Lyophilized whole human melanoma cells stimulate human PBMC proliferation and enhance suppressive action of PBMC toward survival of the same malignant cell line *in vitro**

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The goal of this work was to determine: a) do lyophilized human melanoma BG or Fem-X cells affect the proliferative capacity of normal human peripheral blood mononuclear cells (PBMC) and b) does the PBMC six-days preincubation in nutrient medium with FBS with, or without lyophilized human melanoma BG or Fem-x cells, affect their suppressive action on the survival of the same malignant cell line *in vitro*. In the aim to avoid any stimulating effect of FBS, other group of experiments were done in nutrient medium with human AB serum in order to determine: c) does the PBMC six-day-preincubation with lyophilized human melanoma BG or Fem-x cells affect their antiproliferative action on the corresponding malignant cell line *in vitro* and d) does the PBMC six-day preincubation with *lyophilized normal PBMC,* obtained from healthy volunteer (as a source of allogenous, but not of tumor antigens), affect their suppressive action on the survival of both melanoma BG and Fem-x cell lines *in vitro*.

Results obtained in the presence of FBS in nutrient medium, showed that lyophilized BG cells induced a proliferation of the healthy PBMC, depending on the number of stimulating lyophilized cells. Lyophilized Fem-x cells induced healthy PBMC proliferation in lesser degree than lyophilized BG cells. This stimulation was almost constant, not dependent on the number of stimulating lyophilized Fem-x cells. Six-day stimulation *in vitro* by both lyophilized melanoma cells enhanced the suppressive action of PBMC on the survival of the corresponding malignant cell line.

Experiments done in nutrient medium with normal human AB serum showed that six-day stimulation with lyophilized melanoma cells enhanced, again, the suppressive action of PBMC on the survival of the corresponding malignant cell line. Contrary, six day preincubation of normal PBMC with the lyophilized healthy PBMC (obtained from other healthy person) *inhibited* their suppressive action on the survival of both malignant cell lines *in vitro*.

Key words: Immunostimulation, Fem-x cells, BG cells, human PBMC, MTT.

Suppressed immune response in patients with malignant diseases is documented in many publications. Stimulation of nonspecific, as well as of specific immune response in patients with malignant disease, was the main goal of many immunotherapeutic trials [3, 5, 8, 12, 13]. Publications on the use of tumor infiltrating lymphocytes and interleukin-2 in the immunostimulation showed promising results [9]. As in the recent literature there were no data on the immunogenic properties of lyophilized whole melanoma cells, the aim of this work was to determine a) do lyophilized malignant cells affect the proliferative capacity of normal PBMC, and b) does the PBMC six-day preincubation with, or without lyophilized human melanoma cells affect their suppressive action on the survival of the same malignant cell line *in vitro*. In the aim to remove any stimulating ef-

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Abbreviations used: $PBMC^{6d}n$ – PBMC incubated for six days in nutrient medium only; $PBMC^{6d}{}_{BG}$ – PBMC incubated for six days with lyophilized BG cells; $PBMC^{6d}{}_{F}$ – PBMC incubated for six days with lyophilized Fem-x cells; $PBMC^{6d}{}_{PBMC}$ – PBMC incubated for six days with lyophilized normal PBMC; Sn is supernatant, conditioned medium from sample of $PBMC^{6d}n$; S_{BG} is supernatant from sample of $PBMC^{6d}{}_{BG}$; S_F is supernatant from sample of $PBMC^{6d}{}_{F}$; S_{PBMC} is supernatant from sample of $PBMC^{6d}{}_{PBMC}$; BG and Fem-X cells are human melanoma cell lines.

fect of FBS the other groups of experiments were done in nutrient medium with human AB serum in order to determine: c) does the PBMC six-day preincubation with lyophilized human melanoma BG or Fem-x cells, affect their antiproliferative action on the same malignant cell line *in vitro* and d) does the PBMC six-day preincubation with *lyophilized normal PBMC* obtained from healthy volunteer, as a source of allogeneic, but not of tumor antigens, affect their suppressive action on the survival of both malignant cell lines *in vitro*.

Material and methods

Human melanoma cell lines, Fem-x, and BG, were grown as monolayer culture in nutrient medium, RPMI 1640, with 10% heat inactivated (56°C) FBS, 3 mM of L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution.

Lyophilization of malignant cells as well as of *normal PBMC* obtained from healthy volunteer, was done by freezing the suspension of whole malignant cells (2–4 x 10^6 cells/2 ml), or of normal whole PBMC (8–10 x 10^6 cells/2 ml) in nutrient medium at –80°C. The frost suspension was dehydrated in high vacuum, in lyophilizer. The goal of this treatment was to preserve the immunogenic structures present on the surface of malignant cells and to unable malignant cells to secrete their immunosuppressive factors. Lyophilization of normal PBMC was done to get immunogenic structures with allogeneic, but not tumor antigens for PBMC stimulation.

Reconstitution of lyophilized malignant, or lyophilized normal cell suspensions to original volume, was done with distilled water. Variable dilutions of the suspension were obtained by addition of nutrient medium.

Preparation of peripheral blood mononuclear cells (PBMC). The PBMC were separated from whole heparinized blood of nineteen healthy volunteers (age range 20—50 years) by Lymphoprep™ (Oslo, Norway) gradient centrifugation. Interface cells, washed three times with nutrient medium, were counted and resuspended in the same medium.

Proliferative response of PBMC induced by lyophilized cells. 1.5 x 10^5 PBMC per well, were incubated in quadruplicate, during six days, with or without lyophilized malignant cells Fem-x or BG (10 000, 5000, 2500, 1250, 625 and 0 cells). MTT test [6, 7] was used to determine the PBMC proliferative capacity induced by lyophilized malignant cells. This test was done in nutrient medium with FBS. The results were expressed as stimulation index (i %) [2]:

$$i(\%) = \frac{[(A_{PBMC+lyo} - A_{lyo}) - (A_{pbmc} - A_{nutrient\ medium})]}{(A_{pbmc} - A_{nutrient\ medium})} .100.$$

To get i(%), the absorbance (at 570 nm) of sample with lyophilized cells A_{lyo} was subtracted from the absorbance of sample of PBMC incubated with lyophilized malignant cells $A_{PBMC+lyo}$ and divided by the absorbance of nonstimulated cells ($A_{pbmc} - A_{nutrient\ medium}$) the multiplied by 100.

Cell surface marker analysis. Cell surface marker analysis was done using the direct immunofluorescence staining with specific T cell markers (CD_3/PerCP, CD_4/FiTC, and CD_8/PE) purchased from Becton-Dickinson Immunocytometry Systems (BDIS), San Jose, CA, USA, as instructed by manufacturer, on freshly isolated PBMC from three healthy volunteers. The same was done on PBMC stimulated for six days in nutrient medium with FBS; with or without lyophilized BG or Fem-x cells. Data collection and analysis were done on FacsCalibur™ Flow Cytometer (BDIS) using the Cell-Quest software (BDIS).

PBMC suppression of tumor cells survival

The effect of nonstimulated PBMC on malignant cell survival. The same test was done in nutrient medium independently on the presence of FBS or human serum in the nutrient medium. Live malignant cells were seeded in 50 μl in one group of 96 microtiter flat-bottom wells (10 000 cells per well). The same number of lyophilized cells was added to other group of wells. Lyophilized cells were put into the wells which were used as blank, to avoid any mistake which could arise from the tumor cell induced stimulation of PBMC, which may contribute to the increased formation of formazane; third group of wells contained the same volume of nutrient medium. Increasing numbers of freshly isolated, 'naive ' PBMC were added to the wells four hours after malignant cells seeding; in controls only nutrient medium was added.

The antiproliferative effect of stimulated PBMC

PBMC stimulation. Up to 6 x 10^6 PBMC were incubated in 3 ml of nutrient medium *with FBS* at 37°C for six days, without or with 1 x 10^5 lyophilized malignant BG or Fem-x cells in nutrient medium. Incubation was done in plastic six-well plates (Costar) in humidified atmosphere with 5% CO_2, during six days. The contents of wells were collected, centrifuged, and 2 ml of supernatant, conditioned medium, was put aside and marked as Sn for supernatant from sample of $PBMC^{6d}$ incubated only in nutrient medium; then as S_{BG} for supernatant from sample of $PBMC^{6d}{}_{BG}$ incubated with lyophilized BG cells, and as S_F for supernatant from sample of $PBMC^{6d}{}_F$ incubated with lyophilized Fem-x cells. Then PBMC from each sample were resuspended in remaining supernatant, counted, and further diluted in corresponding conditioned medium, Sn, or S_{BG}, or S_F. Two dilutions of $PBMC^{6d}$ suspension were used in the test: about 1.5 x 10^6 and 0.75 x 10^6 cells/ml.

The same procedure was performed for PBMC stimulation in nutrient medium with human serum, when stimulators lyophilized melanoma BG, or Fem-x cells, or normal PBMC were used. On that way, we have got $PBMC^{6d}_{BG}$, $PBMC^{6d}_{F}$ and $PBMC^{6d}_{PBMC}$, in their corresponding conditioned media.

For determination of *the antiproliferative action* of six-day stimulated PBMC ($PBMC^{6d}n$) in nutrient medium *with FBS*, to one group of wells, of 96 microtiter flat bottomed wells plate, live malignant BG cells (10 000 cells/well) were seeded; while to the others the same number of lyophilized BG cells were put in 50 μl of nutrient medium, while the third group of wells contained only nutrient medium. Four hours later to one group of wells (control samples and their blank), 100 μl of fresh nutrient medium were added; to the other group of wells 100 μl of corresponding supernatant Sn or S_{BG} were added. In the third group of wells, 100 μl of two various dilutions of $PBMC^{6d}n$, in their conditioned medium were added. Two dilutions of $PBMC^{6d}_{BG}$, were put in the fourth group of wells. Control sample were BG cells grown in fresh nutrient medium. The same experiment was set up with live Fem-x cells as target cells; with $PBMC^{6d}n$, and with $PBMC^{6d}_{F}$ in their corresponding conditioned medium, supernatants Sn and S_F, respectively. Control sample were Fem-x cells grown in fresh nutrient medium.

For determination of *the antiproliferative action* of six-day stimulated PBMC (in nutrient medium *with human serum)* to one group of wells, of 96 microtiter flat bottomed wells plate, live malignant BG cells (10 000 cells/well) were seeded; while to the others the same number of lyophilized BG cells were put in 50 μl of nutrient medium, while the third group of wells contained only nutrient medium. Four hours later to one group of wells (control samples and their blank), 100 μl of fresh nutrient medium were added; to the other group of wells 100 μl of corresponding supernatant S_{PBMC} or S_{BG} were added. In the third group of wells, 100 μl of two various dilutions of $PBMC^{6d}_{PBMC}$, in their conditioned medium were added. Two dilutions of $PBMC^{6d}_{BG}$, were put in the fourth group of wells. Control sample were BG cells grown in fresh nutrient medium. The same experiment was set up with live Fem-x cells as target cells; with $PBMC^{6d}_{PBMC}$, and with $PBMC^{6d}_{F}$ in their corresponding conditioned mediums. Control sample were Fem-x cells grown in fresh nutrient medium.

Determination of Fem-x and BG, cell survival. Cell survival was determined by MTT test [6, 7, 10], 24 hours upon addition of the PBMC. (Crystals of formazane were dissolved by addition of 10% SDS, in 0.01 mol HCl.) To get cell survival (%) absorbance of sample with effector cells and lyophilizate ($A_{PBMC+lyo}$) was subtracted from absorbance of sample of target cells + effector cells (A_{T+PBMC}) and this difference multiplied by 100 was divided by the absorbance of target cells grown in fresh nutrient medium, $A_T - A_{\text{nutrient medium}}$

$$S(\%) = \frac{(A_{T+PBMC} - A_{PBMC+lyo})}{(A_T - A_{\text{nutrient medium}})} \cdot 100.$$

Results

Incubation of human PBMC obtained from five healthy volunteers with increasing number of lyophilized malignant cells during six days, induced the proliferation of PBMC (Fig. 1). The extent of stimulation (i%) varied between individual PBMC and was dependent on the lyophilized cell type. Stimulation of PBMC proliferation by lyophilized BG cells was dependent on the number of stimulating lyophilized BG cells; PBMC stimulation by lyophilized Fem-x cells was not the function of the number of stimulating cells.

The action of naive human PBMC and of $PBMC^{6d}n$, and $PBMC^{6d}_{BG}$ (preincubated for six days without or with lyophilized malignant melanoma BG cells) in 50% of the corresponding conditioned medium on the survival of the BG cells was investigated. BG cells are very resistant to the action of naive PBMC in nutrient medium with FBS. A mild PBMC stimulation of BG cells survival was observed at higher E/T ratios. Six-day incubation of PBMC with nutrient medium led to the increased expression of their cytostatic action in comparison to the effect of naive PBMC. This enhancement was more pronounced when PBMC were stimulated with lyophilized BG cells. The extent of $PBMC^{6d}_{BG}$ induced suppression of tumor cell survival varied among different individuals. Moreover, in three of five cases 50% conditioned medium from $PBMC^{6d}_{BG}$ induced suppression of the BG cell survival (results not shown).

The effect of naive human PBMC and of $PBMC^{6d}n$, and $PBMC^{6d}_{F}$ (preincubated for six days without or with lyophilized malignant melanoma Fem-x cells) in 50% of the corresponding conditioned medium on the survival of the Fem-x cells was investigated too. While naive PBMC from one person induced marked decrease in Fem-x cells survival (approximately 50% at E/T ratio 20) PBMC of other four persons expressed very mild cytostatic action to target cells. Six-day incubation of PBMC in nutrient medium with or without lyophilized Fem-x cells, enhances their antiproliferative action in four from five cases, in comparison to the control, naive PBMC, and $PBMC^{6d}n$ (results not shown).

Incubation of PBMC from another group of three healthy volunteers in nutrient medium with, or without lyophilized melanoma BG or Fem-x cells for six days lead to some increase in the percent of T cells, both, with surface markers CD_3+CD_8+ and CD_3+CD_4+, in two analyzed cases, compared to the percent of these cells in untreated, naive, PBMC (data not shown).

The next group of experiments performed were done in the presence of human AB serum in nutrient medium, to avoid any stimulative action of fetal bovine antigens on PBMC. As the data obtained in previous experiments could be result of an alloreactivity, the new set of experiments was done in the order to compare the antiproliferative action of normal

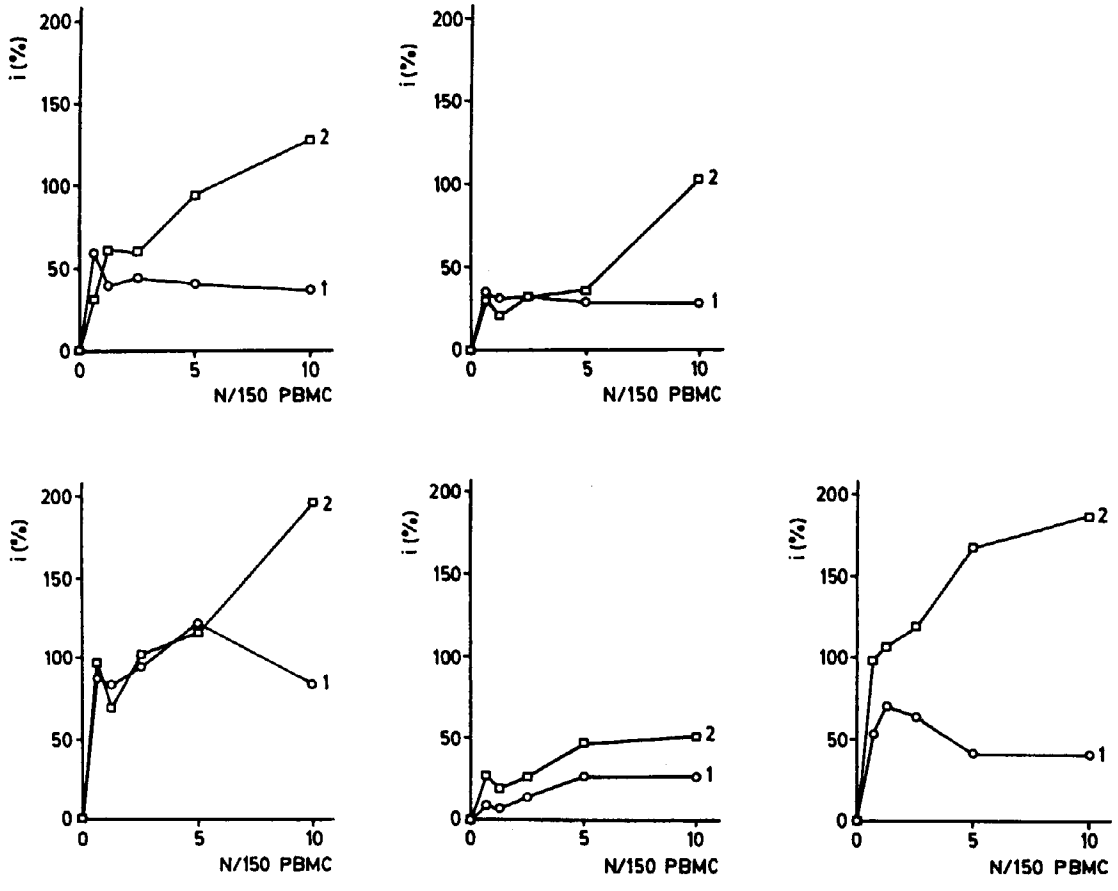

Fig. 1. Index (i %), of stimulation of PBMC proliferation induced by different number of lyophilized human malignant melanoma Fem-x (1) or BG cells (2). 10 000, 5000, 2500, 1250, 0.625 and 0 lyophilized cells were incubated without or with 1.5 x 10^5 PBMC per well, in quadruplicate for six days in nutrient medium with FBS. Then MTT test was used to determine the percent of PBMC proliferation. Results are mean of quadruplicate. CV was less than 10%.

$PBMC^{6d}_{PBMC}$ (stimulated by nontumor alloantigens) with the action of $PBMC^{6d}_{BG}$ (stimulated with nontumor and tumor alloantigens), and to compare both of these actions with the effect of naive PBMC on the survival of BG cells.

Results obtained in the presence of human serum in nutrient medium shown in Fig. 2 demonstrated that naive PBMC obtained from next group of healthy volunteers suppressed survival of BG cells. The extent of this suppression varied between individuals. $PBMC^{6d}_{BG}$ and even 50% conditioned medium had an increase in their suppression of BG cells survival compared to the action of naive PBMC. Contrary, $PBMC^{6d}_{PBMC}$, even 50% conditioned medium in four from five cases *suppressed* antiproliferative action of PBMC in comparison to the action of naive PBMC and $PBMC^{6d}_{BG}$ on BG cell line.

The data on the antiproliferative action of naive, or of six day stimulated $PBMC^{6d}_{F}$ or by lyophilized $PBMC_{PBMC}$ are shown in Fig. 3. Naive PBMC suppressed Fem-x cell survival more intensively than that of BG cells. Stimulation of the antiproliferative action of $PBMC^{6d}_{F}$ in their conditioned medium on Fem-x cells was observed in two from five investigated cases. Contrary $PBMC^{6d}_{PBMC}$ in their conditioned medium showed decrease in the antiproliferative activity on Fem-x cells, compared to the action of naive PBMC, and of $PBMC^{6d}_{F}$, in four of five cases too.

Discussion

Successful tumor vaccine is a dream for every physician working in oncology and for every patient with malignant disease. Large efforts have been undertaken to develop the tumor vaccine, very few successful accomplishments were reported [1, 3, 5, 11]. Although some reports were published on the use of irradiated whole tumor cells, or tumor cells lysates [3, 5] we have not seen any publication dealing with the

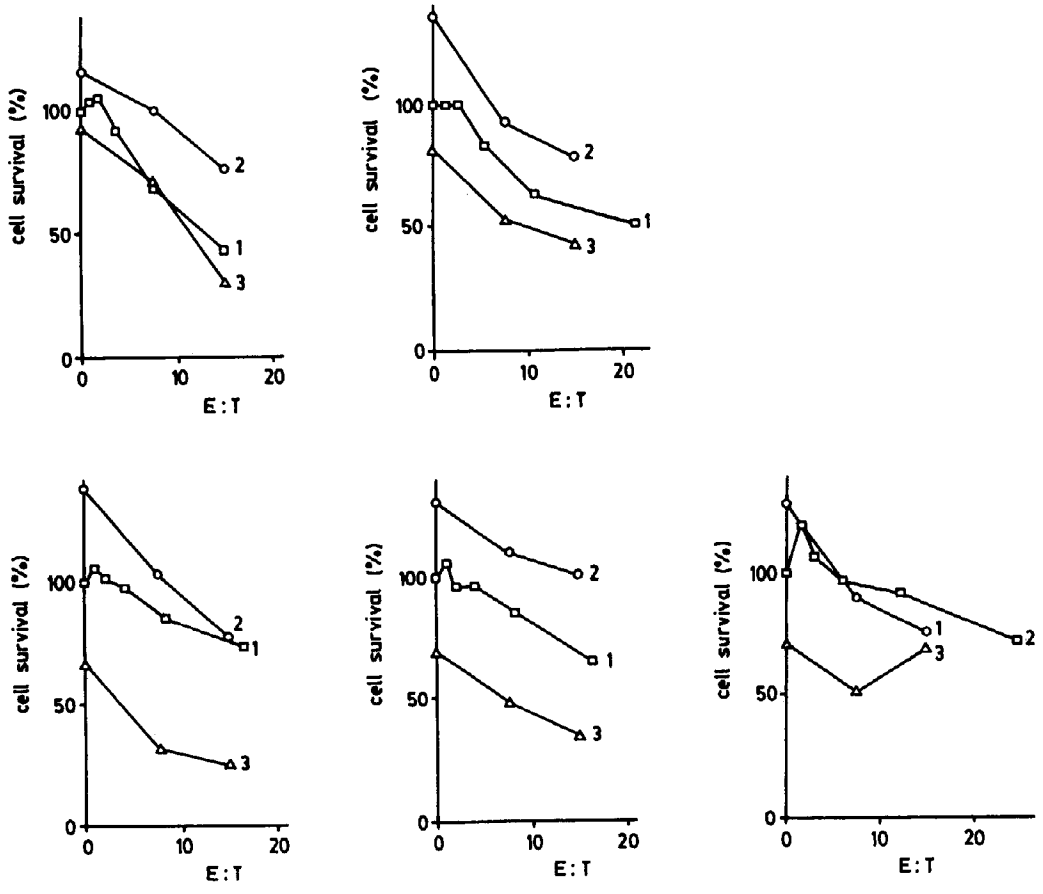

Fig. 2. The survival (%) of human malignant melanoma BG cells (10 000 cells/well) grown in mixed culture with various dilutions of naive PBMC (1), or with $PBMC^{6}_{PBMC}$ (PBMC incubated for 6 days in nutrient medium with human serum with lyophilized normal PBMC (2); or with $PBMC^{6d}_{BG}$, PBMC incubated for six days with lyophilized BG cells in 50% conditioned medium (3). MTT test was used for determination of melanoma cell survival. Results are mean of quadruplicate. CV was less than 7%.

use of lyophilized whole tumor cells as an inducer of the antitumor immune response. Results obtained in this work showed that lyophilized BG and Fem-x cells induced stimulation of proliferation of healthy PBMC grown in nutrient medium with FBS. However this effect was different regarding the lyophilized cell line used. Fem-x lyophilizate induced PBMC proliferation in lesser degree than BG one, this effect was constant, i.e. not dependent on the number of lyophilized cells used. The difference observed could be related to the degree in the allogenicity as well as the difference in HLA Class II expression.

Six-day preincubation of PBMC *in vitro* induced enhancement of their antiproliferative action on the BG melanoma cell line, without respect on the presence or on the absence of lyophilized BG cells in the nutrient medium with FBS. However, this effect was more pronounced when PBMC were preincubated with lyophilized BG cells, then when they were incubated in nutrient medium. Additional experiments performed in the presence of human serum in nutrient medium showing specificity for the enhancement of the antiproliferative action of $PBMC^{6d}_{BG}$ to BG cells compared to the action of allogeneic normal $PBMC_{PBMC}$, may indicate that lyophilized melanoma BG cells stimulate the allogeneic-antitumor immune function of normal PBMC.

Although the influence of lyophilized Fem-x cells stimulation on PBMC induced suppression of Fem-x cell survival was observed, this incubation did not enhance much their antiproliferative action, compared to the effect of PBMC incubated in only nutrient medium (with FBS), and compared to the control, to the action of naive, PBMC. Experiments performed in the presence of human serum in nutrient medium showed specificity for the enhancement of the antiproliferative action of $PBMC^{6d}_{F}$ to Fem-x cells, compared to the action of naive allogeneic normal PBMC and to the action of allogeneic normal $PBMC_{PBMC}$.

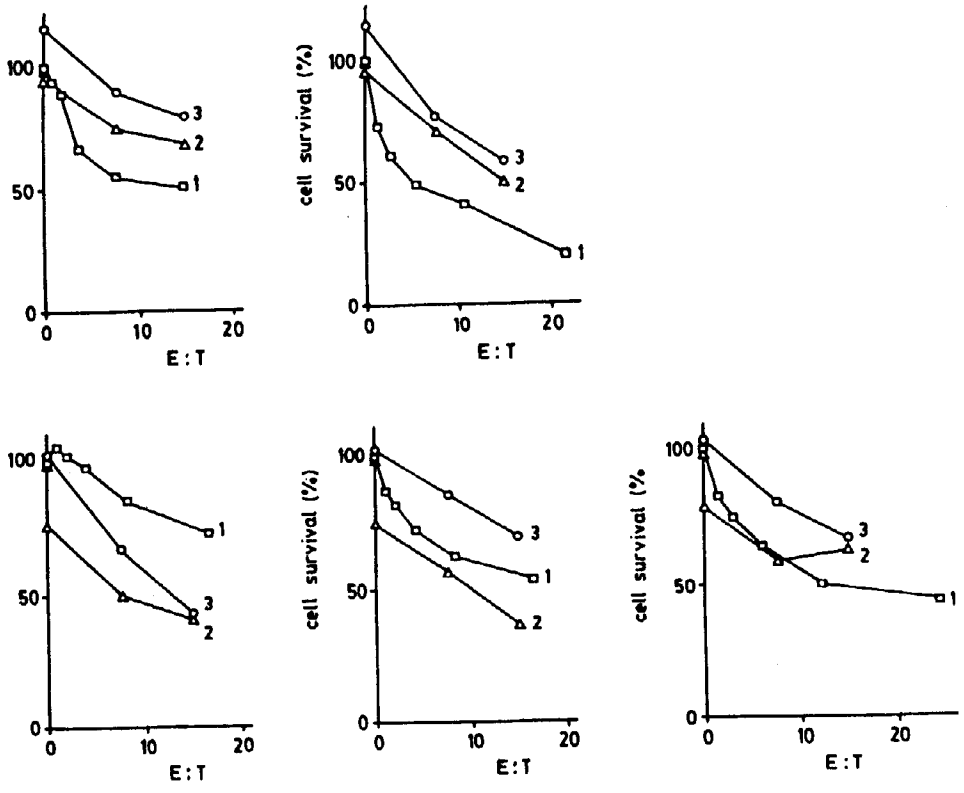

Fig. 3. The survival (%) of human malignant melanoma Fem-x cells (10 000 cells/well) grown in mixed culture with various dilutions of naive PBMC (1), or with $PBMC^{6}_{PBMC}$ (PBMC incubated for 6 days) in nutrient medium with human serum with lyophilized normal PBMC (3) or with $PBMC^{6d}_{F}$, PBMC incubated for six days with lyophilized Fem-x cells in 50% conditioned medium (2). MTT test was used for determination of melanoma cell survival. Results are mean of quadruplicate. CV was less than 6%.

The difference between the antiproliferative action of $PBMC^{6d}_{BG}$ on the BG cell survival and the action of $PBMC^{6d}_{F}$ on Fem-x cell survival could be explained by the possible existence of more immunogeneic structures present on lyophilized BG cells than on lyophilized Fem-x cells. Observation that conditioned medium from $PBMC^{6d}_{BG}$ induced suppression of malignant cell survival could indicate that lyophilized BG melanoma cells, more than lyophilized Fem-x cells, stimulate PBMC for production of cytokines, some factors that suppressed tumor cell survival (TNFα, IFN s ...). This finding is in accordance with another study [4], that tumor associated lymphomonocytes from neoplastic effusions of patients with different primary tumors are able to release cytokines.

In conclusion, results obtained in this work in small number of healthy persons indicate that antigens present on lyophilized whole tumor cells could stimulate allogeneic antitumor immune response; this reaction depends on the immunogenic properties of malignant cell line, as well as on the individuals PBMC. Incubation of normal PBMC with lyophilized normal PBMC as of a source of nontumor allogeneic structures led to the decrease in the antiproliferative action of incubated $PBMC_{PBMC}$, even more to the potentiation of tumor cell growth by corresponding conditioned medium. Further studies on the larger number of normal subjects and in patients with various cancer types particularly are needed. The approach presented in this work offer the chance for the investigation of the possibility for the prediction *in vitro* of the enhancement of antitumor immunity in patient, by autologous, or allogenous vaccine.

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Rerefences

[1] Barrett-Boyes, S.M., Kao, H., Finn, O.J.:Chimpanzee dendritic cells derived *in vitro* from blood monocytes and pulsed with antigen elicit specific immune responses in vitro: J. Immunother., *21*, 1998, 142—148.

[2] Czarnacki, J., Lopez, J.L., Salgado, F.J., Sarandeses, C.S., Erdero, O.J., Nogueira, M.: Effects of prothymosin on PHA stimulated human lymphocytes evaluation of two different methods. Int. J. Thymol., *3*, 1995, 231—238.

[3] Hersey, P.: Melanoma vaccines. Current status and future prospects. Drugs, *47*, 1994, 373—382.

[4] Mantovani, G., Maccio, A., Versace, R., Pisano, M., Lai, P., Esu, S., Ghiani, M., Dessi, D., Cherchi, R., Melis, G.B., DelGiacco, G.S.: Tumor associated lymphomonocytes from neoplastic effusions of patients with different primary tumors are able to release cytokines. J. BUON, *1*,1986, 41—45.

[5] Mastrangelo, M.J., Maguire, H.C. Jr., Sato, T., Nathan, F.E., Berd, D.: Active specific immunization in the treatment of patients with melanoma. Semin. Oncol., *23*, 1996, 773—781.

[6] Mosmann, T.: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods, *65*, 1983, 55—63.

[7] Ohno, M., Abe, T.: Rapid colorimetric assays for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). J. Immunol. Methods, *145*, 1991, 199—203.

[8] Reynolds, S.R., Oratz, R., Shapiro, R.I., Hao, P., Yun, Z., Fotino, M.: Stimulation of $CD8^+$ T cell responses to MAGE-3 and melan. A/MART-1 by immunization to a polyvalent melanoma vaccine. Int. J. Cancer, *72*, 1997, 923—976.

[9] Rosenberg, S.A., Yannelli, J.R., Topalian, S.L., Schwantzertruber, D.J., Weber, J.S., Parkinson, D.R., Seipp, C.A., Einhirn, J.H., White, D.E.: Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin-2. J. Natl. Cancer Inst., *86*, 1994, 1159—1166.

[10] Seung, L.P., Seung, S.K., Schreiber, H.: Antigenic cancer cells that escape immune destruction are stimulated by host cells. Cancer Res., *55*, 1995, 5094—5100.

[11] Soares, M., Finn, O.J.: Phenotypic versus functional maturation of in vitro derived human dendritic cells. Cancer Res. Ther. Contr., *5*, 1998,77—85.

[12] Stanojević-Bakić, N., Milošević, D., Vučković-Dekić, Lj., Šašić, M., Marković, Lj.: Clinical and immunologic effect of T-activin therapy in early stage melanoma patients. Neoplasma, *43*, 1996, 245—252.

[13] Stanojević-Bakić, N., Milošević, D., Vučković-Dekić, Lj., Šačić, M., Marković, Lj.: T-activin therapy in early stage melanoma patients. In vitro and in vivo immunologic effect. Int. J. Thymol., *3*, 1995, 265—272.