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Toxicology in Vitro 19 (2005) 55–61
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

Effects of ozone on isolated peripheral blood mononuclear cells

A. Larini, V. Bocci *

Department of Physiology, University of Siena, Via A. Moro 2, 53100 Siena, Italy

Received 17 March 2004; accepted 25 June 2004

Abstract

We have investigated the release of cytokines from isolated peripheral human blood mononuclear cells (PBMC) exposed to various ozone concentrations for 10 min and the release of both proinflammatory and immunosuppressive cytokine after 24, 48 and 76 h incubation. Ozonation was performed by exposing for 10 min equal cell numbers and volumes of cell suspension to equal volumes of a gas mixture (1:1 ratio) composed of oxygen–ozone with precise ozone concentrations ranging from 1.0 up to 80 μg/ml (0.02 up to 1.68 mM). Markers of oxidative stress showed a significant relationship between ozone doses and both lipid peroxidation and protein thiol groups content. With the exception of the lowest ozone concentration, the cytokine production of PBMC was depressed particularly at concentrations from 40 μg/ml upwards. There was no significant effect on IL-6 production between exposed or unexposed cells, up to 72 h of incubation. IL-4 production was markedly affected by ozone exposure, showing a marked decrease even at the lowest ozone concentration (2.5 μg/ml) already after 24 h incubation. On the other hand, production of IFN-γ and TNF-α was slightly stimulated by the lowest ozone dose either at all times or only after 72 h incubation, respectively. Analysis of the proliferation index (PI) is consistent with these results showing that, while the lowest concentration stimulates it, progressively increasing O_3 concentrations inhibit the PI. These data show that there is a significant relationship between cytokine production and ozone concentrations and that PBMC are very sensitive to oxidation particularly in presence of serum with low antioxidant capacity.

Keywords: Cytokines; Ozone; Oxidants; PBMC; Redox system

1. Introduction

The toxicity of ozone is well known (Pryor, 1993), and yet, after a decade of experimental and clinical work, we have come to regard ozone as a drug that, at appropriate concentrations, can generate interesting biological effects (Bocci, 2002). As other chemical drug, ozone displays a narrow therapeutic window below which there is no activity and above which there are toxic effects so that only a judicious use can be therapeutically useful (Bocci et al., 2001; Lippman, 1989). During the last three decades, millions of ozonated autohaemotherapy (O_3-AHT) have been performed in patients without yielding either acute, or chronic toxic effects.

Cells cultured in antioxidant-poor media, exposed for days even at very low ozone concentrations, owing to a cumulative toxic effect, undergo apoptosis (Leist et al., 1996). Saline-washed erythrocytes resuspended in saline undergo haemolysis after ozone exposure (Goldstein and Balchum, 1967) and similarly, leukocytes present in saline-diluted blood exposed to 5 mM ozone for 1 h display some genetic damage (Diaz-Llera et al., 2002). All of these data are important but they show that ozone toxicity is exerted when cells are incubated in antioxidant-free culture media. In contrast blood is a far more ozone-resistant "tissue" and we have proved that hundreds of human blood samples exposed for 5 min to ozone concentrations ranging from 20 μg/ml

* Corresponding author. Tel.: +39 577 234226; fax: +39 577 234219.
E-mail address: bocci@unisi.it (V. Bocci).

0887-2333/$ - see front matter © 2004 Published by Elsevier Ltd.
doi:10.1016/j.tiv.2004.06.007

(0.42 mM) up to 60 μg/ml (1.26 mM) do not show any damage (Bocci, 2002; Bocci et al., 2001). Moreover the total antioxidant status (TAS), normally in the range of 1.3–1.8 mM, decreases no more than 20% during the first 5 min and returns to the original value after 30 min, clarifying that exposure to ozone causes only a partial and reversible depletion of antioxidants (Bocci, 2002; Bocci et al., 2001).

It has been demonstrated that human blood exposed to appropriate ozone doses, after incubation, can lead to a small release of interferon γ (IFN-γ) (Bocci and Paulesu, 1990), IFN-β, interleukin 2 (IL-2), IL-6, IL-8, tumor necrosis factor α (TNF-α), transforming growth factor β1 (TGF-β1) and granulocyte–monocyte colony stimulating factor (GM-CSF) (Paulesu et al., 1991; Bocci et al., 1993a,b, 1994, 1998a,b). Several authors (Beck et al., 1994; Arsalane et al., 1995; Jaspers et al., 1997) have also observed that ozone can induce the production of cytokines when epithelial cells of the respiratory mucosa have been in contact with ozone.

In the last decade, we (Bocci, 1996; Bocci et al., 1993a,b, 1998a,b, 1999, 2001) have been interested in examining the biological and possible therapeutic effects of ozone after its brief interaction with human blood. Our results were obtained by ozonating blood directly and cytokines were detected in the plasma during the following 4–8 h of incubation. These initial studies shed light on relevant 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 60 μg/ml per ml of blood. However they had limitations because firstly, whole blood can be incubated only for a short time and, most importantly, we could not decide which cell type produced the cytokines.

In order to investigate their viability and the production and type of cytokines released after ozonation, we isolated peripheral blood mononuclear cells (PBMC) from normal blood donors. PBMC were suspended in commercially available human serum and directly ozonated so that cells would undergo the total action of ozone due to immediate effects of hydrogen peroxide (H_2O_2) and other unidentified reactive oxygen species (early ROS), with very short half-life, and late effects, provided by lipid oxidative products (LOPs), with fairly long half-lives.

2. Materials and methods

2.1. 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.

2.2. Isolation of PBMC

After obtaining their informed consent, venous blood was withdrawn from healthy donors who, apparently, had not been affected by any infection for at least one month and had not taken any medication. Donors ranged in age from 24 to 67 years. Only a small volume of blood could be collected in citrate-phosphate-dextrose (CPD) at the Polyclinic Haemotransfusion Centre, thus precluding the possibility of suspending the isolated cells in the respective autologous plasma. PBMC were isolated by Lymphoprep™ (Nycomed Pharma, Oslo, Norway) gradient centrifugation, as described by Boyum (1968), 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). Cell viability was assayed by the trypan blue (Sigma Chemical Co.) exclusion technique and light microscope observation. Immediately after, cells were sedimented at low g and were resuspended in heat-inactivated pooled human serum (from male AB plasma, Sigma Co.), containing heparin 5 U/ml and 5 mM glucose at the final concentration of 1×10^6 viable cells/ml before the ozonation. We had to add pooled serum Sigma that, on further testing, has shown to have a very low antioxidant capacity. In retrospect the addition of this serum has been a drawback but we could not use the original plasma because we could not sample a large volume of blood.

2.3. O_3 delivery to biological samples

A predetermined volume of O_2 (~96%) and O_3 (~4%) gas mixture, at various concentrations, was collected with a syringe and immediately introduced into the second syringe containing the samples via a multidirectional stopcock. We always used a cell suspension/gas volume at a 1:1 ratio. Samples were gently but continuously mixed with the gas mixture at different O_3 concentrations (1.0, 2.5, 20, 40 and 80 μg/ml per ml of cells corresponding to 0.02, 0.05, 0.42, 0.83 and 1.68 mM, respectively) for 10 min. We have previously ascertained that during this period of time ozone reacts completely

with substrates, implying that cell samples receiving ozone react with the ozone dose totally. 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 final gas pressure remained at normal atmospheric pressure. Control samples were either not treated or mixed with an equal volume of pure medical O_2. It is worth emphasising that O_2 represents at least 96% of the O_2–O_3 mixture and that the O_2 control is important.

2.4. PBMC proliferation

The various samples of PBMC suspension were added per well in duplicate to 96 well flat bottomed tissue culture plates (Costar, Cambridge, MA) and again cultured at 37 °C in air, 5% CO_2 atmosphere for 24, 48 and 72 h, without stimulation or stimulated with phytohemagglutinin (PHA, Sigma Chemical Co.) at a final concentration of 5 μg/ml. Cell viability was assessed with the trypan blue exclusion method at all times. Cell mortality was never greater than 10%, even after 72 h of incubation in all samples. Cell proliferation was evaluated by a colorimetric immunoassay (Boehringer Mannheim, Mannheim, Germany) based on BrdU incorporation. Briefly, after 24, 48 and 72 h of incubation, the cells were labelled with BrdU for 12 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 treated cells and untreated ones, after subtraction of the corresponding blanks. The BrdU incorporation test does no allow to discriminate if, after ozone exposure, there is a variation of phase S cells or an increase of DNA repair.

2.5. Determination of cytokines

Aliquots of all blood samples were layered on sterile tissue culture wells that were incubated in air–CO_2 (5%) for 24, 48 and 72 h. After 48 h, 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 Th-1 type cytokines (IL-2; IFN-γ and TNF-α) or Th-2 type 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%.

2.6. Biochemical determinations

(a) Total antioxidant status (TAS) in plasma samples was carried out according to Rice-Evans and Miller (1994).

(b) Protein thiol groups (PTG) were measured in plasma according to 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 (1994). Values are reported (μM) as TBA reactive substances (TBARS).

2.7. Statistical analyses

Results were expressed as the mean ± the standard deviation of the mean (SD) and the data were analysed using the paired Student's *t*-test. *p* values less than 0.05 (*), 0.01 (**) and 0.001 (***) were considered significant. Either the oxygenated sample or the treated ones (O_2–O_3) were compared to the untreated sample (control).

3. Results

Our aim was to investigate how isolated human PBMC suspended in human serum reacted after being exposed for 10 min to a gas mixture composed of oxygen–ozone, with ozone concentrations ranging from 1.0 μg/ml (26.25 μM) up to 80 μg/ml (1.68 mM) of gas per ml of cell suspension against two controls, of which one was not exposed to any gas (C) and one exposed to oxygen only (O_2). After the 10 min period of gas exposure, cells were incubated in air–CO_2 for 24, 48 and 72 h.

Levels of TBARS and PTG, as a marker of peroxidation, are shown in Fig. 1. TBARS and PTG values, as expected, increase and decrease, respectively, by progressively increasing ozone concentration (in both,

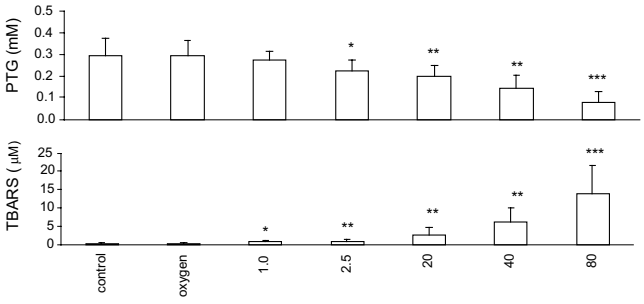

Fig. 1. Values of TBARS and PTG measured in PBMC suspension either unexposed (control) or exposed to O_2 or to O_2–O_3 at increasing concentrations (1.0, 2.5, 20, 40, 80 μg/ml). Diagrams represent the average of 12 experiments.

$p < 0.001$ at O_3 80 μg/ml) making sure that ozonation has been performed correctly.

The proliferation index (PI) of PBMC at different times of incubation is reported in Fig. 2: only the minimal ozone concentration (1.0 μg/ml) stimulated cell proliferation significantly and raised the PI above 1, particularly evident after 48 h of incubation. Oxygen on its own was hardly effective, while PHA treatment, as expected, showed that PBMC were highly responsive to the mitogen. Higher O_3 concentrations, from 20 up to 80 μg/ml, were progressively inhibitory showing significant decrements already after 24 h of incubation for cells exposed even to 20 μg/ml ozone concentration. This mild cytotoxic effect was unexpected because previous data obtained after ozonation of whole blood at this concentration did not show an oxidative damage (Bocci et al., 1993b). However in the present work we have used sterile human stored serum that has a markedly lower antioxidant capacity than fresh human plasma. Indeed the TAS value of the human serum used was 0.64 mM (±0.19; $n = 9$), a value markedly lower than the usual range found in fresh human plasma (1.4–1.8 mM). Moreover PTG values were also as low as 0.27 mM (±0.07; $n = 7$) while are about 0.4 mM in fresh human plasma. This consideration can explain why even after only 24 h incubation, cells suspension exposed to an ozone concentration of 20 μg/ml showed a significant decrease of proliferation in comparison to control (Fig. 2).

To ascertain if this effect was due to immediate effects by very short half-life ROS (among which H_2O_2) rather than by LOPs, mainly 4-hydroxy-2,3-transnonenal, with fairly long half-life, in parallel experiments we studied if ozonated serum (10 min ozonation, 20 min before suspending PBMC) was still active on the cells. The pattern of peroxidation, PTG oxidation and proliferation index was very similar suggesting that LOPs are mainly responsible for the observed cytotoxicity.

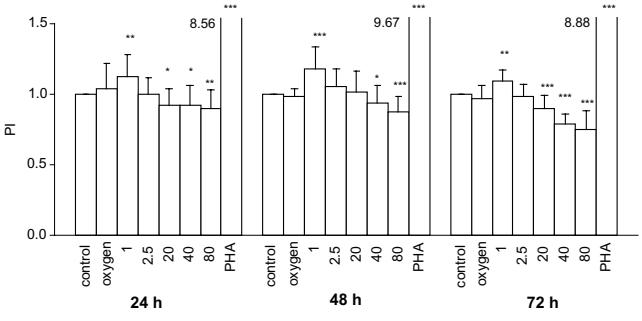

Fig. 2. Values of proliferation index (PI) of PBMC suspended in human serum before ozonation. Symbols are equivalent to unexposed (control), O_2 alone, O_2–O_3 at increasing concentrations (1.0, 2.5, 20, 40, 80 μg/ml) and PHA addition (5 μg/ml). Time of incubation was 24, 48 and 72 h. Diagrams represent the average of 16 experiments.

To further investigate the effect of increasing ozone concentrations on lymphocyte function, levels of several cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-6 and IL-10) were measured at different times in culture supernatants, following ten separate experiments (Figs. 3–7).

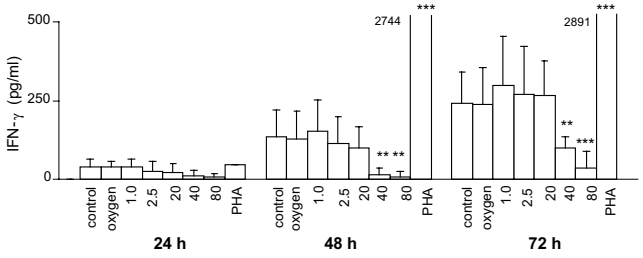

Fig. 3. Pattern of IFN-γ production by PBMC suspended in human serum before ozonation. Samples were not exposed (control) or exposed to either O_2 alone or O_2–O_3 at increasing concentrations (1.0, 2.5, 20, 40, 80 μg/ml). PHA indicates values after mitogen addition. Diagrams represent the average of 9 donors. Time of incubation was 24, 48 and 72 h.

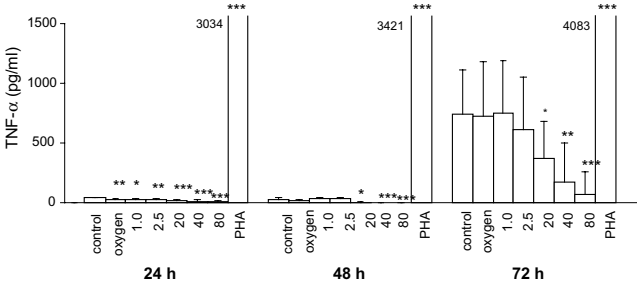

Fig. 4. Pattern of TNF-α production by PBMC suspended in human serum before ozonation. Samples were not exposed (control) or exposed to either O_2 alone or O_2–O_3 at increasing concentrations (1.0, 2.5, 20, 40, 80 μg/ml). PHA indicates values after mitogen addition. Diagrams represent the average of 9 donors. Time of incubation was 24, 48 and 72 h.

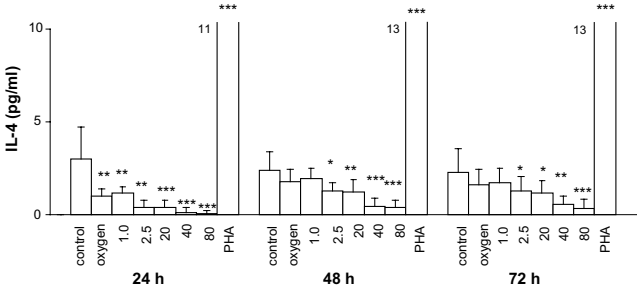

Fig. 5. Pattern of IL-4 production by PBMC suspended in human serum before ozonation. Samples were not exposed (control) or exposed to either O_2 alone or O_2–O_3 at increasing concentrations (1.0, 2.5, 20, 40, 80 μg/ml). PHA indicates values after mitogen addition. Diagrams represent the average of 9 donors. Time of incubation was 24, 48 and 72 h.

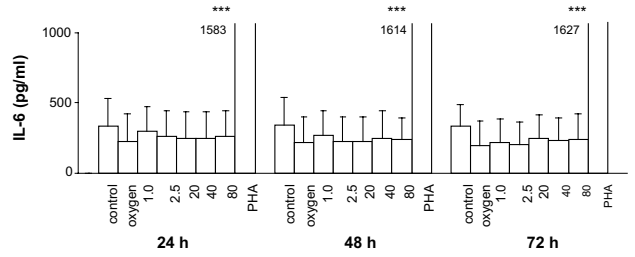

Fig. 6. Pattern of IL-6 production by PBMC suspended in human serum before ozonation. Samples were not exposed (control) or exposed to either O_2 alone or O_2–O_3 at increasing concentrations (1.0, 2.5, 20, 40, 80 μg/ml). PHA indicates values after mitogen addition. Diagrams represent the average of 9 donors. Time of incubation was 24, 48 and 72 h.

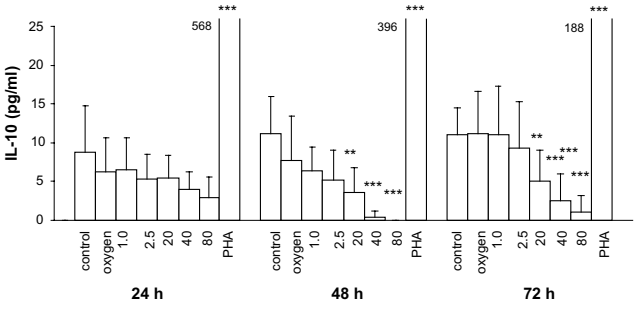

Fig. 7. Pattern of IL-10 production by PBMC suspended in human serum before ozonation. Samples were not exposed (control) or exposed to either O_2 alone or O_2–O_3 at increasing concentrations (1.0, 2.5, 20, 40, 80 μg/ml). PHA indicates values after mitogen addition. Diagrams represent the average of 9 donors. Time of incubation was 24, 48 and 72 h.

IL-2 production was always negligible (data not shown). A release of IFN-γ (Fig. 3) and TNF-α (Fig. 4) above basal levels could be measured only at the lowest ozone concentration. During the last interval (48–72 h) we observed a progressive cytokine reduction ($p < 0.001$) from ozone concentrations of 20 μg/ml upwards. This trend appeared also with regard to IL-10 (Fig. 7) and more markedly IL-4, showing a significant decrease also at very low ozone concentrations, already after 24 h incubation. The brief exposure of PBMC to either oxygen or O_2–O_3 appeared to inhibit the release of IL-6 in the medium.

4. Discussion

The main purpose of this work was to clarify if isolated human PBMC treated with ozone modified their proliferation pattern and produce cytokines. Previous studies have been performed using whole blood but this approach was limited by a short incubation and the impossibility of deciding which cells were responsive to ozone. In the present study we appraised two main disadvantages: the first was that we needed to isolate PBMC, which implied some cellular stress and the second was that the isolated PBMC had to be resuspended in human stored serum, which, compared to the donor plasmas, had a TAS capacity ranging between 35% and 40%. This second drawback could not be avoided because we could not withdraw enough blood to use fresh plasma.

Ozonation of serum is characterised by a series of reactions during which the formation of ROS, mainly H_2O_2, is accompanied by the formation of LOPs among which are peroxyl radicals, hydroxyl alkenals and malonyldialdehyde. H_2O_2 is an early product and has a lifetime of about 2 min in plasma, because it rapidly diffuses into cells and undergoes enzymatic reduction (Bocci, 2002). Once in the cytoplasm, H_2O_2 activates a tyrosine kinase that, by phosphorylating the IkB moiety of the NF–kB complex, allows the heterodimer p50-p65 to move into the nucleus where it regulates the gene expression of several types of proteins including cytokines (Sen and Baltimore, 1986; Li and Verma, 2002). This biochemical pathway is now well known to be modulated by antioxidants, such as intracellular reduced glutathione (GSH), ascorbic acid, GSH peroxidase and catalase and, as a consequence, too low O_3 concentrations generate very low levels of H_2O_2 unable to activate NF–kB (Sen and Packer, 1996). On the other hand, too high O_3 concentrations produce high levels of H_2O_2 that, by overwhelming intracellular antioxidants, lead to cell damage. On the basis of our previous results using fresh blood (Bocci et al., 1993a, 1998a,b), we postulated an activation on a range of concentrations between 1 and 20 μg/ml but, because of the low TAS capacity of the serum, only the lowest ozone concentration (1.0 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 μg/ml) may act as positive signals, while concentrations higher than 1 μg/ml can be cytotoxic. This result is indeed what we have seen either after direct ozonation of the cell suspension or by adding, after a delay of 20 min, the ozonated serum to PBMC.

Previous experiments using the direct ozonation of blood has yielded a small but constant increase of TNF-α, IFN-γ and IL-8 (Bocci et al., 1993a, 1998a,b) that allowed us to conclude that ozone could behave as a week inducer of cytokine production, albeit at a far lower level in comparison to typical mitogens. It is now clear that a direct ozonation of blood implies a minor risk of cytooxidation due to the potent antioxidant capacity of fresh human plasma and the enormous number of erythrocytes that, on one hand, can mop up great amounts of oxidants and, on the other hand, can promptly reduce and cycle back dehydroascorbate to ascorbate as well as GSSG to GSH (May et al., 1996). This is what happens during ozonation of blood using

the therapeutic range of ozone concentrations between 10 and 60 μg/ml per ml of blood (Bocci, 2002). Thus, although the present results do not allow a comparison, they are still instructive because they have shown: firstly, that blood samples from human healthy donors exposed to ozone yield great variability of cytokine titres. Present results are the mean of 12 donors but three of these did not respond at all. This fact is relatively known and indeed, as far as interferon is concerned, donors have been distinguished between low and high responders (Bocci, 1981). Regarding this phenomenon, it is unclear which is the role of genetics, of low or high antioxidant capacity of plasmas, or of previously unnoticed infections. The dishomogeneity in cytokine production must be kept in mind because in practice immunosuppressed patients may not be responsive to ozone therapy.

Secondly, we noted that even the exposure of the cell suspension to hyperoxygenation slightly, but consistently, depressed cytokine production. In the present experimental conditions only the lowest ozone concentration (1.0 μg/ml) seems to slightly stimulate IFN-γ and hardly TNF-α and IL-10 production.

The third observation is that, beside the slight stimulatory effect of 1.0 μg/ml ozone concentration, there is an ozone dose-dependent inhibition of cytokine release that fits with the concept that a progressively increasing ozone dose can block the protein synthesis in spite of only a modest inhibition of the PI (Fig. 2).

In retrospect a limitation of the present experiments has been caused by the very low antioxidant capacity of the human commercial serum. The difference with blood is important and can explain why, at variance with previous results (Bocci and Paulesu, 1990; Bocci et al., 1993a,b, 1994, 1998a,b; Paulesu et al., 1991), we found a decrease of cell proliferation, already after 24 incubation, with ozone concentration of 20 μg/ml (Fig. 2).

Owing to the fact that human serum contains hydro- and liposoluble antioxidants, growth factors and particularly albumin notoriously containing –SH groups, a 60% difference in this antioxidant capacity may well increase the toxicity due to either direct oxidation or adduct formation with relevant proteins or enzyme (Larini and Bocci, 2004). Another possibly negative factor is that incubation is always carried out at 37 °C in air–5% CO_2, so that the pO_2 in the incubation is equivalent to 147 instead of the physiological 100 mm Hg. However, a higher oxygen tension may not as detrimental as the lack of serum or of antioxidants.

It is unfortunate that some cell biologists overlook this parameter that we have come to consider crucial by observing the damage procured by ozone on washed erythrocytes simply resuspended in saline (Goldstein and Balchum, 1967; Freeman et al., 1979; Freeman and Mudd, 1981; Fukunaga et al., 1999). On the contrary, it is now well proven that serum or plasma are highly protective and although these remarks were made by us (Naldini et al., 1993, 1995, 1997) and Leist et al. (1996) several years ago, it has remained unnoticed. In line with this comment, Halliwell (2003) has very recently reviewed this problem and he also concluded that the often unphysiological conditions used for cell culture studies likely lead to erroneous conclusions.

A final remark regards the interesting phenomenon defined with the term "hormesis" described by several authors (Olivieri et al., 1984; Wolff, 1996; Goldman, 1996; Calabrese and Baldwin, 2001). With the benefit of hindsight, it appears obvious that we should have explored the effect of ozone concentrations lower than 1.0 μg/ml, to determine whether ozone can display more significant stimulatory effects than the one observed in the present study. We are now planning to perform such study that, at the same time, must take into account an ample range of antioxidant concentrations.

Acknowledgments

This work was partly supported by PAR fundings to Velio Bocci. We are grateful to Mrs. Helen Carter for revising and editing the manuscript.

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