INVESTIGATION OF NATURAL KILLER CELL ACTIVITY, ANTIOXIDANT ENZYMES AND ARYLSULFATASE-A ACTIVITY IN THE HUMAN NEWBORN

Ph.D. Thesis

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1 PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

Full-length papers

I. Georgeson, G.D., Szőny, B.J., Streitman, K., Kovács, A., Kovács, L., László, A. (2001) Natural killer cell cytotoxicity is deficient in newborns with sepsis and recurrent infections. *Eur. J. Pediatr.*, **160**, 478-482.

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II. Georgeson, G.D., Szőny, B.J., Streitman, K., Varga, I.Sz., Kovács, A., Kovács, L., László, A. (2002) Antioxidant enzyme activities are decreased in preterm infants and in neonates born via caesarean section. *Eur. J. Obstet. Gynecol. Reprod. Biol.* (in press)

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III. Georgeson, G.D., Szőny, B.J., Streitman, K., Sallay, E., Kovács, A., Kovács, L., László, A. (2002) Arylsulfatase-A in umbilical cord blood: gestational age and way of delivery do not influence enzyme activity. *Bone Marrow Transpl.* (in press)

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I. Berk, L.S., Georgeson, G.D., Eby, W.C., Peabody, J., Nehlsen-Canarella, S.L. (1987) Natural killer cell cytotoxicity in human cord and newborn blood. *Clin. Res.*, **35**, 89.

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Oral presentations (international congresses)

I. Berk, L.S., Georgeson, G.D., Eby, W.C., Nehlsen-Canarella, S.L., Swarner, O.W., Demming, D.D., Sancho, N., Vorce, D., Bailey, L.L. Human cord and neonate blood are decreased in natural killer cell cytotoxicity relative to normal adult blood. XVIII International Congress of Pediatrics Honolulu-Hawaii-USA, July 7-12, 1986.

CONTENTS

2 ABBREVIATIONS

ASA	arylsulfatase-A
BU	Bergmeyer unit
CAT	catalase
CML	cell-mediated lysis
CTL	cytotoxic T lymphocyte
DXR	delayed xenograft rejection
EDTA	ethylene diamine tetraacetic acid
ES	embryonic stem
FCS	fetal calf serum
FT-CS	full-term caesarean section
FT-NSVD	full-term normal spontaneous vaginal delivery
GPX	glutathione peroxidase
GSH	reduced glutathione
GVH	graft-versus-host
HAR	hyperacute rejection
HLA	human leucocyte antigen
HMO	Health Maintenance Organization
IFN	interferon
IL	interleukin
LDL	low density lipoprotein
LGL	large granular lymphocyte
MHC	major histocompatibility complex
NK	natural killer
PGE	prostaglandin-E
PT-CS	preterm caesarean section
PT-NSVD	preterm normal spontaneous vaginal delivery
ROS	reactive oxygen species
SOD	superoxide dismutase
TBA	thiobarbituric acid
TFPI	tissue factor pathway inhibitor
TNF	tumor necrosis factor
XNA	xenoreactive natural antibody

3 INTRODUCTION

The transplantation of organs and tissues in humans has achieved great success in the last 35 years, such that heart, kidney, liver and bone marrow grafts now have high 5-year survival rates. However, the ever-increasing need for transplantable organs, tissues and cells vastly exceeds their availability. As a direct consequence of refined transplantation techniques and better immunosuppressive regimens, the waiting lists for transplants continue to increase, and unfortunately many potential recipients succumb while waiting for a transplant. These imperative needs are the impetus for researchers to find alternative sources of organs for human transplantation.

Xenotransplantation, which is the transplantation of organs or tissues from one species into another species, is the focus of a growing field of research, primarily a result of the increasing shortage of suitable donor organs for human organ transplantation.

Xenografts that are likely to be used for human transplantation are normally destroyed by the host in a matter of minutes or hours by the process of hyperacute xenograft rejection. However, with improved understanding of immune recognition and the development of a model for the immunopathogenesis of xenograft rejection, new therapies, which can partially overcome the violent immune response and fulminant rejection associated with xenogenic tissue transplantation, are being tested. Implementation of these new therapies will in most, if not all, instances involve genetic modification of the donor animal.

In transplantation such as pig organs into humans, the universal presence in humans of naturally pre-formed antibodies that bind to donor cells leads to HAR. HAR is initiated by antibodies attaching to the vascular endothelium of the donor organ, resulting in the activation of the classical pathway of complement, endothelial cell activation, and a prothrombotic environment within the graft [89, 93]. HAR in the pig-to-primate model is characterized pathologically by interstitial haemorrhage and diffuse thrombosis [99]. Once initiated, the process destroys xenografts within minutes to hours.

DXR or acute vascular xenograft rejection is observed (when HAR is controlled) within 2 to 4 days of transplantation. It is characterized by type II donor-organ endothelial cell activation, with the up-regulation of pro-inflammatory genes and infiltration into the graft of host monocytes and NK cells, thrombosis and a complex cytokine response [6, 41]. However, if HAR and DXR are controlled, enabling graft survival beyond five days, specifically stimulated T cells and increasing titres of antibodies attack the graft. Therefore it appears that each type of xenograft rejection has a distinct array of effector cells which eliminate the transplanted tissue. In the case of xenotransplantation the DXR seems to be the most critical event and the role of NK cells in this reaction is obvious but both the mechanisms and the extent of NK cell activation and cytotoxicity persists to be a matter of violent debate in the scientific community.

3.1 NK cell cytotoxicity

NK cells are a distinct subpopulation of lymphocytes [36, 45] which in contrast with CTLs (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 [62]. They typically are larger than most lymphocytes and contain azurophilic granules in their cytoplasm, therefore they are often called LGLs. About 5% of lymphocytes in the spleen and peripheral blood can be classified as LGL and approximately 75% of these can function as NK cells [45]. NK cells are unique among the cytotoxic cells of the immune system. They do not recognize specific antigens on their target cells and thus neither require an immune response nor specific immunologic memory for their action.

The precise ontogenic origin of NK cells is not known however there are three major theories addressing this question [45]. Some authors consider that there is a common stem cell for NK cells and other lymphocytes. Others postulate that T, B and NK cells diverge from other hematogeneous elements early during ontogenesis whilst other scientists favor the hypothesis that there might be a common progenitor cell for T and NK

cells but NK cells diverge from the T cell lineage prior to rearrangement of the T cell antigen receptor genes during fetal development.

NK cells can kill a variety of tumor cells and virally infected cells [15, 42, 44, 51, 53, 67, 122]. Apparently they have a role in hematopoiesis, suppressing the development of hematopoietic progenitors [30, 39, 101, 106]. NK cell activity seems to be the first line mechanism in transplanted organ rejection and graft survival, as well [68, 70, 113, 120]. The mechanism of lysis is not well understood. Osmotic lysis mediated by perforins [11, 75, 76], necrosis and apoptosis are the most important mechanisms of target cell killing [123].

NK cytotoxicity is subject to a complex regulation. Pro-inflammatory mediators such as IL-2, and IFN γ were shown to be NK activity augmenting cytokines, both in adults and neonates [12, 49]. 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 [83, 84]. In contrast IL-4 a known Th2-type cytokine is a potent suppressor of NK cell mediated necrotic and apoptotic cytotoxicity [34]. 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 PGE_2 has been well documented [16, 57, 69, 78, 86, 102].

Fetal and neonatal NK cell activity is significantly decreased in comparison with adults [37, 73] 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 anesthetic drugs given to the mother during cesarean 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 the possible influence exerted by the mode of delivery.

3.2 Antioxidant defence

Following the initial infiltration into the grafted organ of host monocytes and NK cells, the next phase of rejection occurs over a period of days to weeks and is a complex process involving multiple factors. One damaging factor believed to be involved in this process is oxidative stress. Under normal conditions, a balance between oxidants and antioxidant mechanisms is maintained due to the presence of antioxidant enzymes such as SOD, CAT and GPX. It is tempting to speculate that if this balance is disrupted to favour the overproduction of oxidants, as occurs during transplantation of organs, the antioxidant enzymes will no longer be able to cope with the removal of excess oxidants and thus loss of the donor organ will occur. Thus increasing the expression of antioxidant enzymes in the donor animal using current genetic technology would protect the organ from injury caused by oxidants and thus prolong survival.

DXR apparently mostly mediated by macrophages and NK cells, however will not be solely a reaction to antigens on the graft (as occurs in allografts), as diverse molecular incompatibilities between the xenogeneic donor and recipient will also set the stage for stimuli that activate cellular responses. In addition, factors such as thrombin ROS activate endothelial cells and precipitate rejection [4]. NK cells have receptors that apparently overlap in specificity with sugars recognized by XNA [50] and monocytes up-regulate lectins that bind sugar epitopes upon exposure to xenogeneic cells [40]. In summary, there are multiple possible stimuli that could activate donor-organ endothelial cell activation and precipitate rejection once natural antibodies and complement have been removed.

Aerobic organisms possess antioxidant defense systems that deal with ROS as a result of aerobic respiration and substrate oxidation. ROS, including hydroxil radicals (•OH), superoxid anions (O_2•—) and hydrogen peroxide (H_2O_2), are generated in response to external and internal stimuli [48, 52, 79]. 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 [65]. 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 LDL 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 [88].

The enzymatic antioxidant defenses include SOD, GPX and CAT. SOD (EC1.15.1.1) is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to O_2 and H_2O_2. 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 [94]. CAT and GPX play an important role in the detoxification of H_2O_2. CAT (EC1.11.1.6) reacts very efficiently with H_2O_2 to form water and molecular oxygen and with hydrogen donors with peroxidase activity thus protecting cells against the H_2O_2 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 [108].

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 [110]. 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 [74]. 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 [63, 66, 85]. 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.

3.3 Arylsulfatase-A

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 [60]. 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 GVH disease. The latter provides the ability to use umbilical cord blood even if there is a greater HLA disparity [61, 118, 119].

Human lysosomal 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[117]. 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[87].

Pulkkinen *et al.*[98] 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

[81]. 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 [90]. 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 [23]. 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 caesarean section.

4 SPECIFIC AIMS

4.1. As NK cells play an important role in graft rejection the first aim of our study was to establish normative data concerning NK cell cytotoxicity in human newborns.

4.2. The aim of our further work was to investigate 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.

4.3. Because antioxidant enzyme activity seems to have a role in the transplanted organ rejection we proposed to establish normative data for the enzymatic antioxidant defense of the healthy newborn such as CAT, GPX, SOD. The experiments were set out 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.

4.4. Because of the advent of cord blood transplantation as a possible therapeutic approach of patients with metachromatic leukodystrophy we proposed to determine the ASA activity in normal neonates as a function of gestational age and way of delivery, in order to establish which newborn category would qualify as a potential donor.

5 MATERIALS AND METHODS

5.1 Patients

5.1.1 Investigation of NK cell cytotoxicity

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: FT-NSVD (n=55), FT-CS (n=51), PT-NSVD (n=34) and 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 have been established for the study of NK cell cytotoxicity related to neonatal infection. The first group consisted of 15 newborns, which were diagnosed with sepsis by positive blood cultures. The second pathological population consisted of 8 different newborns who presented a primitive, early onset infection at birth or the first days of life and during hospitalization developed a secondary infection such as bacterial sepsis, pneumonia and/or fungal infection occuring 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* generalized 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 five-minute Apgar score ≥7, at 38-42 and 31-37 weeks of gestation, respectively (mean gestational age ± SD was 39.5 ± 1.1 weeks for FTNSVD, 39.6 ± 1.5 weeks for FTCS, 34.4 ± 1.8 weeks for PTNSVD and 34 ± 1.7 weeks for PTCS). Septic newborns and those having had recurrent infections were appropriate for gestational age, born at 31-42 weeks of gestation with five-minute Apgar scores of 6-8

and with a maternal history of fever (temperature >38 °C) and/or premature rupture of membranes greater than 24 hours (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 have been treated with antibiotics.

Neonatal NK activity has been compared to healthy adults aged between 22-42 years (n=89).

5.1.2 Investigation of enzymatic antioxidant defence systems in the human newborn

According to the mode of delivery and gestational age four study groups were established: FT-NSVD (n=24), FT-CS (n=19), PT-NSVD (n=15) and 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).

5.1.3 Investigation of ASA activity

The four study groups established according to the mode of delivery and gestational age were: FT-NSVD (n=38), FT-CS (n=22), PT-NSVD (n=26) and PT-CS (n=21). 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.

5.2 NK cell cytotoxicity assay

The NK cell cytotoxicity was measured using the standard 4 hours ^{51}Cr release cytotoxicity assay as previously described [95]. Briefly, peripheral blood mononuclear cells were isolated from heparinized venous blood samples on Fycoll-Hypaque gradient (Pharmacia, Piscataway, NJ) Blood samples were processed within 12 hours from the time obtained. After washing the cell count was adjusted to 2×10^6 viable cells per ml in RPMI 1640 containing 10 mM HEPES, 10% FCS, 100 IU/ml Penicillin and 100 μg/ml Streptomycin (all from Sigma, St. Louis, MO) As targets we used ^{51}Cr-labelled K562 human erythroleukemic 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 labeling, 10^6 target cells in 100 μl of medium were incubated with 100 μCi of ^{51}Cr (sodium chromate) in physiological saline solution. The labeled cells were washed 3 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 cocultured 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 × *g* for 1 min and placed in a 37 °C humidified 5% CO_2 incubator for 4 hours. 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:

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

5.3 Determination of antioxidant enzyme activities

Cu/Zn-SOD activity was determined via inhibition of the epinephrine-adrenochrome autooxidation at 480 nm [80]. CAT activity was measured spectrophotometrically at 240 nm in aliquots of hemolysates. Enzyme activities were expressed in BU. One BU is the amount of CAT that decomposes 1000 mg H2O2/min. GPX activity was determined in supernatant aliquots. As substrates GSH and cumene hydroperoxide were used. GSH degradation was measured using Ellman's reagent [17, 103].

5.4 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.*[72] and for the direct measurement of ASA activity.

ASA was assayed by the method of Singh *et al.* [109]. 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.

5.5 Other measurements

Lipid peroxidation was determined with the TBA method [103, 92] 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 [71].

5.6 Statistical analysis

Statistical analysis of the data was made by ANOVA. The Student`s t test was used (where appropriate) to determine differences in NK cell cytotoxicity at the effector-target cell ratio at 50:1 and differences in slopes. 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.

6 RESULTS

6.1 NK cell cytotoxicity in the human newborn

NK cytotoxicity of healthy neonates proved to be significantly lower than in adults as indicated by our ^{51}Cr-cytotoxicity assays (44.15 ± 5% kill for adults and 26.56 ± 3.21% kill for the healthy neonatal population; $p<0.01$) (Figure 1.). The possible role of the prematurity in influencing the cytotoxic activity of NK cells was also investigated. On the basis of % 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 ($p<0.05$) (Figure 2.). The influence of the way of delivery 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.

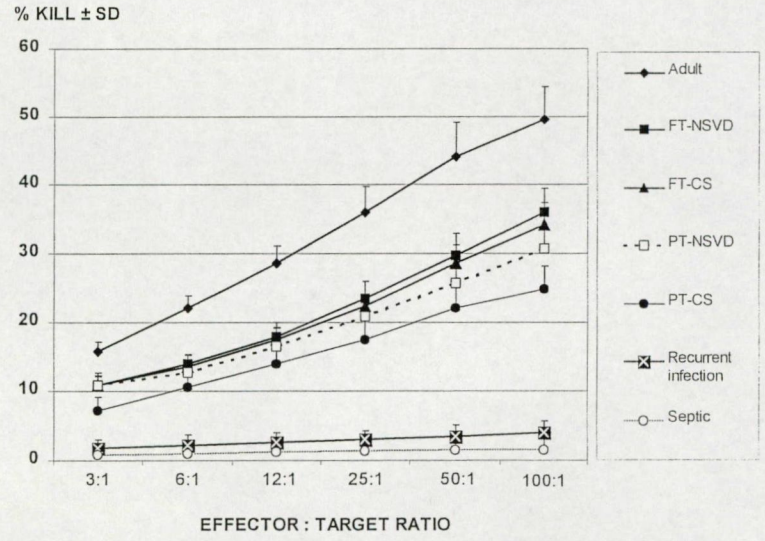


Figure 1. NK cell cytotoxicity of FT-NSVD (n=55), FT-CS (n=51), PT-NSVD (n=34), PT-CS (n=28) and newborns diagnosed with recurrent infections (n=8) or sepsis (n=15) contrasted to healthy adults (n=89), expressed as percent kill values at different effector-target ratios.

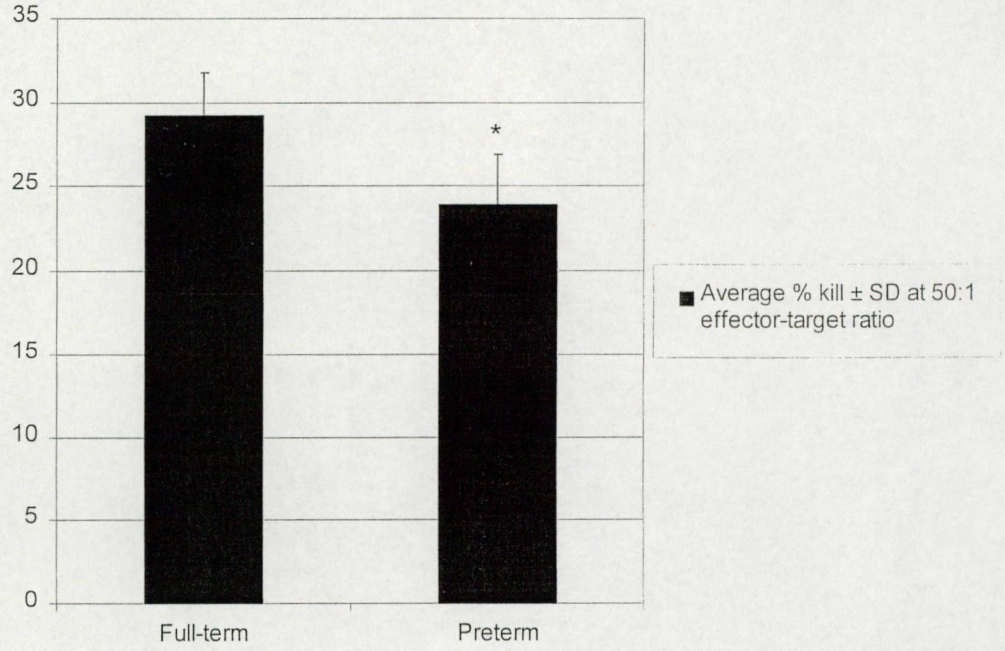


Figure 2. Comparison between average percent kill values for full-term (n=106) and preterm (n=62) healthy neonates at 50:1 effector-target ratio, showing a significant decrease of NK cell cytotoxicity of preterm neonates.

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 % 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; Figure 1.). At lower concentrations of the effector cells the difference between % 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 analyzed no statistically significant difference was found.

6.2 Enzymatic antioxidant defence systems in the human newborn

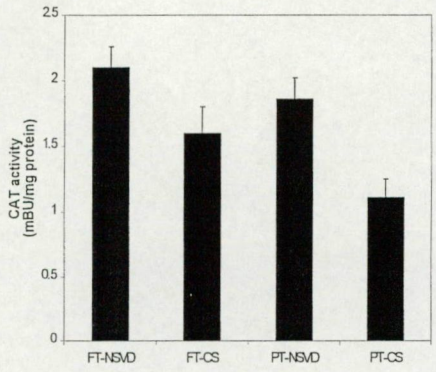
CAT activity in healthy neonates proved to be significantly higher in the FT-NSVD category in comparison to PT-NSVD (average ± SE was 2.10 ± 0.16 mBU/mg protein for full-term healthy neonates and 1.86 ± 0.16 mBU/mg protein for the preterm healthy neonatal population). We found that the neonates born via cesarean section had a significantly lower CAT activity than the vaginally delivered babies in both the full-term and the preterm category. (Figure 3.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 3.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 3.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).

A.



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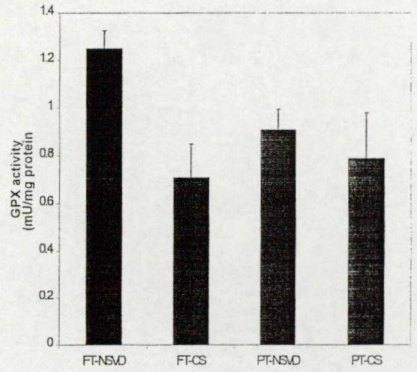

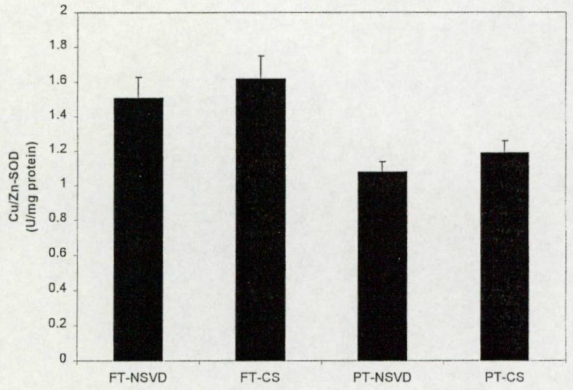

C.

Figure 3. Antioxidant enzyme activities in FT-NSVD (n=24), FT-CS (n=19), PT-NSVD (n=15) and preterm caesarean section (PT-CS, n=21) newborns. Enzyme activities were measured in cord blood hemolysates. Statistical 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.

6.3 Arylsulfatase-A activity in cord blood

The mean values of ASA activity in cord blood leukocytes are indicated in Figure 4. 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) (Figure 4A.). 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 (Figure 4B.). 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).

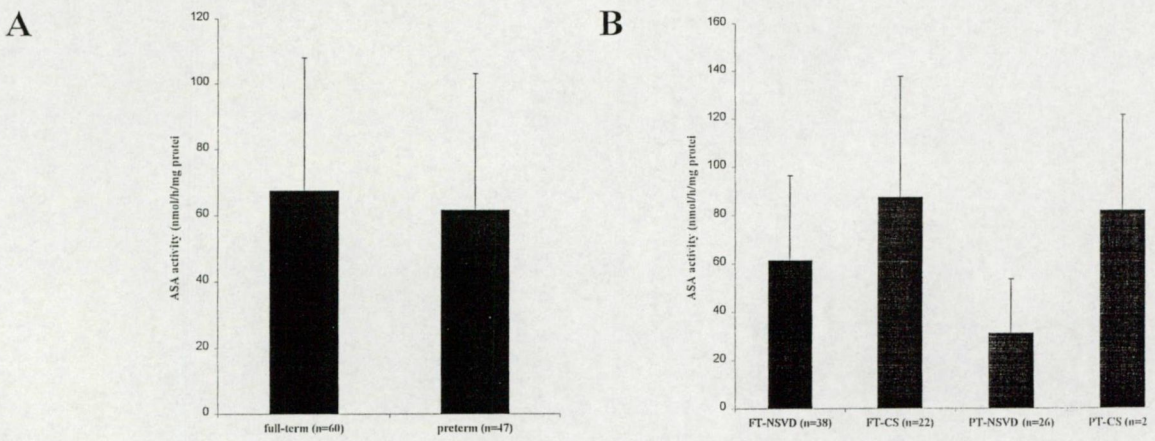

Figure 4. Arylsulfatase A activity in human umbilical cord blood leukocytes.
The bars represent the mean ± 1 SD values. (**A**) Comparison between average enzyme activity for full-term and preterm healthy neonates. (**B**) Comparison between average enzyme activities in umbilical cord blood samples obtained 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)

7 DISCUSSION

7.1 NK cell cytotoxicity in the human newborn

The role of NK cells in normal immunity is not clearly established. These cells were attributed to antiviral and antineoplastic defense, however neither viral infection associated nor tumor 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 GVH disease in recipients of bone-marrow transplants. The NK effector are involved in renal allograft rejection. Concomitantly with their appearance in the graft, the NK activity is depleted from spleen, and when this activity declines from the graft it reappears in the spleen. Most of the *in situ* LGL are spleen-derived, and that once they leave the graft they home again in the spleen.

The NK activity appears in the graft prior to the CTLs, and disappears from the graft before the peak CTL activity is reached *in situ.* This may indicate that the NK cells are involved in the maturation of the CTLs, possibly acting as precursor cells for CTLs.

Previous experiments in untreated rat kidney recipients demonstrated a simultaneous decrease in splenic NK activity and an increase in graft NK activity 4-6 days after transplantation, suggesting that endogenous NK cells migrate to the graft and contribute to rejection [82].

During fetal development the 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 [112, 114]. Previous studies have shown that at birth healthy newborns have significantly lower NK activity than adults [25, 47]. Similarly the decreased NK cytotoxicity of preterm versus full-term newborns has also been demonstrated [77, 100]. 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, antitumoral and anti-graft defense. Recently considerable attention has been payed 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 whithout any evidence of phagocytosis [33]. Other studies proposed that NK cell-bacteria contact is not necessary for efficient killing [32]. 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 leukocytes 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 was found significantly lower than in normal adults and this functional deficiency was paralleled by a severe decrease in NK cell number [96, 97]. 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 insuficiency 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 behind the scope of the present study and waits for further investigation. According to our best knowledge this is the first publication showing a defectiveness of NK cell cytotoxicity in bacterial sepsis and recurrent infection of the neonate.

Significant progress has been made in surmounting hyperacute rejection, and this has led to an examination of underlying mechanisms of DXR. One of these delayed mechanisms concerns the potential role of graft recipient NK cells. NK cells can cause variable, low-level cytotoxity of xenogeneic endothelial cells *in vitro* that may be enhanced in the presence of xenoreactive IgG. The specificity of NK cell-mediated cytotoxicity appears to overlap with a major subset of xenoreactive natural antibodies. These cytotoxic interactions can be regulated by "humanizing" the endothelial cells through expression of the appropriate human MHC class I genes. More important, NK cells induce endothelial cell activation, which results in changing the nature of the endothelial cell surface from an anticoagulant surface to a procoagulant one. [22]

NK cells have been shown to prevent the incidence and severity of GVH disease. Understanding the mechanisms of beneficial effects of NK cells after bone marrow transplantation may lead to significant increases in the efficacy of this procedure [55].

Immunological tolerance would allow the elimination of chronic rejection (*e.g.* graft arteriosclerosis), which is possibly more important than that of acute rejection as there is no effective treatment even in allografts. If tolerance could be achieved, pharmacological immunosuppressive therapy would not be necessary and the accompanying risks of opportunistic infection, malignancy and drug toxicity would be avoided [18].

A possible limitation to the induction of tolerance is that stable mixed chimerism in mice has required pre-treatment of the recipient with monoclonal antibodies against natural killer (NK1.1) cells, Thy-1^+ cells and mature T-cells (CD4 and CD8),

administration of non-lethal whole-body irradiation and extra-selective thymic irradiation to deplete thymic T-cells [105]. Kozlowski *et al.* [58] have shown T-cell tolerance induction in a pig-to-primate model using myeloablative and nonmyeloablative irradiation and splenectomy to be beneficial in reducing the anti-αGal antibody response to porcine bone marrow. Alternative and less rigorous induction regimes are being sought.

7.2 Enzymatic antioxidant defence systems in the human newborn

Oxygen species are important participants in damage caused by virus infections, progression to cancer and neurodegenerative processes [27]. They can be toxic at the molecular level and they are important effectors in ageing and lifespan determination.

Oxygen radicals have been detected in a variety of stimulated blood cells [8]. For instance, during various biological processes as inflammation or septic shock, free radical damages are not only caused by a direct generation of oxygen radicals by phagocytes, but also by a TNF-α-mediated generation in target cells. The oxidative effect of TNF-α is beneficial in physiological conditions as it can destroy cancerous or virus infested cells. But this effect can be deleterious in a situation of deficiency in some antioxidants. TNF-α-induced free radicals can indirectly destroy immunocompetent cells such as T cells. This last action explains the defect in cellular immunity observed in oxidative stress and the immunostimulatory effect of many antioxidants. In this event, CAT has the better protecting effect whereas Cu,Zn-SOD has little effect [28]. So, antioxidants have been demonstrated as protective against TNF-α cytotoxicity.

The mechanism of natural killing as well as the effect of ROS seems to be an apoptotic process. In this process initial stress-induced damage does not kill cells directly, rather it triggers an apoptotic signalling programme that leads to cell death [31].

Apoptosis can be initiated by a variety of stimuli, including hyperthermia, growth-factor or hormone withdrawal, glucocorticoids, oxidants, ionizing radiation and multiple classes of chemotherapeutic agents [10, 46]. Following an apoptopic signal, cells sustain progressive lipid peroxidation. Thus, ROS and oxidative damage have been implicated in

the induction of apoptosis [3, 21, 24, 111]. The Bcl-2 proto-oncogene is unique among cellular genes for its ability in many contexts to block apoptotic deaths. Moreover, a mechanism has been proposed in which Bcl-2 regulates antioxidant pathways at sites of free radical generation [46]. The protein Bcl-2 protects against apoptosis by blocking cytochrome *c* release (preventing superoxide production when it is overexpressed) hence this protein may have an antioxidant function [13].

Apoptosis of neutrophils may be mediated by endogenous oxidative products. This suggestion is confirmed by observation that apoptosis of normal neutrophils is markedly inhibited by reduction of intracellular hydrogen peroxide levels. Inhibition of apoptosis in normal neutrophils by addition of catalase also occurs [54]. Activation of cell death is blocked by a variety of antioxidants. Although reactive oxygen intermediates do not act as mediators in the execution phase of CD95-mediated apoptosis, they are involved in the transcriptional regulation of CD95L expression [9]. A potential role of CD in oxidative stress-mediated cell death, ischemia / reperfusion and other diseases characterised by a disturbed redox balance has been recenlty reported [116]. Caspar-Bauguil *et al.* [14] show that activated T-lymphocytes are present in early atherosclerotic lesions, which occur in the transplanted organ during rejection.

We studied antioxidant enzyme levels in the human neonate and we determined normative data, as well 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 [38]. 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 [115]. 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 [85], 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 [63]. 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. Having determined reference data of antioxidant enzymes in the human newborn we might have contributed with "a piece to the pie" to the proposed xenograft rejection model, according to which xenografts surviving a week or more express numerous protective genes, including hemoxygenase and anti-apoptotic genes of the *bcl* family including A20, *bcl*-2, *bcl*-xL and A1 in endothelial and smooth muscle cells [5, 19, 29, 59]. Conversely, similar studies show rejected grafts do not express protective genes but do express pro-apoptotic genes, including Bax, Bad and CPP-32 [4]. Expression of anti-apoptotic genes protects the cells from TNF-mediated apoptosis in addition to suppressing the activation of NF-kB which is necessary for induction of pro-inflammatory genes [43]. Transgenic expression of protective genes can reduce DXR mediated rejection and may minimize the development of arteriosclerosis (chronic rejection) in xenografts [5].

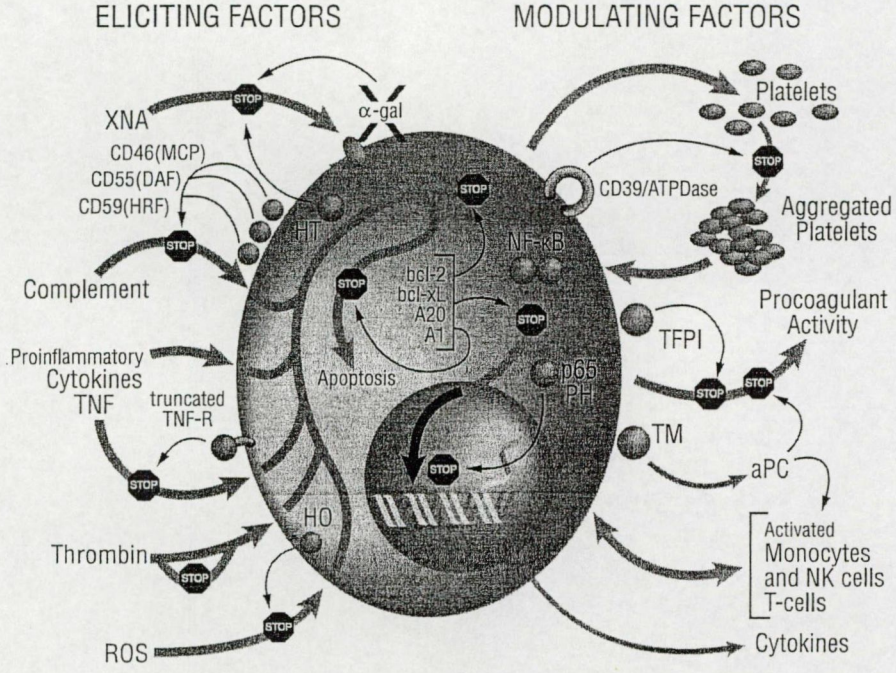

Figure 5. Transgenic strategies for promoting xenograft survival (adapted from 4)

An alternative approach to controlling DXR involves suppression of the pro-inflammatory cytokines that activate endothelial cells. TNF is likely to be a major candidate for blockade and Bach *et al.* [5] have shown that expression of a truncated p55 subunit of the TNF receptor in endothelial cells makes them resistant to activation by TNF.

Direct inhibition of NF-kB activation and thus endothelial cell activation could suppress the inflammatory response and thereby remove stimuli that are likely to contribute to rejection [4]. While the natural inhibitor of NF-kB, I-kBα, sensitizes cells to TNF-mediated apoptosis and thwarts this approach [4], a truncated mutant of p65 (p65RHD) has recently been identified which inhibits the action of NF-kB as effectively as I-kBα but without sensitising cells to apoptosis [20].

Endothelial cells produce an inhibitor of the tissue factor-initiated coagulation pathways known as TFPI. Human TFPI specifically regulates pro-coagulation by inhibiting human factor X, but porcine TFPI does not inhibit human factor X. Expression of human TFPI in xenografts would therefore have a minimal effect on the donor animal while helping to maintain xenograft survival. Thrombomodulin functions as an important anticoagulant suppressor of monocyte activation and infiltration and is a sink for thrombin generation [26]. Activated protein C generated by thrombomodulin suppresses activation of NK cells [104]. Over-expression of thrombomodulin in xenografts would decrease production of thrombin and lead to higher levels of activated protein-C, suppressing both monocyte and NK cell activation [35, 121]. Functional incompatibilities between human porcine thrombomodulin, thrombin and protein C also exist [56, 64, 107] and may provide opportunities to reduce xenograft rejection using transgenic strategies.

An overview of the current vascular-based transgenic strategies required to overcome both HAR and DXR rejection and promote xenograft survival is provided in Figure 5. Factors on the left elicit endothelial cell activation, whereas those on the right modulate or play a role in xenograft rejection. Genetic interventions required to ameliorate the rejection process are illustrated by red stop signs. Transgenic strategies to prevent endothelial cell-activated HAR include: elimination of αGal epitopes by homologous

recombination ('knockout') or expression of H transferase (competitive glycosylation); expression of complement regulatory proteins including CD55, CD46 and CD59 (to block activation of complement); expression of a truncated p55 subunit of the TNF receptor (to render enodothelial cells resistant to the pro-inflammatory cytokine, TNF); overexpression of thrombomodulin (to remove thrombin and reduce activation of infiltrating monocytes and NK cells); expression of hemoxygenase (a potent anti-oxidant to mop up reactive oxygen species involved with endothelial cell activation); expression of p65RHD (to suppress the action of NF-kB) or alternatively expression of one or more of the protective genes that inhibit apoptosis. Additional genetic interventions might target the factors that mediate DXR via platelet aggregation (such as overexpression of CD39/ATPDase) or coagulation (such as expression of human TFPI).

It is possible that a number of these genetic interventions either alone or in combination may have the indirect effect of muting T-cell-mediated responses. High expression of complement regulators, a reduction in Gal epitope levels, and increased thrombomodulin are each predicted to reduce the inflammatory milieu, which should lessen the immunogenic stimulus.

7.3 Arylsulfatase-A activity in cord blood

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 [23]. 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 leukodystrophy 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 [90]. 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 GVH 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 % [7]. The very recent data reported by de Gasperi and co-workers [23] 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.* [23]. 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. Special attention is needed for metachromatic leukodystrophy patients to be transplanted from cord blood (and equally from adult volunteer marrow or family members) because of the high incidence of pseudodeficiency of the normal population. Even though the pseudodeficient person is not affected with the disease, cord blood (or marrow) from such an individual may not be suitable for providing changes of the disease manifestations and should not be used [91]. For instance, the arylsulfatase-A activity was not determined in an adult marrow donor prior to transplant for one patient. This random marrow donor was later determined to be homozygous for the arylsulfatase A pseudodeficiency allele [91]. The result of the latter transplantation indicated no positive response even though engraftment was complete. Therefore the conclusion reached for metachromatic leukodystrophy is to be aware that a potential donor may be pseudodeficient.

Given all the above considerations, even though the incidence of carriers for the above disorders is generally low (in both cord blood and adult marrow volunteer sources), the better part of appropriate transplantation planning should include enzyme data on the 'normal' donor. Therefore, integrating into the transplant system the knowledge of donor relative to enzyme level, although an extra challenge, will be beneficial for the long-term outcome of the recipient.

8 CONCLUSIONS AND PERSPECTIVES

8.1 Practical results

1. Since natural killer (NK) cell cytotoxicity and antioxidant enzyme activity play a role in delayed xenograft rejection (DXR), being important steps in the xenograft rejection model of Bach et al. [4] which suggests the need for transgenic strategies such as: gene manipulation, embryonic stem (ES) cell transfer and nuclear transfer in order to promote xenograft survival we proposed to determine NK cytotoxicity and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) levels in the potential human newborn candidates categorized in four study groups according to gestational age and way of delivery, because these data in this organized fashion prior to our study did not exist. In case of rejection these data could be used as comparative.

2. At the advent of human cord blood transplantation, as a novel modality of treatment, we focused our attention to application of this method in metachromatic leukodystrophy. This patients being deficient in arylsulfatase-A (ASA), benefit from ASA containing cord blood transplantation. We determined ASA normative data in the same fashion as for the above mentioned investigation to serve as parameter for available donor selection for transplantation.

8.2 New results

3. During the accomplishment of our primary goal we intentionaly deviated our scientific attention to clinically important and as well pioneering entities such as:

a) NK cytotoxicity and bacterial sepsis, recurrent infections in the newborn

b) CAT, SOD, GPX activity and caesarean section (CS) delivery

c) ASA activity and pseudodeficiency

4. I consider that our research team did indeed contribute with "a piece to the big pie" of xenograft rejection model adding to two major sites on the model and furnishing normatives for the clinical practice.

Experimental pig-to-primate organ transplantation is currently under way, and results show increased transplant function from minutes to days and weeks. The final therapeutic xenotransplantation regimen is likely to involve a combined approach incorporating genetically modified donor organs or tissue, the development of immunological tolerance and administration of therapeutic agents that can control NK cell- and monocyte-mediated responses.

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