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Expression of FasR, Fas-L and Bcl-2 in $CD4^+$ and $CD8^+$ subpopulations of T lymphocytes in the cord blood of healthy full-term newborns, is gender of influence?

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ABSTRACT

Purpose: The expression of FasR, Fas-L and Bcl-2 on $CD4^+$ and $CD8^+$ T lymphocytes subpopulations from the cord blood were assayed. The results in blood from boys and girls were analyses separately and compared.

Material and Methods: Twenty four full-term newborns: 13 females and 11 males were included into the study. Blood from the umbilical vein was collected immediately after cutting the umbilical cord. The staining with monoclonal antibodies against CD4, CD8, FasR, Fas-L and Bcl-2 was performed within 2 hours after collection and followed with flow cytometry acquisition and analysis.

Results: The percentage of $CD4^+$ and CD8+ T lymphocytes and $CD4^+$:$CD8^+$ ratio was within normal range. The expression of FasR, Fas-L was higher on $CD4^+$ T lymphocytes than on $CD8^+$ T lymphocytes (10,36% vs 6,79% and 6,66% vs 5,63% respectively). The expression of Bcl-2 was comparable (91,9% and 93,75% respectively). The comparison between males and females showed higher percentage of $CD4^+$ lymphocytes on lymphocytes from girls' blood (56% vs 38,69%, p=0.0003). The expression of FasR and Fas-L on $CD4^+$ T lymphocytes was higher on $CD4^+$ T lymphocytes from girls' blood (13,8% vs 7,53% and 6,8% vs 6,52% respectively) but without statistical significance. Bcl-2 expression was higher on $CD4^+$ T lymphocytes from boys' blood (99,65% vs 89,7%) but without statistical significance. Similar pattern of FasR, Fas-L and Bcl-2 expression was noted on $CD8^+$ T lymphocytes analysed separately for girls' and boys' blood origin cells. The difference in Bcl-2 expression was more prominent than on $CD4^+$ T lymphocytes and reached statistical significance.

Conclusions: The lymphocytes from cord blood of boys showed the more immature immunophenotype than T lymphocytes from cord blood of girls'. Impaired apoptosis (as a consequence of low expression of FasR, Fas-L) in neonatal cells may contribute to prolonged inflammation in newborns after oxidative stress or infection.

Key words: Markers, Apoptosis, Lymphocytes T, Newborns, Gender

INTRODUCTION

Activated T lymphocytes differentiate into effector cells: T helper (T_H) lymphocytes $CD4^+$ and cytotoxic T lymphocytes (CTL_S) $CD8^+$. Both effector cells secrete inflammatory cytokines and CTL_S kill infected cells directly. The main role of $CD4^+$ in immune response is the activation of antigen presenting cells (APC) by CD40-CD40L interactions and by the secretion of cytokines to promote a B lymphocyte response. $CD4^+$ T cells provide help for $CD8^+$ T cell responses [1,2]. Our hypothesis is strengthened by the clinical observations that male newborns are at a higher risk for serious bacterial infections than female newborns [3]. There is a widespread acceptance that apoptosis of activated lymphocytes is vitally important for adequate functioning of the immune system [4]. The cell surface receptor CD95 (Fas/APO-1) [5] is located on the long arm of chromosome 10 [6] and specific ligand CD95-L/Fas-L play a crucial role in the control of cell death and survival [7]. Activated lymphocytes express Fas ligand [8]. Therefore, active lymphocytes may be both - effectors

and targets, in a Fas antigen – Fas ligand system [9]. FasL is a 40-kD type II transmembrane protein that is homologous to TNF. FasL induces apoptosis by binding to its membrane receptor, Fas [9]. FasL also works as a cytotoxic effector molecule of CTL (cord T lymphocytes) [10]. FasL to FasR binding results in the induction of cell apoptosis by activating a caspase cascade, altering mitochondria [11] and fragmentation of nuclear DNA, which is considered to be a hallmark of apoptosis [12]. In particular, Fas/FasL-mediated cell death has been implicated in the elimination of unwanted or aberrant cells, and in the maintenance of lymphocyte homeostasis [13]. The interaction between these compounds is responsible for the preservation of peripheral tolerance by the induction of apoptosis of activated T lymphocytes [14]. Apoptosis of T lymphocytes is a fundamental process regulating antigen receptor repertoire selection during T cell maturation and homeostasis of the immune system [15]. Bcl-2 is located in the inner mitochondrial membrane [16] and perinuclear endoplasmic reticulum [17]. This protein blocks apoptosis [16] and controls mitochondrial membrane integrity [18]. Cellular homeostasis of T cells may be sustained by apoptosis, which is related to reduction of Bcl-2 protein [8].

This study is dedicated to the analysis of the expression of FasR, Fas-L and Bcl-2 in $CD4^+$ and $CD8^+$ lymphocytes in cord blood. Additionally, the percentage of $CD4^+$ and $CD8^+$ in cord blood was assayed. The effect of patients' gender on lymphocytes characteristics was of concern.

MATERIALS AND METHODS

24 full-term neonates: 13 females and 11 males were included into our study. They met all the criteria for full-term newborns, including clinical state and laboratory findings. Birth weight ranged from 3 070 g to 4 750 g for female neonates and from 2 740 g to 4 500 g for male neonates. All neonates were born between 38 and 41 weeks of gestation. The Apgar score ranged from 8 to 10 points between the 1^{st} and 5^{th} minutes of life. No known congenital or acquired disorders were noted. No mother had been given antibiotics, or anti-inflammatory agents.

Blood was collected from the umbilical vein immediately after the cutting of the umbilical cord into an EDTA test tube 2 ml (Sarstedt, Germany, Nümbrecht). Blood preparation for CD4, CD8, Fas, FasL and Bcl-2 assays was done within 2 hours from collection. Cell viability was checked each time before cytometric analysis. In order to assess lymphocyte viability, trypan blue vital staining was performed. Cord blood samples containing at least 95% viable cells were approved for further analysis.

CD4, CD8 and Fas (CD95) assay preparation: 10 µL of antibody (appropriately CD4 FITC, CD8 FITC, CD95, IgG1 RPE - DakoCytomation, Denmark) was added to 100 µL of whole blood, vortex stirred for 5 s, incubated for 15 min in room temperature and darkness and lysed with Uti-Lyse Erythrocyte-Lysing Reagent (DakoCytomation, Denmark).

CD4, CD8 and FasL (CD95L) assay preparation: 10 µL of antibody (appropriately CD4 FITC, CD8 FITC, CD95L, IgG1 RPE - DakoCytomation, Denmark) was added to 100 µL of whole blood, vortex stirred for 5 s, incubated for 15 min in room temperature and darkness, lysed with Uti-Lyse Erythrocyte-Lysing Reagent (DakoCytomation, Denmark), vortex stirred for 5 s with 2 mL of Phosphate Buffered Saline and centrifuged (300 g, 5 min). The residue was suspended in 500 µL of Phosphate Buffered Saline.

CD4, CD8 and Bcl-2 assay preparation: 10 µL of antibody (appropriately CD4 RPE, CD8 RPE - DakoCytomation, Denmark) was added to 100 µL of whole blood, vortex stirred for 5 s, incubated for 15 min in room temperature and darkness, and fixed and permeabilized with IntraStain Kit (DakoCytomation, Denmark) with an addition of 4 µL Bcl-2 FITC, IgG1 FITC antibodies, vortex stirred for 5 s with 2 mL of Phosphate Buffered Saline and centrifuged (300 g, 5 min). The residue was suspended in 500 µL of Phosphate Buffered Saline.

Compatible isotypic control antibodies were used. The percentage of CD4, CD8, Fas and FasL and Bcl-2 expressed in the lymphocytes was determined using a flow cytometer (Beckman Coulter PC500, Krefeld).

The study was approved by the parturients and the Ethical Committee of the Medical University of Białystok according to the guidelines for Good Clinical Practice, permission number R-I-003/291/2006.

Data distribution in groups was verified with the Kolmogorov-Smirnov Goodness of Fit Test. We found a distribution abnormality, therefore, we used the Mann-Whitney U test. P-value of less than 0.05 was considered significant.

RESULTS

In newborns, we found 3,85 x 10^3/µL T lymphocytes, female newborns demonstrated a lower count of T lymphocytes 3,56 x 10^3/µL in relation to male newborns 4,15 x 10^3/µL.

The percentage of $CD4^+$ lymphocytes 50,4 is evidently higher than $CD8^+$ lymphocytes 18,83. CD4+ lymphocytes express more Fas 10,36% and FasL 6,66% than $CD8^+$ lymphocytes Fas 6,79% and FasL 5,63%. The exception is the Bcl-2, which is expressed less in $CD4^+$ lymphocytes Bcl-2 91,9% than in $CD8^+$ lymphocytes Bcl-2 93,75% (Tab. 1).

Female newborns demonstrate a higher $CD4^+$ lymphocyte count 56% in relation to male newborns 38,69%, p=0.0003. It is interesting to note, that $CD4^+$ lymphocytes expressed more Fas on their surface 13,8% in females in comparison to male newborns 7,53%, p= NS. FasL was demonstrated in female 6,8% and 6,52% male newborns, p=NS. Bcl-2 demonstrates in female newborns 89,7% in males newborns 99,6%, p= NS (Tab. 1).

The percentage of $CD8^+$ lymphocytes is practically the same in female 19% as in male newborns 18%, p=NS. Fas

Table 1. **Expression of FasR, FAS-L and Bcl-2 in CD4+ and CD8+ of T lymphocytes in the cord blood of healthy full-term newborns.**

N	Gender	Lymph T	% Median (min-max)		FasR% Median (min-max)		FasL% Median (min-max)		Bcl-2% Median (min-max)		Lymph T count x $10^3/\mu L$ x ± SD	
24	F + M	CD4+	50,4 (30,5-68)		10,36 (3,1-24,9)		6,66 (2,6-12,59)		91,9 (81-99,96)		F + M	3.83 ± 0,74
		CD8+	18.83 (12-26)		6,79 (2,71-15,8)		5,63 (1,15-16,4)		93,75 (50,3-99,56)			
13	F	CD4+	56 (32-68)	p=0.0003***	13,8 (6,02-24,9)	NS	6,8 (4,18-12,5)	NS	89,7 (86-97,8)	NS	F	3.56 ± 0,66
11	M		38.69 (30,5-51)		7,53 (3,1-20,12)		6,52 (2,6-12,59)		99,6 (81-99,96)			
13	F	CD8+	19 (13-26)	NS	8,4 (4,68-15,8)	p=0.045*	6,7 (1,15-16,4)	NS	83,4 (50,3-98,9)	p=0.0064**	M	4.15 ± 0.56
11	M		18 (12-24,3)		5,56 (2,71-12,9)		4,94 (3,58-13,39)		97,87 (80,04-99,56)			

F – female
M- male
NS – not statistically significant
p < 0,05 - statistically significant*
p < 0,01- high statistical significance**
p < 0,001- very high statistical significance ***

is more expressed in $CD8^+$ lymphocytes in female 8,4% in comparison to male newborns 5,56%, p=0.045. FasL in $CD8^+$ in female 6,7% and in male newborns 4,94%, p=NS. The percentage of Bcl-2 is lower in female newborns 83,4% in relation to male newborns 97,87%, p=0.0064 (Tab. 1).

DISCUSSION

In newborns, we found a high deficit of $CD8^+$, only 18,83% compared to 57,6% in adults [19]. Potestio et al. [20] found $CD8^+$ 21,5%; these findings are in line with our results.

The question of whether gender effects the count of $CD4^+$ and $CD8^+$ arises. In $CD4^+$ expression, female newborns demonstrate a higher number (56%) compared to male newborns (38,69%) p=0.0003. It is reasonable to postulate a decreased immune response in male newborns. Therefore, a decreased count of $CD8^+$ cytotoxic lymphocytes may disturb the immune system in newborns.

Umbilical cord T lymphocytes secrete less cytokines and have a decreased cytotoxicity. It probably results from a relative dominance of immature lymphocyte subsets [21]. Naive T cells constitute only 0,2 to 0,8% of total human $CD4^+$ cells, whereas in cord blood most T cells belong to naive cells [22]. They exhibit a low CD95 expression [23] and are resistant to apoptosis induction [24]. Because the healthy fetus is usually not challenged with foreign antigens, activation of T cells may reflect the early interaction of T cells with self antigens in the pathogen – free organism [25]. Cord blood lymphocytes are functionally immature [26] and have deficient immune responses.

The count of T lymphocytes in our findings was close to the data demonstrated by Potestio et al. [20] and Banasik et al. [27]. Our results concerning the count of T lymphocytes are higher than in adults [28].

Our data have shown a low expression of Fas in $CD4^+$ 10,36% and in $CD8^+$ 6,79%. Gupta S. [29] found 28,8% and 35,5% in adults, respectively. Our findings are in line with other authors [8, 30]. Male newborns demonstrated lower expression of Fas in $CD4^+$ as well as in $CD8^+$ in comparison to female newborns. A prominent feature of T lymphocytes in male newborns is a more expressed immaturity than in female newborns. A deficit of Fas antigen may disturb the process of cellular homeostasis between $CD4^+$ and $CD8^+$ T cells [8]. Fas is constitutively expressed in mature T cells [31], therefore, a low expression of Fas may indicate that T lymphocytes in cord blood are immature. Fas antigen acts in different processes of cellular homeostasis between $CD4^+$ and $CD8^+$ T cells [8].

FasL is poorly represented in $CD4^+$ and in $CD8^+$. Gender seems not to affect the expression of this marker but lower values were found in male newborns. These data may indicate a low activation of T lymphocytes since FasL is expressed as a result of activation of T cells [9].

Bcl-2 expression was less represented in $CD4^+$ 91,9% in comparison to $CD8^+$ 93,75%. Shinohara et al. [8] found over 95% expression of Bcl-2 in $CD4^+$ and $CD8^+$ in neonates. Oganse [32] found the same expression of Bcl-2 in adults, $CD4^+$ T 88,6%, $CD8^+$ T 87,1%. In $CD8^+$ T cells in females, Bcl-2 positive cells were diminished to 83,4%, which may indicate that apoptosis may be more expressed. After activation and subsequent clonal expansion, cellular homeostasis of T cells may be maintained by apoptosis, which is related to reduction with Bcl-2 protein [8]. Impaired apoptosis in neonatal cells may contribute to prolonged inflammation after oxidative stress or infection [33].

There are no studies in literature that address the possibility of a defect, concerning count and function, of T cells of male newborns in comparison to female newborns. Our hypothesis is strengthened by the clinical observations that male newborns are at a higher risk for serious bacterial infections than female newborns [3].

CONCLUSIONS

A prominent feature of T lymphocytes in male newborns is a more expressed immaturity than in female newborns. Impaired apoptosis in neonatal cells may contribute to prolonged inflammation in newborns after oxidative stress or infection.

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