# OZONE AS A MODULATOR OF THE IMMUNE SYSTEM

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## Abstract

In order to clarify the immunomodulating properties of ozone, we have investigated: a) the effects of stimulation on isolated peripheral human blood mononuclear cells (PBMC) from normal donors with either ozone or ozonated serum; b) the range (in terms of $O_3$ concentrations) of the therapeutic window; c) the stimulatory and toxic effects and d) the pattern, of both proinflammatory and immunosuppressive cytokine production up to 86 hours after exposure to $O_3$. Results show that ozone can act as a weak inducer of cytokines producing IL-6, IL-4, TNF-$\alpha$, IFN-$\gamma$, IL-2 and IL-10 and, most importantly, there is a significant relationship between cytokine production and ozone concentration. Analysis of the proliferation index shows that progressively increasing $O_3$ concentrations inhibit IP and therefore appear cytotoxic.

## Introduction

Leukocytes comprise a heterogeneous cell population composed of lymphocytes (20-25%), monocytes (about 5%) and three type of granulocytes of which the neutrophils are about 70%. We have been the first to show that an appropriate ozone dose can induce a small release of interferon $\gamma$ (IFN-$\gamma$) from human blood (Bocci and Paulesu, 1990). Later on the number of cytokines has expanded to IFN-$\beta$, interleukin 2 (IL-2), IL-6, IL-8, tumor necrosis factor $\alpha$ (TNF-$\alpha$), transforming grow factor $\beta$1 (TGF-$\beta$1) and granulocyte-monocyte colony stimulating factor (GM-CSF) (Paulesu et al., 1991; Bocci et al., 1993a, b; 1994; 1998a, b). Later on several authors (Beck et al., 1994; Arsalane et al., 1995; Jaspers et al., 1997) have confirmed that ozone can induce the production of cytokines after that epithelial cells of the respiratory mucosa have been in contact with ozone.

Our results were obtained by ozoning blood directly and cytokines were detected in the plasma during the following 4-8 hours of incubations. These initial studies shed light on several aspects such as the protective effect of blood antioxidants, the dissimilar production of different cytokines and the progressive inhibitory activity of increasing ozone concentrations, particularly above 80 $\mu$g/ml per ml of blood. However they had limitations because firstly, whole blood can be incubated only for a limited time and, most importantly, we could not decide which cell type produced the cytokines.

During the last year we decided to isolate from normal blood donors either peripheral blood mononuclear cells (lymphocytes and monocytes, PBMC) in order to investigate their viability and the production and type of cytokines released after two different ozonation modalities. The first is a direct ozonation of PBMC suspended in human serum, so that cells undergo the total action of ozone due to <u>immediate</u> effects by hydrogen peroxide ($H_2O_2$) and other unidentified reactive oxygen species (early ROS), with very short half-life, and <u>late</u> effects, provided by lipid oxidative products (LOPs), with fairly long half-life. The second approach has examined the effect of ozonated serum 20 min before addition to PBMC and therefore ozone activity is expressed only by "late LOPs".

## Materials and Methods

### Ozone generation and measurement

$O_3$ was generated from medical-grade $O_2$ using electrical corona arc discharge by the $O_3$ generator (Model Ozonosan PM 100K, Hansler GmbH, Iffezheim, Germany), which allows the gas flow rate and $O_3$ concentration to be controlled in real time by photometric determination at 253.7 nm, as recommended by the Standardisation Committee of the International $O_3$ Association. Tygon polymer tubing and single-use silicon treated syringes were used throughout the reaction procedure to ensure containment of $O_3$ and consistency in concentrations.

### $O_3$ delivery to biological samples

A predetermined volume of $O_2/O_3$ gas mixture at various $O_3$ concentrations was collected with a syringe and immediately introduced into a second syringe containing an equivalent volume of human serum via a multidirectional stopcock. The final gas pressure remained at normal atmospheric pressure. In order to obtain reproducible results, it needs to be emphasised that $O_3$ is a very reactive gas so that extremely rapid and precise handling is required. The sample is gently but continuously mixed with the gas for twenty minutes and afterwards dispensed into test tubes for various analyses. Control samples were either not treated or mixed with an equal volume of $O_2$. It is worth mentioning that $O_2$ represents at least 95% of the $O_2$-$O_3$ mixture.

### Collection of human blood samples and purification of PBMC

After obtaining their informed consent, venous blood was withdrawn from healthy donors who had not been affected by any infection for at least one month and not taken any medication. Donors ranged in age from 24 to 67 years. Blood was collected in 0.14 ml citrate-phosphate-dextrose (CPD) per ml of blood. PBMC were isolated by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) gradient centrifugation, washed twice in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 20 mM HEPES, spun down at low speed to remove platelets, and resuspended in RPMI-1640 medium supplemented with 2 mM HEPES, 10% heat-inactivated fetal calf serum (FCS, Sigma Chemical Co.), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies, Gaithersburg, MD) at the final concentration of $1 \times 10^6$ viable cells/ml. The cells viability was assayed by the trypan blue exclusion technique and light microscope observation.

### Experimental approaches

The aim was to examine two aspects of the ozonation process:
(a) In the first case PBMC were suspended in sterile human serum (from male AB plasma, Sigma Co.) and directly treated with ozone at different concentrations, in order to evaluate the effect of early and late ROS including LOPs.
(b) In the second case, PBMC were suspended in sterile human serum after that this has been ozonized for 20 min before cell addition. In this case PBMC could undergo only the effect of late ROS and LOPs, as early ROS decay in a few minutes during the ozonation process.

In both cases all samples underwent successively the same procedure for incubation and related measurements.

## PBMC proliferation

The various sample of PBMC suspension were added per well in triplicate wells to 96 well flat bottomed tissue culture plates (Costar, Cambridge, MA). PBMC were cultured without stimulation or stimulated with phytohemagglutinin (PHA, Sigma Chemical Co.) at final concentration of 5 µg/ml. Cell proliferation was evaluated by a colorimetric immunoassay (Boehringer Mannheim, Mannheim, Germany) based on BrdU incorporation. Briefly, after 48 h of incubation at 37°C in air-$CO_2$ (5%) and 100% humidity, the cells were labelled with BrdU for 24 h (10 UI/well). The cells were then fixed, anti-BrdU-POD antibody was added and the immune complexes were detected by the subsequent substrate reaction. The proliferative index (PI) was obtained by calculating the ratio between PHA-stimulated cells and unstimulated ones, after subtraction of the corresponding blanks.

## Determination of cytokines

Aliquots of all blood samples were layered on sterile tissue culture wells that were incubated in air-$CO_2$ (5%) for 38, 62 and 86 hours. After 62 hours, 20 µl of sterile glucose solution were added to readjust glucose level at about 5 mM. At the end of each incubation period, samples were centrifuged at high speed and the plasma supernatants were kept at −70°C until determinations of cytokines were carried out. Immunoassays of either proinflammatory cytokines (IL-2; IFN-γ and TNF-α) or suppressive cytokines (IL-4; IL-6 and IL-10) were carried out using Cytoscreen immunoassay kits produced by Biosource Intern. All plasma samples were diluted 1:1 with the appropriate diluent. A three-cycle automatic washing was routinely performed. Negative plasma samples, in absence or presence of haemoglobin, were spiked with the cytokine's standards to assess the reliability and precision of the various assays. Yields ranged between 93% and 105%.

## Biochemical determinations

(a) Total antioxidant status (TAS) in plasma samples was carried out according to Rice-Evans and Miller (Rice-Evans and Miller, 1994).
(b) Protein thiol groups (PTG) were measured in plasma according to Hu (Hu, 1994) using procedure 1 with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) dissolved in absolute methanol. Values are expressed as mM.
(c) The thiobarbituric acid (TBA) assay was carried out in plasma as described by Buege and Aust's method (Buege and Aust, 1994). Values are reported (µM) as TBA reactive substances.

## Statistical analyses

Results were expressed as the mean ± SD and the data were analysed using the Student's $t$-test. $P$ values less than 0.05 (∗), 0.01 (∗∗) and 0.001 (∗∗∗) were considered significant.

# Results and Measurements

First of all we examined the effect of ozonation performed directly on isolated human PBMC resuspended in human serum. The TAS value of the serum was 0.526 mM and this is a value markedly lower than the usual range (1.4-1.8 mM) found in fresh human plasma. In preliminary experiments we estimated the pattern of both the peroxidation (TBARS) and of thiol (PTG) oxidation that represent the usual markers indicating an effective ozonation. As it was expected, TBARS and PTG values increase and decrease, respectively, by progressively increasing ozone concentration (**Fig. 1**).
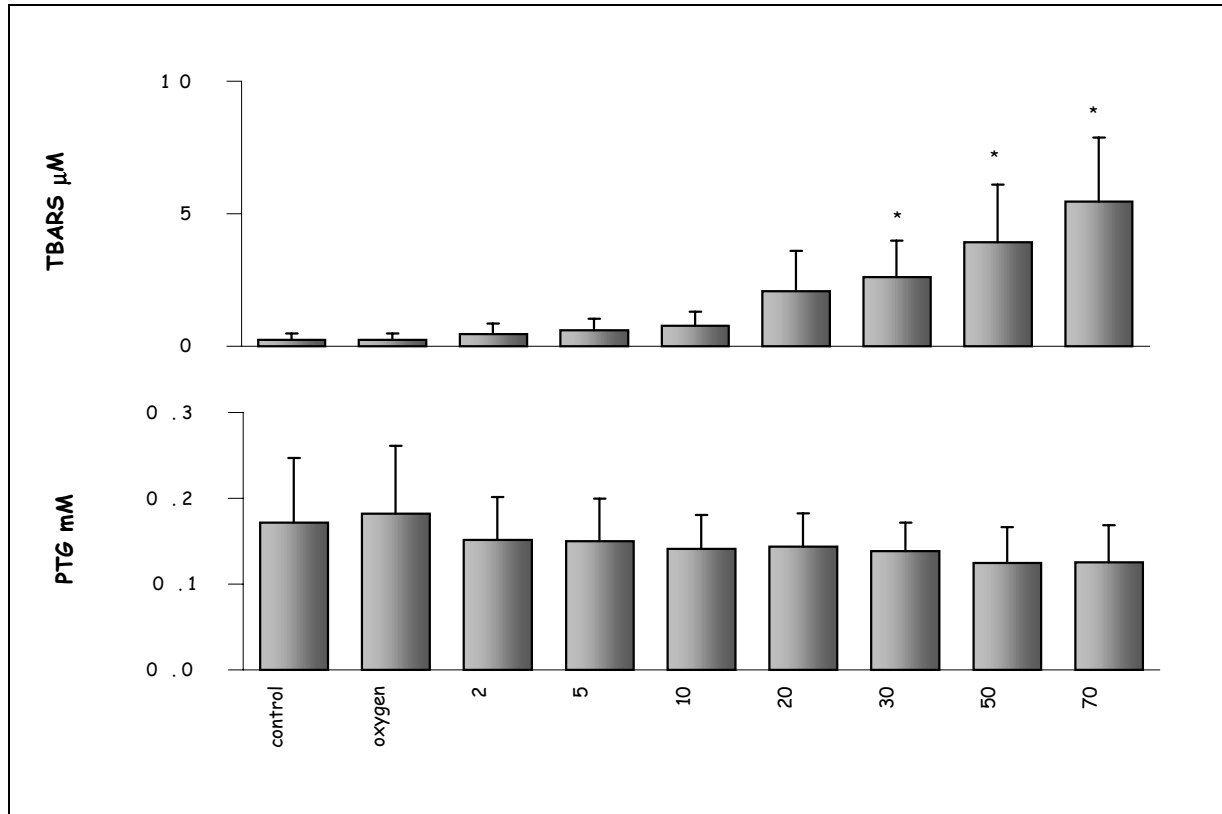


**Fig. 1.** Values of TBARS and PTG measured in human serum either unexposed (control) or exposed to $O_2$ or to $O_2$-$O_3$ at increasing concentrations ($\mu$g/ml). PBMC were suspended in serum before ozonation.

Then we examined the proliferation index (PI) of PBMC at different times of incubation: ozone acts as a very weak stimulus for proliferation only after 62 and 86 h of incubation and only low ozone concentration (2.5-10 $\mu$g/ml) are able to rise the PI slightly above 1. Oxygen on its own is hardly effective while PHA shows that PBMC under test were highly responsive to the mitogen. Higher $O_3$ concentrations, from 2 to 70 $\mu$g/ml, are progressively inhibitory suggesting a cytotoxic effect (**Fig. 2**).
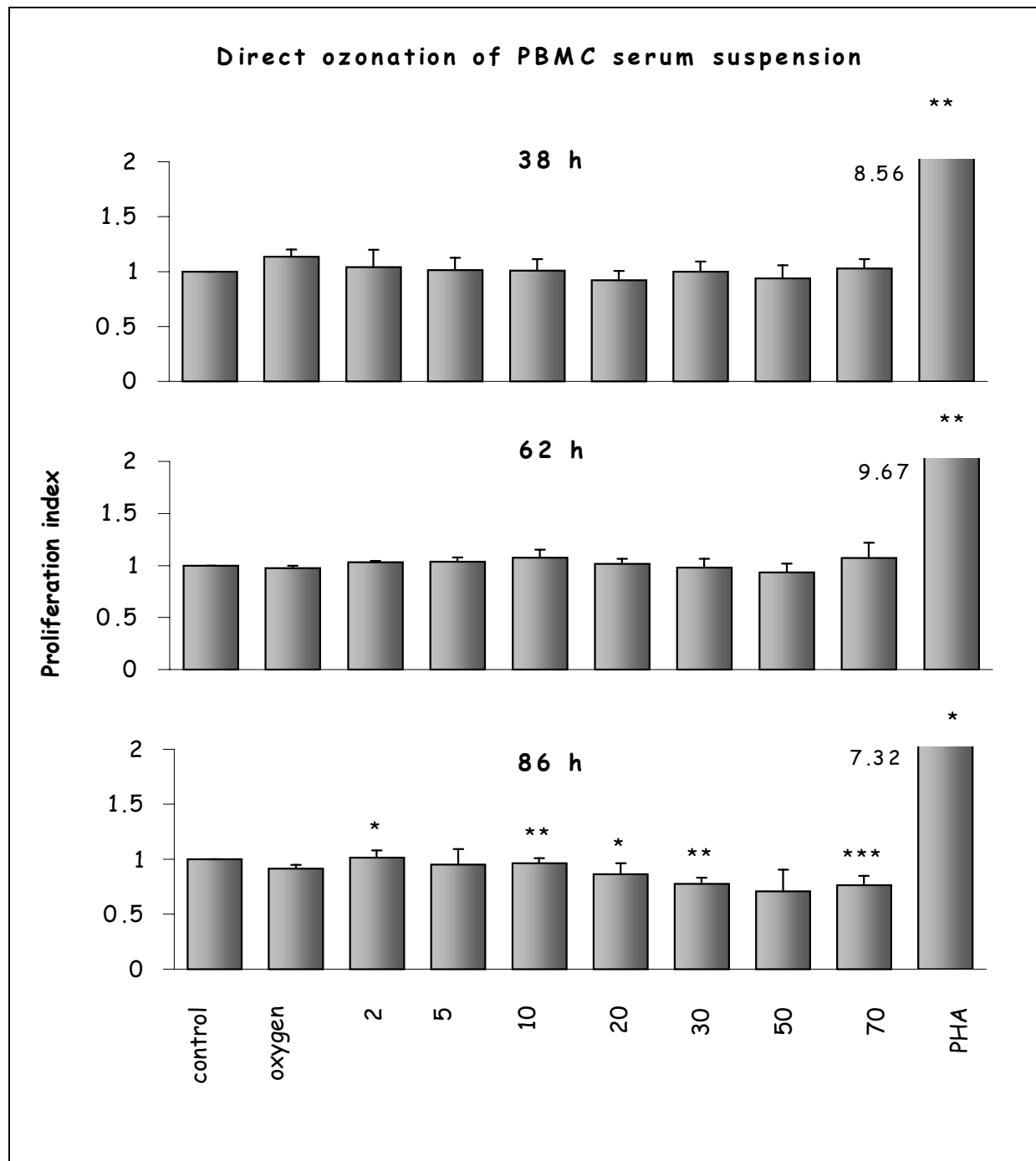
**Fig. 2.** Values of proliferation index are reported of PBMC suspended in human serum before ozonation. Symbols are equivalent to no gas (control), $O_2$ alone and $O_2$-$O_3$ at increasing concentrations (µg/ml). Times of incubation were 38, 62 and 86 hours.

One of the aims of this investigation was to ascertain if previously ozonated serum (20 min before suspending PBMC) is still active on cells. The pattern of peroxidation and PTG oxidation is very similar and, for sake of space, it is not shown. However we must bear in mind that $H_2O_2$ is no longer present while LOPs with long half-life are still active (Bocci et al., 1998b). **Fig. 3** shows that the PI is practically overlapping the one presented in **Fig. 2**.
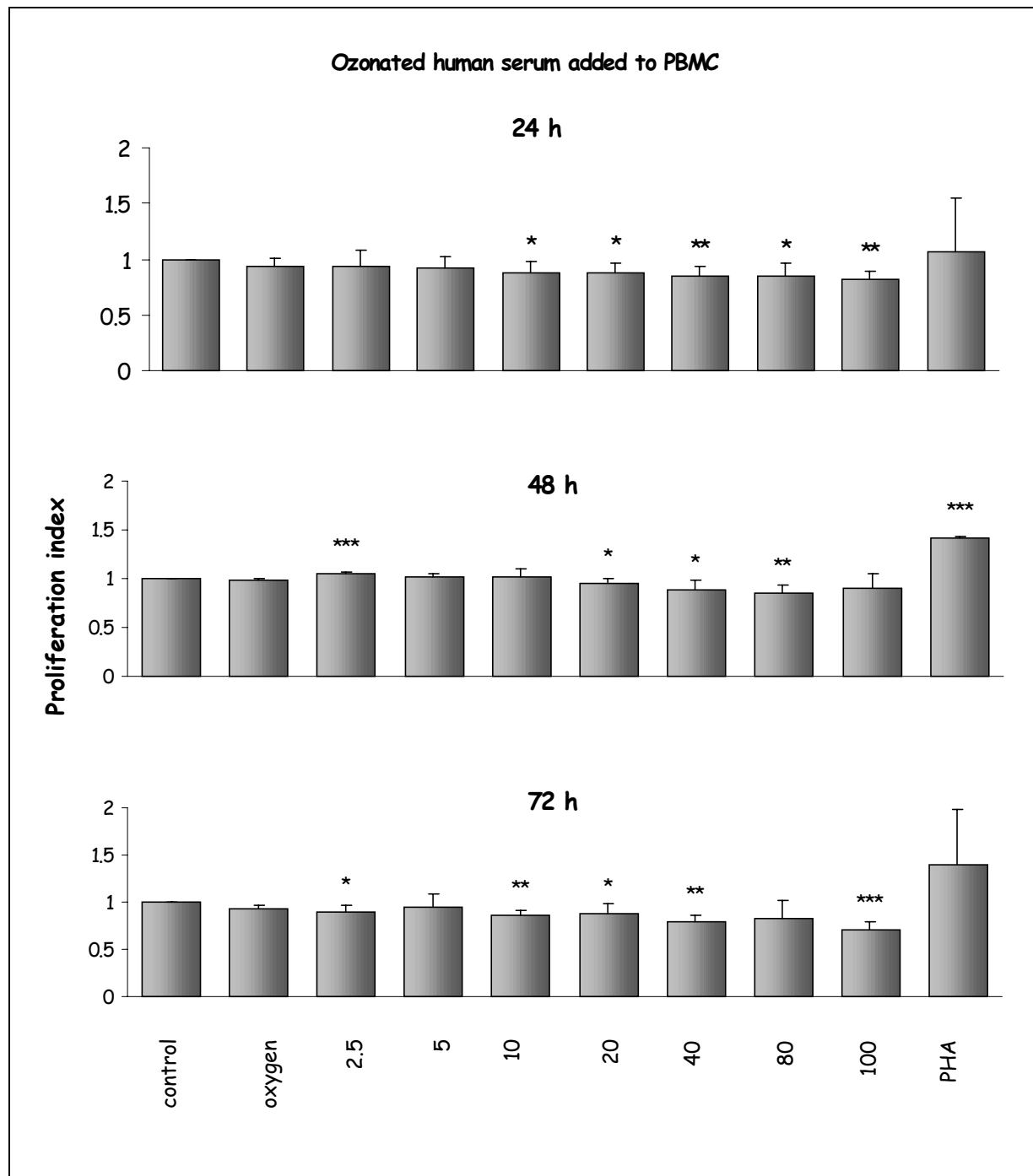
**Fig.3.** Values of proliferation index are reported of PBMC suspended in human serum after that this had been exposed to no gas (control), $O_2$ alone and $O_2$-$O_3$ at increasing concentrations (µg/ml). Times of incubation were 24, 48 and 72 hours.

These experiments have confirmed that very low $O_3$ concentrations are slightly stimulatory while high concentrations are inhibitory. In the past direct incubation of ozonized blood leads to a rapid release of several cytokines but it was no possible to distinguish the contribution of PBMC from granulocytes. The study now performed has yielded disappointing results (**Fig. 4**).
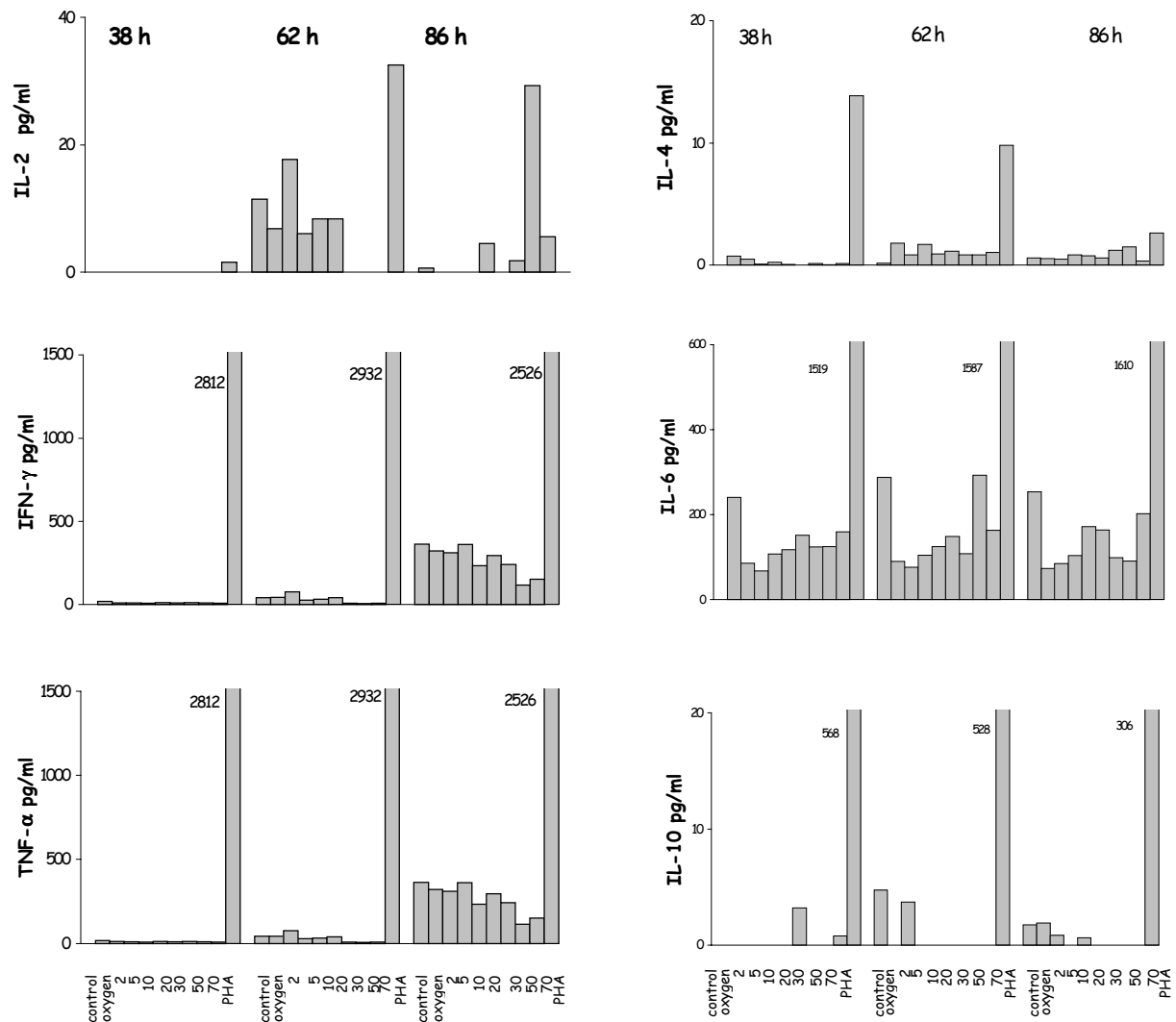
**Fig. 4.** Pattern of cytokine production by PBMC suspended in human serum before ozonation. Samples were not exposed (control) or exposed to either $O_2$ alone or $O_2$-$O_3$ at increasing concentrations (μg/ml). PHA indicates values after mitogen addition. Th-1 type cytokines in the left panel and Th-2 type in the right panel. Diagrams represent the average of four donors of which three were very low responders.

Except for IL-6, production of cytokines during the first 38 h is practically nil and becomes evident for IL-2 and IL-4 between 38-62 hours. During the last interval (62-86 h) IFN-γ, TNF-α and IL-4 could be measured but without any relation to $O_3$ concentration. There is a weak indication that at 62 h, 2 and 5 μg/ml $O_3$ could be more effective for IL-2 and IFN-γ but this does not hold true for IL-6. Induction of IL-10 is practically negligible. It must be mentioned that results presented in **Fig. 4** are the mean of four different buffycoats. Although blood was drawn from healthy donors, three of this hardly responded and had to be considered very low responders. This problem greatly affected the final results showing an enormous variability. For the sake of space we have not shown individual results except buffycoat of donor H who has acted as a good responder (**Fig. 5**).
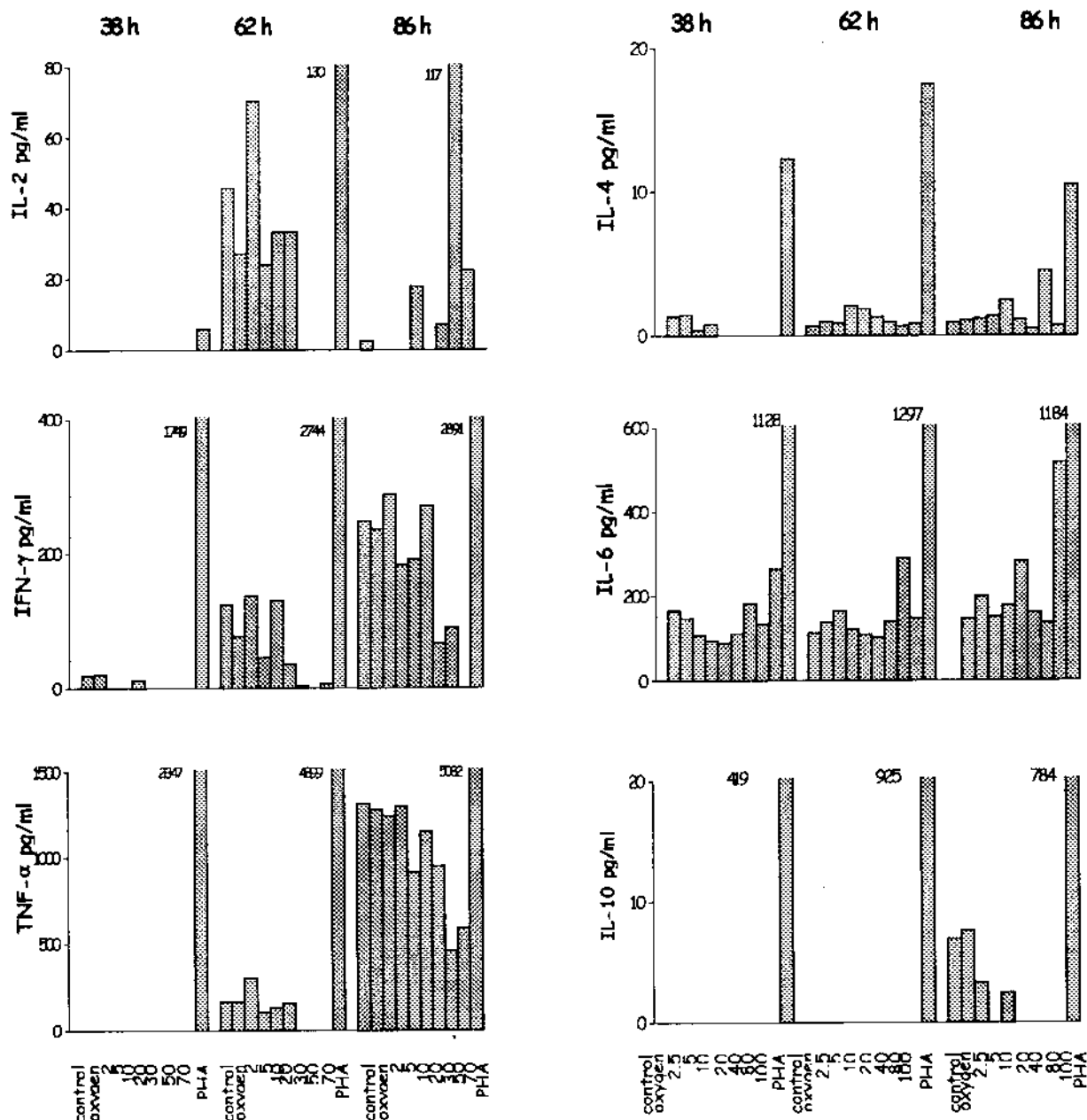
Fig. 5. Pattern of cytokine production by PBMC suspended in human serum before ozonation. Samples were not exposed (control) or exposed to either $O_2$ alone or $O_2$-$O_3$ at increasing concentrations ($\mu g/ml$). PHA indicates values after mitogen addition. Th-1 type cytokines in the left panel and Th-2 type in the right panel. Diagrams represent the values of one donor (H) that was a high responder.

The pattern of cytokine production was also similar but quantitatively less evident when PBMC were additioned to previously ozonized serum indicating that direct ozonation of cell suspension is preferable.

## Discussion

The purpose of this work was two-fold: firstly, we wanted to clarify if isolated human PBMC treated with ozone modify their proliferation pattern and produce cytokines. Previous studies have been performed using whole blood but this approach has the limit of a short incubation and the impossibility of deciding which cells are activated. Two obvious disadvantages are that we need of isolating PBMC that implies some cellular stress and that PBMC are resuspended in human serum that compared to original plasma has a rather low TAS capacity. Secondly, ozonation of serum is characterised by a series of reactions during which the formation of $H_2O_2$ is accompanied by the formation of lipid oxidation products (LOPs) among which peroxyl radicals, hydroxyl alkenals and malonaldehyde. $H_2O_2$ is an early product and has a lifetime of 2.5 min but, by diffusing into the cell, may activate NF-kB responsible of switching on the synthesis of several types of proteins, including cytokines. This biochemical pathway is modulated by antioxidants, such as intracellular reduced glutathione (GSH), ascorbic acid, GSH peroxidase and catalase and, as a consequence, too low $O_3$ concentrations mean very low levels of $H_2O_2$ unable to activate NF-kB. On the other hand, too high $O_3$ concentrations mean high levels of $H_2O_2$ that may overwhelm antioxidants and lead to cell damage. In our experimental approach, on the basis of previous results, we postulated an activation on a range of concentration between 5 and 20 μg/ml but, actually, because of the low TAS capacity, only 2.5 μg/ml was able to increase the PI. LOPs can be considered rather stable products at least in vitro, and at very low concentrations (below 1 μM) may act as positive signals, while concentrations higher than 1 μM may be cytotoxic. This results is indeed what we have seen either directly ozonising the cell suspension or adding the ozonated serum to PBMC. Inhibition of PI has been noted in both cases at the ozone concentration above 70 μg/ml. This trend has appeared also in regard to cytokine production for IFN-γ, TNF-α and IL-10.

A rather unexpected finding has been the extremely poor cytokine response of three out of four PBMC samples. The phenomenon of low and high responders to IFN inducers is well known but we could not anticipate its relevance during ozonation and it has never been so evident in blood samples. This dishomogeneity in cytokine production must be kept in mind in practice, because particularly immunosuppressed patients may not be responsive to ozone therapy.

Next step will be to examine production of cytokines by isolated human granulocytes in relation to the same ozone concentrations because granulocytes represent almost 75% of the leukocyte mass and may explain the observed discrepancy between whole blood and isolated cell components. This work is in progress.

## Acknowledgements

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