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Hypoxia Affects Cytokine Production and Proliferative Responses by Human Peripheral Mononuclear Cells

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We have shown that hypoxia (2% $O_2 \approx pO_2$ 14 mmHg) as opposed to O_2 atmospheric pressure (20.9% $O_2 \approx pO_2$ 140 mmHg) can deeply affect the production of cytokines in human peripheral mononuclear cells (PBMC) in the presence or absence of a specific T-cell activator such as phytohemagglutinin (PHA). In hypoxia, interleukin (IL)-2, IL-4, and interferon (IFN)-γ production increased by 110, 70, and 50% over that of controls, respectively, in PHA-stimulated PBMC ($P < 0.05$). Moreover, in hypoxia, IL-6 production was significantly enhanced in both resting and PHA-stimulated PBMC by 36 and 37%, respectively ($P < 0.05$). However, in hypoxia, IL-10 production decreased in both resting and stimulated PBMC, being 80 and 67% of controls, respectively ($P < 0.05$). PBMC proliferation was not significantly affected by hypoxia, although PBMC susceptibility to PHA was about 80% of that of the control ($P < 0.05$) after 40 hr of treatment, whereas the cycle progression of hypoxic PBMC was delayed. From an evaluation of these results, hypoxia apparently modifies the production of cytokines by PBMC. These results have both theoretical and practical interest because local hypoxia is very common in several conditions, such as inflammation and local ischemia, and is a host-nonspecific defense against infection. Furthermore, these results suggest a differential pattern of cytokine production in vivo in hypoxic tissues. **J. Cell. Physiol. 173:335-342, 1997.** © 1997 Wiley-Liss, Inc.

Cytokines are regulatory proteins well known for their pleiotropic actions, including many effects on immune cells and a variety of other cells. Another feature of cytokines is their ability to stimulate or inhibit the production of other cytokines and the proliferation of T cells and other cell types (Powrie and Coffman, 1993). Interleukin (IL)-2 and IFN-γ exert stimulatory effects on T cells and promote their growth (Grabstein et al., 1986), whereas IL-10 is a cytokine whose major biological function is to inhibit action (Mosmann, 1994). Moreover, whereas IL-2 and IFN-γ are secreted by Th_1 cells, IL-10 is secreted mainly by Th_2 cells and inhibits cytokine production in Th_1 cells and monocytes (Fiorentino et al., 1991) and T-cell proliferation (Taga and Tosato, 1992). IL-6 and IL-4 also are secreted by Th_2 cells and are involved in regulating the growth and differentiation of T and B cells (Callard and Turner, 1990; Hirano et al., 1990). Local hypoxia is a very common condition at different levels (Moulder and Rockwell, 1987), and this situation, associated with other factors, may control the release of cytokines that are physiologically relevant in that context. As a result, a microenvironmental change of a specific cytokine may be relevant in controlling T-cell and other cell-type proliferation. When cells are in hypoxic conditions, they accumulate in the G_1 phase of the cell-growth cycle (Taylor and Hodson, 1984; Amellem and Pettersen, 1993; Graeber et al., 1994), this phase is usually inversely proportional to the cell growth rate. It has been already shown that either the antiviral or the antiproliferative activity of a cytokine such as IFN is enhanced in noncycling human cells (Horoszewicz et al., 1979), and other data have suggested that intracellular events are critical for determining the full biological responses to IFN (De Maeyer and De Maeyer-Guignard, 1979). Moreover, we have shown that hypoxia affected the antiviral (Naldini et al., 1993b) and the antiproliferative activity of IFN (Naldini et al., 1995b) and the cytotoxicity of tumor necrosis factor (TNF) (Naldini et al., 1994). All these effects can be related to the oxygen availability, and there is a relationship between oxygen availability and sensitivity to IFN or TNF of different cell types (Aune and Pogue, 1989). For these reasons, we studied the production of cytokines by resting and PHA-activated PBMC at either normal (20% O_2) or lower (2% O_2) oxygen tension. The lower O_2 tension resembles the hypoxic state revealed at the level of the necrotic area at the tumor site or in inflammatory condition (Vaupel et al., 1981). At the same time, the cell-cycle progression of hypoxic PBMC, resting and PHA-activated, was evaluated as a possible establishment of apoptosis. We also

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performed some metabolic determinations measuring glucose consumption and lactate production to address the question of whether cellular glycolytic flux is preferentially shifted to the anaerobic pathway, resulting in an increase of both glucose consumption and lactate production.

MATERIALS AND METHODS

Venous blood was obtained from healthy blood donors of both sexes. Reagents and their sources were Lymphoprep from Nycomed Pharma (Norway); medium RPMI 1640, Dutch modification, glutamine and penicillin/streptomycin, heat-inactivated dialyzed fetal calf serum, mitogen tested (FCS), phytohaemagglutinin (PHA), and trypan blue from Biochrom KG (Germany); 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide from Sigma (St. Louis, MO); human IL-2-Easia, human IL-4-Easia, human IL-6-Easia, human IL-10-Easia, human IFN-γ-Easia, and human TNF-α-Easia from Medgenix (Belgium); and glucose and lactate test combinations from Boehringer Mannheim (Germany).

Isolation of PBMC

Human PBMC were isolated from heparinized venous blood of healthy subjects by a gradient of Lymphoprep, as previously described (Naldini et al., 1993a). The gradients were spun at 400g, and the PBMC at the interface were removed, washed twice, and resuspended in RPMI-1640 containing 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. PBMC were cultured at 2×10^5 cells/200 μl in 96-well, flat-bottomed tissue culture plates (Costar, Cambridge, MA). All the experiments were performed with two different incubators, as previously described (Naldini et al., 1994). In the control experiments, we used an incubator (KW Officine Meccaniche, Siena, Italy) set to 5% CO_2, 20% O_2 (atmospheric oxygen) and 37°C in a humidified environment. The experiments under hypoxic condition were performed with a water jacketed incubator (Forma Scientific, Marietta, OH) designed to provide a customized and stable humidified environment; CO_2 (5%), O_2 and temperature (37°C) were controlled electronically. In the present experiment, the O_2 tension was set and maintained constantly at 2% (14 mmHg) by injecting N_2 automatically into the chamber to bring the O_2 level to the set point. The PBMC were cultured for 16 or 40 hr in the presence or absence of 5 μg/ml of PHA under hypoxic or normoxic conditions.

Cell viability and proliferation experiments

To assess the effect of hypoxia on cell viability and on the proliferative response of PBMC, the experiments were performed with the colorimetric method (Mosmann, 1983), based on the tetrazolium salt MTT. Briefly, 2×10^5 cells/well incubated in 200 μl of culture medium were exposed to either hypoxia (2% O_2) or normoxia (20% O_2) in the presence or absence of PHA. After 16 or 40 hr of culture, 100 μl of medium were harvested to be assayed later for cytokine release, and 10 μl of a solution of MTT (5 mg/ml) were added to each well and incubated at 37°C. After 4 hr, 100 μl of acid propan-2-ol (0.04 M HCl in propan-2-ol) were added to each well; after all the formazan crystals were dissolved, the plate was read on a microelisa reader (Multiskan, Titertek, Flow). The optical density values (OD) were obtained by using a test wavelength of 570 nm and a reference wavelength of 630 nm. The proliferation index (PI; Naldini et al., 1995a), used to quantitate the PBMC response to PHA, was calculated as follows:

$$PI = \text{PHA-treated lymphocyte activity (OD)} / \text{Untreated lymphocyte activity (OD)}$$

DNA fragmentation assay

Because apoptosis is associated with DNA fragmentation, DNA from hypoxic and normal PBMC was analyzed by agarose gel electrophoresis (Petronini et al., 1996). Briefly, at the appropriate time, cells were pelletted and washed, and DNA was extracted and purified with a commercially available kit (Easy DNA™ Kit, Invitrogen, San Diego, CA). Electrophoresis was performed in 1.2% agarose gels in TAE (0.04 M Tris acetate, 0.001 M EDTA) buffer, containing ethidium bromide (0.5 μg/ml) for 2 hr at 50 V. Agarose gels were then photographed on a transilluminator (Fotodyne, Hartland, WI) with a Polaroid camera (FCR-10).

Flow cytometry

All flow cytometric analyses were performed with a Becton Dickinson FACScan equipped with an argon ion laser at 488 nm and 250 mW light output. For analysis, 10,000 events were collected in list mode fashion and stored by Lysis II, version 1.1 (Becton Dickinson, San Jose, Ca). For data acquisition of propidium iodide emission signals, the doublet discrimination module was used, and a polygonal gating was drawn on dot plot histograms; events were collected in FL2 width (x axis) and FL2 area (y axis) that exclude the presence of doublets. The propidium iodide gate regions were then plotted in one-parameter histograms, with red fluorescence emission values on a linear x axis and cell number on the y axis. Percentages of cells in each region were obtained by using the histogram statistical data (H-stats) of the Lysis II system. DNA content analysis was performed as suggested by the manufacturer. Briefly, a total of 2×10^6 PBMC were washed once in cold phosphate buffered saline (PBS), fixed with ethanol 70%, and kept at −20°C for at least 30 min. Ethanol was then washed out, and cells were resuspended with 100 μl of PBS and RNAse (100 U/ml) and were kept for 20 min at room temperature. Subsequently, 100 μl of staining solution, containing 10 mg/ml propidium iodide, 0.1 ml Triton X-100, 3.7 mg EDTA, and 90 ml PBS were added, and cells were incubated in the dark at room temperature for 30 min. Samples were then filtered through 35-μm nylon mesh and analyzed within 1 hr.

Cytokine measurements

Cytokine concentrations were assessed from PBMC culture supernatants by commercially available ELISA kits, as previously described (Naldini et al., 1995a). Briefly, cell-free supernatants were obtained at the appropriate juncture. After centrifugation, aliquots from supernatants were frozen at −20°C, pending assay. Neither assay showed cross reactivity with other cytokines. Minimum detectable doses were 5 pg/ml for IL-

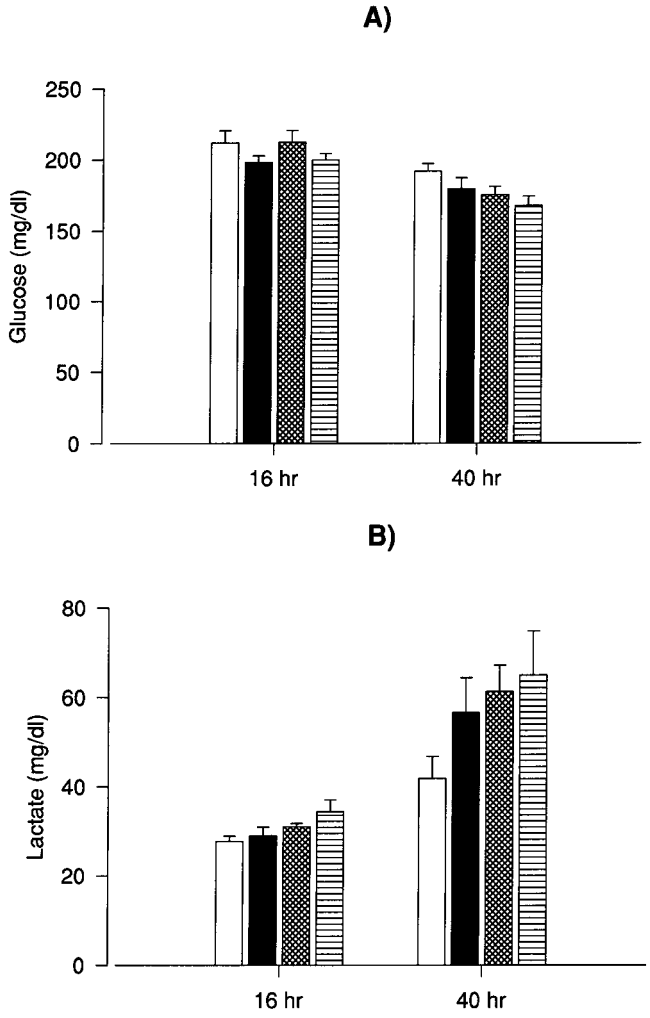

Fig. 1. Effect of hypoxia on PBMC glucose consumption (**A**) and lactate production (**B**). PBMC were cultured at a concentration of 2×10^5 cells/well with medium or PHA (5 μg/ml) and exposed to either a hypoxic (solid bars and striped bars, respectively) or an aerobic (open bars and crosshatched bars, respectively) environment under the conditions described in Materials and Methods. After 16 and 40 hr of treatment, cell-free supernatants were obtained, and glucose and lactate were measured as described in Materials and Methods. Data presented is the mean ± SEM of six independent experiments.

2, 2 pg/ml for IL-4, 2 pg/ml for IL-6, 1 pg/ml for IL-10, 0.03 IU/ml for IFN-γ and 3 pg/ml for TNF-α. Coefficients of variations for intraassay precision were 4–6% for IL-2, 3% for IL-4, 5–6% for IL-6, 3–4% for IL-10, 3–4% for IFN-γ, and 2–5% for TNF-α. Those for interassay precision were 6–8% for IL-2, 3–5% for IL-4, 2–7% for IL-6, 3% for IL-10, 7–8% for IFN-γ, and 8–10% for TNF-α.

Glucose and lactate experiments

Glucose and lactate concentrations were measured as previously described (Naldini et al., 1993b). Briefly, 2×10^5 cells/200 μl of culture medium were exposed to either hypoxia (2% O_2) or normoxia (20% O_2) in the presence or absence of PHA. Cell-free supernatants were obtained at predetermined intervals (16 and 40 hr); after centrifugation, aliquots from supernatants were frozen at −20°C pending assay. Culture medium contained the components previously described except for phenol red, which was eliminated to avoid interference with the enzymatic kit assays.

TABLE 1. Effect of hypoxia on PBMC viability[1]

Incubation time	Culture conditions	Normoxia	Hypoxia
16 hr	Medium	0.182 ± 0.009	0.176 ± 0.019
	PHA	0.177 ± 0.007	0.167 ± 0.014
40 hr	Medium	0.187 ± 0.008	0.196 ± 0.014
	PHA	0.249 ± 0.011	0.232 ± 0.014

[1]PBMC were cultured at a concentration of 2×10^5 cells/well with medium or PHA (5 μg/ml) and were exposed to either a hypoxic or an aerobic environment under the conditions described in Materials and Methods. After 16 and 40 hr of treatment, cell viability was assessed by the MTT method and expressed as $O.D._{570/630}$. Data are presented as the mean ± SEM of 10 independent experiments.

Statistical analysis

Results are presented as the mean ± SEM. Statistical evaluation of the data was done by Student's two-tailed paired t-test. A P value of <0.05 was considered significant.

RESULTS

Effect of hypoxia on lactate and glucose concentrations

Although the effect of hypoxia on cellular glucose metabolism is well known (Robin et al., 1984), we have determined the glucose and lactate concentrations in the cultures of resting or PHA-activated PBMC at both 20% and 2% O_2 tension. Figure 1 shows glucose consumption and lactate production at 20% O_2 and at 2% O_2 in both resting and PHA-activated PBMC. In hypoxic conditions, glucose concentration decreased and, as expected, the lactate production followed an inverse pattern. However, these differences were never significant, indicating that critical metabolic derangements were avoided. In fact, derangements such as glucose deprivation may have a direct effect on PBMC cytokine production. However, the amount of glucose present in culture was never below the physiological level of 100 mg/dl.

Effect of hypoxia on PBMC viability and activation

PBMC viability and activation were established with the MTT method, as reported by Mosmann (1983). Resting and PHA-activated PBMC were treated as reported in the Materials and Methods section in normoxic and hypoxic conditions. No significant differences were observed on the viability of PBMC under hypoxic conditions when compared with controls (Table 1). The PI was almost identical in hypoxia and normoxia after 16 hr of incubation (Fig. 2). However, at 40 hr, the PI was significantly lower in hypoxia than in the controls (80% of controls), indicating that PBMC are less susceptible to PHA activation in hypoxia. This result reflects the fact that, under the hypoxic condition, cell-cycle progression is delayed.

To exclude the presence of apoptosis, we performed a gel electrophoresis of DNA isolated from both normal and hypoxic PBMC. The DNA fragmentation did not

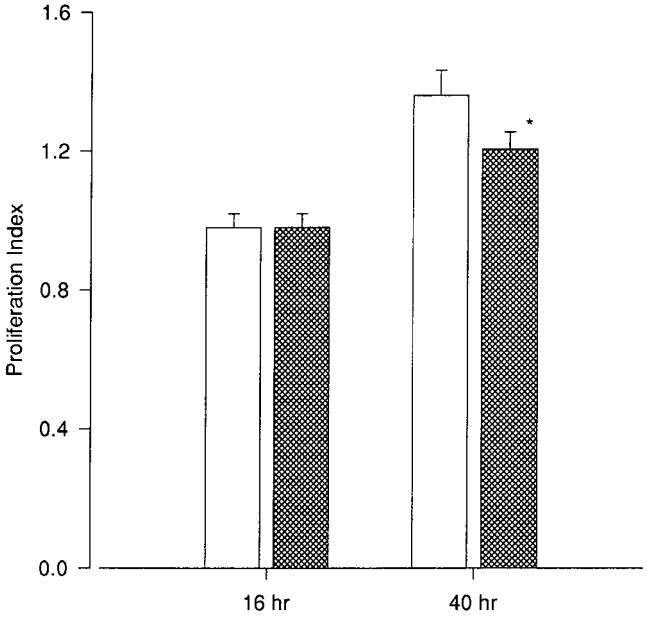

Fig. 2. Effect of hypoxia on PHA stimulation of PBMC. PBMC were cultured at a concentration of 2×10^5 cells/well with medium or PHA (5 μg/ml) and exposed to either a hypoxic (solid bars) or an aerobic (open bars) environment for 16 and 40 hr. Data are presented as the proliferation index (PI), calculated as described in Materials and Methods. Data are presented as the mean ± SEM of 10 independent experiments. Asterisk indicates statistically significant ($P < 0.05$) differences between the PI of PBMC obtained in hypoxia vs. aerobic controls.

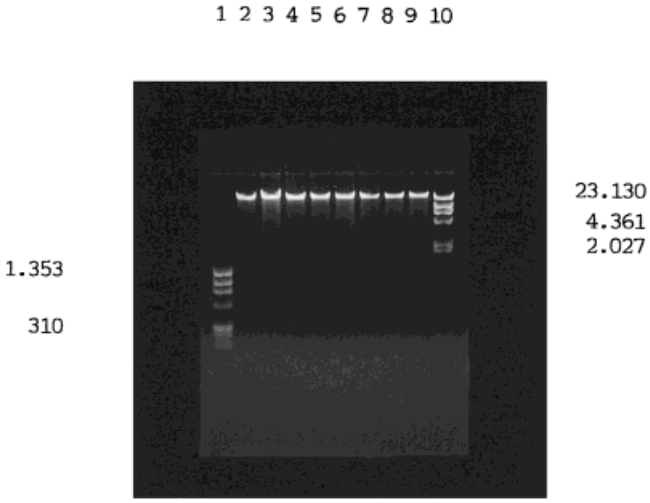

Fig. 3. Agarose gel electrophoresis of DNA from PBMC maintained in culture in either aerobic or hypoxic condition. PBMC were cultured at a concentration of 1×10^6 cells/ml with medium or PHA (5 μg/ml) and exposed to either a hypoxic or an aerobic environment for 16 and 40 hr. DNA was extracted, isolated, and analyzed on a 1.2% agarose gel. Lane 1: ϕX174RF DNA/Hae III fragments as size markers. Lanes 2 and 3: Resting and PHA-activated PBMC in normoxia at 16 hr. Lanes 4 and 5: Resting and PHA-activated PBMC in hypoxia at 16 hr. Lanes 6 and 7: Resting and PHA-activated PBMC in normoxia at 40 hr. Lanes 8 and 9: Resting and PHA-activated PBMC in hypoxia at 40 hr. Lane 10: λDNA/Hind III fragments as size markers. Data presented are the results of one among four similar experiments.

occur, suggesting that apoptosis was not present under these conditions (Fig. 3). This result agrees with the cell viability data that showed no difference between normal and hypoxic PBMC.

Effect of hypoxia on cell-cycle progression

To understand further whether the cell-cycle progression of PBMC is affected by hypoxia, flow cytometric analyses were performed. Exposure of PBMC to 5 μg/ml of PHA initiated cell-cycle progression (Fig. 4A). This treatment induces significative levels of IL-2, and a few cells entered S phase. In contrast, in hypoxia, although IL-2 production is increased, cell-cycle progression is delayed, and cells did not enter S phase. This result is evident in Figure 4B, which represents a flow cytometric analysis (dot plot) of PBMC exposed to either normoxia or hypoxia for 40 hr, but the effect is more striking in Figure 4C (contour plot).

Effect of hypoxia on cytokine production by human PBMC

Because hypoxia affects the differentiation and proliferation of a variety of cell types (Sahai et al., 1994) and the production of IL-1 by lipo poly sacchariole (LPS)-stimulated human monocytes (Ghezzi et al., 1991), we decided to evaluate any possible change in cytokine production by resting or PHA-activated PBMC. First, we determined the amounts of IL-2, IFN-γ, and IL-4 released in hypoxia by PHA-activated PBMC. At 16 hr, hypoxia significantly increases the production of IL-2 (100% higher than controls), and the increase of IL-2 production was still significant after 40 hr of incubation (110% higher than controls) (Fig. 5). IFN-γ and IL-4 production also were increased after 16 hr of hypoxic treatment (70 and 50% higher than controls, respectively; Fig. 6); however, at 40 hr, the difference between hypoxia and controls were no longer significant (data not shown). The latter result may be related to either a decreased susceptibility of PBMC to PHA activation after 40 hr of incubation or an inhibitory effect.

Because IL-2 and IFN-γ are produced mainly by Th_1 cells and IL-4 by Th_2 cells (Powrie and Coffman, 1993), we investigated whether Th_2-type cytokine production was affected by hypoxia. Table 2 shows that IL-6 production was markedly increased by hypoxia in both resting and PHA-activated PBMC. Hypoxic treatment of resting PBMC resulted in a higher production of IL-6 (36% higher than controls); in PHA-activated PBMC, the IL-6 release was 37% higher than controls. Similar results were obtained after 40 hr of treatment. In contrast with other findings (Ghezzi et al., 1991), in our

Fig. 4. Effect of hypoxia on the cell-cycle progression of PBMC. PBMC were cultured at a concentration of 1×10^6 cells/ml with PHA (5 μg/ml) and exposed to either a hypoxic (right) or an aerobic (left) environment for 40 hr. Cell-cycle progression was monitored by flow cytometric measurements of DNA content. Data are presented as a histogram plot (**A**), a dot plot (**B**), and a contour plot (**C**), according to the manufacturer's instructions. Data presented are the results of one among four similar experiments.

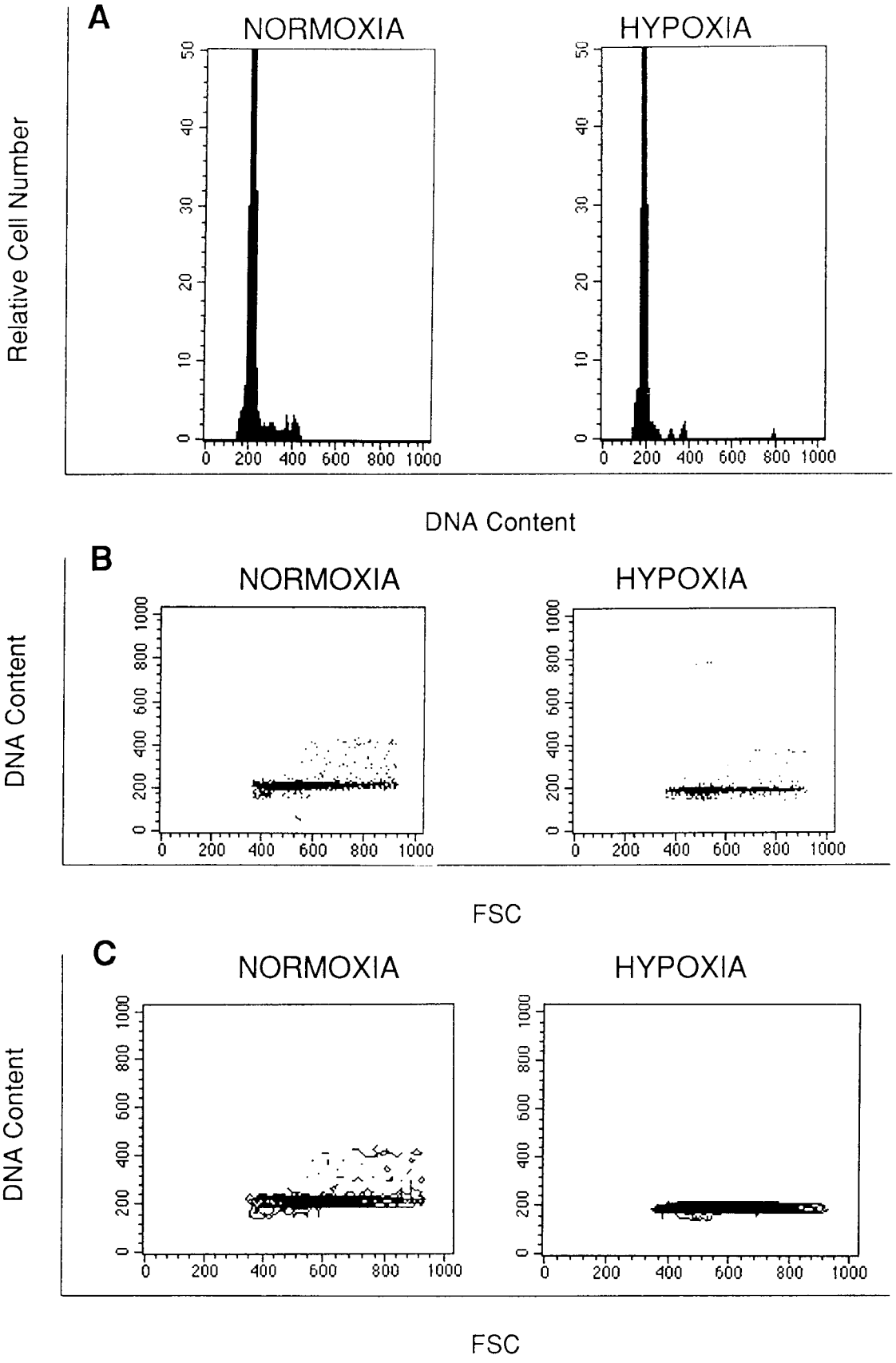
A
NORMOXIA
HYPOXIA
Relative Cell Number
DNA Content
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Fig. 4.

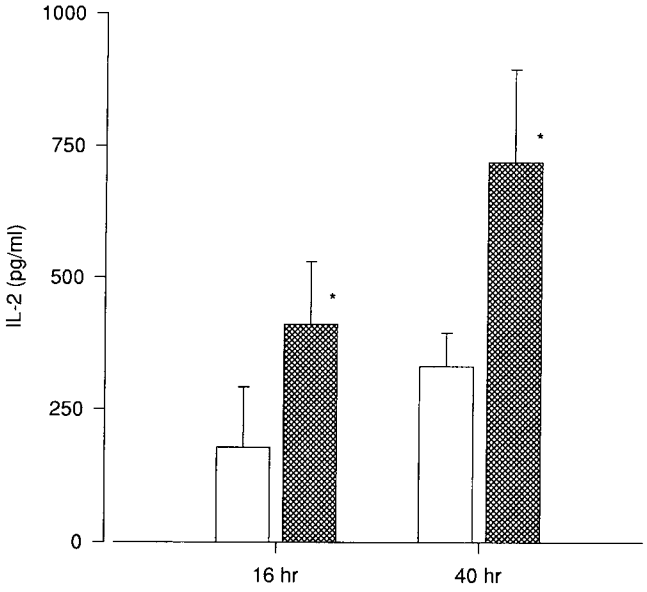

Fig. 5. Effect of hypoxia on IL-2 production from PHA-stimulated PBMC. PBMC were treated with PHA (5 μg/ml) and exposed to either a hypoxic (solid bars) or an aerobic (open bars) environment. After 16 and 40 hr of culture, cell-free supernatants were obtained, and IL-2 present in the supernatants was determined by ELISA. Data presented is the mean ± SEM of six independent experiments. Asterisk indicates statistically significant ($P < 0.05$) differences between IL-2 released by PBMC incubated in hypoxia vs. aerobic controls.

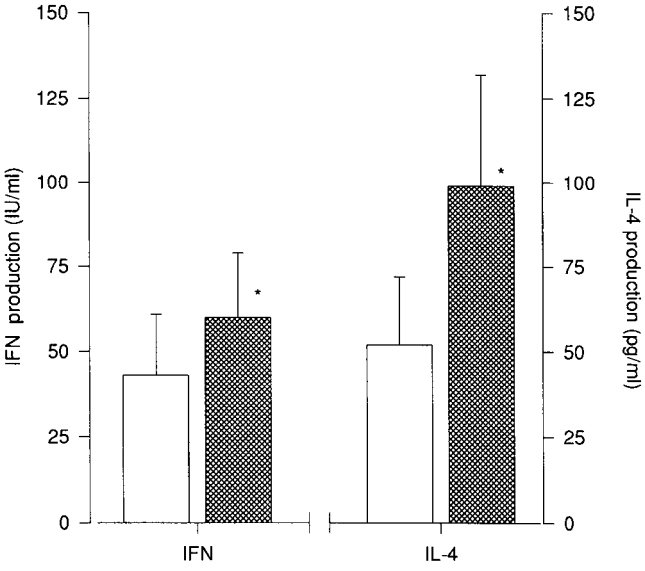

Fig. 6. Effect of hypoxia on IL-4 and IFN-γ production from PHA-stimulated PBMC. PBMC were treated with PHA (5 μg/ml) and exposed to either a hypoxic (solid bars) or an aerobic (open bars) environment. After 16 hr of culture, cell-free supernatants were obtained, and IL-4 and IFN-γ present in the supernatants were determined by ELISA. Data presented are the mean ± SEM of six independent experiments. Asterisk indicates statistically significant ($P < 0.05$) differences between IL-4 and IFN-γ released by PBMC incubated in hypoxia vs. aerobic controls.

experiments, the production of TNF-α was not significantly affected by hypoxia (Table 3). Because IL-2, IFN-γ, IL-4, and IL-6 are known mostly for their stimulatory activities, we decided to investigate the effect of hypoxia on the production of a cytokine with inhibitory activities, such as IL-10. Table 4 shows that at 16 hr the production of IL-10 was significantly inhibited by hypoxia in both resting and PHA-activated PBMC, and the IL-10 releases in both were 72% and 83% that of controls, respectively. Similar results were obtained after 40 hr of hypoxic treatment.

Thus, hypoxia affects the production of cytokine differently with the stimulatory or inhibitory activities of a specific cytokine (Fig. 7). At 16 hr, the release of IL-2, IFN-γ, IL-4, and IL-6 were significantly higher than controls, whereas the release of IL-10 was significantly inhibited.

TABLE 2. Effect of hypoxia on IL-6 production from PBMC[1]

Incubation time	Culture conditions	Normoxia	Hypoxia
16 hr	Medium	4,174 ± 758	5,658 ± 841*
	PHA	4,474 ± 926	6,148 ± 1,157*
40 hr	Medium	4,689 ± 961	6,976 ± 558*
	PHA	5,051 ± 1,142	8,525 ± 1,415*

[1]PBMC were cultured at a concentration of 2×10^5 cells/well with medium or PHA (5 μg/ml) and were exposed to either a hypoxic or an aerobic environment under the conditions described in Materials and Methods. After 16 and 40 hr of treatment, cell-free supernatants were obtained, and IL-6 present in the supernatants was determined by ELISA. Data presented is the mean ± SEM of six independent experiments.
*Statistically significant ($P < 0.05$) differences between IL-6 released by PBMC incubated in hypoxia vs. aerobic controls.

DISCUSSION

The present results indicate that PBMC kept in hypoxia display a different pattern in the production of cytokines than that of the controls kept at normal O_2 tension. We have shown that hypoxia enhances the production of stimulatory cytokines and inhibits the release of IL-10. Our results also confirm that PBMC kept at 2% O_2 tension are in a marked hypoxic state and that their susceptibility to a mitogen such as PHA is lower than controls. Although there is a progressive decrease of glucose level in the culture medium during hypoxia, PBMC cultures never reached a glucose level below the physiological concentration of 100 mg/ml. Thus, the variations in the production of cytokines by PBMC were not due to glucose deprivation.

Moreover, we have shown the viability of PBMC in hypoxia is very similar to that of control cells, demonstrating that a tension of 2% O_2 is still enough to allow cell replication. At the same time, values of pO_2 tension lower than 2% are not sufficient for cell replication (Gupta and Eberle, 1984).

In fact, in the present experimental condition, we did not observe a spontaneous apoptic cell death, although hypoxia causes a delay in the cell-cycle progression of PHA-activated PBMC. However, from the present results, we cannot state that hypoxia will not cause apoptosis in PBMC after a prolonged treatment (Stefanelli et al., 1995). We chose this incubation time to keep the cells viable and to maintain the pH and the levels of glucose and lactate in the media within a physiological range (Naldini et al., 1993b). Moreover, even if a delay in the cell-cycle progression of hypoxic PBMC is already

TABLE 3. Effect of hypoxia on TNF-α production from PBMC[1]

Incubation time	Culture conditions	Normoxia	Hypoxia
16 hr	Medium	2,096 ± 377	2,040 ± 359
	PHA	3,316 ± 655	3,683 ± 753
40 hr	Medium	1,602 ± 283	1,760 ± 318
	PHA	3,672 ± 397	3,601 ± 665

[1]PBMC were cultured at a concentration of 2×10^5 cells/well with medium or PHA (5 μg/ml) and were exposed to either a hypoxic or to an aerobic environment under the conditions described in Materials and Methods. After 16 and 40 hr of treatment, cell-free supernatants were obtained and TNF-α present in the supernatants was determined by ELISA. Data are presented as mean ± SEM of six independent experiments.

TABLE 4. Effect of hypoxia on IL-10 production from PBMC[1]

Incubation time	Culture conditions	Normoxia	Hypoxia
16 hr	Medium	1,289 ± 202	936 ± 146*
	PHA	2,522 ± 336	2,113 ± 236*
40 hr	Medium	807 ± 84	669 ± 116*
	PHA	2,946 ± 393	1,973 ± 138*

[1]PBMC were cultured at a concentration of 2×10^5 cells/well with medium or PHA (5 μg/ml) and were exposed to either a hypoxic or to an aerobic environment under the conditions described in Materials and Methods. After 16 and 40 hr of treatment, cell-free supernatants were obtained and IL-10 present in the supernatants was determined by ELISA. Data are presented as the mean ± SEM of six independent experiments.
*Statistically significant ($P < 0.05$) differences between IL-10 released by PBMC incubated in hypoxia vs. aerobic controls.

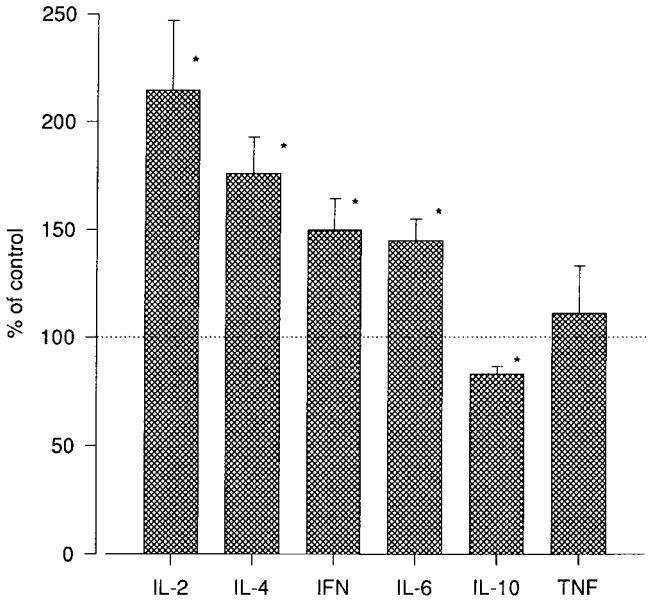

Fig. 7. Effect of hypoxia on cytokine production from PHA-stimulated PBMC. PBMC were treated with PHA (5 μg/ml) and exposed to a hypoxic or an aerobic environment. After 16 hr of culture, cell-free supernatants were obtained, and the cytokine present in the supernatants was determined by ELISA. Data presented are the mean ± SEM of six independent experiments and are expressed as percentage of control cultures exposed to an aerobic environment. Asterisks indicate statistically significant ($P < 0.05$) differences between the cytokines released in hypoxia vs. aerobic controls.

evident after 40 hr, the short incubation time did not allow us to distinguish the single phase of the cell cycle. Unfortunately, an exposure of PBMC to PHA for 72 hr, a protocol usually followed in cell-cycle analysis of propidium-iodide-stained cells, would have lead to a derangement of pH, glucose, and lactate concentration.

The present results describe the effect of hypoxia mainly on the change of cytokine production by PBMC. Different cytokines were selected to explain the behavior of PBMC in hypoxic conditions with regard to cytokine production. Hypoxia is a condition common in the necrotic area of solid tumors, in the inflammatory response, in generalized local ischemia, and at the level of the wound-healing process (Kallinowski et al., 1989). Many cytokines play important roles in the conditions described above. Indeed, TNF-α and IFNs control the proliferation of tumor cells, and these cytokines, with IL-1, IL-8, and others, regulate the inflammatory response. IL-6 is involved in the wound healing process, being produced by fibroblast and epithelial cells (Sower et al., 1995), and local ischemia is related to the production of certain cytokines related to an oxidative stress (Ghezzi et al., 1991). We recently have shown that hypoxia inhibits the antiproliferative activities of IFNs and TNF-α and that this inhibition is potentiated by antioxidants such as glutathione (Naldini et al., 1994). In this study, we show that hypoxia also affects the release of cytokines by immunocompetent cells such as PBMC. Hypoxia enhances the production of cytokines such as IL-2, IL-6, IFN-γ, and IL-4, which are well known for their stimulatory activities on the proliferation and differentiation of immunocompetent cells. As expected, hypoxia inhibits the production of IL-10, a cytokine well known for its inhibitory effects on the proliferation of T cells and in the production of other cytokines, by T cells and monocytes. In contrast, PBMC are less sensitive to the activation induced by PHA, and the overall effect is a decreased proliferation. In fact, this is a cell-cycle-associated effect because the cell-cycle progression is delayed by hypoxia.

Apparently, hypoxic immune cells release an increased amount of stimulatory cytokines, such as IL-2, but the overall effect is to inhibit cell growth. In hypoxia, the amount of IL-2 released may not be sufficient to allow PBMC to enter the S phase (Firpo et al., 1994). This notion agrees with previous reports that have shown that the effects of cytokines on cell growth is associated with cell cycle (Horoszewicz et al., 1979; Darzynkiewicz et al., 1984) and that in hypoxia IL-2-induced proliferation in T lymphocytes is significantly inhibited (Loeffler et al., 1992). However, other reports have shown that hypoxia induces the release of IL-6, the activation of the nuclear factor-IL-6 (NF-IL-6) site (Yan et al., 1995), and activation of NF-kappa B (Koong et al., 1994), which is associated with the production of several cytokines. Thus, hypoxia induces the release of stimulatory cytokines, but, because the cell cycle progression is delayed, a consequent increase of proliferation is not observed.

The production of IL-10, which inhibits the proliferation of T cells, is markedly inhibited, but an increase in PHA-induced cell proliferation is not associated with it. However, hypoxia markedly affects the production of cytokines and the response of PBMC, with regard to cytokine production and proliferation, is the result of stimulatory and inhibitory activities of the cytokines involved, which are associated with the cell cycle

events. For example, in the wound-healing process, the production of fibrogenic cytokines such as IL-6 is enhanced (Kovacs, 1991), and this enhancement may be potentiated by the local hypoxia present. In contrast, IL-2-induced T-cell proliferation is reduced by hypoxia (Loeffler et al., 1992) and the antiproliferative activities of TNF and IFN (Naldini et al., 1994, 1995b), with consequent negative effects on the antitumoral activities of these cytokines. Thus, the present study is important because of the pathophysiological significance of hypoxia (in inflammation, wound healing, cancer, and cancer treatment), with particular regard to cytokine production and biological activities.

An interesting remark concerns the biological relevance of experiments that employ cells that, in vitro, are always incubated in air and 5% CO_2 and therefore at a concentration of atmospheric O_2 (20.9%) or a partial pressure of 140 mmHg. In strict terms, these experiments may be difficult to interpreter biologically because arterial pO_2 is at best about 98 mmHg and, in the tissues, it ranges between 40 and 98 mmHg, which approximately 50% of the atmospheric value. Thus, traditional experiments in vitro, using atmospheric O_2, are performed in a somewhat unphysiological condition and could imply a higher production of oxygen reactive species than that occurring in cells "in vivo." The present study is important because, for the first time, it describes the effects of a continuous exposure of PBMC to hypoxia and may help in understanding the complex modifications that occurs in several pathophysiological conditions where hypoxia is involved.

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