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Full Length Research Paper

Propranolol effect on proliferation and vascular endothelial growth factor secretion in human immunocompetent cells

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Beta-blockers are widely used drugs in cardiovascular diseases. Propranolol is a non selective beta-adrenergic blocker extensively used in treatment of arrhythmias, hypertension and several other cardiac problems. The inhibitory effects of propranolol on tumor growth, inflammation and angiogenesis have been shown by many investigations. Peripheral blood mononuclear cells (PBMCs) play important role in inflammation and vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis. The present study was conducted to evaluate the propranolol effect on human PBMCs proliferation and VEGF secretion *in vitro*. PBMCs from healthy adult volunteers were isolated by ficoll-hypaque-gradiant centrifugation. Then the cells were cultured in complete RPMI-1640 medium and after that incubated with different concentrations of propranolol (0.00034 - 0.34 mM) in the presence or absence of phytohaemagglutinin (PHA) (10 µg/ml) for 12, 24 and 48 h. The cell viability was then assessed by trypan blue dye exclusion and also 3-[4, 5-dimethyl thiazol-2, 5-diphenyltetrazoliumbromide (MTT) reduction methods. Amount of VEGF produced by PBMCs was quantified by enzyme-linked immunosorbent assay (ELISA). Propranolol significantly reduced the viability of human PBMCs at ≥ 0.17 mM concentration, after 12 hours incubation onwards, compared to untreated control cells. Moreover propranolol significantly decreased the VEGF production in PHA-stimulated human PBMCs at 0.034 mM concentration of drug. Our results suggest that propranolol with inhibitory effect on PBMCs proliferative activity and VEGF production may be useful in immunoproliferative, inflammatory and angiogenesis related disorders. Moreover, anti-inflammatory characteristic of propranolol may be other beneficial effect of this drug in treatment of inflammatory based cardiovascular diseases.

Key words: Propranolol, peripheral blood mononuclear cells, proliferation, VEGF.

INTRODUCTION

Inflammation has an important role in numerous disorders including some cardiovascular diseases such as hypertension and atherosclerosis (Duan et al., 2009; Full et al., 2009). Also increased incidence of cardio-vascular disorders in some inflammatory conditions like rheumatoid arthritis has been reported (Full et al., 2009). Proinflammatory cytokines are regarded as potential therapeutic target in some cardiovascular diseases (Bielecka-Dabrowa et al., 2007). Propranolol as a non selective beta-adrenergic blocker, is among the most extensively used drugs in treatment of many cardiac disorders such as arrhythmias and arterial hypertension (Koelemay and Legemate, 2008; Degoute, 2007). Anti-tumor effects of propranolol have also been shown by several studies (Benish et al., 2008; Gibson et al., 1987; Schuller et al., 2000). In addition the cytotoxic effects of propranolol on leukemic cell lines (Hajighasemi and Mirshafiey, 2009), lung cells (Kastelova et al., 2003), skin keratinocytes and fibroblasts (Cheong et al., 2008) have been demonstrated. Also inhibitory effect of propranolol on proliferation of PHA- stimulated lymphocytes of duodenal

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ulcer patients (Malec et al., 1990) has been reported. Moreover, an increase in splenocyte apoptosis rate (Schmitz et al., 2007) and decrease of proliferative capacity of splenocytes (Oberbeck et al., 2004) after administration of propranolol in septic mice have been demonstrated. Anti-inflammatory properties of propranolol (Benish et al., 2008; Nguyen et al., 2008; Kato et al., 2009) and its attenuating effect on proinflammatory cytokines, has been shown (Deten et al., 2003; Tang et al., 2008). Moreover, blocking effect of propranolol on epinephrine induced shift to TH2 cytokine profile in PBMCs has been reported (Agarwal et al., 2000). Furthermore, the inhibitory effects of propranolol on norepinephrine mediated angiogenesis (Yang et al., 2006), norepinephrine stimulated release of functional angiogenic factors (Fredriksson et al., 2000) and surgical stress-enhanced angiogenesis (Lee et al., 2009) have been described. Modifying of angiogenes (neovascularization) showed potential implication for treating a great number of disorders such as cancers and ischemic heart disease (Göőz et al., 2009). One important regulator of angiogenesis is vascular endothelial growth factor (VEGF) (Ferrara, 2009) which has a critical role in inflammation (Tortora et al., 2008). In this study we assessed the effects of propranolol on human peripheral blood mononuclear cells (PBMCs) proliferation and VEGF production *in vitro*.

MATERIALS AND METHODS

Propranolol preparation

Propranolol was dissolved in RPMI-1640 medium and stored at -20°C until use in experiments. Drug was diluted in culture medium to prepare the required concentrations before use.

Reagents

RPMI-1640 medium, penicillin, streptomycin, phytoheamagglutinin (PHA), Ficoll-hypaque and trypan blue (TB) were from Sigma (USA). Fetal calf serum (FCS) was from Gibco (USA). VEGF standard ELISA kit was obtained from R&D company (USA). Propranolol was a kind gift from HAKIM Pvt. Co. Ltd (Tehran, Iran). Microtiter plates, flasks and tubes were from Nunc (Falcon, USA).

PBMCs isolation

PBMCs from the venous blood of healthy adult volunteers were isolated by ficoll-hypaque-gradiant centrifugation. Subsequently, the cells were washed three times in phosphate buffer saline (PBS). Then the cells resuspended in RPMI-1640 medium supplemented with 10% FCS and were incubated in 5% CO_2 at 37°C.

Cell culture and treatment

PBMCs were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO_2. The cells were seeded at a density of 10^5 cell/well and then incubated with different concentrations of propranolol (0.00034 - 0.34 mM) in the presence or absence of PHA (10 µg/ml) for 12, 24 and 48 h. The supernatants of cell cultures were collected, centrifuged and stored at -80°C for next experiments. All experiments were done in triplicate.

Cell proliferation assay

To evaluate the effect of different concentrations of drug on viability of human PBMCs, we used trypan blue dye exclusion (TB test) (Moldeus et al., 1978) and MTT assay (Mosmann, 1983).

TB test

Principle of TB test is exclusion of dye by viable cells and taking it up by dead cells. Viability is evaluated by direct counting of viable and dead cells. Percentage of the number of viable cells to the total number of cells is considered as viability percentage.

MTT assay

In MTT assay the conversion of yellow water soluble MTT to a blue-insoluble formazon was assessed according to the method developed by Mosmann (1983). At the end of incubation time, the medium was replaced with 100 µl of fresh medium. Then 10 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated at 37°C for 4 h. Subsequently 100 µl of the SDS-HCl solution (100 mg SDS was dissolved in 1 ml HCl), was added to each well and incubated at 37°C for 4 h. So the insoluble formazon derivative was dissolved and absorbance at 570 nm was measured using a microplate reader (Awareness Technology INC). The results were expressed as cell numbers per control.

VEGF enzyme-linked immunosorbent assay (ELISA)

Amount of VEGF secreted by PBMCs in the cell culture supernatants was measured by the Quantikine human VEGF enzyme-linked immunosorbent assay (ELISA) standard kits (R&D systems) according to the manufacturer's instructions.

Statistical analysis

Effect of the drug on human PBMCs proliferation and VEGF secretion was performed in three independent experiments and the results were expressed as mean ± SEM. Statistical comparisons between groups were made by analysis of variance (ANOVA). $P < 0.05$ was considered significant. Test of multiple comparison of Tukey was applied (5%) for statistically significant differences. The software Spss 11.5 and Excel 2003 was used for statistical analysis and graph making respectively.

RESULTS

Propranolol effects on proliferative activity of PHA-stimulated human PBMCs

Propranolol effect on proliferative activity of PHA-stimulated human PBMCs in different concentrations and time intervals are shown in Figures 1A and B. In Figure 1A represents TB dye exclusion method and B represents MTT assay. Propranolol profoundly decreased

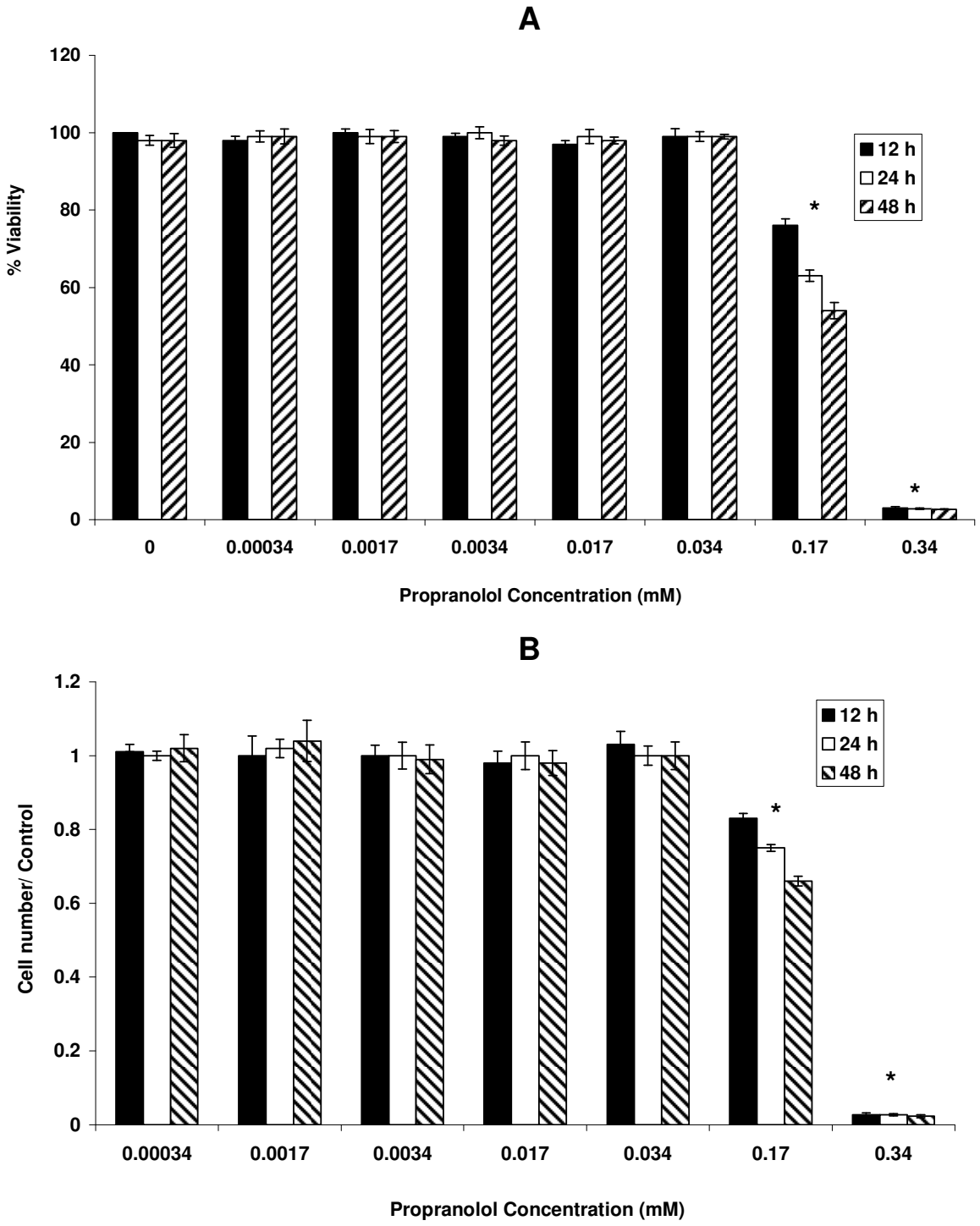

Figure 1. Effect of propranolol on proliferative responses of human PBMCs, demonstrated by (A) trypan blue dye exclusion (TB) test and (B) cell proliferation by MTT assay. The human PBMCs were treated with different concentrations of propranolol (0.00034 - 0.34 mM) for 12, 24 and 48 h. The results are presented as % of viability (A) and cell number/control (B). Data are mean ±SEM of three independent experiments. $P < 0.05$ was considered significant.

proliferative responses of human $PBMC_S$ in both staining methods at all time intervals ($p < 0.001$). The results depicted in Figures 1A and B showed that cytotoxicity of propranolol on human $PBMC_S$ is dose and time

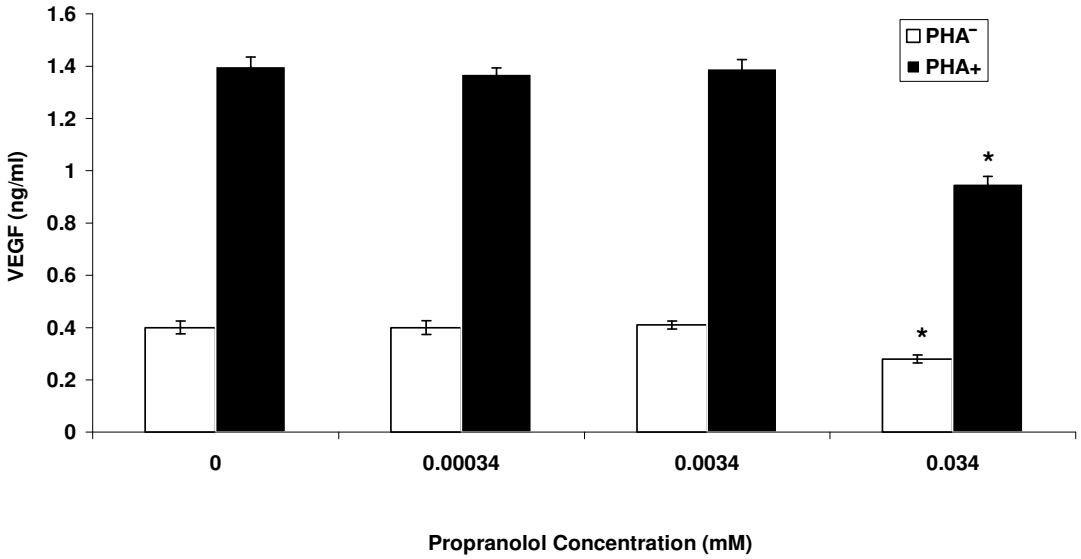

Figure 2. Effect of propranolol on human PBMCs VEGF secretion. The PBMCs were treated with different concentrations of propranolol(0.00034 - 0.34 mM) for 12, 24 and 48 h in the presence or absence of PHA (10 µg/ml). At the end of treatment, VEGF concentration in conditioned medium was measured by ELISA. Data are mean ± SEM of three independent experiments. $P < 0.05$ was considered significant.

dependent.

Propranolol effects on viability (%) of PHA-stimulated human PBMCs

As observed in Figure 1A, propranolol significantly decreased the viability of PHA-stimulated human PBMCs dose dependently at all time intervals which was determined by TB dye exclusion method. These results revealed that percent of viability of human PBMCs significantly decreased by propranolol at ≥ 0.17 mM drug concentration after 12 h incubation time onwards compared with untreated control cells ($P < 0.05$). The decrease of viability of human PBMCs at 0.17 mM dose of propranolol increased with time, 48 > 24 > 12 h respectively as illustrated in Figure 1A.

Propranolol effects on proliferation of PHA-stimulated human PBMCs

As illustrated in Figure 1B, propranolol significantly reduced the proliferation of PHA-stimulated human PBMCs dose-dependently at all time intervals which was determined by MTT assay. These results revealed that MTT reduction in human PBMCs significantly decreased by propranolol at ≥ 0.17 mM drug concentration after 12 h incubation time onwards compared with untreated control cells ($P < 0.05$). The decrease of proliferation of human PBMCs at 0.17 mM dose of drug increased with time, 48 > 24 > 12 h respectively as illustrated in Figure 1B.

Propranolol effect on VEGF secretion in PHA-stimulated human PBMCs

VEGF production was rather low in unstimulated human PBMCs, but PHA (10 µg/ml) significantly increased VEGF production in human PBMCs after 48 h incubation time as can be seen in Figure 2 ($P < 0.05$).

Propranolol significantly decreased VEGF production by human PBMCs in the presence or absence of PHA (Figure 2). This decrease was shown at 0.034 mM concentration of the drug after 48 h incubation time ($P < 0.05$).

DISCUSSION

Anti-inflammatory effect of beta-blockers may have potential implication in some immune-based disorders such as autoimmune diseases (Nguyen et al., 2008). The anti-inflammatory effects of propranolol, as a non selective beta-adrenergic blocker, have been shown by several studies (Benish et al.,2008; Nguyen et al., 2008; Kato et al., 2009). PBMCs have essential role in inflammatory reactions (Hagikura et al., 2009). In the present study we assessed the propranolol effect on proliferative response and VEGF production in human PBMCs.

As our results demonstrated, propranolol profoundly decreased the proliferation of PHA-stimulated human PBMCs at ≥ 0.17 mM concentration after 12 h incubation time onwards (Figures 1A and B). These results are in concordance with our prior study (Hajighasemi and

Mirshafiey, 2009) in which propranolol showed a cytotoxic effect on some human leukemic cell lines at ≥ 0.2 mM concentration after 12 h incubation time onwards *in vitro*. Cytotoxicity of some beta-blockers including propranolol on different cell lines has been reported by other investigators (Hajighasemi and Mirshafiey, 2009; Kastelova et al., 2003; Cheong et al., 2008). In Kastelova et al. study, propranolol caused a cytotoxic effect at 0.001 - 1 mM concentration in rat type II pneumocytes, rat and human alveolar macrophages and human lung adenocarcinoma cell line A549 (Kastelova et al., 2003). The toxic dose of propranolol in present study (≥ 0.17 mM) is almost near to that in our previous study (≥ 0.2 mM) (Hajighasemi and Mirshafiey, 2009). In Kastelova et al. study, different cells showed different sensitivities to propranolol. The discrepancy between our results and Kastelova et al. (2003) study may be due to a number of facts including type of cells and time intervals used in these studies. In the present study, human PBMCs were utilized and assessments were done at 12, 24 and 48 h incubation time, whereas in Kastelova et al study, different cell types (tumoral and normal) with different origin (human and rat) were evaluated after 3 and 20 h incubation with propranolol. Hence Kastelova et al. began assessment of propranolol cytotoxicity after 3 h incubation time, 9 h prior to our earliest time interval assessment (12 h).

In Kastelova et al. (2003), elongation of incubation time, increased propranolol cytotoxicity at ≥ 0.5 mM concentration of the drug. In our study longer incubation time increased propranolol cytotoxicity only at ≥ 0.17 mM concentration of the drug, 48 > 24 > 12 h, respectively and at 0.34 mM concentration of drug, more than 99% of the cells were dead after 12 h incubation time. Any way in the present study there was no significant difference in propranolol cytotoxicity between different incubation times at 0.34 mM concentration of drug. These results are in agreement with our previous study in which propranolol cytotoxic effect on human leukemic cell lines significantly increased with time only at 0.2 mM concentration of the drug in the following order: 48 > 24 > 12 h and at 0.4 mM concentration of propranolol, almost all the cells died after 12 h incubation time onwards. Furthermore, longer incubation time did not cause any significant difference in propranolol cytotoxicity at 0.4mM concentration of the drug (Hajighasemi and Mirshafiey, 2009). The results of present study compared with our previous findings (Hajighasemi and Mirshafiey, 2009) and with Kastelova et al. (2003), indicated that human PBMCs are less sensitive than human and rat alveolar macrophages, and rat type II pneumocytes and more sensitive than human leukemic Molt-4, Jurkat and U937 cell line and human lung adenocarcinoma cell line A549 to propranolol. These comparative results may demonstrate that different cell types have different sensitivity to propranolol and also that normal cells are more sensitive to propranolol than tumoral cells. In Kastelova et al. (2003) study, elongation of incubation time with propranolol from 3 to 20 h, increased the sensitivity of human alveolar macrophages from ≥ 1×10^{-4} M to ≥ 5×10^{-5} M drug concentration respectively while longer incubation time did not change the sensitivity of human lung adenocarcinoma cell line A549 (Kastelova et al., 2003).

We also examined the propranolol effect on human PBMCs proliferation in the absence of PHA and the results were the same as in the presence of PHA [similar to our previous work (Hajighasemi and Mirshafiey, 2009)]. These results suggest that PHA dependent proliferative mechanisms are also sensitive to propranolol. This is in agreement with reports by other researchers of inhibitory effect of propranolol on proliferation of PHA-stimulated lymphocytes in ulcer patients (Malec et al., 1990). Also increase in splenocytes apoptosis rate (Schmitz et al., 2007) and decrease of proliferative capacity of splenocytes (Oberbeck et al., 2004) after administration of propranolol in septic mice have been demonstrated. Regarding the important role of PBMCs in inflammation (Hagikura et al., 2009), the anti-inflammatory role of propranolol may be in part due to its cytotoxic effect on PBMCs. We also showed in this study that propranolol (0.034 mM) significantly decreased VEGF secretion in human PBMCs after 48 h incubation time. Our findings are in concordance with our previous study in which propranolol significantly decreased VEGF production in a number of human leukemic cell lines (Hajighasemi and Hajighasemi, 2009) and also with other studies reported the inhibitory effect of propranolol on norepinephrine mediated VEGF expression (Yang et al., 2006) and norepinephrine stimulated release of angiogenic factors (Fredriksson et al., 2000). Further-more, blocking effect of propranolol on surgical stress-increased VEGF expression, angiogenesis and tumor growth in ovarian cancer of mouse model has been reported (Lee et al., 2009). Additionally, epinephrine-induced shift toward type-2 cytokines in PBMCs has been blocked by propranolol (Yang et al., 2006). Anti-inflammatory effect of propranolol has also been shown (Benish et al., 2008; Nguyen et al., 2008; Kato et al., 2009). VEGF has an important role in inflammation (Tortora et al., 2008). Thus anti-inflammatory properties of propranolol may be in part due to its inhibitory effect on VEGF production. In this study, the cytotoxic concentration of propranolol (≥ 0.17 mM) was much higher than its inhibitory dose effect on VEGF production (≥ 0.034 mM) in human PBMCs. So the inhibitory effect of propranolol on VEGF production in human PBMCs may not be due to its cytotoxic effect and therefore reduction of cell viability. Overall, our results indicated that propranolol inhibits human PBMCs

proliferation and VEGF secretion, which may explain its anti-inflammatory properties reported by several studies (Benish et al., 2008; Nguyen et al., 2008; Kato et al., 2009). As inflammation has important role in some cardiovascular diseases (Duan et al., 2009), anti-inflammatory effect of propranolol might be another

beneficial effect of this drug in cardiovascular diseases along with its main therapeutic effects on such disorders (Bielecka-Dabrowa et al., 2007; Koelemay and Legemate, 2008). Moreover, propranolol with inhibitory effect on VEGF secretion may be a potential anti-angiogenic agent and so a promising modulator of angiogenesis related disorders.

Further studies on propranolol effect on angiogenic factors production *in vivo* as well as in different cell populations of immunocompetent cells *in vitro* are warranted.

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