**INTRODUCTION**

*Helicobacter pylori* (H. pylori) bacteria have been recognized as a major cause of chronic gastritis, gastric and duodenal ulcers and gastric cancer. Macrophages are the targets of lipopolysaccharide (LPS), which is a constituent of the outer membrane of Gram-negative rods. In this study we focused on a potential role of macrophages in the proliferation of human peripheral blood mononuclear leukocytes (PBML) in the milieu of *H. pylori* LPS and standard *E. coli* LPS. First, we found that *H. pylori* and *E. coli* LPS induced proliferation of total PBML (tPBML) from 5 out 21 healthy blood donors (LPS responders). In the LPS milieu, tPBML from the majority of volunteers (LPS non-responders) showed a significant decrease in the [3H]-thymidine incorporation as compared to tPBML in medium alone. The decreased cell proliferation was associated with a diminished metabolic activity of non-adherent lymphocytes. Then, non-adherent lymphocytes were stimulated with autologous macrophages pulsed with bacterial LPS. Still, the lymphocytes from the non-responders did not proliferate in the cultures with LPS exposed macrophages. In the group of LPS responders, the macrophages pulsed with *H. pylori* LPS significantly reduced the proliferation of non-adherent lymphocytes. The possible mechanism regulating the responses of PBML to bacterial LPS with an implication for the outcome of *H. pylori* infections is discussed.

**Key words:** *Helicobacter pylori, lipopolysaccharide (LPS), macrophages, lymphocyte proliferation*
persistent dyspepsia, peptic ulcer or malignancy. They remained free of use of antibiotics, bismuth salts, non-steroidal anti-inflammatory or immunosuppressive agents.

The H. pylori status was estimated by 13C urea breath test-UBT (11) and anti-H. pylori antibodies detected by ELISA as previously described (12).

The volunteers were divided into two groups, one with UBT and anti-H. pylori IgG positive – H. pylori infected – H.p.(+) and the other with UBT and anti-H. pylori IgG negative – H. pylori noninfected – H.p.(-).

Leukocyte cultures

1. Single-stage cultures of total peripheral blood mononuclear leukocytes (tPBML)

In the preliminary experiments the samples of peripheral blood (30 ml) from 21 donors, 10 H. pylori positive H.p. (+) and 11 H. pylori negative - H.p. (-) were separated by Lymphoprep-gradient centrifugation as recommended by the manufacturer (Nycomed Pharma AS, Norway). The total PBML population was prepared by treatment with RNase, DNase and proteinase K, and purified by treatment with phenol-Water technique after pretreatment of LPS (Sigma, St. Louis, Michigan, US) or H. pylori LPS, or 2.5 µg/well of phytohemagglutinin – PHA (Sigma) as a positive control for the lymphocyte proliferation. The H. pylori LPS was prepared by phenol-Water technique after pretreatment of bacterial biomass with pronase. Then the LPS preparation was purified by treatment with RNase, DNase and proteinase K, and by ultra centrifugation, as previously described (13). The experiments were performed in triplicate in the RPMI-1640 medium containing 10% fetal calf serum (FCS), 200 mM L-glutamine and 50 µg/ml gentamicin (complete medium). At 18 h before the end of cultivation, 5 µCi [3H]-thymidine was added to each well to estimate cell proliferation.

2. Two-stage mixed cultures of mature macrophages prestimulated with H. pylori or E. coli LPS and autologous lymphocytes

For two-stage cultures the samples of peripheral blood were harvested from the same donors who gave blood for the one-stage cultures after two months. To obtain adherent monocytes, tPBML (4x10^5 in 200 µl complete medium/well in the 96 well tissue culture plates) were incubated for 1 hour, 37°C, 5% CO₂. The nonadherent cells were washed out. The adherent macrophages in 200 µl complete medium/well were allowed to mature for 5 days with a half volume medium exchange on the 3rd day of the culture. The monolayers of mature macrophages were stimulated with H. pylori LPS or E. coli LPS (1 µg/well) for 24 hours (37°C, 5% CO₂) or they were not stimulated (complete medium alone). At the end of macrophage stimulation the samples of blood were harvested from the volunteers. The suspension of tPBML (4x10^5 in 200 µl complete medium/well) was incubated for 1 hour (37°C, 5% CO₂) to remove adherent macrophages. The nonadherent lymphocytes in the culture medium (200 µl/well) with or without PHA (2.5 µg/well) were added to the monolayers of autologous macrophages, which were or were not stimulated with LPS. The cells were incubated for 5 days. At 18 h before the end of cultivation, 5 µCi [3H]-thymidine was added to each well to estimate cell proliferation.

Cell proliferation

The incorporation of thymidine was measured using microbeta scintillation counter after harvesting the cells on fibre filters. All cultures were settled in 3 repeats. The results are expressed as mean cpm/culture ± standard deviation (S.D.).

The cell viability assays

The tPBML (4 x 10^5 in 200 µl/well) were incubated for 5 days (37°C, 5% CO₂) in 96-well microplates with 1 µg/well of E. coli or H. pylori LPS, PHA (2.5 µg/well), staurosporin (0.25 mM solution, 5 µl/well) or RPMI-1640 complete medium alone. After 5 days of cultivation the viability of adherent and nonadherent cells was estimated. A metabolic activity of PBML was estimated colorimetrically on the basis of the ability of the live cells to reduce MTT tetrazolium salt (cell proliferation and viability assay, TACSTM MTT assays, R&D System, Minneapolis, USA). The Mito Light™ Apoptosis Detection Kit (Chemicon International, Temecula, Canada) was used for the indication of the appearance of early apoptotic changes in the cells. The staurosporin (Sigma, 0.25 mM, 5 µl/well) was used as a positive control in the apoptosis assay. Both tests were performed as recommended by the manufacturers. The intensity of MTT reduction was estimated spectrophotometrically at 600 nm wave length. The cell viability was expressed in the optical density units OD₅₇₀. There was a correlation between the viable cell number, used for preparing the standard curve, and the absorbance intensity.

The enhancement of green versus yellow/red fluorescence allowed detecting apoptotic and live cells, respectively, as measured with Victor 2 reader at 480/530 excitation/emission filters. The results were expressed as relative fluorescence units (RFU).

Cell viability in the cultures was additionally estimated by standard trypan blue exclusion assay.

Statistical analysis

Statistica 5.5 PL program with non-parametric tests was used. Mann-Whitney U test (for impaired data) to verify the hypothesis that the two compared samples came from two statistically different populations; Chi-square χ² test for the comparison of the prevalence of the analyzed parameters in the studied groups.

RESULTS

The proliferation of lymphocytes in response to H. pylori and E. coli LPS (one-stage cultures)

Monocyte derived macrophages (MØ) are the main target of LPS and the key immunocompetent cells in the immune response to Gram negative bacteria. In this study we assumed that the monocyte derived MØ exposed to H. pylori LPS may interfere with a response of lymphocytes to H. pylori bacteria and be involved in the inhibition of antibacterial cellular immunity with a result of defective eradication of H. pylori infection. To verify our hypothesis, we used two culture models to measure the proliferation of lymphocytes responding to H. pylori and E. coli LPS. First, the samples of total peripheral blood mononuclear leukocytes (tPBML) from 21 healthy volunteers, with or without H. pylori infection, were stimulated for 5 days with H. pylori LPS, E. coli LPS or PHA. The cell proliferation was estimated on the basis of [3H]-thymidine incorporated into dividing cells.

The tPBML from all volunteers responded to PHA with intensive proliferation (51200±40420 cpm/culture for H.p.(+) and H.p.(-) donors, respectively), when they were stimulated with this mitogen for 5 days. On the contrary, there was a natural variation in the LPS driven responses of tPBML from both H.p.(+) and H.p.(-) volunteers (Fig. 1). The tPBML from three out of ten H.p.(+) and
two out of eleven H.p.(-) volunteers responded simultaneously to \( H. pylori \) and \( E. coli \) LPS with 2-3 fold more \([3H]\)-thymidine having been incorporated as compared with the cells cultivated in the medium without LPS. The volunteers whose tPBML responded to LPS will be further referred to as responders. There was no difference in the proliferation responses to \( H. pylori \) and \( E. coli \) lipopolysaccharide of tPBML from H.p. (+) and H.p. (-) responders. The tPBML from the majority of the volunteers responded neither to \( H. pylori \) LPS nor \( E. coli \) LPS. Interestingly, in tPBML from both H.p. (+) and H.p. (-) non-responders, stimulated for 5 days with \( H. pylori \) or \( E. coli \) LPS, the DNA synthesis was below the values measured for tPBML in the medium without LPS (Fig. 1). Individual differences in the responsiveness of tPBML to bacterial LPS were not related to cell death. The cell viability in all cultures was in the range 80-98% as estimated by trypan blue exclusion. However, the extremely low incorporation of \([3H]\)-thymidine into \( H. pylori \) LPS-treated PBML was associated with significant impairment of the cell metabolic activity which was demonstrated by the MTT test (Table 1). To check if the decrease in the MTT

![Fig. 1. The total PBML (tPBML) proliferation in 5-day cultures stimulated with \( H. pylori \) LPS, \( E. coli \) LPS or cultivated in a complete medium without stimuli (one-stage cultures). Cell proliferation is expressed as the ratio of cpm value for the culture in medium alone defined as 1, to cpm value for the culture with LPS. PBML from \( H. pylori \) infected (white circles and triangles) and uninfected (black circles and triangles) donors.]

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Cell viability counts</th>
<th>Separated PBML</th>
<th>Apoptosis (RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total population of PBML</td>
<td></td>
<td>Adherent M(0)</td>
</tr>
<tr>
<td>Proliferation</td>
<td>[3H]-thymidine incorporation</td>
<td>MTT reduction (OD&lt;sub&gt;600&lt;/sub&gt;)</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>24810±17580 (p=0.002)</td>
<td>1790±380 (p=0.02)</td>
<td>nt</td>
</tr>
<tr>
<td>Stauroporin</td>
<td>nt</td>
<td>nt</td>
<td>1360±560 (p=0.012)</td>
</tr>
<tr>
<td>( H. pylori ) LPS</td>
<td>590±430 (p=0.018)</td>
<td>860±140 (p=0.012)</td>
<td>1000±240 (p=0.004)</td>
</tr>
<tr>
<td>( E. coli ) LPS</td>
<td>490±320 (p=0.004)</td>
<td>930±190</td>
<td>970±250 (p=0.04)</td>
</tr>
<tr>
<td>RPMI-medium</td>
<td>1000±600</td>
<td>1100±210</td>
<td>960±112</td>
</tr>
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</table>

PBML, peripheral blood mononuclear cells; MTT, tetrazolium salt; OD, optical density; PHA, phytohemagglutinin; RFU, relative fluorescence units; LPS, lipopolysaccharide; nt, non tested. The total population of PBML was stimulated for 5 days in the RPMI-1640 medium alone or in the medium supplemented with \( H. pylori \) or \( E. coli \) LPS, PHA or staurosporin. The proliferating activity of the cells was estimated on the basis of \([3H]\)-thymidine incorporation. After 5 days of cultivation the metabolic activity and apoptotic changes of adherent and non-adherent cells were estimated. The MTT reduction assay allowed the measurement of cellular metabolic activity. The features of apoptosis were detected on the basis of fluorochrome accumulation in the mitochondrial cell membrane. The results were expressed as the number of cpm/culture (cell proliferation), optical density units - OD<sub>600</sub> (metabolic activity of the cells) and the relative fluorescence units (cell apoptosis).
reduction should be attributed to the macrophages or lymphocytes, we separated the tPBML from 5-day cultures stimulated with LPS into non-adherent lymphocytes and adherent macrophages. Then the cell fractions enriched in lymphocytes or macrophages were examined with the MitoLight Apoptosis Detection Kit. No apoptotic changes were found in the population of adherent cell fractions. The early apoptotic changes were identified in non-adherent lymphocytes (Table 1) with the increase in RFU from the value 887±200 for the cell cultures in complete medium alone to 1220±260 (p=0.034) and 1470±430 (p=0.004) for the cultures stimulated with H. pylori LPS and E. coli LPS, respectively.

The proliferation of lymphocytes in response to H. pylori and E. coli LPS (two-stage cultures)

The extent of macrophage maturation is known to affect their activity. Thus, we used a two step culture model in the study. Peripheral blood was obtained from four healthy donors whose non-separated PBML responders (responders) with proliferation to H. pylori LPS and E. coli LPS, two H.p (+) and two H.p, (-), and from four volunteers whose non-separated PBML did not proliferate in the cultures stimulated with the bacterial LPS (non-responders), two H.p. (+) and two H.p. (-). The adherent monocytes were obtained and allowed to mature in complete medium for 5 days. Then the mature macrophages were pulsed for 24 hours with H. pylori LPS or E. coli LPS, or incubated in complete medium alone.

The samples of blood were harvested from LPS responders and non-responders. The suspensions enriched in non-adherent lymphocytes were prepared and added to autologous LPS-pulsed or non-pulsed macrophages. The mixed cultures were incubated for 5 days and cell proliferation was measured. For each individual volunteer the intensity of DNA synthesis in the cultures of non-adherent lymphocytes stimulated with LPS-pulsed MØ was compared with the uptake of [3H]-thymidine in the cultures of tPBML stimulated with LPS itself. In both H.p. (+) and H.p. (-) LPS responders the uptake of [3H]-thymidine by non-adherent lymphocytes stimulated with autologous H. pylori LPS - pulsed MØ was significantly impaired as compared to DNA synthesis in tPBML incubated directly with H. pylori LPS (Fig. 2). Individual variations were marked in the group of LPS responders with regard to E. coli LPS driven cell proliferation responses. There was one H.p (+) LPS responder whose non-adherent lymphocytes incorporated significantly less [3H]-thymidine on the fifth day of stimulation with autologous E. coli LPS – pulsed MØ than was the score for tPBML stimulated directly with E. coli lipopolysaccharide (Fig. 2). For three other LPS responders, similar levels of DNA synthesis were observed in non-adherent lymphocytes stimulated with autologous E. coli LPS – pulsed MØ and tPBML stimulated directly with E. coli LPS. In both H.p (+) and H.p (-) LPS non-responders, the incorporation of [3H]-thymidine remained on very low levels in the cultures of non-adherent lymphocytes stimulated with autologous MØ pulsed with H. pylori or E. coli LPS and in the cultures of tPBML stimulated directly with H. pylori or E. coli lipopolysaccharide.

**DISCUSSION**

Activation of monocytes/macrophages, both murine and human is a well established property of LPS lipid A component. LPS has also been found to be able to activate the murine B and T lymphocytes (14, 15), but its influence on human T lymphocytes is not clear. An important H. pylori adaptation is the synthesis of LPS which has a much lower endotoxic activity, and also a lower ability to stimulate macrophages to produce the proinflammatory cytokines, nitric oxide, prostaglandins as compared to E. coli LPS (16). Data on the interactions of purified H. pylori LPS with a family of pathogen-recognition Toll-like receptors (TLRs) suggest that H. pylori LPS is not effectively recognized by TLR4-MD2-CD14 complex, which is critical in the activation of macrophages, granulocytes and dendritic cells (17). In this paper we observed a similar mitogenic activity of H. pylori LPS and E. coli LPS in the cultures of total PBML (tPBML) from a few volunteers. The H. pylori LPS driven proliferation of tPBML was independent of the H. pylori infection but it was probably determined by the susceptibility of host cells to bacterial LPS. In fact, tPBML from the majority of donors responded with proliferation to neither H. pylori LPS nor E. coli LPS. In the study by Mattern et al., PBML...
from approximately 50% of healthy donors proliferated in response to standard E. coli LPS (18). It seems that humans may be classified into LPS responders and non responders, and a type of LPS driven cell response may have an implication for the outcome of the infections with Gram negative bacteria including H. pylori. In the previous study by Rudnicka et al., H. pylori LPS alone expressed a very weak, if any, capacity to stimulate the proliferation of PBML from dyspeptic patients (19). However, in the presence of IL-2, which is a lymphocyte growth factor, H. pylori lysates or LPS were effective stimuli for these cells (19, 20). The role of interaction between IL-2 and macrophage CD14 antigen as an LPS-binding site, in the activation of human monocytes has been reported by Basco et al. (21).

The PBML populations from the majority of volunteers included into the study, who have been classified as non-responders, showed a deep and significant LPS driven decrease in the [3H]-thymidine incorporation as compared with the uptake of nucleic acid precursor into the cells cultivated in complete medium without any stimuli (Fig. 1). This decrease was associated with a significant alternation in the metabolic activity of non-adherent lymphocytes proved by the inhibition of MTT reduction and appearance of apoptotic changes (Table 1). These results allowed us to think that H. pylori LPS and E. coli LPS expressed an inhibitory effect towards lymphocytes from the majority of volunteers. Previously, the H. pylori VacA and CagA-induced immunosuppression has been suggested as a possible reason for the chronic character of H. pylori related infections (5, 6, 22, 23). In the study by Zabaleta et al., H. pylori decreased the expression of the TCR CD3ε-chain on T lymphocytes and the H. pylori L-arginase was responsible for the inhibition of T cell proliferation (24). Schmees et al. (25), identified the secreted gamma-glutamyl transpeptidase of H. pylori (HPGGT) as a factor responsible for the disruption of T cell Ras-dependent signaling during cell division. Also, other not fully-classified low molecular weight H. pylori compounds may cause the cell cycle arrest (26).

In this study, the deep and significant LPS driven decrease in the [3H]-thymidine incorporation observed for non-separated PBML from the majority of healthy volunteers (Fig. 1) could be explained as a result of a direct influence of H. pylori LPS and E. coli LPS on non-adherent lymphocytes or could have been a consequence of the indirect effect of LPS preparations on monocyte derived macrophages known as a main target of bacterial LPS. Thus, we used a two-stage culture model to explore a possible role of macrophages’ maturation in the LPS driven proliferation of PBML. In this model we pulsed mature macrophages with H. pylori LPS or E. coli LPS, and then we used the pulsed macrophages for stimulating autologous non-adherent lymphocytes. Great individual differences were seen between the reactions of non-adherent lymphocytes from LPS responders and non-responders regardless of H. pylori infection. The incorporation of [3H]-thymidine into non-adherent lymphocytes from the non-responders, stimulated with autologous mature macrophages, pulsed with H. pylori LPS or E. coli LPS, remained much below the [3H]-thymidine incorporation into the cells cultivated with not pulsed macrophages (Fig. 2). Most interestingly, in the group of LPS responders the mature macrophages pulsed with H. pylori LPS were found to be a weak stimulus for autologous non-adherent lymphocytes. The incorporation of [3H]-thymidine into non-adherent lymphocytes stimulated with H. pylori LPS-pulsed mature macrophages was significantly lower than the incorporation of the nucleic acid precursor into total PBML populations stimulated directly with H. pylori LPS itself. The cultures of non-separated PBML contained immature macrophages which allowed the intensive H. pylori LPS driven proliferation of PBML from the LPS responders. Thus, our results have revealed that in the milieu of H. pylori LPS, only mature macrophages from the LPS responders expressed the inhibitory activity towards non-adherent lymphocytes. We could see no evident inhibitory activity in E. coli LPS pulsed mature macrophages from the majority of LPS responders. Our results do not allow saying if the inhibitory activity of H. pylori LPS pulsed mature macrophages from the LPS responders should be attributed to the increase in the expression of surface co-stimulatory molecules such as CD80, CD86, CD40 and MHC antigens or adhesion and cytokine receptors. Alternatively, the inhibitory activity of mature macrophages from the H. pylori LPS responders could result from the changes in cytokine production, interleukin 1 (IL-1), IL-6, IL-8 and tumor necrosis factor (TNF) as well as non-protein mediators, for instance reactive oxygen species and bioactive metabolites of arachidonic and linoleic acids.

We should consider a possible H. pylori driven change in the expression of macrophage TLR4, which is the main LPS signaling receptor, as well as other receptors such as heat shock proteins - Hsp (Hsp 70, Hsp 90), CXXCR4, GDF-5, TREM proteins, a decay accelerating factor - DAF, CD11b/CD18 β-integrins and scavenger receptors (27). It has been shown that H. pylori augmented the growth of gastric cancers via the LPS- TLR4 pathway, whereas it attenuated the antitumor activity and IFN-γ - mediated cellular immunity of mononuclear cells (28). Caramlho et al., showed that the stimulation of CD4+CD25+ T regulatory cells by LPS via TLR4 receptor may promote their proliferation and suppressory activity (29). Raghavan et al., found that elimination of regulatory lymphocytes from the pool of H. pylori specific memory T cells increased their proliferating activity in response to H. pylori antigens, whereas the restoration of T regulatory cells abolished the blastogenic activity of these lymphocytes (30). Stuller et al. (31) demonstrated that the presence of T regulatory cells at the time of T cell activation in the gastric mucosa results in the generation of population of CD25 H. pylori specific anergic T cells. Moreover, once stimulated in the presence of T regulatory cells, this hyporesponsive CD25 population remains anergic even in the absence of CD25 regulatory T cells. It has been suggested that gastric epithelial cells expressing B7-H1, a costimulatory molecule, which is upregulated during H. pylori infection, may play a role in the induction of T cell anergy and the development of T regulatory cells from naive T lymphocytes.

Further studies are needed to explore a cause/s of H. pylori LPS driven decrease in [3H]-thymidine incorporation and metabolic activity of non-adherent lymphocytes from the LPS non-responders. This decrease was observed for the mixed cultures of non-adherent lymphocytes and H. pylori LPS pulsed mature macrophages as well as for total non-separated PBML incubated with H. pylori LPS itself. The difference in the responses to H. pylori LPS of peripheral blood mononuclear leukocytes from the LPS-responders and non-responders may be attributed to a various state and dynamics of macrophage maturation but it may also be caused by a difference in the susceptibility of non-adherent lymphocytes to a cytotoxic effect of bacterial LPS or macrophage products appearing under the influence of LPS.

In this paper a significant proportion of human non-adherent PBML exhibiting the features of apoptosis were found in the cultures treated with H. pylori LPS. It is possible to suggest a role of apoptotic changes in LPS-dependent antiproliferative activity of H. pylori bacteria towards T cells. It can be speculated that H. pylori LPS by an alternation of macrophage-lymphocyte interactions causes the persistence of lifelong H. pylori infection.

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