

Autohaemotherapy After Treatment of Blood with Ozone. A Reappraisal

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Autohaemotherapy, involving bland treatment *ex vivo* of blood with ozone and prompt reinfusion into the donor, is a procedure mainly performed in central Europe, which is claimed to have therapeutic value in circulatory disorders, viral diseases and cancer. This practice is mostly performed in private clinics, and good clinical trials have not been published, which has understandably given rise to prejudice and scepticism. By analysing possible mechanisms of action and current hypotheses, this report attempts to explain how this procedure can be useful in such disparate diseases. The current state of the art is presented objectively, the lack of toxicity is documented, and the rationale and therapeutic advantages are discussed, with the aim of eliciting interest in carrying out controlled clinical trials.

KEY WORDS: AUTOHAEMOTHERAPY; ARTERIOPATHIES; OZONE; CYTOKINES; IMMUNOTHERAPY; VIROSIS; NEOPLASIA

INTRODUCTION

Major autohaemotherapy using about 100 ml of human blood treated with a gaseous mixture of oxygen and ozone has been used since the 1950s particularly in central Europe and, by 1980, no less than 240 000 treat-

ments had been performed without any untoward effects.¹ A variety of unrelated diseases such as acute and chronic viral diseases,²⁻⁴ neoplasia,⁵ vascular disorders such as obstructive arteriopathies, venous insuf-

iciency and vascular degenerative diseases,^{2,6,7} ulcers and cutaneous infections⁸ have been treated with apparently good results, but the mechanisms of action are uncertain, and clinical trials have been conducted in an uncontrolled fashion. This raises questions about how or why this procedure can benefit such heterogeneous pathologies and raises doubts about the claimed therapeutic efficacy. Another serious problem is the growing awareness that ozone is an extremely reactive and toxic gas,⁹ and is probably the worst pollutant of the troposphere.¹⁰ It is not surprising, therefore, that official medicine has always regarded this treatment with scepticism and lack of interest. Work carried out in this laboratory since 1988,¹¹⁻¹³ involving a comprehensive re-evaluation of the several facets of the problem,¹⁴ has stimulated a reappraisal that may be useful either for the critical advancement, or the rejection of the procedure. The aim of this review is to analyse possible mechanisms of action, to increase understanding of how the same procedure can be beneficial to different diseases and finally to encourage an objective clinical assessment before finally deciding whether the treatment is valid and worth pursuing.

HOW CAN AUTOHAEMO-THERAPY BE USEFUL IN MANY DIFFERENT DISEASES?

The key to understanding the value of this autohaemotherapy treatment in a variety of diseases lies in the heterogeneity of blood components (Table 1) and in the possibility that after oxygen/ozone exposure, different blood cells undergo different trophic, biochemical^{15,16} and immunological¹⁶ changes that have beneficial effects on unrelated diseases. It is unfortunate that basic research has been concentrated more on evaluating the damaging effects of ozone^{17,18} than on showing the metabolic changes after ozone exposure that may result in therapeutic efficacy. Both proven and hypothetical mechanisms of action are discussed below for each of the various blood components.

THE ROLE OF ERYTHROCYTES

Erythrocytes are probably ozone's main target because the erythrocytes present in 100 ml of blood expose an approximate surface area of 70 m². Ozone decomposes in a matter of seconds and generates reactive species that are firstly in part quenched by antioxidant compounds present in plasma (Tables 1

Blood components affected by ozone treatment

Percentage of blood volume	Blood component	No. in 1 mm ³ of blood
55	Plasma (a reservoir of antioxidant components)	
45	Erythrocytes	~5 000 000
	Neutrophils, basophils and eosinophils	~4500
	Lymphocytes	~1200
	Monocytes	~300
	Platelets	~300 000

and 2),¹⁰ secondly, or simultaneously, act by oxidizing cell membrane phospholipids, glycolipids and glycoproteins,²⁰ and thirdly, may act by inactivating intracellular components such as enzymes and DNA after exhausting the reserve of intracellular reduced glutathione. Thus the deleterious effects of ozone largely depend on the dynamic equilibrium between ozone concentration, duration of exposure and extra-intracellular antioxidants. Only by using low concentrations for a very short time can the ozone attack be limited to the cell membrane where it exerts its non-specific action.

Oxidant effects can now be estimated by several assays but the most readily available and simple criterion is the extent of haemolysis. A range of ozone concentrations between 5-75 µg/ml of normal blood provokes a progressive increase of haemolysis between either 1.2 and 3.1% or 1.5 and 3.4%, compared with controls treated with normobaric air, respectively, depending upon whether the anticoagulant used is citrate-phosphate-dextrose or heparin (25 IU/ml).¹⁹ It is likely that erythrocytes that are 100 days old or more (about 20% of the mass) become the most susceptible to the oxidative action of ozone, which may accelerate their disappearance from the circula-

tion.²¹ Obviously, it is essential to use the ozone concentration that, while improving the rheology and biochemical characteristics of erythrocytes, causes minimal damage to them. Should any amount of haemoglobin become free, it would readily bind to haptoglobin, thus minimizing the generation of harmful hydroxyl radicals, promoters of cell damage and generators of chain reactions.²²

There are several mechanisms of action (Table 3) through which ozonized blood can improve the circulation and oxygenation of hypoxic tissues. It is not yet clear whether the increased amount of 2, 3-diphosphoglycerate reported either after microaerotherapy,² or after insufflation of ozone into the colon²³⁻²⁵ remains at higher levels than normal for the remaining cell life-span. A permanently higher content of 2, 3-diphosphoglycerate in successive cohorts of erythrocytes undergoing the usual bi-weekly procedure may significantly improve oxygen availability to hypoxic tissues, thus rationalizing this approach in ischaemic arteriopathies.

Another useful aspect is represented by the improved rheology of erythrocytes at the capillary level seemingly due to their improved flexibility and charge modification. An intriguing new development under study in this laboratory is the micro-

Antioxidants present in human plasma capable of preventing lipid peroxidation

Proteins	Small molecular weight chemicals	Vitamins and provitamins
Albumin	Glucose	Ascorbic acid
Haptoglobin/haemopexin	Uric acid	α-Tocopherol
Ceruloplasmin	Bilirubin	β-Carotene
Transferrin	Reduced glutathione	Lycopene
	Cysteine	
	Cysteamine	
	Taurine	

Possible mechanisms of action through which anti-coagulated blood blandly treated with a O_2/O_3 mixture can ameliorate ischaemic diseases

Mechanism

Improvement of blood rheology

Increased amount of 2,3-DPG in erythrocytes

Rightward shift of the HbO_2 dissociation curve with consequent improvement of O_2 delivery to hypoxic tissues

Release of ATP

Release of eicosanoids (prostaglandin E17)

Release of haemopoietins

Release of angiogenic and/or trophic factors

DPG, diphosphoglycerate; HbO_2 , oxygenated haemoglobin; ATP, adenosine triphosphate.

release of adenosine triphosphate (ATP) from ozonized erythrocytes.²⁶ Pharmacological infusion of ATP is known to cause hypotension,²⁷ depriving hypoxic tissues of an already critical blood supply. A microrelease of ATP in the ischaemic environment may, on the other hand, cause a local vasodilatation, thereby improving blood flow. At this stage other hypothetical, yet likely, agents are some of the eicosanoids, nitric oxide, angiogenic and/or growth factors from platelets and endothelial cells.

All of these possibilities may be relevant to the clinical responses seen after autohaemotherapy:^{26,27} it appears likely that a combination of these factors concurs in improving the circulation and oxygen delivery in the hypoxic microenvironment.

THE ROLE OF BLOOD MONONUCLEAR CELLS

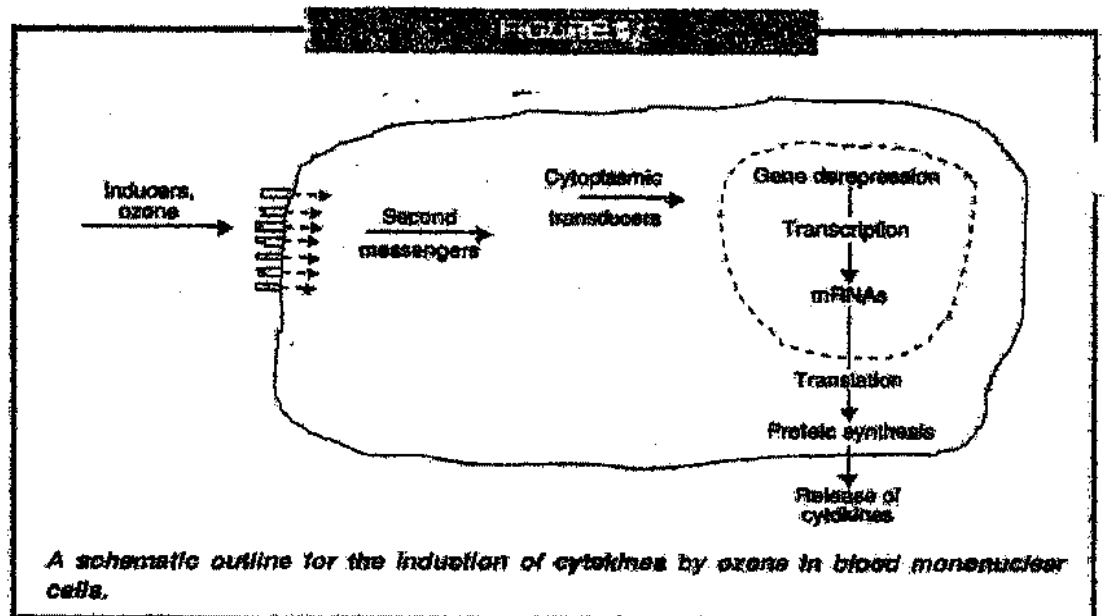
By far the most exciting development of the field has been to find that ozone, used in appropriate concentrations, can act as a mild cytokine inducer.¹⁴ Major autohaemotherapy was considered useful for the treatment of acute, chronic viral diseases (herpes,

hepatitis) and neoplasia, because it induced leukocytosis, and improved the phagocytic and bactericidal activity of leukocytes with concomitant enhancement of immunoglobulin production.¹⁶ It has also been postulated that ozone may both inactivate viruses in blood and accentuate the lysis of infected cells as these become poorly equipped to counteract the action of peroxides.²³ It is true that ozone expresses virucidal activity in plasma,²⁸⁻³⁰ but it is unrealistic to suppose that this may represent an important mechanism for the therapy of viral diseases. The biological effects just outlined seem to be the consequence of a more profound immunological activation. Indeed the breakthrough came with the discovery that ozone can activate monocytes and lymphocytes, and induce the production of an array of cytokines¹⁴ such as interleukin(IL)-1, IL-2, IL-6, interferon(IFN) α , IFN γ , granulocyte-macrophage colony stimulating factor (GM-CSF), tumour-necrosis factor (TNF α) and transforming growth factor (TGF β). Other cytokines will probably be detected as these studies continue. After the seminal reports by Novogrodsky *et al.*¹⁴ and Dianzani *et al.*¹²

A simplified list of cytokine inducers for blood mononuclear cells

Mitogens	Antigens	Antibodies	Proteinases	Interleukins	Oxidizing Agents	Ca ²⁺ Ionophores
PHA	Viruses	anti CD's, etc	Trypsin	Interleukin 1	Periodate	A23187
Con A	Endotoxins		Bromelain	Interleukin 2	Hydrogen peroxide	
PWM	Tumoral proteins		Thrombin	TNF α	Galactose oxidase	
SEB					Ozone	Ozone

PHA, Phytohaemagglutinin; Con A, Concanavalin A; PWM, Pokeweed Mitogen; SEB, Staphylococcal enterotoxin B; CD, Cluster of differentiation; TNF α , tumour necrosis factor; A23187, Ca²⁺ ionophore.



It was logical to advance the hypothesis that ozone, among other agents (Table 4), may act as a cytokine inducer (Fig. 1), triggering a number of immunological mechanisms that are crucial for clearing chronic viral infections and possibly neoplastic cells.

THE ROLE OF PLATELETS

At this stage it is not known if and how platelets react with ozone. Several types of study are warranted as platelets are likely to release several factors, among which TGF β has already been measured in plasma (Boc et

et al. manuscript in preparation) but could be partly of lymphocytic origin.

THE ROLE OF POLYMORPHONUCLEAR LEUKOCYTES

Besides the enhancement of phagocytosis mentioned above, it is not known whether ozone activates polymorphonuclear leukocytes directly, or indirectly via the release of cytokines, such as IFN γ , TNF α and IL-8, but it is certain that either neutrophils or eosinophils are able to release an array of cytokines such as IL-1 β ,²³ IL-3,²⁴ IL-6,²⁵ IL-8,²⁶ TNF α ,²⁷ and various types of CSFs²⁸ as well as TGF β .²⁹

THE ROLE OF PLASMA COMPONENTS

No information is available on the possible effect (activation or inactivation?) of ozone, particularly on lipoproteins and coagulation factors.

A number of investigations are needed to clarify the factors present in ozonized blood and thus assist in understanding, and possibly improving, this therapeutic approach.

THE OPTIMIZED PROCEDURE USED IN OUR LABORATORY

Minimizing blood cell damage during ozone treatment is of the utmost importance. As would be expected, there is a dose-effect relationship between ozone concentration, duration of exposure and the production of cytokines.^{12,16} The biological response to ozone treatment varies widely between blood samples, not only because blood mononuclear cells of different individuals vary in their ability to respond to certain stimuli, but also because each blood sample has a variable level of antioxidants. Attempts have, therefore, been made to improve the procedure currently used¹² as follows: for

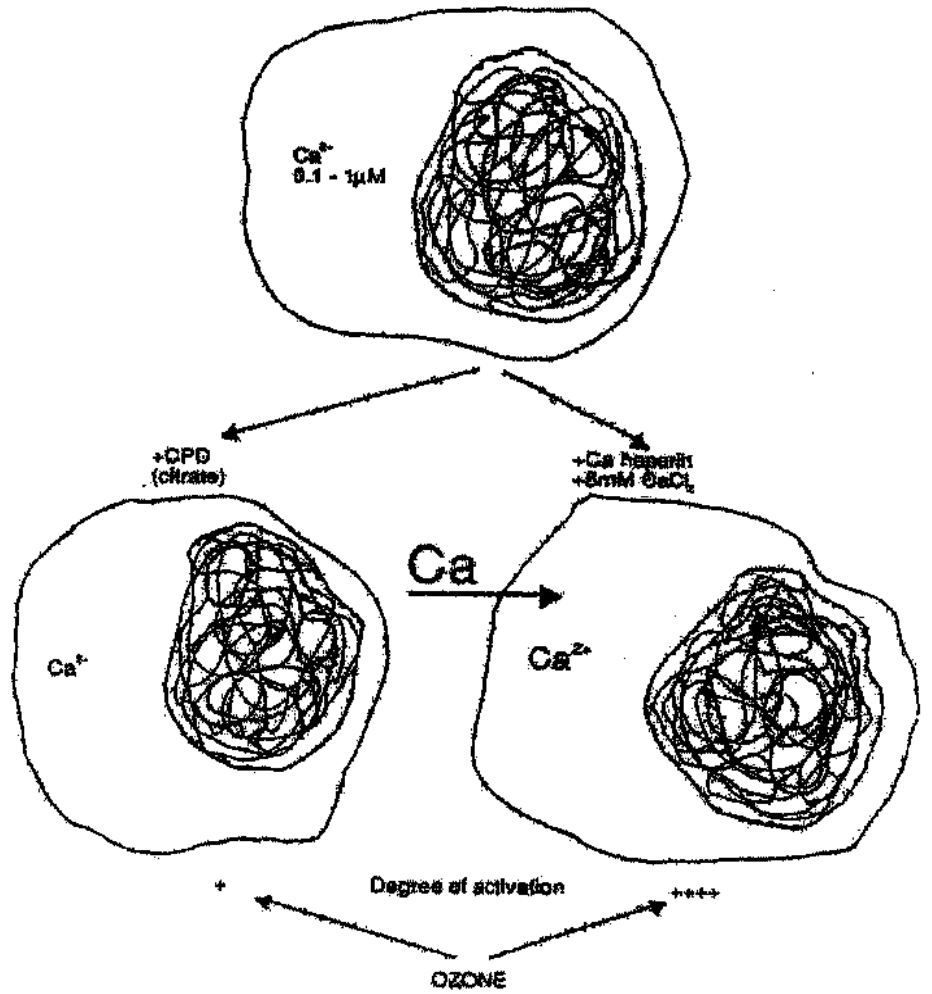
each autohaemotherapeutic treatment 250 ml of blood is collected in a bag containing 6250 IU of heparin (25 IU/ml of blood), plus 5 mM Ca²⁺ (CaCl₂) after total removal of citrate-phosphate-dextrose. The increase in extracellular Ca²⁺ greatly enhances cytokine production as recently reported in detail,¹⁹ and depicted schematically in Fig. 2. The involvement of Ca²⁺ in signal transduction events leading to T-cell lymphokine gene expression has been reviewed recently.³⁰

The oxygen/ozone gas mixture, containing a total of 14.75 mg of ozone (50 μ g O₃/ml of blood), is rapidly added with gentle mixing, after which the blood is slowly reinfused into the donor. Using this procedure, haemolysis does not exceed 2.8 \pm 0.7% and intraserythrocytic-reduced glutathione decreases no more than about 8%. In normal volunteers, 2 - 3 days after autohaemotherapy, there is a distinct increase in the expression of the Mx protein in circulating blood mononuclear cells, that is a specific marker of IFN production.^{19,21} This is not in contrast with the lack of modification of cytokine levels in plasma, a fact in itself explaining the excellent tolerability of the treatment. Subjects undergoing the treatment receive by mouth, daily, a multivitamin supplement (including vitamins E and C). Patients who are hypersensitive to heparin, under anticoagulant therapy, or prone to the haemorrhagic syndrome should be given autohaemotherapy simply using citrate-phosphate-dextrose.

IS OZONE A DOUBLE-EDGED SWORD AND DOES AUTOHAEMOTHERAPY CAUSE SIDE-EFFECTS?

These issues are important and results so far obtained need to be emphasized to convince clinicians that the haemotherapeutic treatment is absolutely safe.

Physiological calcium
(total 2.5 mM; ionized, 1.1 mM)



One possible mechanism of superinduction of blood mononuclear cells. CPD, citrate-phosphate-dextrose.

During the last 3 years both the role of ozone concentrations and the timing of exposures have been investigated by following four parameters, namely the extent of haemolysis, intracellular reduced glutathione,¹² blood mononuclear cell viability (tested by Moorman's method),¹³ and cytokine production.¹⁴ It has previously been shown that ozone expresses dualistic effects, at high concentrations, impairing the cellular and humoral immune response in animals and subjects undergoing chronic exposure.¹⁵⁻¹⁸ Indeed, when high concentrations (above 78 µg/ml) of ozone were used, and particularly when blood was exposed to a constant insufflation of ozone for periods longer than 30 sec, there was a progressive increase in haemolysis up to 52%, a decrease of intracellular reduced glutathione by as much as 47%, significantly reduced blood mononuclear cell viability and erratic production of cytokines.¹⁹ On the other hand, in practice, when the ozone contact with the blood is for a few seconds and the ozone concentration is lower than 78 µg/ml of blood, haemolysis is no higher than 8%, intracellular reduced glutathione decreases by only 8.3%, blood mononuclear cell viability is not impaired and there is significant production of cytokines. These results must be so first, by virtue of the relevant antioxidant properties of plasma and second, because all metabolically active cells display antioxidant mechanisms by means of several enzymatic systems such as catalase, superoxide dismutase and glutathione reductase. The efficiency of glutathione homeostasis is impressive: within about 30 min after ozone treatment, intracellular reduced glutathione levels are almost completely restored.²⁰⁻²¹ It was previously shown that glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are the two key enzymes that, by metabolizing glucose to ribulose-5-phosphate, generate

reduced nicotinamide dinucleotide phosphate, the fundamental substrate for the glutathione redox cycle.^{22,23} Considering the enormous surface area exposed by erythrocytes, it appears that ozone action is likely to be dispersed on an almost infinite number of targets on the plasma membrane, hardly reaching the cytoplasm, as documented by the negligible and transient reduction of intracellular reduced glutathione.

It appears that the potential toxicity of ozone should not prevent its use if a judicious concentration is used and because blood can minimize the formation of free radicals¹⁹ and convert oxidants to less toxic species. At the correct dose, ozone, like any other drug, can do more good than harm and in the biomedical sciences, the concept that even a poison may represent a useful drug at a particular concentration, is a familiar one. Further, a revision of the, so far, dogmatic conclusion that oxidants are always deleterious, is in progress; indeed, the physiological activity of nitric oxide is a paradigmatic example²⁴ and the production at low levels of highly reactive species may serve important roles in cell proliferation, defence and regulation of the immune system.

Experience in normal volunteers and in patients, suggests that autohaemotherapy does not cause any side-effects and gives a sense of well-being in about 20% of subjects. Whether this is a placebo effect, or a result of the suspected release of certain hormones, remains to be determined. There is no risk of cross-infections because each donor must receive his/her own blood and the procedure is safe, simple and easily performed in about an hour, after which the patient can return home. Transfusion-related acute lung injury, a fairly rare complication of allogeneic transfusion,²⁵ has never been reported after autohaemotherapy. The only problem, noticed by the present author, is that the majority of terminally ill patients, particularly after pro-

longed chemotherapy, have poor venous access, and this may occasionally complicate blood collection and reinfusion. Otherwise patients comply quite well with two-weekly treatments continued for 5 - 8 months. Usually treatment is carried out, here, either in the late morning or early afternoon for practical reasons and because, if treatment does cause an elevation of plasma cortisol, this should not markedly affect its nadir at night.

Although the cost of a treatment ought not to be an important criterion, in the case of autohaemotherapy it becomes irrelevant since the value of a collection bag is less than five pounds and therefore almost negligible compared with treatment with biological response modifiers such as IFNs, IL-2 and thymic hormones. On the other hand, the issue of effectiveness is of crucial importance and the existing data, although very encouraging, are too preliminary to support a full discussion. Published reports^{22,27} include claims that autohaemotherapy yields "satisfactory" results, but the reason for the present report has been to review the state of the art and show the urgent need for appropriate and controlled studies.

WHY IS AUTOHAEMOTHERAPY FREE OF SIDE EFFECTS?

Activation either *in vivo* or *ex vivo* of blood mononuclear cells with potent cytokine inducers, such as a polyribonucleic-polyribocytidylic complex,²⁴ or endotoxin,²⁵ or muramyl tripeptide,²⁶ invariably causes typical toxic effects characterized by chills, fever, dyspnoea, nausea, fatigue, hypotension, etc. usually within a few hours after administration. When the evaluation of the optimized procedure using Ca^{2+} as a superinducing factor,²⁴ was started, the lack of typical toxicity was at first puzzling. On the basis of *in vitro* studies, however, it was soon

realized that in order to minimize cell damage, very low ozone concentrations were used, causing a very mild activation of blood mononuclear cells, as deduced by the consistent but low production of cytokines. Moreover, the volume of blood used for each treatment is 250 ml and this represents about 1/20th of the blood volume, that is, 0.1% of the total number of lymphocytes, assuming that only 2% of the lymphocyte mass in the body are present in the blood at any given time.²⁸ If this estimate is correct, it means that only a minimal fraction of blood mononuclear cells undergo stimulation during each treatment. Depending upon their recirculation pattern, the ozonized immune cells (at the most, 2.5×10^6) will rapidly home into various lymphoid and non-lymphoid microenvironments and there will begin to release a number of cytokines. These will react with neighbouring cells, either priming or activating them, with consequent amplification of the primary stimulus (the ozonation of the immune system) and minimal spill-over of released cytokines, if any, into the circulation, thus explaining the lack of fever or other toxic effects. In conclusion, autohaemotherapy will have a mild, yet progressive immunoadjuvant effect, mostly due to cellular interactions very much resembling the physiological process of maintaining the immune system in an active state.²⁷ On the basis of this interpretation, autohaemotherapy will result in a slow process of activation, with potential therapeutic efficiency and without side-effects.

CURRENT USES AND FUTURE PROSPECTS

Autohaemotherapy appears to be a versatile therapeutic approach acting in various different diseases by virtue of the effects of ozone treatment on several blood components, which, after treatment, induce

metabolic changes and release various cytokines. The following four main categories of diseases may benefit from the treatment:

1. *Vascular disorders* from critical limb to heart, brain and retinal ischaemia.
2. *Chronic viral diseases* favoured by immunodepression either due to genetic deficiency, or cytotoxic treatments and/or ageing. All of these situations share either a reduced number of total T lymphocytes (CD3), or of cytotoxic T lymphocytes (CD8), or of natural killer cells, or of neutrophils with depressed cytolytic activity and phagocytosis. Production of thymic hormones, of lymphokines and antibody formation may also be depressed to some extent. Autohaemotherapy may represent an almost physiological stimulation for rejuvenating or reprogramming the immune system.
3. *Autoimmune diseases*. How autohaemotherapy can be beneficial in autoimmune diseases such as rheumatoid arthritis remains a matter of speculation. At the moment it is considered that local release of either IFN γ causing apoptosis of auto-cytotoxic T lymphocytes, or of immunosuppressive factors such as IL-10, TGF β , IL-1 β -TNF α antagonists and eicosanoids may suppress reactive clones.
4. *Minimal residual disease*. As cancer kills the host mostly via metastasizing cells, it is necessary to eliminate them and immunotherapy is now considered the fourth modality of therapy, after surgery, radiation and chemotherapy. To this end several approaches are being actively pursued, such as:
 - (a) exogenous administration of cytokines and thymic hormones;²⁰
 - (b) adoptive immunotherapy with autologous lymphokine-activated killer cells or tumour-infiltrating lymphocytes with exogenous IL-2, or cells cloned with human genes coding for TNF α or

IL-2;²¹ and

(c) therapy with monospecific or bispecific antibodies.²²

In this context autohaemotherapy is an approach based on using inducers able to elicit endogenous production of cytokines. The advantages of autohaemotherapy are its lack of toxicity and the resulting equilibrated, although slow, stimulation of cytokine production, accompanied by improved oxygenation and metabolism.

There are other approaches involving treatments with oxygen/ozone mixtures either *in vivo* or *ex vivo*.²³ *In vivo* treatments include either intravenous, or intraarterial (particularly in critical limb ischaemia) administration of a gaseous oxygen/ozone mixture, or of ozone-saturated isotonic solution. Colorectal insufflation of oxygen/ozone (up to 800 ml) has been used, mainly in Germany, to treat colon cancer, and to treat intractable diarrhoea in AIDS patients in the USA.

Ex vivo treatment could be intensified either by carrying out autohaemotherapy very frequently (it has been repeated three times in the same session), or by extracorporeal circulation of the whole blood volume against oxygen/ozone. Finally, ozonation of erythrocytes may be avoided by specifically collecting large numbers of autologous leukocytes via several procedures. These, unfortunately, are somewhat lengthy with a potential risk of contamination and at this stage are not being pursued.

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