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Dynamics of Cytokine Production in Human Peripheral Blood Mononuclear Cells Stimulated by LPS or Infected by *Borrelia*

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Summary

To specify the role of individual cytokines in the immune response to pyrogens, isolated and cultivated human peripheral blood mononuclear cells (PBMC) were used for the experiments. Different pyrogens (lipopolysaccharide from *Escherichia coli* - LPS and live *Borrelia afzelii*) were applied and the time course of changes in concentrations of different cytokines in the medium was followed using the ELISA method. It was found that nonstimulated human PBMC proliferate under *in vitro* conditions and produce IL-6, TNF-α, IL-10 and finally also IL-1β. Productions of IL-12 and INF-γ are not changed. Proliferation of PBMC is potentiated after incubation with LPS or live *Borrelia*. PBMC stimulated by LPS increase the net production (stimulated minus unstimulated) of IL-1β and TNF-α significantly, while production of IL-6 was smaller. A delayed increase in the production of IL-10 was also observed. Productions of IL-12 and INF-γ were not influenced. In contrast to LPS, stimulation of PBMC with live *Borrelia*, increases also the production of IL-12 and IFN-γ, besides IL-1β, TNF-α, IL-6 and IL-10. Productions of IL-1β, IL-6 and TNFα increased immediately after incubation with both LPS and *Borrelia*, while productions of IL-12 and INF-γ begin to increase 8 hours and production of IL-10 12 hours after stimulation. Data indicate that stimulation with different pyrogens may activate the cells of the immune cascade in a different way. Stimulation of BPMC by LPS seems to activate the initial steps of the immune response (macrophages and granulocytes) only, while infection with live *Borrelia* also stimulates the later phase of the immune response, probably due to effect of initially produced cytokines.

Key words
Fever • Cytokines • Lipopolysaccharide • *Borrelia*

Introduction

Although it is well documented that production of cytokines is stimulated after *Borrelia* infection (for the most recent paper see Häupl *et al.* (1997), further data are given in the Discussion), the effect of live *Borrelia* on the detailed time course of cytokine production in normal human peripheral blood mononuclear cells (PBMC) has not been studied yet. Similarly, details about dynamics of cytokine production in nonstimulated or LPS-stimulated cells are not available, although the general characteristics of the cytokine response to LPS have also already been described (Cavaillon *et al.* 1990, De Groote *et al.* 1992).

Our unpublished results (Vybíral and Janský) have indicated that infection by *Borrelia* induces fever in rabbits, similar to that induced by LPS. To extend our knowledge about the effect of different pyrogens on

responses of the immune system and to specify the role of individual cytokines in the induction of fever, the time course of production of different cytokines by human PBMC after stimulation by LPS or *Borrelia* was studied.

Methods

Healthy volunteers of both sexes (22-68 years old) served as blood donors. No differences in responses were observed in persons of different age. Blood was withdrawn under authorized supervision from subjects who had given their consent.

Venous blood (18 ml) was collected into heparinized tubes and immediately processed. Peripheral blood mononuclear cells (PBMC) were isolated using gradient centrifugation in Ficoll-Paque solution (Pharmacia) (Boyum 1968). Isolated PBMC were incubated in RPMI medium-1640 (Sigma R-8005) with heat inactivated 10 % fetal bovine serum, 5x 10^{-5} M 2-mercaptoethanol and antibiotics (RPMI-10 Sigma) at microtitration desks in concentrations 10^6 cells per well. LPS from *Escherichia coli* (K 235, Sigma) was added in final concentration 5 μg/ml. Live *Borrelia afzelii* were added in concentration 10^7 cells per well. The CB-43 strain of *Borrelia afzelii* isolated from an *Ixodes ricinus* female was grown in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma) supplemented with 6 % rabbit serum at 34º C. The number of spirochaetes was calculated by dark-field microscopy. The fourth passage was used in the experiments.

Control experiments were also performed on nonstimulated PBMC. Cells used as controls and cells stimulated by LPS and by *Borrelia* were taken from the same subject. Samples of culture medium were harvested at different times after stimulation by LPS or with *Borrelia*. Cytokines were measured using specific immunoassays from DPC Milenia (IL-6, TNF-α, IL-1β), Bender Medsystems (IL-10, IL-12), Immunotech (IFNγ) by ELISA method. Cell viability was assessed with the Trypan blue exclusion method. Data on cytokine production, corrected for 10^6 of cells, are expressed as means ± standard deviations. Statistical differences were estimated by Student's T test.

Results

The results indicate that, under experimental conditions used, the nonstimulated PBMC proliferate and produce cytokines. The number of nonstimulated cells increases by about 40 % within 12 hours of cultivation. TNF-α release, when corrected for the same number of cells, increases immediately after the start of cultivation, reaching maximal level after 4 hours and then slightly falls to the level corresponding to about 200 pg/ 10^6 cells within 24 hours. Release of IL-6 also reaches maximum (about 250 pg/ 10^6 cells) after 4 hours of cultivation and then stabilizes. Release of IL-10 starts to increase after 4 hours and reaches maximum after 12 hours of cultivation (about 230 pg/ 10^6 cells), while the release of IL-1β does not significantly increase until after about 24 hours (36 pg/ 10^6 cells). On the other hand, releases of IL-12 and IFN-γ are very low and remain unchanged during 24 hours of cultivation (Fig. 1).

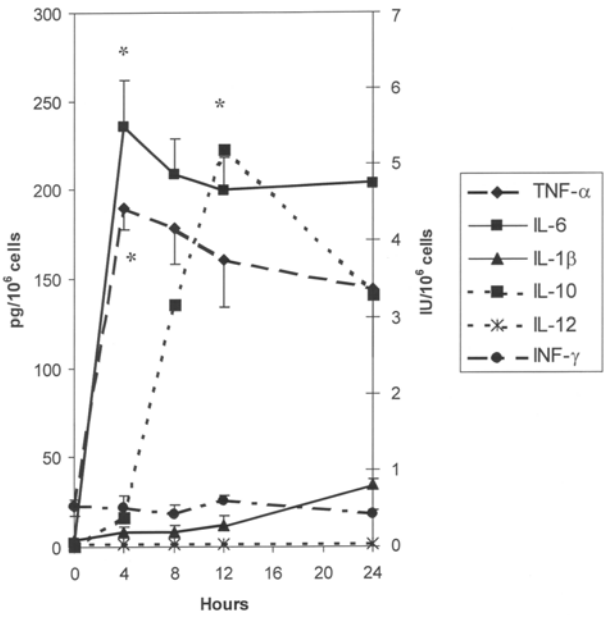

Fig. 1. *Time course of TNF-α (n=10), IL-6 (n=12), IL-1 (n=12), IL-10 (n=5), IL-12 (n=5), and IFN-γ (n=10) production (expressed per number of cells) of nonstimulated human PBMC. Asterisk denotes significant differences compared to controls (p=0.01 for TNFα, IL-6, IL-10). Differences for IL-12 and INFγ were not significant.*

Stimulation of PBMC by LPS or with *Borrelia* increases the number of cells by about 90 % within 8 hours. Kinetic studies show different patterns of cytokine production after incubation with LPS or *Borrelia*. Net release of IL-1β and TNF-α (stimulated minus unstimulated) increases immediately after the start of incubation with both LPS and *Borrelia*, reaching 195-245 pg/ 10^6 cells within 4 hours and then remains stable during the 24-hour test. The net release of IL-6 also reaches the peak within 4 hours (55-75 pg/ 10^6 cells) and

then tends to decrease (Fig. 2). Release of IL-10 starts to increase after 12 hours and reaches the maximum (230 pg/ 10^6 cells within 24 hours after the start of incubation with LPS or *Borrelia* (Fig. 3), while the release of IL-12 and INF-γ startes to increase after 8 hours of incubation with *Borrelia*, but was not influenced by LPS (Fig. 4).

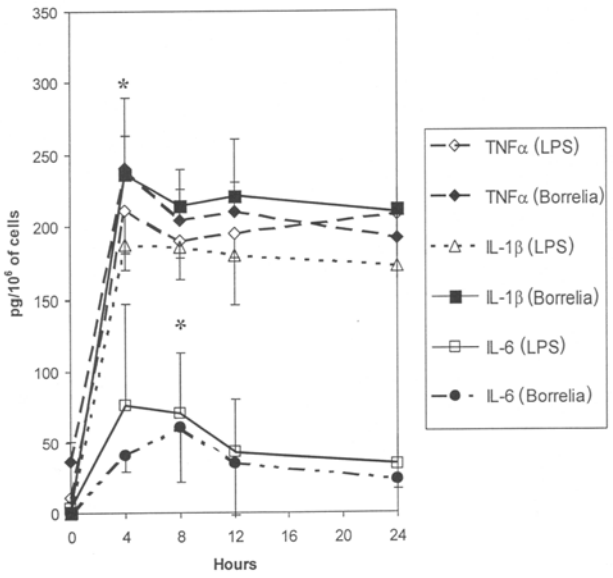

Fig. 2. *Time course of net production of TNF-α (n=10), IL-6 (n=10) and IL-1β (n=5) (expressed per number of cells) by human PBMC after application of LPS (broken lines) or Borrelia (solid lines). Asterisk denotes significant differences compared to controls (p = 0.01 for all cytokines).*

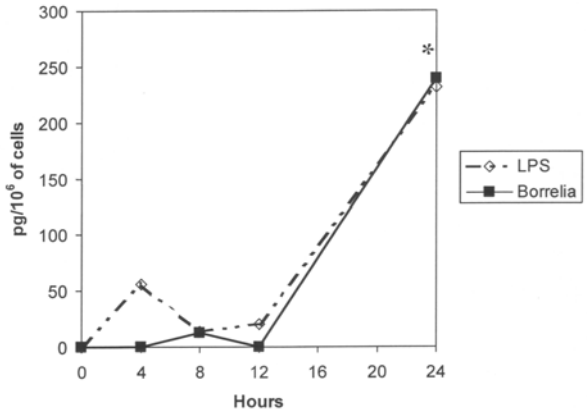

Fig. 3. *Time course of net production of IL-10 (n=5), (expressed per number of cells) by human PBMC after application of LPS (broken lines) or Borrelia (solid lines). Asterisk denotes significant difference compared to controls (p=0.01).*

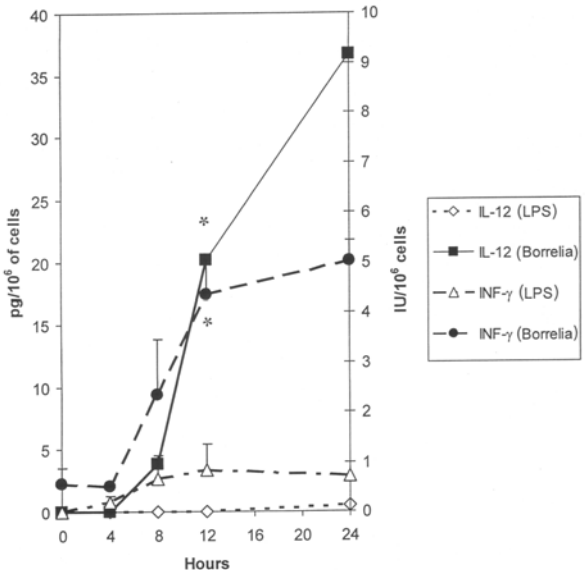

Fig. 4. *Time course of net production of IL 12 (n=5) and IFNγ (n=5) (expressed per number of cells) by human PBMC after application of LPS (broken lines) or Borrelia (solid lines (n=2). Asterisks denote significant differences compared to controls (p=0.01 after stimulation with Borrelia).*

Discussion

The data presented in this publication concerning cytokine production in human PBMC stimulated by LPS are consistent with data of De Groote *et al.* (1992), Cavaillon *et al.* (1990) and Flegel *et al.* (1989) and complement these observations by showing the detailed time course of cytokine production. It appears that TNF-α and IL-1β are the first cytokines to be produced, followed by IL-6, which is being produced in a smaller extent (Fig. 2). After about 12 hours of stimulation, the production of IL-10 also starts to increase steadily (Fig. 3), while that of INF-γ and IL-12 does not change (Fig. 4). Evidently, monocytes and macrophages are activated in the first place after stimulation by LPS in the concentration used. The finding that in PBMC stimulated by LPS the IL-1β and TNFα are being produced in higher quantities than IL-6 may indicate a smaller role of peripheral IL-6 in inducing bacterial fever. This is consistent with the observation of Vybíral *et al.* (in press) showing that peripheral administration of IL-6 does not induce febrile response in rabbits. Furthermore, the fact that relatively high levels of IL-6 can be found in the blood of nonfebrile guinea pigs (Janský *et al.* 1995) supports this view indirectly. On the other hand, since the

patterns of IL-1β and TNF-α production are similar, both the peripheral IL-1β and TNF-α can be designated as fever inducers. Thus, the febrile response after bacterial infection could be induced by a synergic action of several cytokines. Vybíral *et al.* (in press) showed that the mode of action of IL-1β and TNF-α on hypothalamic thermoregulatory centers is identical.

Live *Borrelia burgdorferi* also stimulate cells of the immune system to produce cytokines, in spite of the fact that they lack lipopolysaccharides (LPS) (Takayama *et al.* 1987, Weiss *et al.* 1994). Outer surface proteins (Osp´s A,B) from cell membranes of *Borrelia burgdorferi* seem to be responsible for this effect. Osp´s from *Borrelia burgdorferi* are 50-500 times more effective as cytokines inducers and B-cells mitogens than *Escherichia coli* lipoproteins (Ma and Weiss 1993).

Radolf *et al.* (1991) were the first to report that infection with *Borrelia* activates TNF-α synthesis. Subsequently, several authors demonstrated that different types of cultured cells increase production of TNF-α (Radolf *et al.* 1991, Ma and Weiss 1993, Radolf *et al.* 1995, Häupl *et al.* 1997, Straubinger *et al.* 1998, Brown *et al.* 1999), IL-1β (Miller *et al.* 1992, Radolf *et al.* 1995, Häupl *et al.* 1997, Straubinger *et al.* 1998, Giambartolomei *et al.* 1999), IL-6 (Ma and Weiss 1993, Tai *et al.* 1994, Radolf *et al.* 1995, Häupl *et al.* 1997, Brown *et al.* 1999, Giambartolomei *et al.* 1999), IL-12 (Radolf *et al.* 1995, Filgueira *et al.* 1996, Infante-Duarte and Kamradt 1997), IL-8 (Sprenger *et al.* 1997), IL-10 (Giambartolomei *et al.* 1998) and IFN- γ (Ma *et al.* 1994, Häupl *et al.* 1997) after Osp application. In general, our results confirm earlier observations that the spirochaete infection increases production of IL-1β, IL-6, IL-12, IL-10, TNF-α and IFN-γ. We have found that the cytokine release reaches a maximum within 4 hours after stimulation, while other authors observed the maximum release after about 12 hours. In contrast to our results Giambartolomei *et al.* (1998) and Häupl *et al.* (1997) found that that IFN-γ gene expression and IFN-γ release were not affected by *Borrelia* and OspA.

A novel observation concerns the fact that infection by *Borrelia* induces a different pattern of cytokine production compared to stimulation by LPS in spite of the finding that both are pyrogenic. We have shown that *Borrelia* infection induces not only the immediate releases of IL-1β, IL-6, TNF-α (Fig. 2), but also delayed releases of IL-12 and INF-γ (Fig. 4), while incubation with LPS increases release of IL-1β, IL-6 and TNF-α only. Both pyrogenic substances also induce a delayed release of IL-10 (Fig. 3). This may indicate that *Borrelia* infection has a more extensive effect on immune cells than bacterial stimulation. Since it was shown that IL-1β, TNF-α and IL-6 are produced by monocytes and granulocytes (Häupl *et al.* 1997, Giambartolomei *et al.* 1999), while IFN-γ is produced by NK and T cells (Ma *et al.* 1994), it may be concluded that infection with live *Borrelia* in the used concentrations activates not only the initial steps of the immune response (macrophages and granulocytes), but also the later phase of the immune response due to activation of T cells and NK cells. However, the specificity of this response is still questionable since the possibility that higher concentrations of LPS could also induce increased production if IFN-γ and IL-12 cannot be excluded.

The possibility should also be considered that the signal transmission pathway within the target cells may be different after *Borrelia* infection compared to LPS stimulation. It was documented that activation of monocytes/macrophages and production of cytokines by Osp proceeds *via* the CD 14 receptor (Giambartolomei *et al.* 1999), similarly as in case of LPS (Tobias *et al.*1986, 1993). Recent data indicate that LPS is then transferred to the transmembrane signaling receptor toll-like receptor 4 (TLR4). Several other pathways are also activated that include different protein kinases (Guha and Mackman 2001). In contrast, infection by *Borrelia* seems to include TLR2 and TLR6 (Wooten *et al.* 2002, Bulut *et al.* 2001).

Further, it should be mentioned that the time course of in vitro cytokine production after *Borrelia* infection corresponds to the time course of fever development induced by i.v. administration of live *Borrelia* to rabbits (Vybíral and Janský, unpublished results).

Furthermore, it was found that PBMC proliferate after LPS stimulation or *Borrelia* infection. Since it has been found that the B lymphocytes, but not the T lymphocytes, proliferate when incubated with Osp A (Tai *et al.* 1994, Ma *et al.* 1994), it appears that cell proliferation after *Borrelia* infection may occur in different types of cells than the cytokine production.

Our data also provide information about the time course cytokine production in nonstimulated PBMC (Fig. 1). An earlier paper also indicates increased production of cytokines in nonstimulated PBMC (De Groote *et al.* 1992), but generally this fact is being neglected in the literature. The reason for the increased production of cytokines in nonstimulated PBMC was not clarified in this paper. However, it should be mentioned that the release of TNF-α occurs first in nonstimulated PBMC, followed by IL-6, by IL-10 and finally by IL-1β.

The sequence of cytokine production in nonstimulated PBMC is different from that induced by LPS or *Borrelia* and, therefore, the effect of some unknown stimulatory substances cannot be excluded. Our unpublished experiments show that activation of resting PBMC also occurs during cultivation in the fetal serum albumin-free medium, thus indicating that the fetal serum albumin is not the cause of the increased production of cytokines. On the other hand, it should be taken into consideration that 2-mercaptoethanol, which was used in our experiments, increases proliferation of PBMC (Larsson *et al.* 1992).

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Reprint requests

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