peters C, Shapiro EG, Anderson J *et al.* Hurler syndrome: Outcome of HLA-genotypically identical sibling and HLA haploidentical related donor bone marrow transplantation in fifty-four children. *Blood* 1998; **91**: 2601-2608.

10. deGasperi R, Raghavan SS, Gama Sosa MA *et al.* Measurement from umbilical cord blood of four lysosomal enzymatic activities: α-L-iduronidase (Hurler), galactocerebrosidase (globoid cell leukodystrophy, arylsulfatase A (metachromatic leukodystrophy), arylsulfatase B (Maroteaux-Lamy). *Bone Marrow Transpl* 2000; **25**: 541-544.

11. Lowry OH, Rosebrough NJ, Farr AL *et al.* Protein measurement with the Folin reagent. *J Biol Chem* 1951; **193**: 265-275.

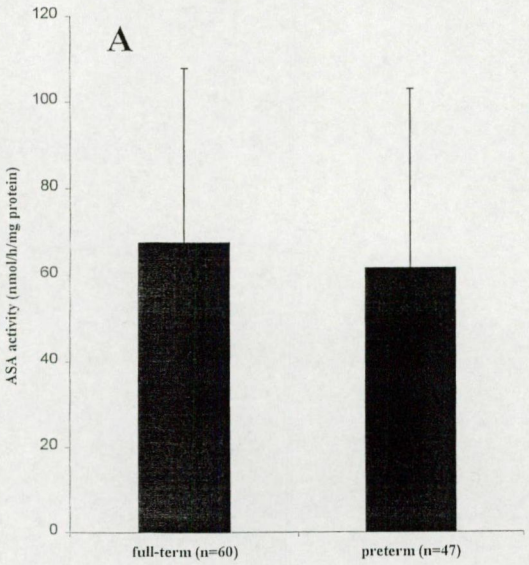
12. Singh J, Tavella S, Di Ferrante N. Measurements of arylsulfatases A and B in human serum. *J Pediatr* 1975; **86**: 574-576.

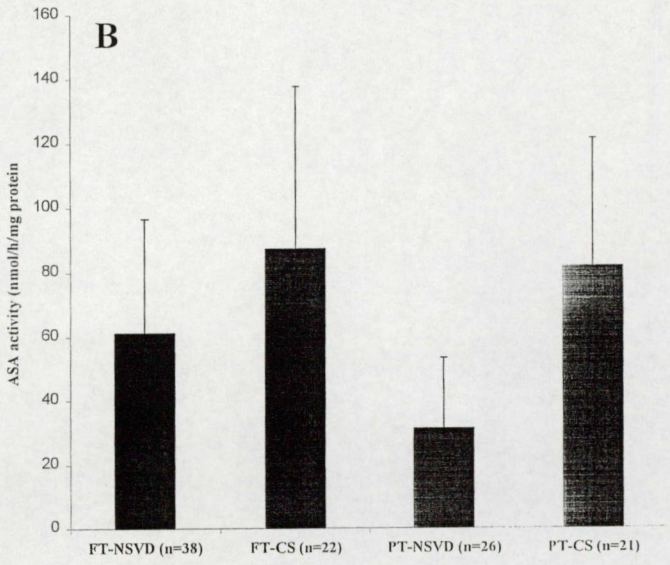
13. Baldinger S, Pierpont ME, Wenger DA. Pseudodeficiency of arylsulfatase A: a counseling dilemma. *Clin Genet* 1987; **31**: 70-76.

Titles and legends to figures

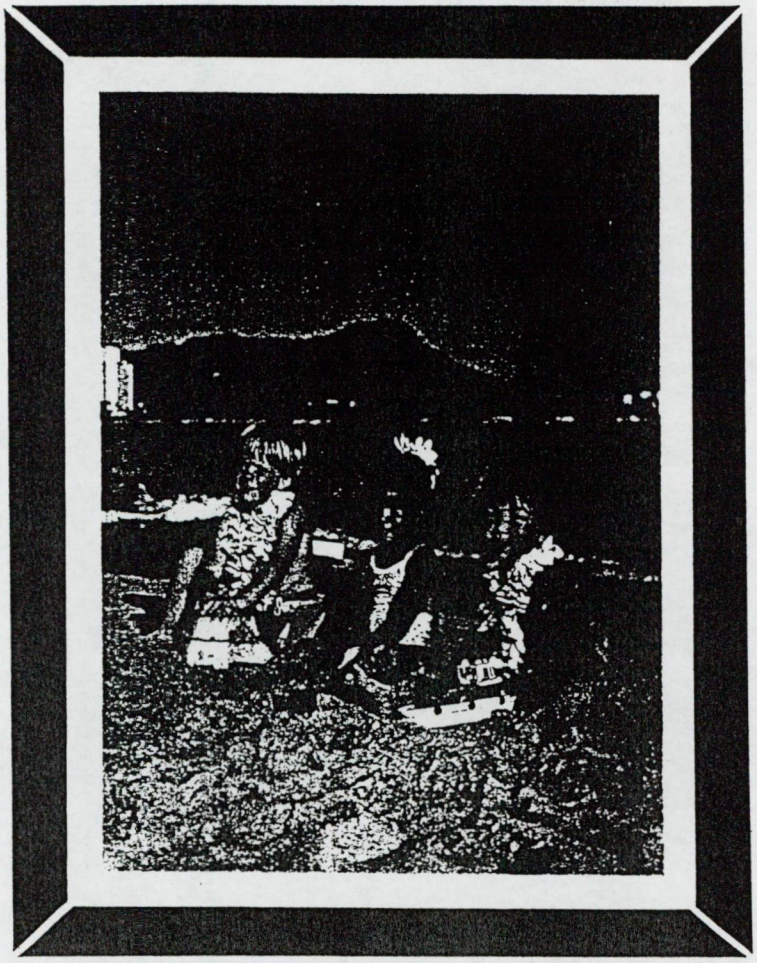
Arylsulfatase A (ASA) activity in human umbilical cord blood leukocytes.

The bars represent the mean ± 1 SD values. **A.** Comparison between average enzyme activity for full-term and preterm healthy neonates. **B.** Comparison between average enzyme activities in umbilical cord blood samples obtained from full-term normal spontaneous vaginal delivery (FT-NSVD), full-term caesarean section (FT-CS), preterm normal spontaneous vaginal delivery (PT-NSVD) and preterm caesarean section (PT-CS) neonates. (n, number of samples)


A
ASA activity (nmol/h/mg protein)
120
100
80
60
40
20
0
full-term (n=60)
preterm (n=47)


B
ASA activity (nmol/h/mg protein
160
140
120
100
80
60
40
20
0
FT-NSVD (n=38)
FT-CS (n=22)
PT-NSVD (n=26)
PT-CS (n=21)

LA SANTE ET LE BIEN-ETRE L'ENFANT: UN ENGAGEMENT MONDIAL
SALUD Y BIENSTAR DEL NINO: UN COMPROMISO MUNDIAL
CHILD HEALTH AND WELL-BEING: A WORLD COMMITMENT



ABSTRACTS OF SCIENTIFIC PRESENTATIONS

XVIII INTERNATIONAL CONGRESS OF PEDIATRICS
HONOLULU — HAWAII — U.S.A.
JULY 7-12, 1986

PRINTED IN U.S.A.

MALARIA DURING PREGNANCY AND ITS EFFECTS ON THE NEWBORN

Harbans Lal* and V. K. Ahuja from Department of Pediatrics
Medical College, Patiala, Punjab, India

The effects of malaria during pregnancy as related to placental infection, transplacental transmission to the newborn and its impact on foetal growth were studied in 102 malarial mothers and their newborns and the results compared with normals. Malaria mostly due to P.vivax complicated 37.8% of the 448 consecutive deliveries. Placental infection, present in 68% of malarial pregnancies, was attended with higher incidence of placental and cord abnormalities. Transplacental transmission of infection was demonstrated in 4 cases. Perinatal deaths increased by 23% in the babies born to the malarial mothers. The newborns of malarial mothers as compared to normals showed that their mean gestational age was shortened by 3.1 weeks along with 18.5% of increase in the incidence of preterm deliveries; the mean birth weight decreased by 395 gms. with 24.3% increase in the incidence of low birth weight deliveries. The mean values of various anthropometric measurements were significantly less in the newborns of malarial mothers as compared to normals. Maternal malaria is still a significant factor in the high perinatal morbidity and mortality in the tropics.

HUMAN CORD AND NEONATE BLOOD ARE DECREASED IN NATURAL KILLER CELL CYTOTOXICITY RELATIVE TO NORMAL ADULT BLOOD.

Lee S. Berk, George D. Georgeson*, William C. Eby, Sandra L. Nehlsen-Cannarella, O. Ward Swarner, Douglas D. Demming, Nancy Sancho, Daryl Vorce, and Leonard L. Bailey, Departments of Pathology and Laboratory Medicine, Surgery and Pediatrics, Loma Linda University, Loma Linda, CA U.S.A. 92350.

Because natural killer cell cytotoxicity (NKCC) is postulated to be involved in transplant rejection and we are involved in neonate cardiac allo- and xenograft transplantion, it is important to understand NKCC in neonates. Therefore, the purpose of this study was to determine normal values of NKCC in cord and neonate (2-4 days) blood for full term normal spontaneous vaginal delivery(FTNSVD) n=15, full term cesarean section(FTCS) n=8, and preterm normal spontaneous vaginal delivery(PTNSVD) n=4, and to compare these with normal adult blood (NA), age 22 - 42 yrs, $\bar{X}$=30. NKCC was measured by a standard 4 hr. 51-Cr release assay with the human tumor cell line K-562 as target cells. With target cells used at 2 x 10^3/well, 6 different ratios of effector to target cells were studied with doubling dilutions from 3:1 to 100:1. The student's t-Test was used to determine differences in NKCC at the effector to target ratio of 50:1 and differences in slopes of NKCC vs. effector to target ratios. The results were as follows: Comparing at a 50:1, FTNSVD, FTCS and PTNSVD cord blood with NA, the NKCC levels were all decreased ($p<0.01$); comparing at 50:1 FTNSVD and PTNSVD neonate blood with NA, NKCC levels were decreased ($p<0.03$), but FTCS was not significant. For the three neonate groups there was no statistically significant difference between cord and neonate bloods The slopes of NKCC vs. effector to target ratios studied for PTNSVD cord and PTNSVD neonate in comparison with NA were decreased ($p<0.05$ and $p<0.01$, respectively). Although the slopes of NKCC vs. effector to target ratios for FTNSVD and FTCS were decreased, they were not statistically significant. In conclusion, cord and neonate blood for the groups studied were found to be significantly less in NKCC than that of normal adult blood. This provides a better understanding of normal NKCC maturation in the neonate relative to transplantation and immunosuppression.

Clinical Research

Official Publication of The American Federation for Clinical Research

VOLUME 35, NUMBER 1, JANUARY 1987

● NATURAL KILLER CELL CYTOTOXICITY IN HUMAN CORD AND NEWBORN BLOOD.
LS Berk,* GD Georgeson,* WC Eby,* J Peabody, and SL Nehlsen-Cannarella.* Departments of Pathology, Pediatrics and Surgery, Loma Linda University, Loma Linda,CA 92350

With the advent of heart, liver, and bone marrow transplantation in newborns and infants, a better understanding of normal newborn immunity is necessary. Natural Killer Cell (NKC) Cytotoxicity has been implicated in transplant rejection and is a potential marker for following newborn and infant response to not only immunosuppressants and the rejection process, but also in immunopathology. However, no normal values for NKC activity in newborns exist. Therefore, we studied NKC in cord and newborn (2-4 days) blood and compared these with normal adult blood (NA) (22-42 yrs). Infants were grouped according to maturity and mode of delivery. NKC was measured by a standard 4 hr 51-Cr release assay with K562 as the target cell line at $2x10^3$/well. The Student's t-test was used to determine differences in NKC. The results at the 50:1 ratio were significantly different, $p<0.01$, between NA and both cord and newborn blood for all groups. The slopes for all these groups represented from a 3:1 to 100:1 ratio, with doubling dilutions were also significantly different from NA, $p<0.01$. No statistically significant difference was found between cord and newborn bloods within their respective group. However, preterm NKC was significantly lower than term NKC for cord natural spontaneous vaginal delivery and newborn cesarean section, $p<0.01$. Within a maturity group, there is no statistically significant difference in NKC between modes of delivery. In conclusion, NKC was found to be significantly decreased in cord and newborn blood, compared to adult normal values. These normal values may now be applied to studies of newborns and infants undergoing organ transplantation and in immunopathogenesis.

● ELEVATED AMNIOTIC FLUID EPIDERMAL GROWTH FACTOR DURING LABOR.
C Callegari,* JA Leake,* S Alvarez,* N Laborde, and DA Fisher, UCLA School of Medicine, Department of Pediatrics, Harbor-UCLA Medical Center, Torrance, CA.

It has been postulated that prostaglandin (PG) production by the amnion may trigger parturition, and epidermal growth factor (EGF) has been reported to stimulate PGE2 production by amnion cells in culture. To date no data are available describing human EGF (hEGF) levels during labor. We measured hEGF, creatinine and osmolarity in amniotic fluid (AF) before birth and/or the first (urine) void from newborns delivered by elective cesarean section (ECS) (AF 16, urine 7-total n=16) or normal vaginal delivery (NVD) (AF 12, urine 10-total n=20). AF was collected from the ECS patients by syringe aspiration when rupturing the membranes and from a pressure catheter during labor (118± 66 min; X±SD before delivery) in the NVD subjects. hEGF was measured by RIA, using a recombinant DNA antigen (Chiron Corp.) with a sensitivity of 15 pg/ml. The groups studied were similar by gestational age; fetal sex, body weight, body length and apgar scores; AF and urine osmolarity, and creatinine levels. Mean (±SEM) AF hEGF levels were

Pediatric Research

Volume 21, Number 4/Part 2/April 1987
ISSN 0031-3998 PEREDL

PROGRAM ISSUE

APS-SPR

Disneyland Hotel
Anaheim, California
April 27–30, 1987

1396 NATURAL KILLER CELL CYTOTOXICITY IN HUMAN CORD AND NEWBORN BLOOD. Lee S. Berk, George D. Georgeson, William C. Eby, Joyce L. Peabody and Sandra L. Nehlsen-Cannarella. Departments of Pathology, Pediatrics and Surgery, Loma Linda University, Loma Linda CA 92350 (Spon. by Stephen Ashwal).

With the advance in cardiac transplantation in newborns and infants, a better understanding of their normal immunity is necessary. Natural killer cell cytotoxicity (NKC) is implicated in transplant rejection and is a potential marker for following newborn and infant response to not only immunosuppressants and the rejection process, but also in immunopathogenesis. However, no normal values for NKC activity in newborn exist. Therefore, we studied NKC in cord and newborn (2-4 days) blood and compared these with normal adult blood (NA) (22-42 yrs). Infants were grouped according to maturity and mode of delivery. NKC was measured by a standard 4 hr 51-Cr release assay with K562 as the target cell. The Student's t-test was used to determine differences in NKC. Results at the 50:1 ratio were significantly different, $p<0.01$, between NA and both cord and newborn blood for all groups. Slopes for all these groups represented from a 3:1 to 100:1 ratio were also significantly different from NA, $p<0.01$. Preterm NKC was significantly lower than term NKC for cord normal spontaneous vaginal delivery and newborn cesarean section, $p<0.01$. Within a maturity group, there is no statistically significant difference in NKC between modes of delivery. In conclusion, NKC was found to be significantly decreased in cord and newborn blood, compared to adult. These normal values may now be applied to studies in newborns and infants undergoing organ transplantation and in immunopathology.

1397 SERIAL STUDIES OF CELL MEDIATED IMMUNITY IN INFANTS OF BIRTHWEIGHT LESS THAN 1300 GRAMS. James Bussel, Michael Giuliano, Walter Greenfield, and Susanna Cunningham-Rundles (Spon by Margaret W. Hilgartner). Cornell Medical Center-New York Hospital, Department of Pediatrics. New York, NY.

Limited studies exist of cell-mediated immunity (CMI) in very premature infants (VPI). We report here serial biweekly immune studies in 58 VPI of birthweight 700 to 1300 grams including proliferative responses to PHA (standard methods using 50,000 cells per well in triplicate) and determinations of lymphocyte subpopulations using % staining with OKT3, OKT4, and OKT8 (flow cytometry). The mean PHA response in VPI was 16,000 cpm ± 5.7k cpm (n=103), significantly decreased compared to term infants and adult normals 23.0k±4.5k; 41% of VPI were <14.0k cpm. The mean OKT3 was 64.6%±15 (n=89), significantly decreased compared to adult normal 75%±7. OKT8 was also low (18.6 vs 27%) but OKT4 was

Scand. J. Immunol. 26, 303–335, 1987

Eighteenth Annual General Meeting of the Scandinavian Society for Immunology

Uppsala, Sweden, 2–4 June 1987

Abstracts of Papers Presented

DETECTION OF ANTIBODYPRODUCTION AT SINGLE CELL LEVEL IN MYASTENIA GRAVIS: Richard Ahlberg, Meta Andersson and Ann-Kari Lefvert. Dept. of Internal Medicine Karolinska Institutet Box 60500 10401 Stockholm Sweden.

In patients with Myastenia Gravis(MG) there is a poor correlation between the levels of the specific autoantibodies in serum and the clinical symptoms. This is probably due to the large heterogeneity of the Ab-pooulation in an individual with MG, and to the fact that the Ab's may be rapidly eliminated from serum by binding to antigen(Ag) or to complementary Ab's.

This implicates the need for a method that detects the Ab-formation at the single cell level. Such a method has recently been described. The method, the ELISPOT-assay (Enzyme Linked Immuno Spot), is a modification of the ELISA and is based on the following principle; Ag's are bound to a membrane. A cellsuspension of Ab-forming cells is incubated on the membrane and the secreted Ab's binds to the Ag. A second Ab, conjugated to an enzyme, is added to this Ag-Ab-complex. Adding the substrate produces visuable spots corresponding to Ab-forming cells. In this way the prevalence of cells producing specific Ab's can be estimated.

We are now about to adapt this method to study the specific Ab-produktion from lymfocytes originating from peripheral blood, bonemarrow and thymus.

PROLIFERATIVE CAPACITY FROM LEUKEMIC CELLS. Aguilar-Santelises, M., García, C. A., Jondal, M. & Mellstedt, H. from: Department of Immunology, Karolinska Institute & Department of Oncology, Karolinska Hospital.

In order to study growth characteristics of leukemic cells from chronic lymphocytic leukemia (B-CLL) such cells have been stimulated with defined factors or chemicals. The cellular responsiveness to IL-1, IL-2 BCGF (MP6 supernatant), Ca^{++} ionophore and phorbol ester TPA has been tested either with factors alone or with different combinations thereof.

Isolated B-CLL cells were found to proliferate in response to IL-1, BCGF and, in some cases, recombinant IL-2. This proliferation was potentiated by TPA. In contrast, normal B lymphocytes demonstrated a different pattern of responsiveness. The enhanced proliferative capacity of B-CLL cells *in vitro* may reflect an altered growth control *in vivo* at selected restrictions points in the cell cycle.

FACS ANALYSIS OF LYMFOCYTES FROM MINK INFECTED WITH ALEUTIAN DISEASE VIRUS. Bent Aasted, The Department of Veterinary Virology and Immunology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Aleutian disease (AD) is a slowly progressing disease in mink. It is caused by persistent infection with Aleutian Disease Virus (ADV), a nondefective parvovirus. The disease is characterized by plasmacytosis, hypergammaglobulinemia and immune complex mediated glomerulonephritis and arteritis.

The cellular response during the infection has barely been studied. We have developed staining methods of ficoll-paque separated mink lymphocytes for FACS analysis, including surface-immunoglobulin staining, PHA (peanut agglutinin) staining and staining with a monoclonal anti-human CD8 antibody which happened to stain quite strongly some mink lymphocytes.

The FACS analysis of lymphocytes of normal mink as well as mink infected with Aleutian Disease virus will be presented.

IL-1 IN VITRO PRODUCTION BY LEUKEMIC CELLS. Aguilar-Santelises, M., García, C. A., Jondal, M. & Mellstedt, H., from: Department of Immunology, Karolinska Institute & Department of Oncology, Karolinska Hospital.

We have recently described IL-1 like spontaneous production in leukemic cells. This factor was *in vitro*, co-mitogenic with Con A and with non mitogenic combinations of phorbol ester and calcium ionophore for mouse thymocytes. We have now analyzed supernatants from PBL either from patients with Chronic Lymphocytic Leukemia (B-CLL) or a benign variant which we have called Monoclonal Lymphocytosis of Undetermined Significance (MLUS). We have found production of IL-1 like activity in the supernatants from cultured cells as determined by the thymocyte assay and confirmed the presence of IL-1 by radioimmunoassay. There is also correlation with the thymocyte assay. The concentration in the majority of the samples vary between 0.25 and 20 ng/ml. MLUS patients' cells produce spontaneously higher amount of IL-1 than B-CLL patients' cells. The production of IL-1 is increased when either MLUS or B-CLL cells are stimulated with *Staphylococcus aureus* strain Cowan I (SAC) by 24 hours.

31

NATURAL KILLER CELL CYTOTOXICITY IN HUMAN CORD AND NEWBORN BLOOD. Lee S. Berk, George D. Georgeson, Joyce L. Peabody Sandra L. Nehlsen-Cannarella, and William C. Eby, Dept. of Pathology Loma Linda Univ. and Medical Center, Rm 1575, Loma Linda, CA 92350 USA

With the advance in organ transplantation in newborns and infants, a better understanding of their immunogenesis is necessary. Natural killer cell cytotoxicity (NKC) is implicated in transplant rejection and is a potential marker for following response to immunosuppressants and the rejection process. However, no normal values for NKC activity in newborn exist. Therefore, we studied NKC in cord and newborn (2-4 days) blood and compared these with normal adult blood (NA) (22-42 yrs). Infants were grouped according to maturity and mode of delivery. NKC was measured by a standard 4 hr 51-Cr release assay with K562 as the target cell. The Student's t-test was used to determine differences in NKC. Results at the 50:1 ratio were significantly different, $p<0.01$, between NA and both cord and newborn blood for all groups. Slopes for all these groups represented from a 3:1 to 100:1 ratio were also significantly different from NA, $p<0.01$. Preterm NKC was significantly lower than term NKC for cord normal spontaneous vaginal delivery and newborn cesarean section $p<0.01$. Within a maturity group, there is no statistically significant difference in NKC between modes of delivery. In conclusion, NKC was found to be significantly decreased in cord and newborn blood, compared to adult. These normal data may now be applied to clinical care of newborns and infants undergoing organ transplantation and pathology

31

32

SELECTIVE ACCEPTANCE OF MHC CLASS I DEFICIENT TUMOR GRAFTS IN THE BRAIN. H. G. Ljunggren, T. Yamasaki, V. P. Collins, G. Klein and K. Kärre. Dept. of Tumor Biology, Karolinska Institutet, Stockholm, Sweden

From the murine retrovirus induced leukemia RBL-5, readily expressing high amounts of cell surface major histocompatibility complex (MHC) class I antigens, we have selected MHC class I deficient ($H\text{-}2^-$) mutants. In contrast to other parts of the body, we show that the brain selectively will accept MHC class I negative tumor cell grafts. $H\text{-}2^-$ tumor cells inoculated intracranially (i.c.) rapidly grew out as solid tumors whereas the same $H\text{-}2^-$ cells were rapidly and efficently eliminated by a host, T-cell independent, cell mediated immune defence mechanism after intravenious (i.v.), subcutaneous (s.c.) or intraperitoneal (i.p.) inoculation. This was in contrast to the $H\text{-}2^+$ cells which were readily accepted both intracranially and extracranially. This indicates that the extracranially efficiently working immune response, selectively directed against MHC class I negative cells, is either suppressed or totally absent in the brain. These results will be discussed in relation to the different immunological surveillence mechanisms working intracranially and extracranially, with special regard to unusally low expression of MHC class I molecules in the brain, the nature of the particular immuneresponses in the brain and to brain tumors.

32