IMMUNOLOGY AND MICROBIOLOGY

Generation of Reactive Oxygen Species by Umbilical Blood Cells and Immune Status of Newborns at Risk of Infectious Inflammatory Diseases

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We carried out a comparative clinical and immunological examination of newborns whose mothers were at risk of infectious inflammatory diseases. Umbilical blood cell phenotype was evaluated by flow cytofluorometry. ROS level was evaluated by chemiluminescence intensity. Spontaneous production of ROS and phagocytic activity of cells in the whole umbilical blood was reduced in newborns born after complicated pregnancy. Low immunoregulatory index indicating changed $CD4^+/CD8^+$ ratio and low percentage of natural killer cells were observed in children with manifestations of bacterial infection. ROS production by isolated granulocytes and the effects of PI3K and p38 MAPK (kinases involved in the regulation of activity of NADPH oxidase responsible for the production of ROS) in the risk group infants differed from the corresponding parameters in the control group. The results indicate shifts in the phagocytosis system, immune status, and the receptor-conjugated regulatory systems of ROS generation by granulocytes in newborns at risk of infectious inflammatory diseases.

Key Words: *leukocyte; phenotypical characteristics; receptor; signal transduction; chemiluminescence*

The mechanisms of immune reactivity in newborns are not completely understood. The blood from newborns and adults differs by many hematological and immunophenotypical characteristics. Umbilical blood contains populations of not yet completely formed cells and numerous morphologically immature monocytes and lymphocytes. The granulocyte population includes an appreciable percentage of promyelocytes and myelocytes [10]. It is assumed that high liability of newborns to infection is due to functional immaturity of leukocytes and inadequate interactions of cells during the formation of the immune response. Functional immaturity of granulocytes in newborns manifests by poor mobility, decreased expression of receptors [6,11], decreased bactericidal activity, weak response to many ligands of Toll-like receptor [8], and decreased Fas-mediated apoptosis [9]. Specific organization of NADPH oxidase (enzyme responsible for

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ROS generation) was shown for umbilical blood neutrophils. Elevated content of cytochrome b_{558} in the plasma membrane and low levels of $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ cytoplasmic components were detected [4]. The capacity of umbilical blood neutrophil to generate superoxide is different in full-term and preterm newborns and depends on the nature of the stimulus [7]. The umbilical blood neutrophil receptors differ significantly from adult human cell receptors by the capacity to bind bacterial ligands. The data on the organization of signal routes are scanty. It is assumed that the production of superoxide anion radical is emhanced at the level of fMLF receptor-protein kinase C [7] (this is also characteristic of adult cells). The peculiarities of signaling in umbilical blood cells are not yet studied.

Maternal infectious inflammatory diseases lead to disorders in the immune system reactivity in newborns. The functioning of the umbilical blood immune cells is different in healthy newborns and in newborns with infectious inflammatory diseases [1]. Umbilical blood leukocytes of newborns with bacterial infection are characterized by lower bactericidal activity in comparison with infants without infection. The mechanisms, through which the risk of bacterial infection of newborns increases, are little studied. There are no reliable data on the probability of infection transmission from infected mother to the newborn [1]. The molecular basis of the formation of immune reactions in children born from mothers at risk of infectious inflammatory diseases remains little studied.

We carried out a clinical and immunological examination of newborns whose mothers formed a group at risk of infectious inflammatory diseases and evaluated changes in ROS generation in their umbilical and peripheral blood.

MATERIALS AND METHODS

Healthy newborns born after normal gestation (n=20, group 1) and newborns whose mothers were at risk of infectious inflammatory diseases were examined. Children born after complicated pregnancy were divided into 3 groups: with normal course of the neonatal period (n=56, group 2), with risk of infection, receiving antibacterial therapy (n=17, group 3), and with manifest bacterial infection (n=28, group 4).

Granulocytes were isolated from the umbilical blood in double Ficoll-urografin density gradient (1.077 and 1.118 g/ml densities). Cell concentration in the suspension was 10^7/ml (viability ≥95%). The cells were stored at 4°C and used no earlier than 1 h after isolation.

Phenotyping of newborn umbilical blood cells was carried out on a flow cytofluorometer (Becton Dickinson) using CD45/CD14, CD3, CD4, CD8, CD16, and CD19 monoclonal antibodies (Catlag).

The capacity of blood cells to generate ROS was evaluated by chemiluminescent analysis. The samples were prepared and the measurements carried out as described previously [2]. Three blood samples from each patient were prepared: spontaneous chemiluminescence was evaluated in one and the levels of chemiluminescence activated with opsonized zymosan (OZ; 0.25 mg/ml) in two other samples. The suspension of isolated cells (10^6/ml) was pipetted into wells (working volume 200 μl). Control (intact) cells and cells with inhibitors: 1 μM SB 203580, inhibitor of mitogen-activated protein kinase with a molecular weight of 38 kDa (p38 MAPK) and 0.1 μM wortmannin, inhibitor of phosphatidylinosite-3-kinase (PI3K), were tested in parallel. Control cells and cells treated with one of inhibitors were incubated for 30 min at 37°C. Recording conditions were described previously [2]. Spontaneous activity of cells was recorded, after which the cells were activated with chemotactic peptide *N*-formylmethionine-leucine-phenylalanine (fMLF) in a concentration of 1 μM (all reagents from Sigma).

The mean values of the parameters and SEM for the above-mentioned number of independent measurements are presented. The inhibitor effects were estimated as the ratio of ROS production by the cells treated with one of inhibitors to ROS production by intact cells. The differences between the groups were estimated using Student's t test.

RESULTS

Spontaneous production of ROS by nonfractionated umbilical blood cells from group 1 children was significantly higher compared to adult blood cells: 0.69±0.14 (n=20) and 0.17±0.05 (n=14, p<0.02), respectively. In groups 2, 3, and 4 this parameter was lower than in group 1 (Fig. 1, 2). The lowest spontaneous production of ROS was detected in group 3 children. The production of ROS in umbilical blood activated with OZ was also lower in children born after complicated pregnancy in comparison with group 1 children (Fig. 1). Hence, healthy newborns, in contrast to risk group infants, are characterized by high spontaneous and stimulated generation of ROS. The lowest phagocytic activity, evaluated by the response to OZ, was observed in newborns with manifest infection. Granulocytes play the main role in ROS production in whole human blood.

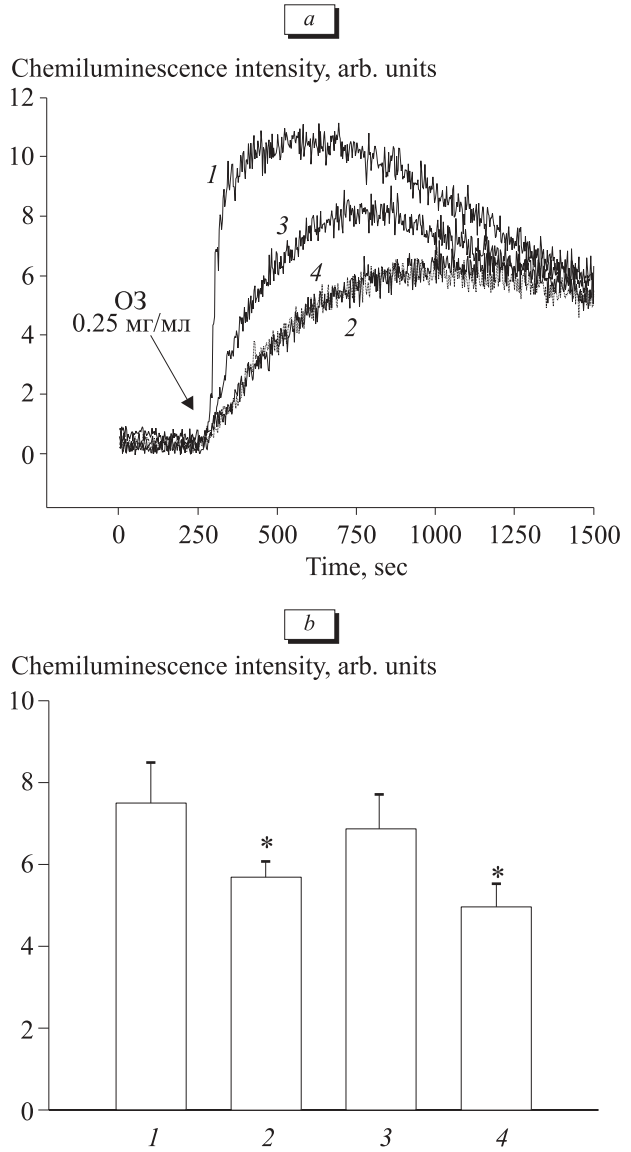

Fig. 1. ROS generation by nonfractionated umbilical blood cells. *a*) original record of response to 0.25 mg/ml OZ; *b*) amplitude of response to 0.25 mg/ml OZ. Here and in Fig. 2: *1*) healthy children born after normal gestation; *2*) children with normal course of the neonatal period, born after complicated pregnancy; *3*) children born from mothers with risk of infection, prescribed antibacterial therapy; *4*) children with manifest bacterial infection from risk-group mothers. **p*<0.05 compared to healthy children.

The production of ROS by isolated granulocytes also differed in different groups of newborns. Spontaneous production was significantly decreased in groups 2, 3, and 4. Activation of neutrophils with 1 μM fMLF resulted in higher intensity of responses in groups 2, 3, and 4 in comparison with group 1 (Table 1), which can indicate primed status of granulocytes.

Pretreatment of granulocytes with many agents, including cytokines, before activation leads to more intensive response to chemotactic factors [13]. Increased response in children from groups at risk of infectious inflammatory diseases reflects previous effects of antigens and cytokines [5,15]. Transduction of the signal from fMLF receptor to NADPH oxidase determining ROS production during the respiratory burst can change in granulocytes from children born after complicated pregnancy. It was found that activation of PI3K and components of the mitogen-activated protein kinase cascade was involved in infectious inflammatory process [3,12]. These enzyme systems are also involved in maturation of peripheral blood cells [3] and regulation of phagocyte function [14]. We used wortmannin (PI3K inhibitor) and SB 203580 (p38 MAPK inhibitor) for evaluation of the role of these kinases in the regulation of ROS generation in newborn granulocytes. The effect of SB 203580 inhibitor was most demonstrative (Table 1). Presumably, p38 MAPK is just slightly involved in cell response to 1 μM fMLF in healthy children (group 1), while in groups 2 and 3 its positive role is more significant. In cells of group 4 children p38 MAPK is involved in negative regulation of NADPH oxidase. Wortmannin significantly inhibited the respiratory burst in cells of groups 1, 2, and 3 children, but was virtually inert towards silent and activated cells of group 4 children (Table 1). The data attest to involvement of PI3K and/or its products in positive regulation of NADPH oxidase in cells from children of groups 1-3, while in group 4 the participation of PI3K in the regulation of NADPH oxidase is minor. Presumably, this latter fact indicates functional immaturity of congenital immune system in children with manifestations of bacterial infection during the early neonatal period.

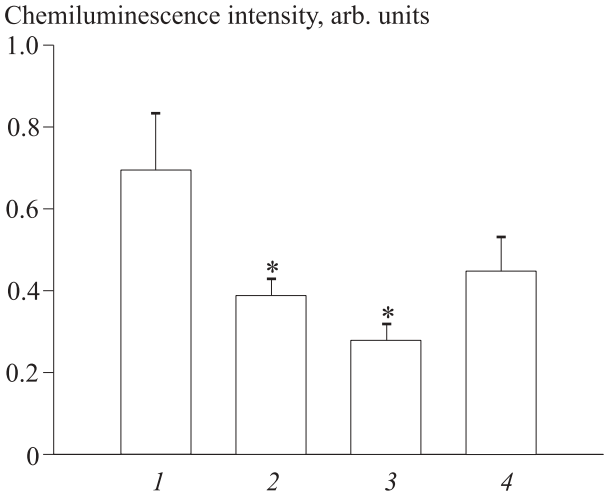

Fig. 2. Spontaneous generation of ROS by nonfractionated umbilical blood cells. **p*<0.01 compared to healthy children.

TABLE 1. Functional Characteristics of Newborn Umbilical Blood Granulocytes (*M*±*SEM*)

Parameter	Group			
	1 (control)	2	3	4
Spontaneous production of ROS, arb. units	6.1±1.8	3.1±0.6*	3.2±0.4*	2.2±0.6*
Amplitude of response to 1 μM fMLF, arb. units	25.2±5.7	38.0±11.4**	41.5±11.6**	34.5±12.8
Effect of inhibitors on spontaneous production of ROS, %				
SB 203580, 1 μM	0.7±0.1	0.8±0.1**	0.6±0.2	1.5±0.6**
wortmannin, 0.1 μM	0.75±0.26	0.9±0.2	0.7±0.1	1.0±0.4
Effect of inhibitors on respiratory burst induced with 1 μM fMLF, %				
SB 203580, 1 μM	0.9±0.1	0.64±0.10*	0.3±0.1*	1.5±0.1*
wortmannin, 0.1 μM	0.8±0.2	0.5±0.1*	0.7±0.4	1.04±0.10

Note. Here and in Table 2: *$p<0.01$, **$p<0.05$ compared to group 1.

TABLE 2. Phenotypical Characteristics of Newborn Umbilical Blood Cells (*M*±*SEM*)

Group	Lymphocyte content, %		Immunoregulatory index, $CD4^+/CD8^+$
	$CD16^+$	$CD19^+$	
1 (*n*=9)	18.9±3.6	16.5±2.9	2.5±0.4
2 (*n*=27)	14.1±1.7*	16.9±2.5	2.6±0.6
3 (*n*=9)	15.2±1.9	11.0±3.1*	2.4±0.3
4 (*n*=6)	7.8±2.0*	28.2±2.4**	1.4±0.5*

The percentage of $CD3^+$ and $CD4^+$ lymphocytes in the umbilical blood of group 1 children was 45.4±5.0 and 30.3±3.9, respectively. The values in groups 2 and 3 were virtually the same as in group 1. The percent of $CD3^+$ and $CD8^+$ lymphocytes tended to increase in group 4 newborns. Decreased content of $CD16^+$ cells was detected in groups 2, 3, and 4, the lowest values were detected in group 4 (Table 2). The percentage of $CD19^+$ cells was lowered in group 3 children and elevated in group 4. The immunoregulatory index ($CD4^+/CD8^+$ ratio) was significantly lowered in group 4 children (Table 2). Hence, the parameters of the immune status in children at risk of infectious inflammatory diseases were characterized by changed ratio of immunoregulatory subpopulations of T-cells, decreased percentage of cytotoxic cells ($CD16^+$), and increased level of B cells ($CD19^+$). However, statistically significant shifts in all these parameters were observed in children with realized infectious process. The parameters of ROS production and content of natural killer (NK) cells were in high correlation (r=0.80). $CD16^+$-NK cells are an important component of congenital immunity. They produce many cytokines, activating or priming other cells, including macrophages and granulocytes, in response to infection. Activation of granulocytes stimulates the production of ROS. It seems that in group 4 children the content of effector cells and their activation were decreased, which promoted the development of infectious process.

The detected deviations in the immune status, phagocytosis system, and conjugated systems regulating the neutrophil respiratory burst in newborns at high risk of infectious inflammatory diseases indicate suppression of congenital immunity, formed under unfavorable conditions of complicated gestation.

REFERENCES

1. M. V. Degtyareva, N. N. Volodin, K. K. Bakhtikyan, *et al.*, *Med. Immunol.*, **2**, No. 1, 69-76 (2000).
2. V. G. Safronova, N. K. Matveeva, V. N. Mal'tseva, *et al.*, *Byull. Eksp. Biol. Med.*, **140**, No. 8, 173-176 (2005).
3. K. Q. Chen, P. Iribarren, W. H. Gong, *et al.*, *Cell. Mol. Immunol.*, **2**, 241-252 (2005).
4. U. H. Chudgar, G. W. Thurman, and D. R. Ambruso, *Pediatr. Blood Cancer*, **45**, 311-317 (2005).
5. A. Davoren, K. Saving, J. G. McFarland, *et al.*, *Transfusion*, **44**, 1041-1046 (2004).
6. S. K. Kim, S. E. Keeney, S. K. Alpard, *et al.*, *Pediatr. Res.*, **53**, 132-136 (2003).
7. H. Komatsu, K. Tsukimori, K. Hata, *et al.*, *Early Human Develop.*, **65**, 11-19 (2001).

8. O. Levy, K. A. Zarember, R. M. Roy, *et al.*, *J. Immunol.*, **173**, 4627-4634 (2004).
9. D. Luo, K. O. Schowengerdt, J. J. Stegner, *et al.*, *Pediatr. Res.*, **53**, 859-864 (2003).
10. T. Mikami, M. Eguchi, H. Kurosawa, *et al. Med. Electron. Microsc.*, **35**, 96-101 (2002).
11. M. Nybo, O. Sorensen, R. Leslie, *et al.*, *Arch. Dis. Child. Fetal Neonatal Ed.*, **78**, F129-F132 (1998).
12. L. Pollak, T. Hanoch, M. J. Rabey, *et al.*, *Neurol. Sci.*, **26**, 324-329 (2005).
13. F. R. Sheppard, M. R. Kelher, E. E. Moore, *et al.*, *J. Leukoc. Biol.*, **78**, 1025-1042 (2005).
14. L. Stephens, C. Ellson, and P. Hawkins, *Curr. Opin. Cell. Biol.*, **14**, 203-213 (2002).
15. J. S. Wang, H. Chen, and Q. R. Zhu, *World J. Gastroenterol.*, **11**, 3582-3585 (2005).