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ANTI-INFLAMMATORY ACTIVITY OF SUPEROXIDE DISMUTASES: COMPARISON OF ENZYMES FROM DIFFERENT SOURCES IN DIFFERENT MODELS IN RATS: MECHANISM OF ACTION

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Comparison of the anti-inflammatory properties of superoxide dismutases from different sources using different models (carrageenan and adriamycin induced inflammation, adjuvant-induced arthritis) in rats shows a very wide range of activity from extremely good to zero. Neither circulating life time nor intracellular penetration are of importance. The mechanism of biological activity of the SODs is discussed in detail, and binding to an interphase situation on the outer cell surface is postulated. As a consequence of these various considerations it is predicted that clinical application of human Cu-SOD in humans may well be much less spectacular than is commonly assumed, and indeed may be somewhat disappointing.

Key words: Anti-inflammatory activity; superoxide dismutases, mechanisms; carrageenan; adriamycin; adjuvant; arthritis

INTRODUCTION

"All superoxide dismutases are equal, but some are more equal than others".

This concept[1] has not previously been developed in any detail, but nevertheless merits considerable attention. Apart from an importance with respect to the fundamental problems associated with the mechanism of anti-inflammatory activity of bovine copper superoxide dismutase *in vivo*, financial aspects concerning tens of millions of

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dollars are also involved in a possible future use of human Cu-SOD (obtained by genetic engineering techniques) in humans. It may be recalled that no clinical studies of human Cu-SOD have been reported (at this time of writing) and indeed animal studies with this enzyme are limited to those of the present authors, despite the fact that this SOD is readily available from human erythrocytes (expired blood transfusion stocks), liver or placenta. Indeed, essentially all other animal studies have used bovine Cu-SOD and uniquely bovine Cu-SOD, particularly in studies of ischemia.

We have recently presented comparative studies of SODs from different sources with respect to biological activity in the carrageenan induced edema of the rat paw[2], in the rat adriamycin model[3] and in the adjuvant-induced arthritis model in Lewis Inbred rats[4]. Despite the fact that these SODs all had the same enzymic activity (and hence were equal) extremely pronounced differences in anti-inflammatory properties were observed. Other studies[5] have shown the clinical utility of bovine SOD as a depolymerising agent in cases of radio-induced fibroses, a biological activity which is associated, but independent of the anti-inflammatory properties.

RESULTS

The relative anti-inflammatory activities of 18 different SODs in the rat paw carrageenan model are resumed in Fig. 1. As previously reported[2], maximum activity is shown by *E. coli* Mn-SOD, bovine Cu-SOD shows good properties, human Cu-SOD is a poor anti-inflammatory agent and the homologous rat Cu-SOD has a pro-inflammatory action rather than anti-inflammatory. A continuous grading of activity from anti- to pro-inflammatory is seen over the range of different SODs studied and it is evident that different SODs do not have identical anti-inflammatory properties. This is confirmed in the rat adriamycin model[3] as shown in Fig. 2 (at 1 hr after injection) and Fig. 3 (at five days). With particular reference to the results at five days, both bovine Cu-SOD and *E. coli* Mn-SOD show very strong anti-inflammatory activity, human Cu-SOD is poor and the homologous rat Cu-SOD has zero properties. As in the carrageenan model, *E. coli* Fe-SOD is active whereas *P. leiognathi* Fe-SOD is not. Yeast Cu-SOD shows no activity at 5 days but is pro-inflammatory at the early phase.

The relatively poor properties of human Cu-SOD (and of human Mn-SOD) are also demonstrated in the adjuvant-induced arthritis model[4] as shown in Fig. 4 (plethysmometric changes) and Fig. 5 (arthritic index), Again the homologous rat Cu-SOD is inefficient, whereas *E. coli* Mn-SOD is a most impressive anti-arthritic drug. Bovine Cu-SOD has quite moderate activity but is very greatly improved by encapsulation in liposomes.

Thus, at least in these three models, and despite reports to the contrary[6] bovine Cu-SOD does possess a definite anti-inflammatory activity, though relatively moderate compared with *E. coli* Mn-SOD. In contrast, human Cu-SOD shows very poor properties and the homologous rat Cu-SOD (at the dose levels used) shows no significant activity whatsoever.

It may be noted that rats have an extremely high (248 ng/ml) level of circulating Cu-SOD[7]. This does not invalidate any of the three models of inflammation which we have studied. In any case, circulating exogenous SOD 1 hr after i.p. injection (i.e. at maximum level) is about 0.1%/ml of the total injected[8]. This corresponds to an increase of 10 ng Cu-SOD per ml i.e. a 4% augmentation, whether the SOD is

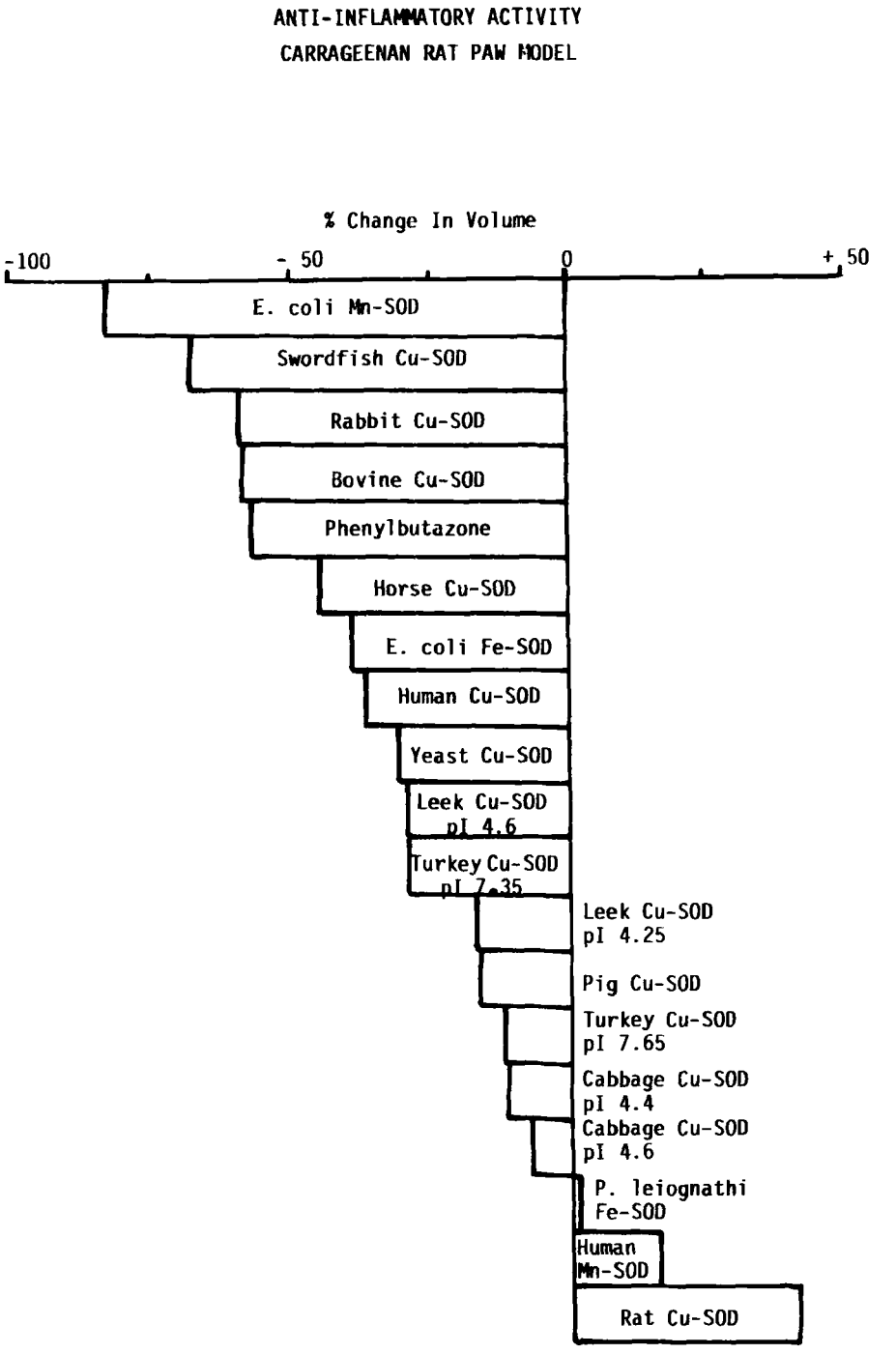

FIGURE 1

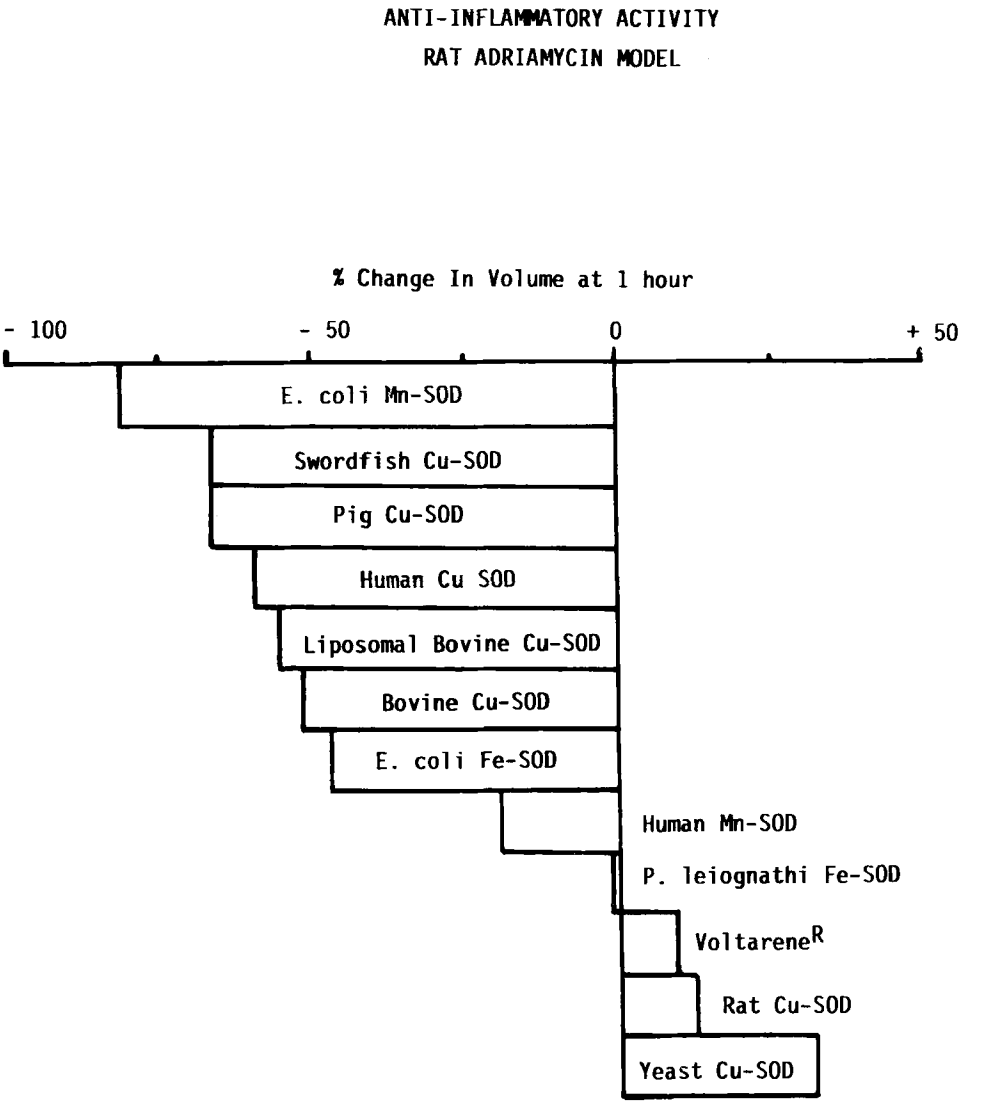

FIGURE 2

homologous rat Cu-SOD or a heterologous enzyme from other sources. It is unlikely that this increase of circulating SOD after administration (reduced to about 0.4% after 6 hr) can play a significant part in anti-inflammatory activity at least at the dose levels (33.3 μg/kg, approximately 10 μg/rat) we have found to be highly efficient for *E. coli* Mn-SOD, liposomal bovine Cu-SOD or other SODs.

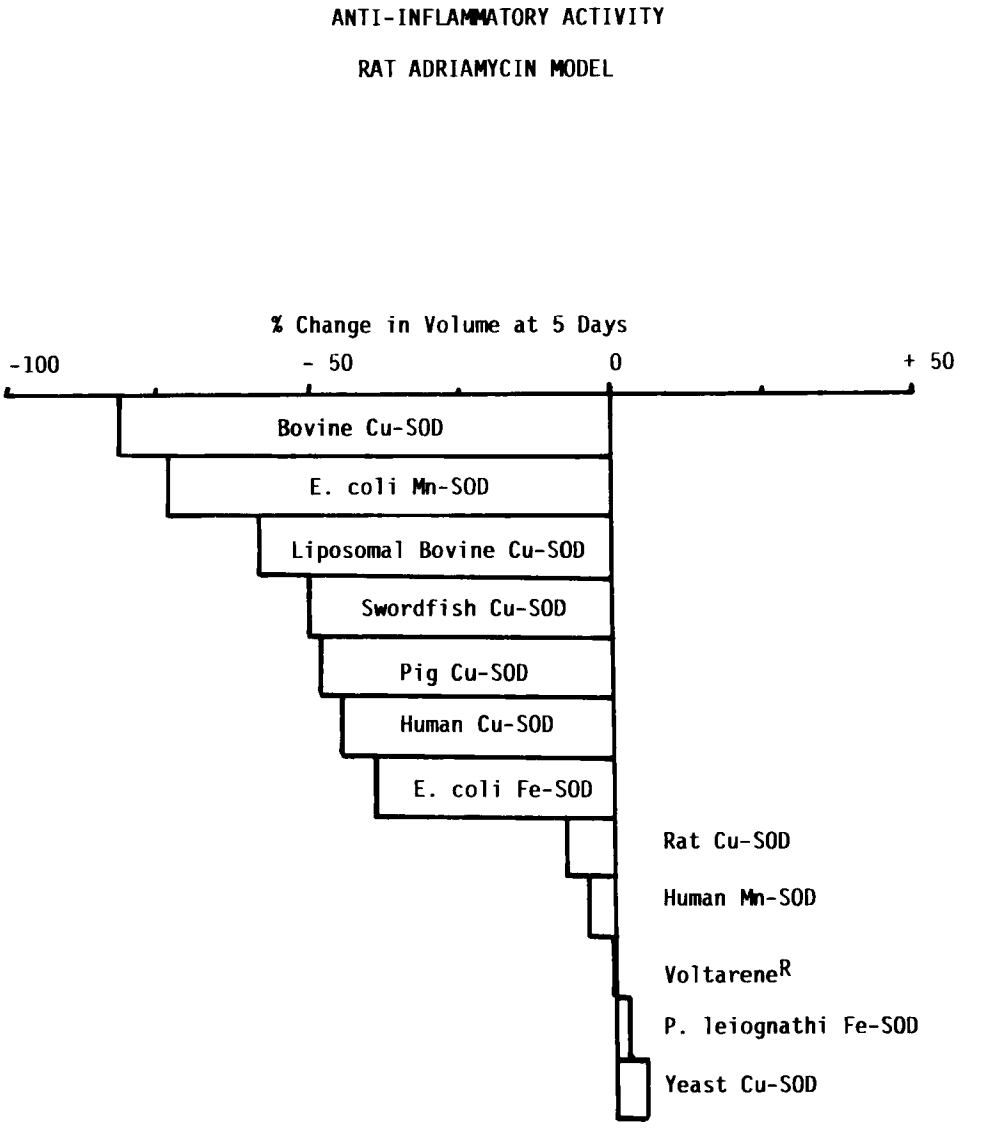

FIGURE 3

DISCUSSION

Any attempt to explain the anti-inflammatory activities of certain, but not all, SODs must necessarily be related to the biological activities of the substrate, superoxide radical anions. Despite the marked reticence shown by many non-biologists to accept the reality of O_2^- toxicity *in vivo* a rather impressive panoply of events can be initiated and maintained by this free radical, either directly or indirectly, leading to

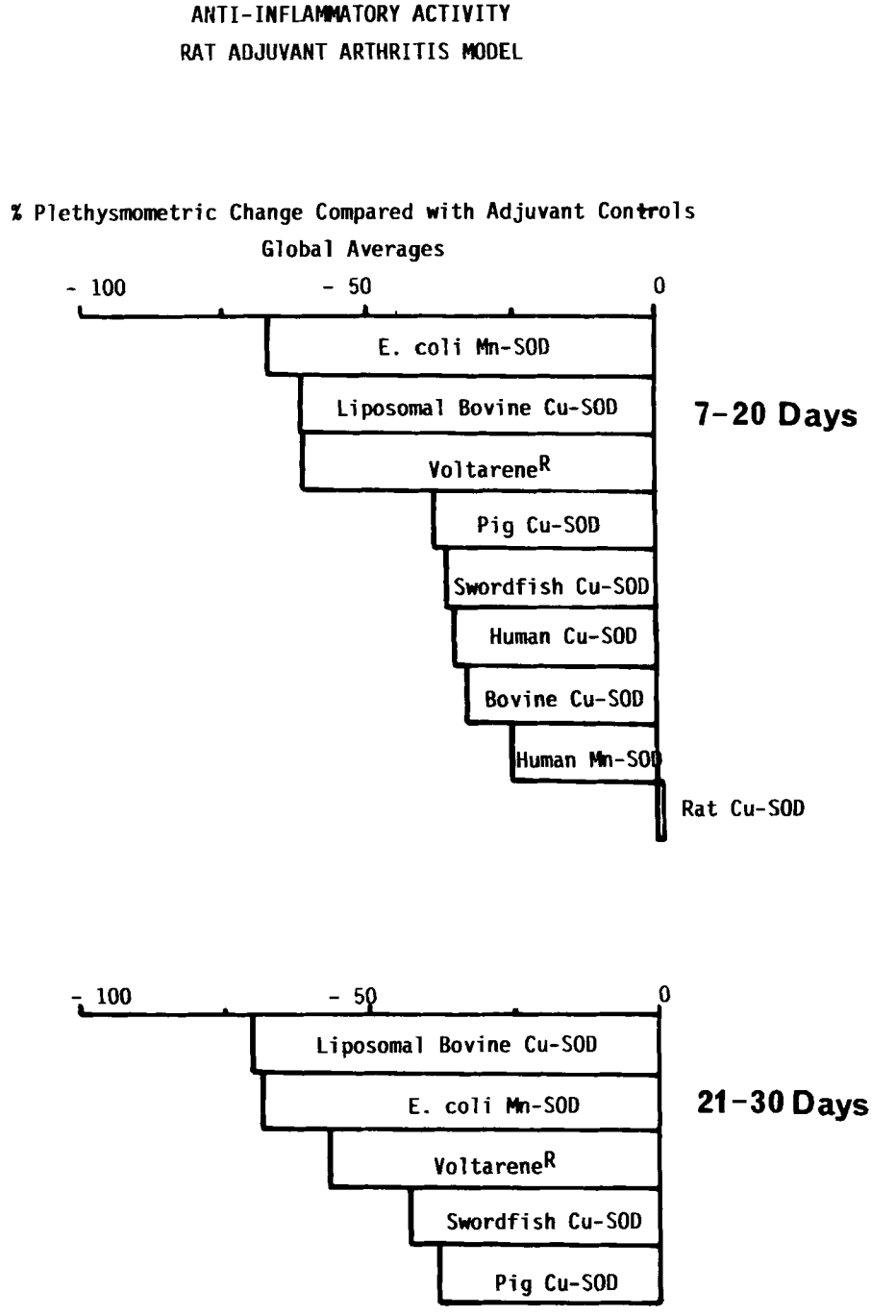

FIGURE 4

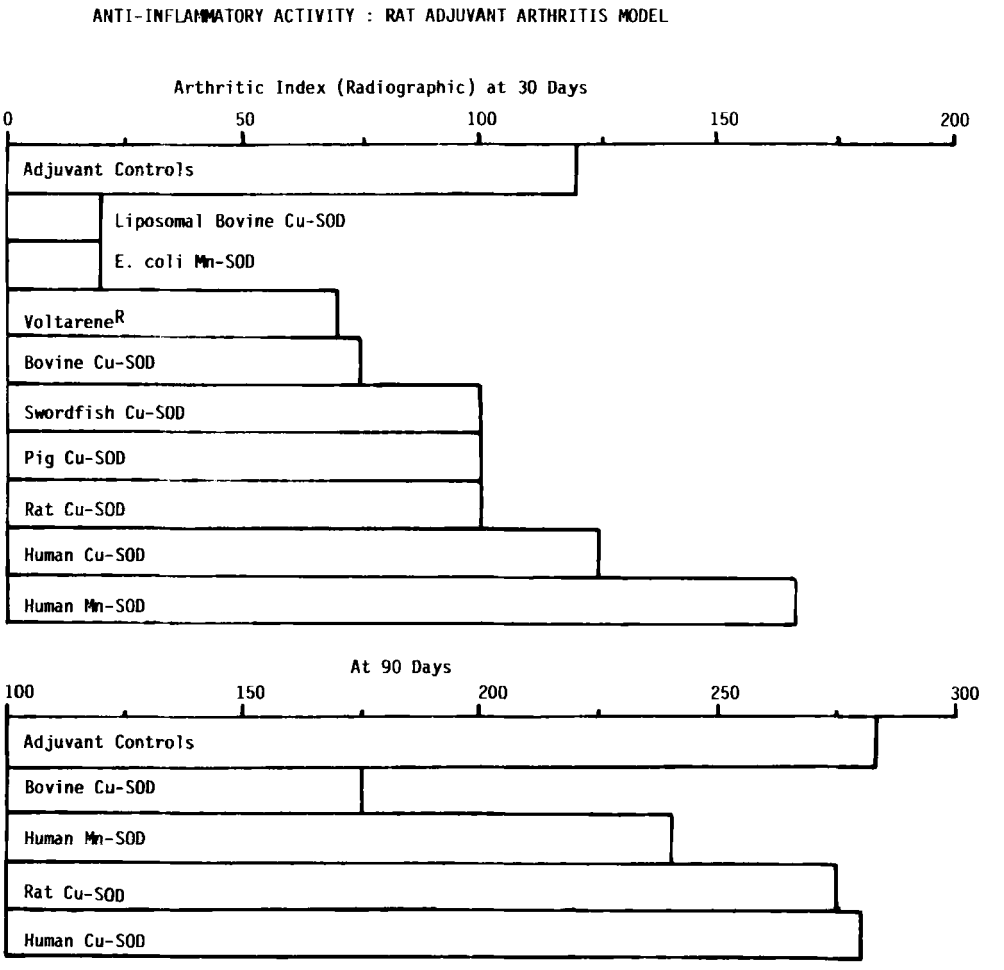

FIGURE 5

pathological conditions and cell death as well as to liberation of circulating cytotoxic factors.

It is now well established that *in vivo* exogenous SOD can act (at various levels) in a wide variety of processes, summarized as follows:

1) Reduction of the number of PMNs aggregated at the site of inflammation,
2) Reduction of peroxidation of cell membranes,
3) Inhibition of clastogenic factors, chromosome aberrations and subsequent events,
4) Restoration of the viscosity of interstitial and intra-articular synovial fluids,
5) Reduction of extracellular O_2^- produced by activated PMNs and macrophages, particularly when these are sur-active,
6) Inhibition of macromolecular cross-linking, a cause of polymerisation leading to fibrosis.

Circulation life time. Many reports have claimed that anti-inflammatory activity is dependent on circulation life time. These studies are essentially based on an increase of molecular weight of the SOD by coupling with another polymer such as polyethylene glycol. This indeed increases circulation life times and it is postulated that anti-inflammatory activity is consequently increased, though very high dose levels are used, not at all comparable with clinical applications. The argument is somewhat spurious and involves a logical solecism (A = B: C = D ∴ B = D) in that coupling of SOD with a macromolecule does not uniquely change a single parameter (life time) but also other physical properties of the conjugated enzyme. Similarly, studies *in vitro* have shown that SOD protects the viability of phagocytosing neutrophils and very large differences were required in the concentrations of various SODs to provide equivalent protection. Pig Cu-SOD (basic pI of 6.3 by isoelectric focalisation or 7.6 by chromatofocalisation) was 100 times more effective than bovine Cu-SOD (pI 5.1). These differences were attributed to the net charges of the enzymes and indeed the protective capacity of bovine Cu-SOD was greatly enhanced by coupling with polylysine to give a net positive charge[9], thus facilitating approach of the enzyme to the negatively charged surface.

Anti-inflammatory properties are **not** directly correlated with circulation life times, as shown by the wide difference in activity of bovine, human and rat Cu-SODs, human and *E. coli* Mn-SOD. The bacterial enzyme has a short half life (44 min, i.v.) compared with human Mn-SOD (6.45 hr, i.v.) yet the two SODs are at opposite ends of the scale, the longer circulating SOD showing no significant anti-inflammatory activity. Both are basic proteins with about the same pI, but the human enzyme has a molecular weight twice that of *E. coli* Mn-SOD. In addition, increase in molecular weight (and increase in circulation life time) of bovine Cu-SOD by covalent fixation to human albumin does not improve activity which is practically the same as that of the parent molecule[2]. If circulation life times were important, then anti-inflammatory properties should be a function of the method of administration, i.e. s.c. > i.m. > i.p. > i.v.

Further, there is no correlation between anti-inflammatory activity and the **absolute level** of circulating exogenous SOD. For example homologous (radioactively marked) rat Cu-SOD is present in the plasma at 10 fold higher levels over the first few hours after injection compared with SODs which are highly active[8].

Biological activity *in vivo* is **not** a direct function of the isoelectric point of the protein. In general, an acidic pI of 4.5 (isoelectric focalisation) or less is associated with poor or zero anti-inflammatory properties but a basic pI does not necessarily confer activity. The value, whether acidic or basic, of pI does not of course rigorously reflect the real surface charge of a globular protein, and simple concepts of positive or negative charges with respect to fixation of a cell surface do not explain the anti-inflammatory activity of *E. coli* Fe-SOD (pI 4.4) and the total lack of such properties in *P. leiognathi* Fe-SOD (pI 4.1).

Intracellular penetration. Anti-inflammatory activity is limited, in the various models studied, to heterologous SOD; homologous SOD is not efficient. The activity is indirect and unrelated to maintenance of high extracellular or circulating levels. Given the amounts of total endogenous SOD per kilogram of rat, it is clear that at the doses used (33 μg/kg) intracellular penetration in a general sense cannot be considered seriously. Although limited in scope, studies with erythrocytes show that bovine, human and rat Cu-SODs are similar with respect to penetration of cells.

Mechanism. It is clear that the concept of removal of extracellular superoxide radicals by exogenous circulating SOD as an explanation for the anti-inflammatory activity of bovine Cu-SOD must be abandoned. Likewise, the postulated effect (particularly with respect to hyperbaric oxygen models) of increased intracellular SOD content is not tenable as a general explanation, particularly in clinical application where at maximum an increase of less than one thousandth of the total endogenous enzyme can be expected of which less than one percent would in fact reach an intracellular localisation.

Since the site of action is neither extracellular nor intracellular, this implies that the anti-inflammatory properties of certain SODs are due to attachment of the enzyme to cell membranes. This fixation does not appear to be influenced to a major extent by pI, nature of the metal at the active centre or molecular weight. However, amino acid sequence and hence minor topological, physical, antigenic determinant or binding site changes are important in the definition of anti-inflammatory activity of a given SOD when used for a specific animal. Isoenzymes (identical metal centre) from a given species do not have identical activity though differences are not large, again reflecting possible effects of minor conformational changes, which could play a role in fixation to a given cell membrane.

Possible explanations for the increased anti-inflammatory activity of heterologous SODs could involve differences in metabolic stability compared with **extracellular** homologous SOD. However, a more likely explanation is that part of the injected SOD is attached to semi-specific "receptor" sites on external cell membranes. Homologous SOD of the host is not readily recognised by these sites and indeed membrane binding is possibly a function of non-homologous sequences in the protein. This would certainly explain the wide variation of anti-inflammatory activity of SODs from different species at least in the rat. Since external secondary and tertiary structure is similar in the several SODs examined it is possible that minor changes in loops or outside parts of the β barrel, topologically in a similar position in the otherwise highly conserved homology of SODs from different species (with respect to amino acid sequence and tertiary structure as well as active site environment) are responsible for the variations of anti-inflammatory activity. Such regions could well correspond to, or be related to, antigenic determinant sites in each individual SOD.

Anti-inflammatory properties would thus be related to a specific "interphase" attachment of the SOD to the outside membrane of cells, this attachment being a function of recognition sites on the cell wall and a given region of amino acid sequence in the SOD, masking of which does not interfer with the enzymic activity. This gives maximum protection against cellular damage, particularly when production of O_2^- by neutrophils clustered to a particular endothelial region is involved, or indeed any other situation in which close contact of the O_2^- producing system with the target cell occurs. That at least immuno-competent cells do not recognise homologous SOD is evident; this differentiation of self and non-self may be envisaged, perhaps to a lesser degree, for other cell populations. In this case a certain distance between homologous (endogenous) and exogenous enzyme in terms of sequence homology is necessary for the expression of anti-inflammatory properties. This difference must not be excessive however as shown by the lack of activity of yeast and other Cu-SODs when the series of copper-containing SODs is considered.

That membrane attached SOD is more significant than extracellular free enzyme with respect to protection is shown by perinuclear halo formation in mouse fibroblasts under the influence of UV irradiation and the inhibition of this effect in

presence of added exogenous SOD[10]. Protection is maintained even when the cell culture medium is replaced after pre-incubation by SOD-free medium. In this case very few molecules (10–100) of SOD remain fixed to each cell. The greater efficiency of liposomal SOD compared with the free enzyme, whether for *in vitro* protection of mitochondrial membranes[11] or in animal studies and clinical use[12] is readily explained by an increased fixation to cell walls as shown in earlier studies[13], as well as improved tissue penetration. Protection of lung cells against hyperbaric oxygen conditions by liposomal SOD[14] is likewise related to increased membrane fixation rather than intracellular penetration or longer circulation life times, and indeed better protection is obtained with forms of SOD which do **not** penetrate the cell[14].

Long term effects of SOD have been reported. Certainly in some cases this is an indirect manifestation due to rectification of a defect (for example in the bone marrow) or to reduction of the activity or levels of various systemic factors (such as a clastogenic factor) in which the beneficial effects can continue long after administration of the enzyme (whether free or liposomal). In other cases, the time required to replace a given population of cells can give rise to an apparent continued effect of SOD. However, an important long term effect could arise from the residual SOD molecules attached to the external cell membrane. Quantitatively as a function of time this will depend for each SOD on factors such as rates of fixation and membrane penetration, as well as dissociation constants, the last perhaps being most important. Clearly these values will vary with the amino acid sequence and topology of the critical zones of the various SODs.

A preliminary correlation of sequence and anti-inflammatory activity in the series of Cu-SODs (in the limited measure in which sequences are known) suggests that the critical "binding" area may be localised at amino acids 22–26, with a requisite of maximum homology elsewhere. Loop 6.5 (both the zinc ligand region and the disulphide region) as well as the active site lid loop (loop 7.8) and the β strands, 6d, 7g and 8h must remain relatively unchanged for anti-inflammatory activity (in rats) whereas in active SODs variations are seen in the Greek key loop (loop 4.7, amino acids 100–114) and the remaining β strands 2b and 3c (amino acids 14–36) and 5e and 4f (amino acids 84–99). Thus changes in the active site lid loop (cabbage and yeast Cu-SOD) do not correlate with anti-inflammatory properties whereas modification of amino acids 22–26 (junction of β strands 2b and 3c) appears to be necessary[15]. At present no definitive conclusions can be drawn, but in principle, after refinement with further studies of sequence, topology and activity, it should be possible to design a superoxide dismutase with optimal anti-inflammatory properties by amino acid modification in a specific region and a pI not excessively acidic, and to produce this super SOD by common genetic engineering techniques.

Polyethylene glycol-SOD. Although increased circulation life time is often proposed to explain a postulated improved activity against hyperbaric oxygen or in simple animal inflammation models for this modification, it is more reasonable to suppose that attachment to membranes is greatly improved. This could also be true for the increased protection afforded to neutrophils by polylysine-SOD, that is, physico-chemical binding properties other than net positive charge are of importance. It may be recalled that coupling bovine Cu-SOD with human albumin (a normal protein with properties quite unlike a chemical polymer such as polyethylene glycol) increases circulation life time but has no effect on anti-inflammatory properties[2] if used at the same dose levels of the original enzyme. In any case since somewhat

massive quantities of polyethylene glycol-SOD have generally been used in animal experiments, other explanations such as depletion of various circulating factors may be of importance.

Efficacity of SOD in various diseases. Whereas O_2^- and related free radicals are implicated in many inflammatory conditions the importance of this role varies not only in different pathological manifestations (depending on which of the various parameters are predominant) but also on the stage of development of the disease. If initiation is due to O_2^- then it is clear that very early treatment with SOD or liposomal SOD can be of advantage. However this is not always the case and in certain instances free radicals become of major significance at later stages, that is, the biochemical etiology (with reference to O_2^-, lipid peroxidation, etc.) can vary during the progression of a given pathology. This can give rise to an apparent paradox that enzymotherapy with SOD may be more efficient in severe cases of disease (such as Behçet or arthritic diseases) than in mild forms. A certain variability of efficiency may therefore be expected when similar clinical symptoms are a result of dissimilar biochemical conditions. This suggests that a nosology based on free radical biology may be of more use than the classical approaches, and indeed in certain directions this is, at least by some clinicians, now considered seriously.

A second apparent paradox may be briefly mentioned. Superoxide dismutase can undoubtedly inhibit depolymerisation of synovial fluid macromolecules and collagen surfaces and thus be useful, particularly by intra-articular injection. On the other hand SOD can lead to depolymerisation in cases of radio-induced fibroses. This latter effect is probably due[5] to perturbation of an equilibrium of superoxide-induced cross-linking between macromolecules giving rise to a three dimensional matrix, and dissolution of this mass by normal processes. If build-up of the fibrosis is inhibited, then regression can occur. In the two cases conditions such as concentration and solvent conditions are quite different.

Cascades and long lived auto-sustaining systems. A common misconception is that O_2^- has a very short life time and that the biological activity of SOD is limited to the simple destruction of free molecules of the radical, more or less in circulation. Apart from the fact that at biological concentrations, O_2^- has a very long life time and complex formation with metal ions can give significant stabilisation (and resistance to dismutation by SOD), a much more important contribution of SOD is to prevent amplification and to interrupt cascade processes. Due to initiation of free radical chain reactions, a single molecule of O_2^- can give rise, by interaction with lipids, H_2O_2, hydroquinones, flavins, etc. . . . (and subsequent reaction with molecular oxygen) to a greatly increased number of organic radicals and other oxygen containing species such as a carbonate anion radicals, some of which in turn may produce more superoxide. This can give rise to autosustaining oxidative conditions, which may in certain circumstances rest dormant as a small nucleus of activity (at a localised site) but which can ultimately burst into a highly active, explosive situation induced by a relatively minor change in environment (flux of oxygen) or relative concentration of the different species. This could explain the efficiency of SOD for the treatment of radio-induced lesions, long after the initiating irradiation event, as well as the sometimes tardive appearance of such pathologies.

A second aspect is that such latent centres of activity will not be found in free solution (circulating conditions) but rather on or in the cell membrane. This in itself gives

rise to physico-chemical conditions which increase the life times of certain radicals, but more importantly implies that in order to break into the cascade and thus diminish total radical activity, the SOD must itself be at the interphase of the cell and the interstitital medium and not simply in circulation. The increased efficiency of liposomal bovine Cu-SOD compared with the free enzyme for the treatment of certain pathologies is thus based not only on generally improved pharmacokinetic properties and longer availability in the organism, but also on the all important targetting to the cell surface.

***E. coli* Mn-SOD and Fe-SOD.** Whereas variations of anti-inflammatory properties of different SODs in the copper-containing enzyme series can perhaps be explained by a requisite to be similar, but not too similar, to be different, but not too different, to the host homologous enzyme, the high activity of *E. coli* Mn-SOD and the non-negligible properties of *E. coli* Fe-SOD cannot be covered by this postulate. The sequence of the bacterial Mn-SOD (that of the Fe-SOD is currently under investigation) does not readily lend itself to the concept of a specific loop sequence recognised by receptor sites. However, a particular region, even though composed of an amino acid sequence not to be found in mammalian Cu-SOD (or Mn-SOD) may have the same geometrical qualities and physical properties (hydrophobicity, charge, etc. . . .) necessary for efficient binding and appropriate dissociation constant. In this respect it may be recalled that human Mn-SOD has virtually zero activity in the various rat models. The only other Mn-SOD tested for anti-inflammatory activity is that of *Gluconobacter cerinus*. Apparently this enzyme (unlike that of *E. coli*) shows activity only when it is coupled to polyethylene glycol[16]. This again suggests that both *E. coli* Mn-SOD and Fe-SOD contain a sequence or topology not to be found in *G. cerinus* Mn-SOD or *P. leiognathi* Fe-SOD.

That *E. coli* Mn-SOD is somewhat different from most SODs is demonstrated by a simple experimental observation. Due to extremely high adsorption losses in plastic (polystyrene) tubes, dilutions of the enzyme must be made in glassware. This unusual adsorptive capacity of polystyrene and the protein may reflect a similar cellular phenomenon.

Human Cu-SOD. The availability of large quantities of human Cu-SOD by clonage of the gene in yeast has engendered almost euphoric expectations for the treatment of human disorders, in particular cerebral and cardiac ischemia-induced damage. In the absence of any clinical evidence, are these hopes justified? Clearly this enzyme (in terms of mechanism of action) cannot be compared with proteins and peptides such as insulin and growth factors. Certainly, in human pathologies in which large amounts of **homologous** SOD are found in the plasma (10–20 fold increase in normal levels) no increased protection is observed, and indeed high plasmatic SOD levels are an excellent indication of various hepatic disorders. A large increase of plasmatic Cu-SOD is found in certain cases of hemolytic anemia (for whom injections of liposomal bovine Cu-SOD are beneficent) suggesting that in humans homologous Cu-SOD whether prepared from erythrocytes or produced by genetic engineering techniques will not necessarily be very efficient. It may be recalled that heterologous calcitonin (e.g. from salmon) is much more effective in humans than the homologous human polypeptide. Plasmatic Cu-SOD is also extremely high in patients with Alzheimer's disease (330.6 ± 37.4 ng/ml), Pick's disease (328.2 ± 63.6 ng/ml) and in vascular forms of senile dementia (263.5 ± 43.1 ng/ml) whereas levels of Mn-SOD are normal

(19.4, 13.1 and 29.7 ng/ml respectively). Erythrocyte catalase and glutathione peroxidase are normal in these diseases, whereas red cell SOD is decreased in Alzheimer (453 μg/gHb) and Pick (406 μg/gHb) pathologies[17].

It may appear paradoxal that bovine Cu-SOD should be used to lower circulating levels of homologous Cu-SOD, but this is indeed the case for certain forms of hemolytic anemia[12]. We have begun treatment of a small series of Alzheimer patients with liposomal bovine Cu-SOD and intend to follow the level of circulating SOD as well as possible improvement in clinical symptoms. Again this is based on the concept that heterologous enzyme has a beneficial effect whereas homologous SOD does not.

In the three different models in rats, carrageenan, adriamycin and the long term arthritic systems, human Cu-SOD shows very poor biological properties compared with other SODs, in particular *E. coli* Mn-SOD and to a lesser extent bovine Cu-SOD. This may be due to a species specificity, but if such is the case one might reasonably expect homologous rat Cu-SOD to be efficient in this animal. Whether considered as a short term or long term form of treatment this is not so, and the enzyme shows a total lack of activity. These considerations indicate (but do not prove) that human SOD may in fact be rather disappointing when applied to human clinical disorders, as we have previously suggested[18], and that a heterologous molecule is necessary. Indeed clinical administration of human Cu-SOD, at least at high dose rates, may well give rise to unsuspected secondary effects or be **pro**-inflammatory. The materialistic view that more is better may in this case be counter-productive.

If indeed human Cu-SOD shows poor activity, this can undoubtedly be improved by encapsulation in liposomes. Judging by preliminary results with rats and using rat Cu-SOD as primary protein, other approaches to increase anti-inflammatory properties of a homologous enzyme while avoiding creation of antigenicity, are possible[19].

CONCLUSIONS

The various concepts outlined above have been further developed in studies with more sophisticated animal models[20]. A computer program to produce three dimensional images of the SODs using known sequences of different enzymes for a comparison with anti-inflammatory activity is underway and may prove useful. At the moment we are prepared to predict that Cu-SOD from *Neurospora crassa* will show low activity, and that yeast Cu-SOD, apart from its pro-inflammatory action with adriamycin (and presumably related drugs) will be totally inefficient in models of cardiac or other ischemic conditions. With respect to the application of human Cu-SOD in humans, only the results of unbiased clinical trials will demonstrate whether or not it is disappointing as a powerful and useful drug for the treatment of ischemia and various inflammatory diseases.

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