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Polymorphonuclear leucocyte (PMN)-derived inflammatory cytokines—regulation by oxygen tension and extracellular matrix

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SUMMARY

The kinetics of IL-8, tumour necrosis factor-alpha (TNF-α) and IL-1β release by PMN adhered to fibronectin, laminin or plastic for 24 h in response to continuous stimulation with lipopolysaccharide (LPS; 50 ng/ml), N-formyl-Met-Leu-Phe (fMLP; 100 mM), or phorbol myristate acetate (PMA; 10 ng/ml), was investigated under altered oxygen tension conditions. Cell supernatants were sampled for cytokine content every 6 h and measured by ELISA. IL-8 was the most abundant cytokine, produced in a range of up to 5·4 ng/ml; TNF-α and IL-1β were produced in a range of up to 1 ng/ml. During normoxia, LPS was the most potent stimulus, inducing the release of each cytokine, while fMLP showed a less pronounced effect on IL-8 and IL-1β production and markedly inhibited TNF-α production. PMA markedly suppressed IL-8 and IL-1β release and failed to induce any release of TNF-α. Hypoxia had an overall inhibitory effect on cytokine release except for PMA-induced IL-1β release, and hypoxia/reoxygenation had a significant up-regulating effect except for a further inhibition of fMLP-induced release of TNF-α. Integrin–matrix protein ligation differentiated both spontaneous and externally induced cytokine release and its sensitivity to alteration in oxygen tension. Thus the process of PMN elaboration of inflammatory cytokines is controlled on multiple levels of signal transduction, differentiated by integrin–extracellular matrix interactions, and is sensitive to alterations in microenvironmental oxygen tension.

Keywords PMN matrix proteins oxygen tension

INTRODUCTION

PMN defence mechanisms against invasive sepsis include phagocytosis and intracellular killing, release of various proteases and production of reactive oxygen intermediates [1,2]. Being considered a terminally differentiated cell, the PMN has not been appreciated in terms of *de novo* protein synthesis. Recently, a growing body of evidence has demonstrated that PMN are capable of generating cytokines (tumour necrosis factor-alpha (TNF-α), interferon-alpha (IFN-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-6, and IL-8), which constitutes a marked departure from previous understanding of the capabilities of this cell [3–6].

IL-8, TNF-α and IL-1β have been shown to strongly influence neutrophil function in a variety of experimental and clinical settings [3,7–9]. Further, PMN function is regulated by its interactions with extracellular matrix through integrin molecules [2,10–12]. Another phenomenon implicated in diverse clinical situations is modulation of PMN function by altered oxygen tensions [11,12]. Nonetheless, the effect of altered oxygen tensions on PMN–matrix protein interactions with regard to cytokine production has not been previously investigated. Therefore, the current study was designed to investigate PMN release of inflammatory cytokines as a function of integrin signalling and sequential changes in environmental oxygen tension from normoxia to hypoxia and reoxygenation.

In order to enhance the release of cytokines by PMN surface receptor stimulation we employed lipopolysaccharide (LPS) and N-formyl-Met-Leu-Phe (fMLP) in considering their well established agonist-like effect [3,13]; in order to directly stimulate protein kinase C (PKC) we used phorbol myristate acetate (PMA) at a low concentration [3,5,14].

We now report that purified adhered PMN can release immunologically active forms of IL-8, TNF-α or IL-1β and that this process is subject to regulation at multiple levels, including integrin signalling and alterations in microenvironmental oxygen tension.

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MATERIALS AND METHODS

Buffers and reagents

PBS, Hanks' balanced salt solution (HBSS) with and without Ca^{++} and Mg^{++} ($HBSS^{+}$ and $HBSS^{-}$), penicillin–streptomycin were

obtained from GIBCO Labs (Grand Island, NY). The reagents and their sources were as follows: dextran (United States Biochemical Corp., Cleveland, OH), Ficoll–Hypaque (Litton Bionetics, Kensington, MD), fibronectin (Fn) and laminin (Ln) (Collaborative Biomedical, Bedford, MA), LPS serotype 055:B5, fMLP, PMA, adenine, actinomycin D, cycloheximide (Sigma Chemical Co., St Louis, MO), fetal bovine serum (FBS; HyClone Labs, Logan, UT), cell culture RPMI 1640 IX media with L-glutamine (Mediatech, Washington, DC).

Preparation of human PMN

The starting material was fresh heparinized venous blood obtained from healthy volunteers. The initial cell suspensions were conventionally fractionated on Ficoll–Hypaque and Dextran gradients [6,15] to separate the PMN from lymphocytes, monocytes, and platelets. After a double hypotonic lysis, cells were washed twice. PMN preparations were ≈97% pure as assessed by Giemsa staining and 95% viable as assessed by trypan blue exclusion. At the completion of the 24-h incubation, monocyte and lymphocyte subsets were ≈1·5%, respectively, and PMN viability remained 90–95%.

Preparation of hypoxic or reoxygenated buffer

Aliquots (30–40 ml) of RPMI media supplemented with 20% v/v heat-inactivated FBS to ensure PMN cytokine release [9], 100 μM adenine to ensure PMN viability in culture [16], and 100 μg/ml penicillin–streptomycin, were placed in sterile medical grade silicon tubing (Cole-Parmer, Chicago, IL). The tubing was placed in a clear plexiglass chamber which was filled with a standardized mixture of 93% N_2, 5% CO_2, 2% H_2 (MG Industries, Valley Forge, PA). Buffer was then slowly pumped through this system of tubing by a Masterflex Pump (Cole-Parmer) using tubing adapters (no. 8205; Becton Dickinson, Cambridge, MA). Buffer pO_2 was dropped from 180–200 to 60–70 mmHg after 30 min of exposure to the gas mixture. Buffer was then collected into a syringe and put into a 50 ml conical tube located in a plexiglass chamber with gas in- and outflow valves and then hermetically sealed. The above mentioned gas mixture was then pumped directly through buffer aliquots rendering the buffer hypoxic with levels of pO_2 being $\leqslant$ 20 mmHg. Buffer pO_2 was confirmed prior to all assays and subsequently frequently monitored using a Clark-type oxygen electrode connected to a Dual Channel Differential Oxygen Electrode Amplifier (Instech, Plymouth Meeting, PA). All following manipulations starting from cell resuspension up to sample harvesting were performed inside this chamber in a sustained hypoxic environment, while the above mentioned gas mixture was constantly pumped through the chamber. Reoxygenation was achieved by removing the covers to the plates which were kept on a heating pad at 37°C and the pO_2 of cell suspensions was measured at 5 min intervals. Buffer reoxygenation ($pO_2 > 150$ mmHg) occurred after 20 min of exposure at atmospheric pressure. Therefore, during subsequent assays cells were allowed to reoxygenate for 1 h at 37°C at room barometric pressure.

Cytokine production and immunoassay

After final purification PMN were resuspended at 4×10^6/ml in the above mentioned supplemented RPMI media and then incubated without agonists, LPS (50 ng/ml), fMLP (1×10^{-7} M) or PMA (10 ng/ml). The resulting cell suspensions were then distributed in 250 μl aliquots in 96-well flat-bottomed plates (Costar, Cambridge, MA), precoated for 4 h with buffer, Fn (10 μg/well), or Ln (5 μg/well). The plates were then spun at 800 ***g*** for 5 min to facilitate cell adhesion and PMN were then incubated for 24 h. To determine extracellular cytokine concentrations, cell-free supernatants were collected during every 6 h of incubation, centrifuged at 800 ***g*** for 10 min and stored at −70°C until assayed. Depending on the particular assay, the preparation of final cell suspensions, distribution and sample acquisition were performed using either normoxic buffer, hypoxic buffer, inside the plexiglass chamber, or were rendered reoxygenated for 1 h at 37°C.

Cytokine concentrations were measured using commercially available ELISA kits (R & D Systems, Minneapolis, MN), following the manufacturer-specified quantitative immunometric 'sandwich' ELISA technique. The lower limits of sensitivity for IL-8, TNF-α and IL-1β were 4·7, 4·4 and 0·3 pg/ml, respectively. No cross-reactivity or interference among the above reported cytokines was found. Optical densities (OD) were determined at 450/550 nm on an EL340 microplate reader (Bio-Tek Instruments, Winooski, VT), plotted against a standard curve using DeltaSoft II software (Biometallic Inc., Princeton, NJ), and actual protein concentrations are expressed as pg/ml.

Superoxide anion production

Superoxide anion released by PMN was assayed by quantitative reduction of cytochrome c as described [17]. After 100 μM cytochrome c in 1 ml total volume were added, PMN at 2×10^6/ml in normoxic, hypoxaemic or H/R buffers (70% O_2 saturation) were adhered to buffer, Fn or Ln (1 μg) for 30 min. The reactions were stopped after 30 min at 37°C by immediate immersion of reaction tubes in ice followed by centrifugation at 4°C and reading of supernatants at 550 nm (spectrophotometer model 576ST; Perkin-Elmer, San Diego, CA). Control tubes included superoxide dismutase (SOD; 100 μg/ml). Results are expressed as nanomoles of SOD-inhibitable ferricytochrome c reduced per PMN (2×10^6/ml) per 30 min, using the previously described extinction coefficient of 21·1 m^{-1}/cm, followed by subtraction of calculated values for corresponding SOD and unstimulated control PMN measured simultaneously.

Myeloperoxidase release

The assays were performed as previously described [18], using 96-well U-shaped Immulon 1-μl plates (Dynatech Labs, Chantilly, VA). PMN at 8×10^6/ml were pretreated with cytochalasin B (5 μg/ml) immediately before 25 μl of the PMN were added to each well. The microtitre plates were then incubated for 30 min at 37°C in a 5% CO_2 incubator in normoxic, hypoxic or H/R buffer. Fifty microlitres of 0·2 M sodium phosphate buffer pH 6·2 were then added followed by 25 μl of a solution containing equal parts of 15 mM H_2O_2 combined with 3·9 mM 3′3′ dimethoxybenzidine. A visible colour reaction was stopped after 15 min at room temperature by 1% sodium azide. The OD was assayed at 490 and 630 nm on an MR580 model micro-elisa automatic reader (Dynatech Labs).

Statistical analysis

Data are presented as means ± s.e.m. The obtained results were analysed by one-way ANOVA with post-hoc Scheffe *f*-test to determine significance between groups. $P < 0{\cdot}05$ was considered statistically significant.

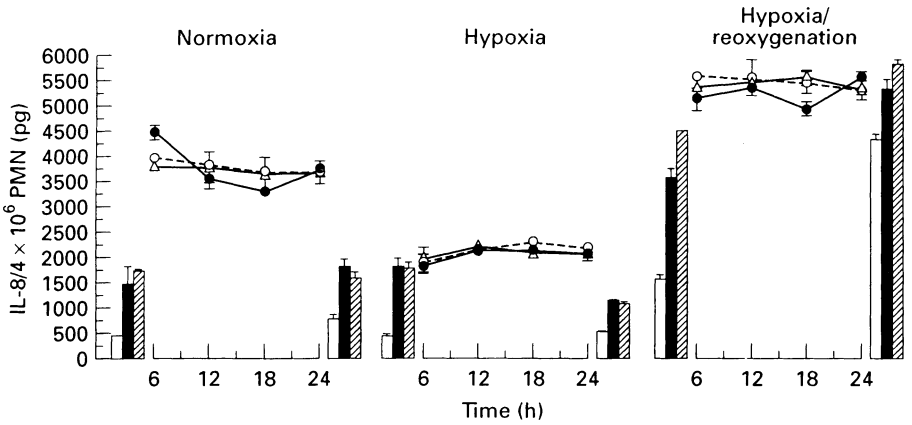

Fig. 1. IL-8 concentration (pg/ml) in cell supernatants under normoxia (left), hypoxia (middle) and hypoxia/reoxygenation (right). PMN at 4×10^6/ml in 250 μl aliquots were placed into 96-well plates and adhered for up to 24 h to blank plastic surfaces, or surfaces precoated either with 10 μg/well fibronectin (Fn) or 5 μg/well laminin (Ln). Cells were continuously stimulated either with buffer (results at 6 h and 24 h are depicted as bar diagrams for each of the oxygen tension conditions) or lipopolysaccharide (LPS) at 50 ng/ml (depicted as linear graphs, samples were collected every 6 h). □, △, blank plastic surface; ■, ●, Fn-coated surface; ▨, ○, Ln-coated surface. Results are shown as mean ± s.e.m., *n* = average of four separate experiments conducted in duplicate.

RESULTS

PMN IL-8 release is regulated by integrin signalling and altered oxygen tensions

Initial experiments were performed to confirm that in this model PMN-derived free radicals were produced to support the contention that anti-oxidants are regulated by free radical production. Both O_2^- and myeloperoxidase (MPO) production were measured as described above. (nM $O_2^-/4 \times 10^6$ PMN/ml/12 h = 3·86 ± 0·31, 0·52 ± 0·03 and 5·92 ± 0·61 during normoxia, hypoxia and H/R, respectively. *n* = results of three experiments performed in quadruplicate.) Neither Fn nor Ln affected O_2^- production. O_2^- production was significantly increased by LPS, fMLP and PMA compared with buffer controls during each oxygen tension (data not shown). (OD MPO production 4×10^6 PMN/ml/12 h = 6·11 ± 0·72, 1·36 ± 0·18 and 9·87 ± 0·90 during normoxia, hypoxia and H/R, respectively. *n* = results of four separate experiments performed in triplicate.) The effects of Fn, Ln, PMA, LPS and fMLP MPO production were similar to those seen for O_2^-. During normoxia, PMN IL-8 release was significantly enhanced by binding of integrin receptors for Fn or Ln *versus* cells adhered to plastic at 6 and 24 h of incubation (Fn, Ln *versus* blank): 1492 ± 369, 1764 ± 11 *versus* 487 ± 1 and 1813 ± 156, 1595 ± 123 *versus* 788 ± 96 pg/ml, respectively (Fig. 1). LPS stimulation induced a marked increase in IL-8 release throughout the entire assay, notably ablating the up-regulating effect of integrin–extracellular matrix (ECM) interactions. No significant kinetic changes were observed in LPS-induced IL-8 release.

In contrast to LPS, for fMLP-stimulated PMN (Fig. 2) IL-8 concentrations in cell supernatants were increased only at the end of the incubation period and only in the cells adhered to plastic and Ln. (Results are given *versus* corresponding buffer-stimulated controls: 1486 ± 184 *versus* 7788 ± 96 and 2172 ± 235 *versus* 1595 ± 123 ($P < 0·05$)). For fMLP-stimulated PMN, only Ln-adhered PMN demonstrated consistently positive increases in the kinetics of IL-8 release from 774 ± 40 pg/ml at 6 h to 2172 ± 235 at 24 h. Further, the Ln-adhered PMN demonstrated significantly increased IL-8 release *versus* plastic- and Fn-adhered PMN during the second half of the incubation period.

In marked contrast to the two previous agonists, PMA-stimulated PMN demonstrated a marked suppression of IL-8 release independent of integrin–ECM stimulation (Fig. 3).

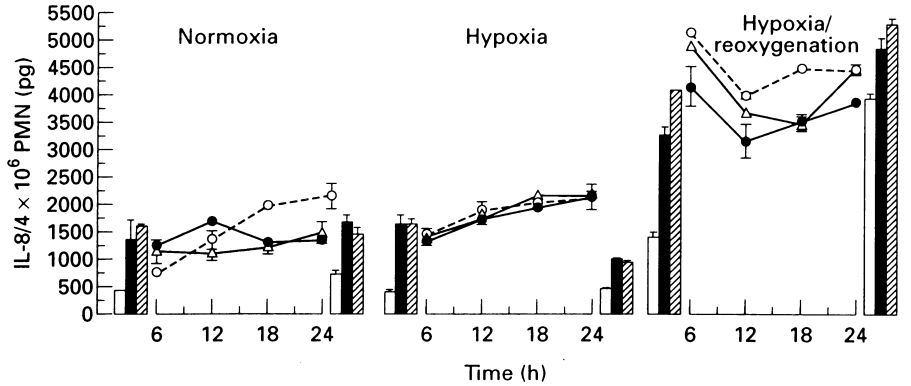

Fig. 2. IL-8 concentration (pg/ml) in supernatants of PMN stimulated either with buffer (bar diagrams for 6 h and 24 h) or N-formyl-Met-Leu-Phe (fMLP) at 100 nM (linear graphs). □, △, blank plastic surface; ■, ●, Fn-coated surface; ▨, ○, Ln-coated surface. Results are mean ± s.e.m., *n* = average of four separate experiments conducted in duplicate.

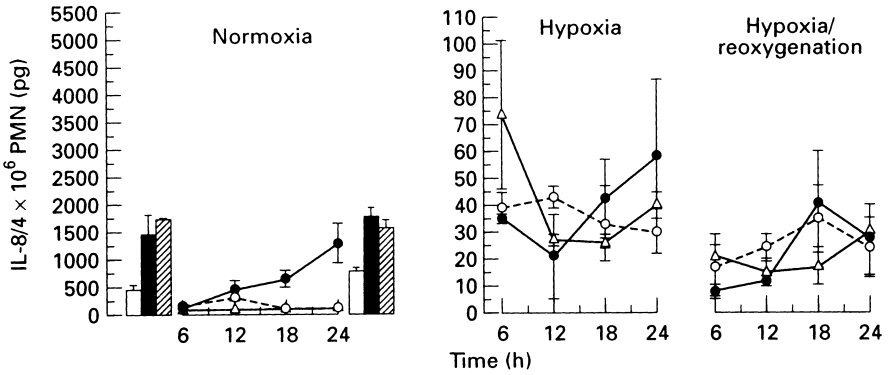

Fig. 3. IL-8 concentration (pg/ml) in supernatants of PMN stimulated either with buffer (bars for 6 h and 24 h) or phorbol myristate acetate (PMA) at 10 ng/ml (linear graphs) under normoxia (left), hypoxia (middle) and hypoxia/reoxygenation (right). Please note the different scales for normoxia and hypoxia, hypoxia/reoxygentation conditions. □, △, blank plastic surface; ■, ●, Fn-coated surface; ▨, ○, Ln-coated surface. Results are mean ± s.e.m., *n* = average of four separate experiments conducted in duplicate.

During hypoxia, IL-8 concentrations in the supernatants of cells adhered without matrix proteins remained close to normoxic levels, with the up-regulating effect of both matrix proteins being preserved. At the same time, LPS-stimulated PMN (Fig. 1) had a marked decrease in IL-8 release *versus* those levels seen during normoxia independent of integrin stimulation throughout the time course of the assay. Hypoxia, in and of itself, did not cause significant changes in fMLP-induced IL-8 release (Fig. 2), but the kinetics of IL-8 release was consistently positive for PMN adhered to any of the surfaces (Fn, Ln or plastic): 1318 ± 23, 1439 ± 21 and 1389 ± 153 at 6 h *versus* 2171 ± 11, 2147 ± 31 and 2157 ± 2 after 24 h of adherence ($P < 0{\cdot}05$). PMA-induced IL-8 release during hypoxia was further suppressed (Fig. 3).

Reoxygenation by itself significantly enhanced IL-8 release compared with levels seen during hypoxia and amplified the up-regulating effect of integrin–ECM stimulation, especially $\alpha_6\beta_1$–Ln interactions on PMN cytokine secretion. Notably, during reoxygenation the up-regulating effect of $\alpha_6\beta_1$–Ln interactions in fMLP-stimulated cells was seen throughout the assay except at 24 h. Hypoxia/reoxygenation further reduced IL-8 secretion in PMA-stimulated cells.

Alterations in oxygen tension regulate PMN-derived TNF-α release

During normoxia, spontaneous TNF-α release (Fig. 4) started from a range of 34–41 ± 21 pg/ml rising significantly up to 315–378 ± 71 pg/ml by the end of the incubation period, with a minimal effect of integrin signalling via Fn or Ln. Stimulation with LPS not only quantitatively elevated TNF-α release, but also revealed a differential regulatory effect of the two separate integrin–ECM complexes: an overall down-regulating effect of the $\alpha_5\beta_1$–Fn complex and an up-regulating effect of its $\alpha_6\beta_1$–Ln counterpart. The values of TNF-α concentrations in supernatants of PMN adhered to Fn or Ln after 24 h of incubation were as follows: 875 ± 71 *versus* 665 ± 37 ($P < 0{\cdot}05$). The kinetics of TNF-α release remained relatively flat during the assay except for a rapid fall in cytokine release in Ln-adhered PMN after 24 h of incubation.

Unlike LPS, fMLP-stimulated cells (Fig. 5) demonstrated low initial TNF-α concentrations, with a significant increase by the end of incubation with an up-regulating effect seen in Ln-adhered PMN. Unlike LPS-treated PMN at 24 h, TNF-α concentrations were lower in the supernatants of Fn- and plastic-adhered PMN *versus* the corresponding buffer controls. PMA failed to induce any detectable TNF-α release during any of the oxygen tensions (data not shown). Hypoxia further reduced the release of this cytokine by PMN adhered in buffer. Both LPS- and fMLP-induced TNF-α production were decreased but remained above buffer control levels without changes in the kinetics of TNF-α release during the assay.

Reoxygenation increased TNF-α release in PMN adhered to Fn or Ln back to initial normoxic levels and increased TNF-α release,

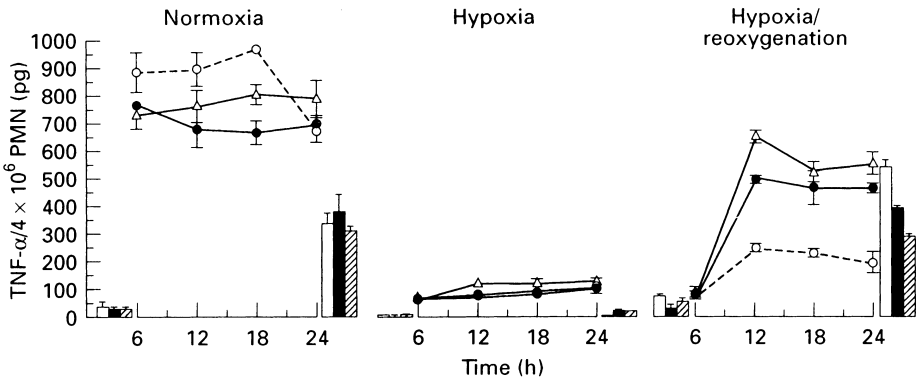

Fig. 4. Tumour necrosis factor-alpha (TNF-α) concentration (pg/ml) induced by buffer (bars for 6 h and 24 h) or lipopolysaccharide (LPS) at 50 ng/ml (linear graphs). □, △, blank plastic surface; ■, ●, Fn-coated surface; ▨, ○, Ln-coated surface. Results are mean ± s.e.m., *n* = average of four separate experiments conducted in duplicate.

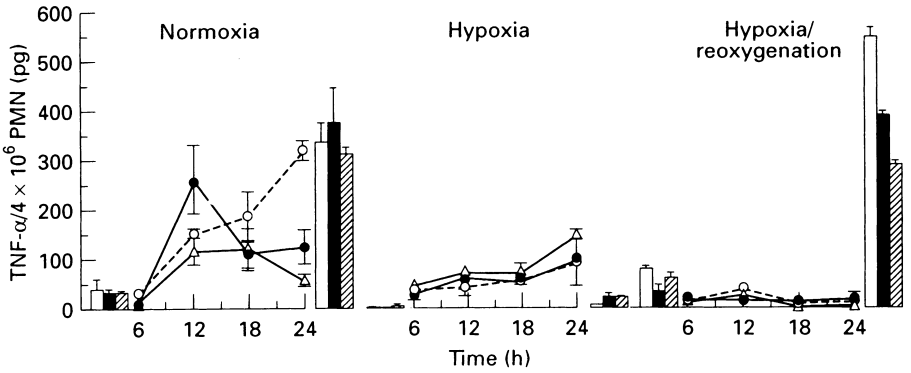

Fig. 5. Tumour necrosis factor-alpha (TNF-α) concentration (pg/ml) in response to stimulation with buffer (bars for 6 h and 24 h) or N-formyl-Met-Leu-Phe (fMLP) at 100 nM (linear graphs). □, △, blank plastic surface; ■, ●, Fn-coated surface; ▨, ○, Ln-coated surface. Results are mean ± s.e.m., n = average of four separate experiments conducted in duplicate.

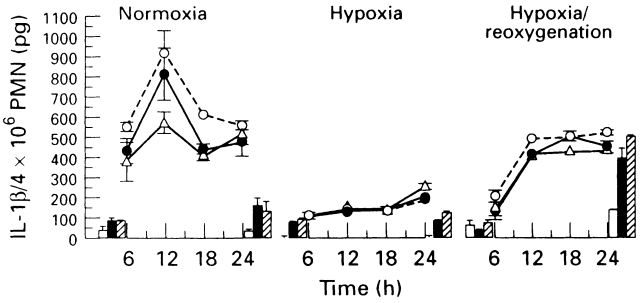

Fig. 6. IL-1β release (pg/ml) by PMN stimulated with buffer (bars for 6 h and 24 h) or lipopolysaccharide (LPS) at 50 ng/ml (linear graphs). □, △, blank plastic surface; ■, ●, Fn-coated surface; ▨, ○, Ln-coated surface. Results are mean ± s.e.m., n = average of five separate experiments conducted in duplicate.

thereby establishing a down-regulating effect of both integrin–ECM complexes. This effect was also exhibited by LPS-stimulated cells. Of note was the change in the effect of $\alpha_6\beta_1$–Ln complex from increasing to decreasing TNF-α release during the normoxia/hypoxia/reoxygenation cycle. After 24 h of incubation the levels of TNF-α concentrations were below both corresponding buffer controls and the initial levels seen during normoxia. Surprisingly, the fMLP-induced response was further suppressed by reoxygenation. Thus, PMN-derived TNF-α release, controlled by the integrin $\alpha_6\beta_1$–Ln complex, was the most sensitive of the three cytokines assayed to alterations in environmental oxygen tension.

PMN-derived IL-1β release is controlled primarily by altered oxygen tensions

The release of IL-1β by PMN exhibited a pattern similar to that for IL-8. During normoxia, both matrix proteins enhanced spontaneous IL-1β response after 24 h of incubation (Fn, Ln, *versus* plastic): 154 ± 40, 133 ± 42 *versus* 33 ± 7 pg/ml ($P < 0{\cdot}05$). LPS and fMLP stimulation increased IL-1β release and this was enhanced by both integrin–ECM complexes (Figs 6 and 7) with the overall up-regulating effect of the $\alpha_6\beta_1$–Ln complex being the most pronounced. Both LPS- and fMLP-induced IL-1β release peaked at 6 h.

PMA-stimulated PMN again demonstrated significant reductions in IL-1β release. Hypoxia practically ablated spontaneous IL-1β release in plastic-, Fn- or Ln- adhered PMN and partially ablated the effect of matrix proteins on IL-1β release. Surprisingly, hypoxia significantly up-regulated all three PMA-induced IL-1β release kinetic curves (Fig. 8), but within each oxygen tension no major kinetics changes were observed.

Reoxygenation significantly increased spontaneous IL-1β release by the end of the incubation time compared with both hypoxic and initial normoxic levels and amplified the up-regulating effect of integrin signalling (Fn, Ln *versus* plastic at 24 h H/R):

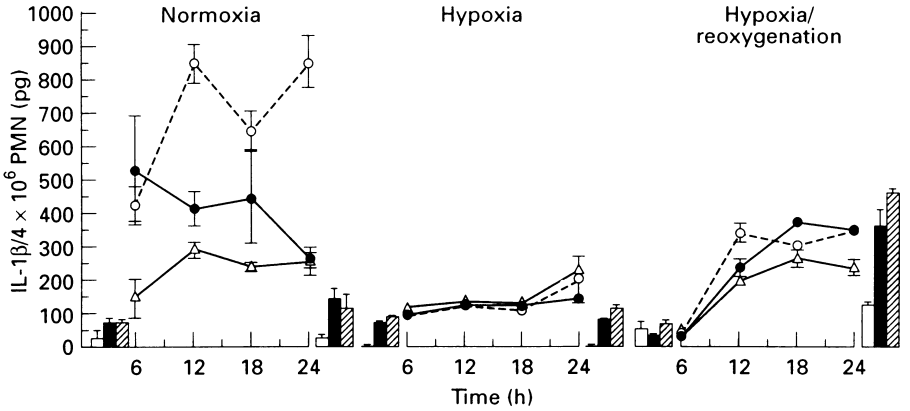

Fig. 7. IL-1β release (pg/ml) by PMN stimulated with buffer (bars for 6 h and 24 h) or N-formyl-Met-Leu-Phe (fMLP) at 100 nM (linear graphs). □, △, blank plastic surface; ■, ●, Fn-coated surface; ▨, ○, Ln-coated surface. Results are mean ± s.e.m., n = average of four separate experiments conducted in duplicate.

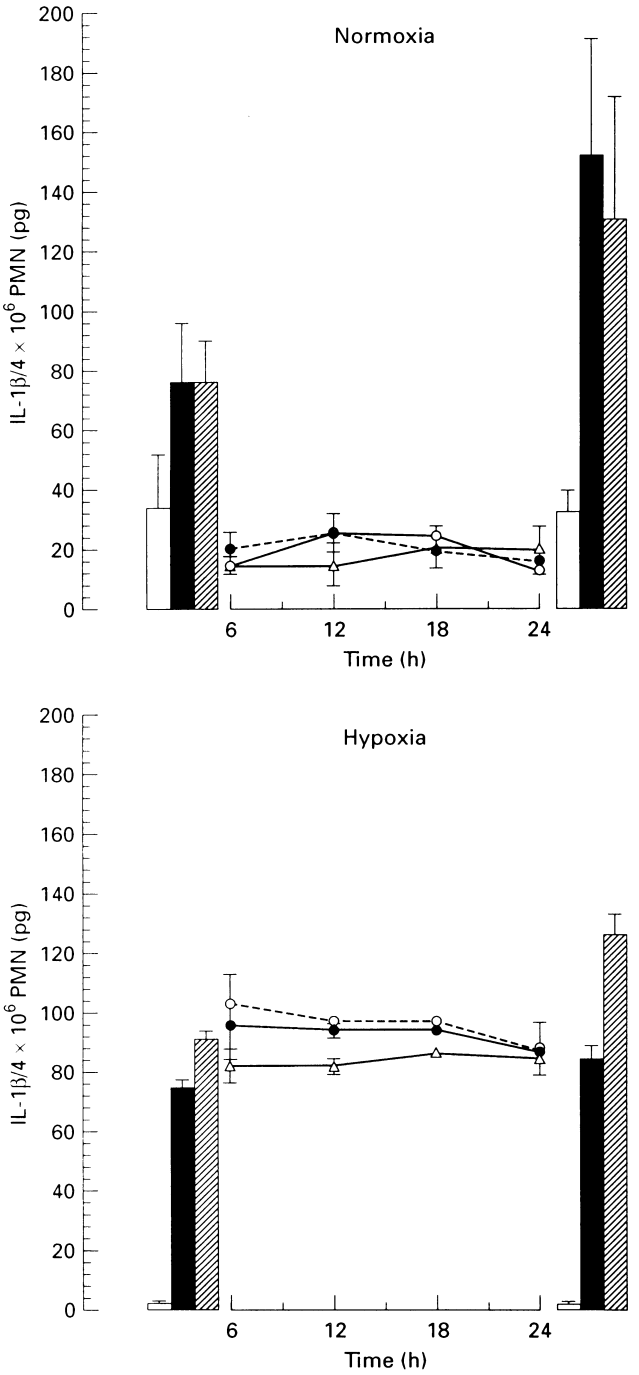

Fig. 8. Concentration (pg/ml) of IL-1β in PMN supernatants stimulated with buffer (bar diagrams for 6 h and 24 h) or phorbol myristate acetate (PMA) at 10 ng/ml (linear graphs) under normoxia and hypoxia. □, △, blank plastic surface; ■, ●, Fn-coated surface; ▨, ○, Ln-coated surface. Results are mean ± s.e.m., n = average of four separate experiments conducted in duplicate.

456 ± 60, 526 ± 16 *versus* 135 ± 6 pg/ml ($P < 0{\cdot}05$). However, for LPS- and fMLP-stimulated PMN, IL-1β release was only partially restored compared with both corresponding normoxic levels after 24 h of incubation and with matrix protein stimulation. Thus the differential sensitivity of IL-1β release to external stimulation was only partially restored by the normoxia/hypoxia/reoxygenation cycle. During reoxygenation, PMA failed to induce IL-1β release (data not shown). Of note, neither actinomycin D nor cycloheximide significantly altered IL-8, TNF-α or IL-1β release induced by altered oxygen tensions or matrix proteins, demonstrating that the cytokines measured in these assays represented pre-formed proteins.

DISCUSSION

PMN are an important cell line in the host defence cascade against infection. Its multiple functions with regard to inflammation are well recognized: phagocytosis, degranulation with release of various proteases and production of reactive oxygen intermediates. Traditionally, the PMN has been considered to be terminally differentiated with minimal capacity to produce *de novo* proteins. Recently, the neutrophil has been shown to have the ability to produce a number of immunomodulators, cytokines in particular, that may regulate the inflammatory cascade [3–6]. Among those, IL-8, TNF-α and IL-1β have particular importance [3,7–9,19–21]. At sites of inflammation, as well as in the systemic circulation, leucocytes elaborate cytokines in response to various proinflammatory agonists. LPS, fMLP and PMA have been initially shown as potent stimulators of monocyte-macrophage cytokine production, but more recently PMN as a leucocyte sharing the same stem cell have also been appreciated as a responder to these agonists [3,5–7,9,14,22,23]. Though the whole blood model in terms of investigating cytokine concentrations is considered relevant in terms of the diversity of biological interactions occurring *in vivo* [5,23], there is increasing experimental evidence regarding the modulating effect of ligation of integrins by ECM proteins—in particular Fn and Ln [24,25]. The importance of ECM–PMN interactions has previously been documented [2,26].

In our experiments we ensured adequate PMN adhesion to either of these two main matrix proteins (Fn or Ln) or plastic, because it has been previously demonstrated [2,25,27,28] that binding of integrin receptors ($\alpha_5\beta_1$ for Fn and $\alpha_6\beta_1$ for Ln) [26,27] up-regulates cellular bioactivity.

In these studies we measured cytokine concentrations in cell supernatants as a measure of cytokine secretion by PMN in response to the presence or absence of external stimulation. *De novo* protein synthesis and the release into the extracellular space (i.e. translational regulation) is the most sensitive part in cellular cytokine elaboration induced by external stimulation or integrin signalling, though some transcriptional changes also take place [3,6,14].

Inflammatory sites frequently undergo changes in environmental oxygen tension from normoxia to hypoxia and reoxygenation. The importance of altered oxygen tensions in regulating PMN function is well established and has been implicated in ischaemia/reperfusion injury [12,29]. Alterations in oxygen tension have been shown to effect cell adhesion and interactions with extracellular matrix [11], endotoxin tolerance [20], and macrophage cytokine elaboration [17,30]. Therefore, we designed our studies to investigate the dynamics of the modulating effect of the normoxia/hypoxia/reoxygenation transition on the integrin-controlled sensitivity of PMN cytokine elaboration with external stimulation at the level of cell surface receptor–ligand interactions (fMLP and LPS) as well as at the post-receptor level (direct stimulation of PKC by PMA).

Under normoxic conditions, LPS turned out to be the most potent stimulus for each of the cytokines measured, and induced a

general (up to 5·4 ng/ml) increase in cytokine concentrations over control, which is consistent with previous reports [3,6,31]. In contrast, fMLP used at a physiological concentration of 100 nM failed to increase TNF-α release, and its effect on IL-8 and IL-1β was much less pronounced—up to 1 ng/ml. Previous investigators have reported the dose-dependent mode of fMLP-induced cytokine production [3]. In marked contrast to the overall stimulatory effect of LPS and fMLP surface receptor ligation, direct stimulation of PKC with PMA at 10 ng/ml significantly suppressed IL-8 and IL-1β production, while TNF-α production was suppressed below the level of assay sensitivity. While some investigators have achieved substantial TNF-α release with higher doses of PMA [14], we deliberately used 10 ng/ml because of the inhibitory *versus* stimulatory effect on PKC with prolonged high-dose PMA challenge [14].

Hypoxia exerted an overall down-regulating effect on cytokine release (except for the up-regulation of PMA-induced IL-1β production) and subsequent reoxygenation restored cytokine production, increasing IL-8 levels even above initial normoxic levels. In spite of restored or even up-regulated absolute values of cytokine concentrations following hypoxia/reoxygenation, the overall extent of the external stimulating effect *versus* corresponding buffer controls was reduced. These results demonstrate that while the normoxia/hypoxia/reoxygenation cycle increases baseline levels of cytokine release, this cycle of altered oxygen tension decreases PMN sensitivity to additional stimulation. The fact that PMN viability remained >90% for the incubation period suggests that changes in cytokine levels were not due to release of mediators from cell death, but to an up-regulation of cytokines within the cell induced by free radicals. Throughout the entire incubation period and during the normoxia/hypoxia/reoxygenation transition, integrin signalling differentiated both spontaneous and stimulated cellular responsiveness to alterations in environmental oxygen tension laminin. Adherence of PMN to laminin induced an overall up-regulating effect on the production of each of the cytokines, and integrin control of cytokine production by itself turned out to be highly sensitive to changes in oxygen tension. The demonstrated differences in profiles of cytokine concentrations support earlier observations regarding different signal transduction pathways employed in governing the production and release of IL-8, TNF-α, or IL-1β [7,14].

Considering all data together, PMN can participate in the host defence process against infection by elaborating inflammatory cytokines. This process is controlled on multiple levels of signal transduction, differentiated by integrin–ECM interactions, and is sensitive to alterations in microenvironmental oxygen tension. Considering the multiplicity of factors that regulate the PMN cytokine response, efforts to delimit the PMN inflammatory response by manipulation of a single aspect in the cellular control of cytokine production is unlikely to be effective. Therefore, potential therapeutic approach to manipulating the PMN inflammatory response will probably have to be multifactorial.

ACKNOWLEDGMENTS

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