THE EXPRESSION AND EFFECT OF PROGESTERONE INDUCED BLOCKING FACTOR IN PERIPHERAL LYMPHOCYTES AND IN DECIDUAL TISSUE IN NORMAL AND PATHOLOGICAL PREGNANCIES

Ph.D. Thesis

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I. LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
ConA	Concanavalin A
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocytes
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetate
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
GM-CSF	Granulocyte macrophage-colony stimulating factor
HRPO	Horse radish peroxidase
Hsp	Heat shock protein
IFNγ	Interferon gamma
IL	Interleukin
LAK	Lymphokine activated killer
LGL	Large granuled lymphocytes
LIF	Leukaemia inhibitory factor
MHC	Major histocompatibility antigens
P	Perforin
PAGE	Polyacrilamide gel electrophoresis
PBS	Phosphate buffered saline
PBL	Peripheral blood

PGE2	Prostaglandin E2
PHA	Phytohaemagglutinin
PIBF	Progesterone induced blocking factor
RSA	Recurrent spontaneous abortion
TBC	Target binding cells
TBS	Tris buffered saline
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TH	T helper
TNFα	Tumor necrosis factor alpha

II. INTRODUCTION

1. Immunology of normal pregnancy

Despite the HLA disparity between the mother and the fetus, the maternal immune system allows, and even promotes the development of the semiallogeneic fetus during the 9 months of pregnancy and at the same time protects the mother and the fetus from infections.

In an ideal situation, maternal immunosuppression should function in a selective manner to efficiently prevent antifetal responses and leave other protective features of the immune system intact. This optimal requirement is not completely fulfilled since pregnancy results in an increased susceptibility to infections[1,2] by viruses, such as hepatitis virus[3], Epstein-Barr virus[4], and rubella virus[5]. In addition, there is a decreased *in vitro* immunoreactivity to viral antigens e.g., cytomegalovirus[6].

The most profound immunological alterations occur locally at the blastocyst implantation site in the decidua, at the syntytiotrophoblast cover of the chorionic villi and at the endovascular trophoblast replacing endothelial cells in the maternal spiral arteries. Many suppressive factors, such as progesterone[7], transforming growth factor beta (TGFβ)[8], prostaglandins[9], AFP[10,11] and TJ6[12] are produced locally. In general, there is evidence to suggest that

pregnant women have depressed cell-mediated immunity and increased humoral immunity[13]. This is consistent with the concept that pregnancy is biased towards the production of T helper (TH) 2 cytokines which promote B-cell development and at the same time down regulate TH1-derived interleukin2, interferon γ and tumor necrosis factor which are considered deleterious for pregnancy[14,15]. This paradigm helps to explain why autoimmune diseases mediated primarily by T cells undergo temporary remission during pregnancy[16], while those involving excessive autoantibody production tend to be exacerbated[17]. TH1 cytokines promote NK cell reactivity which can be detrimental to the fetus[18,19]. However, NK-cell activity is reported to be diminished in the blood of pregnant women and in the fetus and newborn[20,21,22], whereas spontaneous abortion is associated with increased systemic NK activity[23]. Resorbing murine embryos are infiltrated with NK cells. Adoptive transfer of high NK activity spleen cells to pregnant mice induces abortion. NK activity is increased before term[24] in humans.

NK cells are abundant at the implantation site in many species. In the first trimester of pregnancy the majority of decidual lymphocytes are NK cells. These are nonclassical (CD3-, CD56+, CD16-) NK cells with low cytolytic activity[25,26,27,28], and are thought to play a role in the control of placentation.

2. Progesterone dependent immunomodulation

Progesterone is essential for the maintenance of pregnancy. It inhibits the contractions of myometrial smooth muscle[29] , blocks the activity of uterine collagenase[30] and modifies the activity of proteolytic enzymes. It is produced first by the corpus luteum later by the placenta. Progesterone production gradually rises during gestation, to reach a level of 3000 ng/g of placental tissue[31] . The serum concentrations of progesterone range from 100 to 500 nM during pregnancy. Blocking of progesterone binding sites by progesterone receptor antagonists causes abortion[32,33,34]. Progesterone in high local concentrations prolongs the survival of uterine skin homografts in rats[35] and in rabbits as well as renal allografts in dogs [36,37].

Peripheral lymphocytes of pregnant women develop specific progesterone receptors [38,39,40]. These receptors are different from the classical progesterone receptors[41] and they are not present on nonpregnancy lymphocytes, but become expressed after lymphocyte activation[38]. The progesterone receptor positive cells belong to the $CD8^+$ lymphocyte subset. In the presence of progesterone these cells produce a mediator protein called the Progesterone Induced Blocking Factor (PIBF).

PIBF is a 34 kDa protein with complex immunological effects. PIBF inhibits NK activity as well as the proliferation of allogeneically stimulated lymphocytes in vitro[42]. PIBF inhibits arachidonic acid release from mononuclear cells, thus inhibiting prostaglandin

synthesis[43]. Recently it has been demonstrated that PIBF influences cytokine production of lymphocytes. PIBF induces a significant increase in interleukin (IL) 10, IL3 and IL4 production by concanavalin A (ConA) activated murine spleen cells[44] and inhibits IL 12 synthesis of activated lymphocytes. Anti-PIBF treatment of pregnant mice reduced splenic IL-10 production and resulted in increased NK activity and resorption rate[45].

In healthy pregnant women there is an increase in the level of asymmetric antibodies[46], which have a mannose-rich olygosaccharide linked to one of the Fab arms of the molecule. These Ig molecules are generally unable to activate effector functions, such as complement fixation, phagocytosis and cytotoxicity, however, they can block the antigen and thus might play a role in the protection of the fetus. Data from our laboratory showed that PIBF enhances asymmetric antibody production of hybridoma cells[44]. The sera of pregnant mice treated with either RU486 or anti-PIBF antibody contained four times less asymmetric antibodies than those of normal pregnant animals[47], suggesting the role of progesterone in regulation of nonprecipitating antibody production.

3. Immunocompetent cells in the decidua

The human 1st trimester decidua is richly vascularised and contains three major cell populations:

- cuboidal cells of the glandular epithelium lining the endometrial glands
- large stromal cells with glycogen rich cytoplasm
- leucocytes

At the time of implantation a single-cell outer layer of the blastocyst, the trophectoderm, and its cellular descendant, the cytotrophoblast invade the uterine epithelium and come into direct and increasingly intimate contact with maternal decidua and blood. As the cytotrophoblast cells come into contact with blood they fuse to form nonproliferating syntitium. At other sites cytotrophoblasts invade into the decidua and form anchoring columns and penetrates the decidua as extravillous trophoblasts[48].

Maternal lymphomyeloid cells constitute a significant part of decidual cells [49]. Two main lymphocyte populations were identified in human first trimester decidua: large granular lymphocytes and as a minor component, T lymphocytes[50]. Flowcytometric analysis of separated decidual lymphocytes revealed 15% of CD3+, 11% of CD8+ and 48% of CD56+ cells[51]. Decidual NK cells express almost a three fold increase in the levels of the CD56 molecule than do peripheral blood lymphocytes. The prevalent phenotype of decidual NK cells is CD2+, CD3-, CD8-, CD56 $^{bright+}$, CD16- and perforin$^{bright+}$. Only 2% of

decidual lymphocytes show the classical CD56+, CD16+ phenotype[51]. Decidual CD56+ cells exert a low cytotoxic activity and release biologically active TGFβ in vitro[52]. Van den Heuvel at al.[53] found that 28% of mouse uterine NK cells express progesterone receptors, which raises the possibility of their participation in progesterone dependent immunomodulation.

Although CD3+ cells are a minor subset in decidual tissue, recent studies suggest that these cells are also important for maintaining pregnancy because of their ability to secrete cytokines[54,55]. Heyborne et al.[56] demonstrated a twofold increase in the frequency of γδT cells relative to all T cells in pregnant vs. non pregnant uteri, and showed that this population reacts with heat shock protein 60 (Hsp60) and responds to trophoblasts in a T cell receptor (TCR) dependent fashion[57]. These cells are only weakly cytotoxic[26] but secrete cytokines that could play a role in immunoregulation, host defence, placental growth and/or endometrial remodelling. These include colony stimulating factor 1 (CSF1), Tumor necrosis factor alpha (TNFα), interferon γ (IFNγ), TGFβ, leukaemia inhibitory factor (LIF) and granulocyte macrophage-colony stimulating factor (GM-CSF)[58]. Arck et al.[59] demonstrated that after formation of a distinct placenta γδT cells produce TGFβ-like molecules as well as IL10. The ratio of γδTCR+ lymphocytes is significantly increased in peripheral blood of pregnant women and data from our laboratory show that 97% of these express progesterone receptors[60]. CD8+ T cells were found

to bear progesterone receptors during pregnancy and in the presence of progesterone they produced PIBF.

T cells expressing αβ receptors are rare in the decidua and B cells are essentially absent except in pathologic conditions such as preeclampsia and infections[61].

The pregnant endometrium also contains macrophages, which surround, but do not damage basal plate trophoblast[62]. Haller et al.[63] found that the adherent fraction of separated decidual leucocytes (mostly macrophages) exerted a potent immunosuppressive effect on phytohaemagglutinin (PHA) induced lymphocyte proliferation. Others demonstrated, that first trimester human decidual cells and decidual macrophages suppress T lymphocyte alloreactivity in a major histocompatibility antigens (MHC) - unrestricted manner by secreting prostaglandin E2 (PGE2), which blocks the generation of IL2 receptors and production of IL2 by lymphocytes, but does not interfere with the interaction between IL2 and IL2 receptor or the lytic function of cytotoxic T lymphocytes (CTL), once generated [9, 64,65,66].

4. Immunological background of pathological pregnancies

Habitual abortion is defined as the occurrence of two or more consecutive pregnancies that have resulted in miscarriages. The risk for a new abortion increases with the number of previous miscarriages experienced by the same woman, being 20% after two, 40% after three, and at least 54% after four or more previous miscarriages[67].

In human recurrent spontaneous abortion (RSA), the abortion tissue is in most of the cases chromosomally abnormal and these women have normal blood NK cells. Approximately 60% of recurrent spontaneous abortions are unexplained by conventional genetic, anatomical, infectious and endocrinological criteria[68]. The majority of these pregnancies are characterised by immunological disorders. Immunological theories have been proposed to account for these otherwise unexplained reproductive losses since embryo and trophoblasts are semiallogenic and potential targets for rejection. In normal pregnancy, $CD8^+$ T cells upregulate their progesterone-receptors, but in RSA patients this upregulation fails to occur[69,70]. Generation of $TGF\beta_2$ related suppressor activity in decidua may also be defective in about 50% of women with RSA [71].

Recurrent spontaneous abortion is associated with increased systemic NK activity[23]. In mice, a direct relationship has been established between high NK activity and pregnancy termination. Resorbing murine embryos are infiltrated with NK cells. Adoptive

transfer of high NK activity spleen cells to pregnant mice induces abortion.

Leucocytes with cytolytic activity (cytotoxic T lymphocytes, NK cells, γδ T cells and lymphokine activated killer (LAK) cells) express perforin, which mediates the lytic activity[72]. Perforin is stored and released by cytoplasmic granules. The granules are vectorially secreted into the intracellular space formed during conjugation of the cytotoxic cells and target cells[73]. The release and the execution of the lytic function is inextricably dependent on the presence of Ca^{2+} ions. The perforin monomers can bind and insert into the target membrane and polymerise to form aggregates comprising of various numbers of monomers. Perforin monomers are thought to aggregate into a central opening (pore), which grows in diameter through the progressive recruitment of additional monomers. The perforin pores may perturb the permeability of the target membrane and lead to osmotic lysis of target cells. The protein is partially homologous to the terminal components of the membrane attack complex of complement and produces pores of up to 20 nm in diameter on target membranes[74].

Under physiological conditions perforin-containing cells in vivo are not seen in normal tissues but are found in tissues undergoing viral diseases, autoimmune diseases, or allogeneic transplant rejection, where they clearly appear to be a part of an ongoing cell-mediated immune response resulting in tissue injury[75]. The first tissue that was found to contain perforin under physiological conditions was the metrial gland. The number of perforin synthesising granulated metrial gland cells is larger than that observed in any other

pathological condition[76]. It is not yet clarified, which mechanisms ensure that this potential cytolytic activity does not disturb the development of the semiallogeneic fetus. We investigated, whether progesterone and PIBF play a role in the regulation of perforin content and perforin liberation from NK cells.

III. AIMS OF THE STUDY AND RESULTS

1. PIBF expression in peripheral blood lymphocytes of healthy pregnant women and recurrent spontaneous aborters

- Szekeres-Bartho J, Faust Zs, Varga P: The expression of a progesterone-induced immunomodulatory protein in pregnancy lymphocytes Am J Reprod Immunol 1995; 34:342-348.

Earlier data from this laboratory show that PIBF prevents abortion in murine models[41] and that neutralisation of the biological activity of PIBF in pregnant mice induces fetal loss[45]. In this study we investigated the relationship between PIBF production and the outcome of pregnancy in humans.

Lymphocytes of 96 healthy pregnant women were tested for PIBF expression by immunocytochemistry, using a PIBF-specific rabbit IgG. The mean - 2SD (13%) was considered as the cut-off between normal and lower than normal values. The percentage of PIBF-positive lymphocytes was 67 ± 2,99% in peripheral blood of healthy pregnant women. *In 62 women with pathological pregnancies as well as in 10 samples taken during spontaneous pregnancy termination, we found a significantly lower rate of positivity* (6,5 ± 1%). These values were comparable to those of nonpregnant controls.

The ratio of PIBF positive lymphocytes at different weeks of gestation did not significantly differ from each other within either the "normal" or "pathological" group. On the other hand, the values obtained in corresponding weeks of gestation were significantly higher in healthy than in pathological pregnancy.

Lymphocytes from ten patients undergoing spontaneous pregnancy termination (spontaneous abortion or preterm labour) at the time of sampling, showed a significantly lower rate of PIBF positivity than those of healthy pregnant women. We found lower than normal values in 88% of women showing clinical symptoms of threatened preterm pregnancy termination.

In order to investigate the relationship between PIBF positivity and NK activity of the lymphocytes, we simultaneously determined the two parameters. *NK activity was inversely related to the % of PIBF positive cells.* Our previous data suggested, that in normal uneventful pregnancy NK activity was lower than 40%. Therefore, we divided our risk patients into two groups: those with NK activity higher than 40% and lower than 40%. The percentage of PIBF-positive lymphocytes in the low NK activity group was significantly higher than of those with >40% NK activity.

2. The expression of PIBF in decidual lymphocytes

- Faust Zs , Laskarin G, Rukavina D, Szekeres-Bartho J: Progesterone induced blocking factor inhibits degranulation of NK cells. Am J Reprod Immunol (accepted for publication)

Decidual lymphocytes are mostly nonclassical NK cells with low cytotoxic activity. We examined whether PIBF producing cells are present in decidua, and if they might be at least partly responsible for inhibiting uterine NK activity.

We separated lymphocytes from decidual tissue and labelled them for PIBF as well as leucocyte (CD45) and NK (CD56) markers with immunocytochemistry. Sixty two per cent of isolated decidual cells with lymphocyte morphology reacted with CD45 antibody. *Ninety % of CD45+ lymphocytes were PIBF positive and 60% of the lymphocytes were double positive for CD45 and CD56.* No single CD56 positive cells were found. These data suggest that NK cells constitute 60% of all decidual lymphocytes and practically all decidual NK cells are PIBF positive.

3. The effect of progesterone and PIBF on perforin production and release by NK cells.

- Faust Zs , Laskarin G, Rukavina D, Szekeres-Bartho J: Progesterone induced blocking factor inhibits degranulation of NK cells Am J Reprod Immunol (accepted for publication)

- Laskarin G, Faust Zs, Strbo N, Sotosek V, Szekeres Bartho J, Podack ER, Rukavina D: The role of progesterone in regulation of perforin expression in decidual and peripheral blood lymphocytes. Am J Reprod Immunol (accepted for publication)

PIBF inhibits degranulation of NK cells.

In spite of their high perforin content decidual NK cells exert a low lytic activity. The latter might be due to the inability of the lymphocytes to release perforin, upon contact with the target cells. Since PIBF is present in the decidua, we investigated its effect on perforin synthesis and degranulation of NK cells.

Degranulation of NK cells was induced by incubation with the appropriate target cells. The lymphocyte-target conjugates were centrifuged on glass slides and labelled with an anti-perforin antibody. The rate of degranulation was estimated by counting perforin positive lymphocytes that were bound to the target cells under light microscope.

Among lymphocytes bound to K-562 cells, the rate of perforin positive cells was significantly higher in PIBF treated samples than in untreated ones. We found no difference between the ratio of perforin positive cells among PIBF treated and untreated, unstimulated lymphocytes suggesting that PIBF does not act on the perforin content of resting peripheral blood lymphocytes.

The effect of PIBF on the regulation of perforin content of decidual and peripheral lymphocytes

Freshly isolated decidual NK cells express high amounts of perforin. However, both the perforin content of individual lymphocytes and the ratio of perforin positive cells decreases during 18 hours of culture. Similar phenomena are observed in cultured peripheral blood lymphocytes. Coculturing of decidual lymphocytes with decidual or peripheral monocytes or their supernatants prevents the loss of perforin. We investigated the possible role of progesterone and PIBF in this regulation.

Progesterone acts on perforin expression of decidual lymphocytes both directly and indirectly. At a concentration of 20 μg/ml, progesterone inhibits perforin synthesis of decidual lymphocytes. Lower concentrations of progesterone have no direct effect on perforin expression, but exert an indirect effect via monocytes. Five μg/ml of progesterone counteracts the perforin synthesis increasing effect of decidual monocytes. Higher

progesterone concentrations further decrease the rate of perforin expression under the level of freshly isolated decidual lymphocytes.

PIBF, which mediates the immunological effects of progesterone, does not directly affect the perforin level of decidual lymphocytes, but inhibits the increasing perforin expression effect of decidual monocytes.

PIBF fails to influence the effect of decidual monocytes on perforin expression of peripheral blood lymphocytes or of peripheral monocytes on either peripheral blood or decidual lymphocytes.

IV. CONCLUSIONS

Progesterone-dependent immunomodulation is one of the mechanisms that enables the immunologically incompatible fetus to survive until term. Earlier data from our laboratory show that progesterone exerts immunological effects during pregnancy and that these effects are manifested via a 34 kDa mediator protein: the progesterone induced blocking factor (PIBF)[43]. PIBF blocks NK activity in vitro[42] and inhibits NK mediated pregnancy loss in mice[41]. In human pregnancies a direct relationship between high NK activity and pregnancy termination has not been established, however, NK activity of healthy pregnant women is significantly lower than that of non-pregnant individuals or of women with threatened premature pregnancy termination[24]. This study was aimed at investigating the role of progesterone-dependent mechanisms in this phenomenon.

In our hands the percentage of PIBF-expressing lymphocytes was significantly higher in peripheral blood of healthy pregnant women than in that of RSA patients. Eighty eight per cent of women showing clinical symptoms of threatened preterm pregnancy termination and 90% of the patients undergoing spontaneous pregnancy termination had a lower than normal rate of PIBF expression in peripheral lymphocytes.

Our findings suggest an association between pregnancy termination and the lack of lymphocytic PIBF positivity, as well as a

negative correlation between PIBF expression and NK activity. Since PIBF blocks NK activity, the lack of this protein would result in an increased NK-cell function, threatening the maintenance of pregnancy. The decrease in the rate of PIBF positive lymphocytes and the increase of NK activity occurs earlier than clinical symptoms appear.

Low PIBF expression is predictive of premature pregnancy termination. In patients whose pregnancies resulted in miscarriage or preterm delivery the % of positive lymphocytes was lower than normal in all cases and this was accompanied with high NK activity. Low PIBF values were detected as early as 10 weeks before pregnancy termination.

Altered function of peripheral lymphocytes is possibly a reflection of the events taking place at the feto-maternal interface. Decidual tissue is rich in NK cells, which, in spite of the fact that they contain the cytotoxic mediator - perforin, they exhibit no cytotoxic activity[52].

We found that CD56 positive (NK) cells present in decidual tissue strongly express PIBF. This is in line with the findings of Van den Heuvel et al.[53], who demonstrated progesterone receptors in murine decidual NK cells. On the other hand, King et al.[77] could not find progesterone receptors in human decidual large granuled lymphocytes (LGL) and T lymphocytes, whereas progesterone receptors were detected on decidual stromal cells. Therefore, PIBF could either be produced by the NK cells themselves or alternatively

PIBF produced by other nonlymphoid cells would be internalised by CD56+ cells.

Petrovic et al.[78] reported that decidual NK cells suppress PHA induced peripheral blood lymphocyte activation. Since PIBF has been shown to inhibit PHA-induced lymphocyte proliferation[42], the above effect might be due to PIBF production by decidual NK cells.

Decidual NK cells express high levels of perforin ($P^{bright+}$), thus they are potentially cytotoxic[51], yet they exert a low cytotoxic activity. It is likely that there are multiple mechanisms to hold this high cytolytic activity under control. Our experiments with peripheral lymphocytes revealed that PIBF inhibits degranulation of NK cells, but does not act on the perforin content of resting peripheral lymphocytes. Since our experience showed, all decidual NK cells were PIBF positive and PIBF inhibited degranulation of peripheral blood NK cells, we assume that this mechanism might operate locally as well and at least partly account for the low lytic activity of decidual NK cells, in spite of their high perforin content.

We found that PIBF does not influence the ratio of perforin positive decidual lymphocytes cultured alone but blocks upregulation of perforin content in decidual lymphocytes by decidual monocytes. Progesterone has a direct perforin downregulating effect in high concentrations (20 μg/ml) on decidual lymphocytes and blocks the upregulating effect of decidual and peripheral monocytes on lymphocyte perforin expression. This blocking effect is stronger on decidual lymphocytes.

The fact that supernatants of decidual monocytes exerted an effect similar to that of the cells, suggests that monocyte derived soluble factors are responsible for upregulation of perforin synthesis. The effect of decidual lymphocytes on perforin expression is abrogated by IL15 (Laskarin et al. ms in preparation), suggesting that IL15 is involved in the regulation of perforin synthesis or decidual lymphocytes.

Progesterone blocked the effect of both decidual and peripheral monocytes on perforin synthesis of decidual lymphocytes. This can be due to an inhibition of cytokine production by monocytes, or alternatively, to decreased responsiveness of decidual lymphocytes to the cytokines. The latter possibility is more likely, since high concentrations of progesterone completely counteracted the effect of decidual monocyte supernatants.

Our studies establish a relationship between progesterone, NK activity and the maintenance of normal pregnancy. The progesterone induced immunomodulatory protein, PIBF is produced by lymphocytes of healthy pregnant women, but not by those of recurrent aborters. PIBF expression in pregnancy lymphocytes is inversely related to NK activity, and the presence or absence of PIBF correlates with the outcome of pregnancy.

PIBF is present in the decidua and regulates perforin synthesis as well as perforin release from decidual NK cells. These effects might contribute to the low NK activity of decidual lymphocytes, and consequently, to the maintenance of pregnancy.

V. PAPERS

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The Expression of a Progesterone-Induced Immunomodulatory Protein in Pregnancy Lymphocytes

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The expression of a progesterone-induced immunomodulatory protein in pregnancy lymphocytes. Am J Reprod Immunol 1995; 34:342–348 © Munksgaard, Copenhagen

PROBLEM: The immunological effects of progesterone are mediated by a protein, named the progesterone-induced blocking factor (PIBF). The PIBF blocks NK activity in vitro and therefore prevents the abortive effect of high NK activity in mice. Increased NK activity has been suggested to play a role in pregnancy termination; thus NK inhibitory effect of the PIBF should contribute to the maintenance of normal gestation. This study was designed to investigate the relationship between in vivo PIBF-producing capacity and in vitro cytotoxic activity of pregnancy lymphocytes, as well as the clinical status or the outcome of pregnancy.

METHOD: Lymphocytes of 168 pregnant women (96 normal pregnancies, 16 showing clinical symptoms of threatened preterm pregnancy termination, 46 recurrent aborters, and 10 women sampled at the onset of spontaneous abortion or preterm delivery) were isolated on Ficoll-Paque gradient. The lymphocytes were tested for reactivity with a PIBF-specific antibody by immunocytochemistry, and simultaneously for cytotoxic activity to human embryonic fibroblast targets.

RESULTS: The percentage of PIBF-positive lymphocytes in peripheral blood of healthy pregnant women was significantly higher than in that of women at risk for premature pregnancy termination. In peripheral blood of patients undergoing spontaneous pregnancy termination at the time of sampling, and in those of women showing symptoms of premature pregnancy termination we found lower than normal percentage of PIBF-positive cells. PIBF expression of the lymphocytes showed an inverse correlation with NK activity, and the rate of PIBF positive lymphocytes was related to the outcome of pregnancy.

CONCLUSION: These data suggest a strong relationship between PIBF producing capacity as well as NK activity of the lymphocytes and the success of gestation.

Key words:
Progesterone-dependent immunomodulation, NK activity, pregnancy termination

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INTRODUCTION

An increasing body of evidence suggests that immunological mechanisms play a key role in the maintenance of pregnancy. The homeostasis created by a bidirectional interaction between the immune and endocrine systems guarantees the success of gestation.

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The reproductive steroid, progesterone is an important component of this system. The presence of this hormone is indispensable for normal gestation in humans;[1,2] furthermore, its supportive immunological effects have been demonstrated both in vitro and in vivo.[3,4]

The immunomodulatory effects of progesterone are mediated by a protein named the progesterone-induced blocking factor (PIBF), which 1) inhibits the release of arachidonic acid,[5] 2) is endowed with immunomodulatory properties,[6] and has 3) antiabortive[7] properties.

The immunological effects of the PIBF include inhibition of NK activity and proliferation of allogeneically stimulated lymphocytes,[6] induction of cells with suppressor function[6] as well as the establishment of a Th2 biased immune response.[8]

Nonspecific immune effectors play a key role in pregnancy termination. Gendron and Baines[9] have demonstrated murine resorptions with considerable NK infiltration. DeFougerolles and Baines[10] have successfully influenced resorption rates by modulating NK activity in mice. Kinsky et al.[11] have induced resorptions in Balb/c mice by adoptive transfer of spleen cells from poly I:C treated mice. In the latter system, resorptions could be prevented by simultaneous administration of either anti NK serum[11] or PIBF.[7] In normal human pregnancy, NK activity is significantly lower than in nonpregnant individuals, whereas idiopathic spontaneous abortions are often associated with increased NK activity. Finally, there is a significant increase in NK activity before human parturition.[12]

The PIBF blocks NK activity in vitro[5] and prevents resorptions induced by transfer of high NK activity spleen cells.[7] These effects might be one of the factors controlling the rate of NK activity during pregnancy, thus PIBF-producing capacity of pregnancy lymphocytes might be crucial for the outcome of pregnancy.

The aim of this study was to investigate PIBF expression on pregnancy lymphocytes as well as the relationship between PIBF expression and NK activity. An additional goal of the project was to correlate these factors with clinical symptoms and the outcome of pregnancy.

MATERIALS AND METHODS

Patients

One hundred seventy-five pregnant women were included in the study (Table I). Lymphocytes of 103 women between the 9th and 40th week of gestation (7 in the 1st trimester, 35 in the 2nd trimester, and 61 in the 3rd trimester) were tested, in order to establish normal values of PIBF positivity during pregnancy.

Ten women were sampled at the onset of spontaneous abortion or preterm delivery. The study group at risk for premature pregnancy termination consisted of 62 patients. Sixteen of them showed clinical symptoms (bleeding or regular uterine contractions) of threatened abortion or threatened preterm delivery.

Forty-six pregnant women between the 8th and 31st week of gestation had a history of two or more unexplained spontaneous miscarriages and no living child. Ten healthy nonpregnant individuals formed a control group.

Production of PIBF-Specific IgG

Ten million/ml lymphocytes of healthy pregnant women were incubated with 20 μg/ml of progesterone overnight at 37°C in 5% CO_2 atmosphere. Supernatants were collected and dialysed against 400× volume of PBS to remove progesterone. The dialyzed supernatants were concentrated on Amicon PM10 filters 1,000-fold, and 100 μg of the concentrated supernatant was subjected to electrophoresis on a 10% linear SDS-polyacrylamide gel and the separated proteins were blotted to nitrocellulose filters. The 34 kDa band was dissolved in DMSO and emulsified with 1 ml of complete Freund's adjuvant (Difco). This was divided into three parts and administered i.m., s.c., and i.p. to rabbits weighing 4 kg each. The animals were boosted with the antigen in incomplete Freund's adjuvant at 2-week intervals. Blood samples were drawn at the 10th day after each boost and the antibody content of the serum was determined by ELISA. IgG was purified on protein A Sepharose columns.

Immunocytochemistry

Lymphocytes were isolated from heparinized venous blood on Ficoll-Paque gradient, washed once, and

TABLE I. Gestational Age of Pregnant Women Included in the Study

	Weeks of Gestation		
No. of patients	<14	<28	>28
Normal Pregnancy (N=103)	7	35	61
Pathological Pregnancy (N=72)	29	32	11

centrifuged on glass microscope slides. After drying at room temperature, the cells were fixed for 5 min in cold acetone and washed in phosphate buffered saline (PBS).

All incubations were performed at room temperature in a humidified atmosphere. The primary antibody (anti-PIBF IgG) was applied in a concentration of 10 μg/ml for 1 h. Peroxidase labeled anti-rabbit IgG (Dakopatts, Hungary) was used in a dilution of 1:200 for 45 min. The reaction was developed by aminoetylcarbasol, nuclei were counter stained with hematoxylin, and slides were mounted with gelatine-glycerol. The percentage of positive cells was determined by light microscopy counting 300 cells.

Cytotoxicity Assay

This technique has been described elsewhere in details.[7] Briefly, human embryonic fibroblasts derived from 10 to 12 week embryos were used as targets. Cells were seeded on ninety-six well Nuclon tissue culture plates at a density of 5,000 target cells/well in 0.2 ml of medium 199 supplemented with 10% fetal calf serum. The target cells were allowed to attach by overnight incubation. The following day the medium was replaced by 0.2 ml of lymphocyte suspension containing 5×10^5 lymphocytes in 0.2 ml of the same medium. After 16 h of incubation, the plates were washed with PBS three times in order to remove lymphocytes and damaged target cells. Then the substrate of alkaline phosphatase (Sigma tablets No. 104) was added to the wells in diethanolamine buffer at a concentration of 1 mg/ml. The plates were incubated for 10 min at 37°C in the dark and the resulting yellow reaction product was quantitated photometrically at 405 nm. The percentage reduction in enzyme activity relative to the target cell control was considered as a measure of cytotoxicity.

Statistical Analysis

The two tailed Student's t-test was used for statistical analysis of the data. Mean ± SEM are indicated in the tables and figures. Differences were considered to be significant at $P < 0.05$.

RESULTS

PIBF Expression on the Lymphocytes in Uneventful and Pathological Pregnancies

Lymphocytes of 96 healthy pregnant women were tested for PIBF expression by immunocytochemistry, using a PIBF-specific rabbit IgG. The mean – 2SD (13%) was considered as a cutoff between normal and lower than normal values. The percentage of PIBF-positive lymphocytes was 67 ± 2.99% in peripheral blood of healthy pregnant women. In 62 women with pathological pregnancies (clinical symptoms of threatened preterm pregnancy termination or a history of habitual abortions) as well as in 10 samples taken during spontaneous pregnancy termination, we found a significantly lower rate of positivity (6.5 ± 1%) (Fig. 1). These values were comparable to those of nonpregnant controls (data not shown). Eight of the 72 patients had higher than 13% PIBF positive cells.

The ratio of PIBF positive lymphocytes at different weeks of gestation did not significantly differ from each other within either the "normal" or "pathological" groups, on the other hand the values obtained on corresponding weeks of gestation were significantly higher in healthy, than in pathological pregnancy (Table II).

PIBF Expression on Lymphocytes During Pregnancy Termination

Lymphocytes of ten patients undergoing spontaneous pregnancy termination (spontaneous abortion or preterm labor) at the time of sampling, showed a significantly lower rate of PIBF positivity than those of healthy pregnant women (Fig. 2). Reduced PIBF expression in these patients was accompanied with an elevated NK activity. In 90% of the cases, the percentage of PIBF-positive lymphocytes was lower than 13% and NK activity values were above 40% (Table 3).

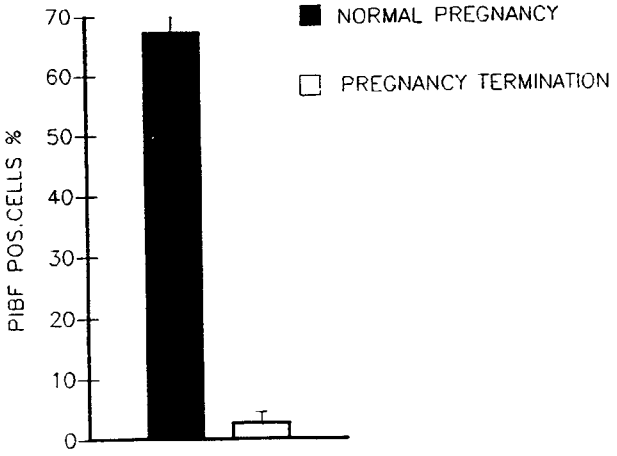

Fig. 1. The percentage of the PIBF positive cells in peripheral blood of healthy pregnant women is significantly higher than in that of women with pathological pregnancies. The bars represent mean ± SEM of 103 and 72 determinations, respectively. *$P < 0.001$.

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TABLE II. PIBF Positivity of Pregnancy Lymphocytes In Different Weeks of Gestation

Healthy Pregnant Women	Weeks of Gestation	Pathological Pregnancies
	5–8	8.7 ± 2.4% (N=9)
69 ± 5.0% (N=3)*	9–11	12.4 ± 5.0% (N=9)
64 ± 3.0% (N=4)*	12–14	5.5 ± 1.7% (N=11)
74 ± 5.9% (N=16)*	15–18	6.2 ± 2.1% (N=12)
	19–21	2.7 ± 1.8% (N=5)
68 ± 8.0% (N=17)*	23–25	3.7 ± 1.9% (N=10)
63 ± 6.2% (N=26)*	26–28	6.4 ± 1.5% (N=5)
58 ± 11% (N=14)*	29–32	2.5 ± 2.1% (N=7)
67 ± 8.0% (N=11)*	33–36	4.9 ± 2.1% (N=4)
74 ± 5.0% (N=12)	38–40	

*Significantly different from pathological pregnancies, $P < 0.01$.

Threatened Premature Pregnancy Termination and PIBF-Producing Capacity of the Lymphocytes: Relationship Between Clinical Symptoms and PIBF Positivity

Lymphocytes of 16 women with clinical symptoms of premature pregnancy termination (bleeding or uterine contractions) were tested for reactivity with the PIBF-specific antibody. The percentage of PIBF-positive cells in PBL of women with threatening symptoms at the time of sampling was significantly lower (5.19 ± 2.2%), whereas NK activity was significantly higher (59 ± 7.37%, not shown on the figure) than those values measured in healthy pregnant women (Fig. 2). In 2 out of 16 patients the PIBF values fell in the normal range, whereas 3 of 16 patients demonstrated an NK activity of <40% (Table III).

Due to the small number of completed pregnancies the predictive value of the test could not be determined. However, in five patients whose pregnancies resulted in miscarriage or preterm delivery, the % of PIBF-positive peripheral lymphocytes was < 13% in all cases. Additionally, only one out of five women showed NK activity under 40% (Table IV). The low PIBF values were detected as early as 10 weeks before pregnancy termination. In 3 women whose pregnancies resulted in term delivery of mature babies both the PIBF and NK values fell in the normal range (Table IV).

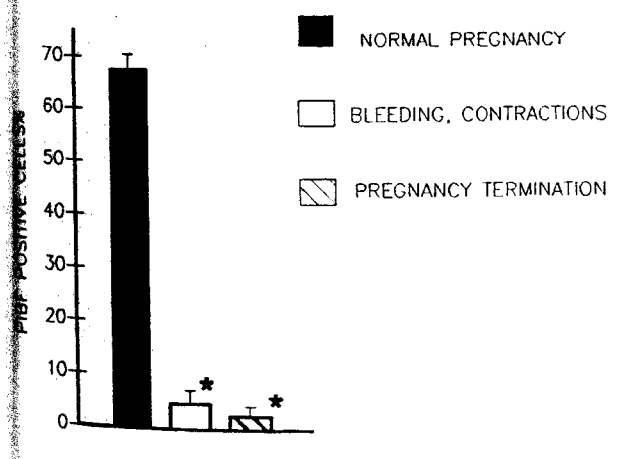

Fig. 2. PIBF-positive cells in peripheral blood of women showing threatening clinical symptoms as well as in samples taken at the termination of pregnancy. The bars represent mean ± SEM of 103, 16, and 10 determinations, respectively. *$P < 0.001$.

PIBF and NK Activity

In order to investigate the relationship between PIBF positivity and NK activity of the lymphocytes, we determined simultaneously the two parameters. NK activity was inversely related to the % of PIBF positive cells ($y = 18.46\text{-}0.15\times$ $r = -0.72$). Our previous data suggested that in normal uneventful pregnancy NK activity was lower than 40%. Therefore, we divided our risk patients into two groups; those with lower and with higher NK activity than 40%. The percentage of PIBF-positive lymphocytes in the low NK activity group was significantly higher (29 ± 3%) than of those with >40% NK activity (5.8 ± 1.5%) (Fig. 3). In the <40% NK activity group, 6 out of 18 cases showed PIBF expressions lower than normal. In the >40% NK activity group 6 out of 27 patients possessed PIBF values within the normal range.

TABLE III. The Incidence of Higher Than Normal NK Activity and Lower Than Normal PIBF Positivity in Pathological Pregnancies

Labor, Spontaneous Abortion		Bleeding, Contractions		Recurrent Spontaneous Abortion	
NK>40%	PIBF<13%	NK>40%	PIBF<13%	NK>40%	PIBF<13%
a/b	a/b	a/b	a/b	a/b	a/b
9/10	9/10	13/16	14/16	40/46	41/46

[a]Number of patients with out of range values.

[b]Total number of patients.

DISCUSSION

Lymphocytes of pregnant women develop specific progesterone receptors.[13–15] Steroid hormones act according to the "hit and run" mechanism. Binding to the receptor induces a structural alteration or "transformation" of the receptor, which in turn, enables the complex to bind specific DNA sequences. This might result in selective transcription of target genes, with ultimate synthesis of key mediator proteins.[16]

Critical immunomodulatory effects of progesterone are manifested via a 34 kDa protein,[5,6] which exerts an anti-abortive effect in mice.[7] Recently Beaman et al. described a similar, steroid hormone-dependent immunoregulatory factor TJ6,[17] expressed predominantly on CD19+B cells of pregnant, but not of non-pregnant women.[18]

In this study we established a relationship between in vivo PIBF-producing capacity and in vitro cytotoxic activity of pregnancy lymphocytes. In peripheral blood of healthy pregnant women, the percentage of PIBF-positive lymphocytes was significantly higher—in all trimesters of pregnancy—than in that of women showing clinical symptoms of threatened preterm pregnancy termination. Similarly, lower than normal PIBF positivity was found in recurrent spontaneous aborters and among patients undergoing spontaneous pregnancy termination (spontaneous abortion, preterm labor or labor) at the time of sampling.

TABLE IV. The % of PIBF-Expressing Lymphocytes in Peripheral Blood and the Outcome of Pregnancy

Pregnancies Ending up in Term Labor		Pregnancies Resulting in Miscarriage or Preterm Delivery	
NK%	PIBF%	NK%	PIBF%
0	22	62	0
27	23	35	9
19	13	57	0
		95	0
		58	0

By now it is well established that nonspecific immune effectors, e.g., NK cells, play a part in fetal wastage in mice. Gendron and Baines[9] as well as de Fougerolles and Baines[10] suggested the involvement of increased NK activity in murine abortions. In human pregnancy, we demonstrated a significant increase of NK activity prior to term.[12] Taken together, these data suggest a relationship between pregnancy termination and increased NK activity. The mechanism through which increased NK activity might contribute to pregnancy termination is not completely understood. It might involve the action of various cytokines since, although the trophoblast resists NK mediated lysis in vitro, it is susceptible to lysis by lymphokine activated killer (LAK) cells.[19,20] NK like cells may cause a fetal loss by being converted into lymphokine activated killer cells in the presence of TNFα and IL-2.[21] The PIBF blocks NK mediated lysis in vitro[5] and counteracts the abortive effect of high NK activity in mice.[7] Therefore, PIBF production might well correlate with, and also indicate the rate of NK activity.

Our findings suggest a close relationship between threatening clinical symptoms and the lack of PIBF positivity. Since the PIBF normally blocks NK activity, the lack of this protein would result in increased NK activity. In fact, increased NK activity is associated with term as well as pre-term labor.

We found an inverse correlation between the percentage of PIBF-positive lymphocytes and NK activity. At present there is no direct proof for the direct role for increased NK activity in the etiology of human spontaneous pregnancy termination. However, data obtained in murine models suggest a direct cause-effect relationship.[9–12]

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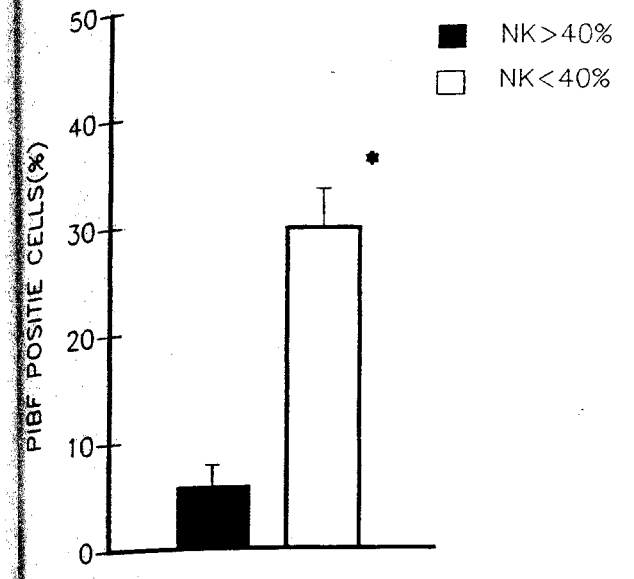

Fig. 3. PIBF positivity in peripheral blood of women with high and low NK activity. The bars represent mean ± SEM of 27 and 18 determinations respectively. $*P < 0.001$.

Roussev et al.[14] have demonstrated progesterone receptor positive lymphocytes in placental cell suspension. In placental supernatants we identified a substance reactive with PIBF-specific antibody (unpublished). These findings make it conceivable that the PIBF is produced locally at the fetomaternal interface. During labor, spontaneous abortion, or premature labor, there is a drop in progesterone receptor expression of pregnancy lymphocytes.[7] Since functional progesterone receptors are required for PIBF production, reduced PIBF positivity of lymphocytes might be an indicator of threatened premature pregnancy termination.

Pregnancy lymphocytes are permanently exposed to progesterone, thus if they express functional progesterone receptors they should produce the PIBF. Therefore, a high rate of PIBF positivity is expected in normal pregnancy. The PIBF blocks NK activity in vitro; consequently, PIBF-producing lymphocytes should be less cytotoxic than nonproducing ones. Miscarriage is characterized by a high rate of NK activity. In our experience, 89% of women sampled during pregnancy termination had a lower than normal % of PIBF positive cells.

These data prompted the following hypothesis: Due to the expression of progesterone receptors, pregnancy lymphocytes produce an immunomodulatory protein (PIBF). Among other effects, the PIBF blocks NK activity in vitro and in vivo. Preceding pregnancy termination, progesterone receptors disappear from peripheral pregnancy lymphocytes, thus, even if progesterone is present at biologically sufficient concentrations, they are unable to produce the PIBF.

Acknowledgments

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Progesterone-Induced Blocking Factor Inhibits Degranulation of Natural Killer Cells

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Faust Z, Laškarin G, Rukavina D, Szekeres-Bartho J. Progesterone-induced blocking factor inhibits degranulation of natural killer cells. AJRI 1999; 00:00–00 © Munksgaard, Copenhagen

PROBLEM: During the first trimester of pregnancy, nonclassical ($CD3^-$, $CD56^+$, $CD16^-$, perforin $[P]^{bright+}$) natural killer (NK) cells comprise the major decidual lymphocyte population. These cells, in spite of their high perforin content, exert a low cytolytic activity. Peripheral blood lymphocytes of healthy pregnant women produce progesterone-induced blocking factor (PIBF), which inhibits NK activity. PIBF-producing cells are likely to be present in decidua and might contribute to low decidual NK activity.

METHODS: Decidual cells obtained from elective pregnancy termination were double labeled for CD56 and PIBF. We tested the effect of PIBF on perforin liberation by activated peripheral blood NK cells.

RESULTS: Sixty percent of decidual lymphocytes were CD56+ and expressed PIBF at the same time. PIBF-treated and untreated peripheral blood NK cells were incubated with K-562 cells, and perforin content of target conjugated NK cells was detected with immunocytochemistry. PIBF treatment of peripheral blood lymphocytes significantly reduced lysis of K-562 cells. Among target bound lymphocytes in PIBF-treated samples, we found a significantly ($P < 0.01$) higher rate of P^+ cells than in untreated samples.

CONCLUSIONS: These data suggest that PIBF inhibits cytotoxicity of NK cells via a block of degranulation, and since decidual NK cells are $PIBF^+$, it cannot be ruled out that this effect of PIBF contributes to low decidual NK activity.

Key words:
Decidua, degranulation, NK cells, perforin, PIBF

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INTRODUCTION

In normal human pregnancy, natural killer (NK) activity is significantly lower than that in nonpregnant individuals, and there is a significant increase of NK activity before human parturition.[1] Furthermore, the level of perforin expression at the time of parturition is significantly higher than in the first trimester of pregnancy.[2] One of the mechanisms by which NK cells kill their target cells is exocytosis of perforin and serine esterase-containing granules in the contact area between effector and target cells.[3] In contrast to the 25–27% perforin positivity of first-trimester peripheral blood pregnancy lymphocytes, in the decidua, 55% of lymphocytes were simultaneously found to be perforin

$[P]^{bright+}$.[4] Perforin-expressing decidual lymphocytes are prevalently nonclassical NK cells ($CD3^-CD56^{bright+}CD16^-$).[3] In spite of the high perforin content, these cells exert a low cytotoxic activity.[5-7] The question of how the strong cytotoxic machinery of decidual lymphocytes is held under restraint is as yet unanswered.

Alteration of cell-mediated immunity during pregnancy involves regulation by serum factors[8] and substances present in placental supernatants.[9] Progesterone is indispensable for the maintenance of pregnancy in humans. The immunological effects of progesterone are manifested via mediators.[10,11] Peripheral blood lymphocytes of pregnant women develop specific progesterone receptors.[12-14] In the presence of progesterone, these cells produce progesterone-induced blocking factor (PIBF), which has complex immunological effects. PIBF enhances asymmetric antibody production of hybridoma cells[15] and inhibits interleukin (IL)-12 expression on activated lymphocytes (manuscript in preparation). Anti-PIBF treatment reduced splenic IL-10 production in mice and resulted in increased resorption rate.[16] PIBF inhibits NK activity and proliferation of allogeneically stimulated lymphocytes *in vitro*.[17] NK activity in pregnant women is inversely related to the rate of PIBF positivity of pregnancy lymphocytes.[18]

In the peripheral blood of healthy pregnant women, the percentage of $PIBF^+$ lymphocytes was significantly higher in all trimesters of pregnancy than in women showing clinical symptoms of threatened preterm pregnancy termination. Similarly, lower than normal PIBF expression was found in recurrent spontaneous aborters.[18]

In this study we investigated the possible presence of PIBF in decidual tissue, where it could be one of the factors affecting NK activity. The high perforin content of NK cells, but low cytotoxic activity, might be a result of inhibition of perforin liberation from the granules of cytolytic cells. We examined the possibility that PIBF can account for the inhibition of NK cell degranulation.

MATERIALS AND METHODS

Preparation of Decidual Leukocytes

Human decidua was obtained from first-trimester elective pregnancy termination. Decidua separated from fetal and placental tissue was excessively washed in Roswell Park Memorial Institute (RPMI) medium 1640. The tissue was cut into small pieces and exposed to 0.125% trypsin and 0.02% EDTA at 37°C for 90 min. The resulting cell suspension was filtered through sterile nylon mesh to eliminate tissue debris. The cells were centrifuged on Ficoll–Hypaque gradient ($800 \times g$, 20 min). Cells at the interface were removed and washed twice in RPMI 1640. The cells were resuspended at a concentration of 10^6 cells/mL in RPMI and 100-μL aliquots of the suspension were centrifuged on glass microscope slides (500 rpm, 5 min). After drying at room temperature, the cells were fixed for 10 min in cold acetone. The slides were stored at 4°C until labeling.

Preparation of Peripheral Blood Lymphocytes

Heparinized peripheral blood was obtained from ten nonpregnant women. Mononuclear cells were separated by Ficoll–Hypaque gradient centrifugation, washed twice in RPMI 1640 medium containing 10% fetal calf serum, and resuspended in the same medium at a concentration of 10^6 cells/mL.

Test for Degranulation

Peripheral blood lymphocytes were incubated with or without PIBF for 2 hr at 37°C in a humidified atmosphere with 5% CO_2. Both PIBF-treated and untreated lymphocytes were incubated with or without K-562 cells (10^6 cells/mL) at a ratio of 1:1 for an additional 2 hr. After the incubation, the cells were carefully resuspended, centrifuged on microscope slides, and fixed as described above. The rate of degranulation was estimated by counting P^+ target-bound lymphocytes.

Immunocytochemistry

All incubations were performed at room temperature in a humified atmosphere. Endogenous peroxidase was blocked with H_2O_2. Cell smears were first incubated with 1% bovine serum albumin (BSA) for 20 min. Each sample was incubated with appropriately diluted primary antibody for 60 min and with the secondary antibody for 30 min. The reaction was developed with aminoethylcarbasol or with diaminobenzidine (DAB). After DAB, we applied silver intensification. The nuclei were counterstained by hematoxylin.

The following primary antibodies were applied: anti-human perforin (IgG2b purified from Balb/c ascites) at a dilution of 3 μg/100 μL; anti-human PIBF (IgG, rabbit)[19] at a dilution of 2 μg/100 μL; anti-human CD45 (mouse ascites fluid, Sigma-Aldrich, Hungary) at a dilution of 0.2 μg/100 μL; and anti-human CD56 (mouse ascites fluid, Sigma-Aldrich, Hungary) at a dilution of 8 μg/100 μL. Goat anti-mouse (1 μg/100 μL) or goat anti-rabbit (0.125 μg/100 μL)

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peroxidase-conjugated antibodies (Dakopatts, Hungary) were applied as the secondary antibodies.

RESULTS

CD56+ Decidual Cells Produce PIBF

Sixty-two percent of isolated decidual cells showing a lymphocyte morphology reacted with CD45 antibody. Decidual leukocytes were double labeled with anti-PIBF and with anti-CD56. Approximately 60% of these cells were double positive for both PIBF and CD56, 26.6% for PIBF, and only 2.16% of $CD56^+$ cells did not express PIBF (Table I and Fig. 1).

PIBF Inhibits Perforin Liberation from the Granules of Stimulated NK Cells

In PIBF-treated peripheral blood lymphocytes, the rate of P^+ cells among those conjugated with K-562 was significantly higher than in untreated samples (Figs. 2 and 3) We found no difference between the ratio of P^+ cells among PIBF-treated and untreated unstimulated lymphocytes, suggesting that PIBF does not act on perforin content of resting human lymphocytes (not shown).

DISCUSSION

Bone-marrow-derived cells infiltrating the decidua mediate both specific immune responses and immunosuppression.[20–23] Both the adherent and nonadherent fraction of human decidual cells were found to exert a potent immunosuppressive effect on PHA-induced proliferation of peripheral lymphocytes.[24]

Immunohistochemical study of leukocytes in the endometrium and decidual tissue revealed a significant increase of $CD56^+$ cells in the first trimester and a dramatic decrease in the number of these cells in the third trimester.[25] Similar phenomena were observed in rodents and humans; uterine NK cells increase in number during estrus and proestrus in rodents[26] or during the postovulatory luteal phase in humans,[25] and these changes appear to be under hormonal control. In these species, uterine NK cells are present during early gestation, particularly at sites where fetal trophoblast cells invade the maternal uterine lining.[26,27] Contrary to these results, our immunohistochemical analysis of perforin-expressing lymphocytes in both decidua basalis and decidua parietalis showed a higher number of P^+ cells in decidua parietalis in

TABLE I. The Percentage of Progesterone-Induced Blocking Factor (PIBF)-Positive Natural Killer Cells Among Decidual Lymphocytes

$PIBF^+CD56^-$ cells (%)	$CD56^+PIBF^-$ cells (%)	$PIBF^+CD56^+$ cells (%)
26.6 ± 7.2 $n=3$	2.16 ± 0.9 $n=3$	60.05 ± 2.8 $n=3$

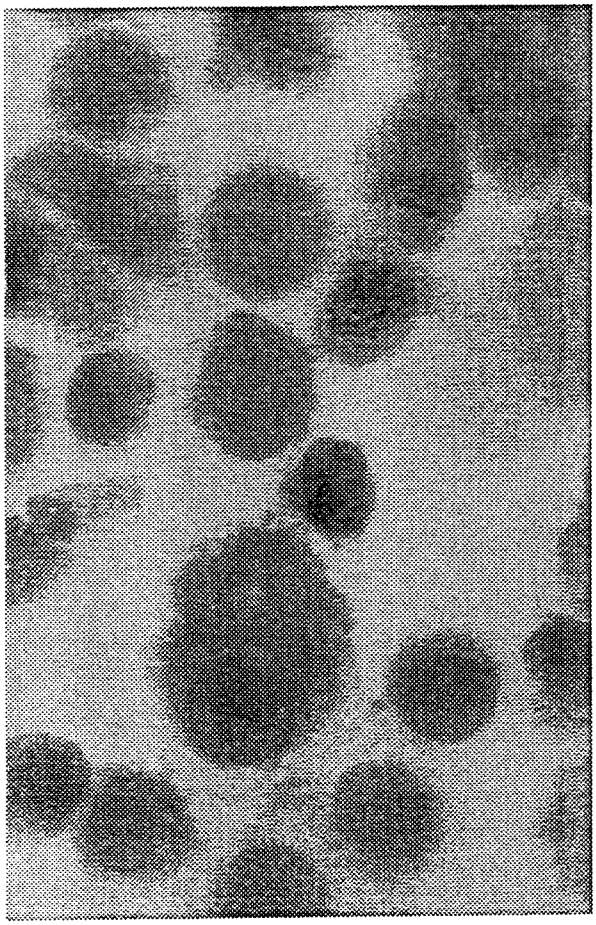

Fig. 1. Freshly isolated decidual cells labeled with anti-CD56 (silver) and anti-PIBF (AEC) antibody. 1000 × magnification.

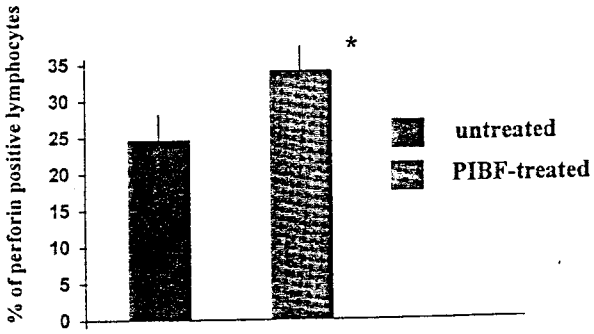

Fig. 2. The percentage of P^+ cells among target bound, PIBF-treated and untreated peripheral blood lymphocytes. The bars represent the mean ± SD of ten experiments. * Significantly different from the control at $P < 0.01$.

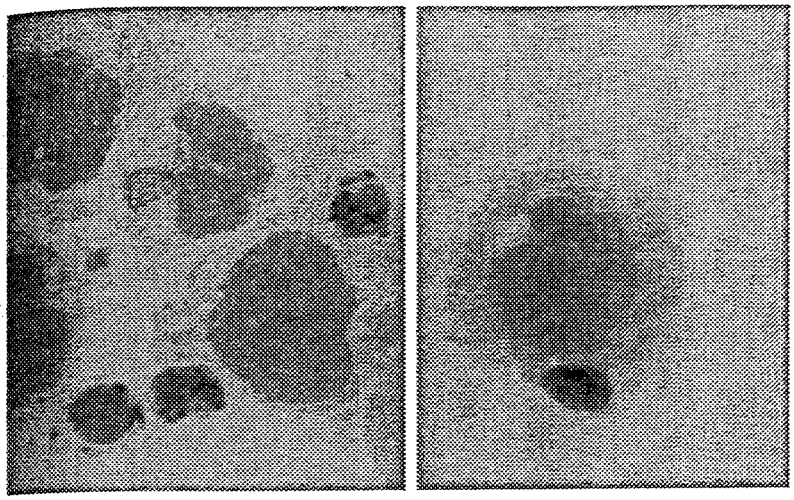

Fig. 3. Perforin positivity of peripheral blood lymphocytes conjugated to K-562 cells. (a) PIBF-treated lymphocytes; (b) untreated lymphocytes. 1000× magnification.

the vicinity of noninvasive trophoblast than in decidua basalis. The prevalent phenotype of these cells is $CD3^-$, $CD4^-$, $CD8^-$, $CD56^+$, $CD16^-$, $P^{bright+}$. Hameed et al.[28] found P^+ cells in the normal cycling endometrium and showed that recruitment of these cells is hormonally responsive and correlates with progesterone levels. Perforin-containing endometrial lymphocytes increased in number in mid through late secretory phase, but were completely absent in postmenopausal atrophic endometrium. We have found that activated cytolytic cells (P^+) are reduced in pathological pregnancies both in prevalence and the level of perforin expression per cell compared to normal pregnancy.[29]

Many potentially immunosuppressive factors, e.g. TJ6,[30] PGE2,[23] progesterone,[31] and Th2 type cytokines,[32–34] are present in decidual tissue.

Our present data show that CD56+ cells in the decidua strongly express PIBF. This is in line with the findings of van den Heuven et al.,[35] who demonstrated progesterone receptors in a murine decidual NK cell line. On the other hand, King et al.[36] could not find progesterone receptors in human decidual LGL and T lymphocytes, whereas progesterone receptors were detected on decidual stromal cells. Therefore, PIBF, which was found in $CD56^+$ cells in our experiments, could have been produced by other nonlymphoid cells and internalized by $CD56^+$ cells. Petrovic et al.[37] reported that decidual NK cells suppress PHA-induced peripheral blood lymphocyte activation. Since PIBF has been shown to inhibit PHA-induced lymphocyte proliferation,[17] the above effect might be due to PIBF production by decidual NK cells

Decidual NK cells express a high level of perforin ($P^{bright+}$); thus, they are potentially cytotoxic,[4] yet they exert a low cytotoxic activity. It is likely that there are multiple mechanisms to hold this high cytolytic content under control.

In our study, PIBF inhibited perforin liberation from activated peripheral NK cells. Since all decidual NK cells were $PIBF^+$ and PIBF inhibited degranulation of peripheral blood NK cells, we assume that this mechanism might operate locally, as well and at least partly accounting for the low lytic activity of decidual NK cells in spite of their high perforin content.

Acknowledgments

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PROGESTERONE DIRECTLY AND INDIRECTLY AFFECTS PERFORIN EXPRESSION IN CYTOLYTIC CELLS

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Running head: Progesterone affects perforin expression in decidual lymphocytes

Problem: Decidual lymphocytes expressing the cytolitic molecule perforin (P) represent approximately 55% of decidual lymphocytes in the first trimester of human pregnancy. Progesterone dominates this phase of pregnancy and controlling the production of uterine cytokines and growth factors. The aim of this study was to investigate the role of progesterone and progesterone induced blocking factor (PIBF) on perforin expression in decidual and peripheral blood lymphocytes (DL and PBL).

Method: Perforin expression was analysed in PBL and DL incubated either in culture medium or with decidual adherent cells (DAC) and peripheral blood adherent cells (PBAC) and their supernatants with or without progesterone or PIBF. P was detected by flow cytometry in PB and in decidual first trimester pregnancy lymphocytes.

Results: Progesterone in high concentrations directly affects P expression in decidual lymphocytes but not in peripheral blood lymphocytes. Progesterone in a concentration dependent manner indirectly blocks P expression in DL and PBL cultured with adherent cells or their supernatants. PIBF blocked upregulation of P expression of DL cultured with DAC, but non of those cultured with PBAC. Similarly, PIBF was inefficient when PBL or DL were cultured with PBAC.

Conclusion: Progesterone present in a high concentration locally at the maternal-fetal interface modulates P expression in the FTP decidual lymphocytes.

Key words: decidual granular lymphocytes, first trimester pregnancy, perforin, peripheral blood lymphocytes, progesterone, PIBF

INTRODUCTION

Recent studies showed that perforin (P), granzyme (G) and FasL/Fas-based mechanisms are used by cytolitic effector cells, both by cytolitic T lymphocytes (CTL) and natural killer (NK) cells, as rapid cytolitic pathways.[1-4] The accumulation of P+ cells in the metrial gland of pregnancy decidua in mice[5] and human first trimester pregnancy decidua,[6-8] suggests potential physiological and pathological significance of perforin-mediated cytotoxicity. Hameed et al.[9] found P+ cells in the normal cycling endometrium and showed that recruitment of P+ cytolitic cells is hormone responsive and correlates with progesterone stimulation in first trimester pregnancy. Progesterone is produced locally, at the maternal-fetal interface in much higher concentrations (3 μg/g of tissue) than that found in peripheral blood of pregnant women.[10] Progesterone has many physiological functions. Progesterone is important in the control of production of a host uterine cytokines and growth factors.[11,12] Progesterone itself appears to favour the development of human Th2 type cells.[13] It is well accepted that the prevalence of Th2 type response over Th1 at the maternal/fetal interface is a characteristic of human pregnancy.[14]

Immunosuppressive actions of progesterone are mediated by progesterone-induced blocking factor (PIBF), a 34 kDa molecule produced by human pregnancy lymphocytes[15,16,17] or activated peripheral blood lymphocytes.[10] PIBF suppresses mitogen induced lymphocyte proliferation, activation of NK cells and TNF production by NK cells.[10] The fact that P+ cells are accumulated in first trimester decidua in quantities much higher than in any other physiological or pathological condition opens not only the question of the role of these cells but also whether or not female steroid hormones, primarily progesterone which is present locally in high concentrations, in the first trimester, has a direct or indirect role as one of the environmental signals which can modulate perforin expression in the decidual lymphocytes.

MATERIALS AND METHODS

Isolation of decidual leukocytes. The Ethics Committee of the Medical Faculty, University of Rijeka approved the study. Decidual tissue for preparation of single cell suspension was obtained by curettage from elective pregnancy termination of normal human 6-10 weeks old pregnancies. Decidual tissue was washed in RPMI 1640 medium (Institute of Immunology, Zagreb, Croatia), cut to pieces of 2x2 mm and exposed to trypsin digestion (0.125% trypsin - DIFCO+EDTA) at 37°C for 90 minutes. Trypsinisation was stopped by adding human AB serum up to the 10% of the entire volume. The cell suspension was separated from non-digested tissue, passing twice through the nylon mesh. The cells were centrifuged (600g/10 min) and the supernatant was aspirated. RPMI 1640 medium was added to the pellet and the resulting cell suspension was overlaid on Ficoll-Hypaque and centrifuged (800g/20 minutes). The cells at the interface were collected, washed twice in RPMI 1640 medium and viability was assessed by trypan blue dye. Viability was always above 90%.

Separation of decidual adherent cells (DAC). Decidual leukocytes obtained by Ficoll-Hypaque separation, were resuspended in tissue culture medium [RPMI 1640 containing 10% Fetal Calf Serum (FCS) (GIBCO, Gaithersburg, MD), L-glutamine (2 mM), penicillin ($1x10^5$ IU/l), gentamicin sulphate (0,05 g/l) and streptomycin sulphate (0,1 g/l), 2-mercaptoethanol ($5x10^{-5}$ mol/l), Hepes (pH 7,2, 10 mM)]. The cells were incubated overnight in tissue culture Petri dishes (Grainer, Frickenhausen, Germany) at 37°C in an 5% CO_2 atmosphere to allow adherence of decidual adherent cells (DAC). Next morning decidual lymphocytes (DL) were collected by pipette and the decidual adherent cells (DAC) were washed in RPMI 1640 and removed from the plastic surface by short-term trypsin treatment. Detaching of adherent cells was optically controlled under an inverted microscope. The process of trypsinisation was stopped by adding 10 ml RPMI 1640 with 10% FCS. Cells from both non-adherent and adherent fractions (DL and DAC) were centrifuged (600g/10min), and the pellet was resuspend in 1ml of RPMI 1640.

Preparation of peripheral blood . Heparinized peripheral blood was obtained immediately before the pregnancy termination procedure initiation (syngeneic peripheral blood leukocytes). Mononuclear cells were separated by Ficoll-Hypaque centrifugation, washed twice with RPMI 1640 medium and counted. The same procedure for separation of peripheral blood lymphocytes (PBL) from peripheral blood adherent cells (PBAC) was performed with blood, as it is described for decidual tissue.

Cell cultures. Decidual and peripheral blood lymphocytes (DL and PBL) were cultured for an additional 18h at a cell count of 10^6 cells/ml tissue culture medium (described above) alone or in the presence of DAC (30%), DAC (30%) + Progesterone (5, 10 or 20 μg/ml), Supernatant of DAC (10^6 cells/ml), Supernatant of DAC + Progesterone (5, 10 or 20 μg/ml), PBAC (30%), PBAC (30%) + Progesterone (5, 10 or 20 μg/ml), PIBF, DAC (30%) + PIBF, PBAC (30%) + PIBF. All cells suspensions were cultured in tissue culture Petri dishes (Grainer, Germany) at 37°C in a humidified atmosphere containing 5% CO_2.

Perforin detection. The perforin content of decidual and peripheral blood lymphocytes was tested by indirect immunofluorescence after overnight culture (procedure for separation of adherent cells) and an additional 18h culturing of non-adherent cells by themselves or in the presence of DAC, supernatant of DAC, PBAC, progesterone or PIBF at a concentrations mentioned above. The procedure was described in details elsewhere.[19] Briefly: 10^6 cells per sample were washed in FACS buffer and fixed with 4% paraformaldehyde. After two washes in FACS buffer, the cells were permeabilized with 0,1-% saponin buffer. Mouse anti-perforin monoclonal antibody was added to each cell sample (3μg diluted in 200 μl) and incubated 30 minutes at +4°C. Secondary antibody fluorescein-conjugated goat anti-mouse IgG (Becton Dickinson) was added after two washing steps, for further 30 minutes, again at +4°C. The samples were washed twice in 0,1-% saponin buffer and resuspended in 1ml FACS buffer to restore the integrity of the membranes. A minimum of 10^4 cells was analysed on FACScan (Becton Dickinson) using the Lysis II software (Becton Dickinson).

Immunocytochemistry. Immunocytochemistry was performed to follow cell suspensions: DL + DAC, DL + DAC + Progesterone 5 μg/ml to detect the number of perforin positive cells and their granularity. The method was described in details elsewhere.[19] Briefly: the cells

were cytospun onto glass microscope slide, dried and fixed in cold acetone at room temperature. Before labelling the cells were rehydrated in Tris Buffered Saline (TBS) medium, endogenous peroxidase was blocked by H_2O_2 and non-specific binding was blocked by preincubation with 1% bovine serum albumin (BSA). The primary antibody, mouse anti - human perforin was applied (3μg per sample, diluted 1:100) for 1 hour. After two washing steps in TBS, peroxidase labelled anti-mouse IgG (Boehringer Mannheim, Germany) was applied at a concentration 10μg per ml for 45 minutes. After two additional washing steps in TBS, the reaction was developed by diaminobenzidine (DAB) solution and stopped by redestilled water. Nuclei were counter stained with hematoxylin. Slides were mounted with gelatine-glycerol.

Statistical analyses. Results were analysed using the Sigma Plot for Windows, version 1.02 (Jandel Scientific). Statistical analyses were performed using a student's t-test one-way analysis.

RESULTS

1. Progesterone directly affects P expression in decidual lymphocytes (DL) but not in peripheral blood lymphocytes (PBL)

As it is described elsewhere[20] and shown in the Figure 1A and B, the percentage of P+ cells significantly decreases in the suspension of fresh unstimulated DL and PBL cultured for 18 hours. Further and significant decrease ($p<0,001$) of the percentage of P+ cells was obtained in suspensions of DL, but not PBL, treated with a high dose of progesterone (20μg/ml). Decrease of the percentage of P+ cells was accompanied by a simultaneous and highly significant ($p<0.001$) decrease of the P content/cell (AFI values for P - Table 1), in decidual, but not in peripheral blood lymphocytes.

2. Progesterone blocks upregulation of P expression in decidual lymphocytes (DL) and peripheral blood lymphocytes (PBL) cocultured with adherent cells.

We investigated the effect of progesterone on P expression in decidual and peripheral blood lymphocytes cultured with DAC. DAC prevent downregulation of P expression in non-adherent cells (Figure 2A and B). Progesterone, in a low dose (5 μg/ml) prevented the effect of DAC on P expression in DL, and in higher concentrations strongly downregulated the percentages of P+ cells (Figure 2A, $p<0.001$, and Figure 3), as well as perforin content per cell (AFI values). By immunocytochemistry we obtained similar results and have found fewer P+ cells in the suspension of DL cocultured with DAC at the concentration of 5 μg/ml of progesterone, than in the suspensions of DL and DAC in absence of progesterone (Figure 4). In the suspensions of DL cocultured with DAC progesterone diminished the content of P per cell in a dose-dependent manner (Table II). Twenty μg/ml of progesterone was needed to block the effect of DAC on PBL (Figure 2B, $p< 0.05$). Similarly, progesterone blocked the effects of DAC supernatants on both DL and PBL (Figure 5), as well as that of PBAC on both DL and PBL (Figure 6).

3. Regulation of P expression by progesterone induced blocking factor (PIBF).

PIBF - the mediator of progesterone actions, blocked the effects of DAC on P expression in decidual lymphocytes, but was not able either to block effects of DAC on P expression in PB lymphocytes, nor the effects of PBAC on either peripheral blood or decidual lymphocytes (Figure 7).

DISCUSSION

Both local endocrine and immune mechanisms contribute to the success of pregnancy in mammals. Decidual lymphocytes with a high expression of the cytolitic molecule perforin[7, 8] display a very low cytolitic potential towards trophoblast cells.[21] One possible explanation for low decidual NK activity is a fact that these cells are at the maternal-fetal interface subjected to high concentrations of progesterone (approximately 3 μg/g of decidual tissue). [10] Similarly, PBL of pregnant women could not lyse fetal fibroblast target cells, but their cytotoxicity was enhanced by removing progesterone from peripheral blood samples by dialysis.[15] Therefore, progesterone as a natural immunosuppressive substance could play a role in regulation of cytotoxicity.[17] Progesterone receptor is a marker of cell activation, because PBL following contact with allogeneic antigens (for example following blood transfusion or transplantation) express progesterone receptor.[10] Upregulation of progesterone receptor expression is also found in PBL during pregnancy which might result as a consequence of increasing concentrations of progesterone and allogeneic stimulation by trophoblast cells, appearing in maternal blood.[10] Approximately 14% of peripheral blood lymphocytes belonging to the CD8+ subpopulation (T cells and some NK cells) during pregnancy are progesterone receptor positive, comparing to negligible appearance of this marker in PBL of non pregnant women.[22] PIBF is the mediator of progesterone actions and in peripheral blood is secreted from progesterone receptor positive cells in the presence of progesterone.[16,17] Recent investigations in our laboratory clearly showed that all CD56+ decidual lymphocytes are PIBF positive,[23] and previously we have proved that P expression in the decidual $CD56^+$ LGL is constitutive.[6] PIBF positivity of decidual LGL cells indicates that these cells are under progesterone influence, but the question whether these cells internalise exogenous PIBF, secreted by stromal cells or even produce their own PIBF is not yet clear. King et al.[24] were not not able to determine progesterone receptors in human uterine T and NK cells in first trimester pregnancy decidua although the accumulation and survival of these NK cells appear to be hormonally dependent. On the other hand, van den Heuvel M et al.[25] reported the presence of progesterone receptor in the GWM hybridoma, which represents murine uterine NK-like cells. Therefore, in humans the effects of progesterone in physiological concentrations and enviroment at the beginning of pregnancy

might be indirect, and possibly is mediated by PIBF secreted from progesterone receptor positive glandular and stromal cells.[26]

Our studies clearly showed that perforin expression in first trimester decidual lymphocytes cultured by themselves is influenced by progesterone in a dose dependent manner. Both the number of P+ cells and the content of P per cell (AFI values) decreased during 18 hours culture of decidual lymphocytes with increasing concentrations of progesterone (5-20 μg/ml). The highest concentration of progesterone (20μg/ml), which is even higher than progesterone concentrations in decidua, could upregulate progesterone receptor and directly stimulate PIBF production in the perforin positive decidual lymphocytes, as has been discussed above for PBL. These cells could become suppressive, because due to this direct effect of high concentration of progesterone on LGL cells they could not upregulate P expression, even following stimulation by other stimuli (for example PBAC or supernatant of DAC). Contrary to decidual lymphocytes, direct effects of progesterone on perforin expression in PBL were not significant. To explain this difference it would be necessary to analyse separately progesterone effects on perforin expression in $CD8^+$ T lymphocytes and $CD56^+$ NK cells, because these effects could be different.

Progesterone at the dose of 5 μg/ml, which is comparable to its concentrations at the maternal-fetal interface, efficiently blocks upregulation of perforin expression in decidual lymphocytes cocultured with DAC. This effect was additionally and clearly illustrated by immunocytochemistry (Figure 4). Higher concentrations of progesterone further decreased both the number of P+ cells and perforin content per cell (AFI values) (Table II). In peripheral blood lymphocyte suspensions progesterone could block the effect of DAC only at the concentration of 20 μg/ml, which is many times higher than physiological concentrations of progesterone in plasma of pregnant women (30-160 ng/ml).[10] Therefore, progesterone probably could not be as efficiently involved in physiological mechanisms of regulation of perforin expression in PBL, as it is involved in that of decidual lymphocytes during pregnancy.

There are at last two cell populations (macrophages and stromal cells), present in the suspensions of decidual adherent cells, which could be candidates for regulation of perforin expression in decidua. We have shown recently that decidual macrophages upregulate P expression in decidual lymphocytes, but macrophage depleted suspensions of decidual stromal cells were inefficient.[27] Decidual stromal cells (approximately 80% of decidual

adherent cells) are progesterone receptor positive[26] and therefore probably could secrete PIBF, which could block upregulation of P expression. Studies *in vitro* confirmed that many effects of progesterone on PBL activity are mediated by PIBF. PIBF blocks NK activity, cytotoxicity and proliferation of mitogen stimulated PBL.[10] Our results showed that PIBF *in vitro*, similarly as progesterone, blocks upregulation of P expression mediated by DAC in decidual lymphocytes, but not in peripheral blood lymphocytes. This result is in the line with the described difference in progesterone effects on perforin expression in DL and PBL. Finally, the observed absence of the direct effect of decidual stromal cells on P expression in PBL could be explained by results showing that PIBF, which is the potential mediator of their action, can not directly influence P expression either (Figure 7).

Recent results in our laboratory [20] show that decidual LGL are likely to be exposed to a cocktail of various cytokines and hormones with both synergistic and antagonistic actions on the level of P expression. We are still far from completely understanding the cytokine network operating at the maternal-fetal interface, but could conclude that progesterone and PIBF, secreted from progesterone receptor positive cells in the presence of progesterone, modulate P expression more profoundly in the maternal-decidual lymphocytes than in the peripheral blood lymphocytes. In other words progesterone predominantly acts on perforin expression as a local immunosuppressive substance.

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LEGENDS TO THE FIGURES

Figure 1. Progesterone downregulates perforin expression in cultured decidual lymphocytes (DL), but has no effect on peripheral blood lymphocytes (PBL). Perforin expression is shown in percentages (Mean +/- SE) of positive cells at time 0, and after 18 hours culture in the medium with or without various concentrations of progesterone.

Figure 2. Progesterone prevents upregulation of perforin expression in decidual and peripheral blood lymphocytes (DL and PBL) mediated by decidual adherent cells (DAC). Perforin expression shown in percentages (Mean +/- SE) of positive cells at time 0, and after 18 hours culture in the medium, or cocultured with DAC without or with addition of progesterone.

Figure 3. Progesterone blocks in a dose-dependent manner upregulation of perforin expression in decidual lymphocytes (DL) cocultured with decidual adherent cells (DAC). Flow cytometric analysis shown by percentages of perforin positive cells and average fluorescence intensities (AFI) for perforin in one sample of decidual lymphocytes (DL) cocultured with DAC (A), or with DAC in the presence of various concentrations of progesterone (B, C, D). IgG 2b monoclonal antibody was used as a negative control. Note decrease in the percentage of perforin positive cells and AFI values for perforin in the suspensions cultured in the presence of progesterone in a dose-dependent manner.

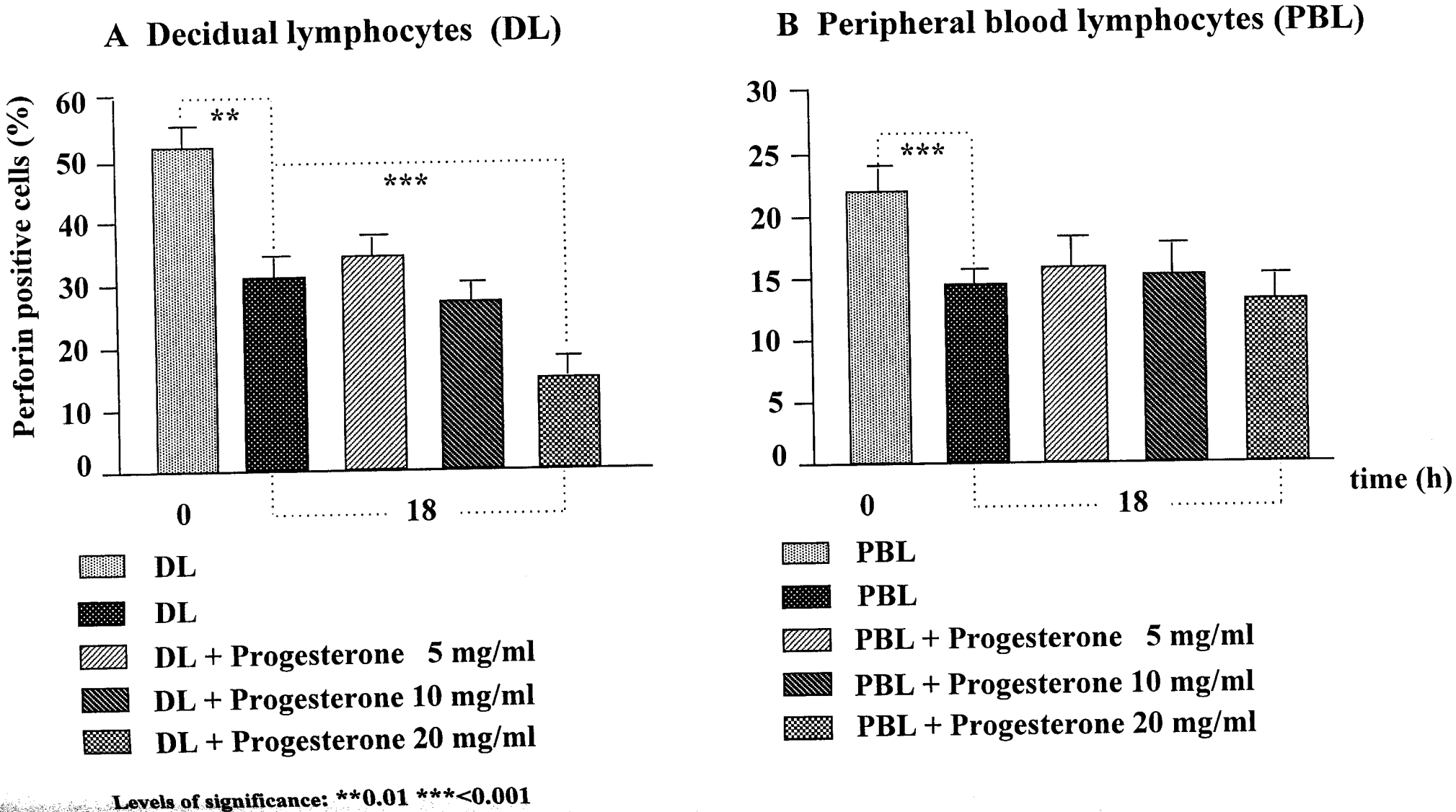
Figure 4. Immunocytochemistry of perforin expression in decidual lymphocytes (DL) cultured with decidual adherent cells (DAC) (A) or DAC + Progesterone 5 μg/ml (B). Photomicrographs (400x magnification) showing perforin positive cells (arrows).

Figure 5. Progesterone blocks more profoundly the effects of the supernatant of decidual adherent cells (Sn DAC) in decidual lymphocytes than in peripheral blood lymphocytes. Perforin expression shown in percentages (Mean +/- SE) in the suspension of DL (A) or PBL (B) at time 0, and after 18 hours culture in the medium or in the supernatant of DAC (Sn of DAC) with or without various concentrations of progesterone.

Figure 6. Progesterone more efficiently prevents upregulation of perforin expression mediated by peripheral blood adherent cells (PBAC), in decidual lymphocytes (DL), than in peripheral blood lymphocytes (PBL). Perforin expression shown in percentages (Mean +/- SE) in the suspension of DL (A) or PBL (B) at time 0, and after 18 hours culture in the medium or cocultured with peripheral blood adherent cells (PBAC) with or without progesterone.

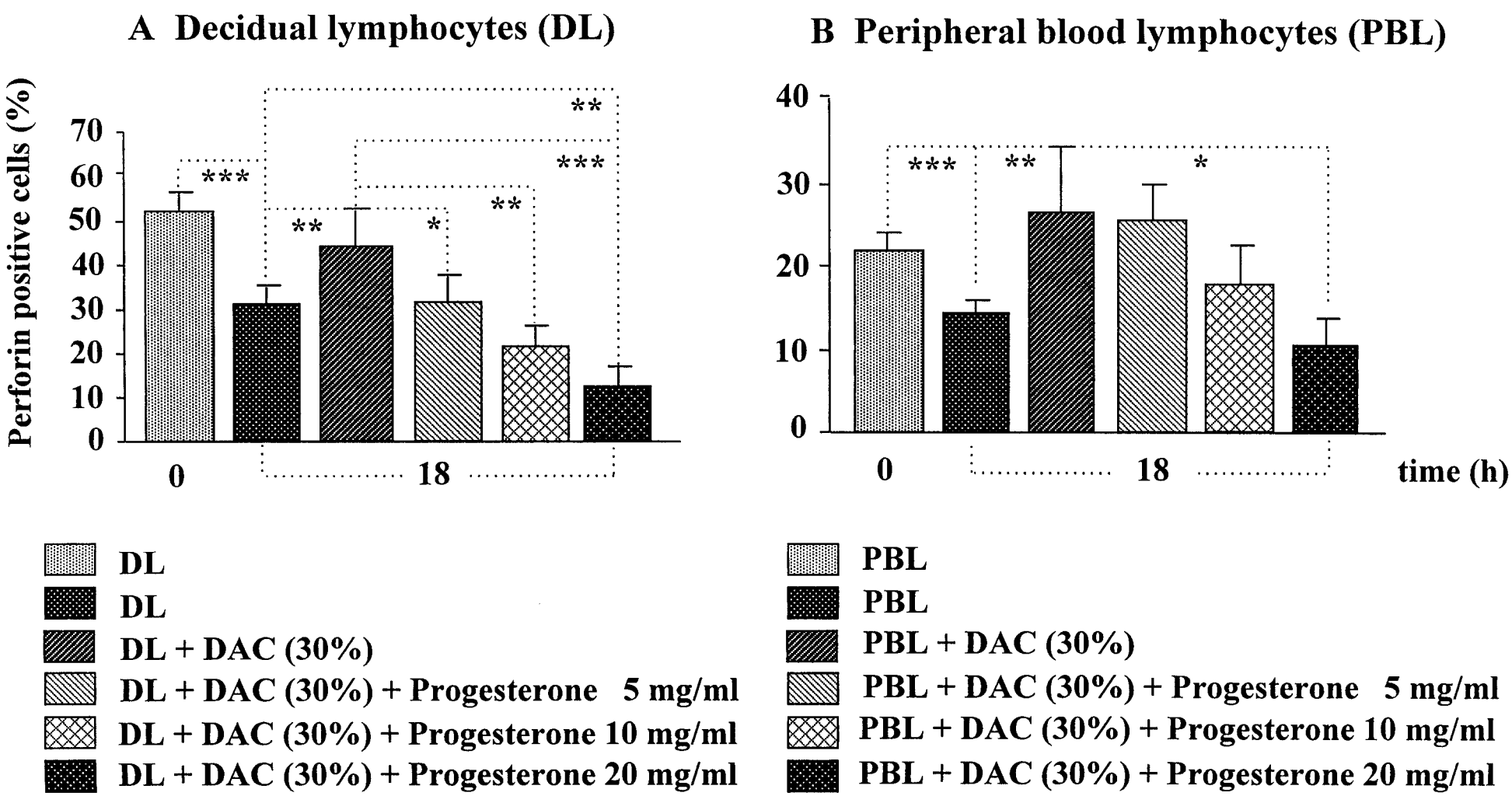
Figure 7. Progesterone induced blocking factor (PIBF) prevents upregulation of perforin expression in decidual lymphocytes (DL) only, and does not modulate effects of peripheral blood adherent cells (PBAC). Perforin expression shown in percentages (Mean +/- SE) in the suspension of DL (A) or PBL (B) at time 0, and after 18 hours culture in the medium with or without PIBF or cocultured with DAC or PBAC with or without PIBF.

Fig. 1.



Levels of significance: **0.01 ***<0.001

Fig.2.

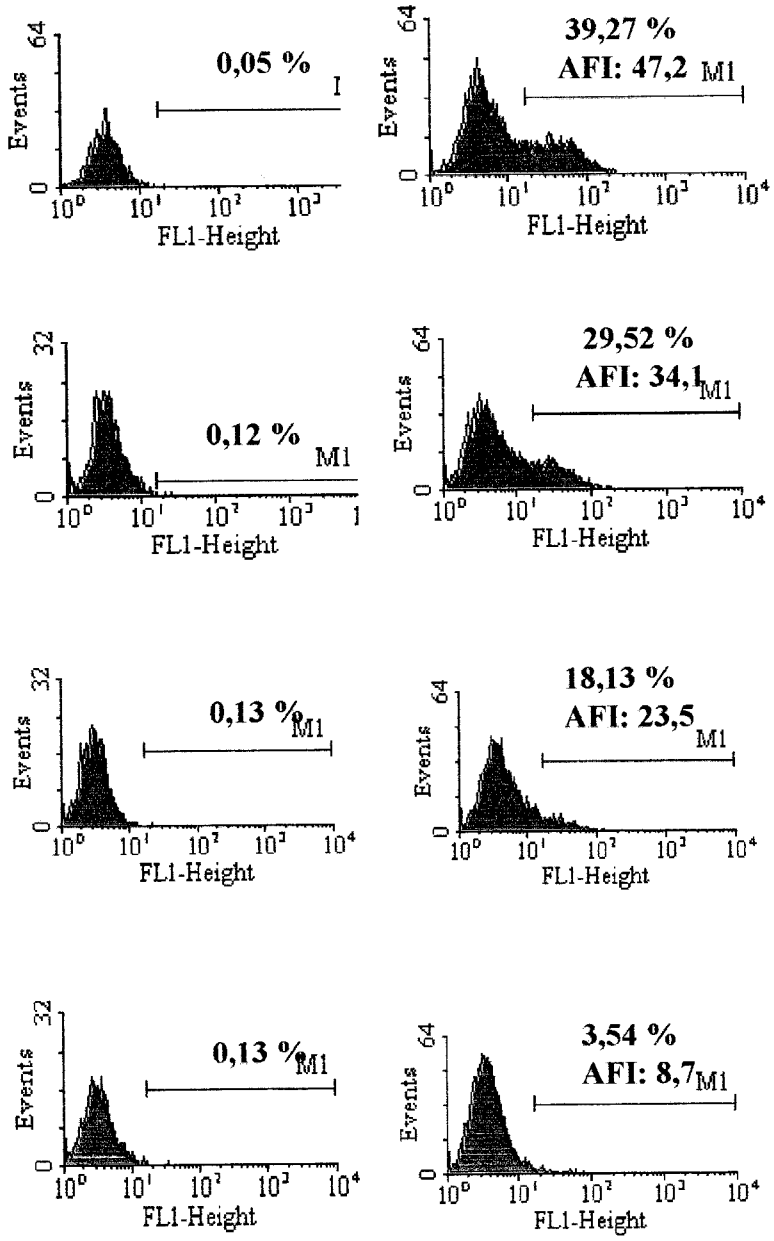


Levels of significance: *<0.05 **<0.01 *<0.001**

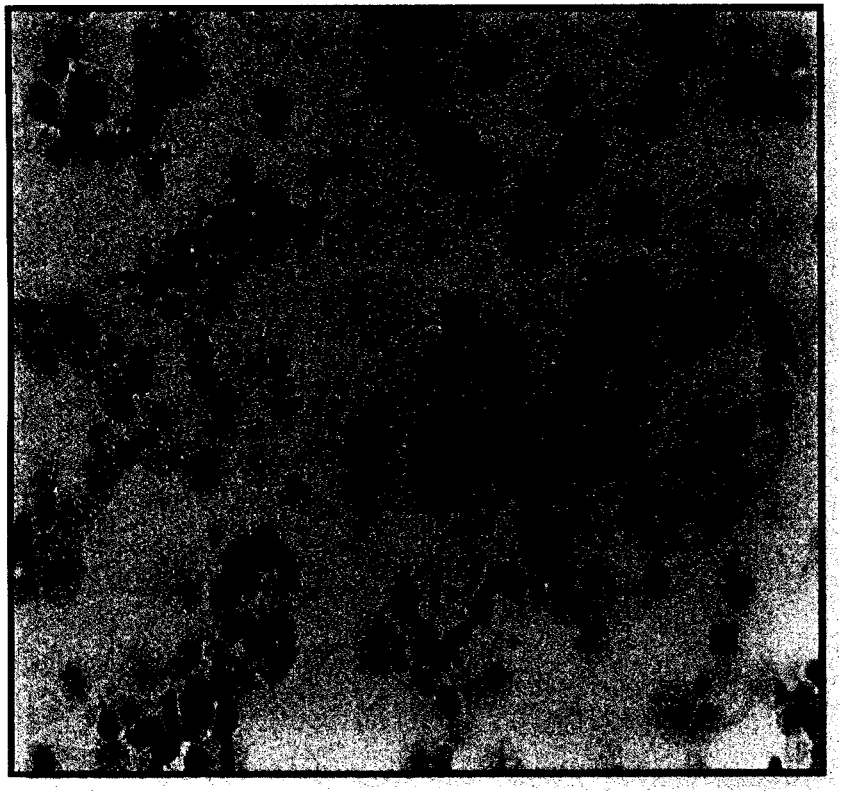
Fig. 3.

A
mouse IgG2b/GAM FITC

B
mouse α-human perforin/GAM FITC



A



B

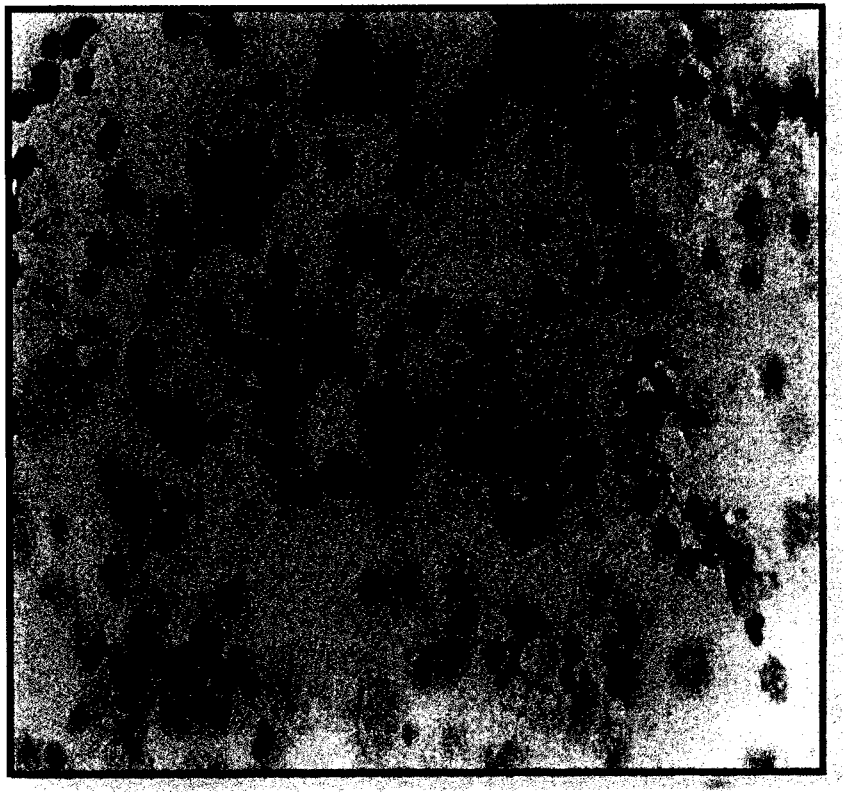
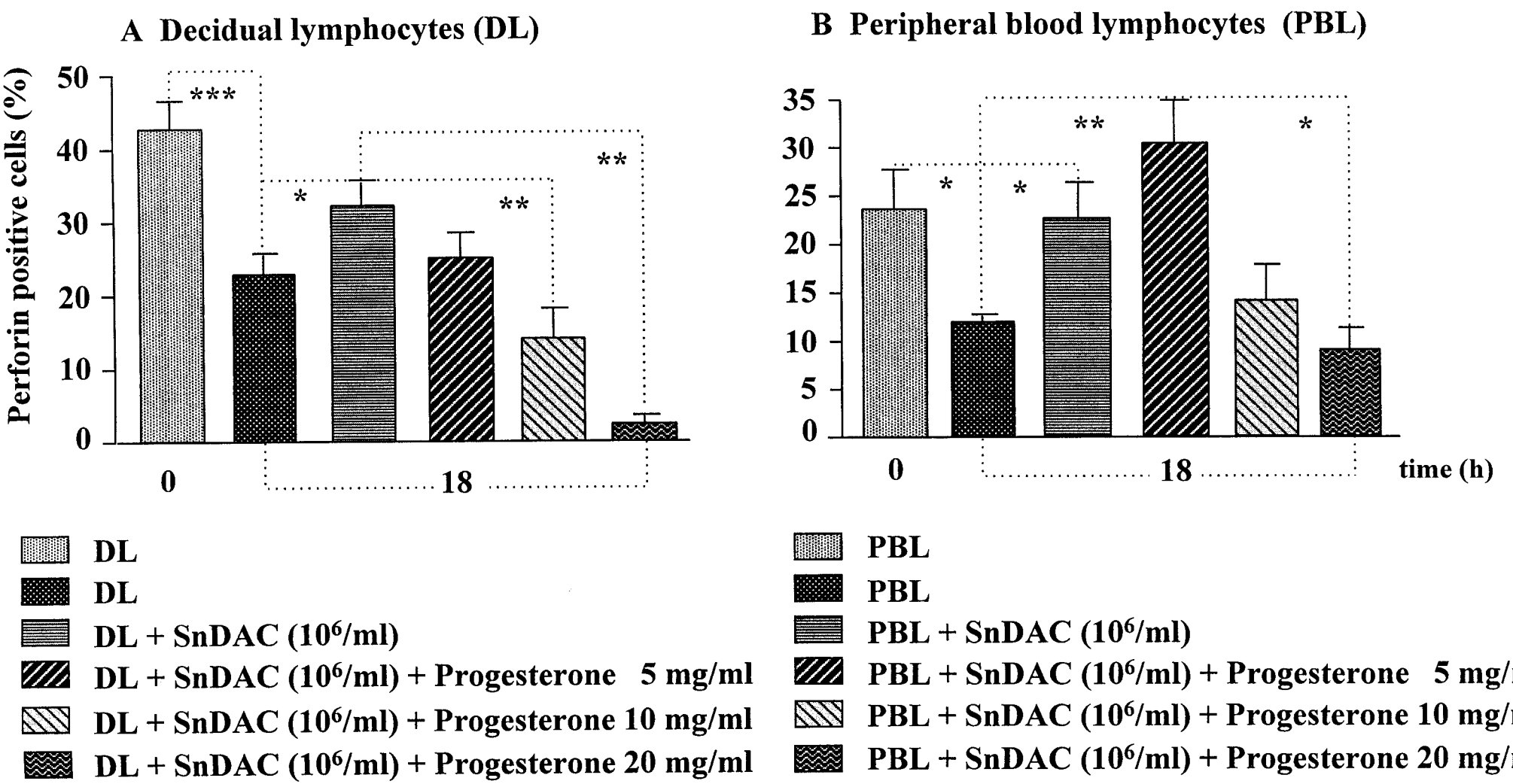
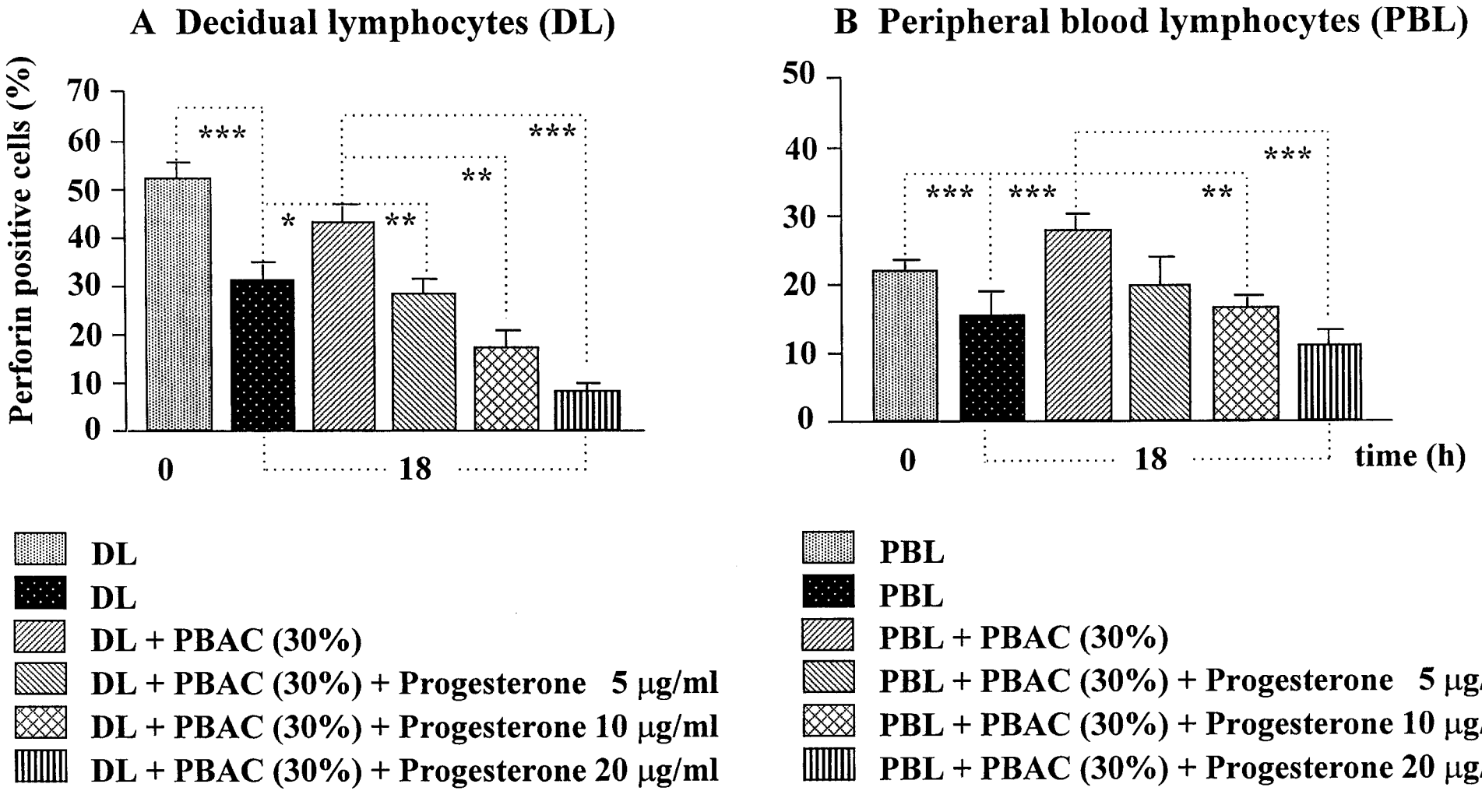


Fig. 5.



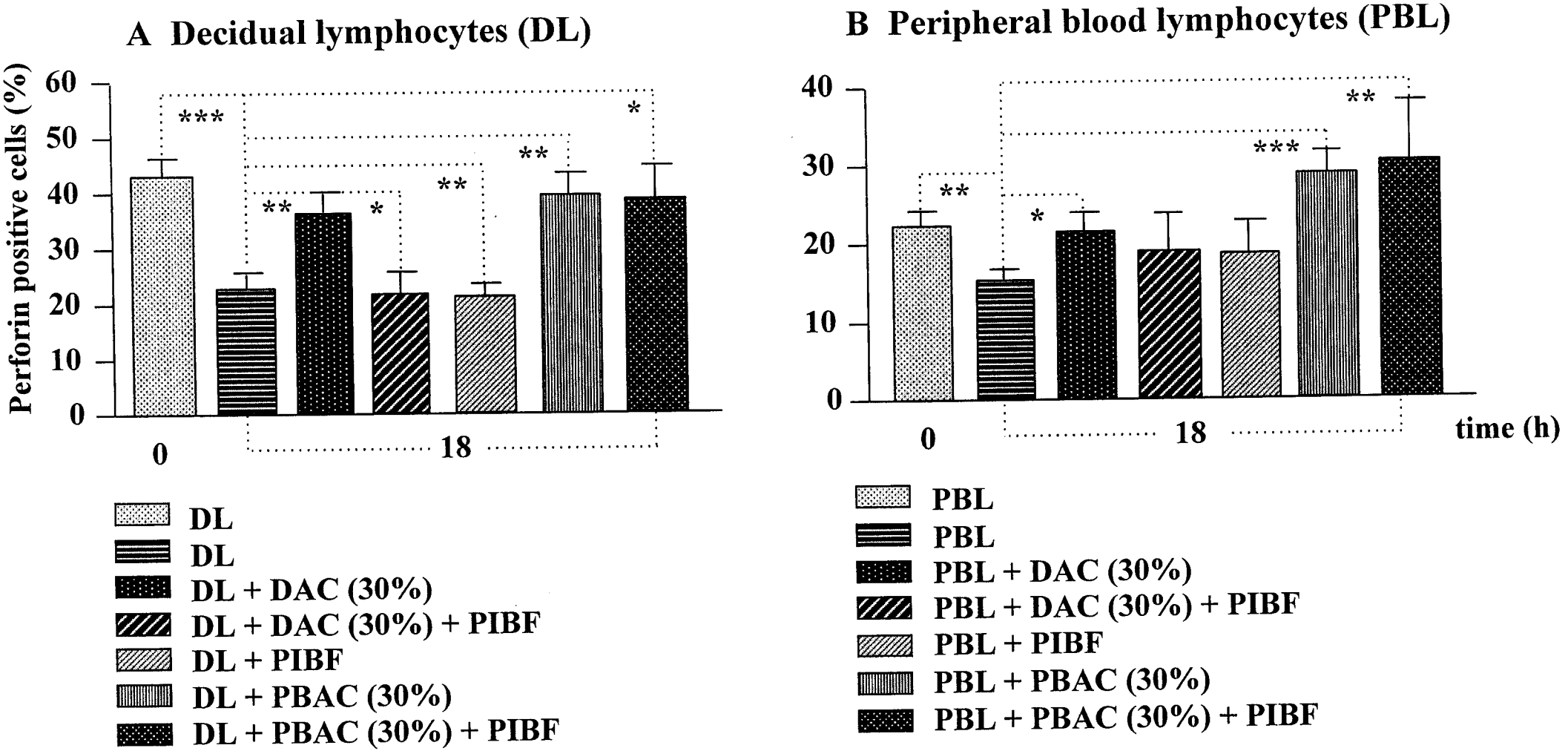
Levels of significance:* p<0,05, ** p<0,01, p*<0,001**

Fig.6.



Levels of significance: *<0.05 **<0.01 ***<0.001

Fig. 7 .



Levels of significance: *<0,05, **<0,01, ***<0,001

Table I Average fluorescence intensities (AFI) for perforin in decidual and peripheral blood lymphocytes cultured (18 hours) in the medium or with various concentrations of progesterone

	without progesterone	Progesterone		
		5 μg/ml	10 μg/ml	20 μg/ml
Decidual lymphocytes	* **39.52 +/-6.36**	**30.30 +/-7.42**	**30.22 +/-8.31**	** **16.86 +/-9.65**
Peripheral blood lymphocytes	**24.21 +/-4.30**	**25.54 +/-6.30**	**27.5 +/-2.12**	**25.57 +/-6.37**

* Mean +/- SD

** $p<0.001$

Table II Average fluorescence intensities (AFI) for perforin in decidual and peripheral blood lymphocytes cultured (18 hours) with decidual adherent cells (DAC) or with DAC and various concentrations of progesterone

	DAC	DAC + Progesterone		
		5 mg/ml	10 mg/ml	20mg/ml
Decidual lymphocytes	[a] 45.72 +/-10.15	33.95 +/-12.0	[b] 25.36 +/-7.78	[d] 14.9 +/-4.3
Peripheral blood lymphocytes	22.43 +/-5.4	23.9 +/-10.0	21.1 +/-8.6	[c] 14.67 +/-6.5

[a] Mean +/- SD

[b] $p<0.05$ [c] $p<0.01$ [d] $p < 0.001$

VI. APPENDIX

MATERIALS AND METHODS USED IN THIS STUDY

1. Immunocytochemistry

Lymphocytes were isolated from heparinized venous blood on Ficoll-Paque gradient. The cells were washed once in RPMI 1640 medium and centrifuged on glass microscope slides. The slides were dried in room temperature, the cells were fixed for 5 min in cold acetone and washed in tris buffered saline (TBS). All incubations were carried out at room temperature in a humid chamber. After blocking of endogenous peroxidase activity with 1% H_2O_2 the cells were further incubated in TBS containing 1% bovine serum albumin (BSA, Sigma) for blocking nonspecific protein-binding sites.

The primary antibodies were diluted in TBS supplemented with 0.5% BSA. The second antibodies (HRPO-labelled) were purchased from Dakopatts and Sigma, Hungary, and applied at a proper dilution respectively for 30 min. The reaction was developed by aminoethilcarbasol or by diaminobenzidine and intensified with silver staining.

The percentage of positive cells was calculated after counting 300 lymphocytes in the microscope at high power magnification.

In some cases (Fig. 1.) we observed CD56 labelling above the nuclei of the lymphocytes. Since CD56 is a membrane structure we performed con-focal microscopy in order to clarify the localisation of

the label and to exclude the possibility of nonspecific DAB binding. Fig. 2 shows the localisation of the silver particles in a series of horisontal sections from a CD56+ lymphocyte. It clearly demonstrates that the granules bind to the membrane.

Fig 1.

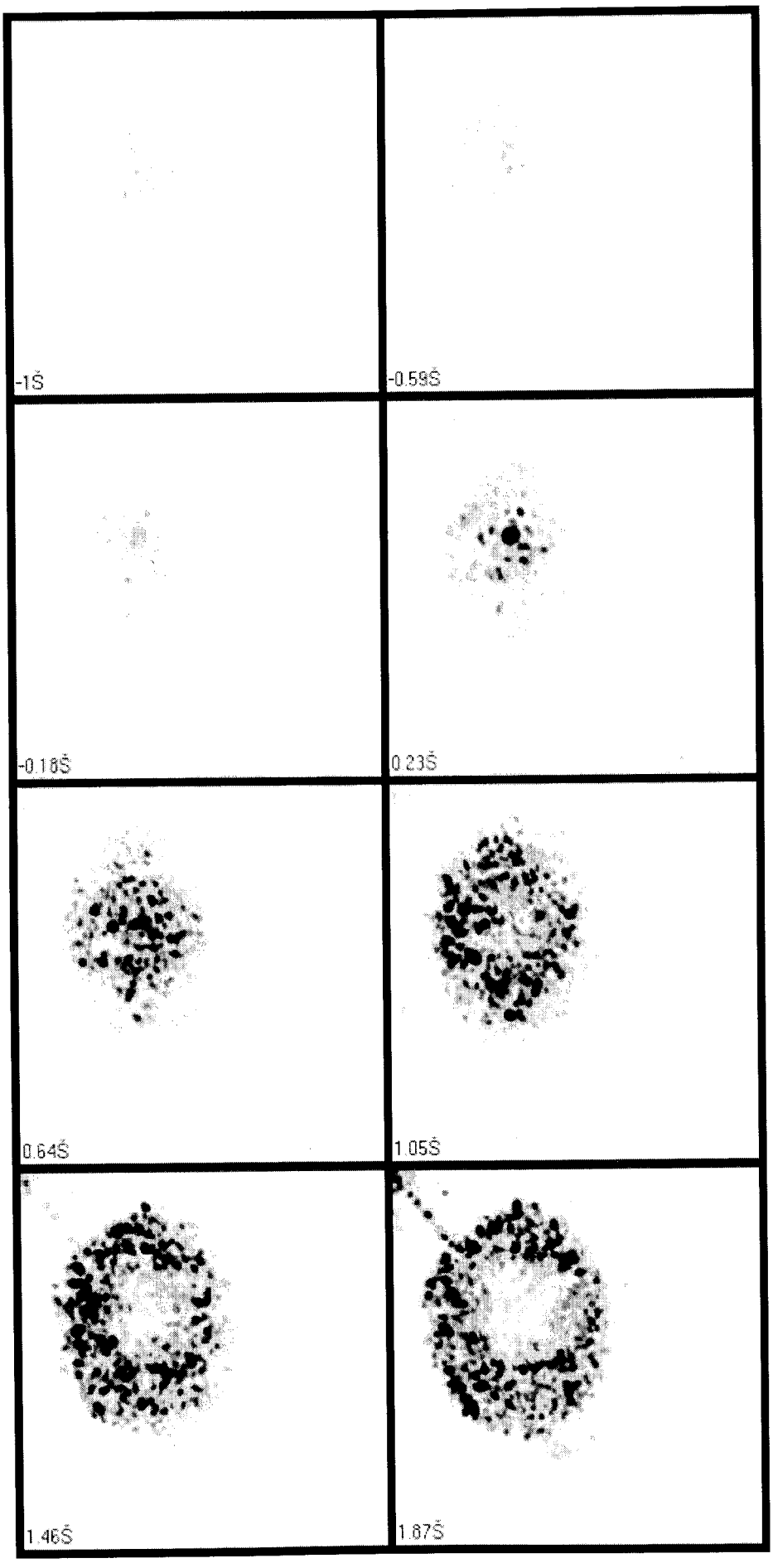


Fig. 2.

2. Production of PIBF

Peripheral lymphocytes of pregnant women were adjusted to a cell count of 10 x 10^6/ml in RPMI supplemented with 10% fetal calf serum (FCS; both from Gibco, Grand Island, NY) and the cells were incubated with 20 μg/ml of progesterone for 16 hr. At the end of the incubation the supernatants were collected. Progesterone was removed by dialysis. The supernatants were used as the source of the human PIBF.

3. Production of PIBF-specific IgG

Spleen cells of pregnant mice were treated with 20μg/ml of progesterone overnight. The supernatants were collected, concentrated on Amicon filters, and subjected to polyacrilamide gel electrophoresis (PAGE) on 12% polyacrilamide gels. The separated bands were blotted onto nitrocellulose filters. The 34 kDa band was cut out, dissolved in DMSO and injected to rabbits weighing 4kg each together with complete Freund adjuvant. Boosters with incomplete Freund adjuvant were given at 2-week intervals. Immunoglobulin G (IgG) was purified on protein A columns.

4. Test for measuring degranulation

Peripheral blood lymphocytes were incubated with or without PIBF for 2 hours at 37^0C in humidified atmosphere with 5%CO_2. Both PIBF treated and untreated lymphocytes were incubated with or without K562 target cells (10^6/ml) for 2 h. In the former case, the lymphocyte to K562 ratio was 1:1. After 2 hours of incubation, the cells were carefully resuspended and centrifuged on microscope slides and fixed as described above.

5. Separation of decidual lymphocytes

Human decidua was obtained from first trimester induced termination of pregnancy. The decidua was separated from fetal and placental tissue and washed in RPMI 1640. The tissue was cut into small pieces and exposed to 0,125 % trypsin and 0,02% EDTA at 37^0C for 90 min. The resulting cell suspension was then filtered through sterile fine gauze to eliminate tissue debris. The cells were centrifuged on Ficoll-Hypaque gradient 800 g for 20 min. Cells at the interface were removed and washed twice in RPMI 1640. The cells were resuspended at a concentration of 1 million/ml in RPMI and 100ml of the suspension was centrifuged on glass microscope slides (500 rpm, 5 min). After drying at room temperature the cells were fixed for 10 min. in cold acetone. The slides were stored at $+4^0$C till labelling.

6. Separation of peripheral lymphocytes

Lymphocytes were isolated from heparinised blood on Ficoll-Paque gradient, washed twice with RPMI 1640 medium and counted. The cells were centrifuged for glass microscope slides or cultured at the concentration of 10^6 cells/ml RPMI1640 + 10% FCS.

7. Flow cytometry

For immunofluorescent labelling of perforin in separated lymphocytes, 10^6 cells were washed in FACS buffer and fixed with 4% paraformaldehyde. After two further washes in FACS buffer, the cells were permeabilised with 0,1% saponin buffer. Mouse anti perforin monoclonal antibody was added to each sample (3 μg diluted in 200μl buffer) and incubated for 30 min. at +4°C. A secondary antibody, fluorescein conjugated goat anti mouse IgG, was added after two washing steps for a further 30 minutes at +4°C. The samples were washed twice in 0,1% saponin buffer and resuspended in 1 ml FACS buffer to restore the integrity of the membranes. The cells were analysed on FACScan using the Lysis II software.

8. 4-hr single cell cytotoxic assay for NK activity

We used the assay originally described by Grimm and Bonavida[81]. One hundred μliters of lymphocytes and the same amount of K562 target cells ($2x10^6$/ml each) were centrifuged at 40g for 5 min, and incubated at 37 °C, in 5% CO_2 for 10 min. The pellets

were then resuspended and 100 μl of 1% agarose (Serva) in RPMI 1640 (Gibco) was added to the mixture. One hundred μliters of this suspension was spread over microscope glass slides previously coated with 1% agar. Target cells alone were used to detect spontaneous lysis. The gel was allowed to solidify and was submerged in RPMI 1640. The slides were incubated for 4 hr at 37 °C in 5% CO_2. Then the gels were stained with 0.5% trypan blue for 1 min. After 2 min washes with phosphate buffered saline (PBS), the gels were fixed in 2% formaldehyde for 5 min and desalted in destilled water. The slides were read using light microscope with 400x magnification. The proportion of lymphocytes bound to the target cells was expressed as a percentage of total lymphocyte population by counting 300 lymphocytes. Results are expressed as a percentage of target binding cells (TBC). Dead conjugates were scored as a percentage of the total number of conjugates by counting 50 conjugates and results are expressed as a percentage of dead conjugates (cytotoxic TBC%). The percentage of NK cells was calculated according to the formula NK%=TBC% x cytotoxic TBC% / 100. All results for cytotoxic TBC% were corrected for the proportion of target cells that died spontaneously in control plates.

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