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# The suppressive effect of gelatin-conjugated superoxide dismutase on disease development and severity of collagen-induced arthritis in mice

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## SUMMARY

We studied the effect of superoxide dismutase (SOD) on murine collagen-induced arthritis (CIA), an animal model of human rheumatoid arthritis (RA). Among SOD derivatives studied, only gelatin–SOD conjugate which has prolonged half life *in vivo* was effective to suppress the development of CIA, while native SOD or gelatin carrier alone was ineffective. Interestingly, pyran polymer-conjugated SOD which also has a long half life showed no suppressive effect on the disease. No significant effect on immune response against type II collagen (CII) was found in any of the experimental groups. In addition, induction of suppressor cells was not detected in spleen or lymph node cells of the gelatin–SOD-treated group. Therefore, these results suggest that oxygen radicals may have an important role in the effector phase of the immune response to manifest this chronic autoimmune polyarthritis. Thus, the use of appropriate antioxidants for the treatment of human RA may be rationalized.

**Keywords** collagen-induced arthritis superoxide dismutase

## INTRODUCTION

The role of oxygen radicals in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (RA) has been debated for over a decade [1–3]. While there is little question that oxygen radicals are produced in inflamed rheumatoid joints [4–6], the lack of success of antioxidant therapy and difficulties in quantifying oxygen radical production and damage *in vivo* have made it difficult to establish unequivocally whether these radicals play any significant role in the disease process [3]. Since a free radical is known to damage lipids, protein and DNA, causing chronic inflammation and mutagenesis leading to hyperplasia and cancer, it is reasonable to assume oxygen radicals cause damage to connective tissues such as collagen, cartilage and bone, and also alter the metabolism of chondrocytes and synovial cells composing the joint microenvironment.

Collagen-induced arthritis (CIA) is a chronic polyarthritis induced in rodents and monkeys by immunization with type II collagen (CII) abundant in cartilage [7,8]. This is well known as a useful and possibly the best experimental model of RA so far developed. In this model, humoral and cell-mediated autoimmune responses to CII seem to be involved in the development of arthritis. Actually, anti-CII antibody or T cells, or preferably antibody and T cells together could transfer the disease to naive animals [9–11]. However, the passive disease is usually mild, and inflammatory cells infiltrated in the joints of actively induced CIA contain many polymorphonuclear leucocytes (PMN) as well as lymphocytes in rats [12]. The infiltration of PMN is more dominant in mice and usually begins as early as 7–10 days after immunization, remaining even as late as 60 days of arthritis.

In order to evaluate the role of oxygen radicals in CIA, we administered superoxide dismutase (SOD) to see the effect on the development of the disease. In the present study, gelatin–SOD which had a long *in vivo* half life showed significant reduction of the disease without affecting the anti-CII immune response.

These results suggest that superoxide anion, possibly derived from infiltrated PMN in the joint microenvironment, is involved in the pathogenesis of CIA together with immunological mechanism(s) by lymphocytes.

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## MATERIALS AND METHODS

*Animals*

Female DBA/1 mice, 7–8 weeks old, were purchased from Seiwa Breeding Company for Experimental Animals (Fukuoka, Japan) and used throughout the study.

*Type II collagen*

Human CII was purified from costal cartilage according to the method reported by Miller [13].

*Induction of CIA*
According to a modification of the method of Courtenay *et al.* [8], mice were immunized intracutaneously in the right hind footpad with CII (200 μg/mouse) emulsified in Freund's complete adjuvant (FCA). Three weeks later, these mice were injected intracutaneously into the tail root with the same amount of CII in Freund's incomplete adjuvant (FIA). After the second immunization, mice were examined daily for the onset and severity of arthritis.

*Anti-CII antibody response*
ELISA was used to titrate serum antibody against CII [14]. Rabbit anti-mouse IgG conjugated to alkaline phosphatase (Tago Inc., Burlingame, CA) was used as the second antibody. Sera were collected on day 35 after primary CII immunization and stored at −20°C until the assay.

*Anti-CII cell-mediated immunity*
DTH against CII was measured as a parameter of cell-mediated immunity [15]. CII antigen was challenged on day 20 and the DTH response was measured 48 h later using microcalipers.

*SOD and its polymer conjugates*
Native SOD (recombinant human Cu, Zn-SOD), pyran–SOD (I) (mol. wt 41 000), pyran–SOD (II) (mol. wt 65 000), succinyl gelatin-conjugated SOD (referred to as gelatin-SOD, gelatin content 55·7%, mean mol. wt 110 000), succinyl gelatin and polymer gelatin were used. Pyran-conjugated SOD was prepared by the reaction of SOD and pyran copolymer anhydride in an aqueous milieu as described previously [16].

Gelatin–SOD was prepared as follows. In order to block free amino group and increase solubility, the gelatin was succinylated with excess amount of succinic anhydride as described previously [17]. The degree of succinylation of gelatin was 98·0%. Succinyl gelatin (160 mg) was dissolved in 5 ml of 0·1 M potassium phosphate buffer (pH 6·0) containing 50 mg of SOD, and then 100 mg of 1-ethyl-3-(3-dimethylamino propyl) carbodiimide were added. The reaction continued for 1 h at room temperature and for 16 h at 4°C under stirring. It was then concentrated to about 2 ml using an Amicon system (YM-30 membrane) and chromatographed on a column of Sephacryl S-200 (3·5 × 70 cm) (Pharmacia-LKB, Uppsala, Sweden) with 50 mM potassium phosphate buffer (pH 7·0). The eluted solution was collected in fractions of 5 ml and the absorbance at 280 nm was measured. Fractions 50–70 were pooled and used as gelatin–SOD. Fractions containing the conjugates were washed repeatedly with 10 times distilled water by ultrafiltration and the final concentrate of 5 ml was lyophilized. This process gave 64·5 mg of gelatin–SOD. The amount of SOD and its derivatives given were determined on the basis of specific SOD activity.

*Administration of SOD derivatives*
Mice were injected intraperitoneally every other day with each derivative of 200 U of SOD activity per mouse for 15 days, starting on day 0 (the day of CII immunization) (3000 U in total amount). Gelatin contained in 200 U of gelatin-SOD was 0·15 mg, being equivalent to 0·09 mg of polymer gelatin. Seven to 10 mice were used in each group.

*The assay for suppressor activity of lymphocytes*
Suppressor cell activity was evaluated by suppressive effect on indirect plaque-forming cell (IgG anti-CII PFC) detected in the culture of lymph node cells obtained from mice immunized with CII 3 weeks previously. Lymph node cells ($1{\cdot}5 \times 10^7$) were cultivated for 14 days with CII (20 μg/ml) and varied numbers of cells described below. Spleen or lymph node cells derived from animals treated with various SOD derivatives were tested for their suppressive effect, and the cells from mice orally administered CII were used as a positive control of the suppressor cells. For oral administration, each mouse was given 500 μg CII (0·2 ml/mouse), twice a week for 5 weeks (5 mg/mouse in total). PFC assay for anti-CII IgG antibody was done using ELISA-plaque assay [18]. We previously found that suppressor cells were induced in spleen of mice administered with CII orally by gastric tube, and these cells were Thy-$1^+$, Lyt-$1^+$, Lyt-$2^+$, L3T4$^-$ as revealed by the treatment with specific antibody plus complement (K. Kakimoto, unpublished data).

*Evaluation of arthritis*
Clinical evaluation of arthritis was done according to the method of Wood *et al.* [19], a method originally developed for scoring adjuvant arthritis in rats. Lesions of the extremities distal to the elbow or knee were graded on a scale of 0–4, based on the number of joints involved and the degree of erythema and swelling. The maximal score was thus 12, because lesions on the injected foot were not included in the numerical score. Histological examination was done by killing mice on day 56 and by staining with haematoxilin–eosin. Histopathological findings of arthritis were classified as mild, moderate, or severe based on the following criteria [20]: mild, minimal synovitis, cartilage loss, and bone erosions at limited discrete foci; moderate, synovitis and erosions present but retains normal joint architecture; severe, synovitis, extensive erosions, and joint architecture disrupted.

*Paw swelling*
The thickness of each affected hind paw was measured using calipers. The results were expressed as the per cent of increment in paw width relative to the paw width before the onset of arthritis.

*Statistical analysis*
Statistical significance was analysed by Student's *t*-test and $\chi^2$-test.

## RESULTS

*Effect of SOD compounds on the incidence and severity of CIA*
As shown in Fig. 1a, gelatin–SOD showed a suppressive effect on the incidence of CIA (75% suppression) compared with that of the non-treated group. On the other hand, treatment with native SOD showed only a slight delay in the onset of the disease, if any. In this case, groups treated with native gelatin and polymer gelatin served as controls. Severity of arthritis of diseased mice was also reduced by gelatin–SOD treatment compared with other groups, although this effect was not so remarkable (Fig. 1b) as on the incidence. Paw thickness was also reduced in the group treated with gelatin–SOD (Table 1) compared with other groups. The suppressive effect of gelatin–SOD was further confirmed in another experiment, in which

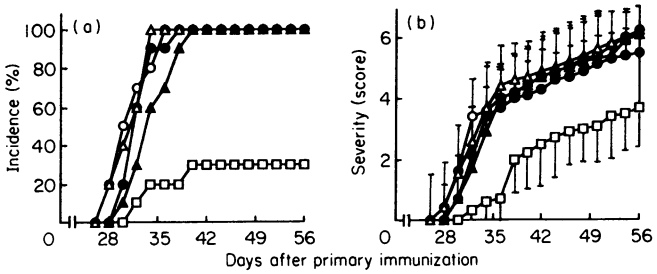


**Fig. 1.** Effect of various superoxide dismutase (SOD) derivatives on the incidence (a) and severity (b) of collagen-induced arthritis (CIA). Mice treated with PBS (○), native SOD (●), succinyl gelatin (△), polymer gelatin (▲) and gelatin–SOD (□). Ten mice were used for each group. Suppression of incidence by gelatin–SOD is statistically significant ($P < 0.05$). Bars show s.e.m.

other mice including those treated with pyran–SOD(I) or pyran–SOD(II) showed no effect (Table 1). Histological examination also showed that mice treated with gelatin–SOD developed milder synovitis than other groups (Table 1, Fig. 2a, b, c).

*Effect of SOD on the immune response to CII*

Since it is known that anti-CII immune responses are responsible for the development of CIA, we studied the effect of the administration of SOD derivatives on the immune response against CII. As shown in Fig. 3, no significant difference was detected between each of the groups tested in serum antibody titre (Fig. 3a) as well as in cell-mediated immunity (Fig. 3b).

*Effect of SOD derivatives on suppressor cell induction*

The involvement of suppressor T cells in the suppressive effect of gelatin–SOD on CIA was studied, focusing on the regulatory effect of the spleen and the lymph node cells of mice treated with various SOD derivatives on the anti-CII antibody response of the cultured lymph node cells from CII-immunized DBA/1 mice. As demonstrated in Fig. 4, the splenic cells induced by oral administration of CII revealed a suppressive effect on anti-CII IgG PFC response of the CII-immunized lymph node cells stimulated with CII in culture. In contrast, neither spleen nor lymph node cells treated with native SOD or gelatin–SOD, as well as gelatin alone (controls), showed any suppressive effect in this assay system.

## DISCUSSION

Several lines of evidence exist for the increased generation of oxidants *in vivo* in patients with active RA, but the contribution of these oxidants to the disease process is still uncertain [21–24]. The clinical and pathological resemblance of CIA to human RA led CIA to become one of the most useful animal models of this intractable autoimmune disease [7,8]. Previously, Beauchamp *et al.* gave a brief account of the use of polyethylene glycol (PEG)-conjugated SOD in rat CIA, showing some efficacy [25]. The present study used pharmacologically much improved SOD derivatives as well as native SOD. Among the various polymer–SOD conjugates, we found gelatin–SOD to be one of the best in view of the least inactivation of protease inhibitor ($\alpha_1$-PI) (Kojima *et al.*, unpublished data). We have recently demonstrated that native and PEG–SOD conjugates exhibit rapid inactivation of $\alpha_1$-PI following the liberation of $Cu^{++}$ from Cu, and Zn–SOD upon exposure of the SOD to $H_2O_2$, the reaction product of SOD plus superoxide ($O_2^-$) [26].

It was found that gelatin–SOD showed a suppressive effect on the incidence of CIA, while neither native SOD nor two different types of pyran–SOD conjugates (I and II) showed any such effect. This different effect may not reflect their turnover rate *in vivo*. The half life of native SOD was shown to be about 5 min, whereas those of pyran–SOD (I) and gelatin–SOD were six times longer. Actually, pyran–SOD was much more effective in protecting mice from the pathological effect of influenza virus

**Table 1.** Effect of various superoxide dismutase (SOD) derivatives on collagen-induced arthritis (CIA)*

| Parameter | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | None | Native SOD | Pyran–SOD (I) | Pyran–SOD (II) | Succinyl gelatin | Polymer gelatin | Gelatin–SOD |
| *Clinical evaluation* | | | | | | | |
| 1. Incidence of arthritis | 18/18 | 16/16 | 7/7 | 8/8 | 10/10 | 10/10 | 5/18 |
| 2. Day of onset | 26·1±1·2 | 28·6±1·5 | 29·3±1·1 | 31·2±1·0 | 26·5±1·6 | 29·3±1·8 | 30·2±1·3 |
| 3. Clinical score | 4·7±1·0 | 4·6±1·2 | 4·9±1·5 | 5·0±1·0 | 5·0±1·3 | 4·9±1·8 | 2·7±1·7 |
| 4. Maximum paw swelling | 51±3 | 50±2 | 53±6 | 55±8 | 52±5 | 51±2 | 39±7† |
| *Histological evaluation* | | | | | | | |
| Total no. of joints assessed | 18 | 16 | 10 | 10 | 15 | 15 | 20 |
| 1. Mild, no. | 2 (11) | 2 (13) | 1 (10) | 2 (20) | 3 (20) | 2 (13) | 8 (40) |
| 2. Moderate, no. | 5 (28) | 2 (13) | 3 (30) | 3 (30) | 5 (33) | 1 (7) | 7 (35) |
| 3. Severe, no. | 11 (61) | 12 (74) | 6 (60) | 5 (50) | 7 (47) | 12 (80) | 5 (25)‡ |

Paw thickness was measured every 2 days after the onset of arthritis. Joints from mice (five to 10 mice per group) that showed clinical arthritis were histologically examined (killed on day 48). Data for the day of onset, clinical score and maximum paw swelling are mean+s.e.m. of seven to 10 mice. Data in parentheses indicate per cent.

* Cumulative results of two separate experiments.

† $P < 0.05$ ($\chi^2$-test; gelatin–SOD-treated *versus* other derivative-treated animals); ‡ $P < 0.01$ (two-sample *t*-test; gelatin–SOD-treated *versus* other derivative-treated animals).

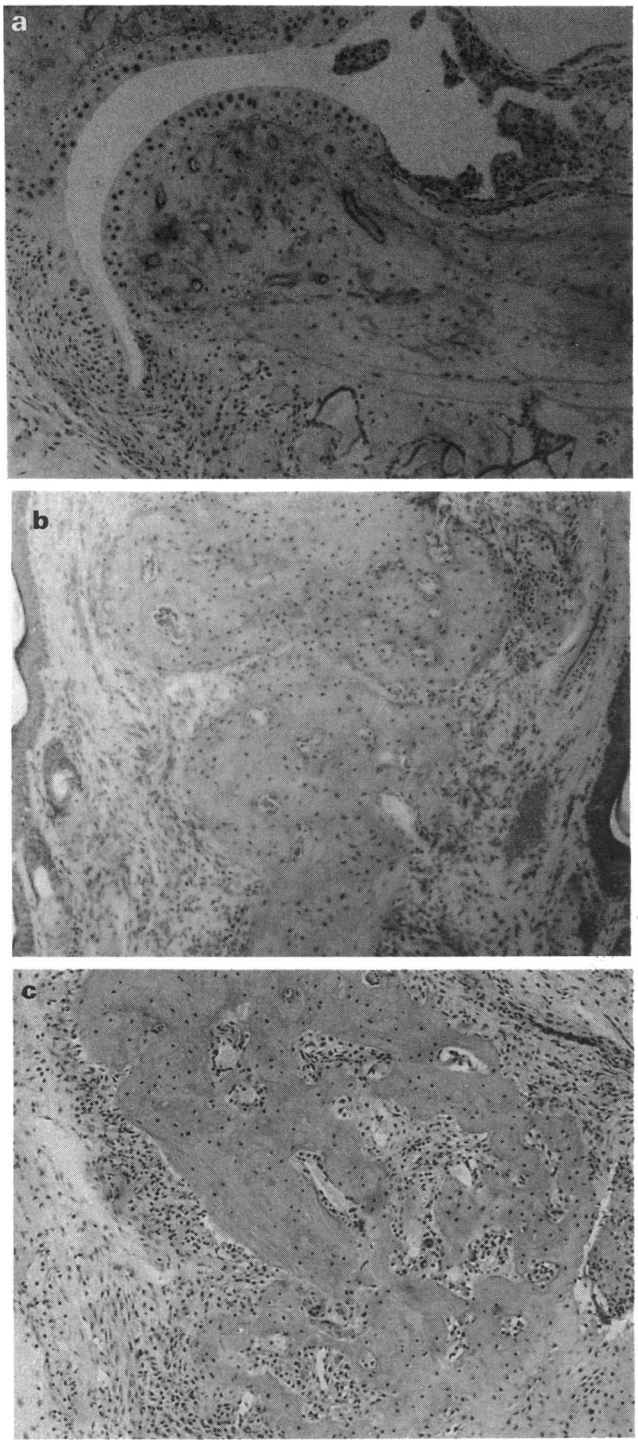


**Fig. 2.** Histological findings of collagen-induced arthritis (CIA) in the mice treated with various superoxide dismutase (SOD) derivatives. (a) Mild arthritis of mice treated with gelatin-SOD. (b) Moderate arthritis of mice treated with pyran-SOD. (c) Severe arthritis of mice treated with native SOD. See Materials and Methods for details.

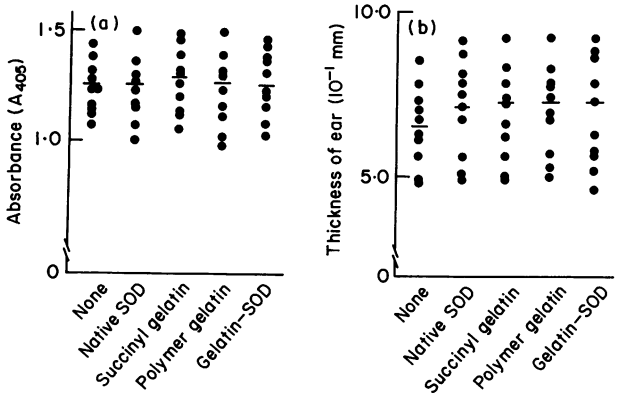


**Fig. 3.** Anti-type II collagen (CII) immune response of mice treated with various superoxide dismutase (SOD) derivatives as studied by ELISA for IgG antibody titre (a) and DTH response of the ear for cell-mediated immunity (b). No significant difference was observed among each group.

infection than native SOD [27]. Similarly, pyran-SOD did not inactivate $\alpha_1$-PI in the presence of $H_2O_2$, a great contrast to PEG- or native SOD. However, in CIA studied in this report, pyran-SOD showed no suppressive effect in spite of the same length of half life as that of gelatin-SOD (Fig. 1a). The reason for this discrepancy may be attributed to the fact that pyran-SOD induces host reaction such as induction of interferons, cytokines and activated macrophages, etc., although the molecular weight of pyran-SOD used in such an experiment was much higher than that used in our study [28]. Other factors than the half life, such as the nature of carrier (pyran and gelatin), especially the difference in the immunogenicity, may be responsible for the different effect on CIA. Severity of arthritis was also reduced by gelatin-SOD (Fig. 1b), although not so remarkably compared with the effect on the incidence of the disease.

On the other hand, humoral and cell-mediated immunity against CII were not affected by the administration of gelatin-SOD nor of other derivatives compared with the non-treated group. These results suggest that the effect of gelatin-SOD

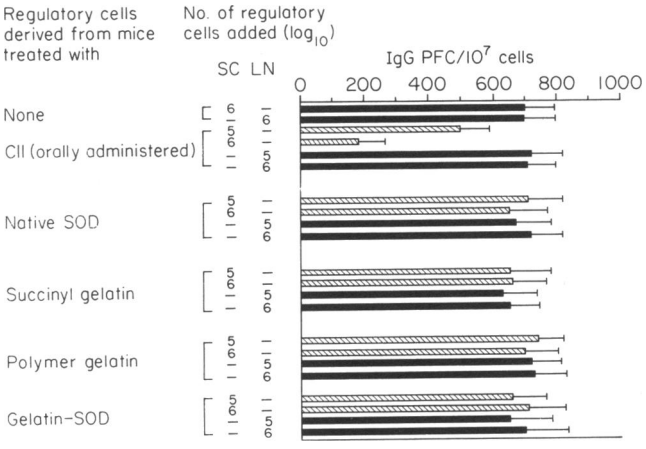


**Fig. 4.** Study for suppressive effect of the cells obtained from gelatin-superoxide dismutase (SOD)-treated mice on collagen-induced arthritis (CIA). Effect of the addition of spleen or lymph node cells from mice treated with various SOD derivatives on anti-type II collagen (CII) IgG plaque-forming cells (PFC) of CII-sensitized lymph node cells ($1·5 \times 10^7$ cells/well) stimulated *in vitro* with CII in culture was studied by ELISA plaque assay. Note that a clear suppressive effect was shown by the spleen cells from mice which received oral administration of CII, while no suppressive effect was revealed by lymphocytes of the mice treated with various SOD derivatives. SC, Spleen cells; LN, lymph node cells.

might be attributable to the efferent pathway of autoimmune response to CII. However, gelatin, a denatured form of type I collagen, is known to contain a cross-reactive epitope with type II collagen [29]. It is possible that the immune response to the gelatin carrier itself might evoke the suppressor T cells which may be responsible for the suppressive effect of gelatin–SOD, where SOD becomes carrier and gelatin, epitopic determinant [30]. This is unlikely, however, since the administration of gelatin–SOD did not affect the anti-CII immune response. Consistently with this notion, no suppressor cell activity was detected in the spleen or lymph node cells of the animals treated with any SOD-related compounds, while clear suppressor cell activity was revealed in the spleen cells of mice orally administered native CII antigen (Fig. 4).

These results suggest that gelatin–SOD itself has the suppressive effect that has nothing to do with the afferent immunological mechanisms. Several possibilities exist to explain this. For example, predominant infiltration of PMN is noted in animals with collagen arthritis (CA) [12]. These PMN should generate active oxygen species in the hosts with inflammatory reactions. Superoxide release and production of arachidonate metabolites by a certain number of PMN may serve as a signal to attract other PMN to the inflammatory foci, resulting in the vicious circle of inflammation as suggested by Petrone *et al.* [31]. SOD would remove superoxide and thus act as an antioxidant agent against the oxygen radicals. It is also possible that SOD protected polymer hyaluronic acid from degradation by the exposure to hydroxy radical derived from $H_2O_2$ and superoxide [32], and also protected CII in the joint cartilage from direct attack by these hydroxy radicals [33].

Whatever the reason for the effect of gelatin–SOD may be, the results reported in this study may provide a rationale for the use of gelatin–SOD as an antirheumatic agent. However, when SOD is used as a therapeutic agent, two problems should be taken into consideration. First is a possible deleterious effect, as reported recently. Namely, in the presence of $H_2O_2$, which is a reaction product of SOD, SOD could generate hydroxy radicals ($\cdot OH$) from $H_2O_2$ [26,33]. $\cdot OH$ has a potent chemical reactivity, and hence toxic effect against cells and tissues. In this regard, our demonstration of the inactivation of $\alpha_1$-PI brought about by Cu, Zn–SOD will have a significant consequence because this is a major inhibitor for elastase derived from PMN. Therefore, it would be beneficial to design SOD derivatives that are resistant to the inactivation by $H_2O_2$ and not generate $\cdot OH$. Second, it is important for SOD derivatives to have a long half life for the treatment of chronic diseases such as RA. A prolonged plasma half life would permit higher accumulation of the agent at the site of lesion, where vascular permeability is enhanced for macromolecules and enzymes [16].

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