Effect of "Profetal" on Differentiation and Functional Activity of Human Mononuclear Leukocytes

O. V. Lebedinskaya, N. P. Velizheva*, F. V. Donenko*, V. A. Chereshnev, S. Yu. Rodionov**, N. K. Akhmatova*, I. Zh. Shubina*, E. A. Lebedinskaya, and M. V. Kiselevskii***

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The effect of "Profetal", a preparation containing human α-fetoprotein, on proliferative activity and differentiation of mononuclear leukocytes isolated from donor peripheral blood was studied in cultures with serum-free media. The results suggest that human α-fetoprotein in effective doses stimulates proliferation and blast transformation of cultured peripheral blood mononuclear leukocytes with the formation of $CD34^+/CD45^+$ hemopoietic precursor cells. This phenomenon of *ex vivo* generation of hemopoietic precursor cells using human α-fetoprotein can be important for clinical oncology, specifically, for the development of adaptive cell therapies.

Key Words: *α-fetoprotein; "Profetal"; mononuclear leukocytes; precursor cells*

The findings of Russian and foreign studies suggest that α-fetoprotein (AFP) is a fine regulator of homeostasis in health and disease [3,11,12]. The functions of AFP have been studied since the middle of the last century [14,17], but its immunoregulatory activity remains not quite clear. AFP acts as a growth regulator, biological response modifier, and as a factor transporting serum ligands, such as steroid hormones, folic acid, fatty acids, bilirubin, and metal ions [27-29]. Depending on cell activation, AFP stimulates or suppresses certain clones. Recent studies showed that some AFP conformations can function as double regulators of growth (stimulators or inhibitors) [23,28]. AFP stimulates the growth, differentiation, and proliferation of fetal fibroblasts [18]. The effects of AFP are realized at the level of complex regulation of cell proliferation, apoptosis mechanisms triggering, energy and plastic material supply to cells, induction of regulatory signals through intensification of receptor expression and provision of synthesis of prostaglandins, thromboxanes, and leukotrienes, interactions with the extracellular matrix structures, and immunomodulating effects [2,10,12,13,22,25].

Many data on immunomodulating effects of AFP are contradictory. According to some reports, it inhibits tmaturation of dendritic cells (DC), which is seen from reduced expression of costimulatory molecules on the surface of cell membranes [28]. According to other reports [35], AFP stimulates DC apoptosis. However, the capacity to stimulate apoptosis is a sign of functional maturity of cells, including dendritic ones, whose maturation in cultures, according to our data, is induced under the effect of definite doses of AFP [9].

Suppressive effect of AFP on lymphokine-activated killers (LAK), generated *in vitro* during incubation of blood mononuclear leukocytes (ML) with interleukin-2 (IL-2) was described [28]. On the other hand, we noted that AFP in certain doses stimulated the cytotoxic activity of ML towards K562 tumor cells and was a mitogenic factor for

Perm State Medical Academy, Ministry of Health of the Russian Federation, Perm; *N. N. Blokhin National Oncological Research Center, Russian Academy of Medical Sciences, Moscow; **Institute of Immunology and Physiology, Ural Division of Russian Academy of Sciences, Ekaterinburg. ***Address for correspondence:* lebedinska@mail.ru. O. V. Lebedinskaya

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donor blood ML, capable of inducing their blast transformation [5,6,8,9].

The data on the possibility of liver cell dedifferentiation under the effect of human AFP with expression of AFP and CD34 receptors deserve special interest. Cells with fetal phenotype were detected in the liver during tumor growth. The appearance of these cells is attributed to hyperexpression of AFP on hepatocytes [19,20,26,32]. Two subpopulations of stem cells are detected in the liver of experimental animals in this case. One of these subpopulations consists of hemopoietic cells ($CD34^+$, $CD45^+$), while the other is qualified as oval cells or hepatoblast type cells ($CD34^+$, $CK19^+$, AFP^+). $CD34^+$ cells are not present in intact liver of adults, but are detected in fetal liver. The majority of these cells have the characteristics of hemopoietic stem cells expressing CD34 and AFP markers on their membranes during long-term culturing of fetal hepatocytes. The authors hypothesised that during tumor growth $CD34^+$ cells were recruited into the liver from the bone marrow for tumor angiogenesis, and are subsequently differentiated under the effect of microenvironment of the mesenchymal origin. On the other hand, dedifferentiation of cells was hypothesized [20]. AFP plays an important role in these processes; it can act as a biological modifier and growth factor for stem and tumor cells. A relationship between AFP level, erythropoiesis activity, and erythropoietin synthesis was demonstrated [15], AFP functioning as a regulating factor [16].

The structure of surface receptors to AFP is actively studied [33,36,39]. The available data provided the basis for the concept of autocrine AFP/AFP-receptor-mediated system of growth regulation, demonstrated for human monocytes, T-lymphocytes, and tumor cell strains [33,34,37,38].

Hence, indirect evidence of AFP potentiality to induce blast transformation of mature lymphocytes was reported.

Complete culture medium with fetal calf serum (FCS) containing fetal proteins was used in our previous studies of the functional and immunophenotypical characteristics of human peripheral blood ML activated by AFP (the main active component of "Profetal", PFT) [6,8,10].

Now we studied the effect of PFT, containing human AFP, on the proliferative activity and immunophenotype of donor peripheral blood ML cultured in serum-free media.

MATERIALS AND METHODS

Isolation of mononuclear leukocytes. ML were isolated from heparin-stabilized (25 U/ml) peripheral blood of 30 donors by centrifugation (400*g*, 30 min) in a single-step Ficoll gradient (1.077 g/cm^3; Pharmacia). Mononuclear cells forming the interphase ring were collected and washed 3 times in medium 199 (Institute of Poliomyelitis and Viral Encephalitis, Russian Academy of Medical Sciences); after each washing in 10-fold volume of the medium the cells were precipitated by centrifugation at 200*g*.

Preparations. The main component of "Profetal" (Institute of New Medical Technologies) is lyophilized and dextrane-stabilized human AFP. The doses of 0.001, 0.01, 0.1, 1.0, and 10.0 μg/ml were studied previously [5,6,8]. The concentrations of 0.01-10.0 μg/ml were found to be optimal for stimulation of mitotic activity, their effect on peripheral blood ML being almost the same. We therefore used the optimal doses of the preparation (0.1, 1.0, and 2.0 μg/ml).

Generation of PFT-activated mononuclear leukocytes. ML isolated from the peripheral blood were resuspended in RPMI 1640 (ICN) with 10% FCS, 2 mM glutamine, streptomycin with penicillin (5000 U/ml each, in a concentration of 1×10^6 ml medium); PFT (2 μg/ml) was then added. The cells were incubated at 4.5% CO_2 and 37°C for 4 days.

Analysis of ML phenotype. The phenotype of generated cells was studied using monoclonal antibodies (Catlag Laboratories) to the corresponding antigens. The cells were washed in cold phosphate buffer saline (PBS) and stained with fluorescein isothiocyanate (FITC) and phycoerythrin-labeled (PE) antibodies as recommended by the manufacturer. Expression of CD3, CD4, CD8, CD16, CD25, CD38, CD56, CD57, CD58, and HLA-DR molecules on the cells isolated from donor peripheral blood mononuclears was determined. In addition, the cells were stained with monoclonal FITC-labeled antibodies to CD45 and with PE-labeled antibodies to CD34 separately and in combination, after which they were washed twice in cold PBS. The results were evaluated on a FacsCalibur flow cytometer (Becton Dickinson).

The gate of cell population was established on the basis of combination of direct and lateral light scattering and cell size. A total of 10,000 cells/gate were counted. The data were statistically processed using WIN MDI 2.8 software.

Evaluation of proliferative activity of generated ML. Proliferative activity of ML under the effect of PFT was evaluated in the colorimetric test using AlamarBlu vital stain (Biosours) under sterile conditions, using a laminar box with a horizontal air flow (JuanVFS 906). Suspension of ML treated with PFT in doses of 0.1, 1.0, and 2.0 or by IL-2

(in an optimal dose of 1000 U/ml) in RPMI 1640 without 10% FCS (in order to rule our the probable effects of fetal proteins) was pipetted into 96-well plates (1×10^4 cell/well). ML suspension in RPMI 1640 without 10% FCS served as the control. The cultures were then incubated for 24 h in a CO_2 incubator (Binder). After incubation AlmarBlue stain (20 μl) was added into the wells. Optical density was measured after 24-h incubation at 37°C and 5% CO_2 on an MS multiscanner (Labsystems) at excitation wavelengths λ=540 and λ=620 nm. The increase in proliferative activity of AFP-activated ML (in percent) in comparison with intact ML and LAK was calculated by the formula:

$$1-\frac{(\varepsilon_{ox})\lambda_2 A\lambda_1-(\varepsilon_{ox})\lambda_1 A\lambda_2}{(\varepsilon_{ox})\lambda_2 A_0\lambda_1-(\varepsilon_{RED})\lambda_1 A_0\lambda_2}\times100\%,$$

where $(\varepsilon_{ox})\lambda_2=34.798$, $(\varepsilon_{ox})\lambda_1=47.619$; $(\varepsilon_{RED})\lambda_1=5.494$, A is optical density of the test object; A_0 optical density of the control; $\lambda_1=540$ nm; and $\lambda_2=620$ nm.

Mathematical data processing for ML proliferative activity. The data were processed using special software realizing the method of gradient decline and exponential curves were plotted, corresponding to the exponential regression equation with a linear member [4]:

$$E=a_1\times k+a_2\times k^m\times \exp^{(-nk)},$$

where E is increase (%) in proliferative activity of PFT-stimulated ML in comparison with intact ML or LAK cells; k is PFT concentration (μg/ml); A_1, A_2, m, n are regression coefficients and indexes of the degree, determined by the method of least squares.

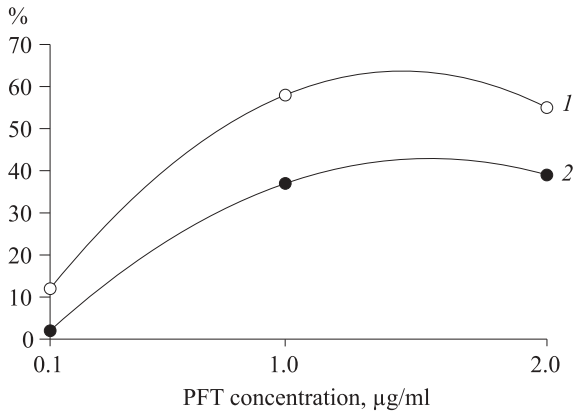

Fig. 1. Increased proliferation of mononuclear leukocytes (ML) incubated with "Profetal" (PFT) in comparison with proliferative activity of intact ML and LAK cells. Ordinate: increase in proliferation (%) of ML incubated with PFT in comparison with proliferative activity of intact ML (*1*) and LAK cells (*2*).

Morphohistochemical study. After 3 and 7 days of culturing, smears were made from centrifuged supernatants of PFT-activated peripheral blood ML. The smears were stained with eosin-Azur after Romanowskii—Giemsa, with methyl green-pyronine after Brachet for RNA with control RNase treatment, with Schiff's reagent after Shabadash with amylase control for glycogen and neutral glycosaminoglycans, and Alcyan Blue for acid glycosaminoglycans [7].

Light and phase contrast microscopy and photographs of cells generated from peripheral blood mononuclears in suspension and in stained smears were carried out using AxioVision 4 system (Carl Zeiss).

RESULTS

PFT in the studied doses activated the functional properties of human peripheral blood ML and differentiation and maturation of dendritic cells generated from during culturing in complete culture medium with FCS [6,8,10].

In serum-free media PFT in all studied concentrations stimulated mitotic activity of ML (Fig. 1).

Proliferative activity of ML increased significantly (by 55-58%) after treatment with PFT in concentrations of 1.0 and 2.0 μg/ml in comparison with the control (spontaneous proliferation of ML). The increase in mitotic activity of AFP-activated ML in serum-free culture medium in comparison with cells stimulated with IL-2 (LAK) was less demonstrative (by 37-39%).

AFP-activated ML formed two cell subpopulations, one consisting predominantly (50-79%) from $CD3^+$ lymphocytes and the other, consisting of large cells, containing macrophage $CD14^+$ and $CD34^+$ precursor cells (Fig. 2, *a*). The counts of these latter cells in this subpopulation reached 28% (Fig. 2, *b*). Cultured intact ML form a population consisting of $CD3^+$/CD34 cells (Fig. 2, *c*).

Analysis of culture suspension of PFT-activated ML stained with monoclonal antibodies to CD34 (PE-labeled) and CD45 (FITC-labeled) detected $CD34^+/CD45^+$ large round or oval cells fluorescencing bright yellow after double staining (Fig. 3, *a*). Microscopic examination of control ML (not treated with PFT) showed solitary $CD34^+$ cells (red fluorescence; Fig. 3, *b*) and an appreciable amount of $CD45^+$ ML fluorescing green (Fig. 3, *c*). No double staining was observed (Fig. 3, *d*).

Colonies detected in PFT-stimulated ML suspension by phase contrast microscopy in the light and dark fields consisted of large round cells with a wide cytoplasmic rim (Fig. 4, *a*, *b*).

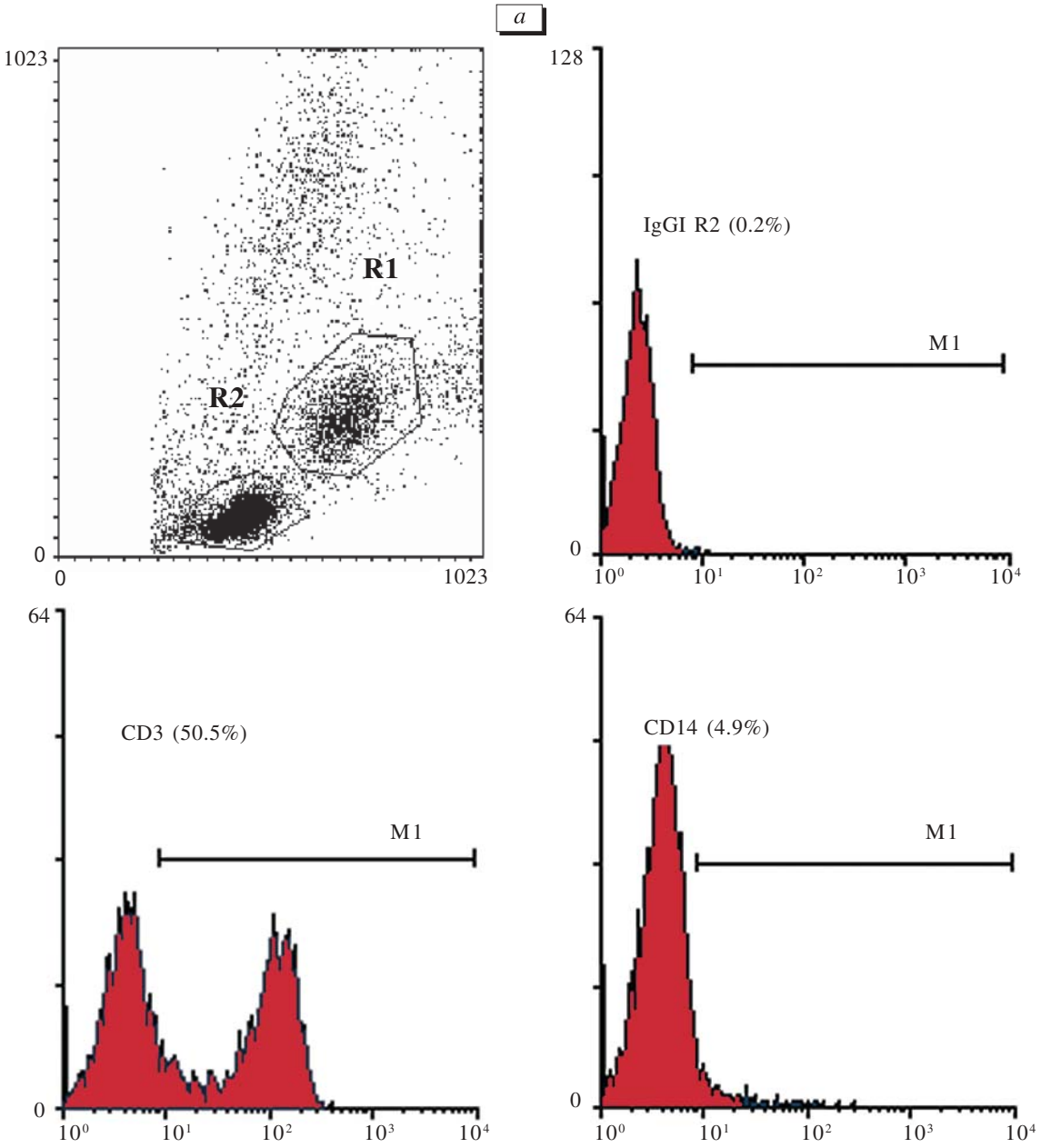

Fig. 2. Histograms reflecting expression of ML surface molecules (determination clusters). *a*) day 5 of ML incubation with PFT (2 μg/ml). Dotplot: light scattering (population of mature dendritic cells (DC) in the distinguished area); left peak on histograms: cell autofluorescence in isotypical control cultures; right peak: fluorescence (FITC: fluorescein isothiocyanate; PE: phycoerythrin) after staining with respective antibodies. Ordinate: cell count; abscissa: fluorescence intensity. CD: differentiation antigens.

Morphohistochemical analysis of smears from culture suspension of ML co-incubated with PFT showed predominance of immunoblast type cells (the highest percentage), prolymphocytes, and activated (pyroninephilic) lymphocytes (Fig. 5). Blast cells were large, with large nuclei with predominating euchromatin and numerous pyroninephilic nucleoli, a wide rim of basophilic cytoplasm (with bright pyroninephilic staining) detected by Brachet's test and PAS-positive component remaining in amylase control samples after staining after Shabadash (Fig. 5, *a-c*). Prolymphocytes were smaller, with more compact nucleus and pyroninephilic cytoplasm. Bright pyroninephilia of activated lymphocyte cytoplasm (similarly as of blasts and prolymphocytes) was weaker after RNase treatment, indicating increased level of RNA in the cells. Cells in a state of mitosis (Fig. 5, *d*) and apoptosis (Fig. 5, *e*) were often seen in the smears.

Hence, PFT in the studied concentrations stimulated functional activity of human peripheral blood ML during culture growth. This does not contradict

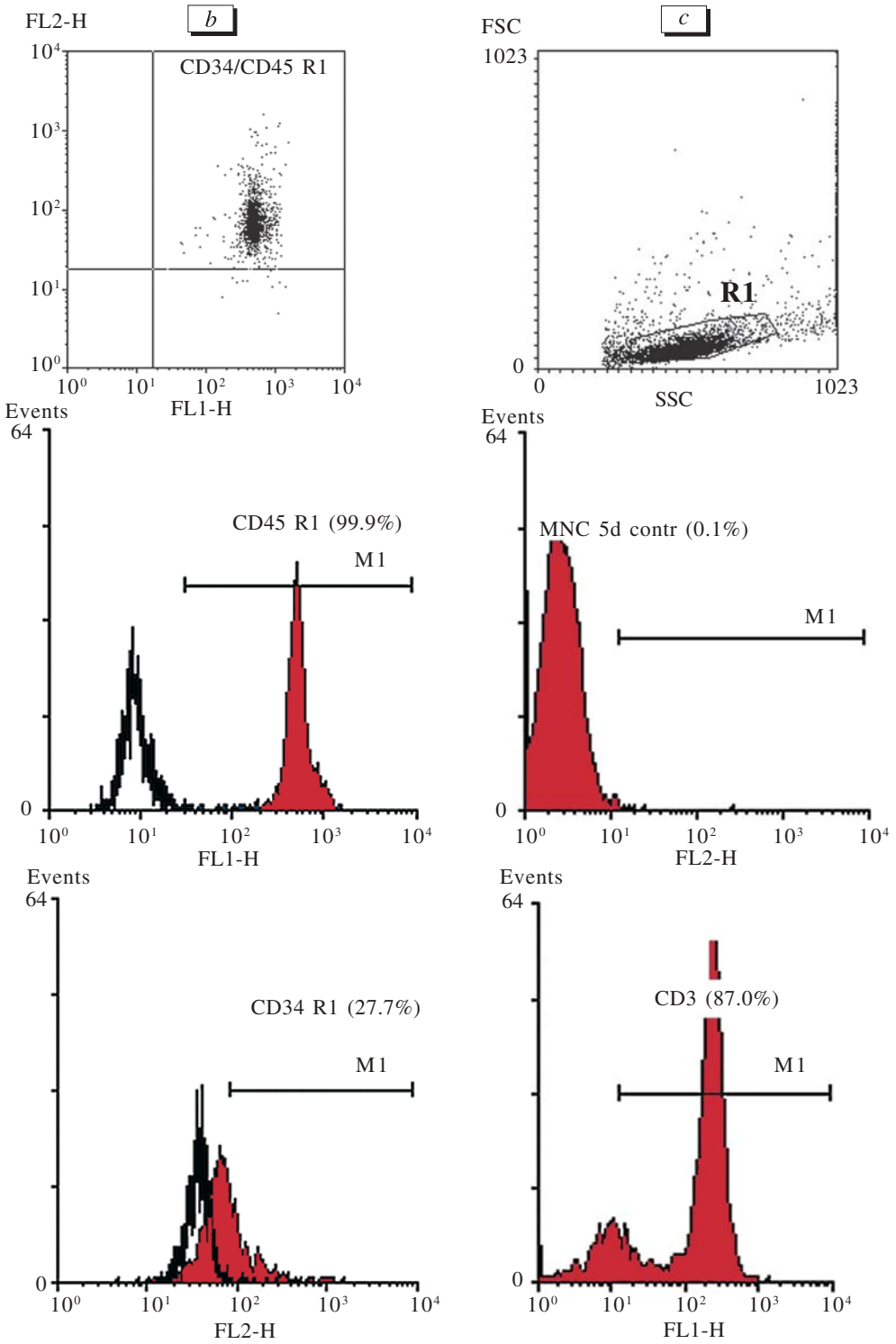

Fig. 2. Histograms reflecting expression of ML surface molecules (determination clusters). *b*) day 5 of ML incubation with PFT (2 μg/ml); *c*) day 5 of intact ML incubation.

published data, as reports published during recent decade confirmed that AFP served as a double regulator, stimulating and inhibiting cell growth [27-31]. AFP capacity to activate and suppress cell growth and differentiation as its dose-dependent function was demonstrated for cells of different types, including ovarian, uterine, testicular, and lymphoid [21,24].

Increase in ML cytotoxicity towards K562 tumor cells, which we described previously, indicated generation (under the effect of human AFP) of acti-

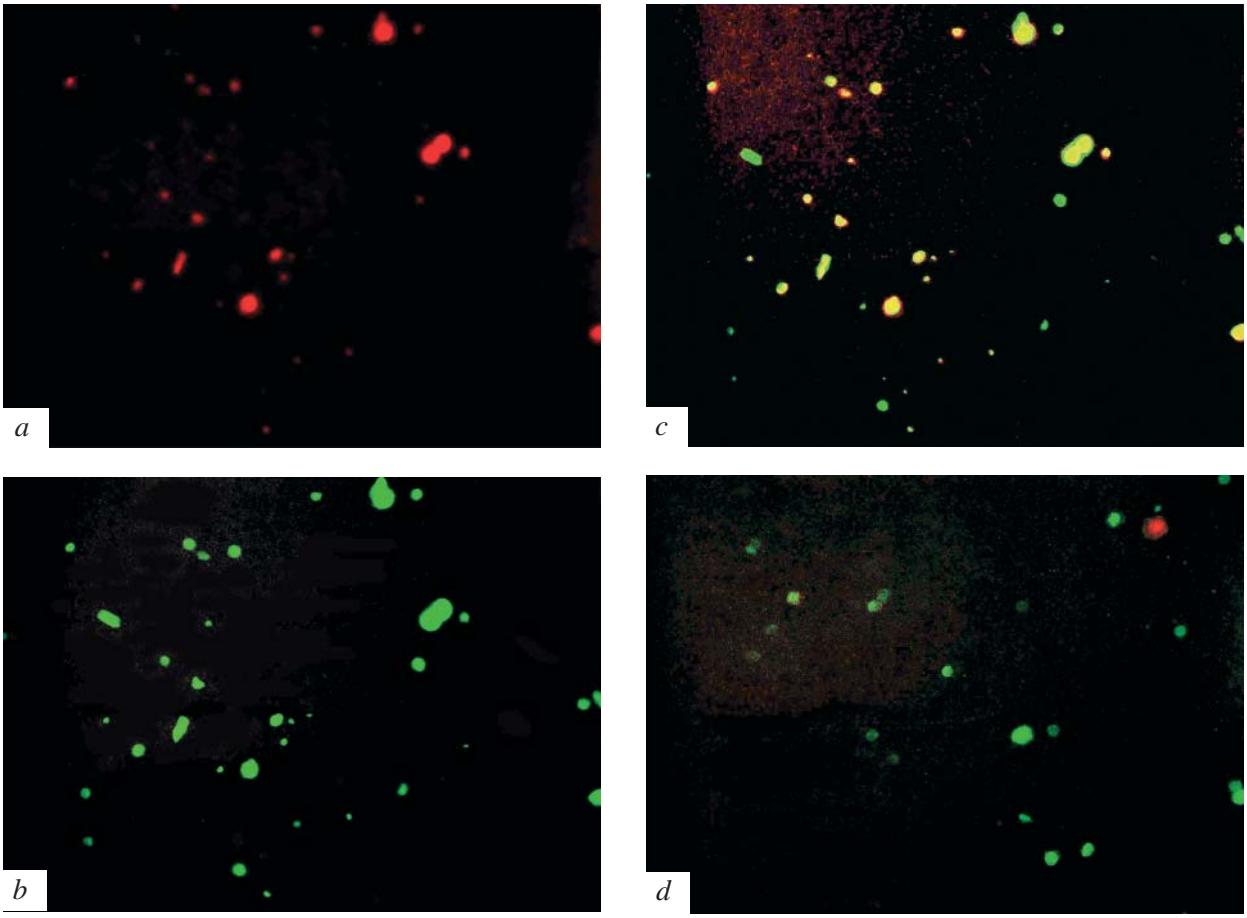

Fig. 3. Donor peripheral blood ML labeled with fluorescent antibodies in culture suspension on day 5 of incubation. *a-c*) PFT-stimulated ML, phase contrast microscopy, ×400. *a*) $CD34^+$ ML. FITC staining; *b*) $CD45^+$ ML. PE staining; *c*) $CD34^+/CD45^+$ ML. FITC+PE staining; *d*) intact ML ($CD34^+/CD45^+$) not treated with PFT. Phase contrast microscopy. FITC+PE staining.

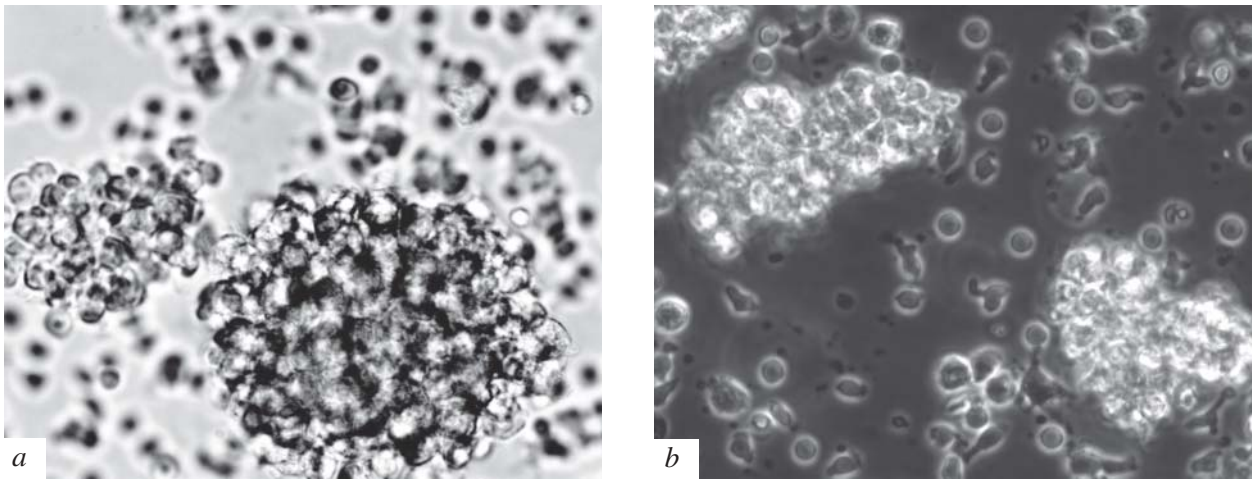

Fig. 4. Human peripheral blood ML activated with PFT (2 μg/ml) on day 5 of culturing. ML colonies in culture suspension, phase contrast microscopy (×400). *a*) in clear field; *b*) in dark field.

vated lymphoid elements including not only cytotoxic lymphocytes and natural killers, but also natural T killer cells (NTK cells) characterized by high cytotoxic activity [5,6,8].

"Profetal" increased mitotic activity of donor peripheral blood mononuclears cultured in serum-free media by 55-58% in comparison with spontaneous proliferation of ML and with proliferative

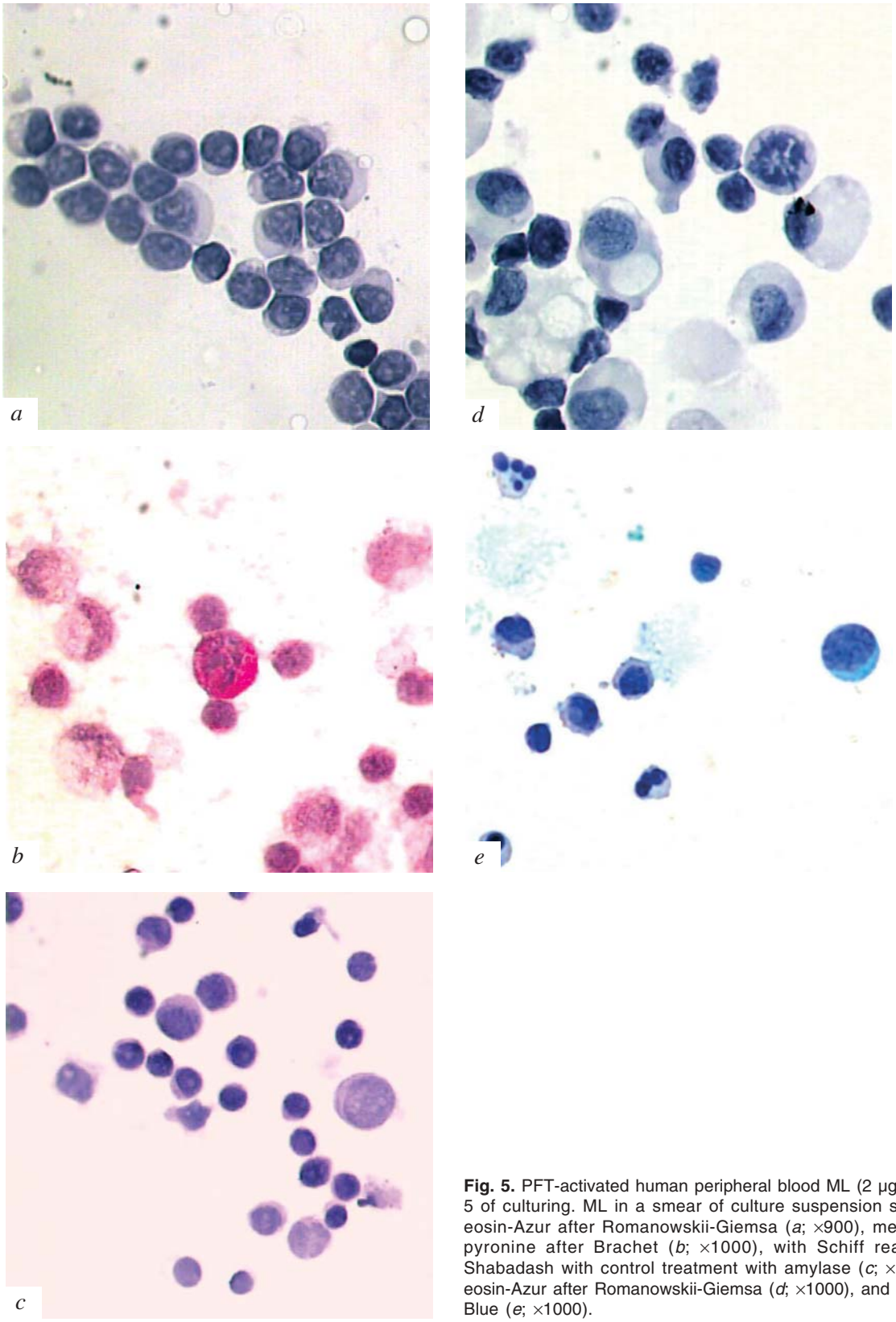

Fig. 5. PFT-activated human peripheral blood ML (2 μg/ml) on day 5 of culturing. ML in a smear of culture suspension stained with eosin-Azur after Romanowskii-Giemsa (*a*; ×900), methyl green-pyronine after Brachet (*b*; ×1000), with Schiff reagent after Shabadash with control treatment with amylase (*c*; ×1000), with eosin-Azur after Romanowskii-Giemsa (*d*; ×1000), and with Alcyan Blue (*e*; ×1000).

capacity of LAK cells (by 37-39%), which can be regarded as a manifestation of blast transformation of peripheral blood mature lymphocytes under the effect of human AFP, which was also paralleled by the appearance of $CD34^+/CD45^+$ cells (up to 28%). PFT-activated ML exhibited double staining with

monoclonal antibodies to these antigens, which was not observed in control ML cultures not treated with the preparation.

Microscopic examination of peripheral blood ML cultures co-incubated with PFT showed clusters of large cells not adhering to glass starting from day 3. Morphohistochemical analysis of smears of this culture suspension showed predominance of blast forms characterized by high RNA content and a certain amount of neutral glycosaminoglycans in the cytoplasm (indicators of active synthetic function of the cells) in PFT-activated cultures. High proliferative potential of ML is confirmed by the presence of numerous cells in a state of mitotic division. On the other hand, apoptotic cells were abundant in smears. This phenomenon indicates that AFP can regulate ML differentiation via several mechanisms, presumably including apoptotic regulation, modulation of cytoplasmatic signal transfer, and receptor desensitization.

Hence, our results suggest that human AFP in effective doses stimulates proliferation and blast transformation of cultured peripheral blood ML with the formation of $CD34^+/CD45^+$ hemopoietic precursor cells. The detected phenomenon of hemopoietic precursor cells generation *ex vivo* under the effect of AFP can be significant for clinical oncology, specifically, for the development of methods of adaptive cell therapy. The need in precursor cells which can be used for preparation of antitumor vaccines and activated cytotoxic lymphocytes is significant in this sphere. In addition, this method for generation of hemopoietic stem cells can replace the expensive and labor-consuming procedure used at present for autotransplantation of peripheral stem cells in patients after courses of myeloablative high-dose chemotherapy.

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