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Modulatory effect of interleukin-10 on the production of platelet-activating factor and superoxide anions by human leucocytes

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SUMMARY

We observed that human monocytes (MO) and polymorphonuclear neutrophils (PMN) stimulated by lipopolysaccharide (LPS) produce platelet-activating factor (PAF) in a pattern characterized by an early and a delayed peak of synthesis. The early peak of PAF synthesis was due to a direct stimulation of these cells through mCD14 receptor as it was inhibited by anti-CD14 monoclonal antibody. The delayed and sustained peak of PAF synthesis was dependent on protein synthesis and cytokine production as shown by the inhibitory effect of cycloheximide on both MO and PMN, and of anti-tumour necrosis factor-α (anti-TNF-α) and of anti-interleukin-8 (anti-IL-8) neutralizing antibodies on MO and PMN respectively. IL-10 completely prevented this second, cytokine-dependent peak of PAF synthesis. In contrast, IL-10 markedly enhanced the first peak of PAF synthesis both in MO and PMN. Moreover, IL-10 was shown to modulate the production of superoxide anions (O_2^-) on both MO and PMN. As suggested by previous studies, IL-10 inhibited the delayed production of O_2^-. In the present study, we observed that IL-10 directly stimulated an early production of O_2^-. In addition, IL-10 enhanced the synthesis of O_2^- by MO and PMN challenged with LPS. The IL-10-induced O_2^- production was dependent, at least in part, from its effect on PAF synthesis, as it was inhibited by the PAF receptor antagonist WEB 2170. These results suggest that IL-10 may upregulate the early synthesis of PAF and O_2^- triggered by direct LPS stimulation, whereas it may downregulate the delayed production of these mediators.

INTRODUCTION

Numerous inflammatory mediators produced by the host have been implicated in the pathogenesis of Gram-negative septic shock.[1] Among these mediators, a relevant role has been ascribed to a phospholipid mediator showing diverse and potent biologic actions, known as platelet-activating factor (PAF).[2] PAF belongs to a family of acetylated phosphoglycerides and acts through specific receptors.[3–6] The involvement of this mediator in inflammation and shock induced by bacterial lipopolysaccharide (LPS) has been suggested by the beneficial effect of PAF receptor antagonists on hypotension, renal insufficiency and lethality determined by infusion of LPS in rats.[2] Moreover, an intravascular release of PAF has been detected in patients with Gram-negative sepsis and in experimental animals injected with LPS.[7–8] The induction of PAF synthesis during Gram-negative sepsis may be triggered either by direct cell stimulation of LPS or by indirect stimulatory effect mediated by cytokines released from LPS-activated cells. In particular, tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) induce PAF synthesis in monocytes (MO), polymorphonuclear neutrophils (PMN), endothelial and glomerular mesangial cells.[9,10]

It has recently been shown that interleukin-10 (IL-10) modulates the development of LPS-induced inflammation and shock by acting on lymphoid and myeloid cells.[11] On MO and PMN, IL-10 acts mainly as a suppressive cytokine.[12,13] In fact, IL-10 inhibits the synthesis and release of several proinflammatory cytokines such as TNF-α, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) from LPS-activated cells.[11–13] Moreover, IL-10 was suggested to downregulate the production of other chemical mediators involved in the pathogenesis of LPS-induced hypotension and inflammation such as nitric oxide[14] and reactive oxygen intermediates.[14,15] *In vivo*, IL-10 has been shown to limit the duration and the extent of inflammatory response induced by LPS and to reduce lethality caused by septic shock.[16,17]

The aim of the present study was to evaluate whether IL-10 may modulate the synthesis of PAF and of superoxide anions (O_2^-) by MO and PMN stimulated with LPS.

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MATERIALS AND METHODS

Materials

Polymyxin B, phospholipase A2, phosphlipase A1, bovine serum albumin (BSA) fraction V (tested for not more than

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1 ng endotoxin per mg), fMet-Leu-Phe (FMLP), sphingomyelin and lyso-2-phosphatidylcholine (lyso-PC), cycloheximide were purchased from Sigma Chemical Company (St Louis, MO). Synthetic PAF (1-O-octadecyl- and 1-O-exadecyl-3 phosphorylcoline) and 1-O-octadecyl-2-lyso-glycero-3-phosphocholine (lyso-PAF), phosphorylcholine and phosphatidylethanolamine were from Bachem Feinchemikalien (Bubendorf, CH). WEB 2170 was obtained from Boehringer (Ingelheim, Germany). CV 3988 was from Takeda Chemical Industries (Kyoto, Japan). Anti-CD14 monoclonal antibody Leu M3 was from Beckton-Dickinson (Milano, Italy). The rabbit polyclonal anti-human TNF-α antibody (IgG fraction) was from Cetus (Emerville, CA); the monoclonal anti-human IL-8 antibody (isotype IgG2) and the goat polyclonal anti-human IL-10 antibody (IgG fraction) were from Sigma. Irrelevant polyclonal rabbit antibody anti-human von Willebrand (IgG fraction) and monoclonal anti-rabbit IgG (RG 96) were from Sigma. As isotypic controls, goat IgG and mouse IgG2b were also used (Cedarlane, Hornby, Ontario, Canada). IL-10 and the enzyme-linked immunosorbent assay (ELISA) kits for detection of human IL-8 and TNF-α were purchased from Genzyme Corporation (Cambridge, MA).

LPS from *Escherichia coli* (0111:B4) was purchased from Sigma. Stock solution of LPS were prepared by suspending 10 mg of LPS in 2 ml 20 mM EDTA and sonicating until clarified (3–5 20-sec bursts at maximum intensity using a W375 sonicator with a No. 419 microtip, Heat Systems-Ultrasonics, Farmingdale, NY). Aliquots of LPS stocks (200 μl) were stored at −20°, and when thawed for use were sonicated for 15 sec using a microsonicator (Microson, Heat Systems-Ultrasonics). LPS working dilutions were prepared in 10 mM HEPES saline formulated using 1 M HEPES stock (Gibco Laboratories, Grand Island, NY) and sterile, non-pyrogenic saline. Quantitative chromogenic LAL assay kit (COATEST-Endotoxin, Stockholm, Sweden) was from KabiVitrum (Stockholm, Sweden). C3b-coated Baker's yeast particles (C3b-BY) used as substrate for phagocytosis were prepared as previously described.[18] Human serum (HS) complement inactivated at 56° for 1 hr and acidified with HCl (2N) at pH 3·5 for 3 min to inactivate PAF-specific acetyl-hydrolase, was used as source of LPS-binding protein (LBP).

Preparation of human MO

Leucocytes were obtained from peripheral blood of normal donors collected in siliconized plastic tubes containing 5×10^{-3} M EDTA. MO were purified after centrifugation on a gradient of Fycoll–Hypaque and subsequent adhesion to plastic dishes as described by Valone & Epstein.[19] Non-adherent cells were removed by vigorous washing and 1 ml RPMI-1640 (Gibco, Paisley, UK) containing 0·25% BSA was added to each well. Adherent cells in representative wells were removed by scraping with a rubber-policeman and counted. The number of cells recovered per well was $2{\cdot}5 \pm 0{\cdot}2 \times 10^6$ (mean ± 1 SD for 10 consecutive studies). The adherent cells were >90% MO as detected by non-specific esterase staining and positivity by immunofluorescence for Leu M3 anti-CD14 monoclonal antibody. Less than one platelet for every 10 MO were detected. The viability of MO was more than 90% as assessed by Trypan blue dye exclusion test.

Preparation of human PMN

Human PMN were isolated from venous blood of health donors by sequential centrifugation and gelatin sedimentation (2·5% gelatin in PBS, pH 7·2, for 30 min at 37°) as previously described.[18] Contaminating erythrocytes were removed by hypotonic lysis and the cells were resuspended in RPMI-1640 medium at the final concentration of 5×10^6/ml. The percentage of neutrophils in cell preparation used in this study was 95–97%. The cell viability as determined by trypan blue exclusion was 96–98%.

Purification and quantitation of PAF

PAF was quantified after extraction and purification by thin layer chromatography (TLC; silica gel plates 60 F254, Merck, Darmstardt, Germany) and high-pressure liquid chromatography (HPLC; μPorasil column, Millipore chromatographic division, Waters, Milford, MA) by aggregation of washed rabbit platelets as previously reported.[20,21] The biologically active material extracted from cells and supernatants in different experiments was characterized by comparison with synthetic PAF by the following criteria:[20–23] (a) induction of platelet aggregation by a pathway independent both from ADP and from arachidonic acid/thromboxane A2-mediated pathways; (b) specificity of platelet aggregation as inferred from the inhibitory effect of 5 μM WEB 2170 or CV 3988, two different PAF receptor antagonists; (c) TLC and high-pressure liquid chromatography (HPLC) behaviour and physico-chemical characteristics such as inactivation by strong bases and by phospholipase A2 treatment, but resistance to phospholipase A1, acids, weak bases and 5 min heating in boiling water;[22] (d) the chemical identity with synthetic PAF was evaluated by HPLC-tandem mass spectrometry.[23]

Cytokine production

The quantitative determination of IL-8 or TNF-α in the supernatants of PMN and MO was performed by ELISA using the human IL-8 and TNF-α kits (Genzyme).

O_2^- assay

Production of O_2^- was measured as the superoxide dismutase inhibitable reduction of ferricytochrome C.[24] MO and PMN were incubated at 37° with Tyrode's buffer (2·6 mM KCl, 1 mM $MgCl_2$, 137 mM NaCl, 6 mM $CaCl_2$, 0·1% glucose, 1 mM Tris, pH 7·4) containing 80 μM cytochrome C and appropriately stimulated at specified time. Basal O_2^- production was assessed in the absence of stimulating factors. Supernatants were removed and centrifuged, and the absorbance was measured in a spectrophotometer at 550 nm. The extinction coefficient of ferricytochrome C at 550 nm was taken as $2{\cdot}1 \times 10^4$ $M^{-1}\,cm^{-1}$. Protein content of the MO was measured by the Lowry technique. O_2^- production was expressed as nanomoles of cytochrome C reduced/mg protein/time.[24]

Chemiluminescence

Luminol (50 μM) enhanced chemiluminescence was measured on 2×10^6 PMN suspended in HEPES-buffered Krebs medium containing 0·05% BSA using a LKB 1250 luminometer. Data are expressed in mVolt.[25]

Experimental protocols

For PAF and cytokine production, PMN or adherent MO washed three times with Tris-buffered Tyrode, were

equilibrated for 15 min in Tris-buffered Tyrode containing 0·25% delipidized BSA (fraction V) as previously described.[18–20]

Cells were incubated at 37° for the indicated time with different stimuli: LPS, 2% HS as source of LBP plus LPS, 2% HS alone, 2% HS-LPS plus IL-10, IL-10 alone. In selected experiments, the role of protein synthesis, of TNF-α or IL-8 production or of mCD14 was evaluated by stimulating the cells in the presence of 5 μg/ml cycloheximide or 5 μg/ml anti-TNF-α neutralizing antibodies or 100 μg/ml anti-IL-8 neutralizing antibodies or 2 μg/ml anti-CD14 Leu M3. As irrelevant antibodies the rabbit antibody anti-human von Willebrand, the monoclonal anti-rabbit IgG, the goat IgG, or the mouse IgG2 were used where appropriate. Each individual experiment was performed in duplicate. After the incubation of cells with various stimuli the supernatants and the cell pellets were extracted according to a modification of the Bligh & Dyer procedure,[26] with formic acid added to lower the pH of the aqueous phase to 3·0.

In selected experiments, MO or PMN were stimulated with LPS or LPS plus IL-10 in the presence of 2% HS as a source of LBP, IL-10 or vehicle alone or 2% HS alone to evaluate the production of O_2^-. Phagocytosis of C3b-BY or FMLP were used as positive controls. The treatment with 5 μg/ml Polimixin B for 30 min at 37° was used to evaluate the specificity of the effects ascribed to LPS. Moreover, as control for IL-10 effects, the cytokine was inactivated by boiling for 10 min or was preabsorbed with 10 μg/ml anti-IL-10-neutralizing antibodies. After preabsorbtion with anti-IL-10 antibody, IL-10-anti-IL-10 IgG complexes were removed by affinity chromatography on protein A Sepharose (Sigma Co).

RESULTS

PAF synthesis by human MO and PMN stimulated with LPS

Human MO

The ability of LPS to induce an early synthesis of PAF (15 min) from human MO trough stimulation of mCD14 has been recently reported.[27] In the present study the time course of PAF synthesis was extended up to 18 hr. Figure 1 (a and b) shows the total amount of PAF synthesized by human MO stimulated with LPS. A biphasic synthesis of PAF, characterized by an early transient peak at 15 min and a delayed-sustained peak at 6–12 hr, was obtained challenging MO with LPS in the presence of 2% HS as source of LBP, but not with LPS alone (Fig. 1a). PAF synthesized at 15 min was 95% cell associated, and only 5% released in the supernatant (Fig. 1c). The amount of PAF released in the supernatant progressively increased to reach the 65–75% at 6–12 hr (Fig. 1c). Pretreatment of LPS with polymyxin B (5 mg/ml at 37° for 30 min) abrogated both the early transient and the delayed sustained synthesis of PAF (data not shown). Also, anti-CD14 monoclonal antibody (Leu M3), but not an irrelevant monoclonal anti-rabbit IgG, completely prevented the early and delayed peak of PAF synthesis (Fig. 1a). The second but not the first peak of PAF was abrogated by neutralizing anti-TNF-α antibody and by pretreatment of the cells with cycloheximide (5 μg/ml), an inhibitor of protein synthesis (Fig. 1b). PAF synthesis induced by LPS was not affected by an irrelevant polyclonal rabbit antibody anti-human von Willebrand factor or by neutralizing anti-IL-8 antibody (data not shown).

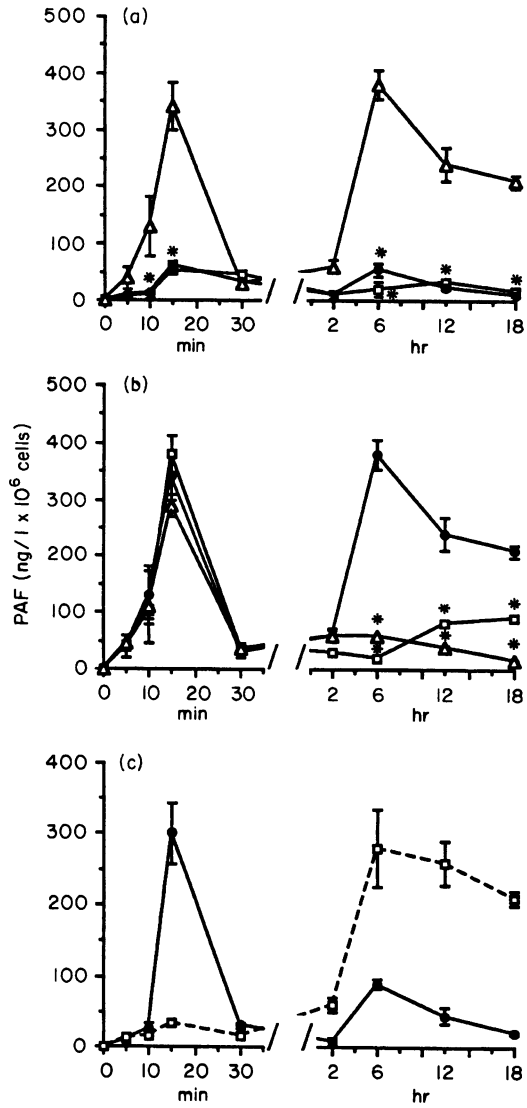

Figure 1. (a) Total amount of PAF synthesized by 1×10^6 MO stimulated with: 10 ng/ml LPS alone (●), 10 ng/ml LPS plus 2% HS as a source of LBP (△), 10 ng/ml LPS–2% HS plus 2 μg/ml Leu M3 anti-CD14 antibody (□). Data are mean ± SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among MO stimulated with LPS–2% HS versus LPS alone or versus LPS–2% HS plus anti-CD14 (*$P < 0.05$). (b) Total amount of PAF synthesized by 1×10^6 MO stimulated with: 10 ng/ml LPS plus 2% HS as a source of LBP (●), 10 ng/ml LPS–2% HS plus 10 μg/ml cycloheximide added at 37° for 30 min before LPS–2% HS (△) and 10 ng/ml LPS–2% HS plus 5 μg/ml anti-TNF-α neutralizing antibody (□). Data are mean ± SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among MO stimulated with LPS–2% HS versus LPS–2% HS plus cycloheximide or versus LPS–2% HS plus anti-TNF-α (*$P < 0.05$). (c) Time course of PAF synthesis from 1×10^6 MO challenged with 10 ng/ml LPS plus 2% HS. PAF was detected as cell associated (●) and as released in the supernatant (□). Data are mean ± SD of four individual experiments.

Human PMN

As observed for MO, PMN stimulated with LPS and 2% of HS as source of LBP synthesize PAF with a biphasic pattern (Fig. 2). The first peak was observed at 10 min and the second

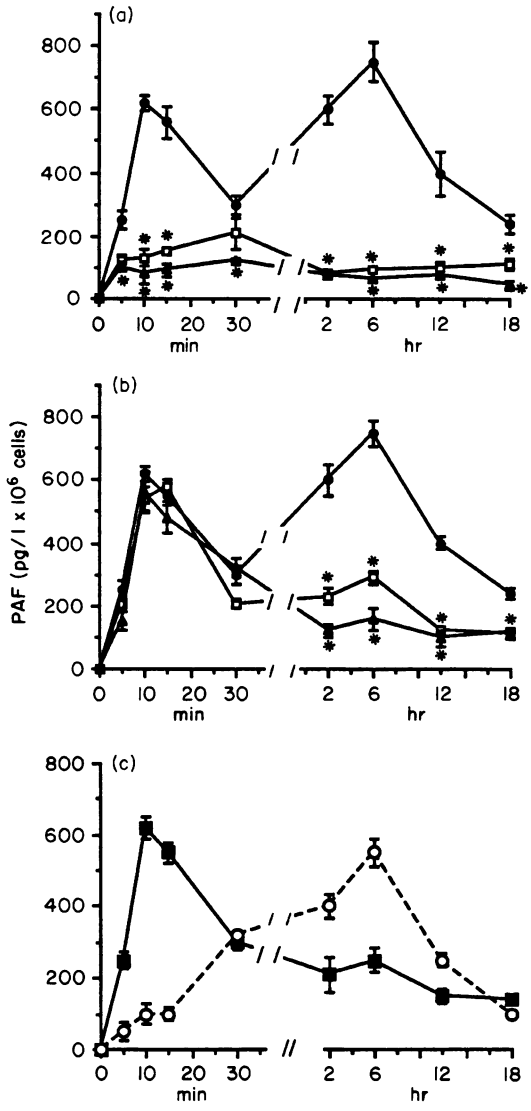

Figure 2. (a) Total amount of PAF synthesized by 1×10^6 PMN stimulated with: 10 ng/ml LPS alone (□), 10 ng/ml LPS plus 2% HS as a source of LBP (●) and 10 ng/ml LPS–2% HS plus 2 μg/ml Leu M3 anti-CD14 antibody (▲). Data are mean ± SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among PMN stimulated with LPS–2% HS versus LPS alone or versus LPS–2% HS plus anti-CD14 (*$P < 0{\cdot}05$). (b) Total amount of PAF synthesized by 1×10^6 PMN stimulated with: 10 ng/ml LPS plus 2% HS as a source of LBP (●), 10 ng/ml LPS–2% HS plus 10 μg/ml cycloheximide added at 37° for 30 min before LPS–2% HS (▲) and 10 ng/ml LPS–2% HS plus 100 μg/ml anti-IL-8 neutralizing antibody (□). Data are mean ± SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among PMN stimulated with LPS–2% HS versus LPS–2% HS plus cycloheximide or versus LPS–2% HS plus anti-IL-8 (*$P < 0{\cdot}05$). (c) Time course of PAF synthesis from 1×10^6 PMN challenged with 10 ng/ml LPS plus 2% HS. PAF was detected as cell associated (■) and as released in the supernatant (○). Data are mean ± SD of four individual experiments.

peak at 6 hr after stimulation with LPS in the presence of 2% HS as source of LBP. LPS alone had no effect on PAF synthesis (Fig. 2a). PAF synthesis at 10 min was mainly cell associated, while at 6 hr, PAF was mainly released in the supernatant (Fig. 2c). Anti-CD14 monoclonal antibody, but not an irrelevant monoclonal anti-rabbit IgG, completely prevented the early and delayed peak of PAF synthesis (Fig. 2a). Previous studies have shown that stimulation of PMN with LPS induced synthesis of IL-8 which increases from 2 to 20 hr.[28] Since IL-8 has been reported to stimulate PAF synthesis,[29] we tested the effect of anti-IL-8 neutralizing antibodies on PAF synthesis by PMN stimulated with LPS. Pretreatment with anti-IL-8 neutralizing antibody almost completely abrogated this second peak, but not the first (Fig. 2b). Similar results were obtained by treating the cells with cycloheximide (Fig. 2b). In contrast, anti-TNF-α antibodies or an isotype control IgG2b did not inhibit the synthesis of PAF by PMN (data not shown).

Control MO and PMN incubated with 2% HS did not produce PAF.

Effect of IL-10 on PAF synthesis by MO and PMN stimulated with LPS

IL-10 inhibited the total PAF synthesis (cell associated and released) of the second delayed-sustained peak of PAF induced by LPS and 2% HS both on MO and PMN (Fig. 3). No variation in the ratio of cell associated to released PAF was observed (data not shown). In contrast, IL-10 markedly increased the first peak of PAF synthesis. IL-10 alone did not stimulate PAF synthesis. Figure 4 shows the potentiating effect of IL-10 on the first peak of PAF synthesis observed after incubation of MO or PMN with various concentrations of LPS. In this peak, PAF was entirely detected as cell associated. On PMN, this effect of IL-10 was evident only for the lower concentrations of LPS (Fig. 4b). Pretreatment with IL-10 was shown to inhibit 95% and 67% respectively of the production of TNF-α from MO (unstimulated MO: 35 ± 10 pg/ml, LPS-stimulated MO: 1400 ± 65 pg/ml, IL-10-pretreated LPS-stimulated MO: 80 ± 23 pg/ml) and of IL-8 from PMN (unstimulated PMN: 0·5 ± 0·1 pg/ml, LPS-stimulated PMN: 50 ± 4 pg/ml, IL-10-pretreated LPS-stimulated PMN: 17 + 2 pg/ml) stimulated with 10 ng/ml LPS and 2% HS.

Inactivation of IL-10 by 5 min boiling or preabsorption with 10 μg/ml of neutralizing anti-IL-10 antibodies completely prevented both the stimulatory and inhibitory effect of this cytokine (data not shown).

Effect of IL-10 on O_2^- production by MO and PMN

As shown in Fig. 5, IL-10 alone induced O_2^- production by MO which peaked 8 min after the addition of IL-10. However, when MO were stimulated with FMLP after 24 hr incubation with IL-10, they failed to produce O_2^-. These data suggest that IL-10 stimulated an early synthesis of O_2^- but inhibited the subsequent ability of these cells to respond to FMLP or phagocytosis (data not shown). LPS, in the presence of 2% HS as a source of LBP, induced only a slight production of O_2^- 5 min after stimulation of MO. The addition of IL-10 anticipated at 2 min and enhanced the response to LPS. MO incubated with LPS, but not with LPS and IL-10, were able to produce O_2^- when challenged 24-hr later with FMLP. These effects were abrogated by 5 min boiling of IL-10 as well as by preabsorption of the cytokine with 10 μg/ml of anti-IL-10 neutralizing antibody (see Fig. 7 later).

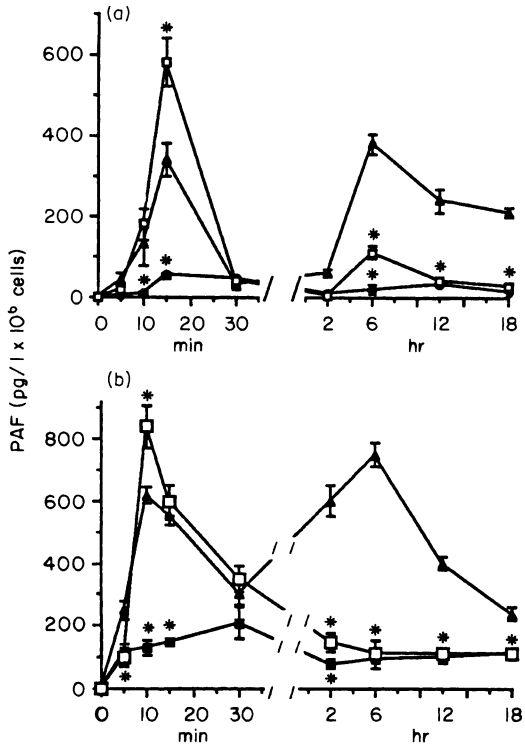

Figure 3. (a) Total amount of PAF synthesized by 1×10^6 MO stimulated with: 20 ng/ml IL-10 alone (■), 10 ng/ml LPS–2% HS (▲) and 10 ng/ml LPS–2% HS plus 20 ng/ml IL-10 (□). Values are given as mean ± 1 SD of six individual experiments. ANOVA with Dunnet's multicomparison test was performed among MO stimulated with LPS–2% HS versus LPS–2% HS plus IL-10 or versus IL-10 alone (*$P < 0.05$). (b) Effect of IL-10 on total production of PAF by 1×10^6 PMN. PMN were stimulated with 10 ng/ml LPS–2% HS (▲), 20 ng/ml IL-10 alone (■) and 10 ng/ml LPS–2% HS plus 20 ng/ml IL-10 (□). Values are mean + 1 SD of six individual experiments. ANOVA with Dunnet's multicomparison test was performed among PMN stimulated with LPS–2% HS versus LPS–2% HS plus IL-10 or versus IL-10 alone (*$P < 0.05$).

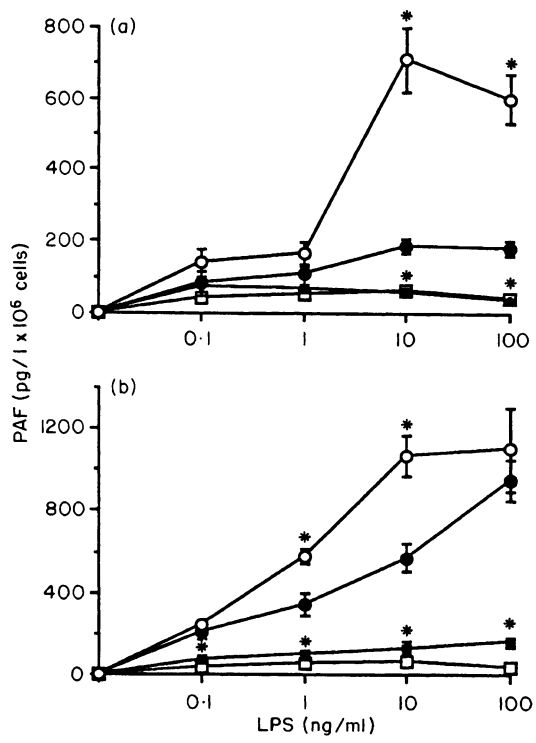

Figure 4. (a) Effect of IL-10 on PAF synthesis by 1×10^6 MO incubated with different doses of LPS. PAF was all detected as cell associated from MO incubated for 15 min at 37° with 2% HS alone (□) or stimulated with LPS alone (■), LPS–2% HS (●) and LPS–2% HS plus 20 ng/ml IL-10 (○). Values are mean ± 1 SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among MO stimulated with LPS–2% HS versus LPS alone or versus LPS–2% HS plus IL-10 (*$P < 0.05$). (b) Effect of IL-10 on PAF synthesis by 1×10^6 PMN stimulated with different doses of LPS. PAF was all detected as cell associated from PMN incubated for 10 min at 37° with 2% HS alone (□) or stimulated with LPS alone (■), LPS–2% HS (●) and LPS-2% HS plus 20 ng/ml IL-10 (○). Values are mean ± 1 SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among PMN stimulated with LPS–2% HS versus LPS alone or versus LPS–2% HS plus IL-10 (*$P < 0.05$).

Similar experiments were performed on PMN. PMN, when challenged with LPS alone did not produce a significant amount of O_2^-. However, when incubated with IL-10 and LPS, PMN produced a relevant amount of O_2^- already detectable after 1 min of incubation (Fig. 6). This effect of IL-10 was synergistic rather than addictive as the peak of O_2^- production observed at 1 min largely exceeded the sum of O_2^- produced by the individual stimulation of PMN with LPS or IL-10 alone. The preabsorption with 10 μg/ml of anti-IL-10 neutralizing antibody abrogated all these effects of IL-10 (Fig. 7). As previous studies suggested a role of PAF in LPS-induced priming of PMN superoxide secretion,[30] we evaluated whether the effect of IL-10 on PAF synthesis was related to the effect of IL-10 on O_2^- production. For this purpose cells were pretreated for 2 min with the PAF receptor antagonist WEB 2170. WEB 2170 significantly reduced the priming effect of IL-10 on the production of O_2^- by MO and PMN (Fig. 7a and b).

The stimulating effect of IL-10 on the production of oxygen radicals from PMN was also tested by chemiluminescence. As shown in Fig. 8, the synergic effect of IL-10 on LPS-induced chemiluminescence was significantly reduced by WEB 2170.

DISCUSSION

The results of the present study suggest that IL-10 modulates direct and cytokine-dependent synthesis of PAF as well as O_2^- production by human MO and PMN stimulated with LPS.

Several studies indicate that the synthesis of PAF from LPS-stimulated cells may be triggered either by a direct stimulatory effect or by indirect stimulation via proinflammatory cytokines such as TNF-α, IL-8 and IL-1.[20,21,29] In fact, these cytokines can induce PAF synthesis by MO, PMN and endothelial cells.[20,21,29] The direct stimulation of the cells of the host by LPS is triggered by a receptor-dependent mechanism.[31–33] It has been shown that the membrane glycerophosphatidyl-inositol anchored CD14 molecule (mCD14) mediates the specific LPS-dependent stimulation of MO/macrophages.[31–33] The mechanism of LPS-cell interaction involves the formation of complexes of LPS with a plasma protein named LPS-binding protein (LBP), that may catalyse the interaction of LPS with the cell surface receptor mCD14.[31–33] Cells, directly stimulated by LPS, may synthesize inflammatory

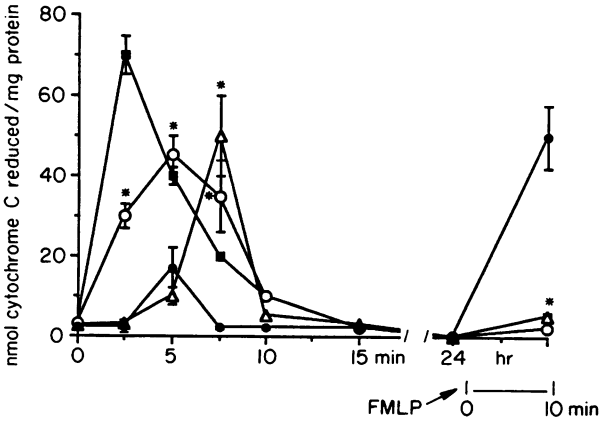

Figure 5. (a) Production of O_2^- from MO (1×10^6 cells) incubated with 10 ng/ml LPS–2% HS (●), 10 ng/ml LPS–2% HS plus 20 ng/ml IL-10 (○), 20 ng/ml IL-10 alone (△) or stimulated with phagocytosis of 1×10^7 C3b-BY (■). The figure shows the direct production of O_2^- within the early 15 min incubation and the effect of stimulation with 0·1 μM FMLP 24 hr after the beginning of the experiments. Values are mean ± 1 SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among MO stimulated with LPS–2% HS versus IL-10 alone or versus LPS–2% HS plus IL-10 (*$P < 0.05$).

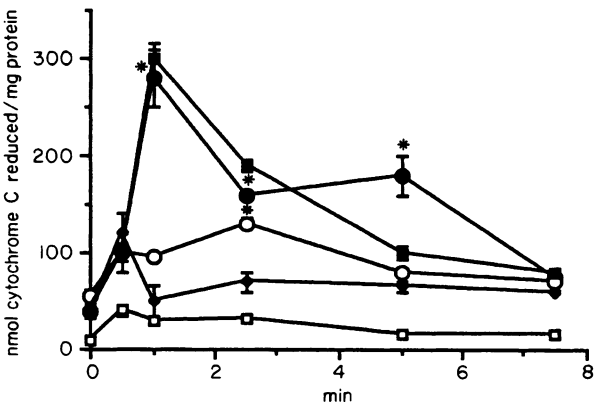

Figure 6. Production of O_2^- from PMN (1×10^6 cells) unstimulated (□) or stimulated with 20 ng/ml IL-10 alone (○), 1 ng/ml LPS–2% HS (◆), 1 ng/ml LPS–2% HS plus 20 ng/ml IL-10 (●) or with phagocytosis of 1×10^7 C3b-BY (■). Values are mean ± 1 SD of four individual experiments. ANOVA with Dunnet multicomparison test was performed among PMN stimulated with LPS–2% HS versus IL-10 alone or versus LPS–2% HS plus IL-10 (*$P < 0.05$).

cytokines including TNF-α, IL-1, IL-6 and IL-8.[32,33] Moreover, we recently demonstrated that the stimulation of mCD14 receptor by LPS-LBP complexes promotes an early synthesis of PAF by MO.[27]

The results of the present study indicate that also PMN, which bear mCD14,[31] can be stimulated to synthesize PAF by the same mechanism. In fact, the synthesis of PAF triggered by LPS on PMN was abrogated by anti-CD14 antibodies and required serum as a source of LBP. Moreover, we observed that the pattern of LPS-induced synthesis of PAF by both MO and PMN was biphasic. Similar results were previously reported for mesangial cells and for monocytes stimulated with cytokines.[27,34] The early peak of PAF synthesis was observed at 10 min for PMN and at 15 min for MO. The

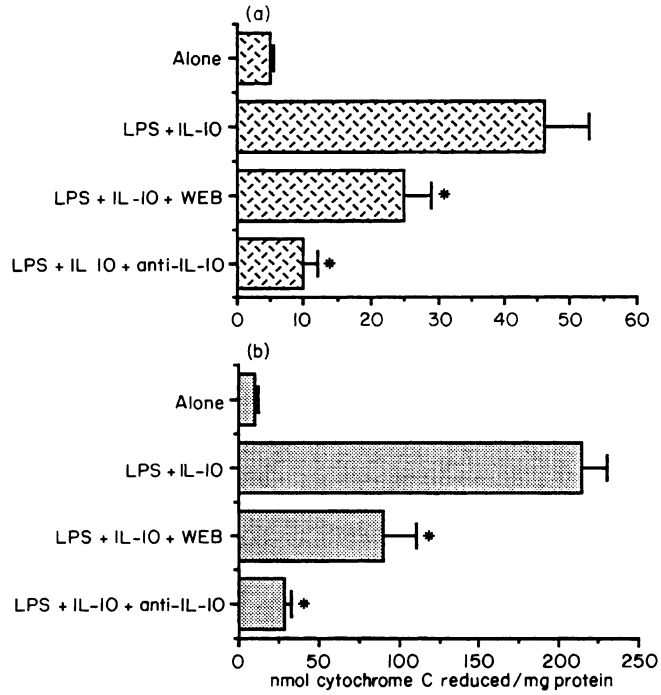

Figure 7. (a) Production of O_2^- at 5 min from MO (1×10^6 cells) unstimulated (Alone) or stimulated with 10 ng/ml LPS–2% HS plus 20 ng/ml IL-10 (LPS + IL-10), 10 ng/ml LPS–2% HS plus 20 ng/ml IL-10 after preincubation for 2 min with WEB 2170 (LPS + IL-10 + WEB) and 10 ng/ml LPS–2% HS plus 20 ng/ml IL-10 preabsorbed with 10 μg/ml anti-IL-10 neutralizing antibody (LPS + IL-10 + anti-IL-10). (b) Production of O_2^- at 5 min from PMN (1×10^6 cells) unstimulated (Alone) or stimulated with 1 ng/ml LPS–2% HS plus 20 ng/ml IL-10 (LPS + IL-10), 1 ng/ml LPS–2% HS plus 20 ng/ml IL-10 after preincubation for 2 min with WEB 2170 (LPS + IL-10 + WEB) and 1 ng/ml LPS–2% HS plus 20 ng/ml IL-10 preabsorbed with 10 μg/ml anti-IL-10 neutralizing antibody (LPS + IL-10 + anti-IL-10). Values are given as mean ± 1 SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among MO or PMN stimulated with LPS–2% HS plus IL-10 versus LPS–2% HS plus IL-10 plus WEB 2170 or versus LPS–2% HS plus IL-10 plus anti-IL-10 antibodies (*$P < 0.05$).

delayed and sustained peak of PAF synthesis was detected at 3–6 hr for PMN and 6–12 hr for MO. PAF detected in the first peak was almost completely cell associated, whereas in the second peak PAF was mainly released in the supernatant.

The first peak of PAF synthesis was because of direct stimulation of mCD14 as it was abrogated by anti-CD14 monoclonal antibody and did not require protein synthesis. In contrast, the second peak of PAF production was strictly dependent on protein synthesis as it was inhibited by cycloheximide. Moreover, anti-TNF-α and anti-IL-8 antibodies significantly inhibited the second peak of PAF synthesis respectively from MO and PMN. In contrast, anti-TNF-α and anti-IL-8 neutralizing antibody did not prevent the first peak of PAF synthesis.

Several studies indicate that TNF-α is a central mediator in septic shock.[35] The synthesis of TNF-α temporarily precedes production of other cytokines both in experimental animals and in humans.[35] The lethal toxicity of TNF-α also depends on production of other secondary mediators including regulatory cytokines, reactive oxygen radicals and eicosanoids such

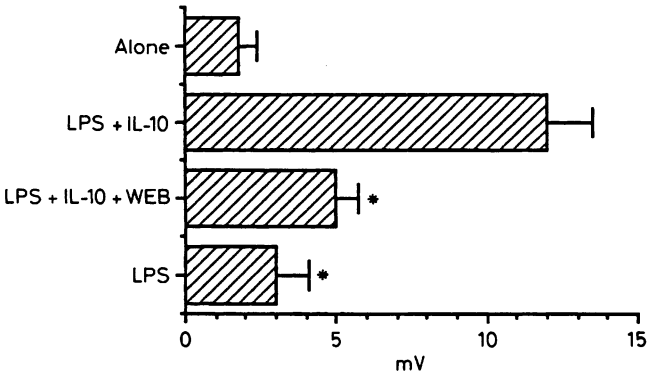

Figure 8. Chemiluminescence of PMN recorded 5 min after stimulation with 10 ng/ml LPS–2% HS (LPS), with 10 ng/ml LPS–2% HS plus 20 ng/ml IL-10 (LPS + IL-10), with 10 ng/ml LPS–2% HS plus 20 ng/ml IL-10 after preincubation for 2 min with a PAF receptor antagonist WEB 2170 (LPS + IL-10 + WEB). Alone indicates chemiluminescence of unstimulated PMN incubated for the same period of time. Values are given as mean ± 1 SD of four individual experiments. ANOVA with Dunnet's multicomparison test was performed among PMN stimulated with LPS–2% HS plus IL-10 versus LPS–2% HS or versus LPS–2% HS plus IL-10 plus WEB 2170 (*$P < 0.05$).

as leukotriens and PAF.[35,36] PAF, in particular, mediates several of the biological effects of TNF-α.[36] IL-8 is also produced during septic shock.[1] This cytokine is a potent activator of PMN and can induce PAF production from these cells.[29] The synthesis of TNF-α, IL-1, IL-8 and other proinflammatory cytokines triggered by the direct stimulation of mCD14 receptor on MO and PMN has been shown to be prevented by IL-10.[13,37] This inhibitory mechanism has been ascribed to transcriptional and post-transcriptional effects.[12,13,38,39]

In the present study we tested the effect of IL-10 on PAF synthesis by MO and PMN stimulated with LPS. It was found that the second cytokine-dependent peak of PAF synthesis was completely abrogated by IL-10 which also prevented the release of cytokines induced by LPS. This result further indicates the dependency on cytokine production of the delayed and sustained synthesis of PAF by MO and PMN stimulated with LPS. Unexpectedly, the first peak of PAF synthesis was markedly enhanced by IL-10 both in MO and PMN. These data suggest a 'priming' effect of IL-10 on the early production of PAF triggered by LPS direct cell stimulation. Moreover, previous studies have shown that IL-10 downregulated the generation of reactive oxygen intermediates when macrophages were preincubated with IL-10 for 48 hr and then stimulated with phorbol-myristate acetate.[15] On the basis of the present observation that IL-10 is stimulatory on the early production of PAF, we extended the time course of oxygen radicals generation also to the early times. Our results confirm the inhibitory effect of IL-10 on the delayed production of O_2^- by MO stimulated with FMLP. However, we found that IL-10 possessed an early stimulatory effect. Indeed, IL-10 directly stimulated an early production of O_2^- by MO and to a minor extent by PMN. In addition, IL-10 added in association with LPS, which *per se* was scarcely or totally ineffective, anticipated and enhanced the synthesis of O_2^- by MO and PMN. The effect of IL-10 on early O_2^- production by LPS-stimulated MO and PMN can, at least in part, be ascribed to the 'priming' effect of this cytokine on PAF production, as the PAF receptor antagonist WEB 2170 significantly reduced the IL-10-enhanced generation of oxygen radicals. These results are consistent with the previously reported observation that the synthesis of PAF mediates the 'priming' of superoxide secretion on human PMN.[30]

In conclusion, the results of the present study indicate that IL-10 may upregulate the early production of mediators by inflammatory cells, whereas it downregulates the delayed and persistent inflammatory response. Our data are consistent with a recent study that limits IL-10 inhibitory effect to the synthesis of new mediators, but not to the release of preformed inflammatory products.[40] Therefore, IL-10 may have a biphasic action in the development of inflammation and shock: it may potentiate the early inflammatory response that can be beneficial for the host defence, and it may suppress the excessive production of proinflammatory mediators detrimental for the organism.

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