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Treatment of adjuvant arthritis in mice with yeast superoxide dismutase

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Yeast Cu/Zn superoxide dismutase (SODy) was used for treatment of adjuvant-induced arthritis in mice. SODy was applied intraperitoneally (i.p.) in doses of 10 mg/kg (30000 U/kg) and 30 mg/kg (90000 U/kg) one or three times daily on consecutive days. It was very effective in reducing the paw swelling whether administered before or immediately after induction or when the treatment began at the onset of inflammation or at the peak of the arthritic process. The effect of yeast SOD was compared to that of commercial SOD from bovine erythrocytes (SODb), as well as with indomethacin treatment. Histological data confirmed the antiinflammatory effect of yeast SOD. The schedules and doses tested did not elicit anti-SOD antibodies in serum.

1. Introduction

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide, thus helping to protect cells from toxic by-products of aerobic metabolism. Reactive oxygen species, such as superoxide or hydroxyl radicals, are involved in the pathogenesis of various inflammatory disorders, including rheumatoid arthritis [1, 2]. SOD has been postulated to be useful as an anti-inflammatory agent and for reducing the tissue damage that occurs during ischemia reperfusion [3–8]. We have previously observed the inhibitory effect of Cu/Zn SOD from yeast cells and bovine erythrocytes on zymosan-induced inflammation in mice [9]. Data on the antiinflammatory properties of bovine Cu/Zn SOD have been reported by some authors who have reviewed the therapeutic benefit of bovine SOD in osteoarthritis, rheumatoid arthritis and periarticular inflammation [3, 5, 10]. Adjuvant arthritis (AA) is an experimental autoimmune disease in rats and mice that shares certain clinical and immunological features with rheumatoid arthritis (RA) in humans. This experimental model has been commonly used to study new anti-inflammatory and anti-arthritic drugs [11–14].

In this study we examined the effect of a new thermostable Cu/Zn superoxide dismutase from yeast cells (SODy) on joint inflammation during the development of arthritis and compared it to that of SOD from bovine erythrocytes (SODb).

2. Investigations and results

Adjuvant arthritis was induced by a single injection of heat-killed *Mycobacterium tuberculosis* into the right hind paw of ICR mice. This strain appeared to be highly susceptible as in this experimental model 80–85% of the animals developed paw inflammation about the 3rd day post-induction followed by arthritic alterations in the injected and in the contralateral paw.

When mice were treated with SODy at doses of 10 and 30 mg/kg on 3 consecutive days prior to adjuvant inoculation (−3, −2, −1), a significant reduction of paw thickness was observed until the 14th day (Fig. 1 A). SODy and SODb showed very similar effects in inhibition of arthritic manifestations. Only on the 3rd day at the higher dose of 30 mg/kg was SODb more effective than SODy. To assess the activity of SOD more precisely, paw swelling was measured on the 8th day (at the peak of inflammation) and on the 14th day (in the late phase of inflammation, when arthritic pathology has been already developed). The highest inhibition of paw swelling was observed in mice treated with SODb (30 mg/kg) on 3 consecutive days (−3, −2, −1) prior to *M. tuberculosis* (68.0% at day 8 postinduction) (Fig. 1 B).

In another set of experiments SOD was administered at the same doses of 10 and 30 mg/kg in 3 consecutive days (+1, +2, +3) after the adjuvant challenge. A significant decrease in paw thickness was observed between the 3rd and 14th day of inflammation after treatment with SODy and SODb (Fig. 2 A). The best effect was achieved with

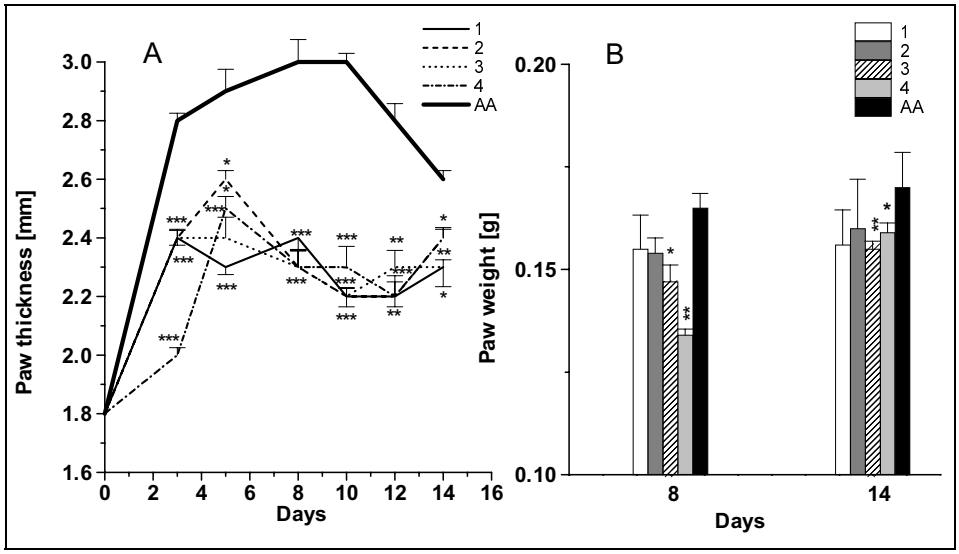

Fig. 1:
Decrease of adjuvant-induced edema in mice pretreated with SOD. Mice received 3 i.p. injections on consecutive (−3, −2, −1) days. Groups: 1. SODy 10 mg/kg; 2. SODb 10 mg/kg; 3. SODy 30 mg/kg; 4. SODb 30 mg/kg; AA − control. **(A)** The thickness of adjuvant-injected paws was measured every other day (n = 8). (**B**) Adjuvant-treated paws were cut at the ankle joint and weighed on the 8th and 14th day (n = 3). Data are presented as mean ±SEM. * − $p < 0.05$; ** − $p < 0.01$; *** − $p < 0.001$, Student's *t*-test

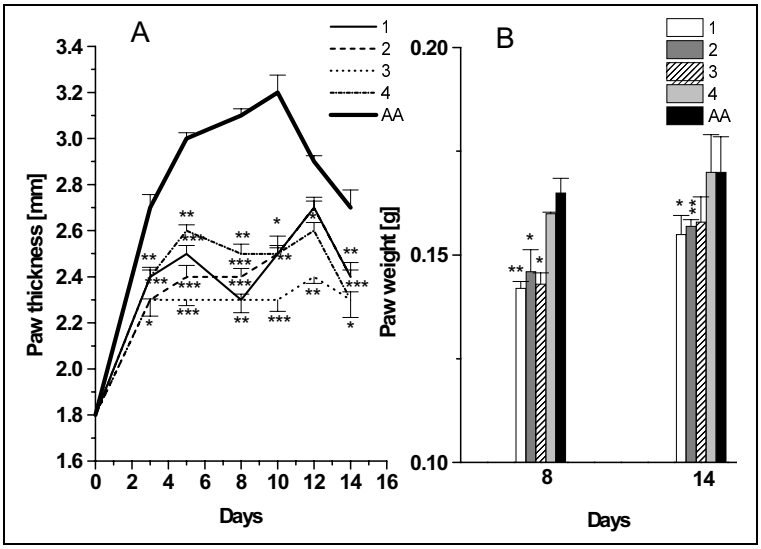

Fig. 2:
Decrease of adjuvant-induced edema after 3 i.p. injections of SOD on consecutive (+1, +2, +3) days. Groups: 1. SODy 10 mg/kg; 2. SODb 10 mg/kg; 3. SODy 30 mg/kg; 4. SODb 30 mg/kg; AA – control. **(A)** The thickness of adjuvant-injected paws was measured every other day (n = 8). (**B**) Adjuvant-treated paws were cut at the ankle joint and weighed on the 8th and 14th day (n = 3). Data are presented as mean ±SEM. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$, Student's *t*-test

SODy at 30 mg/kg. Both enzymes applied from days +1 to +3 at the lower dose of 10 mg/kg were very effective in reduction of paw weight (51.1% for SODy and 42.2% for SODb at day 8) (Fig. 2 B). SODb at the higher dose of 30 mg/kg did not alter the swelling.

Even a single injection of SODy and SODb at a dose of 10 mg/kg one day after the adjuvant challenge caused a statistically significant decrease in paw swelling (Fig. 3 A, B).

SODy treatment was also effective in reducing formation of paw edema when started after the development of arthritis. When SODy was administered on 3 consecutive days at a dose of 10 mg/kg starting on the 3rd day postinduction (the initial inflammation was already evident), the decrease of paw swelling on day 8 was 34% and on day 14 was 56% (Fig. 4 A, B). Even when SODy treatment started from day 8 postinduction (when arthritic pathology already existed), a 55% inhibition in paw swelling was found on day 14.

To assess the antiinflammatory properties of SODy, the well known nonsteroidal drug indomethacin (IM) was included in the next experiment. A dose of 10 mg/kg SODy and IM was administered one or three times (+1, +2, +3 days) postinduction. As shown in Fig. 5 A and B, the effect of SODy was weaker than that of IM, especially after a single application. When both agents were administered on 3 consecutive days their activity was very similar.

Adjuvant-injected paws developed massive cellular infiltration consisting of leukocytes, lymphocytes and histiocytes in the loose connective tissue around the blood vessels and haversian canals on the 8th day. In mice which received 3 consecutive treatments with SODy such infiltration was not observed although a slight hemorrhage was evident (data not shown). On day 14 postinduction the inflammation in control arthritic mice was severe with dense cellular influx and large hemorrhages (Fig. 6 A). In contrast, no signs of inflammation and tissue destruction were found in SODy-treated mice (Fig. 6 B). In adjuvant-injected mice on day 8 numerous macrophages in the white pulp of the spleen were seen, while no histological changes were found in SOD-treated mice (data not shown).

To test for the presence of anti-SOD antibodies that might have been produced in response to therapy, sera from arthritic mice (4 per group) were collected individually on days 7, 14, 21 and 28. There were no anti-SODy or anti-SODb antibodies (IgG and IgM) detected by enzyme-linked immunosorbent assay (ELISA) in the sera from mice treated on 3 consecutive days with either enzyme at doses of 10 and 30 mg/kg.

3. Discussion

Our results demonstrated that i.p. administration of yeast Cu/Zn SOD significantly inhibited inflammation in murine AA. The effects were similar whether enzyme was admi-

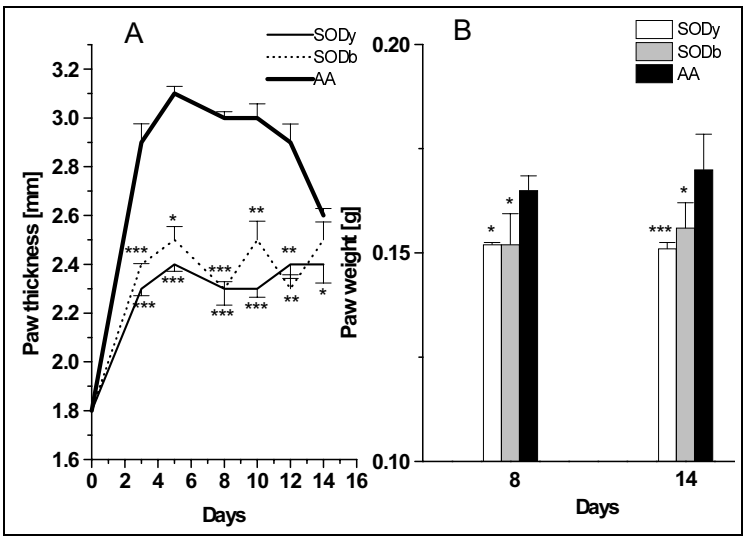

Fig. 3:
Decrease of adjuvant-induced edema after a single i.p. injection with SOD at a dose of 10 mg/kg on the next day after adjuvant inoculation. (**A**) The thickness of adjuvant-injected paws was measured every other day (n = 8). (**B**) Adjuvant-treated paws were cut at the ankle joint and weighed on the 8th and 14th day (n = 3). Data are presented as mean ±SEM. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$, Student's *t*-test

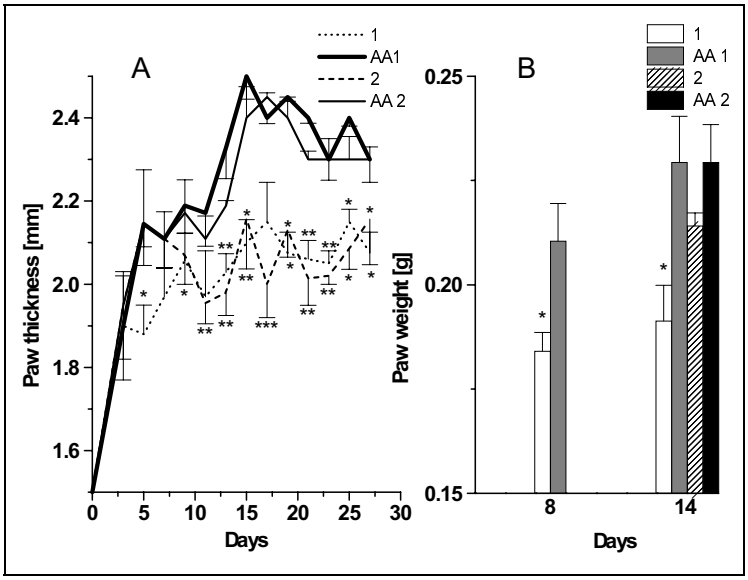

Fig. 4:
Decrease of adjuvant-induced edema when treatment with SODy (10 mg/kg) started after the onset of inflammation. Groups: 1. from the 3rd day postinduction (+3, +4, +5); AA1 − control; 2. from the 8th day postinduction (+8, +9, +10); AA2 − control. **(A)** The thickness of adjuvant-injected paws was measured every other day (n = 8). (**B**) Adjuvant-treated paws were cut at the ankle joint and weighed on the 8th and 14th day (n = 3). Data are presented as mean ±SEM. * − $p < 0.05$; ** − $p < 0.01$; *** − $p < 0.001$, Student's *t*-test

nistered before or after adjuvant inoculation and at both doses used (10 and 30 mg/kg). The doses approximated to those reported in the literature [7, 15]. They were well tolerated after multiple administration without any side effects which was in accordance with data for bovine and recombinant human SOD after intravenous or intramuscular route of application to healthy volunteers [15, 16].

The anti-inflammatory activity of SODb on polyarthritis in rats has been reported by Vaille *et al*. [17], who has established that heterologous but not homologous SOD are active [17–19].

Our comparison between SODy and SODb showed that both enzymes had a similar therapeutic potential when used before and immediately after adjuvant inoculation. SODy also inhibited paw swelling when the treatment started after the onset of inflammation (from the 3rd day) or after the detection of arthritic alterations (from the 8th day). Since SODy treatment appeared to be effective in all schedules tested it could be suggested that the enzyme affected the initial stage of inflammation as well as an arthritic process already developed. Our histological studies also supported the antiinflammatory effect of SODy in this model of AA.

IM is one of the most commonly used nonsteroidal drugs for treatment of inflammatory disorders. It inhibits the cyclooxygenase pathway thus preventing prostaglandin and thromboxane synthesis [20]. IM has been reported to inhibit joint inflammation and to reduce bone lesions in AA [21, 22]. However, long-term administration of IM is strongly associated with toxic effects, the major being gastrointestinal ulceration [23–25]. According to our own experience, application of a high dose of IM to mice (10 mg/kg i.p. daily for more than 6 days) resulted in 40% mortality (data not shown). In this model of inflammation SOD and IM exhibited fairly similar efficacy in suppressing the intensity of joint swelling.

We did not detect any immunological reaction (antibodies) following SOD administration at the schedules and doses tested. In contrast, Vaille et al. found a decrease of SOD treatment efficacy after multiple application (more than 10 times) due to formation of significant anti-SOD antibody response [17].

In our previous experiments on a model of acute inflammation (zymosan-induced paw edema in mice) SODy proved to be less effective than SODb, although it powerfully inhibited both classical and alternative pathways of complement activation and caused a strong decrease of C3 functional activity [9].

Exogenous SODy might influence chronic arthritic inflammation by two possible pathways. As the enzyme has been postulated to bind to the cell surface [26], the direct effect is an enhanced scavenging of the superoxide radi-

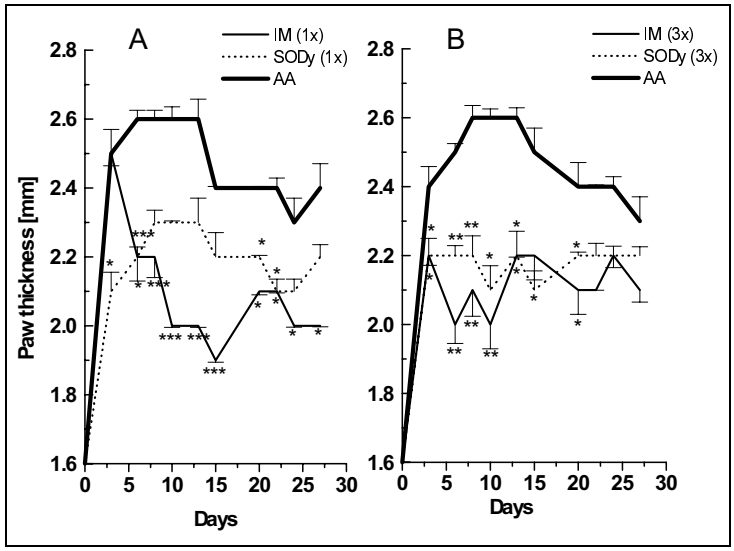

Fig. 5:
Comparison between SODy and IM in reduction of adjuvant-induced edema formation. Mice received (**A**) a single (+1 day) or (**B**) triple (+1, +2, +3 days) i.p. injections of SODy or IM at a dose of 10 mg/kg. Data are presented as mean ±SEM (n = 8). * − $p < 0.05$; ** − $p < 0.01$; *** − $p < 0.001$, Student's *t*-test

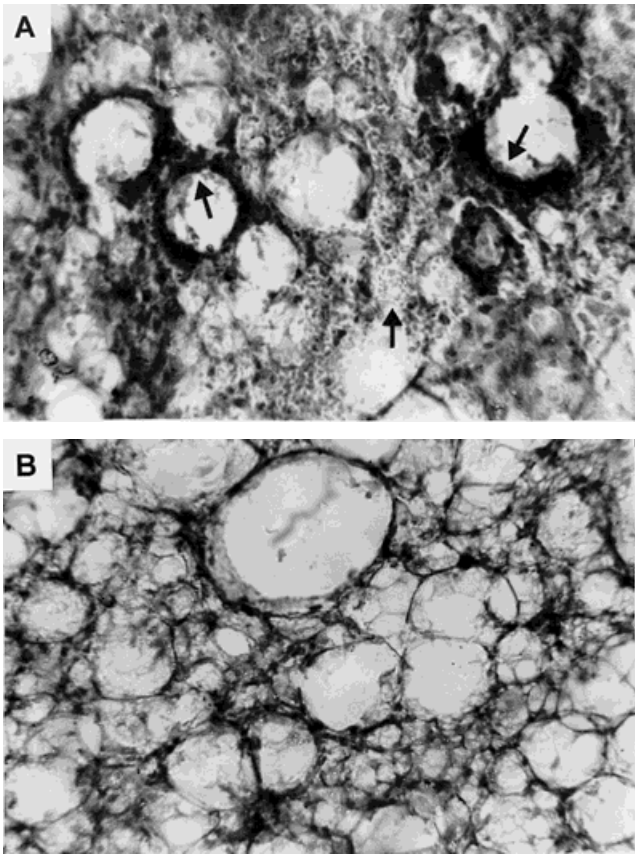

Fig. 6: Hystological section of the mouse ankle joints on the 14th day after induction of AA. (**A**) Dense cellular infiltration (lymphocytes, histiocytes, leukocytes) and tissue inflammation (arrows) among the haversian canals with marked hemorrhage in control arthritic mice; (**B**) No change around the haversian canals and no sign of inflammation in mice treated with 10 mg/kg SODy in 3 consecutive days postinduction. Hematoxylin and eosin staining. Magnification: × 250.

cals released continually by macrophages and polymorphonuclears into the extracellular space of the synovium. This leads to reduced tissue damage in the joint. The possible indirect mechanism affects numerous inflammatory mediators such as nitric oxide, complement fragments, PGE_2, cytokines, etc. It is well known that NO is another important effector molecule responsible for tissue damage in rheumatoid arthritis. Overproduction of human Cu/Zn SOD in transgenic mice has been reported to decrease the release of NO by macrophages, suggesting that oxygen radical and nitro compound metabolisms are closely related [27]. It is likely that SOD treatment of AA influences the existing imbalance between the levels of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8 and anti-inflammatory ones (IL-4, IL-10, IL-13, TGF-β). The possible complex mechanism of SOD action in AA needs additional elucidation. Further studies on the effect of SOD treatment on the level of proinflammatory cytokines might be important and are in progress.

4. Experimental

4.1. Materials

4.1.1. Superoxide dismutases

The Cu/Zn yeast-derived enzyme (SODy) was isolated from *Kluyveromyces marxianus v. bulgaricus* (National Bank for Industrial Microorganisms and Cell Cultures, NBIMCC No 1984, Sofia, Bulgaria) [28]. It possesses an iso-electric point of 6.95, a pH optimum in the alkaline region and is a glycoprotein containing a polysaccharide moiety of 1690 Daltons. The specific activity of the preparation is 3050 U/mg protein, determined by the cytochrome-C method. SOD from bovine erythrocytes – SODb (Serva) with specific activity of 3125 U/mg was included in some experiments for comparison.

4.1.2. Animals

Male ICR mice (14 per group) weighing 18–20 g, 6–8 week-old were obtained from our breeding facilities (Slivnitza, Bulgaria). They were housed in plastic cages with food and water *ad libitum*.

4.2. Methods

4.2.1. Induction and assessment of AA

Adjuvant arthritis was induced by a single intradermal injection into the right hind paw of 25 µl of heat killed *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI, 0.5% w/v) in liquid paraffin on day 0. Hind-paw edema was measured every other day for a period of 28 days using a calliper-guage. On the 8th and on the 14th day 3 animals per group were killed under ether anesthesia and hind paws were cut at the ankle joint. The degree of swelling of adjuvant-treated paws was determined by weighing.

4.2.2. Administration of SOD

SOD was administered i.p. in a volume of 0.4 ml in saline. Mice received single or triple injections of SODy or SODb on consecutive days at doses of 10 mg/kg (30000 U/kg) or 30 mg/kg (90000 U/kg). The treatment began before adjuvant injection (−3, −2, −1 day), on the next day (+1 − single application; and +1, +2, +3 − triple application), on the 3rd day (+3, +4, +5) or on the 8th day postinduction (+8, +9, +10). For each treatment schedule we included its own control group injected with saline instead of SOD.

4.2.3. Indomethacin treatment

SODy was compared with the known antiinflammatory agent indomethacin (IM, Sigma). Animals received single (+1 day) or triple (+1, +2, +3 days) i.p. injections with IM or SODy at a dose of 10 mg/kg.

4.2.4. Histological examination

The right hind limbs and spleens were removed and fixed in 10% neutral formaldehyde. The limbs were decalcified according to the method of Jennings [29], processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Cross-sections of the tibia near the *art. talocruralis* were evaluated.

4.2.5. Detection of anti-SOD antibodies

Sera from arthritic mice treated with SODy and SODb were collected weekly and tested for anti-SOD IgG and IgM antibodies by ELISA. A 96-well microtiter plate was coated with 2 µg/ml SOD in carbonate buffer. After blocking, test sera were added in 1/100 and 1/500 dilutions. Sera from arthritic mice non-treated with SOD served as negative controls. After incubation and rinsing, horseradish peroxidase conjugated antimouse IgG or IgM (Calbiochem) were added to the test samples and negative controls. o-Phenylenediamine (Sigma) was used as a substrate and the plate was read at 492 nm in a microplate reader.

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