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Reproductive Hormones in the Regulation of Apoptosis of Neutrophils

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Abstract—The ability of main reproductive hormones such as chorionic gonadotropin (CG), estradiol, and progesterone to regulate apoptosis of human neutrophils was studied. The hormones were studied separately and in physiological combinations specific for different trimesters of pregnancy. A low dose of CG (10 IU/ml) increased the spontaneous apoptosis of neutrophils, whereas its combination with estradiol and progesterone corresponding to that of trimester III of pregnancy significantly decreased this parameter. The stimulating effect of CG was prevented by an inhibitor of protein kinase A, whereas the hormone-induced suppression of apoptosis depended on the activity of Ca^{2+}-channels. The antiapoptotic effect of the hormonal combination corresponding to that of trimester III was also manifested in the presence of autologous T-lymphocytes and on stimulation of neutrophils by bacterial lipopolysaccharide. The apoptosis induced with monoclonal antibodies to CD95 was significantly suppressed by the hormones studied and their combinations. Thus, apoptosis of neutrophils is effectively regulated by reproductive hormones; this seems to be an important control mechanism of activation of these cells in pregnancy.

Key words: apoptosis, neutrophils, T-lymphocytes, chorionic gonadotropin, estradiol, progesterone, pregnancy

Neutrophils are main effectors of inflammatory reactions of the body and are also important for antitumor and transplantation immunity [1, 2]. Mature neutrophils have a short lifetime, and their apoptosis, i.e., the programmed cell death induced by various stimuli and associated with degradation of chromatin, develops before long [3]. Apoptosis of neutrophils and their subsequent elimination by mononuclear phagocytes are important for regulation of the quantity of these cells and effectiveness of neutrophil-dependent reactions of the body [4, 5].

The membrane molecule Fas (CD95) mainly mediates apoptosis of neutrophils. The binding of this molecule to the appropriate ligand (FasL) both soluble and membrane-associated initiates an apoptotic signal in the cell [6, 7]. Fas is expressed by various leukocyte types, while FasL is constitutively present only on the membrane of neutrophils [6]. The high level of spontaneous apoptosis of neutrophils is associated just with their coexpression of both Fas and FasL [6]. In addition, in response to various stimuli, neutrophils produce soluble FasL [6, 7] which acts as a para- or autocrine factor and also contributes to induction of apoptosis of neutrophils.

In pregnancy neutrophil functions are controlled by reproductive hormones, of which the main peptide placental hormone chorionic gonadotropin (CG) and sex steroid hormones estradiol and progesterone synthesized on stimulation with CG are the most important [8]. Both CG [9, 10] and steroid hormones [10-12] are known to regulate the functional activity of neutrophils, and effects of the hormones are interrelated and in physiological combinations specific for different trimesters of pregnancy they can be either increased or leveled [10]. Apoptosis is an important component of the regulation of neutrophil activity during this period. In pregnancy, the level of neutrophil apoptosis is decreased, especially during the last trimester [13]. Studies on the role of reproductive hormones in the control of apoptosis of neutrophils are important for elucidation of mechanisms of regulation of the quantity and activity of these cells during gestation.

The purpose of this work was to study the effects of CG, estradiol, progesterone, and their physiological combinations on apoptosis of human neutrophils and also

Abbreviations: CD) membrane markers of leukocytes; FasL) Fas-ligand; LPS) lipopolysaccharide; McAb) monoclonal antibodies; CG) chorionic gonadotropin.

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to assess intracellular mechanisms of the hormonal effects using inhibitors of the adenylate cyclase (H-89), calcium (verapamil), and phosphoinositol (lithium hydroxybutyrate) signals. The first signal, as a rule, mediates the effect of CG [14], the other two are involved in realization of non-genomic effects of steroid hormones [15, 16]. Apoptosis was determined in three variants: the spontaneous one and those stimulated by bacterial lipopolysaccharide (LPS), and induced with monoclonal antibodies (McAb) to CD95. With the effective regulation of neutrophil functions by T-lymphocytes [17], the role of T-cells was also studied in the hormone-dependent control of apoptosis of neutrophils.

MATERIALS AND METHODS

The hormones were used in concentrations corresponding to their levels in peripheral blood during trimesters I and III of pregnancy: CG (Profasi, Italy), 100 and 10 IU/ml [18]; estradiol (Sigma, USA), 1.0 and 10 ng/ml [19]; progesterone (Sigma), 20 and 100 ng/ml [20]. Doses of the hormones were combined according to their presence in pregnancy, i.e., the high dose of CG was introduced into the culture together with the low dose of steroids, and *vice versa*.

Intracellular mechanisms of the hormonal effects were analyzed using the following reagents: the protein kinase A inhibitor H-89 (ICN, USA) (1.0 μg/ml) [21], the inhibitor of Ca^{2+}-channels verapamil (Knoll, AG; Germany) (0.025 mg/ml) [22], the inhibitor of inositol-1-monophosphatase lithium hydroxybutyrate (Tallinn Chemical Pharmaceutical Plant, Estonia), 1.5 mg/ml [23].

Neutrophils and T-lymphocytes were isolated from peripheral blood of non-pregnant women of reproductive age [24, 25].

Neutrophils at the concentration of $2 \cdot 10^6$ cells/ml were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Serva), L-glutamine (300 μg/ml; Serva), 0.01 M HEPES (Sigma), and gentamicin (100 μg/ml; Pharmacia, Sweden) at 37°C in the presence of 5% CO_2 for 24 h. As stimulators we used bacterial LPS (Sigma; 100 ng/ml) and apoptosis inducing McAb to the CD95 molecule (Fas) which were kindly presented by Dr. A. V. Filatov (State Research Center - Institute of Immunology, Ministry of Health of Russia, Moscow). The neutrophils were incubated with the hormones for 1 h, and then an appropriate stimulator was introduced into the culture. In experiments with the inhibitors, the cells were preincubated with them for 1 h, supplemented with the hormone, incubated for 1 h, and washed before the addition of the stimulator.

In the mixed cultures neutrophils ($1.5 \cdot 10^6$ cells/ml) and autologous or allogenic T-lymphocytes ($0.5 \cdot 10^6$ cells/ml) were taken at the ratio 3 : 1 [25]. Apoptosis of leukocytes was determined by cytofluorimetry [25]. Apoptosis of neutrophils in the mixed culture with T-lymphocytes was determined by two-color immunofluorescence using McAb to the membrane molecule CD16 of neutrophils. The antibodies were labeled with fluorescein isothiocyanate (FITC; Sorbent, Russia) (LNK16·FITC).

The CD95 expression was determined by immunofluorescence with a FACSCalibur cytometer using McAb LT95 labeled with FITC.

Data were processed using Student's *t*-test.

RESULTS AND DISCUSSION

The spontaneous apoptosis of neutrophils in the absence of hormones was high (Table 1) and was significantly decreased on activation of the cells with LPS that modeled a bacterial invasion (Table 1). This is consistent with the literature data about the antiapoptotic effect on neutrophils of proinflammatory factors including LPS [26]. The stimulation of neutrophils with agonistic McAb to the CD95 significantly increased the percent of apoptotic cells in the culture (Table 1).

The low physiological concentration of CG (10 IU/ml) specific for trimester III of pregnancy significantly increased the spontaneous apoptosis, whereas the appropriate combination of CG with estradiol and progesterone decreased it (Table 1). The stimulating effect of CG was not changed in the presence of inhibitors of the calcium or phosphoinositol signals (verapamil or lithium hydroxybutyrate, respectively), but was abolished in the presence of the protein kinase A inhibitor H-89 (Fig. 1), suggesting an cAMP-dependent mechanism of this effect. However, the hormone-mediated suppression of apoptosis did not depend on the activity of protein kinase A but was abolished by the inositol-1-monophosphatase inhibitor lithium hydroxybutyrate and by the inhibitor of L-type Ca^{2+}-channels verapamil (Fig. 1). Moreover, alongside with inhibition of calcium channels the hormonal combination displayed a stimulatory effect instead of inhibition (Fig. 1).

The low dose of CG increased the expression of the membrane molecule CD95 (Fas) by the cells (Table 2), and this appeared to be a mechanism of the CG-dependent stimulation of apoptosis of neutrophils. Nevertheless, the CD95 expression was also significantly increased in the presence of the high dose of progesterone (100 ng/ml) which failed to regulate apoptosis and in the presence of the physiological hormonal combination specific for trimester III of pregnancy (Table 2), which suppressed apoptosis of neutrophils under similar conditions.

On stimulation of cells with LPS, none of the hormones had a modifying effect separately, but their physiological combinations specific for trimester III of pregnancy significantly suppressed apoptosis of the LPS-

Table 1. Role of reproductive hormones in regulation of apoptosis of human neutrophils

Group number	Experiment conditions	Percent of apoptotic cells (M ± m)		
		spontaneously (n = 8)	LPS (n = 10)	anti-CD95 McAb (n = 4)
1	Control	32.1 ± 4.43	26.6 ± 3.44*	79.1 ± 3.32*
2	CG, 10 IU/ml	41.7 ± 6.51 ***p(2–1) < 0.05***	28.7 ± 3.58	63.1 ± 2.81 ***p(2–1) < 0.05***
3	CG, 100 IU/ml	31.7 ± 4.42	28.2 ± 4.59	53.4 ± 3.67 ***p(3–1) < 0.01*** ***p(3–2) < 0.05***
4	Estradiol, 1.0 ng/ml	34.6 ± 5.06	31.5 ± 3.88	55.2 ± 2.56 ***p(4–1) < 0.01***
5	Estradiol, 10 ng/ml	31.7 ± 4.91	31.2 ± 4.62	55.3 ± 2.82 ***p(5–1) < 0.01***
6	Progesterone, 20 ng/ml	29.9 ± 5.10	30.8 ± 4.98	61.6 ± 1.38 ***p(6–1) < 0.05***
7	Progesterone, 100 ng/ml	31.1 ± 3.43	28.7 ± 5.34	51.1 ± 4.13 ***p(7–1) < 0.005*** ***p(7–6) < 0.05***
8	CG (100 IU/ml) + estradiol (1.0 ng/ml) + progesterone (20 ng/ml) (trimester I of pregnancy)	29.7 ± 4.98	28.7 ± 5.11	48.4 ± 4.41 ***p(8–1) < 0.05*** ***p(8–4) < 0.05*** ***p(8–6) < 0.05***
9	CG (10 IU/ml) + estradiol (10 ng/ml) + progesterone (100 ng/ml) (trimester III of pregnancy)	25.8 ± 3.00 ***p(9–1) < 0.01*** ***p(9–2) < 0.01***	22.4 ± 3.42 ***p(9–1) < 0.05*** ***p(9–2) < 0.01***	49.8 ± 9.61 ***p(9–1) < 0.01***

Note: All groups studied were processed statistically. The tables present p only for significant differences.
* $p < 0.05$ (compared to the spontaneous variant).

stimulated neutrophils (Table 1). Unlike the spontaneous variant, the inhibitory effect of the hormonal combination was not abolished by verapamil (control/verapamil ratio was 24.0 ± 4.08%; hormones/verapamil ratio was 18.3 ± 3.27%; $p < 0.05$) and by lithium hydroxybutyrate (control/lithium hydroxybutyrate ratio was 28.3 ± 2.78%; hormones/lithium hydroxybutyrate ratio was 26.9 ± 2.14%; $p < 0.05$).

On induction of apoptosis with agonistic McAb to CD95 the hormones studied and their physiological combinations significantly inhibited it, and the inhibitory effects of CG and progesterone were dose-dependent and maximal at the high doses (Table 1).

Functions of neutrophils in peripheral blood significantly depend on their interactions with other leukocytes, especially with lymphocytes, which are main effectors of

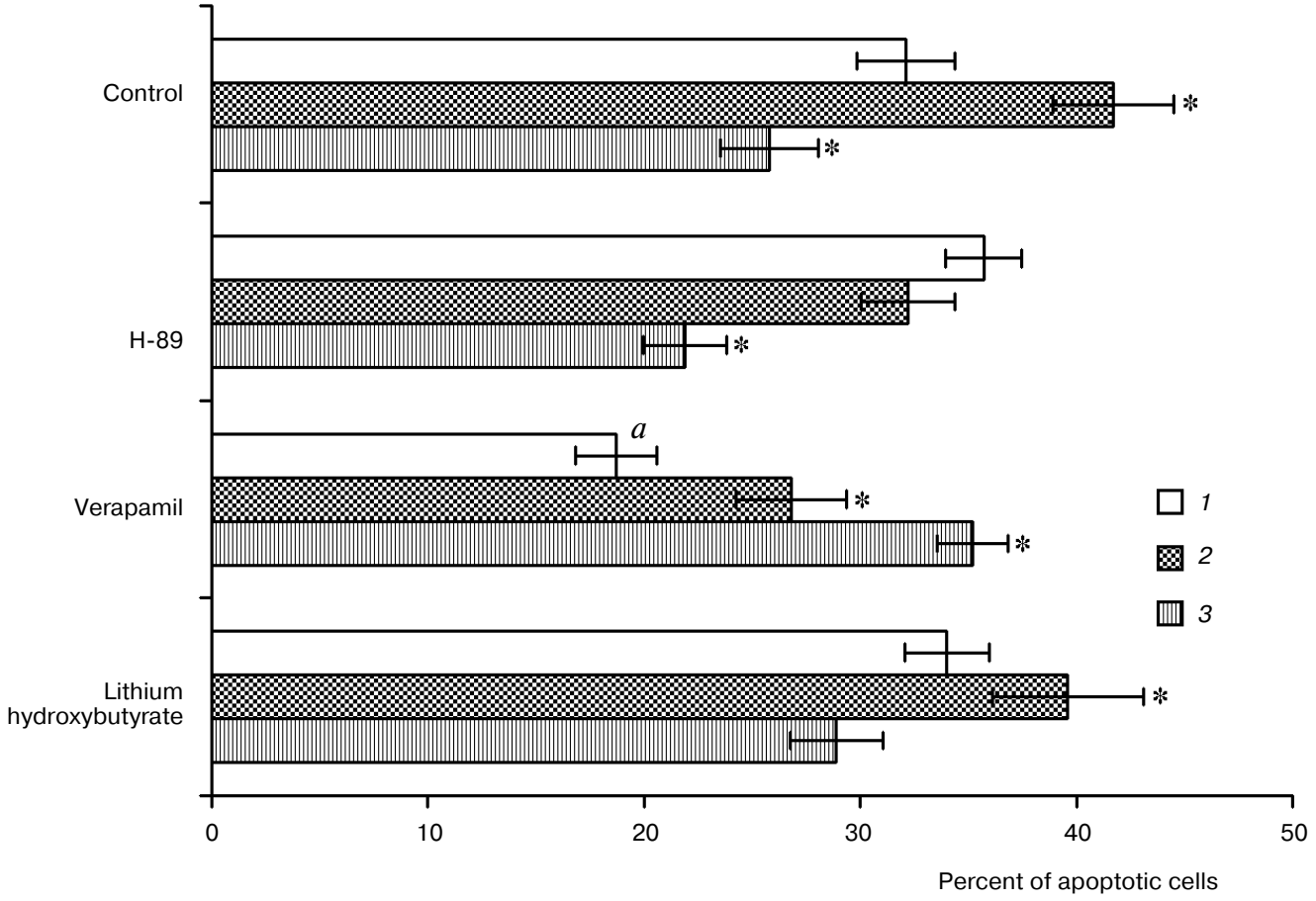

Fig. 1. Mechanisms of the hormone-dependent regulation of spontaneous apoptosis of neutrophils: *1*) hormone-free control; *2*) CG, 10 IU/ml; *3*) CG (10 IU/ml) + estradiol (10 ng/ml) + progesterone (100 ng/ml) (trimester III of pregnancy). * $p < 0.05$ (as compared to the hormone-free control); [a] $p < 0.05$ (as compared to the appropriate inhibitor-free control).

adaptive immunity. Modeling the *in vivo* conditions, we studied the role of T-cells in the hormone-dependent regulation of apoptosis of neutrophils. In the presence of autologous T-lymphocytes, the level of spontaneous apoptosis of neutrophils was virtually unchanged (Fig. 2a). And the inhibitory effect of the hormonal combination corresponding to trimester III of pregnancy was retained and significantly more pronounced. This effect was also reproduced in a trans-well system (Fig. 2a) that suggested its mediation by humoral factors of T-cells or neutrophils themselves in the mixed culture.

Apoptosis of neutrophils induced by anti-CD95 McAb was significantly decreased in the presence of autologous T-lymphocytes (Fig. 2b). The inhibitory effect of physiological hormonal combinations was retained, but unlike the neutrophil monoculture, it was dose-dependent and significantly more pronounced for the hormonal combination corresponding to trimester III of pregnancy (Fig. 2b).

Similar experiments with allogenic T-lymphocytes revealed a significantly decreased spontaneous apoptosis of neutrophils as compared to that in the monoculture (Fig. 3) that seemed to be associated with production by the activated T-lymphocytes of cytokines, especially γ-interferon, which has an antiapoptotic effect on neutrophils [26]. The hormonal regulation of apoptosis of neutrophils was also changed: the combination of reproductive hormones specific for trimester III of pregnancy failed to suppress it but, on the contrary, abolished the inhibitory effect of allogenic T-lymphocytes (Fig. 3).

In total, only the low dose of CG of the hormones studied displayed a proper proapoptotic effect on intact neutrophils, and this effect was mediated by cAMP. The effectiveness of the low concentration of CG along with the lack of effect of the high dose of the hormone suggested that the neutrophil membrane had two types of CG receptors with different affinity. Therefore, it should be noted that the level of cAMP in neutrophils was shown to increase under the influence of the low concentration of CG [24].

The absence of proapoptotic effect of CG under conditions of stimulation with LPS could be also associated

Table 2. Effect of reproductive hormones on the CD95 expression by human neutrophils

Group number	Experiment conditions	Percent of $CD95^+$-cells (M ± m)	
		spontaneously ($n = 5$)	LPS ($n = 5$)
1	Control	74.3 ± 3.34	61.3 ± 6.15
2	CG, 10 IU/ml	84.2 ± 6.04 ***p(2–1) < 0.05***	63.4 ± 4.79
3	CG, 100 IU/ml	76.6 ± 6.32	65.4 ± 6.02
4	Estradiol, 1.0 ng/ml	74.7 ± 1.39	64.8 ± 2.96
5	Estradiol, 10 ng/ml	79.7 ± 3.51	57.5 ± 5.95
6	Progesterone, 20 ng/ml	87.7 ± 7.72	66.0 ± 3.68
7	Progesterone, 100 ng/ml	88.3 ± 5.72 ***p(7–1) < 0.05***	53.9 ± 5.76
8	CG (100 IU/ml) + estradiol (1.0 ng/ml) + progesterone (20 ng/ml) (trimester I of pregnancy)	83.7 ± 4.04	63.2 ± 4.63
9	CG (10 IU/ml) + estradiol (10 ng/ml) + progesterone (100 ng/ml) (trimester III of pregnancy)	92.7 ± 2.07 ***p(9–1) < 0.01***	52.7 ± 4.38 ***p(9–1) < 0.05***

with both non-identity of apoptotic signals in the intact and stimulated neutrophils and the appropriate difference in the hormonal regulation of these signals and the LPS-dependent abolishment of the effect of CG. Thus, LPS is known to inhibit the steroidogenic effect of a structural analog of CG, luteinizing hormone, which acts through the same receptor in the traditional targets, gonads [27]. And we have shown [28] an inverse effect: gonadotropin abolished the immunostimulatory effect of LPS.

Notwithstanding the stimulatory effect of CG on the spontaneous apoptosis of neutrophils and the lack of effects of steroid hormones separately, the appropriate combination of CG with steroids inhibited apoptosis. The inhibitory effect of the hormonal combination seemed to be provided for by both the interaction of steroid hormones with each other and of each steroid with CG, and this interaction could result in both the regulation of expression of the appropriate hormone receptors (their density and affinity) and the overlapping of the hormonal signals on the intracellular level. In particular, CG is known to stimulate the synthesis and expression of progesterone receptors by the cells of gonads [29, 30], and, consequently, it can increase (or promote) their sensitivity to this steroid. No such effects are found for estradiol, but they cannot be excluded. Moreover, estradiol was earlier shown to change the immunomodulatory effects of CG for the opposite ones [11].

Note, that although the physiological combination of hormones corresponding to trimester III of pregnancy inhibited apoptosis of both intact and LPS-stimulated neutrophils, the inhibition mechanisms in these two cases were different: in the spontaneous variant they were mediated through the phosphoinositol and calcium signals and in the case of stimulation they were not mediated. Ca^{2+} is known to play an important role in the induction of apoptosis of neutrophils [31] that is also confirmed by the decrease in this parameter with the verapamil-induced inhibition of calcium channels (Fig. 1). Nevertheless, we found that the antiapoptotic effects of the hormones on neutrophils was not increased but, by contrast, abolished in the presence of verapamil (the spontaneous variant). This effect seemed to be associated

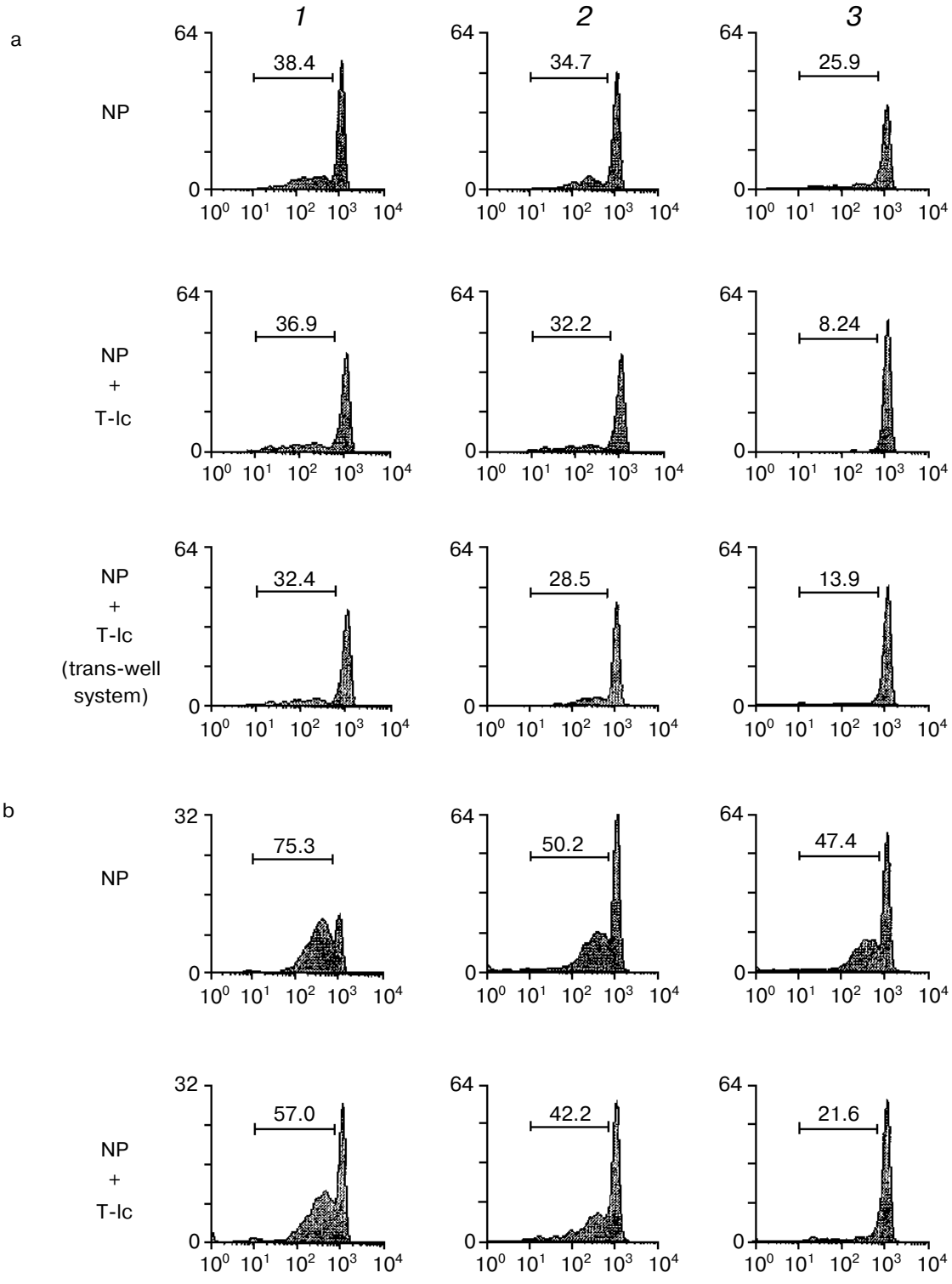

Fig. 2. Role of autologous T-lymphocytes in the hormone-dependent regulation of apoptosis of neutrophils, the spontaneous one (a) and that induced by anti-CD95 McAb (b). Here and in Fig. 3: along the abscissa axis the intensity of luminescence (arbitrary units), along the ordinate axis the number of cells; the figures show the percent of apoptotic cells in the sample; *1*) control; *2*) CG (100 IU/ml) + estradiol (1.0 ng/ml) + progesterone (20 ng/ml) (trimester I of pregnancy); *3*) CG (10 IU/ml) + estradiol (10 ng/ml) + progesterone (100 ng/ml) (trimester III of pregnancy). NP, neutrophils, T-lc, T-lymphocytes. The figure presents results of one of five similar experiments, the statistical processing of which revealed the following significant differences: a) (NP + T-lc)/control ratio was 34.7 ± 2.47, (NP + T-lc)/hormones (trimester III) ratio was 8.81 ± 2.03, $p < 0.01$; (NP + T-lc) (trans-well system)/control ratio was 31.9 ± 1.74, (NP + T-lc) (trans-well system)/hormones (trimester III) ratio was 13.3 ± 3.02, $p < 0.01$; b) (NP + T-lc)/control ratio was 68.8 ± 1.87, (NP + T-lc)/hormones (trimester III) ratio was 20.9 ± 5.45, $p < 0.05$.

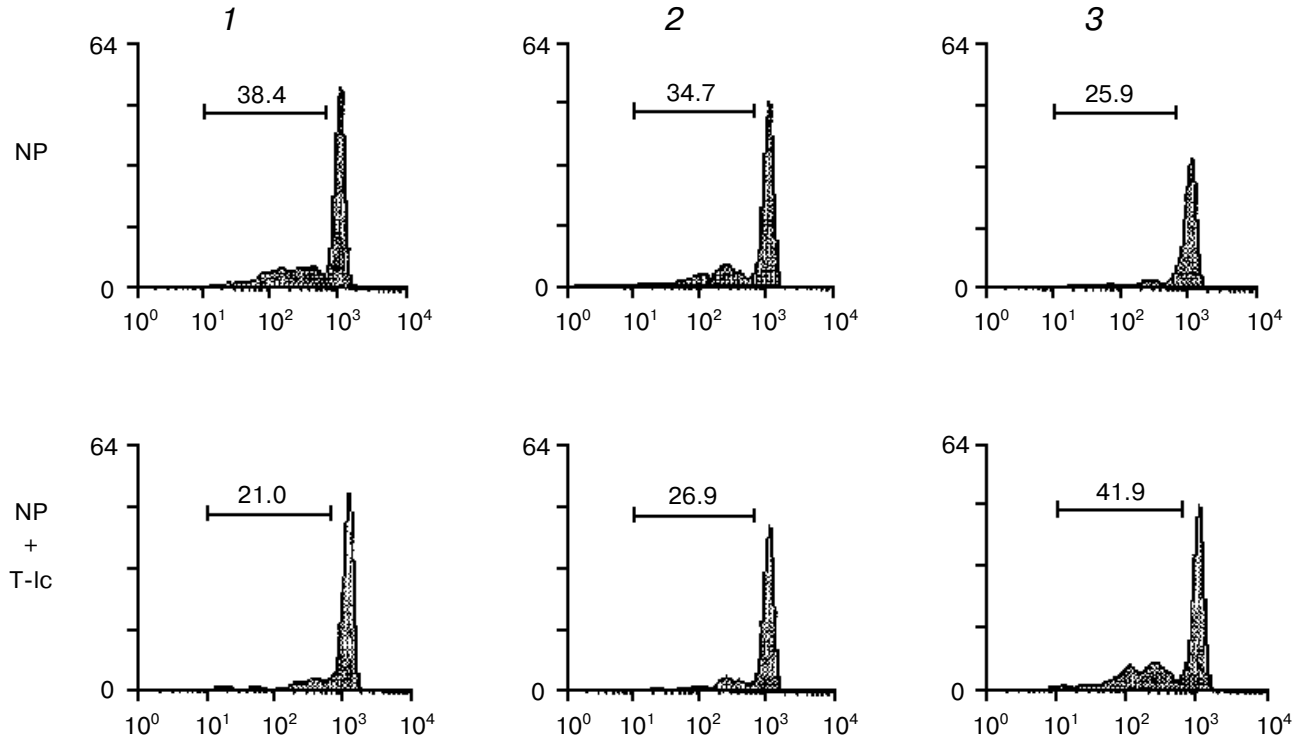

Fig. 3. Role of allogenic T-lymphocytes in the hormone-dependent regulation of the spontaneous apoptosis of neutrophils. The figure presents the results of one of three similar experiments, the statistical processing of which revealed the following significant differences: NP/control ratio was 32.1 ± 4.43, (NP + T-lc)/control ratio was 20.3 ± 2.78, $p < 0.05$; (NP + T-lc)/control ratio was 20.3 ± 2.78, (NP + T-lc)/hormones (trimester III) ratio was 37.7 ± 3.38, $p < 0.05$.

with the ability of verapamil to inhibit only the L-type of calcium channels [32], without prevention of alternative mechanisms providing for an increase in the Ca^{2+} level in the cytoplasm. Moreover, the L-type calcium channels in some cells have been shown to be controlled by hormones and directly stimulated by Gs-proteins or intracellular protein kinases [32], thus, the hormone-dependent signals can abolish or level the inhibitory effect of verapamil.

Unlike physiological conditions modeled in the spontaneous and LPS-stimulated variants, in the case of directed activation of the Fas-dependent apoptosis the reproductive hormones studied and their combinations had pronounced antiapoptotic effects.

In the presence of autologous T-lymphocytes, the inhibitory effects of the hormones on both spontaneous and anti-CD95-McAb-induced apoptosis of neutrophils were reproduced and even more pronounced. The lack of similar effects of the reproductive hormones in the presence of allogenic T-lymphocytes seemed to be associated with a significantly suppressed apoptosis under these conditions.

The inhibitory effect on apoptosis of neutrophils of the hormonal combination corresponding to trimester III of pregnancy suggests that they are the most important factors responsible for survival of these cells during trimester III of pregnancy [13]. In this connection, it should be noted that during gestation neutrophils in addition to their protective functions play an important role in the initiation of labor [33], and the hormone-dependent maintenance of viability of these cells by the end of pregnancy can be necessary just to provide for these processes. The antiapoptotic effects of the hormones studied are specific, depend on the presence and character of the neutrophil activation, and are associated with both direct effect of the hormones on the target cells and mediated by T-lymphocytes. In total, the findings suggest that the control of apoptosis is another important mechanism of the hormone-dependent regulation of neutrophils which compensates their decreased functional response [9, 10] and provides for the level of activation required for successful gestation and the body defense against infection during this period.

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