ANTI-INFLAMMATORY ACTIVITY OF VARIOUS SUPEROXIDE DISMUTASES ON POLYARTHRITIS IN THE LEWIS RAT

A. VAILLE,* G. JADOT† and A. ELIZAGARAY‡

*Laboratoire de Pharmacodynamie, Faculté de Pharmacie and †Laboratoire de Pharmacologie, Faculté de Médecine, 27 boulevard Jean Moulin, 13385 Marseille Cedex 5; and ‡C.E.R.B.H.I.A. Sainte-Anne, 83800 Toulon - Naval, France

Abstract—The anti-arthritic activity of four superoxide dismutases (SODs) has been compared by using the adjuvant-induced polyarthritis rat model. Many of the clinical signs observed in the rat closely resemble those of human rheumatic diseases and the Fiessinger–Leroy–Reiter syndrome. An original protocol and various approaches allowed study of the evolution of long term (30–90 days) SOD treatment. Results are relevant to clinical application: human and bovine Cu-SODs are fully active during secondary and tertiary arthritic reaction; homologous rat Cu-SOD is active only transiently at the end of the secondary reaction; human Mn-SOD is active only on the second stage of arthritic reaction. It should be noted that bovine and human SODs slightly delay the appearance of bony damage. These data were confirmed by the scintigraphic study. Finally it is noteworthy that drug pharmacological activity decreases when the blood level of anti-SOD antibodies increases. This indicates the existence of an immunological reaction following SOD administration.

Rheumatoid arthritis is a common illness characterized by a chronic symmetrical inflammation with a predilection for the more peripheral joints, which in its final stages results in joint deterioration. The disease occurs throughout the world, affecting 0.3–1.5% of the population. Seventy per cent of cases begin between the ages of 25 and 54. It is a syndrome that takes a high toll in human suffering, lost working hours and medical costs.

Adjuvant-induced arthritis in rat, described by Pearson in 1956[1], is a systemic disease which involves articular and visceral manifestations. Chronic arthritis is induced in the rat by injection of *Mycobacterium tuberculosis*. This polyarthritis which affects mainly smooth-skin extremities, is analogous to joint lesions observed in rheumatoid arthritis and in the Fiessinger–Leroy–Reiter syndrome. The rachis lesions in animals are highly similar to those found in ankylosing spondylitis. Adjuvant-induced polyarthritis is characterized by a chronic evolution with recurrent inflammatory bouts resulting in peri-articular, articular, and bony evolutive trophic lesions. In the clinical context, these aspects are identical with those of rheumatoid arthritis.

The relative anti-inflammatory activities of different superoxide dismutases (SODs)§ have already been compared using the carrageenan [2] and Adriamycin® [3] models in rats. In order to further this comparison and to elucidate the mechanisms of SOD action, the present study was undertaken to evaluate

the activity of rat, bovine and human Cu-SODs and that of human Mn-SOD on a model of chronic inflammation.

MATERIALS AND METHODS

Human, bovine and rat Cu-SODs were purified from erythrocytes according to the method of McCord and Fridovich [4], with a slight modification. The specific enzymatic activities were measured by using the riboflavin NBT method described by Beauchamp and Fridovich [5]. Human Mn-SOD was purified from liver according to the technique of McCord *et al*. [6]. The purity of the preparation used was checked by electrofocussing on polyacrimide gel (Multiphor LKB). All SODs were stored at −80° and diluted extemporaneously in normal saline solution at 37°. The biochemical characteristics of the various SODs used are given in Table 1.

Animals. Male adult Lewis Inbred rats aged exactly 7 weeks with a weight of 200 ± 20 g (Charles River, MA U.S.A.) were used. The Lewis Inbred rat is the only strain capable of developing immunological polyarthritis in 100% of the animals. In addition, the gnotoxenic Lewis rat is maintained under sterile conditions, and thus provides stable and reproducible biological material [7].

"Microclimate" conditions in germ-free animal rooms were rigorously controlled and the following parameters held constant : temperature, $24 \pm 2°$; humidity, 50%; isolation from noise and direct sunlight; alternance of illumination, light 06.00 a.m.–06.00 p.m., darkness, 06.00 p.m.–06.00 a.m. (this alternance was chosen as the major synchronizer), standardized diet (Rat-Entretien U.A.R.) and tap water were available *ad lib*.

---

§ Abbreviations: SOD, superoxide dismutase; Cu-SOD, copper-containing superoxide dismutase; Mn-SOD, manganese-containing superoxide dismutase; NBT, nitroblue tetrazolium; IP, isoelectric point; MDP-Tc, methylene diphosphonate (Sn) - 99ᵐ Technetium.

Table 1. Biochemical characteristics of SODs

| SODs | Mol.wt | Isoelectric Points | | Plasmatic half-life (hr) | Enzymatic activity units/mg |
		Isoelectric focusing	Chromato-focusing		
Rat Cu-SOD	33,000	5.0	6.3	1.5	3050
Human Cu-SOD	33,000	4.8		1.6	2900
Bovine Cu-SOD	33,000	5.0	5.3	3.0	3200
Human Mn-SOD	80,000	7.4		6.5	2300

Two weeks before beginning an experiment, the animals were randomized and grouped five per cage. All injections and manipulations began at 09.00 a.m. to avoid any nycthemeral variation. Grading of arthritic lesions and plethysmographic measurements were performed before SOD injection, in order to reduce stress to a minimum.

Adjuvant: a mixture of 6 mg *Mycobacterium tuberculosis* H37 Ra (Difco) in 1 ml of paraffin oil, distilled water and Tween 80 (6:4:1) was emulsified in an MSE mixer (OSI France) for 2 × 90 min, then sterilized twice for 20 min at 120°.

Before use, the adjuvant was warmed and magnetically stirred to maintain homogeneity of the emulsion. A volume of 0.1 ml at 38° was injected in the right hind paw pad (previously cleaned with ether) of the animal.

Tested groups. Modifications of adjuvant-induced polyarthritis were studied in 62 animals distributed as follows:

Group A: Non-induced controls, N=11
Group B: Adjuvant-induced controls, N=11
Group C: Rat Cu-SOD treated, N=10
Group D: Human Cu-SOD treated, N=10
Group E: Bovine Cu-SOD treated, N=10
Group F: Human Mn-SOD treated, N=10.

Animals in each group were divided into two subgroups:

Subgroup 1 : animals 1–5 with an 11-day treatment beginning at day 7 (treatment 1);

Subgroup 2: animals 6–10 with an 11-day treatment beginning at day 7, then no treatment until day 30 and 30-day treatment beginning at day 30, i.e. a total of 41 days (treatment 2).

In the A group, the antigen was not injected. This group was used as reference for the X-ray film study, scintigraphies, assays and electrophoresis of total proteins, and detection of anti-SOD antibodies.

During the treatment periods, B group was given a daily intraperitoneal injection of normal saline solution. Similarly, each animal in Cu-SOD groups (C,D,E) was given a daily intraperitoneal dose of 33

μg/kg (about 100 units/kg) of Cu-SOD dissolved in normal saline solution. Mn-SOD group (F) was given intraperitoneal injection of 43.5 μg/kg (about 100 units/kg). Daily volume injected was 1 ml.

Periods of treatment and observations.

Day 0: antigen injection;
day 0–day 7: no treatment during primary reaction;
day 7–day 17: treatment during secondary reaction;
day 17–day 30: no treatment during secondary reaction, which reached its maximum at day 30 (X-ray film study, scintigraphy, and biochemical controls were performed at day 30);
day 30–day 60: treatment during the tertiary reaction (appearance of evolutive trophic lesions);
day 60–day 90: no treatment during the quaternary reaction during which the appearance of trophic lesions indicates chronic polyarthritis (X-ray film study, scintigraphy, and biochemical controls were performed at day 90). Thus this protocol allowed us to observe the effects of SOD therapy on evolutive polyarthritis and on chronic fixed polyarthritis.

Evaluation of arthritis. Since it was difficult to evaluate visceral lesions in animals treated or untreated by various SODs, we retained the arthritis grading scale of Jouanneau [8] (Table 2).

The intensity of the inflammatory reaction was measured at the level of joints of the tail and of the distal segments of each hind limb, including knees.

We separately evaluated primary (right limb) and secondary (left limb or tail) articular damage according to the above mentioned scale. For each measurement day, we compared the index mean of each group to the index mean of group B by using Student's unpaired *t*-test.

Plethysmographic study. Plethysmographic study is a convenient way to monitor arthritic edema. It reflects the development and active stage of the adjuvant-induced inflammation, in particular modification of soft tissue, which is not seen on X-ray films.

We used a plethysmographic measurement of the

Table 2. Arthritis grading scale

Grade	Articulations	Tail
0	Normal	Normal
1	Erythema	Swelling at the root, or a nodule
2	Erythema plus swelling	Extended stiffness or edema, with several nodules
3	Pseudo-phlegmonous aspect	Swelling of the whole tail
4	Necrosis	Necrosis

Table 3.

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

controlateral left hind limb and the injected ipsilateral right hind limb. These determinations were carried out with a water plethysmometer (Ugo-Basile). The parameters evaluated were inhibition of edema in the injected paw (primary edema) and that in the controlateral paw (secondary edema). Each animal was its own control. Changes in edema volume of each paw were expressed in percentage of variation from the plethysmographic measurement at day 0.

X-ray examinations. Grade of lesions for each hind limb was evaluated at the level of distal tibial ending, tarsal bones, metatarsal bones, phalanges and corresponding joints according to the classification of Dureng et al. [9] (Table 3).

The grades for both hind limbs were summed up; each animal was placed in a category ranging from 0 to 4.

Scintigraphic study. The radionucleid used was MDP-Tc, commercialized by *Commissariat à l'Energie Atomique.* This product, injected by intravenous route, exhibits a marked tropism for normal bone tissue and hyperfixation (localized or diffuse) signifies perturbation of the calcium phosphate metabolism. Moreover, its distribution reliably reflects variations in metabolic exchanges around lesions, a phenomenon which occurs earlier than the accumulation or resorption of bony calcium, which can both be observed on X-ray film. MDP-Tc solution, prepared from extracted technetium, was used within a 90-min delay. MDP-Tc (2 mCi) was injected into the penis vein under ether anesthesia.

Scintigraphy was carried out exactly 24 hr after radionucleid injection, also under ether anesthesia. A gamma-camera connected to an oscilloscope was used to detect radioactivity. Scintillation traces observed on the oscilloscope screen were photographed with a Polaroid camera. Photographs of anterior and posterior sides were obtained. Scintigraphic data were numerized and summed up in order to establish an index of binding i.e. a value corresponding to the intensity of six areas: the right and left femoro-tibial joints, the right and left tarso-metatarsal ones and the right and left scapulo-humeral ones. Results are expressed as the ratio between this value and that obtained from skull.

Blood protein analysis. Blood samples were taken by cardiac punction in unanesthetized rats. Total serum proteins were assayed using biuret method (Weichselbaum reagent), modified and adapted to the Technicon auto-analyser II. Protein electrophoresis was performed according to the technique of Paget and Coustenoble [10].

Detection of blood anti-SOD antibodies. Blood anti-SOD antibodies were assayed with rat serum according to a specific radioimmunological technique described by Baret et al. [11, 12].

RESULTS

Evaluation of arthritis

Figures 1 and 2 show the anti-arthritic properties of Cu-SODs and Mn-SOD. It was not possible to statistically analyse results obtained at the level of
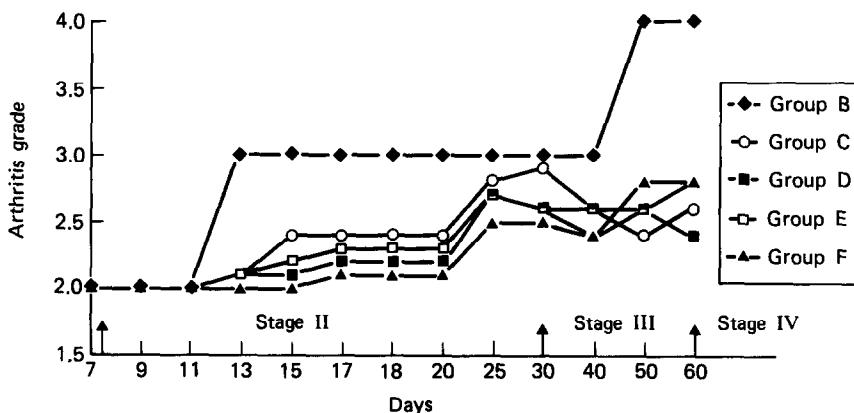

Fig. 1. Time course of arthritis grade on the injected right limb. For clarity, error bars are omitted and each time point expresses the mean grade of groups. Significant decreases in arthritis (calculated using Student's unpaired *t*-test, Group B served as reference) are noted in Results. I, II, III, IV: the four arthritic disease stages. Stage I (day 0–7) is not represented. Group B: Adjuvant-induced controls; Group C: Rat Cu-SOD treated; Group D: Human Cu-SOD treated; Group E: Bovine Cu-SOD treated; Group F: Human Mn-SOD treated.
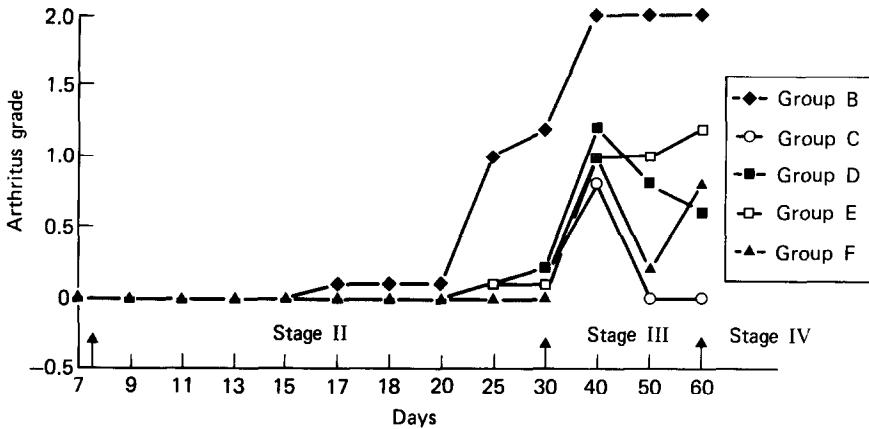
Fig. 2. Time course of arthritis grades on the tail. For clarity, error bars are omitted. Each time point expresses the mean grade of groups. Significant decreases in arthritis (calculated using Student's unpaired *t*-test, Group B served as reference) are noted in Results. I, II, III, IV: the four arthritic disease stages. Stage I (day 0–7) is not represented. Group B: Adjuvant-induced controls; Group C: Rat Cu-SOD treated; Group D: Human Cu-SOD treated; Group E: Bovine Cu-SOD treated; Group F: Human Mn-SOD treated.

the left hind limb because the grading of the B group often gave contradictory and non-reproducible results during the study.

At the level of the right hind limb. Treatment 1: group B showed pseudo-phlegmon aspects on paws starting on day 13. Human, bovine Cu-SODs and human Mn-SOD were active from day 13 to day 30. Only rat Cu-SOD was active from day 13 to day 20. *Treatment 2:* group B showed necrosis on the paw only from day 50. Human Cu-SOD and human Mn-SOD were active from day 30 to day 60.

Bovine Cu-SOD was active from day 30 to day 60; only the results at day 40 are not significant. Rat Cu-SOD was active from day 50 to day 60.

At the tail level. Treatment 1: in group B, nodules

appeared only from day 15. In the four treated groups, efficacy was observed from day 25 to day 30. *Treatment* 2: in group B, all animals exhibited nodules on several segments from day 40. Rat, human Cu-SOD and human Mn-SOD were efficacious from day 30 to day 60. Only bovine Cu-SOD was efficacious from day 30 to day 40.

Plethysmographic measurements

Time course of edema is presented in Fig.3. *Treatment* 1: group B showed a constant inflammation from day 7 to day 30. From day 30 edema decreased slightly. Rat Cu-SOD showed no efficacy during secondary arthritic reaction but became efficacious when this reaction reached its maximum (days 25–
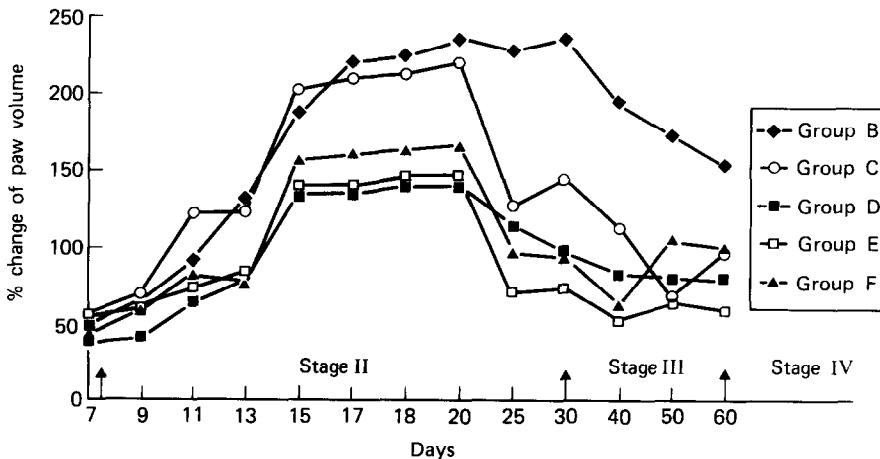

Fig. 3. Time course of paw volume on the injected right paw. For clarity, errors bars are omitted and each time point expresses average change in volume. Significant anti-inflammatory activities (calculated using Student's unpaired *t*-test, group B served as reference) are noted in Results. I, II, III, IV: the four arthritic disease stages. Stage I (day 0–7) is not represented. Group B: Adjuvant-induced controls; Group C: Rat Cu-SOD treated; Group D: Human Cu-SOD treated; Group E: Bovine Cu-SOD treated; Group F: Human Mn-SOD treated.

Table 4. Bony X-ray examination at day 30 and day 90

Grades	0 No damage	1 Slight damage	2 Moderate damage	3 Severe damage	4 Very severe damage	Statistical evaluation
Treatment 1 — *Control at day* 30						
Group A	100%	—	—	—	—	—
Group B	—	80%	20%	—	—	—
Group C	20%	60%	20%	—	—	ns
Group D	—	75%	25%	—	—	ns
Group E	25%	75%	—	—	—	ns
Group F	—	33%	67%	—	—	ns
Treatment 2 — *Control at day* 90						
Group A	100%	—	—	—	—	—
Group B	—	16.7%	—	66.7%	16.6%	—
Group C	—	25%	—	50%	25%	ns
Group D	—	20%	20%	20%	40%	ns
Group E	—	50%	25%	25%	—	ns
Group F	—	40%	—	40%	20%	ns

Percentage of animals in each damage category was compared using non parametric Mann-Whitney and Kruskall-Wallis tests for group B vs group A and vs treated groups.
ns = not statistically significant.
Group A: Non-induced controls.
Group B: Adjuvant-induced controls.
Group C: Rat Cu-SOD treated.
Group D: Human Cu-SOD treated.
Group E: Bovine Cu-SOD treated.
Group F: Human Mn-SOD treated.

30). Human Cu-SOD showed a significant anti-inflammatory effect from day 9 to day 30. Bovine Cu-SOD and human Mn-SOD were active from day 13 to day 30. *Treatment* 2: group B presented constant and slightly decreasing edema until day 60. Evolutive trophic lesions appeared during this period. Rat Cu-SOD was only transiently active during the tertiary reaction (days 30–60). Human Cu-SOD was active only from day 30 to day 40. Bovine Cu-SOD was significantly active during all stages of tertiary reactions. Human Mn-SOD was active only from day 30 to day 40.

X-ray film control

In the first treatment as well as in the second one, corresponding to a developing polyarthritis and to an established polyarthritis respectively, the administration of SODs did not inhibit the damage caused to articular bone and cartilaginous tissues. Group A was taken as the reference group for studies of bone damage since these animals showed no bone lesions. Results at day 30 and day 90 are reported in Table 4.

Scintigraphic control

Means of total binding for each group and changes in right/left ratio are reported in Tables 5 and 6. Results obtained in the first and the second treatments confirmed those of the X-ray study on left and right hind limbs. Further information given by scintigraphy can be summarized as follows: *Treatment* 1: SOD treatment does not prevent extension of polyarthritis at the level of the right femoro-tibial joint. *Treatment* 2: SOD treatment does not inhibit extension of polyarthritis at the level of the right or left tarso-metatarsal joint.

Analysis of results obtained after Treatment 2 allowed us to ascertain that, in group B, the exchanges that occurred in phospho-calcic bone metabolism are stabilized and that in SOD groups (C,D,E,F) these exchanges constantly evolve but significantly so only in group C. These results confirmed those derived from the statistically non significant X-ray film study and showed that bone is less severely affected in groups D, E and F than in group B.

Table 5. Scintigraphic arthritis evaluation expressed using radionuclide binding index

Groups	Number	Day 30	Day 90
A	11	4.31 ± 0.12	4.31 ± 0.12
B	11	5.06 ± 0.08*	4.05 ± 0.08†
C	5 + 5	4.69 ± 0.07†	5.70 ± 0.32*
D	5 + 5	5.07 ± 0.10†	4.61 ± 0.09†
E	5 + 5	5.37 ± 0.10†	4.36 ± 0.12†
F	5 + 5	5.87 ± 0.09*	4.35 ± 0.09†

This mean index was calculated on six areas vs animal skull reference. Values are mean ± SE. Statistical analysis was used to compare treated groups and group B vs group A (Students *t*-test).
* $P < 0.5$,
† not statistically significant.
Group A: Non-induced controls.
Group B: Adjuvant-induced controls.
Group C: Rat Cu-SOD treated.
Group D: Human Cu-SOD treated.
Group E: Bovine Cu-SOD treated.
Group F: Human Mn-SOD treated.

Table 6. Comparison of right/left side ratios

Groups	Number	Day 30	Day 90
A	11	1.02 ± 0.01	1.02 ± 0.01
B	11	1.19 ± 0.10†	1.50 ± 0.22*
C	5+5	1.12 ± 0.13†	1.11 ± 0.11†
D	5+5	1.27 ± 0.12†	1.07 ± 0.11†
E	5+5	1.16 ± 0.80†	1.20 ± 0.12†
F	5+5	1.05 ± 0.04†	1.13 ± 0.14†

Student's t-test was used to compare treated groups and group B vs group A. Values are ratio ± SE.
*P < 0.05,
† not statistically significant.
Group A: Non-induced controls.
Group B: Adjuvant-induced controls.
Group C: Rat Cu-SOD treated.
Group D: Human Cu-SOD treated.
Group E: Bovine Cu-SOD treated.
Group F: Human Mn-SOD treated.

Total protein assay and electrophoresis profile

As the results of group B differed markedly from classical data, probably because of the particular genetics of this rat strain, experimental data appeared difficult to interpret. Change in total protein level and in electrophoresis profile of serum protein cannot be taken, as in human pathology, as a specific indicator of rheumatoid polyarthritis. Thus analysis of the total protein level and the electrophoresis profile cannot be used to determine the evolutive anti-inflammatory state.

Relationship between the level of blood anti-SOD antibodies and plethysmographic data

To study this relationship, we compared the level of anti-SOD antibodies, expressed in picograms of bound SOD per millilitre of serum, and the plethysmographic data, expressed as the percentage of mean change in volume at all time points. Correlation and regression analyses for the groups (D, E, F) producing antibodies revealed a significant linear relationship:

Group D; $r = 0.96$; $F = 93$; $P \leqslant 0.0001$.
Group E; $r = 0.96$; $F = 73$; $P \leqslant 0.005$.
Group F; $r = 0.97$; $F = 89$; $P \leqslant 0.005$.

In each group, values correspond to $r \geqslant 0.96$ and calculation of linear F confirmed the linear regression between the two parameters.

These correlations clearly show that anti-arthritic activity decreases when the blood level of anti-SOD antibodies increases.

DISCUSSION

It is now well established that oxygen free radicals play a fundamental role in the inflammatory process [13]. In fact, any tissue damage attracts polynuclear cells. In phagocyte vacuoles, O_2^- is released by a NADPH oxidase bound to membranes [14]. Excretion of O_2^- and its derived free radicals in extracellular medium might explain the inflammatory process and the microcirculation impairment [15, 16]. The O_2^- induces the lipidoperoxidation of

membrane lipids which are in this way released. Thi phenomenon produces chemotactic derivatives among them the albuminolipidic chemotactic facto reported by Petrone et al. [17]. The oxidizing catab olism of arachidonic acid induces eicosanoids bio synthesis, particularly prostaglandins vi cyclooxygenase and lipooxygenases pathways Experimental microcirculation alterations provoke by O_2^- induce platelet aggregation which in turi triggers arachidonic acid catabolism [18]. The for mation of reactive oxygen species and cytotoxi effects are summarized in the diagram of Kappu and Sies [19].

Thus, the current therapeutics of inflammation are generally limited to the inhibition of prostaglandir formation. Treatment using SODs seems to be a promising alternative since it breaks the sequence o free radical-induced events without modifyin immunological defences. SODs use will undoubtedly contribute to stop inflammation without conco mitantly decreasing essential functions of phago cytes.

Following the results obtained previously on acute models, our work consisted in showing the phar macological activity of SODs in a chronic inflam mation animal model. Treatment of chronic arthritis by SODs gave the following results:

(i) human and bovine Cu-SODs are fully active during secondary and tertiary arthritic reactions;
(ii) homologous rat Cu-SOD is active only tran siently at the end of the secondary reaction;
(iii) human Mn-SOD is active only at the second stage of secondary arthritic reaction.

Moreover, bovine and human SODs delay the appearance of bone damage. These results were confirmed by the scintigraphic study which showed the persistence of bone phospho-calcic metabolism during the quaternary arthritic reaction.

It appears that daily administration of SODs would inhibit the occurrence of bone damage. Thus, the use of this model enabled us to observe the therapeutic effects of SODs on evolutive and chronic poly-arthritis. Analysis of results corroborates data obtained on acute inflammation models [2,3]:

(i) the anti-inflammatory and anti-arthritic activity of bovine and human Cu-SODs;
(ii) the weak activity of human Mn-SOD;
(iii) the inefficacy of homologous rat Cu-SOD.

Thus the pharmacodynamic activity of exogenous SODs is limited to heterologous Cu-SOD in rat. A similar phenomenon may occur in man. Although all studies concord on the safety of this treatment, since SODs are proteins, the risk of immunological complications cannot be ruled out [20]. The use of human SODs would avoid this problem, but its pharmalogical activity in man remains to be established. It can be noted that in the present report, the doses of SODs used correspond to the clinical ones in a range from 30 to 150 $\mu g/kg$, though very much higher levels are used by other authors. Among the first clinical studies on inflammation therapy, must be mentioned the clinical report by Lund-Olesen and Menander [21], the clinical and experimental studies by Huber et al. [22] and other specific clinical studies

such as the one in which 101 subjects suffering from chronic polyarthritis were treated by Orgotein® (bovine Cu-SOD) at the dose of 8 mg (i.e. about 100–133 µg/kg) per day four times weekly during three consecutive months [23, 24].

The widespread concept that much larger amounts of drugs should be administered in animals than in man is perhaps true in chemotherapy, but not necessarily so in enzymotherapy. Other authors have often given SODs in rat at 5–50 mg/kg (i.e. 100–1000 times higher than usual clinical doses), for example in studies on ischemia [25]. The negative aspects of an excess of SOD with respect to protection *in vivo* are well established: increase in amount of i.v. injected SOD suppresses the radio-protective effects observed at low doses [26]. Negative effects or reduced efficacy at high doses have already been noted in previous experimental reports [2, 3, 27], and clinical studies [28]: a clear dose–response relationship is observed for doses ranging from 6.6 to 166 µg/kg (about 20 to 500 units/kg). Thus, previous different clinical and experimental studies allowed us to choose the dose of 33 µg/kg. This dose is situated in the optimal part of the dose–response curve determined by Michelson *et al.* [29] for bovine Cu-SOD. Evidently this curve will be displaced depending on the type of SOD used. The essential feature is the existence of a bell-shaped curve with the presence of negative dose–response relationships at high levels of SODs.

A number of conclusions may be drawn from the results obtained by different animal models of inflammation and clinical studies:

(i) different SODs have no identical anti-inflammatory activity;

(ii) anti-inflammatory activity is not a function of the nature of the metal at the active centre of the enzyme;

(iii) biological activity *in vivo* is not a direct function of the IP of the protein. An acidic IP of at least 4.8 or less is not generally associated with weak or no anti-inflammatory properties but IP does not necessarily confer activity. Furthermore the IP values do not rigorously reflect the real surface charge of a globular protein and a simple concept of positive or negative charges with respect to fixation at cell surface does not explain the anti-inflammatory activity;

(iv) anti-inflammatory efficacy is not a direct function of molecular weight (33,000–80,000) and size of SODs [2];

(v) differences in biological activity cannot be explained by differences in specific enzymatic activity since all the SODs have about the same value (3000 units/mg of protein) [2] and the doses we used for the four SODs are equal to 100 units/kg.

Thus we may speculate on the mechanisms of action responsible for the anti-inflammatory effects of heterologous SODs. Its mechanism of action is not linked to the pharmacokinetic behaviour of these molecules [27] and the anti-inflammatory property of a given Cu-SOD is not directly correlated with its plasma half-life [2], as shown by the wide differences in the activities of bovine, human and rat Cu-SODs, and human Mn-SOD. If plasma half-life is important,

then anti-inflammatory activity should be a function of the route of administration, i.e., s.c. > i.m. > i.p. > i.v. [29]. But Baret *et al.* have demonstrated that pharmacokinetic characteristics do not explain the activity of SODs [27]. However, Oyanagui *et al.* [30] recently noted an irritancy due to i.p. route as well as Baret *et al.* [27] and Jadot *et al.* [2], who observed a slight transitory pro-inflammatory activity with human Mn-SOD and a definitely pro-inflammatory effect with rat Cu-SOD. At all events the clinically useful route must be mainly the i.m. route for free enzymes, using levels of SOD corresponding to human clinical doses (about 60–100 µg/kg) rather than 3–30 mg/kg (i.e. 60–600 times higher than usual clinical doses) which could give rise to other pharmacodynamic effects. These high levels were used by Oyanagui *et al.* [30] who found a maximum edema suppression of only 30% in a dose range from 330 µg/kg to 2 mg/kg with homologous Cu-SOD in rat. In fact these authors have observed the efficacy of SODs and in particular homologous SODs for much higher doses than described in clinical trials and in experimental pharmacological studies of Michelson and co-workers; these latters report discrepant results, mainly in the rat tourniquet poditis model [31]. Oyanagui, using this ischemic model but with a different protocol, reported bell-shape suppression curves of edema with very dispersed data from only four animals for each dose.

Furthermore, there is no correlation between the degree of anti-inflammatory activity and the absolute level of circulating exogenous SOD [27]. The activity is indirect and unrelated to maintenance of high extracellular concentrations.

Given the amount of total endogenous SOD/kg of rat, it is clear that at the dose used (33 µg/kg) intracellular penetration in a general sense cannot be considered seriously. Studies with erythrocytes show that bovine, human and rat Cu-SODs are similar with respect to penetration into cells. The postulated effect by an increase intracellular SOD [32] is not tenable as explanation, particularly in clinical applications where at the most an increase of less than 0.001 of the total endogenous enzyme can be expected, of which less than 1% would in fact reach an intracellular localization.

Than it may only remain that the probable site of action of SODs is neither extracellular nor intracellular; it is more likely linked to the properties of membrane binding. In this respect, it has been demonstrated that protection of human fibroblasts against UV damage by bovine Cu-SOD is unchanged even when excess exogenous SOD is removed by washing [33] and that protection of mouse fibroblasts is shown by a perinuclear halo formation in presence of added exogenous SOD [34].

In agreement with Michelson *et al.* [29], the most likely explanation is that part of the injected SOD is attached to semispecific sites on external cell membrane. Homologous SODs of the host are not recognized by these sites and indeed membrane binding is possibly a function of non-homologous sequences in the protein. Such heterologous regions of the enzyme correspond well to an antigenic determinant site in each individual SOD important for the

activity. Thus, we demonstrated by measuring the relationship between anti-inflammatory activity and the level of blood anti-SODs antibodies that efficacy is linked to the heterologous protein sequence.

Moreover, the increased efficacy of liposomal bovine Cu-SOD compared to the free enzyme for the treatment of some disorders [35, 36], is thus based not only on generally improved pharmacokinetic properties and longer availability, but also on the all-important binding to cell surface, as shown in earlier studies [37]. We can include another argument: the very low range of active doses supports the hypothesis of a surface phenomenon.

In pathology, inflammation is the main symptom in a large number of diseases. Steroid anti-inflammatory drugs inhibit indirectly the activity of phospholipase A_2 by various polypeptides, for example macrocortine [38], whereas non-steroid anti-inflammatory drugs inhibit the activities of cyclooxygenase and also lipooxygenases [39]. A third process, that of oxygen free radicals, is as important as the other two. Indeed these radicals are able to maintain the inflammatory processes where they are not inhibited. SODs are natural systems of defence which limit the toxicity of these free radicals [40]. These enzymes are in particular able to inhibit acute and chronic inflammatory reaction. But the relative inefficacy of heterologous Mn-SOD raises a problem. All reports are in agreement with our observations. Perhaps the weak activity of heterologous Mn-SOD is related to its subcellular distribution in rat, where it is apparently located exclusively in the inner membrane and matrix space of the mitochondria [41].

Our work contributes to justify the choice of heterologous Cu-SOD. Thus we may better understand the efficacy of this therapeutic approach in inflammatory syndrome. It may be recalled, with regards to heterologous SODs activity, that heterologous calcitonin (e.g. from salmon) is much more effective in human than the human homologous polypeptide, and the concept that heterologous enzyme is beneficial, whereas homologous SOD is relatively inefficient, is not entirely new.

The high frequency and the difficulty in treating severe rheumatoid arthritis or polyarthritis have required the use of drugs such as D-penicillamine, methothrexate and cyclosporin, which present serious side effects and even toxicity. A recent open clinical study of treatment of severe rheumatoid arthritis using liposomal bovine Cu-SOD gave positive results with the advantage of no toxicity [42].

REFERENCES

1. Pearson CM, Development of arthritis, periarthritis and periostis in rats given adjuvant. *Proc Soc Exp Biol Med* **91**: 95–101, 1956.
2. Jadot G, Michelson AM, Puget K and Baret A, Anti-inflammatory activity of superoxide dismutases: inhibition of carrageenan induced edema in rats. *Free Rad Res Comms* **1**: 395–403, 1986.
3. Jadot G, Michelson AM and Puget K, Anti-inflammatory activity of superoxides dismutase: inhibition of adriamycin induced edema in rats. *Free Rad Res Comms* **2**: 19–26, 1986.
4. McCord JM and Fridovich I, Superoxide dismutase, an enzymatic function for erythroprotein. *J Biol Chem* **244**: 6049–6055, 1969.
5. Beauchamp CO and Fridovich I, Superoxide dismutases. Improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**: 276–287, 1971.
6. McCord JM, Boyle JA, Day ED, Rizzolo LJ and Salin ML, A manganese-containing superoxide dismutase from human liver. In: *Superoxide and Superoxide Dismutases* (Eds. Michelson AM, McCord JM and Fridovich I), pp. 129–138. Academic Press, London, 1977.
7. Jadot G, *Le Rat de Laboratoire, Tome I Réactif Biologique*, Masson, Paris, 1981.
8. Jouanneau M, Effets des immunosuppresseurs et des serums anti-lymphocytaires sur les polyarthrites expérimentales. Thesis of *Doctorat ès sciences*, Paris VI, 1971.
9. Dureng G, Chevalier H and Lamar J-C, Activité de quatre anti-inflammatoires sur l'arthrite à l'adjuvant de Freund. *IIème Réunion LVSO - Espagnole de Pharmacologie, Coimbra, Portugal, 25-28 May 1983* (personal communication), p. 589. Coimbra, 1983.
10. Paget M and Coustenoble P, Microélectrophorèse des protéines du sérum sur acétate de cellulose gélatineux. *Ann Biol Clin* **23**: 1209–1219, 1965.
11. Baret A, Michel P, Imbert MR, Morcellet JL and Michelson AM, A radioimmunoassay for copper containing superoxide dismutase. *Biochem Biophys Res Commun* **88**: 337–345, 1979.
12. Baret A, Schiavi P, Michel P, Michelson AM and Puget K, A radioimmunoassay for manganese containing superoxide dismutase. *FEBS Lett* **112**: 25–29, 1980.
13. McCord JM, A superoxide-activated chemotactic factor and its role in inflammatory process. *Agents and Actions* **10**: 522–527, 1980.
14. Babior BM, Oxygen-dependent microbial killing by phagocytes. Part I. *New Engl J Med* **298**: 659–668, 1978.
15. McCord JM, Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science* **185**: 529–531, 1974.
16. Sies H and Cadenase E, Biological basis of detoxication of oxygen free radicals. In: *Biological Basis of Detoxication* (Eds. Caldwell J and Jakoby WB), pp. 181–211. Academic Press, London, 1983.
17. Petrone WF, English DK, Wong K and McCord JM, Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma. *Proc Natl Acad Sci USA* **77**: 1159–1163, 1980.
18. Krinsky NI, Sladdin DG, and Levine PH, Effect of oxygen radicals on platelet functions. In: *Oxygen and Oxy-radicals in Chemistry and Biology* (Eds. Rodgers MAJ and Powers EL), pp. 155–160. Academic Press, New York, 1981.
19. Kappus H and Sies H, Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. *Experientia* **37**: 1233–1241, 1981.
20. Kelly K, Boux H, Petkau A and Sehon A, On the safety of treatment with bovine superoxide dismutase: production of a humoral antibody response in rabbits with repeated treatment. *Can J Physiol Pharmacol* **60**: 1374–1381, 1982.
21. Lund-Olesen K and Menander KB, Orgotein: a new anti-inflammatory metalloprotein drug: preliminary evaluation of clinical efficacy and safety in degenerative joint disease. *Curr Ther Res* **16**: 706–717, 1974.
22. Huber W, Menander-Huber KB, Saifer MGP and Dang PH-C, Studies on the clinical and laboratory pharmacology of drug formulations of bovine Cu-Zn superoxide dismutase (Orgotein). In: *Perspectives in Inflammation: Future Trends and Developments* (Eds. Willoughby DA, Giroud JP and Velo GP), pp. 527–540. MTP Press, Lancaster, 1977.
23. Huber W, Saifer MGP and Williams LD, Superoxide

dismutase pharmacology and orgotein efficacy: new perspectives. In: *Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. Developments in Biochemistry* (Eds. Bannister WH and Bannister JV), pp. 395–407, Vol.11b. Elsevier, Amsterdam, 1980.

24. Menander-Huber KB, Orgotein in the treatment of rheumatoid arthritis. *Eur J Rheumatol Inflamm* **4**: 201–211, 1981.

25. Huhn W, Ostling E and Fellenius E, Ischemic damage reduced by bovine Cu superoxide dismutase in heart Rat. In: *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine* (Ed. Rotilio G), pp. 591–597. Elsevier, Amsterdam, 1986.

26. McCord JM and Wong K, Phagocyte produced free radicals: role in cytotoxicity and inflammation. In: *Oxygen Free Radicals and Tissue Damage*. Ciba Foundation Symposium 65 (new series). (Ed. Fritz Simons W), pp. 343–360. Elsevier, Amsterdam, 1979.

27. Baret A, Jadot G and Michelson AM, Pharmacokinetic and anti-inflammatory properties in the rat of superoxide dismutases (Cu-SODs and Mn-SOD) from various species. *Biochem Pharmacol* **33**: 2755–2760, 1984.

28. Flohe L. Biehl G, Hofer K, Kadrnka F, Koelbel R and Puhl W, Effectiveness of superoxide dismutase in osteoarthritis of the knee joint. Results of a double blind multicenter clinical trial. In: *Biological and Clinical Aspects of Superoxide and Superoxide Dismutases. Developments in Biochemistry* (Eds. Bannister WH and Bannister JV), pp. 424–430, Vol.11b. Elsevier, Amsterdam, 1980.

29. Michelson AM, Jadot G and Puget K, Mechanism of anti-inflammatory activity of superoxide dismutases. In: *The Biological Role of Reactive Oxygen Species in Skin*. (Eds. Hayashi O, Imamura S and Miyachi Y), pp. 199–210. University of Tokyo Press, Tokyo, 1987.

30. Oyanagui Y, Sato S, and Okajima T, Suppression of ischemic paw edema in mice, rats and guinea pigs by superoxide dismutases from different sources. *Free Rad Res Comms* **4**: 385–396, 1988.

31. Jadot G and Michelson AM, Comparative anti-inflammatory activity of different superoxide dismutases and liposomal SOD in ischemia. *Free Rad Res Comms* **3**: 389–394, 1987.

32. Freeman BA, Turrens JF, Crapo JD and Young SL, Liposome-entrapped superoxide dismutase and catalase augment enzyme activities in cultured endothelial cells and rat lungs and protect against oxygen toxicity. In: *Oxy-Radicals and their Scavenger Systems, Vol.11, Cellular and Medical Aspects*. (Eds. Greenwald RA and Cohen G), pp. 159–166. Elsevier Biomedical, New York, 1983.

33. Emerit I, Michelson AM, Martin E and Emerit J, Perinuclear halo formation as an indication of phototoxic effects. *Dermatologica* **163**: 295–299, 1981.

34. Emerit I and Michelson AM, Recent advances in Crohn's disease: Chromosomal breakage in Crohn's disease. In: *Developments in Gastroenterology, Vol. I* (Eds. Pena AS, Weterman IT, Booth CC and Strober W), pp. 225–229. Martinus Nijkoff, The Hague, 1981.

35. Niwa Y, Somiya K, Michelson AM and Puget K, Effect of liposomal-encapsulated superoxide dismutase on active oxygen related disorders. A preliminary study. *Free Rad Res Comms* **1**: 137–153, 1986.

36. Baillet F, Housset M, Michelson AM and Puget K, Treatment of radiofibrosis with liposomal superoxide dismutase. Preliminary results of 50 cases. *Free Rad Res Comms* **1**: 387–394, 1986.

37. Michelson AM and Puget K, Cell penetration by exogenous superoxide dismutase. *Acta Physiol Scand* suppl. **492**: 67–80, 1980.

38. Blackwell GJ, Carnuccio R, Dirosa M, Flower RJ, Parente L and Persico P, Macrocortine, a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature* **287**: 147–149, 1980.

39. Sandee I and Schlegel MD, General characteristics of nonsteroidal anti-inflammatory drugs. In: *Drugs for Rheumatic Disease* (Eds. Paulus HE, Furst DE and Dromgoole SH), pp. 203–226. Churchill Livingstone, New York, 1987.

40. Halliwell B, Biochemical mechanisms accounting for the toxic action of oxygen on living organisms: the key role of superoxide dismutase. *Cell Biol Int Rep* **2**: 113–128, 1978.

41. Tyler DD, Polarographic assay and intracellular distribution of superoxide dismutases in rat liver. *Biochem J* **147**: 493–504, 1975.

42. Richard P, Roux H, Mattei JP, Michelson AM and Jadot G, Etude clinique ouverte de la superoxyde dismutase liposomale dans la polyarthrite rhumatoïde sévère. *Thérapie* **44**: 291–295, 1989.