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Modulation of Structural and Functional Properties of Human Lymphocytes by Reactive Oxygen Species

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The effects of ROS on functional properties (cytotoxic activity, antibody-producing activity, TNFα synthesis, and free cytosol calcium level), membrane structure (by expression of some surface markers), and apoptosis of lymphocytic cells were estimated in the peripheral blood of healthy volunteers. $^1O_2$, $O_2^{\bullet}$, $OH^{\bullet}$, and H_2O_2 mostly suppressed cytotoxic activity of lymphocytes against Ehrlich ascites carcinoma cells and inhibited IgG synthesis and expression of receptors and surface markers (Fc receptors, CD3, CD19, and CD56). The exposure of lymphocytes to H_2O_2 (10^{-6} M), $^1O_2$, and $OH^{\bullet}$ was followed by an increase in the level of a secondary messenger, intracellular calcium, in comparison with non-exposed cells. The presence of exogenous calcium in the medium for lymphocyte suspending induced an increase in the number of cells at early and late stages of apoptosis 6 h after exposure to H_2O_2 and $^1O_2$ in comparison with lymphocytes incubated in Ca^{2+}-free medium.

Key Words: *reactive oxygen forms; lymphocytes; structural and functional properties; apoptosis; Ca^{2+}*

Lymphocytes play the main role in the development of specific immune response as they can selectively recognize self and alien antigens and initiate an active response for their elimination. Lymphocytes are directly involved in the neoplastic processes based on the recognition, neutralizing, and elimination of modified cells [10]. The search and development of targeted activation of immune response to developing tumors and enhancement of antigen properties of neoplastic cells are priority directions of modern biomedical investigations.

The role of ROS and activated oxygen metabolites (AOM) in the damage to the tissues, organs, cells, and molecules, functions of phagocytes and lymphocytes, regulation of vascular tone, carcinogenesis, inflammatory processes, ontogenesis and cell proliferation, and aging is extensively discussed [4,9,15]. Possible mechanisms of tumor-promoting effects of ROS at the second stage of carcinogenesis are studied. These mechanisms are mediated via stimulation of proliferation, changes in cell—cell interactions, stimulation of cell migration, and blockage of apoptosis [1]. Inflammation associated with intensive ROS production at the stage of alteration is a risk factor of tumor development. Considering the complex interactions between the tumor and immune cells of the host organism, essentially affected by the microenvironmental factors, including ROS, calcium ions and other mediators, model experiments focused on the analysis of specific features of functioning of immunocompetent and neoplastic cells during the exposure to these factors are needed. Elucidation of possible effects of ROS and ROM on lymphocytes and their components is needed for the analysis and understanding of the mechanisms of functioning of immunocompetent cells and regulation of their properties. This will help to develop the methods of immune correction as components of complex therapy of socially important human diseases, and to broaden modern understanding of targeted correction of functioning of organism cells during pathology and exposure to extreme environmental factors.

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Here we analyzed functional properties (cytotoxic activity, antibody-producing activity, and synthesis of TNFα, and free cytosol calcium level), structure of membranes (by the expression of surface makers), and apoptosis in human peripheral blood lymphocytes after exposure to exogenous ROS (superoxide anion radical, hydrogen peroxide, and singlet oxygen).

MATERIALS AND METHODS

Lymphocytes were isolated from the heparinized blood of volunteers, healthy men aging 18-45 years at the Branch of an Emergency Care Hospital of Voronezh Regional Blood Transfusion Station.

The lymphocytes were prepared by blood centrifugation in Ficoll–Urografin density gradient [6]. The separation of lymphocytes to T and B cells was conducted by the Terasaki method [6]. The method of direct fluorescence was used to control the purity of T- and B-cell suspensions [6]. Lymphocytes not exposed to ROS served as the control.

The following systems of ROS generation were used for the experiments: hydroxyl radical ($OH^{\bullet}$) was produced in the system of univalent H_2O_2 reduction by metal ions [14]; superoxide anion radical ($O_2^{\bullet-}$) was generated in tetramethylethylenediamine-riboflavin system under the influence of visible light [14]; singlet oxygen ($^1O_2$) was produced by exposure of methylene blue to red light (Uloks device, Voronezh Branch of the State Research and Production Company Mirotek; radiation range 630-700 nm; peak 665±15 nm; radiation intensity 20 mW/cm^2). H_2O_2 in a final concentration of 10^{-6} M was used.

Cytotoxic activity of lymphocytes against Ehrlich ascitic carcinoma cells cultured in SHK laboratory mice was estimated by the colorimetric MTT-analysis [11]. To estimate the antibody-producing activity of human lymphocytes, the method of local hemolysis was used [3]. Lymphocyte viability was evaluated using standard trypan blue exclusion test [5].

The expression of Fc receptors CD3, CD8, CD19, and CD56 on the surface of native lymphocytes and lymphocytes modified by ROS was measured by ELISA using special test-systems (Sorbent) [5]. TNFα concentration in the lysates of native and modified lymphocytes was estimated by ELISA test-systems (Proteinovyi kontur).

The intracellular level of free calcium ions was measured using fluorescent probe Fura-2AM (Sigma) on a Shimadzu-1501 spectrofluorometer [13].

The number of apoptotic cells in the lymphocyte suspension was evaluated by the flow cytofluorimetry on a CyFlow Space flow cytofluorimeter (Partec) and Annexin V-FITC Kit for apoptosis detection (Beckman Coulter).

Statistical analysis of the results was performed using StatGraphics software. Significance of differences between the study parameters in the treatment and control groups was estimated using Student's *t* test at significance level of 95% [1].

RESULTS

Cytotoxic activity of lymphocytes *in vitro* is mediated by T-cell-mediated antibody-independent and antibody-dependent mechanisms [10]. Cytotoxic $CD8^+$ T cells determine the T-cell-mediated antibody-independent mechanism. Antibody-dependent cell cytotoxicity is realized by natural killers carrying FcγRIII receptors interacting with the target cell-IgG complex.

We observed a significant decrease in cytotoxic activity against Ehrlich ascitic carcinoma cells and antibody-producing activity (IgG) of lymphocytes exposed to $OH^{\bullet}$, $O_2^{\bullet-}$, H_2O_2 (10^{-6} M), H_2O_2, and $OH^{\bullet}$ in comparison with the control (Table 1).

Analysis of changes in TNFα level (a trigger of apoptosis in tumor cells) in lymphocytes subjected to oxidative modification showed that $O_2^{\bullet-}$ and $OH^{\bullet}$ stimulated production of this cytokine, while H_2O_2 inhibited this process (Table 1).

The exposure of lymphocytes to $O_2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2 led to a decrease in the level of membrane Fc receptors, CD3, CD19, and CD56 receptors mediating cytotoxic activity in comparison with the control (Table 1).

It was found that $^1O_2^{\bullet}$, $OH^{\bullet}$, and H_2O_2 induced an increase in free calcium concentration (universal secondary messenger) in the lymphocytes suspended in Ca^{2+}-containing and Ca^{2+}-free media (Ca^{2+}-free Hanks solution) in comparison with the control (Table 1). Calcium channel blocker verapamil (10^{-6} M) induced a significant reduction in Ca^{2+} concentration in the cells exposed to $^1O_2$ and $OH^{\bullet}$ in Ca^{2+}-containing medium (to 306.0±64.0 and 339.7±34.1 nmol/liter, respectively, in comparison with cells exposed to ROS without verapamil treatment). Hence, the increase in the calcium level in the cytosol caused by ROS is determined by its release from intracellular depots, calcium entry from the medium via Ca^{2+} channels inhibited by verapamil, and impairment of structure of the plasma membranes due to LPO intensification.

The level of free Ca^{2+} in the cytoplasm is an important inducer of apoptosis in many cell types [7,12]. Calcium ions modulate the processes of proliferation of immune and tumor cells as well as effector functions of immunocytes [12].

Flow cytometry allowed assessing quantitative parameters of apoptotic death of lymphocytes after the exposure to $^1O_2$ and H_2O_2 with and without the addition of exogenous calcium to the suspension medium (Figs. 1-3).

TABLE 1. Changes in Structural and Functional Parameters of Lymphocytes Exposed to ROS

Parameter	Control	$O_2^{\bullet}$	H_2O_2	$OH^{\bullet}$	$^1O_2$
Cytotoxic activity of mixed lymphocyte population (65% T cells, 17% B cells, 18% NK) against Ehrlich ascites carcinoma cells, %	100	91.2±2.0*	68.5±0.6*	95.8±1.6*	99.7±0.8
Cytotoxic activity of T-cell suspension (87% T cells, 4% B cells, 9% NK) against Ehrlich ascites carcinoma cells, %	100	90.5±3.0*	85.5±4.3*	88.7±3.1*	93.6±1.2*
Antibody-producing activity of B cells (measured by IgG), %	100	152.0±8.9*	67.0±15.6	70.0±13.3*	100.0±20.0
TNFα production, pg/ml	50.1±0.8	60.1±0.9*	44.2±0.5*	60.7±1.2*	50.3±0.1
Expression of Fc receptors (CD16, CD32, and CD46), opt. density units	0.50±0.01	0.46±0.01*	0.41±0.01*	0.43±0.01*	0.31±0.02*
Expression of CD3 (transmits signal activating cytotoxic apparatus of T lymphocytes and NK), opt. density units	0.46±0.01	0.36±0.01*	0.34±0.02*	0.38±0.01*	0.42±0.01*
Expression of CD8 (co-receptor for T-cell receptor binding with MHC-1), opt. density units	0.38±0.01	0.40±0.01	0.40±0.02	0.38±0.01	0.39±0.01
Expression of CD19 (additional signal-transducing molecule associated with B-cell antigen-recognition receptor), opt. density units	0.50±0.01	0.39±0.02*	0.38±0.01*	0.47±0.01*	0.47±0.02
Expression of CD56 (exclusive marker of NK involved in adhesion and activation), opt. density units	0.30±0.01	0.21±0.01*	0.23±0.02*	0.26±0.01*	0.27±0.02
Intracellular Ca^{2+} concentration in lymphocytes suspended in Ca^{2+}-containing medium	121.9±15.7	—	260.0±43.1*	436.3±48.0*	521.2±61.2*
Intracellular Ca^{2+} concentration in lymphocytes suspended in Ca^{2+}-free medium	114.6±32.7	—	241.2±71.2*	226.2±20.8*	275.0±77.5*
Phosphatidylserine-positive lymphocytes at the early stage of apoptosis after 6-h incubation of modified cells in Ca^{2+}-containing medium, %	7.90	—	9.08	—	20.87
Phosphatidylserine-positive lymphocytes at the early stage of apoptosis after 6-h incubation of modified cells in Ca^{2+}-free medium, %	2.35	—	1.63	—	6.23

Note. The data are presented as *M*±CI; *$p<0.05$ in comparison with the control.

The exposure of lymphocytes, which were suspended in Ca^{2+}-containing and Ca^{2+}-free medium with the following incubation for 1 h, to H_2O_2 did not induce significant changes in the number of cells at early stage of apoptosis and late stages of apoptosis and necrosis in comparison with the control (Fig. 1). Under conditions of $^1O_2$ generation in Ca^{2+}-containing medium, a slight increase in the number of cells at the early stages of apoptosis was noted (4.12% *vs.* 0.80% in the control). Addition of H_2O_2 to the cells suspended in Ca^{2+}-containing and Ca^{2+}-free medium followed by 3 h incubation did not significantly increased the amount of cells at the early stage of apoptosis and late stages of apoptosis or necrosis in comparison with unmodified cells (Fig. 2). The number of cells at the early stage of apoptosis after exposure to $^1O_2$ in Ca^{2+}-containing medium increased to 14.14% (*vs.* 1.8% in the control). Flow cytometry of lymphocytes exposed to $^1O_2$ and H_2O_2 revealed 20.87 and 9.08% cells at the early stage of apoptosis after 6-h incubation in Ca^{2+}-containing medium (Fig. 3); in Ca^{2+}-free medium, the corresponding values were 6.23 and 1.63%. Under control conditions, this parameter was 7.90% in Ca^{2+}-containing medium and 2.35% in Ca^{2+}-free medium.

Thus, $^1O_2$, $O_2^{\bullet}$, $OH^{\bullet}$, and H_2O_2 have predominantly inhibiting effects on the majority of the studied parameters of some lymphocyte components responsible for anti-tumor defense. Cytotoxic activity of lymphocytes against the of Ehrlich ascites carcinoma cells, IgG synthesis, and expression of receptors and surface markers (Fc receptors, CD3, CD19, and CD56) decreased in comparison with the control. Oxidative modifications

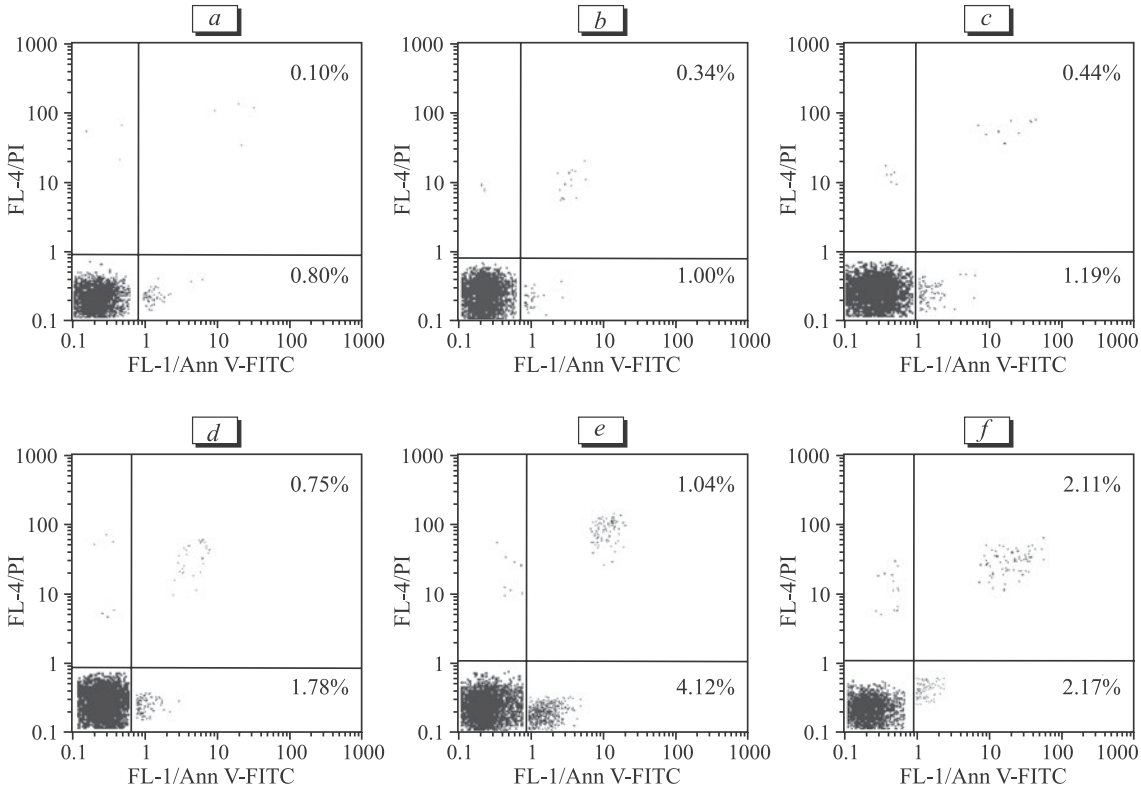

Fig. 1. Flow cytometry analysis of lymphocytes in 1 h after modification. *a*) Control in Ca^{2+}-containing medium; *b*) control in Ca^{2+}-free medium; *c*) H_2O_2 in Ca^{2+}-containing medium; *d*) H_2O_2 in Ca^{2+}-free medium; *e*) $^1O_2$ in Ca^{2+}-containing medium; *f*) $^1O_2$ in Ca^{2+}-free medium.

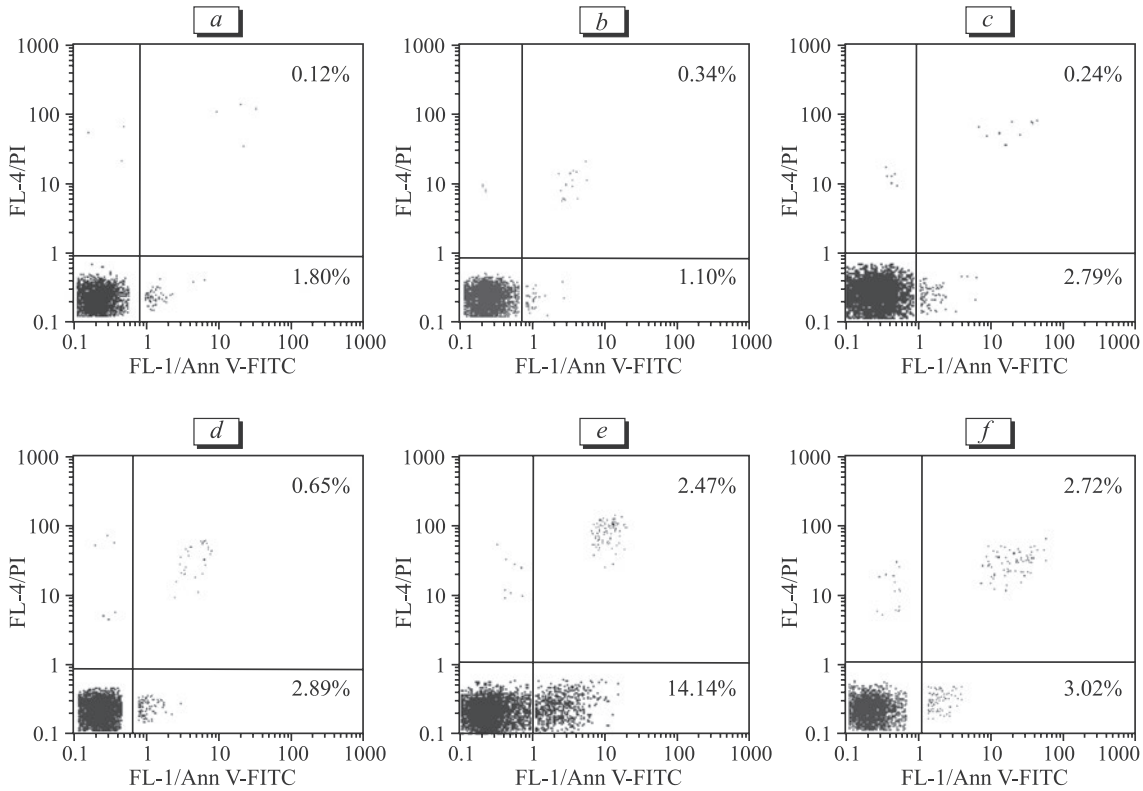

Fig. 2. Flow cytometry analysis of lymphocytes in 3 h after modification. *a*) Control in Ca^{2+}-containing medium; *b*) control in Ca^{2+}-free medium; *c*) H_2O_2 in Ca^{2+}-containing medium; *d*) H_2O_2 in Ca^{2+}-free medium; *e*) $^1O_2$ in Ca^{2+}-containing medium; *f*) $^1O_2$ in Ca^{2+}-free medium.

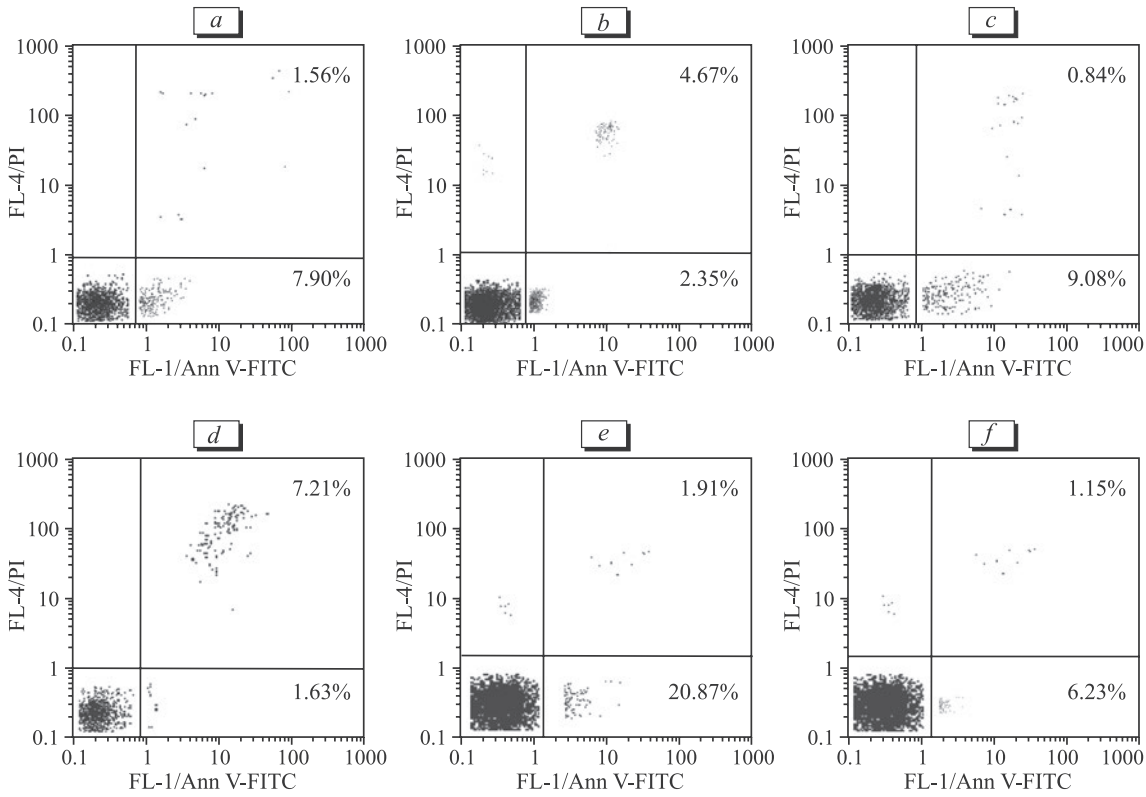

Fig. 3. Flow cytometry analysis of lymphocytes in 6 h after the modification. *a*) Control in Ca^{2+}-containing medium; *b*) control in Ca^{2+}-free medium; *c*) H_2O_2 in Ca^{2+}-containing medium; *d*) H_2O_2 in Ca^{2+}-free medium; *e*) $^1O_2$ in Ca^{2+}-containing medium; *f*) $^1O_2$ in Ca^{2+}-free medium.

of the components of lymphocyte membranes change the expression of receptors and secondary messengers, modify functional properties, and, finally, lead to death of the studied cells. Significant increase in the intracellular concentration of free Ca^{2+} that depends on the level of exogenous calcium observed after exposure of lymphocytes to ROS triggers changes in functional properties of lymphocytes and induces their apoptotic death. The presence of calcium in the medium for lymphocyte suspension during exogenous generation of ROS accelerates apoptotic death of these cells.

Taking into account the fact that certain stages of tumor development are associated with significant intensification of free radical processes [8], it can be suggested that ROS along with problastomic factors suppressing the immunity and accelerating tumor growth inhibit the functions of lymphocytes in the pathological focus. Probably, immunocytes should be protected from oxidative stress under these conditions.

The relative resistance of the study parameters of structural and functional properties of lymphocytes (cytotoxic activity, antibody-producing activity, TNFα level, and expression of CD3, CD19, and CD56) to singlet oxygen observed in our study can be used in the analysis of the mechanisms of photodynamic therapy of tumors, because it can promote stable functioning of immunocytes under conditions of $^1O_2$ generation in the pathological focus.

The obtained data enlarge modern understanding on the changes in structural and functional parameters of human peripheral blood lymphocytes indicating their involvement to the anti-tumor immunity during the exposure to ROS. These results should be taken into account for the development of correction methods for human immunocompetent cell impairments during malignant tumors and pathologies associated with the intensification of free radical processes.

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