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ANTI-INFLAMMATORY ACTIVITY OF SUPEROXIDE DISMUTASES: STUDIES ON ADJUVANT INDUCED POLYARTHRITIS IN RATS

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The anti-arthritic activities of various superoxide dismutases and of liposomal bovine Cu-SOD have been compared in the adjuvant induced Lewis Inbred Rat model. Various approaches, including plethysmometric measurements, red cell sedimentation rates, while cell counts, levels of IgA and IgG immunoglobulins and scoring by visual, radiographic and scintigraphic techniques all concord in a demonstration of different activities for different SODs. The most efficient are liposomal bovine Cu-SOD and *E. coli* Mn-SOD, a moderate activity being shown by free bovine Cu-SOD. Poor or zero results are obtained with human Mn-SOD, human Cu-SOD or the homologous rat Cu-SOD.

Key words: Superoxide Dismutases, Liposomal SOD, Adjuvant Arthritis, Inflammation

INTRODUCTION

Rheumatoid arthritis is a common illness (more than 500,000 cases in France) characterised by inflammation of conjunctive (particularly synovial) tissue. The disease is chronic, occurs three times as often in females as in males, evolves in successive crises and leads to irreversible articular destruction. Apart from pain and swelling of the articulations, typical radiological lesions (decalcification of the bone) and various histological alterations are observed. Among the many modified clinical parameters may be cited an increased rate of erythrocyte sedimentation (in general the more active the disease, the greater the sedimentation rate) and a frequent increase in IgA and IgG immunoglobulins, more rarely IgM. White blood cell counts are increased; with respect to specific cell types, lymphocytes are decreased and polymorphonuclear neutrophils increased.

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The adjuvant-induced chronic form of polyarthritis in the rat[1] has been used since 1956 as an animal model as many of the effects closely resemble those of human rheumatic diseases, ankylosing spondylarthritis and the Fiessenger-Leroy-Reiter syndrome. Thus recurrent inflammatory active phases occur resulting in evolutive articular, peri-articular and bone lesions, with infiltration of lymphocytes. Since certain of the parameters are readily followed by standard techniques such as radiography, scintigraphy, blood protein analysis, cell counts and plethysmography, this model presents a more complete overall test of possible anti-inflammatory activity than does the rat paw carrageenan test[2] or reduction of adriamycin-induced inflammation[3]. In addition, the long term (30–90 days) efficacity can be evaluated and the results have more relevance to clinical application.

We have previously compared the relative anti-inflammatory activities of various SODs from different sources using the carrageenan[4] and adriamycin[5] models in rats. Remarkable differences were observed and the results indicated that the biological activity of a given SOD is highly dependent on the amino acid sequence, but not of circulation life time, molecular weight or nature of the metal at the catalytic centre. In the present report we continue this comparison for seven different SODs, a liposomal form of bovine Cu-SOD, and a commonly used anti-inflammatory drug Voltarene^{R}, using the rat adjuvant-induced polyarthritis model.

MATERIAL AND METHODS

Male adult Lewis Inbred rats aged exactly 7 weeks with a weight of 200 ± 20 g (Charles River Laboratories, Mass., USA) maintained under sterile conditions were used. "Microclimate" conditions in a sterile animal room were rigorously controlled and the following parameters held constant: temperature 24 ± 2°C; humidity 50%; isolation from noise and direct sunlight; alternance of illumination, light 06.00–18.00 hr, darkness 18.00–6.00 hr, this alternance being chosen as the major nycthemic synchroniser; standardized Rat-Entretien U.A.R. alimentation and tap water with no additives *ad libidum*. Two weeks before beginning an experiment the animals were randomized and grouped five to a cage. All injections and manipulations began at 9.00 a.m. to avoid nycthemeral variations. To reduce stress to a minimum, scoring and plethysmographic measurements were performed before injection of the SOD. Ten rats were used for each test of anti-inflammatory activity, 11 animals for adjuvant only injected controls and 11 for non-injected non treated controls.

Adjuvant

A mixture of 6 mg *Mycobacterium tuberculosis* H 37 Ra (Difco) in 1 ml of paraffin oil, distilled water and Tween 80 (6:4:1) was emulsified in an MSE mixer for 2 × 90 min then sterilised twice for 20 min at 120°C. Before use, the adjuvant was warmed and magnetically stirred to maintain homogeneity of the emulsion and 0.1 ml at 38°C was injected in the right rear paw pad (previously cleaned with ether) of the animal.

Treatment

Injection of SOD was intraperitoneal at 33 μg/kg in all cases, the dose being administered in 1 ml of 0.9% NaCl. Adjuvant-induced controls received i.p. injections of 1 ml of 0.9% NaCl with the same time schedule. Non-induced, non-treated animals served as controls for radiography, scintigraphy, blood proteins and circulating cell counts. Voltarene[R] (diclofenac, sodium N-2,6-dichlorophenyl-o-amino phenyl acetate) obtained from Geigy, was injected at 5 mg/kg in 1 ml saline i.p. and served to validate the adjuvant-arthritis test as well as provide comparison with a reference anti-inflammatory drug.

The protocol of injections was as follows: at day zero each animal is weighed and plethysmographic measures of the right paw and left (contralateral) paw performed, followed by injection of adjuvant in the right rear (ipsilateral) paw. After a rest period of 7 days to allow development of the primary reaction, the animals were injected with SOD (or refence drug) daily from the 7th day to the 17th day (i.e. 11 days of treatment) during the secondary arthritic reaction, followed by rest till the 30th day. Plethysmometric, weight and other measurements and arthritic scoring began on the seventh day and continued at days 9, 11, 13, 15, 17, 18, 20, 25 and 30. With certain SODs showing low activity, a second treatment was applied daily from the 30th to the 60th day (during the established arthritis) followed by a one month rest and examination at the 90th day.

Plethysmometric measurements

These were done on the left non-injected contralateral paw and the injected right rear ipsilateral paw using a water plethysmometer equipped with cells of 35 mm and 18 mm diameter. These parameters define the inhibition of the primary edema in the injected paw and secondary edema in the contralateral paw. The increase or decrease of the edema of each paw with reference to the value at day zero (before injection of adjuvant) for each animal (serving as an auto-control) is used to compare the anti-inflammatory activities of different SODs either directly or as a percentage reduction compared with the non-treated controls. For this purpose measurements of both paws were combined to give an overall view.

Estimations of IgA, IgG and IgM immunoglobulins were performed at the 30th day, two weeks after the last day of treatment using a nephelometric (Behring) method with a rabbit anti-sera specific for the rat. Blood was obtained by cardiac puncture of the non-anesthetized animal.

Erythrocyte sedimentation rates were performed on the 30th day using the Sigma SR method[6]. Values at the end of the first hour are given. Non-treated non-adjuvant injected animals as well as adjuvant-induced non-treated animals served as controls.

Scintigraphy

Scintigraphy was done with a gamma camera using a Tc emitter. Polaroid photographs and numeric values were used to assess fixation of right and left femoro-tibial, tarso-metatarsian and scapulohumoral articulations, using the skull as reference zone. A solution of 2 mC of the radio-nucleide, methylene diphosphonate $(Sn)^{-99m}$ Tc, was injected in the vein of the penis of the ether-anesthetized rat. Scintigraphy examination was performed exactly 24 hr after injection with the anesthetized animal.

RESULTS AND DISCUSSION

The Lewis Inbred rat is the only strain capable of developing immunological polyarthritis in 100% of the animals. Other strains respond only in variable proportions of 30–70%. In addition, the gnotoxenic Lewis rat is maintained under sterile conditions, and thus provides stable and reproducible biological material[7] which confers certain advantages over other strains. For optimal results, conditions in a sterile animal room must be rigorously controlled (see Methods).

An inflammatory lesion occurs at the site of injection of the adjuvant within a few hours with redness and edema reaching a maximum at the third day. This non-specific reaction provoked by the adjuvant then regresses and is followed by an active evolutive phase and the immuno-arthritis appears between the 9th and 13th day. The arthritis is severe, and very inflammatory, touching essentially the articulations of the extremities in smooth skin. Lesions of the tail and of the knees are frequent. Adjuvant arthritis is accompanied by tendinitis and tenosynovitis. The maximum of intensity occurs between the third and fourth week after adjuvant injection and decreases slowly after the 30th day leaving various evolutive trophic lesions such as ankylosis and retraction of periarticular conjunctive tissue, demineralisation of peripheral articulations and necrosis of the injected paw and tail.

No attempt was made to estimate lymphatic, cutaneous, ocular, genitourinary or digestive tract lesions. However, in order to have a fairly complete assessment of anti-inflammatory activity using the rat adjuvant-induced arthritis model the following numerical parameters were estimated: weight, plethysmometric volumes, erythrocyte sedimentation rates, levels of IgA, IgG and IgM immunoglobulins, numeration of circulating blood cells (erythrocytes, total leukocytes, polymorphonuclear neutrophils, lymphocytes, eosinophils, basophils and monocytes), hematocrit and mean globular values. In addition, scoring of the severity of the disease by visual, radiographic and scintigraphic examination was performed. The overall global appreciations give a relatively sure estimation of comparative anti-inflammatory efficiencies of the various SODs.

Use of Voltarene^{R} authenticates the validity of the arthritic test and provides comparison with a reference drug. We have preferred to begin treatment at the 7th day after initiation of the arthritis since this appears more realistic as a clinical model, in that treatment of rheumatoid arthritis in humans rarely begins before manifestation of the disease. A limited 11 day schedule was employed in order to observe stability of the results obtained, again with human application in view. We consider that use of massive quantities of bovine SOD (5 to 10 mg/kg) applied daily from the moment of injection of the adjuvant may in fact inhibit initiation more than chronic or acute evolution and at the same time perhaps act by other than enzymic mechanisms e.g. removal of factors from the circulation such as complement. This is unlikely at the dose rates (33 μg/kg) used in this work which correspond more closely with a twice weekly injection of 2.5 mg liposomal bovine SOD for an adult human, rather than daily injections of 600 mg SOD.

The average increase in weight of each batch of rats as a percentage of the original weight at the thirtieth day is shown in Table I. The smallest relative weight losses are shown by rats treated with *E. coli* Mn-SOD or by liposomal bovine Cu-SOD (33 μg/kg of encapsulated SOD), whereas with free bovine Cu-SOD the weight is the same as untreated adjuvant injected controls.

In human clinical studies erythrocyte sedimentation rates are often used as a

TABLE I
Percentage increase in weight at 30 days

			Adjuvant injected, treated animals			
	Non-injected controls (n = 11)	Adjuvant injected controls (n = 11)	Bovine Cu-SOD liposomes + E. Coli Mn-SOD	E. coli Mn-SOD	Bovine Cu-SOD liposomes	Human Cu-SOD
% increase in weight (30 days)	12.3	8.3	11.5	10.8	10.5	9.9
% increase compared with non-injected controls	—	67.5	93.5	87.8	85.4	80.5

	Adjuvant injected, treated animals					
	Rat Cu-SOD	Pig Cu-SOD	Bovine Cu-SOD	Swordfish Cu-SOD	Voltarene®	Human Mn-SOD
% increase in weight (30 days)	9.6	8.6	8.5	7.5	7.5	6.9
% increase compared with non-injected controls	78.0	69.9	69.1	61.0	61.0	56.1

n = 11 for each of the two controls
n = 10 for all other batches.

biological evaluation of inflammation, particularly since increase in rate is greater the more active the rheumatism. As shown in Table II, this parameter allows a comparison of the efficacity of different SODs. It is clear that major effects are observed with liposomal bovine Cu-SOD and *E. coli* Mn-SOD and to a much lesser extent with pig and swordfish Cu-SODs.

Numeration of circulating blood cells and leukocyte analysis was performed. A slight drop in hemoglobin in non-treated arthritic animals compared with controls was restored by *E. coli* Mn-SOD (control 15.40 gHb/100; arthritic 14.48 g/100; *E. coli* Mn-SOD treated 15.24 g/100) and to a lesser extent by swordfish Cu-SOD (15.02 gHb/100) but in general the results were not very significant. No significant differences in hematocrit, eosinophils, basophils and monocytes were observed among the two controls and the series of treated animals. As earlier[8] observed, a decrease in the mean hemoglobin per red cell (colour index) was observed in arthritic rats compared with controls (65.20 → 55.54, $p < 0.005$) but this was not modified significantly by any of the treatments. However, the marked increase in leukocyte count, the large increase in PMNs and the decrease of lymphocytes[8] shown by adjuvant injected rats were all improved to a lesser or greater degree by different SODs as shown in Table III, the use of *E. coli* Mn-SOD plus liposomal bovine SOD being particularly efficient.

Since variations of electrophoretic profiles of serum proteins, total protein or even

TABLE II
Red cell sedimentation rates (day 30)
(n = 10 for each series)

	Mean sedimentation in mm at 1st hour	± SEM	% change	t	P <
A. Controls	1.0	0.1	—	—	—
B. Adjuvant injected arthritic controls	10.0	0.2	$\left(\frac{\text{B-A}}{\text{A}} \times 100\right)$ +900	38.18	0.001
E. coli Mn-SOD	1.95	0.11	$\left(\frac{\text{B-SOD}}{\text{B-A}} \times 100\right)$ −89.4	33.46	0.001
Bovine Cu-SOD liposomes plus E. coli Mn-SOD	1.98	0.21	−89.1	26.24	0.001
Bovine Cu-SOD liposomes	2.05	0.15	−88.3	30.17	0.001
Voltarene®	3.29	0.71	−74.6	8.63	0.001
Swordfish Cu-SOD	4.99	0.41	−55.7	10.42	0.001
Pig Cu-SOD	5.03	0.22	−55.2	15.86	0.001

albumin globulin ratios are not specific tests for rheumatoid arthritis in humans, we have preferred to estimate the levels of IgA, IgG and IgM immunoglobulins as a measure of anti-inflammatory activity. This was done at the 30th day i.e. **two weeks** after the **last** day of treatment, at the maximum of the arthritic reaction.

The analytical assay of these immunoglobulins involved in the humoral immune defense system also allows the evolution of the arthritis to be followed. As in human rheumatoid arthritis, the IgA and to a lesser extent IgG values were increased whereas IgM is unchanged. Reversal of this increase, particularly significant for the IgA provides a convenient measure of the relative anti-inflammatory activity of the different SODs. As shown in Table IV, the liposomal bovine Cu-SOD and *E. coli* Mn-SOD are particularly effective and reduce the levels of IgA and IgG almost to those of non-arthritic controls. This test of active rheumatic inflammation, like estimations of erythrocyte sedimentation rate or leukocyte numeration is thus convenient to follow regression of the disease.

Plethysmometric measurements are a convenient way to follow the arthritic edema which reflects the development and active phases of the adjuvant-induced inflammation, in particular modification of the soft tissue, not seen in radiography. In order to minimise errors, measurements with all the animals (minimum 10 per series) were done on days 7, 9, 11, 13, 15, 17, 18, 20, 25 and 30. The values are given in Table V. From these, global values of average percentage change in volume covering days 7 to 20 and 21–30 were calculated and are presented in Table VI.

With respect to evolution of arthritis in the controls, a pseudo-phlegmonous state of the paw appears at the 13th day and nodes on the tail at the 15th day i.e. the first

TABLE III
White cell counts (n = 10 for each series)

	Total Leukocytes				Neutrophils				Lymphocytes			
	Count ± SEM	% change	t	p<	Count ± SEM	% change	t	p<	Count ± SEM	% change	t	p<
A. Controls	12870 ± 490	—	—	—	22.1 ± 2.0	—	—	—	74.8 ± 2.4	—	—	—
B. Adjuvant injected Arthritic controls	19860 ± 770	$\left(\frac{\text{B-A}}{\text{A}} \times 100\right)$ +54.3	7.2	0.001	55.4 ± 2.1	$\left(\frac{\text{B-A}}{\text{A}} \times 100\right)$ +150.7	10	0.001	41.9 ± 2.3	$\left(\frac{\text{B-A}}{\text{A}} \times 100\right)$ −44.0	9.3	0.001
Bovine Cu-SOD liposomes plus E. coli Mn-SOD	12330 ± 1250	$\left(\frac{\text{B-SOD}}{\text{B-A}} \times 100\right)$ −108	4.9	0.001	28.6 ± 3.9	$\left(\frac{\text{B-SOD}}{\text{B-A}} \times 100\right)$ −80.5	5.7	0.001	66.8 ± 4.1	$\left(\frac{\text{SOD-B}}{\text{A-B}} \times 100\right)$ +75.7	5.0	0.001
Voltarene[R]	14830 ± 951	−72.0	3.9	0.001	35.3 ± 1.6	−60.4	7.3	0.001	64.3 ± 2.9	+68.1	5.7	0.001
E. coli Mn-SOD	15720 ± 937	−59.2	3.2	0.005	43.7 ± 3.0	−35.1	3.0	0.01	54.6 ± 3.5	+38.6	2.9	0.02
Bovine Cu-SOD liposomes	17100 ± 1070	−39.5	2.0	0.1 (NS)	43.4 ± 2.5	−36.0	3.5	0.005	51.8 ± 2.8	+30.1	2.6	0.02
Swordfish Cu-SOD	18860 ± 1253	−14.3	0.64	NS	41.3 ± 2.1	−42.3	4.4	0.001	55.6 ± 1.9	+41.6	4.3	0.001
Pig Cu-SOD	18410 ± 1445	−20.7	0.8	NS	42.1 ± 2.9	−39.9	3.5	0.005	54.8 ± 2.7	+39.2	3.4	0.001

TABLE IV
Variations of IgA and IgG at 30th day
(Controls n = 11; SOD series n = 10)

IgA					IgG			
	Mean (g/l) ± SEM	% change	t	p<	Mean (g/l) ± SEM	% change	t	p<
A. Controls	5.45 ± 0.20	—	—	—	1.50 ± 0.05	—	—	—
B. Adjuvant injected arthritic controls	7.60 ± 0.15	$\left(\frac{\text{B-A}}{\text{A}} \times 100\right)$ +39.4	8.16	0.01	2.07 ± 0.15	$\left(\frac{\text{B-A}}{\text{A}} \times 100\right)$ +38.0	3.42	0.005
E. coli Mn-SOD	6.00 ± 0.20	$\left(\frac{\text{B-SOD}}{\text{B-A}} \times 100\right)$ −74.4	6.07	0.01	1.59 ± 0.20	$\left(\frac{\text{B-SOD}}{\text{B-A}} \times 100\right)$ −84.2	1.82	NS
Bovine Cu-SOD liposomes plus E. coli Mn-SOD	6.15 ± 0.09	−67.4	7.86	0.001	1.60 ± 0.18	−82.5	1.90	NS
Bovine Cu-SOD liposomes	6.27 ± 0.11	−61.9	6.78	0.001	1.68 ± 0.22	−68.4	1.39	NS
Voltarene®	6.60 ± 0.17	−46.5	4.18	0.001	1.86 ± 0.11	−36.8	1.07	NS
Pig Cu-SOD	7.05 ± 0.05	−25.6	3.3	0.005	1.95 ± 0.07	−21.1	0.69	NS
Swordfish	7.48 ± 0.03	−5.6	0.74	NS	1.99 ± 0.08	−14.0	0.45	NS

phase of the secondary arthritic reaction. The second phase occurs at 20–30 days and the tables thus present a treated first phase and an untreated second. The results indicate that anti-inflammatory activity continues over the period of the secondary reaction despite cessation of treatment after the first phase, and a long term effect cannot be separated from immediately positive results. Study of the tertiary reaction, manifested by nodes on several segments of the tail (at the 40th day) and necrosis of the paw (50th day) was not pursued.

A net superiority of liposomal bovine Cu-SOD over the free enzyme can be seen and the exceptional activity of *E. coli* Mn-SOD is confirmed. Human Mn-SOD is poor with respect to anti-inflammatory properties and the homologous rat Cu-SOD has no effect whatsoever (as previously shown in the shorter term carrageenan and adriamycin models).

In addition to the measurable clinical parameters, the severity of the arthritis was scored (necessarily subjectively) by three methods; visual examination, radiography and scintigraphy.

Visual scoring was done using the Jouanneau[9] scale for intensity of inflammation of the articulations and groups of articulations (toes) of the right (adjuvant injected) leg and of the tail. Analysis of the left (non injected) leg was not attempted since

TABLE V
Plethysmometric measurements

Day	Controls	Bovine Cu-SOD liposomes	Pig Cu-SOD	Swordfish Cu-SOD	E. coli Mn-SOD	Voltarene[R]	Bovine Cu-SOD liposomes + E. coli Mn-SOD	Rat Cu-SOD	Human Cu-SOD	Bovine Cu-SOD	Human Mn-SOD
7	50.70 ± 5.29	36.19 ± 3.36	31.33 ± 1.48	40.86 ± 3.46	31.46 ± 2.58	39.26 ± 2.36	35.79 ± 4.01	56.95 ± 6.10	38.34 ± 2.17	55.58 ± 6.57	42.68 ± 3.96
9	65.21 ± 4.23	34.99 ± 5.57	35.06 ± 4.87	37.72 ± 7.12	22.92 ± 4.43	47.67 ± 4.26	39.66 ± 4.37	63.39 ± 6.34	41.71 ± 4.33	60.90 ± 8.50	58.30 ± 8.48
11	92.40 ± 7.61	38.99 ± 7.57	54.06 ± 7.53	59.92 ± 8.06	40.53 ± 5.37	38.13 ± 3.39	42.39 ± 4.81	121.00 ± 11.46	64.50 ± 9.09	74.24 ± 4.86	82.37 ± 9.82
13	131.49 ± 8.15	66.93 ± 8.45	99.33 ± 9.64	94.26 ± 10.18	56.72 ± 7.61	56.29 ± 5.28	48.52 ± 5.01	123.23 ± 7.44	79.66 ± 8.05	84.49 ± 8.21	76.08 ± 8.75
15	187.46 ± 14.73	85.36 ± 12.22	126.46 ± 9.80	124.86 ± 11.80	56.59 ± 5.13	75.66 ± 5.43	69.13 ± 4.84	202.10 ± 9.98	135.93 ± 7.85	137.22 ± 6.20	156.59 ± 13.74
17	220.84 ± 15.34	72.40 ± 9.26	127.40 ± 10.96	139.93 ± 8.82	68.13 ± 4.94	67.80 ± 5.35	69.53 ± 6.25	210.25 ± 12.23	138.88 ± 8.40	140.47 ± 8.49	160.55 ± 12.78
18	224.64 ± 15.35	73.28 ± 10.22	130.58 ± 11.24	142.84 ± 12.49	70.59 ± 5.26	70.99 ± 6.24	70.41 ± 5.25	211.89 ± 14.45	141.23 ± 12.55	143.89 ± 10.51	161.98 ± 8.87
20	235.58 ± 12.28	52.59 ± 6.77	136.06 ± 10.56	125.39 ± 7.09	49.46 ± 2.60	71.19 ± 4.38	40.79 ± 5.22	219.24 ± 15.38	143.64 ± 13.68	145.99 ± 12.58	165.27 ± 7.49
Total 7–20 days	1208.3	460.7	740.3	765.8	396.4	467.0	416.2	1214.1 NS	784.0 $p<0.001$	842.8 $p<0.001$	903.8 $p<0.001$
25	227.15 ± 19.56	60.40 ± 7.73	135.66 ± 11.74	123.33 ± 10.66	65.59 ± 5.22	90.26 ± 9.55	54.22 ± 9.82				

30	235.62 ± 22.22	75.42 ± 8.58	149.54 ± 12.48	138.65 ± 12.22	78.91 ± 10.66	104.69 ± 10.05	68.46 ± 12.28
Total days 25 + 30	462.8	135.8	285.2	262.0	144.5	194.9	122.7
Total 7–30 days ± SEM	1671.1 ± 124.8	596.5 ± 79.7	1025.5 ± 90.3	1027.8 ± 91.9	540.9 ± 53.8	661.9 ± 56.3	538.9 ± 61.9
t		6.9	4.0	3.9	7.9	7.0	7.7
p<		0.001	0.001	0.001	0.001	0.001	0.001

TABLE VI
Percentage change in volume
compared with non-treated arthritic controls

	Global averages	
	7–20 Days	21–30 Days
E. coli Mn-SOD	−67.2	−68.8
Bovine Cu-SOD liposomes plus E. coli Mn-SOD	−65.6	−73.5
Bovine Cu-SOD liposomes	−61.9	−70.7
Voltarene[R]	−61.4	−57.9
Pig Cu-SOD	−38.7	−38.4
Swordfish Cu-SOD	−36.6	−43.4
Human Cu-SOD	−35.1	—
Bovine Cu-SOD	−30.3	—
Human Mn-SOD	−25.2	—
Rat Cu-SOD	+ 0.5	—

scoring of the control animals was not sufficiently homogeneous. The scale used is as follows:

Score	*Articulations*	*Score*	*Tail*
0	Normal	0	Normal
1	Erythema	1	Swelling at the root, or a node.
2	Erythema plus swelling	2	Extended stiffness or edema, with several nodes.
3	Pseudo-phlegmonous aspect	3	Swelling of the whole tail
4	Necrosis	4	Necrosis

Each animal was given a single score covering leg and tail and the average of the 10 rats of each batch (11 for the adjuvant injected controls) noted. In order to have an overall view over several weeks and to reduce fluctuations in a global score, examination was done on days 7, 9, 11, 13, 15, 17, 18, 20, 25 and 30. The results for controls and some of the SOD treated batches are shown in Table VII to indicate the perfect homogeneity of the controls and the small dispersion in the treated animals. Global scores for the 7th to 30th day (average of all the examinations) for the 12th to the 19th day and for the 20th to 30th day are given in Table VIII. The second time period shows results during an active phase of arthritic progression and covering the period of treatment (7th to 17th day) while the last describes subsequent non-treated development. Despite the subjective character of the observations, the number of animals used and the number of examinations justifies certain conclusions which can be drawn from this information.

Thus some SODs do appear to have a longer term effect carried over after the end of treatment in contrast with a chemical drug such as Voltarene[R]. The anti-inflammatory properties of liposomal bovine Cu-SOD and *E. coli* Mn-SOD are again apparent and

TABLE VII
Visual scoring at days 7 to 30

Day	Controls	Bovine Cu-SOD liposomes	Pig Cu-SOD	Swordfish Cu-SOD	E. coli Mn-SOD	Voltarene®	Liposomes plus E. coli Mn-SOD	Rat Cu-SOD	Human Cu-SOD	Bovine Cu-SOD	Human Mn-SOD
7	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
9	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
11	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
13	3.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.10 ± 0.10	2.10 ± 0.10	2.10 ± 0.10	2.00 ± 0.00
15	3.00 ± 0.00	2.00 ± 0.00	2.40 ± 0.16	2.40 ± 0.16	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.40 ± 0.16	2.10 ± 0.10	2.20 ± 0.13	2.00 ± 0.00
17	3.00 ± 0.00	2.00 ± 0.00	2.40 ± 0.16	2.40 ± 0.16	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.40 ± 0.16	2.20 ± 0.13	2.30 ± 0.15	2.10 ± 0.10
18	3.00 ± 0.00	2.00 ± 0.00	2.40 ± 0.16	2.40 ± 0.16	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.40 ± 0.16	2.20 ± 0.13	2.30 ± 0.15	2.10 ± 0.10
20	3.00 ± 0.00	2.40 ± 0.16	2.60 ± 0.16	2.60 ± 0.16	2.40 ± 0.16	2.60 ± 0.16	2.40 ± 0.16	2.40 ± 0.16	2.20 ± 0.13	2.30 ± 0.15	2.10 ± 0.10
25	3.00 ± 0.00	2.40 ± 0.10	3.00 ± 0.00	3.00 ± 0.00	2.00 ± 0.15	3.00 ± 0.00	2.00 ± 0.07	2.80 ± 0.06	2.70 ± 0.15	2.70 ± 0.15	2.50 ± 0.17
30	3.00 ± 0.00	2.40 ± 0.10	3.00 ± 0.00	3.00 ± 0.00	2.00 ± 0.15	3.00 ± 0.00	2.00 ± 0.07	2.90 ± 0.03	2.60 ± 0.17	2.60 ± 0.16	2.50 ± 0.17

TABLE VIII
Global scoring by visual examination

	Days 7–30					Days 12–19		Days 20–30	
	Total score	± SEM	% change	t	p<	Total score	% change	Total score	% change
Arthritic Controls	27.0	0	—	—	—	12.0	—	9.0	—
E. coli Mn-SOD	20.4	0.46	−24.4	13.6	0.001	8.0	−33.3	6.4	−28.9
Bovine Cu-SOD liposomes	21.2	0.36	−21.5	15.5	0.001	8.0	−33.3	7.2	−20.0
Bovine Cu-SOD liposomes plus E. coli Mn-SOD	20.4	0.16	−24.4	39.1	0.001	8.0	−33.3	6.4	−28.9
Voltarene®	22.6	0.16	−16.3	26.1	0.001	8.0	−33.3	8.6	− 4.4
Human Mn-SOD	21.3	0.64	−21.1	8.4	0.001	8.2	−31.7	7.1	−21.1
Human Cu-SOD	22.1	0.90	−18.1	5.2	0.001	8.6	−28.3	7.5	−16.7
Bovine Cu-SOD	22.5	0.99	−16.7	4.3	0.001	8.9	−25.8	7.6	−15.6
Swordfish Cu-SOD	23.8	0.64	−11.8	4.7	0.001	9.2	−23.3	8.6	− 4.4
Pig Cu-SOD	23.8	0.64	−11.8	4.7	0.001	9.2	−23.3	8.6	− 4.4
Rat Cu-SOD	23.4	0.83	−13.3	4.1	0.001	9.3	−22.5	8.1	−10.0

appear to be markedly superior to free bovine Cu-SOD. In this test, both pig and swordfish Cu-SOD show relatively poor scoring as does the homologous rat Cu-SOD. However this kind of examination, despite all precautions, is far from precise and not entirely reliable.

Radiographic examination was performed on the 30th day and severity of bone damage scored for the two rear paws (tibiotarsian articulation, tarsal bones, calcaneum and the ensemble of the metatarses and phalanx) according to the following scale[10]:

0 No damage.
1 Moderate dispersed damage (decalcification).
2 Severe (deformation of bone structure, condensation, and fusion of bones).

The scores for each leg (primary and secondary zones of arthritis) are summed and thus each animal has a category ranging from 0 to 4. Distribution of the scores within each group of animals was compared using the Mann and Whitney[11] and the non-parametric Kruskal-Wallis[12] tests for adjuvant injected rats against non-induced controls (zero damage) and against induced SOD treated groups. The categories correspond to 0, no damage; 1, slight; 2, moderate; 3, severe; and 4, very severe. A final arthritic index was obtained by multiplying the value 0 to 4, by the percentage of animals in each category, and addition. A maximum index is thus 400 and a minimum 0. It may be noted that radiographic scoring reflects bone damage, whereas visual

TABLE IX
Radiographic examination
(percentage of animals in each damage category)

Damage Score	0 Zero	1 Slight	2 Moderate	3 Severe	4 Very severe	Arthritic Index
At 30 days						
Non arthritic controls	100	—	—	—	—	0
Arthritic non-treated controls	—	80	20	—	—	120
Bovine Cu-SOD liposomes	80	20	—	—	—	20
E. coli Mn-SOD	80	20	—	—	—	20
Bovine Cu-SOD liposomes plus E. coli Mn-SOD	80	20	—	—	—	20
Voltarene^{R}	50	30	20	—	—	70
Bovine Cu-SOD	25	75	—	—	—	75
Swordfish Cu-SOD	10	80	10	—	—	100
Pig Cu-SOD	10	80	10	—	—	100
Rat Cu-SOD	20	60	20	—	—	100
Human Cu-SOD	—	75	25	—	—	125
Human Mn-SOD	—	33	67	—	—	167
At 90 days						
Non arthritic controls	100	—	—	—	—	0
Arthritic non-treated controls	—	16.7	—	66.7	16.6	283.2
Bovine Cu-SOD	—	50	25	25	—	175
Human Mn-SOD	—	40	—	40	20	240
Rat Cu-SOD	—	25	—	50	25	275
Human Cu-SOD	—	20	20	20	40	280

examination is influenced more by inflammatory appearance and touch.

As shown in Table IX, a clear order of efficacity is apparent. Thus human, pig, swordfish and homologous rat Cu-SODs and human Mn-SOD have no significant effect. Indeed with the last the situation is worse. Bovine Cu-SOD and Voltarene^{R} have about the same moderate activity and do in fact reduce bone damage. However, by far the most efficient are liposomal bovine Cu-SOD and *E. coli* Mn-SOD (or a mixture of both) which show highly significant, indeed remarkable anti-arthritic properties particularly when the limited nature of the treatment is considered.

In order to determine whether a more prolonged treatment would increase the efficiency of the least active SODs, daily injections (at 33.3 μg/kg) were continued for

days 30–60 for rats treated with rat, human and bovine Cu-SODs and human Mn-SOD. Radiographic examination was performed at day 90, that is when the arthritis in the controls is completely established and permanent. The results are presented in Table IX and it can be seen that homologous rat Cu-SOD has absolutely no protective capacity, as with human Cu-SOD, and human Mn-SOD. Anti-inflammatory activity of bovine Cu-SOD is identical with that shown at 30 days (62.5% of the arthritic control index at 30 days, 61.8% at 90 days) and is not increased with long term use.

Scintigraphy was performed at the 30th day as a complement to radiography for the study of perilesional metabolic exchanges of the bone. Fixation of the radionucleide is more pronounced in certain zones of the skeleton and as in human pathology, hyperfixation (localised or diffuse) in the adjuvant-induced rat arthritis model signifies perturbation of the calcium phosphate metabolism. The adjuvant-injected controls show a net increase of fixation at the level of the left tarso-metatarsian articulation of 255% as well as the right injected leg (+390%) compared with non-arthritic controls. At 90 days this increase is reduced, and stabilised at 70% and 180% respectively.

The results may be resumed briefly as follows. No significant changes compared with arthritic non-treated rats are observed with the homologous rat Cu-SOD or with human, bovine, swordfish or pig Cu-SOD, or human Mn-SOD. In contrast, significant protection against bone destruction is easily seen in rats treated with liposomal bovine Cu-SOD, *E. coli* Mn-SOD or a mixture of both.

It may be noted that a significant additive effect of *E. coli* Mn-SOD and liposomal bovine SOD is not observed. This implies that saturation is obtained at the dose rates used (33 μg/kg for each SOD) and that in fact with these two treatments even lower quantities (preliminary results indicate 5–10 μg/kg) could be equally effective. That 100% efficacity is not obtained is quite reasonable since superoxide (or other) radicals are not the unique explanation of an inflammatory situation. Other factors do indeed have some importance and have been very extensively studied[13].

CONCLUSIONS

A rather complete examination of the anti-arthritic activity of different SODs and of liposomal bovine Cu-SOD shows that certain have high (*E. coli* Mn-SOD and the liposomes), or moderate activity (bovine Cu-SOD) whereas others have essentially zero properties (human Cu-SOD, human Mn-SOD and the homologous rat Cu-SOD). Comparison of the anti-inflammatory properties of various superoxide dismutases using the carrageenan-induced edema[4], adriamycin-induced inflammation[5] and the more extensive adjuvant arthritis models in rats has shown remarkable differences and demonstrated the low or zero activity of homologous rat Cu-SOD. A number of conclusions with respect to the mechanism of anti-inflammatory activity of SOD can be drawn from these studies[14].

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