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Study of interaction between the polyoxidonium immunomodulator and the human immune system cells

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Abstract

Polyoxidonium (PO) is a high-molecular weight physiologically active compound with pronounced immunomodulating activity, an N-oxidized polyethylene–piperazine derivative. The aim of our work was to study cellular and molecular mechanisms of the action of PO on the human peripheral blood leukocytes. By means of flow cytometry it was established that the binding of fluorescein-isothiocyanate-labeled PO (FITC-labeled PO) occurs more rapidly with monocytes and neutrophils than with lymphocytes (7- to 8-fold weaker as compared with monocytes). Using colloidal gold-labeled PO and electron microscopy it was shown with that the preparation penetrates into leukocytes by endocytosis. PO is localized in endoplasmic vesicles of cellular cytosol. Analysis of one of the crucial signal transducer, the intracellular Ca^{2+}, performed with the Fluo-3 fluorescent dye, showed that PO does not induce Ca^{2+} mobilization from the intracellular calcium stores and influx of extracellular Ca^{2+}. The study of the intracellular hydrogen peroxide (H_2O_2) production with the 2′,7′-dichlorfluorescein indicator demonstrated that PO significantly increases the level of intracellular H_2O_2 in monocytes and neutrophils, however, this increase is much less as compared with phorbol myristate acetate stimulation. The analysis of immunomodulating effect produced by PO proved its stimulating activity on some cytokines production in vitro, e.g. interleukin 1β (IL-1β), tumor necrosis factor (TNF)-α and IL-6. A dose-dependent increase in the intracellular killing by blood phagocytes was established under the action of PO.

Keywords: Immunomodulator; Polyoxidonium; Endocytosis; Intracellular Ca^{2+}; Hydrogen peroxide

1. Introduction

Polyoxidonium (PO) is an immunomodulating preparation that is successfully used in Russia to treat chronic recurrent secondary immunodeficiency states [1] resistant to adequate etiotropic therapy. PO

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is also used in therapy of acute infectious processes due to its combination of immunomodulating effect with detoxicating, antioxidant and membrane-protecting activities. PO is a non-toxic, water-soluble, biodegradable synthetic polymer. As to its chemical structure, it is a copolymer of N-oxidized 1,4-ethylenepiperazine and (*N*-carboxyethyl)-1,4-ethylenepiperazine bromide with a molecular weight of 60–100 kDa.

In this paper, the effect of PO on the human peripheral blood leukocytes is studied in vitro. More specifically, the aim of this work was to study the degree of the PO binding with the human peripheral blood cells depending on the dose and time as well as to consider activation of target cells under the effect of this immunomodulator. We used such minimal experimental dose as 1 μg/ml, which corresponds the minimal therapeutic dose (6 mg per injection) of PO.

2. Materials and methods

2.1. Reagents

Polyoxidonium was obtained from National Research Center "Institute of Immunology", Immafarma (Moscow, Russia). Colloidal gold $HAuCl_4$, 0.01%; 10 nm, osmium tetroxide, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCF-DA), Hanks' balanced salts (without phenol red and sodium bicarbonate), RPMI-1640, L-glutamine, saponin, propidium iodide (PI) were products from Sigma (St. Louis, MO, USA). Ficoll-Hypaque, gentamycin were purchased from Pharmacia (Piscataway, NJ, USA). Fluorescein-5-isothyocyanate (FITC, isomer I), Fluo-3 AM fluorescent dye was obtained from Molecular probes (Eugene, OR, USA).

2.2. Leukocyte isolation on gelatin

Leukocyte suspension was obtained from venous heparinized donors' blood after spontaneous sedimentation of erythrocytes in a 1% gelatine solution. The leukocytes were then twice washed in phosphate-buffered saline (PBS, pH 7.4).

2.3. Labeling of PO with fluorescein-5-isothyocyanate

Conjugation of PO was performed as described by Goding [2] in a 0.1 M sodium bicarbonate solution. After that, the FITC–PO preparation was lyophilized. The conjugate obtained had a PO-to-FITC ratio of 1 mg/20 μg.

2.4. Evaluation of leukocytes interaction with PO

The white blood cell suspension (2 Mln/ml) was incubated at a temperature of 37 °C with FITC–PO at five different concentrations (100, 200, 500, 1000, 2000 μg/ml) for 1 to 5 h. The reaction was performed in a 96-well round-bottom dishes in a volume of 200 μl. Non-bound FITC–PO was washed out with PBS. Monocytes in cell suspension were labeled with MAb directed against CD14 (human CD14 R-PE; Caltag laboratories, Burlingame, CA, USA) at 4 °C for 30 min. The samples were analyzed using a FACScalibur flow cytometer (Becton Dickinson) equipped with argon laser (λ=488 nm) and CellQuest software. Three cell clusters, such as monocytes, neutrophils, and lymphocytes, were distinguished in the Dot Plot window according to two parameters (forward scatter signal, FCS, and side scatter signal, SSC). The mean geometric fluorescence intensity (GeoMean) was analyzed for either of them in the FL-1 channel.

2.5. Preparation of samples for electron microscope evaluation

A complex of colloidal gold and PO (Au–PO) was used (200 μg/ml PO, 0.01% colloidal gold, $HAuCl_4$: 30 Au ions per one PO molecule). The leukocyte suspension (6 Mln/ml) was incubated with Au–PO for 60 min at 37 °C (ratio 1:1). The reaction was performed in cytometric tubes (Falcon, Becton Dickinson) in a 2 ml volume. The cells were fixed in 2 ml 2.5% glutaraldehyde (Merck, Darmstadt, Germany) in PBS for 2 h at 4 °C. After that, it was replaced by 2 ml 1% osmium tetroxide solution, the rest of incubation terms in the cold being retained. The material was dehydrated by following changes of 40%, 60%, 70%, 80%, 96% and 100% ethyl alcohol (4 °C, 30 min each). The last incubation before embedding was made in 3 acetone changes for 30 min. The specimen were embedded in Epon 812 (Polyscience, USA)

according to a standard procedure. For some time, the material was kept in a clean embedding medium and was placed into embedding vessels. Polymerization was performed for 24 h at 37 °C and for 48 h at 60 °C. Ultrathin sections were cut with an LKB-IV ultratom (LKB, Sweden), they were stained with aqueous uranyl acetate and lead citrate and were examined using a Jeol-100B electron microscope (Jeol, Japan).

2.6. Evaluation of intracellular calcium production by leukocytes

The production of intracellular calcium (Ca^{2+}) by human peripheral blood leukocytes was examined by means of flow cytometry using a Fluo-3 fluorescent dye [3]. To assess the effect of PO on the intracellular Ca^{2+}, 100 μl of leukocytes suspension was used (2 Mln/ml). The reaction was performed in cytometric tubes. Leukocytes were incubated with 3 μM Fluo-3 AM for 30 min at 37 °C. After the incubation, the leukocytes were twice washed in PBS. The second incubation was conducted in 500 μl warm Hanks' balanced salts for 30 min at 37 °C. A classic stimulator of intracellular Ca^{2+}, calcium ionophore A23187 (1 μg/ml), was used as a criterion of system functioning. This stimulator, like PO (1 and 100 μg/ml), was added to specimen at the moment of analysis that was performed with flow cytometer for 10 min at 37 °C. The Dot Plot window was displayed in two parameters—FL1 and time (s). Detection of spontaneous Ca^{2+} production was done for 2.5 min, then, PO or calcium ionophore A23187 were added and stimulated intracellular Ca^{2+} production by neutrophils and lymphocytes was studied for the following 8 min.

2.7. Evaluation of intracellular H_2O_2 production

Intracellular production of hydrogen peroxide (H_2O_2) was measured using DCF-DA [4,5]. The reaction was performed in 96-well round-bottom culture dishes. Leukocytes were incubated with 5 μM DCF-DA with 5 mM sodium azide added at 37 °C for 20 min. After it, equivalent doses of PBS with various doses of PO (1; 10 and 100 μg/ml) were added, in control samples only PBS was added. The mixture was incubated with PO at 37 °C for 60 min. As a criterion of the system functioning, PMA, a classic stimulator of phagocytes, was used. It was added at a dose of 100 ng/ml 30 min prior to the end of incubation. The samples were analyzed by flow cytometry. The intensity of DCF fluorescence (the GeoMean value) which produced in this reaction and emitting in the green spectral region was measured.

2.8. Evaluation of IL-6 production

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized donors' blood using Ficoll-Hypaque (ρ=1.077) gradient centrifugation as described Bouym [6]. PBMC were adjusted to the concentration of 2 Mln/ml by using a complete cultural medium (RPMI-1640, containing 10% heat-inactivated fetal calf serum (ICN Biomedicals, USA), 2 mM L-glutamine and 40 μg/ml gentamycin. PO was added to the PBMC in the cultural medium at concentrations of 1, 10 and 100 μg/ml. In control samples a complete cultural medium was added. The mixtures were incubated at 37 °C for 48 h in the presence of 5% CO_2 in air. The IL-6 production was assessed in supernatants of PBMC by means of ELISA using a commercial IL-6 test system produced by CytImmune (Maryland, USA).

2.9. Evaluation of intracellular bacteria killing

Reaction was performed using FITC conjugated *Staphylococcus aureus* [7]. The reaction was performed in 96-well round-bottom culture dishes (200 μl/well). A 90 μl leukocyte suspension (2 Mln/ml), 90 μl *S. aureus* (10 Mln/ml), and 20 μl pooled donor serum were incubated for 20 min at 37 C. To remove unbound bacteria, leukocytes were centrifuged at 200×*g* for 1 min at +4 C and twice washed with PBS. After it, leukocytes were re-suspended in 200 μl PBS with polyoxidonium at various doses (only PBS was added to the control well) and incubated at 37 C for 60 min. Leukocytes were centrifuged and re-suspended in 200 μl 0.2% saponin solution in 10 mM carbonate–bicarbonate buffer (pH 9.5). The released bacteria were centrifuged at 1000×*g* for 10 min and re-suspended in 200 μl PBS with 2.5 μg/ml PI. After 10 min, the samples were analyzed by flow cytometer. The percentage of double positive bacteria ($FITC^+PI^+$) among FITC-labeled bacteria ($FITC^+$) was determined.

2.10. Statistical analysis

Data has been presented as mean±standard error of the mean (S.E.M.). Statistical significance between the control and treatment groups was determined by Student's *t*-test. A *p* value of <0.05 was considered significant.

3. Results

3.1. Evaluation of PO interaction with the peripheral blood leukocytes by means of flow cytometry

Using FITC–PO, it was established that the PO immunomodulator interacts with all leukocytes populations, however the degree of this interaction varies. The GeoMean value of FITC–PO was measured that reflects the degree of PO-to-cell interaction for 5 h in 1-h intervals with various PO concentrations (ranging from 100 to 2000 μg/ml). By the 3rd incubation hour the increase in the FITC–PO level (from 100 to 2000 μg/ml) caused an increase in the FITC–PO fluorescence intensity of monocytes from 141±26.4 to 474.3±75.7 relative units ($p<0.01$) (Fig. 1). The GeoMean level of neutrophils was lower by 10–20% (109.1±21.3 and 386.5±74.8 relative units, respectively, $p<0.01$). As for lymphocytes, the fluorescence intensity was 7- to 8-fold lower in comparison with monocytes and 6- to 7-fold lower in comparison with neutrophils (16.4±2.5 and 65.0±13.6, respectively, $p<0.05$).

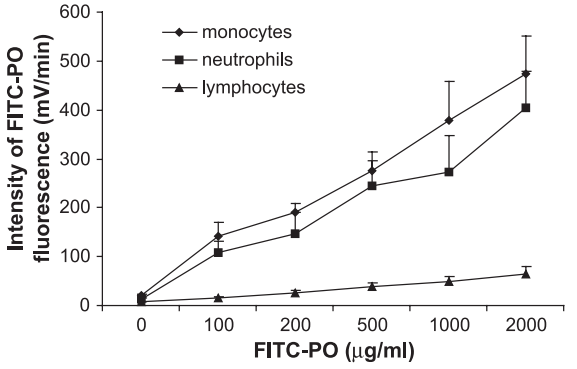

Fig. 1. The "dose–effect" dependence of the FITC–PO fluorescence intensity on leukocytes. The leukocytes were incubated with 100–2000 μg/ml FITC–PO for 3 h (N=10). The samples were analyzed using flow cytometer. Values represent mean±S.E.M. of GeoMean of FITC–PO fluorescence.

Evaluation of the FITC–PO to leukocytes interaction in the course of time showed that within the first 3 h of incubation the FITC–PO fluorescence increased for all three leukocytes populations with all PO concentrations under study. By the 4th and 5th h, the GeoMean level did not change practically (data not shown).

3.2. Electron microscope study of the PO penetration into cells

While studying the possibility for PO which molecules are bound in a complex with colloidal gold (Au–PO) to penetrate cells by electron microscopy, it was established that the Au–PO complex can reside at the phagocytes (neutrophils and monocytes) surface or can penetrate through the cell membrane. Intracellular localization of Au–PO is found in small vesicles with a single membrane (Fig. 2). The number of Au particles in the vesicle varies from 3–4 to 8. In most cases, gold-containing vesicles were located near the nucleus. We could fix the moment of capturing a chain of colloidal gold particles by a neutrophil from the cell surface (Fig. 3). The particles of colloidal gold were also found at lymphocytes surface, however no intracellular localization of Au–PO was noted in this case.

3.3. Study of the PO effect on the level of secondary messengers, intracellular Ca^{2+} and H_2O_2

It is known that different substances can stimulate the initiation of activation pathways cascade. As a result, functional rearrangement occurs: the cell enters a new phase of the cell cycle, changes of secretory activity and expression of surface molecules, etc. [8–10]. The action of the activating agent on the leukocytes lead to mobilizing Ca^{2+} with participation of inositol triphosphates [11,12]. Cell activation can be also provided by Ca^{2+}-independent way [13,14].

In this study, the PO effect on the intracellular Ca^{2+} mobilization was investigated by flow cytometry. For this purpose, the Fluo-3 fluorescent dye was used. The principle of identification of intracellular Ca^{2+} is based on the capture of Fluo-3 by the acetoxymethyl

ether cell with further splitting of ether by cytoplasmic esterases thus causing formation of Ca^{2+}-sensitive dye. Our study of the PO effect on the intracellular calcium production showed that this immunomodulator does not cause activation of Ca^{2+} mobilization from the internal stores and outer media in presence of extracellular Ca^{2+} (data not shown). These experiments were repeated at least 10 times.

Recent publications [15–19] report on the involvement of intracellular reactive oxygen species, such as hydrogen peroxide (H_2O_2), into processes of cell activation as secondary messengers. To detect reactive oxygen species in cells, various fluorochromes are used. Their fluorescent features become evident only when they interact with these radicals directly in cell cytoplasm. As the intracellular H_2O_2 indicator, we used DFC-DA that initially did not have fluorescent characteristics. After passive diffusion of fluorochrome into the cell, hydrolysis of diacetate group occurs under the effect of intracellular esterases. In presence of peroxidase, DFC is oxidized by H_2O_2 and becomes fluorescent [20]. It was shown by flow cytometry that PO augments the fluorescence intensity (GeoMean) after a 1-h incubation in all leukocytes, lymphocytes including (Fig. 4). At a dose of 100 μg/ml, PO showed a statistically significant increase in the level of DFC fluorescence with respect to neutrophils and monocytes. Thus, the GeoMean value of control neutrophils was 91.5±25.8, while in presence of PO it was 120.9±43.7 ($p<0.01$); the same was true for monocytes (25.9±8.9 and 30.9±13.2, respectively, $p<0.01$). On average, the H_2O_2 produc-

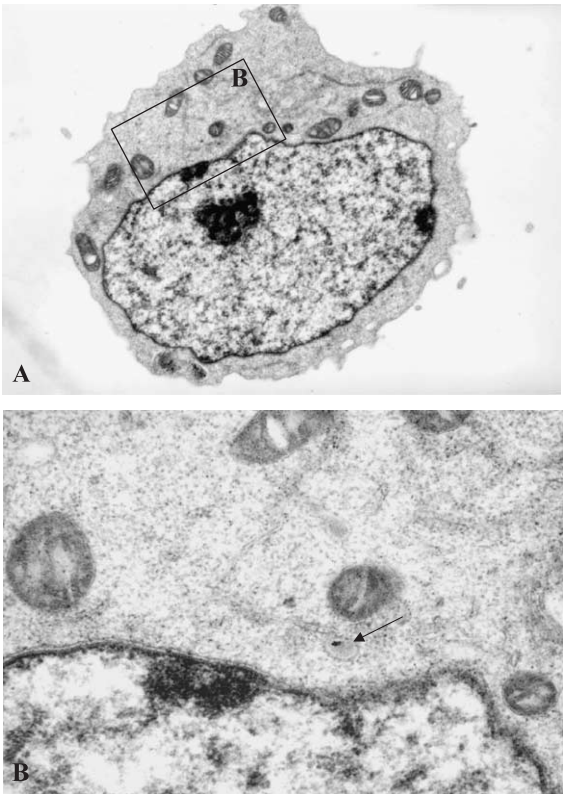

Fig. 2. An Au–PO vesicle in a monocyte. Electron microscopy. Leukocytes were incubated with Au–PO in PBS for 1 h at 37 °C. (A) Phagocyte with a PO-containing vesicle labeled with colloidal gold, magnification ×10,000; (B) Enlarged electron microscopic feature of the monocyte (A): the vesicle is labeled with as arrow; magnification ×30,000.

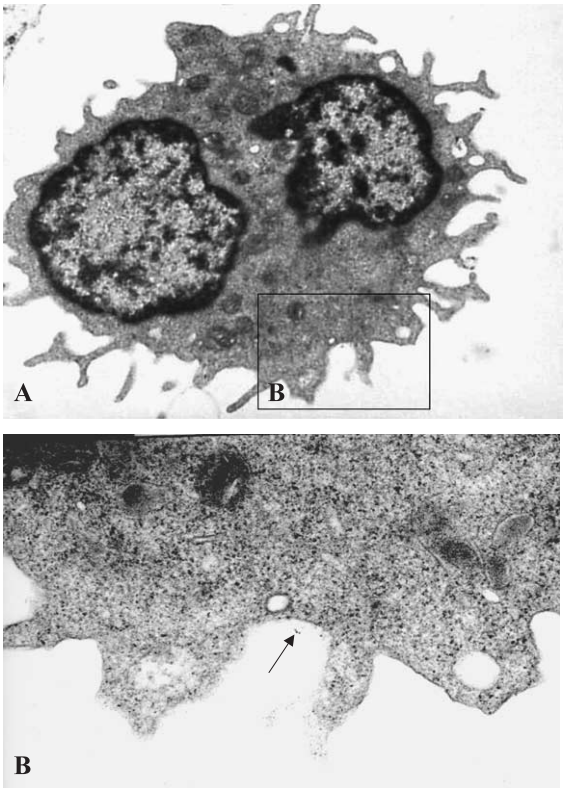

Fig. 3. Capture of the Au–PO complex by a peripheral blood phagocyte. Electron microscopy. Leukocytes were incubated with Au–PO in PBS for 1 h at 37 °C. (A) Neutrophil, magnification ×10,000; (B) Enlarged neutrophil (A): the colloidal gold particles are shown with arrows, magnification ×25,000.

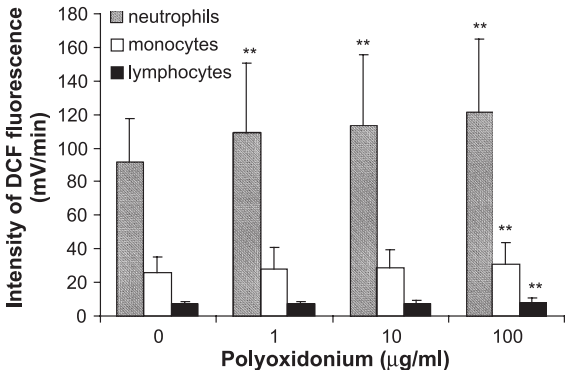

Fig. 4. The effect of PO on the synthesis of intracellular H_2O_2 evaluated by the DCF fluorescence (GeoMean) (N=30). Leukocytes were incubated with 5 μM DFC-DA for 20 min in PBS, followed by a 1-h incubation with 1–100 μg/ml PO at 37 °C. The samples were analyzed using flow cytometer. Values represent mean±S.E.M. Significance level: *p<0.05 compared with the control (without PO), **p<0.01 compared with the control.

tion by neutrophils increased by 20–32%, that by monocytes by 11–19%; as to lymphocytes, an increase was also observed (by 15% at a dose of 100 μg/ml, p<0.01).

3.4. The effect of PO on the production of proinflammatory cytokines

To study the immunomodulating activity of PO, we investigated its effect on the production of several proinflammatory cytokines: IL-1β, tumor necrosis factor (TNF)-α and IL-6. It was shown that PO increases these cytokines production by mononuclear blood cells in a dose-dependent manner. The data obtained with IL-6 were most stable and reproducible. A 1.2- to 3-fold increase in the IL-6 synthesis was observed depending on the PO concentration used (Table 1); p<0.05 at a dose of 100 μg/ml.

Table 1
Stimulating effect of PO on IL-6 synthesis (pg/ml) by mononuclear cells

Cytokine	Polyoxidonium, μg/ml			
	None	1	10	100
IL-6	131±71	161±68	190±113	259±155*

PBMC were isolated using Ficoll-Hypaque gradient centrifugation and incubated with and without 1–100 μg/ml PO in a complete culture medium for 48 h. The level of IL-6 production was assessed in the supernatant of PBMC by ELISA (N=7). Data represent mean±S.E.M.

* Significance level: p<0.05 compared with the control (without PO).

Table 2
The killing level (%, mean±S.E.M.) in leukocyte suspension obtained from donors and patients with chronic granulomatous disease in the presence of polyoxidonium

	Control	Polyoxidonium, μg/ml		
		100	250	500
Donors	33.9±7.3	34.5±8.8	48.7±10.2**	59.3±6.8**
CGD	12.5±4.2	15.6±3.6	22.0±2.4**	36.8±5.2**

Leukocytes (2 Mln/ml) were incubated with *S. aureus* (10 Mln/ml) and pooled donor serum for 20 min at 37 °C. After it, leukocytes were twice washed with PBS and re-suspended in PBS with polyoxidonium at various doses (only PBS was added to the control well) and incubated at 37 °C for 60 min. After it, leukocytes were precipitated and resuspended in 0.2% saponin solution. The released bacteria were precipitated and resuspended in PBS with 2.5 μg/ml PI. Samples were analyzed using flow cytometer.

** Significance level: p<0.01 compared with the control (PBS).

3.5. The effect of PO on the phagocytic activity of the peripheral blood phagocytes

The ability of PO to affect the intracellular killing of some bacteria was studied, e.g. *S. aureus*. It was demonstrated that after a 1-h incubation of PO with *S. aureus*-loaded leukocytes, the percentage of the killed bacteria increases by 44% at a dose of 250 μg/ml, and by 75% at a dose of 500 μg/ml (Table 2). In other words, PO stimulates phagocytic bactericidal activity in a dose-dependent character. Bactericidal activity was also analyzed with phagocytes obtained from patients with chronic granulomatous disease (CGD). It was established that the ability of PO to increase the efficiency of microbe killing becomes even more apparent with phagocytes from CGD patients. If taking into account the extremely low initial bactericidal level in CGD, these values even reach normal levels when PO is added at doses of 250 and 500 μg/ml (Table 2). Cytometric analysis of samples obtained from a CGD patient (histogram in Fig. 3) shows that the level of propidium iodide-positive bacteria (that is, killed bacteria) increases from the initial 9% to 24% under the effect of PO.

4. Discussion

By flow cytometry and FITC-labeled PO it was demonstrated that PO binding to monocytes and neutrophils occurs more intensively than that to lymphocytes. Fig. 1 shows that the PO-to-cells interaction was of a linear dose-dependent character. Of note, statistical analysis revealed differences in the PO binding to cells of different donors. The cause for such data scattering may be individual sensitivity to the immunomodulator. The study of the FITC–PO binding to cells in the course of time showed an increase in the GeoMean value by the 3rd hour, thus evidencing that the PO-to-cells interaction occurs during the first hours and reaches its saturation level.

After 1-h incubation of leukocytes with colloidal gold-labeled PO, vesicles that contain Au–PO could be detected in cytoplasm of neutrophils and monocytes. More often, they are situated close to the nucleus. It may presumably be evidence of PO transportation to the nucleus. Large clusters of surface Au–PO were seen on phagocytic cells and on lymphocytes. We failed to detect the above-mentioned vesicles in lymphocytes. It may testify to the fact that PO does not penetrate into lymphocytes. If it still does, than to a very small extent. It could be speculated that PO enters leukocytes by an active pathway that is by endocytosis.

Following PO interaction and its penetration into the immune system cells a Ca^{2+}-independent cell activation takes place. We showed that PO did not cause an increase in the level of non-bound intracellular Ca^{2+} either by its mobilization from the intracellular compartments, or by its recruitment from outside.

We evaluated the DFC fluorescence intensity by flow cytometry. Evaluating its level, it is possible to assess the intensity of H_2O_2 production, which is a secondary messenger and mediator of intracellular signaling pathways [21–23]. Further study showed that PO stimulates the intracellular H_2O_2 production in a dose-dependent manner. Compared with PMA, the classic inductor of the respiratory burst [11,24,25], which caused a 10-fold increase in the fluorescence intensity, PO-induced elevation of the GeoMean value was not so high (Fig. 5). We believe that some increase in the synthesis of intracellular H_2O_2 after a 1-h incubation with PO is sufficient to initiate activation pathways of leukocytes, in spite of the fact

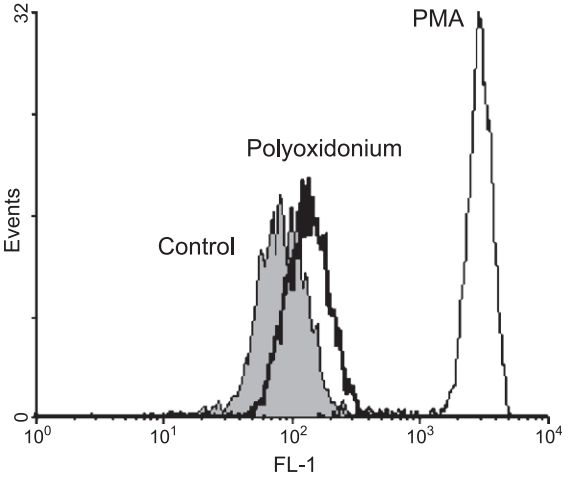

Fig. 5. DCF test cytofluorogram. The effect of PO on the level of intracellular H_2O_2 in donors' peripheral blood neutrophils. Leukocytes were incubated with 100 μg/ml PO for 1 h at 37 °C and with 100 μg/ml PMA for 30 min. The samples were analyzed using flow cytometer.

that this increase is not comparable with in the respiratory burst.

Recent publications [15,19,26] on the production of reactive oxygen species show that a small increase in the H_2O_2 level is crucial in the activation of some signaling molecules and its biological impact is of not less value than the phenomenon of the respiratory burst. The role of H_2O_2 is proved in the activation of the NF-κB nuclear transcription factor [18,27–29]. NF-κB regulates gene transcription, the products of which are responsible for the development of inflammation and immune response, NO synthesis, viral replication, intercellular interaction, proliferation, apoptosis, etc. [29].

As a result of PO-induced cell activation, some processes are activated that transfer the cell to another functioning level. It is shown in stimulation of proinflammatory cytokines production by mononuclear blood cells and augmentation of intracellular killing of bacteria by phagocytic cells.

Using the typical ELISA method, the stimulating effect of PO on the production of proinflammatory cytokines (IL-1β, TNF-α and IL-6) was detected. It is known that IL-6 is the regulator of some proinflammatory cytokines, such as IL-1β and TNF-α, by inhibiting their production; that is, IL-6 is an anti-inflammatory cytokine [30]. That is why stimulation

of IL-6 synthesis by PO can reflect its modulating activity. Thus, the PO effect on the synthesis of pro- and anti-inflammatory cytokines can be regarded as having immunomodulating character.

It is well known that the main feature of the functional activity of phagocytes is the killing of bacteria ingested bacteria. Our study in a group of donors showed that PO increases the intracellular killing of *S. aureus* after a 1-h incubation with the peripheral blood leukocytes. It was also revealed that PO restores bactericidal activity of leukocytes obtained from CGD patients up to the normal level. The activation mechanism of bactericidal function may be O_2-independent bactericidal mechanism, as the genetic defect in leukocytic NADPH-oxidase causes a practically absolute absence of reactive oxygen species production [31,32] in CGD patients. We hypothesize that stimulation of intracellular killing of Staphylococcus may be connected with activation of NO-dependent pathway of bacteria death. NO is of crucial value for the killing of *Mycobacterium tuberculosis*, *Plasmodium falciparum* and some other pathogenic microorganisms [33,34].

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