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There are several reasons explaining why autohaemotherapy after ozone treatment has remained in a scientific limbo: firstly, the biological basis have remained nebulous and have been barely studied [1,2], partly because of lack of financial support by the pharmaceutical industry, secondly the therapeutic schemes have been elaborated on personal bias rather than on the evaluation of objective parameters [3,4], thirdly clinical experience, although vast, had remained limited to private practice with the consequence that results have been reported on a anecdotal fashion and never published in peer-reviewed journals [1,3-8]; even worse, a few quacks have completely discredited ozonetherapy by claiming wonderful results and exploiting patients. Fourthly, ozone has often been used unproperly without knowing exactly its physicochemical properties [9] and fifthly, the awareness that ozone is one of tile worst pollutant and can generate formation of oxidizing compounds [10-14] has comprehensibly originated a strong prejudice for its use. Thus, it is not surprising that the use of ozone for medical purposes is only allowed in Germany, Austria, Switzerland, Italy, Russia, Cuba and in a few States of USA. England and France, although widely using ozone for the treatment of drinking water, refrain from using it in Medicine. This involutive state of affairs has now undergone a critical reappraisal by considering that:

a) of the various routes of ozone administration, the exposure of a volume of blood to a precise ozone dose offers and meaningful and reproducible delivery system.
b) Ozone is now considered a citokine inducer with a definite benefit/risk ratio. As a consequence, autohaemotherapy may favour immunorestoration in secondary cellular immune deficiencies.
c) Blood cellular components display such a marked functional heterogeneity that under ozone action quite different biological responses have to be expected. This does not exclude however, that therapeutic activity in each disease has a multifactorial origin.

Routes of ozone administration in different pathological conditions.

The solubility of ozone in water, is about 50% higher than oxygen and it follows Henry's law [1,15]. However when ozone dissolves in the plasma, Henry's law is no longer valid because ozone decomposes in about one second and generates a cascade of unstable and highly reactive oxygen intermediates (ROI) among which are aldehydes, ozonides, hydrogen peroxide and lipid hydroperoxides that almost instantaneously react with membranous and cytoplasmic components [1,14-17]. On the other hand, cells and body

fluids contain a wealth of antioxidant compounds such as vitamins, low molecular weight compounds and proteins [18] as well as a number of enzymes able to either scavenge ROI or to rapidly regenerate reducing compounds. Both parenteral (intravenous, intra-arterial, intramuscular, subcutaneous and intra-articular) and local (nasal, tubal, oral, vaginal, vesical, colorectal and cutaneous) routes have been used [1,3] for the treatment of a number of pathological states with ozone (Table 1 [19-26]). One is surprised to note that, with the exception of the intravenous route, only minor side-effects have been observed probably because the potential toxicity of ozone is largely quenched by the body antioxidant reservoir and because the initial irritating (burning feeling) action of ozone in tissues is within few minutes followed by analgesia.

Use of the intravenous administration route is extremely dangerous because even if the gaseous mixture of oxygen-ozone is administered very slowly with a pump, it frequently procures lung embolization and serious side effects, particularly when daily dosing is up to 120 ml. Moreover, to the best of my knowledge, this procedure does not yield clinical benefit and no data have been published.

Jacobs [27] has analysed the type of accidents and side-effects after ozonetherapy in Germany in a total of 5,579,238 applications performed in 384,775 patients up to 1982. Although the percentage of accident due to ozone was 0.0007 %, it nonetheless included four fatalities most likely due to lung embolization while the remaining were minor accidents due to technical mistakes. During the last decade the administration of ozone via the intravenous route has been prohibited in Europe but regrettably someone still uses it in USA.

Intraarterial administration of 02-03, useful for treating chronic limb ischemia, is far less risky probably because only a small volume of gas is injected and because there is rapid gas solubilization and absorption at the capillary level [3,4,6,8].

Administration of up to 150 ml of 02-03, via intramuscular and subcutaneous routes is painful for a few minutes, and although therapeutic efficacy is claimed to be good, convincing clinical data are lacking [3]. My prejudice against these routes, particularly when used for immunomodulatory purposes, is that ozone dosing is totally empirical because no stoichiometric relationship can be defined between the ozone dose and the unknown volume of blood exposed in an internal cavity or tissue.

Of the local treatments, the cutaneous one (for torpid ulcers or necrotic lesions due to vascular limb disorders), is free of side effects and effective particularly when combined to autohaemotherapy [3,6,8,15].

Colorectal insufflation over a period of one minute of up to 800 ml of 02-03 (ozone concentration 20 ug/ml) is reported to be free of side-effects and effective in AIDS patients with intractable diarrhoea and in hepatitis patients [28-30]. This route ought to be borne in mind because the procedure of execution is simple, it can be done by the patient

at home and it is a practical alternative when there is not a venous access. However, so far neither endpoints are available for establishing an optimal dosage for this route, nor do we know how ozone insufflated into the colon lumen works. While there will occur an improved oxygenation of portal and peripheral blood [29] and possibly of intestinal lymph, we can only speculate on the activation of the colon- associated lymphoid system. It remains also unknown whether the bactericidal activity of ozone enhances absorption of bacterial compounds, similar to muramyl/ peptides, with immunoenhancing properties.

In conclusion, all of these routes have the disadvantage that ozone dosing has to be defined on a trial and error basis which is difficult and time-consuming to assess. Thus, at present time, ozonated autohaemotherapy, first described by Wehrli and Steinbart [31], represents the best option because it implies an almost stoichiometric relationship between a known dose of ozone versus a volume of blood measured by weight (about 105 g correspond to 100 ml) and because we can correlate several biochemical and immunological parameters with ozone dosing. These are crucial advantages that associated with the simplicity of the procedure, portray ozonated autohaemotherapy as a very important therapeutic possibility. After the discovery [32] that endothelial and other cells synthesize NO, that is an inorganic free radical gas and use it as a mediator and immune modulator, we ought to examine without any bias the biological activity of ozone.

Induction of cytokines from blood mononuclear cells (BMC) during ozonation.

Autohaemotherapy represents an almost ideal model because there is a reasonable stoichiometric relationship between ozone and blood. However, even in this case, among blood samples there are unavoidable uncertainties such as a variable amount of antioxidant compounds [18,33], of intracellular reducing enzymes [33,34], as well as quantitative and qualitative differences in cell components. As we have found [35,36] already great variability among normal donors, we expect an even greater variability among patients and for this reason, rather than suggesting an optimal ozone concentration (around 70 ug/ml ozone per ml of blood at normal barometric pressure), we propose that effective ozone concentrations range between 50 and 80 ug/ml without any evident toxicity when erythrocyte counts range between 3.5-5.0 x 10(6)/mm(3). Within these concentrations there is no formation of metahaemoglobin, either haemolysis or intraerythrocylic reduced glutathione levels remain below 3.0 or 10%, respectively [35,36], cell viability is normal and no morphologic cell damage is evident by electron microscopic analysis (Bocci et al., manuscript in preparation).

The new result which has confirmed the hypothesis that ozone can act as a cytokine's inducer [37] is that blood, after being exposed for a few minutes to concentrations of ozone ranging from 10 up to 90 ug/ml (per ml of blood) at normal barometric pressure, upon the usual incubation in air/CO, (95/5%) for up to 72 hours, progressively releases small amounts of cytokines such as interferon (IFN) B and y, tumor necrosis factor

(TNFx), interleukins (IL) 1B, 2, 4, 6, 8 and 10, granulocyte-macrophage, colony-stimulating factor (GM-CSF) and activated transforming growth factor (TGF) B1 [35,36,38-40]. IFNx has been barely detectable and other cytokines will be measured as soon as we have suitable reagents. At least for IFNs B/y and TNFx there is correspondence between biological and immunoenzymatic activities. In spite of great variability among normal blood samples, that is a normal fact due to the existence of either low or high responders [41], there is an ozone dose/cytokine response effect that tends to level off when ozone concentration reaches the 70 ug/ml mark. At the 105 ug/ml level, that is the maximum ozone concentration delivered by the generator, we have often noted a depressed cytokine production suggesting a possible cytotoxic effect [40]. Cytokine production is markedly depressed when blood is collected in the usual Ca++ chelating solution (citrate-phosphate-dextrose) and incubated in the absence of extracellular Ca.++ This finding led us to test the physiological anticoagulant, i.e., heparin (25 U/ml of blood) additioned with CaCl2, to a final concentration of 5mM [35]. Thus, a five-fold surplus of extracellular Ca++ (physiological Ca++ level is about 1 mM) displays a superinducing effect [2] but it must be noted that further Ca++ addition up to 50 mM, although modestly increasing cytokine's production, yields a prohibitive haemolysis [35]. However, heparin occasionally presents the problem of excessive anticoagulation as it has recently occurred in two hepatitis patients leading us to re-evaluate whether CPD treated blood, once heparinized and recalcified at physiological levels, is sufficiently activated. Data to be reported soon have shown that, after Ca++ addition, the production of cytokine is partially restored whereas blood sample incubated without Ca++ are consistently inhibited. These results have practical importance because they indicate that either CPD- or heparin-treated blood after ozonation and reinfusion will release similar amounts of cytokines in vivo. As a rule now, for patients under anticoagulant therapy, or aspirin, or prone to the haemorrhagic syndrome, or thrombocytopenia or hepatic dysfunction, we collect blood in CPD only thus avoiding any risk of dyscoagulation.

How ozone acts at the cellular level remains uncertain and for the time being, it is a matter of speculation. Probably ozone may oxidize unspecifically some carbohydrates, probably galactose [42,43], present on the cell membrane lectins leading to a coupling with transducer proteins and to an enhanced Ca++ influx. Moreover ROI can passively diffuse through the cell membrane and activate the gene-regulatory Nuclear Factor-Kappa B (NF-Kappa B) that appears to play many roles in the immune cells and certainly causes gene activation for several cytokines [44,45]. At the present our working hypothesis is that ozone acts unspecifically: the bulk of generated ROI is consumed by antioxidant substances in plasma and by the huge amounts of phospholipids present in erythrocytes because their membranous surface is as large as 70 m(2) per 100 ml of blood. As there is about 1 BMC every 3000 erythrocytes, there should occur a transient overproduction of ROI, for them to reach the trans-activating factor in the cytoplams and release the inhibitory subunit I Kappa B. Schreck et al [44] have indicated a threshold of about 30 uM of hydrogen peroxide to be effective in their system, implying that if ROI are not homogenously distributed, BMC will not be activated. We do not envisage and actually we do not notice cell damage because intracellular catalase, superoxide dismutase as well as other reducing enzymes and chainbraking compounds [14,17,18,33],

are capable of quickly neutralizing or scavenging residual ROI terminating the ozone action. In conclusion it would seem that ozone dosing is critical in the sense that if it is too low it is probably ineffective while if it is excessive, it may induce oxidative stress and eventually cell apoptosis [46].

Another interesting characteristic of this approach is that blood, after being thouroughly exposed to O2/O3 ex vivo for 5 minutes can be reinfused in the donor without practically any trace of ozone. Only the pO2 level, from a baseline value of 33-40 mmHg reaches in a few minutes a plateau level of about 400 presumably returning to the original value after gas exchange in the capillaries. Lipid hydroperoxide levels in plasma increase about 3-fold immediately after ozonation and return to baseline values within 3-4 hours when blood is incubated in vitro. However it is most important to note that in vivo, even 5 min after reinfusion, peroxides in the plasma remain at baseline value.

Both CPD- and heparin-treated blood are occasionally mixed to minimize erythrocyte sedimentation and reinfused fairly rapidly without any vascular or respiratory distress. In order to stabilize the plasma levels of antioxidants and to make sure that patients receive a normal vitaminic support we have always described a daily multivitamin (including vitamin C and E) supplement.

Mechanisms of action of ozonated autohaemotherapy.

Until recently it was thought that in chronic viral diseases, the virucidal properties of ozone were of paramount importance [1,3,7,21,47-49] for the therapeutic effect, neglecting the fact that the viral reservoirs are the internal organs (liver for hepatitis, lymph nodes and spleen for HIV infection, neuronal ganglia for herpes viruses etc) and that less viral particles are free in the plasma, implying a minimal direct effect during exposure of a small aliquot of plasma to ozone [50]. Moreover it was reported that after ozonation, leukocytes improved their phagocytic activity and that immunoglobulin levels could increase without underlying the causes of these biological effects [51]. The breakthrough has come with the demonstration that ozone acts as an inducer of cytokine's production [35,36,38-40]. Since then the approach of ozonized autohaemotherapy has gained a rational basis and one can understand why synthesis of antibodies can be stimulated by IL-6. enhanced phagocytic functions and leukocytosis can be due to IL-8 and GM-CSF and how both direct and indirect antiviral activities can be stimulated by IFNB, y and TNFx. In viral diseases, however, it cannot be excluded that small amounts of free virus inactivated in the plasma during ozonization may act either as an endogenous immunogen or/and an activator of cell-mediated immunity. It is worth while mentioning that autohaemotherapy has been surprisingly used also for the treatment of autoimmune diseases such as rheumatoid arthritis [52] and it would be desirable to investigate whether particular ozone concentrations may enhance the release of inhibitory cytokines leading either to the suppression of autoreactive cytotoxic T cell clones or/and to the blocking of inflammatory cytokines by the release of either soluble receptors or/and cytokine antagonists. It is possible that the release of IL-lO [53] and

TGFB1[54,55] can serve the useful purpose of quenching an excessive immune stimulation, thus leading to an orderly reprogramming of immune responses.

Distribution and fate of ozunatcd blood cells after reinfusion.

With the exception of the most aged erythrocytes that, being more susceptible to ozone,,re likely to be taken up by the reticulo-endothelial system [56],it is reasonable to speculate that most erythrocytes will continue to circulate in the vascular system. On the other hand, activated BMC may home in various lymphoid and non-lymphoid organs and we plan to verify this possibility with Indium-III labelled cells as soon as possible. If this happens, as we have shown during in vitro incubation [35,36], BMC will release around the pericellular environment various cytokines which can bind to the appropriate receptors on neighbouring stationary or in transit cells. In comparison to classical mitogens, which can easily activate 10-20% of the isolated BMC causing the synthesis of large amounts of cytokines, depending upon the fairly critical ozone dosing, a smaller percentage of BMC appears activated so that the minute amount of released cytokines is consumed in cellular microenvironments and does not emerge in the general circulation via the lymphatic system. This tentative explanation is supported by two pieces of evidence: firstly, we have never detected any significant change in cytokine's levels in the plasma of healthy volunteers at 1, 3, 6, 9 and 24 hours after reinfusion of ozonated blood [57]. In contrast, after injection of as little as 4 ng/Kg body weight of endotoxin in patients, there is a massive release of pyrogenic cytokines with IL-1, IL-6 and TNFx peaking a few hours after endotoxin administration [58]. The second point is that although autohaemotherapy does not allow the release of endogenous cytokines in the circulation, which is an important advantage. it has a real biological effect because 48-72 hours after reinfusion we have measured [57] in BMC an increase of the Mx protein that is one of the best indicators of IFN release [59]. The progressive enlargement of the area under curve of Mx protein versus time throughout autohaemotherapeutic treatments suggests that in vivo there occurs a progressive amplification of the priming and IFN production [57]. Thus, ozonated autohaemotherapy may be assimilated to a slightly enhanced physiological response [41,60] due to a gaseous inducer which acts rapidly and disappears. Further support to this interpretation is that typical side effects such as chills, fever, fatigue and nausea never occur after autohaemotherapy and actually the patients often report a sense of well-being and euphoria that may be due to improved oxygenation or/and release of hormonal factors not yet identified. As far as the number of treatments to be carried out is concerned, one treatment only can be hardly effective as the number of ozonated BMC present in 300 mi of blood is probably less than 0.1% of the total BMC mass [61]. If this calculation is correct, only the prolonged repetition of autohaemotherapy (two treatments weekly for several months) can allow a progressively amplified activation of the immune system via numerous pathways such as activation of either major histocompatibility complex-restricted cytolysis, or unspecific killing and removal of viral-infected and metastatic cells. Table I reports a list of chronic viral diseases or pathological situations accompanied by either immune deficiency or immune dysregulation, where ozonated autohaernotherapy has and could play a beneficial role.

Autohaemotherapy may prove to be very useful as an adjuvant treatment particularly if either chemotherapy or/and radiotherapy have been effective in reducing the tumor mass. Several immunotherapeutic approaches such as: a) therapy with monospecific or bispecific antibodies [62]; b) adoptive immunotherapy with more or less genetically engineered cells [63]; c) exogenous administration of either an array of immunostimulants [64] or/and d) of recombinant cytokines such as IFNs, IL-1, 2, 3, 6 and 12, TNFx and leukopoietins are being actively pursued. Approaches a to c are in a more or less advanced experimental phase while therapy with cytokines has dominated the scene in the last decade. Unfortunately pharmacological administration of cytokines, thatphysiologically are not present in the circulation, causes a substantial increase of their plasma levels which correlate well with fairly severe toxicity [65]. Clinical results in solid neoplasms have been far below expectations and the cost/benefit ratio is very high [66].

The variety of biological effects depends upon the heterogeneity of blood cells.

While immunomodulatory effects are due to BMC activation, the beneficial effect of autohaemotherapy in chronic limb ischemia, in cardiac, ophthalmologic and cerebral vasculopathies [3,4,6,8,22] in infections and in burns [1,3,4,6,8,15,19,20] can be explained by improved transport of oxygen due either to increased oxygen availability, or oxygen delivery to hypoxic tissues due to an increase of 2-3 diphosphoglycerate in erythrocytes. The rapid healing of torpid ulcers in ischemic disorders is becoming particularly interesting since the demonstration that transforming growth factors (TGF) B1 accelerates the healing process [67-69] and that levels of activated TGF B1 increase substantially after ozonation of blood, probably due to partial degranulation of platelets [40]. There is no doubt that improved oxygenation enhances cell metabolism and proliferation and that the local application of ozone inhibits bacterial infections but, until now, the crucial role of proteins such as TGF B1, vascular endothelial growth factor (VEGF) for enhancing neovascularization [70] and healing has not been taken into account. Thus it appears that clinical benefits possibly derive from the activation of erythrocyte function, the release of either TGF B1, other growth factors and by improved leukocytic functions in terms of removal of necrotic tissue and bactericidal activities.

Final remarks

It has been pointed out that ozonated autohaemotherapy performed with an optimized procedure represents a powerful therapeutic approach. Its main advantages are the lack of toxicity, often a feeling of well-being and the equilibrated, although slow, stimulation of cytokine production accompanied by improved oxygenation and metabolism. Both in the treatment of neoplasia, particularly after chemiotherapy, and of chronic viral diseases the frequent report of well-being after treatment is relevant because the quality of life of

these patients is generally poor. We are planning to investigate the reason of euphoria and we believe that it may be due to an immune-neuroendocrine response elicited by the ozonated blood. On clinical ground there is also the need to carry out extensive and well-controlled clinical trials in several diseases including HIV infection [50]. The treatment is simple to execute, safe, far less expensive that comparable procedures and could be carried out easily also in Third-World Countries where it could be applied also to several parasitic diseases.

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