Evaluation of the Role of FMLF Chemotaxic Peptide Receptors in Umbilical Cord Blood Granulocytes from Newborns at Risk of Infectious Inflammatory Diseases

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We studied the role of receptors with high and low affinity for fMLF chemotaxic peptide in the generation of active oxygen species by umbilical cord blood granulocytes from newborns with normal neonatal period, born after normal or complicated gestation, in children with manifestations of bacterial infection born after complicated pregnancy, and in granulocytes of non-pregnant women with normal reproductive function. Granulocytes of children born after complicated pregnancy exhibited high reactivity in induction of respiratory burst in a wide range of fMLF concentrations. The presentation of receptors with high and low affinity on granulocytes during initiation of the respiratory burst differs in children born after complicated pregnancy and in healthy babies born after normal gestation. Presumably, the detected differences result from high expression of receptors with low affinity for fMLF and disorders or immaturity of mechanisms responsible for receptor inactivation.

Key Words: *newborns; granulocytes; fMLF; high- and low-affinity receptors; respiratory burst*

Defense from bacterial infection in fetuses and newborns is provided by mechanisms of innate immunity against the background of the formation of adaptive immunity [8]. Maternal infectious inflammatory diseases lead to disorders in the reactivity of the immune system newborns. Deviations of the immune status and functions of cells of the innate immune system are observed in newborns at risk of infectious inflammatory diseases [3]. This indicates modified expression of receptors and the receptor signal transduction.

Neutrophils support reactions of the innate immune system and are involved in development of acquired immunity [5]. Functions of neutrophil are governed by strict hierarchical relationships providing fine regulation of cell activity. N-Formyl-Met-Leu-Phe (fMLF) peptide, a product of degradation of bacterial proteins, is the strongest chemoattractant for neutrophils. Concentration-dependent binding of fMLF to specific receptors on the neutrophil membrane activates the motor and cytotoxic functions [1,4,13].

Activity of immune system cells is regulated through ligand-receptor interactions. The sequence of events includes ligand binding, transmembrane and intracellular transfer of the receptor signal to effector molecules, and realization of the appropriate function by the cell. Formyl-peptide receptors (FPR) are 7-domain transmembrane proteins coupled with G proteins. The biological destination of the receptors is provision of defense infection-

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control reactions and, presumably, participation in tissue-specific differentiation, because these receptors are present on mesenchymal stem cells [14]. The physiological role of receptors is the follows: in activated state, they initiate adhesion and migration of phagocytes into the inflammatory focus (chemotaxis) and the cytotoxic functions of cells (degranulation, production of active oxygen species (AOS) and cytokines). The expression of FPR on cells of different tissues (dendritic cells, fibroblasts) and transformed cells (astrocytoma, glioma with high metastatic potential) indicates that they are involved in pathophysiological processes, autoimmune reactions, and inflammation [12,15].

The most potent stimulator of these receptors is fMLF. In addition to fMLF, these receptors bind proinflammatory agents (lipoxin A_4, amyloid peptides, serum amyloid A, annexin I, cathepsin G, catelicidine fragment LL-37, *etc.*) [11]. Detection of endogenous antagonists and synthesis of the receptor peptide inhibitors suggest that FPR can be a pharmacological target in the treatment of inflammatory diseases.

In humans, three genes (*fpr1*, *fprl1*, and *fprl2*) encode specific receptor subtypes: FPR with high affinity (dissociation constant K_d<1 nM); FPR-like receptor 1 (FPRL1R) with low affinity for fMLF (K_d>100 nM), and FPR-like receptor 2 (FPRL2R). Expression of receptors with different affinity for fMLF allows phagocyte functioning in a wide range of fMLF concentrations (10^{-12}-10^{-4} M). However, their role in the realization of different functions in health and disease remains little studied. It is assumed that high- and low-affinity receptors act through heterotrimer G_i protein and a system of second messengers, including phosphatidylinosite and Ca^{2+}. It was found that high- and low-affinity FPR on human neutrophils induce phosphorylation of *L*-plastine (cytoskeleton protein) through different signal-transduction intermediates [10]. Comparison of signal events in neutrophils of $FPR^{+/+}$ and $FPR^{-/-}$ mice showed their similarity [7]. It was also shown that mitogen-activated proteinkinase with a molecular weight of 38 kDa (p38 MAPK) had opposite effects on activity of NADPH oxidase during activation of high- and low-affinity receptors on mouse neutrophils [2]. The presence of receptors with high and low affinity for fMLF on newborn neutrophils was demonstrated [6]. Functional significance of FPR family subtypes in umbilical cord blood neutrophils was not studied.

We evaluated the role of receptors with high and low affinity for fMLF chemotaxic peptide in the respiratory burst of umbilical blood granulocytes of children at a high risk of infectious inflammatory disease.

MATERIALS AND METHODS

Newborns with normal course of the neonatal period, born after normal gestation (group 1; *n*=19) and after complicated pregnancy (group 2; *n*=39), children with symptoms of bacterial infection born after complicated pregnancy (group 3; *n*=23), and non-pregnant women with normal reproductive function (group 4; *n*=20) were examined. Mothers gave informed consent to participation in the study.

Umbilical and peripheral blood granulocytes were isolated in double Ficoll-urografin density gradient (1.077 and 1.119 g/ml). Cell viability was at least 95%. The cells were stored at 4°C and used in experiments not earlier than 1 h after isolation.

Generation of AOS by cells was evaluated by the chemiluminescence method. Cell suspension was pipetted in experimental wells with a working volume of 200 μl (10^6 cell/ml). The effects of receptors with high affinity for fMLF were excluded using fMLF antagonist (N-tert-butoxycarbonyl-Met-Leu-Phe (T-boc)) [9]. Control (intact) cells and cells treated with the inhibitor were incubated for 30 min at 37°C, after which spontaneous activity of cells and respiratory burst induced by fMLF in concentrations of 10^{-11}-5×10^{-5} M were recorded. All reagents were from Sigma.

The differences between the groups were evaluated using Student's *t* test.

RESULTS

Granulocytes act in a gradient of chemotaxic factors, and it is assumed that their functions (adhesion, chemotaxis, degranulation, and respiratory burst) are successively activated [1,13]. Activation threshold is the highest for the respiratory burst, during which AOS toxic for exogenous microorganisms and the host are produced. fMLF peptide is considered to be the most effective of the known chemotaxic factors.

We compared the concentration-dependent responses to umbilical blood granulocyte fMLF from group 1 newborns and adult peripheral blood granulocyte fMLF (Fig. 1, *a*). In both cases, the concentration-response relationships were presented by saturation curves with at similar threshold concentrations (below 10^{-8} M), whereas EC50 were 4.5×10^{-8} and 2.0×10^{-7} M, respectively. Umbilical blood granulocytes were characterized by less intensive response than adult granulocytes, their response varying little at concentrations of 10^{-6}-5×10^{-5} M. The reaction of granulocytes from babies born after complicated pregnancy (groups 2 and 3) to fMLF was more intensive at all concen-

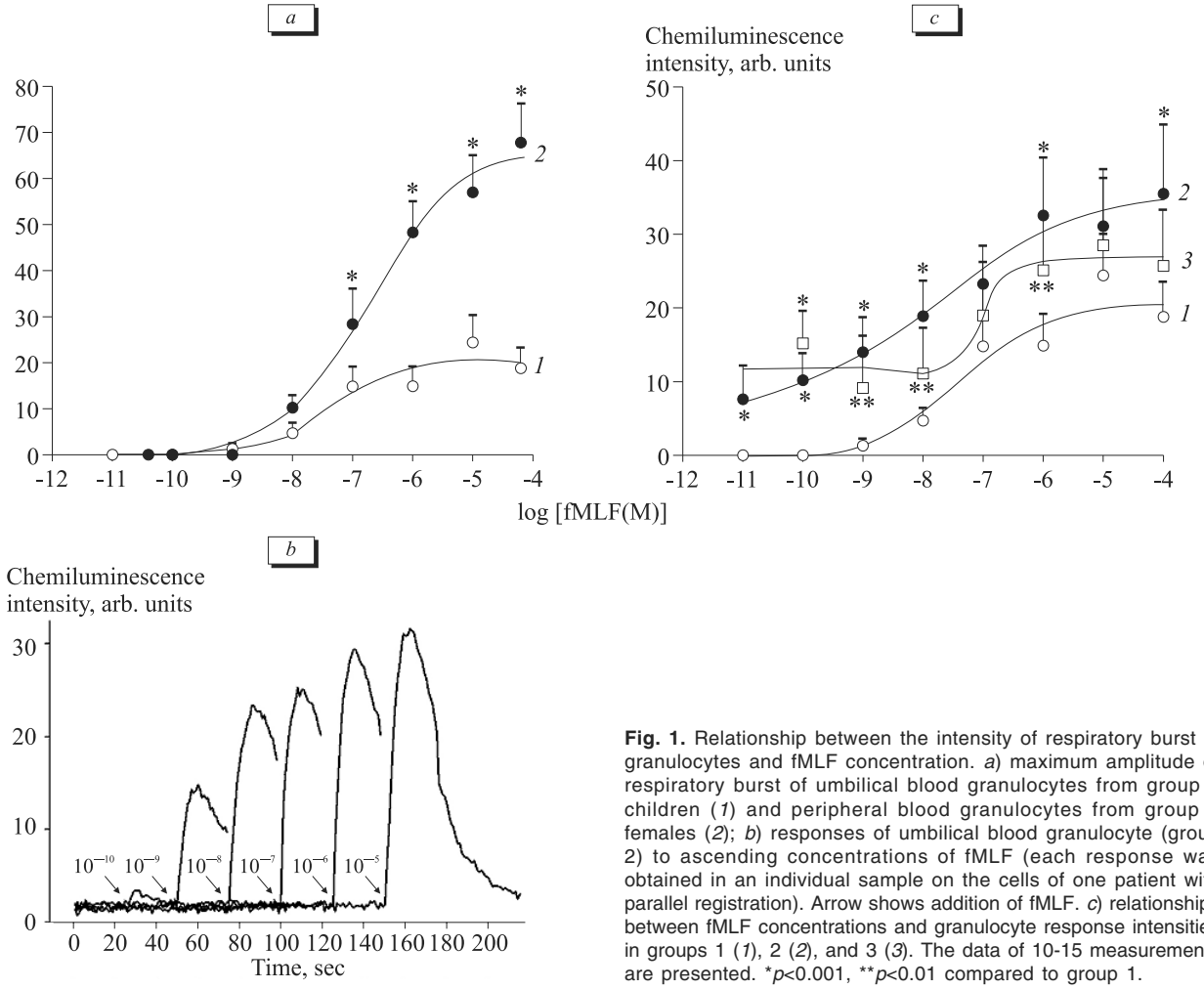

Fig. 1. Relationship between the intensity of respiratory burst in granulocytes and fMLF concentration. *a*) maximum amplitude of respiratory burst of umbilical blood granulocytes from group 1 children (*1*) and peripheral blood granulocytes from group 4 females (*2*); *b*) responses of umbilical blood granulocyte (group 2) to ascending concentrations of fMLF (each response was obtained in an individual sample on the cells of one patient with parallel registration). Arrow shows addition of fMLF. *c*) relationships between fMLF concentrations and granulocyte response intensities in groups 1 (*1*), 2 (*2*), and 3 (*3*). The data of 10-15 measurements are presented. *$p<0.001$, **$p<0.01$ compared to group 1.

trations. Comparison of the concentration curves in different groups of newborns showed that respiratory burst in cells from these children could be induced by fMLF in much lower concentrations (Fig. 1, *b*, c). However, granulocytes of group 1 children virtually did not react to concentrations of 10^{-11}-10^{-9} M, while activities of granulocytes from groups 2 and 3 children were high (Fig. 1, *b*, *c*). This fact suggests the involvement of receptors with high and low affinity for fMLF into respiratory burst activation in granulocytes from children of these groups.

The use of T-boc (antagonist of receptors with high affinity for fMLF) led to inhibition of fMLF-induced respiratory burst in adult peripheral blood granulocytes (group 4) at T-boc concentrations comparable to the concentrations of fMLF (Fig. 2, *a*, *b*), which confirms specificity of the effect of the inhibitor. The inhibition was significant for cells activated with 10^{-7} and 10^{-6} M fMLF, while the response to 5×10^{-5} M fMLF virtually did not change. Hence, in granulocytes from adults respiratory burst is activated by 10^{-7} M fMLF through receptors with high-affinity for fMLF, while the response to 5×10^{-5} M fMLF is mediated mainly by low-affinity receptors. The effect of T-boc on granulocytes of children born after complicated pregnancy was also concentration-dependent. However, maximum sensitivity to T-boc was detected in cells activated with 10^{-10} M fMLF (Fig. 2, *c*). Increasing fMLF concentration to 10^{-8} M significantly attenuated the reaction to T-boc. This means that, similarly as with granulocytes from adults, the role of low-affinity receptors in activation of the respiratory burst increases with increasing fMLF concentration, but the range of fMLF concentrations in this case is much wider.

Then we evaluated the role of high- and low-affinity receptors in respiratory burst activation using T-boc in a concentration of 5×10^{-6} M. The responses of granulocytes from adults and group 1 chil-

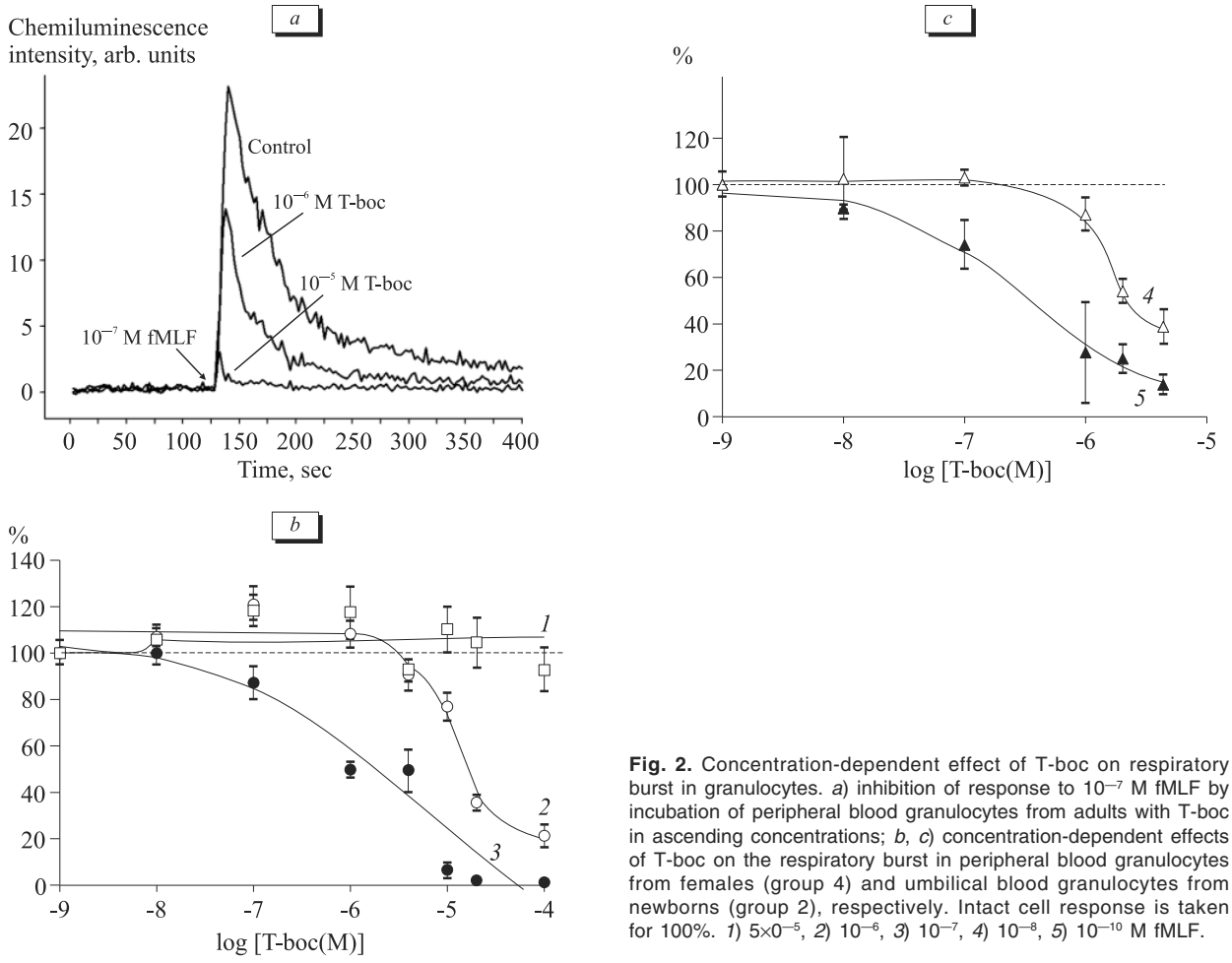

Fig. 2. Concentration-dependent effect of T-boc on respiratory burst in granulocytes. *a*) inhibition of response to 10^{-7} M fMLF by incubation of peripheral blood granulocytes from adults with T-boc in ascending concentrations; *b*, *c*) concentration-dependent effects of T-boc on the respiratory burst in peripheral blood granulocytes from females (group 4) and umbilical blood granulocytes from newborns (group 2), respectively. Intact cell response is taken for 100%. *1*) 5×0^{-5}, *2*) 10^{-6}, *3*) 10^{-7}, *4*) 10^{-8}, *5*) 10^{-10} M fMLF.

dren were virtually completely suppressed at low fMLF concentrations, while granulocytes of children born after complicated pregnancy demonstrated significant response after T-boc treatment (Fig. 3, *c*), which suggests the involvement of receptors with low affinity for fMLF in this response. This concentration of T-boc caused a half-maximum inhibition in granulocytes of children born after complicated pregnancy (group 2) in the presence of fMLF concentration by almost 2 orders of magnitude lower than in granulocytes of children born after normal gestation (group 1). This means that receptors with low affinity for fMLF are involved in activation of the respiratory burst in granulocytes of group 2 children at lower concentrations of fMLF. In addition, the response initiated by fMLF in a high concentration was partially inhibited by T-boc in this group (Fig. 3, *a*, *c*), which confirmed the contribution of high-affinity receptors.

We hypothesized that the mechanisms of receptor function regulation were not yet formed in granulocytes of children born after complicated pregnancy. The responses of cells preactivated with fMLF in low concentrations and of cells incubated with 5×10^{-6} M T-boc to 5×10^{-5} M fMLF differed significantly (Fig. 3, *b*). Desensitization of receptors by repeated doses of fMLF led to attenuation of the response.

Hence, granulocytes of children born after complicated pregnancy are characterized by high reactivity, manifested in activation of the respiratory burst at significantly lower concentrations of fMLF. The presentation of receptors with high and low affinity for fMLF in the initiation of respiratory burst of granulocytes in children born after complicated pregnancy differs from that in granulocytes of healthy newborns born after normal gestation. These differences probably result from high functional expression of low-affinity receptors and disorders or immaturity of mechanisms regulating the receptor processing.

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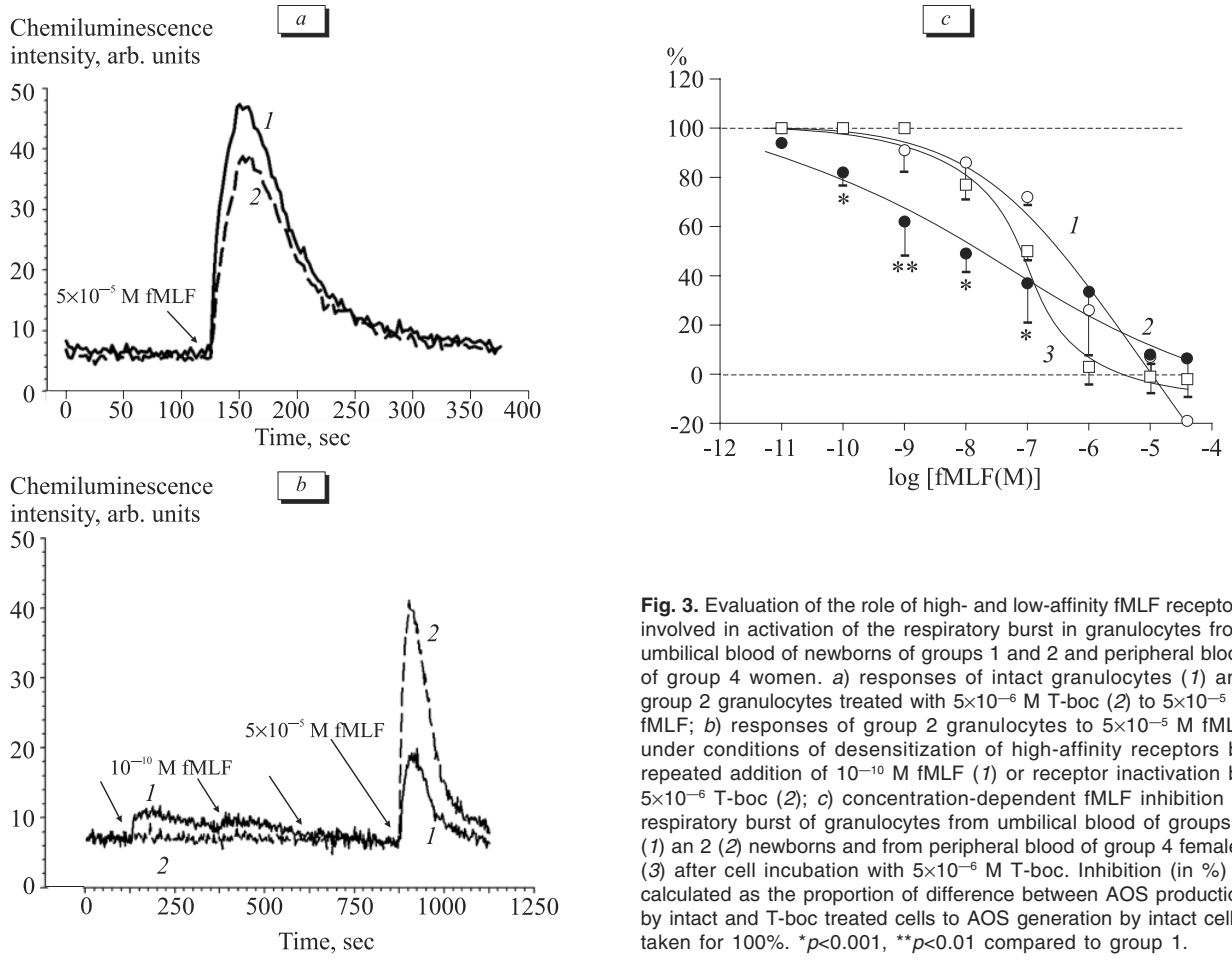

Fig. 3. Evaluation of the role of high- and low-affinity fMLF receptors involved in activation of the respiratory burst in granulocytes from umbilical blood of newborns of groups 1 and 2 and peripheral blood of group 4 women. *a*) responses of intact granulocytes (*1*) and group 2 granulocytes treated with 5×10^{-6} M T-boc (*2*) to 5×10^{-5} M fMLF; *b*) responses of group 2 granulocytes to 5×10^{-5} M fMLF under conditions of desensitization of high-affinity receptors by repeated addition of 10^{-10} M fMLF (*1*) or receptor inactivation by 5×10^{-6} T-boc (*2*); *c*) concentration-dependent fMLF inhibition of respiratory burst of granulocytes from umbilical blood of groups 1 (*1*) an 2 (*2*) newborns and from peripheral blood of group 4 females (*3*) after cell incubation with 5×10^{-6} M T-boc. Inhibition (in %) is calculated as the proportion of difference between AOS production by intact and T-boc treated cells to AOS generation by intact cells, taken for 100%. *$p<0.001$, **$p<0.01$ compared to group 1.

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