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ANTI-INFLAMMATORY ACTIVITY OF SUPEROXIDE DISMUTASES: INHIBITION OF ADRIAMYCIN INDUCED EDEMA IN RATS

G. JADOT

Service de Pharmacologie, Faculté de Pharmacie, 13385 Marseille Cedex 5, France.

A.M MICHELSON† and K. PUGET

Institut de Biologie Physico-Chimique
13, rue P. et M. Curie, 75005 Paris, France.

A. BARET

CERB, HIA Sainte-Anne, 83800 Toulon, France.

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Various superoxide dismutases from different sources, containing Cu, Mn or Fe at the active centre, have been examined with respect to anti-inflammatory activity in a model using adriamycin-induced edema in rats. Very large differences in efficiency are observed, the most active being *E. coli* Mn-SOD and bovine Cu-SOD. The Fe-SOD from *E. coli* is active whereas *P. leiognathi* Fe-SOD is not. Human Mn-SOD shows no significant activity and homologous rat Cu-SOD is totally inactive. Yeast Cu-SOD shows pro-inflammatory properties. Anti-inflammatory activity is **not** a function of molecular weight or circulation life-time.

Key words: Adriamycin, superoxide dismutases, anti-inflammatory activity, liposomes

INTRODUCTION

Although the carrageenan-induced paw edema[1] is a simple and rapid technique for the testing of anti-inflammatory activity of different superoxide dismutases, it is clear that long term effects cannot be evaluated with this model. Further, in order to understand at least partially the physiological mechanisms of action of superoxide dismutase (SOD) *in vivo* and even to have valid comparisons of the relative efficacity of various SODs, restriction to a single model is quite insufficient. We have previously presented a comparison of 18 different SODs from procaryote, plant, fish, bird and mammalian species using the carrageenan model as test. The results demonstrate that

† Correspondence and reprint requests to A.M. Michelson.

anti-inflammatory efficiency of the enzyme (at dose levels where catalytic activity rather than mass of protein is involved) is widely variant as a function of the source, that is, of the amino acid sequence in the protein[2].

The adriamycin-induced paw edema model in mice[3] was introduced in 1980. The test is relatively easy and gives results with only small dispersion, but is not generally used in pharmacological screening since at the major second phase the chemical anti-inflammatory drugs, steroidal or non-steroidal, give totally negative results. An explanation for this lies in the major role of free radicals in the inflammatory (and other) syndromes induced by adriamycin. For this same reason, the test could be of considerable value for the evaluation of the biological activity of different SODs. We have therefore somewhat modified the original technique for application to male Wistar AF-gnotoxenic rats, and studied the efficacity of 10 SODs from different sources, as well as liposomal bovine Cu-SOD. In addition a very active anti-inflammatory drug, Voltarene^{R} (diclofenac), has been tested in this system.

Adriamycin, an anthracyclic antibiotic is widely used as an anti-cancer drug, the clinical use of which is however limited by a dose-dependent cardio-toxicity[4]. Other undesirable side-effects can arise as a result of extravascular filtration[5,6] which apart from local pain, produces tissue necrosis or at best, local reactions such as edema and cutaneous necrosis. The edema lasts several days after initiation and is characterised by induration. Histology of the ulcers shows chronic and acute inflammation and dermal fibrosis; the dermis surrounding the ulcer is hyperplasic[7]. Clinically, the only efficient treatment is excision of the affected area though some slight amelioration is obtained with hydrocortisone[5,6].

Secondary effects of adriamycin are theoretically limited by suitable protocols for administration, though long term actuarial life rates with respect to death not due directly to cancer after treatment with this, or any cancer drug, are not easily available. The first sign of intolerance, stomatitis, appears within two weeks but is greatly reduced with present protocols. Medullar hypoplasy (generally preceded by oral lesions) is an extremely severe side effect manifested by leucopenia, thrombocytopenia and anemia, and may entail cessation of the treatment. This is particularly so if immuno-depressive effects are concurrent. Other acute secondary effects include loss of hair, this alopecia occurring in the large majority of cases, fever, vomitting, diarrhea and abdominal pain. Cardiotoxicity is manifested by tachycardy, extrasystoles, arythmy, etc . . . and a congestive cardiac insufficiency, perhaps due to production of an aglycone metabolite by the action of myocardial hydrolases[8].

The mechanisms by which adriamycin induces tissue inflammation are complex, extensively studied and will not be reviewed here, perhaps because no clear explanations can be drawn at present. It is nevertheless to be noted that adriamycin can potentiate[9] the effects of irradiation (increase of intestinal vascular permeability in the rat); that cardiac mitochondria appear to be more sensitive than those of cancer cells[10]; that free radicals and lipid peroxidation occurs in hepatic microsomes during the redox cycle of the drug[11]; and that *via* production of free radicals and lipoperoxides adriamycin can destabilise the lysosomal membrane, thus liberating the contents which are chemotactic for the polymorphonuclear neutrophils and macrophages[12] and contribute to the tissue damage leading to inflammation[13].

The antitumoral activity of adriamycin is particularly useful for treatment of acute leukemias (lymphoid or myeloid), lymphomes (Hodgkin, lymphosarcomes, reticulosarcomes), lung and bladder cancers, breast carcinomas and bone sarcomas. An efficient technique for reduction of secondary effects in absence of decreased anti-

tumoral activity would thus be of considerable interest. The detailed mechanisms of this activity have merited considerable investments over the past 15 years and the general rather unprecise conclusion is that the drug interfers with nucleic acid metabolism, though recent fashions imply free radical activities. It is possible that autoxidation of reduced adriamycin gives rise to the adriamycin radical or semi-quinone (i.e. an activated form which readily covalently binds to nucleic acids and proteins) and to superoxide radicals (leading to inflammation and other secondary effects). $AH_2 + O_2 \rightarrow AH^{\cdot} + O_2^{-} + H^{+}$; $AH^{\cdot} + O_2 \rightarrow A + O_2^{-} + H^{+}$.

This excessively simplist view is of course presented in order to suggest that reduction of secondary effects in the clinical use of adriamycin can be achieved by use of a suitable form of SOD without interference in the anti-tumoral activity. Preliminary indications are that this is so[14,15,16].

MATERIAL AND METHODS

Adriblastine^{R} (doxorubicine, adriamycin) was freshly prepared at 2.5 mg/ml in double distilled water at 37°C. The right rear paw of male Wistar AF-gnotoxenic rats weighing 300 ± 20 g was cleaned with ether and the pad injected with 0.2 ml of this solution (0.5 mg of adriamycin) at time zero, all experiments beginning systematically at 9.00 a.m. to avoid nycthemeral or chronobiological variations. This dose was determined in preliminary experiments using a range of 0.05 to 1.0 mg per injection, each time in 0.2 ml.

Plethysmometric estimations were performed with a water plethysmometer (Ugo Basile) with a Wheatstone bridge, using variation in resistance to measure change in volume of the immersed paw. Measurements were made at time zero immediately before injection of the adriamycin, at 1 hr and at 5 days after. Percentage increase in volume is given by $\frac{Vt-Vo}{Vo} \times 100$.

Superoxide dismutase was administered intraperitoneally at 33.3 μg/kg (in 1 ml of saline at 37°C) at 30 min **before** injection of the adriamycin and each morning of the second, third, fourth and fifth days (5 injections with a total of 166.5 μg/kg over 5 days). An identical schedule was used with Voltarene^{R} at 5 mg/k per injection (total 25 mg drug).

Blood samples were removed from non-anaesthetized animals by cardiac puncture on the sixth day, i.e. 24 hr after the last injection of SOD. Antibodies were estimated by homologous radio-immunoassay using the radioactively marked SOD as tracer in each case, with previously described techniques[17,18].

Superoxide dismutases were isolated and purified as previously described[2].

Variance was controlled by the Snedecor F test; probabilities were calculated by the Student t technique.

RESULTS AND DISCUSSION

One hour after injection of adriamycin in the rat paw pad an inflammatory response occurs associated with liberation of histamine and serotonin and accumulation of polymorphonuclear neutrophils. This edema lasts 4–10 hr then decreases, leaving a diffuse dermal inflammatory response at 1 day after injection. Histopathological

TABLE Ia
Change in volume at 1 hr and 5 days after injection of adriamycin. Effect of different SODs

SODs at 33.3 μg/kg i.p. 5 daily injections	n	Plethysmometric measure 1 hr	±SEM	5 days	±SEM	% change in volume 1 hr	5 days	t 1 hr 5 days	p < 1 hr 5 days
Controls	10	73.3	7.0	123.9	7.4	—	—	—	—
E. coli Mn-SOD	10	13.7	4.8	33.1	4.0	−81.3	−73.3	6.63 10.24	0.001 0.001
Swordfish Cu-SOD	10	24.4	3.4	61.8	3.9	−66.8	−50.1	5.94 7.04	0.001 0.001
Pig Cu-SOD	10	24.7	2.9	64.1	3.4	−66.3	−48.3	6.05 6.97	0.001 0.001
Liposomal bovine Cu-SOD	10	32.4	3.8	51.7	4.9	−55.8	−58.3	4.85 7.72	0.001 0.001
Controls	10	65.6	5.8	110.7	8.2	—	—	—	—
Bovine Cu-SOD	10	31.5	3.5	20.7	3.3	−52.0	−81.3	4.74 9.58	0.001 0.001
Rat Cu-SOD	10	74.8	5.2	101.0	10.9	+14.0	− 8.7	1.11 0.67	NS NS
Yeast Cu-SOD	10	86.6	6.1	116.2	9.9	+31.9	+ 5.0	2.34 0.40	0.05 NS
Voltarene^{R}	10	71.6	3.2	110.2	4.8	+ 9.0	− 0.4	0.84 0.05	NS NS
Rat Cu-SOD Second batch	10	72.3	5.2	102.6	10.7	+10.2	− 7.3	0.98 0.54	NS NS
Controls	10	70.0	3.3	125.9	8.7	—	—	—	—
Human Cu-SOD	10	28.4	3.2	69.2	7.5	−59.5	−45.0	8.56 4.68	0.001 0.001
Human Mn-SOD	10	56.1	3.3	120.3	3.2	−19.8	− 4.4	2.83 0.57	0.02 NS
E. coli Fe-SOD	10	37.0	4.3	76.5	8.0	−47.1	−39.2	5.80 3.96	0.001 0.001
P. leiognathi Fe-SOD	10	69.1	6.3	128.5	10.0	− 1.2	+ 2.1	0.12 0.19	NS NS

Bovine Cu-SOD, yeast Cu-SOD and *E. coli* Mn-SOD were retested after an interval of two months. The same results were obtained. Plethysmometric measures are expressed as a percentage increase of the readings compared with that at time zero.

examination shows presence mainly of mononuclear cells (monocytes) and a few PMNs together with cellular debris. A second inflammatory phase begins at the fourth day and reaches a maximum on day 5, with a severe diffuse dermal edema containing mononuclear cells and cell residues indicative of necrosis. Hemosiderosis and focalised muscular degeneration indicate hemorrhage.

Four to five days after injection, residual adriamycin at the site is metabolised to semiquinone intermediates which generate superoxide radicals leading to membrane peroxidation[19]; oxidative modification of endothelial cell membranes gives rise to a vascular hyperpermeability. Increased microvascular permeability is induced by generation of oxygen free radicals using the xanthine oxidase system suggesting that permeability changes may be partially related to the release of free radicals from inflammatory cells[20]. An increase in vascular permability may also occur due to an exudation of plasmatic proteins in the interstitial space correlated with the degree of inflammation[3]. In contrast with the first phase at 1 hr for which some anti-inflammatory drugs result in reduction of the edema, anti-serotonins and anti-histaminics are without effect on the second phase and no commonly used steroid or non-steroid drug is effective on both phases. At the end of the sixth day desquamation occurs, and the edema, originally in liquid form, hardens in the region of the paw. Inflammation decreases after the fifth day and disappears at the twentieth day, but the histology is similar.

Plethysmometric measurements after daily treatment with the SODs, with liposomal bovine SOD and with Voltarene^R are presented in Table Ia. The percentage changes in volumes show very strong differences in anti-inflammatory activity among the various SODs tested (all with the same specific activity, except for *E. coli* Mn-SOD and the Fe-SODs which due to a single catalytic site compared with the Cu-SODs have a lower enzymic activity per mg) at both phases of the adriamycin-induced reaction. Doses were held in all cases (except Voltarene^R) to 33 μg/kg in order to avoid non-specific effects occuring when massive amounts of protein or polyethyleneglycol-coupled SOD (1000 times that used in the present study) are used and where the catalytic activity of SOD is somewhat irrelevant. This dose rate also approximates those used in human clinical studies.

As shown in Table II and III which present a decreasing order of efficiency with respect to both the early immediate inflammatory phase and the delayed second phase, the SODs fall into three major groups. Those which are highly efficient at both phases include *E. coli* Mn-SOD, pig, swordfish, and human Cu-SODs, bovine Cu-SOD (and the liposomal form) and *E. coli* Fe-SOD. The pig, swordfish and human Cu-SODs appear to be slightly more efficient at the first phase compared with the second whereas bovine Cu-SOD is very effective at the second phase. The exceptional anti-inflammatory activity of *E. coli* Mn-SOD is to be noted. A second group of SODs show zero or negligible activity such as human Mn-SOD. *P. leiognathi* Fe-SOD

TABLE Ib
Treatment of second inflammatory phase (5 days)
Injection (i.p.) of SOD (33 μg/kg) at days 3, 4 and 5 after adriamycin. Measure at 5 days, 30 min after last injection

	Plethysmometric measure	% Change in volume	t	p <
Control	115.1 ± 6.2	—	—	—
Bovine Cu-SOD	34.6 ± 4.5	−69.9	9.94	0.001
Liposomal bovine Cu-SOD	56.4 ± 3.2	−51.0	7.91	0.001
E. coli Mn-SOD	41.4 ± 5.4	−64.0	8.49	0.001
Yeast Cu-SOD	120.9 ± 4.0	+ 5.0	0.74	NS

(in contrast with the enzyme from *E. coli*) and the homologous rat Cu-SOD. The reference drug Voltarene[R] has no effect on adriamycin induced inflammation. Finally, in this model it is apparent that yeast Cu-SOD is **pro-inflammatory** at the early phase and has a lack of activity at the second.

In order to separate possible long term effects of early treatment with SOD, a second series of animals were injected with adriamycin at day one, but daily treatment began at the third day (Table Ib), after full development of the first inflammatory effects. As shown in Table III, the order of efficiency is unchanged for bovine Cu-SOD, *E. coli* Mn-SOD and liposomal SOD and again yeast Cu-SOD shows zero anti-inflammatory activity.

No circulating antibodies were detected as may be expected in such a short time period.

CONCLUSIONS

The above results confirm the wide variation of anti-inflammatory activity shown by SODs from different sources previously demonstrated in the rat carrageenan model. Again excellent properties are shown by *E. coli* Mn-SOD whereas the homologous rat Cu-SOD is totally ineffective. In this model yeast Cu-SOD is **pro-inflammatory** and thus is counter-indicated as a possible treatment for human inflammatory conditions. The activity shown by Fe-SOD from *E. coli* is of interest since with respect to pI, molecular weight and enzymic properties it closely resembles the Fe-SOD from *P. leiognathi* which is totally inactive (as in the carrageenan model). Again it may be emphasized that circulation life times are **not** of major importance in determining anti-inflammatory activity, nor is molecular weight. The mechanisms involved will be discussed in a subsequent communication[21].

The cardiotoxicity of adriamycin can perhaps be explained by lipid peroxidation in the heart[22], a mechanism which does not appear important in the anti-tumoral action[23]. It is thus reasonable to use the above results as a guide to the clinical application of SOD to reduce secondary effects (in the absence of interference with anti-tumoral activity) of chemotherapy with adriamycin and related drugs. A large scale clinical trial using liposomal SOD in a specific chronological protocol for co-treatment with cancer chemotherapy of patients with malignant lymphoid pathologies such as acute lymphoblastic leukemias has begun.

Comparison of anti-inflammatory properties of different SODs is further developed in studies using the long term (30 day) adjuvant-induced polyarthritis model in the rat[24].

TABLE II
Order of efficiency of SODs at first (Serotonin) phase
Treatment at 30 min before adriamycin injection

SOD	E. coli Mn-SOD	Swordfish Cu-SOD	Pig Cu-SOD	Human Cu-SOD	Liposomal Bovine Cu-SOD	Bovine Cu-SOD	E. coli Fe-SOD	Human Mn-SOD	P. leiognathi Fe-SOD	Voltarene^R	Rat Cu-SOD	Yeast Cu-SOD
% change in volume	−81.3	−66.8	−66.3	−59.5	−55.8	−52.0	−47.1	−19.8	−1.2	+9.0	+12.1	+31.9

TABLE III
Order of efficiency of SODs at second (Free Radical) phase
Treatment at days 1-5

SOD	Bovine Cu-SOD	E. coli Mn-SOD	Liposomal Bovine Cu-SOD	Swordfish Cu-SOD	Pig Cu-SOD	Human Cu-SOD	E. coli Fe-SOD	Rat Cu-SOD	Human Mn-SOD	Voltarene^R	P. leiognathi Fe-SOD	Yeast Cu-SOD
% change in volume	−81.3	−73.3	−58.3	−50.1	−48.3	−45.0	−39.2	−8.0	−4.4	−0.4	+2.1	+5.0

Treatment at days 3-5

SOD	Bovine Cu-SOD	E. coli Mn-SOD	Liposomal Bovine Cu-SOD	Yeast Cu-SOD
% change in volume	−69.9	−64.0	−51.0	+5.0

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