Introduction

Superoxide dismutases (SODs) are metalloenzymes, which are essential for the defense against oxygen toxicity and have been considered as anti-inflammatory agents for reducing the tissue damage (Weber and Bruch, 1992; Bolli, 1991).

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown etiology that affects multiple joints leading to the destruction of cartilage and bones. Cytokines play indispensable roles in development and perpetuation of joint inflammation (Baret et al., 1984). There is an imbalance between pro- and anti-inflammatory cytokines both locally and systemically (Feldmann et al., 1996a; Arend, 2001).

A number of authors have reviewed the therapeutic benefit of SODs from different sources in osteoarthritis, RA, and periarticular inflammation (Weber and Bruch, 1992; Schulze-Koops and Kalden, 2001; Vervoordeldonk and Tak, 2002). In our previous investigations we have compared the effect of yeast Cu/Zn SOD to those of a commercial one from bovine erythrocytes and indomethacin in murine adjuvant arthritis (Ratcheva et al., 2000a). Histological data from arthritic joints have confirmed the anti-inflammatory activity of yeast SOD (Ratcheva et al., 2000b). Yeast SOD was superior to that from bovine erythrocytes in augmentation of arthritis and reduction of pathological alterations in the joints.

In the present work we report the effect of yeast SOD treatment on the production of proinflammation.
flammatory cytokines as well as oxygen and nitrogen metabolites in mice with adjuvant arthritis (AA).

**Material and Methods**

**Animals**

6- to 8-week-old ICR mice, weighing 18–20 g were used. Animals (12 per group) were maintained in the animal house of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria and were used according to the guidelines accepted by the Bulgarian Veterinary Health Control Service.

**Induction and assessment of AA**

AA was induced as previously described (Ratcheva *et al.*, 2000a) by a single injection into the right hind paw with 25 μl of heat-killed Bacillus Calmette-Guerin (BCG) (0.5% w/v) in liquid paraffin on day 0. The hind paw edema was measured every other day for a period of 28 d using a caliper gauge. On the 3rd, 8th and 14th day three animals per group were killed under diethyl ether anesthesia. Serum samples and peritoneal cells were collected. On the 8th and 14th day hand paws were cut at the ankle joint. The degree of swelling was determined by weighing.

**Administration of SOD**

The yeast Cu/Zn-ySOD (kindly provided by A. Kuyumdjieva, Faculty of Biology, Sofia University, Bulgaria) was isolated from a thermotolerant strain, *Kluyveromyces marxianus* var. *bulgaricus*. The enzyme had the specific activity of 3,000 U/mg protein, determined by the cytochrome-C method. Yeast SOD was administered intraperitoneally (i.p.) on 3 consecutive days starting on the day after AA induction (+1, +2 and +3) at a dose of 10 mg/kg body weight (30,000 U/kg).

**Superoxide production by murine peritoneal macrophages**

The concentration of superoxide radicals produced in the culture of peritoneal macrophages was determined by the SOD-inhibitable reduction of ferricytochrome C according to Leslie (1987). Briefly, washed, adherent peritoneal macrophages (2.4 × 10^5) were covered with 60 μl of phenol red-free Hanks’ balanced salt-solution (HBSS) and 100 μl cytochrome C solution (Serva, 75 μM), cytochrome C + phorbol 12-myristate 13-acetate (PMA) (200 ng/ml) or cytochrome C + PMA + SOD (from bovine erythrocytes; 200 U/ml, Serva). After 1 h, the absorbance at 550 nm was read in an ELISA reader (Organon Teknika), and the concentration of the released superoxide radical was calculated.

**Hydrogen peroxide (H_2O_2) production by murine peritoneal macrophages**

The method of Pick and Mizel (1981) was used. Briefly, cells collected by peritoneal lavage were washed, counted, and suspended in HBSS, containing 10% fetal bovine serum at a cell density of 4 × 10^5/ml. Cells were added to the wells of a 96-well plate (Falcon) (4 × 10^5/well). After incubation at 37 °C, 5% CO₂ for 2 h the non-adherent cells were removed by washing with HBSS without phenol red. The reaction mixture, containing 200 μg/ml phenol red and 50 μg/ml horseradish peroxidase type VI-A (Sigma) in HBSS, was added (0.1 ml/well). For *in vitro* stimulation, PMA (200 nm) was added to the cultures. After 45 min the reaction was stopped by addition of 10 μl/well 1 M NaOH, and the absorbance was read at 620 nm in an ELISA reader. For calculations, a standard curve with H_2O_2 concentrations from 5 to 50 μM was used.

**NO production by murine peritoneal macrophages**

The nitrite concentration in cultures was measured as an indicator of NO release. Washed macrophages (4 × 10^5/well) were cultivated for 72 h in cell culture medium, in the presence or absence of rm(IFN-γ) (100 U/ml) and LPS (1 μg/ml from *E. coli* 055:B5, Sigma). Then Griess reagent was added, the absorbance at 550 nm measured, and the nitrite concentrations were calculated using a standard curve with NaNO₂ concentrations from 200 to 3.125 μM.

**Measurement of the serum cytokines levels**

The levels of IL-6, IL-12, TNF-α, and IFN-γ in circulation were measured using ELISA kits (PeproTech Inc., Rocky Hill, NJ, USA) according to the manufacturer’s instructions. The sensitivity of the assays was 32 pg/ml for IL-12 and TNF-α, 16 pg/ml for IFN-γ, and 62 pg/ml for IL-6.
Results

Inflammation

Mice injected with BCG in liquid paraffin for induction of adjuvant arthritis were i.p. treated on three consecutive days with yeast SOD at a dose of 10 mg/kg, starting on the day after arthritic induction. In treated mice, a significant reduction in paw thickness was observed until the 14th day (Fig. 1A). To assess the activity of yeast 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). A significant decrease in the paw weight was observed in both phases of inflammation after yeast SOD treatment (Fig. 1B).

Superoxide production

Three mice from each group were killed on days 3, 8 and 14, peritoneal macrophages were isolated and their superoxide production was studied with and without an additional in vitro stimulation with 200 ng/ml PMA. Results in Fig. 2 show that spontaneous superoxide production in arthritic mice increased highly and this increase lasted till day 8. PMA-induced secretion showed such elevation only on the 3rd day post induction. Although the yeast SOD treatment did not influence the superoxide production in the initial phase of arthritic development, a substantial suppression of spontaneous as well as of PMA-induced superoxide radical secretion was observed on day 14 compared to non-treated arthritic animals and the levels detected were even below that in healthy mice.
Hydrogen peroxide production

As seen in Fig. 3, as a result of arthritic development the spontaneous H$_2$O$_2$ release from peritoneal macrophages was elevated compared to the controls on day 14 and their ability to respond to an additional in vitro stimulation with 200 nM PMA was increased from day 8 thereafter. Yeast SOD treatment substantially decreased the levels of both spontaneous (day 14) and PMA-induced H$_2$O$_2$ secretion at all times, and they differed significantly from the control values.

NO production

Peritoneal macrophages from mice with adjuvant arthritis were studied for their ability to produce NO. Results in Fig. 4 show that very early in the development of inflammation (from day 3) the spontaneous NO secretion increased and remained elevated at least till day 14. A significant increase in IFN-$\gamma$ + LPS-stimulated NO release was detected in arthritic mice compared to healthy controls only on day 8 post induction. Yeast SOD treatment effectively reduced both spontaneous and stimulated NO secretion in arthritic mice on day 14.

Levels of proinflammatory cytokines in serum

Levels of IL-12, IFN-$\gamma$, IL-6 and TNF-$\alpha$ were measured in serum of mice after triple administration of yeast SOD starting on the day after induction of adjuvant arthritis. As can be seen in Fig. 5, a significant decrease in serum levels of all cytokines was detected during the whole period of observation. The only exception was IFN-$\gamma$, which showed no significant decrease on day 8 after arthritic induction.

Discussion

Our investigations revealed some of the mechanisms of action of yeast SOD in the inhibition of acute and chronic inflammation in mice with adjuvant arthritis. Previously, SODs from different sources have been applied with dependable success in various experimental models of rheumatoid arthritis. The empirical treatment has not included investigation of their influence on the important factors of inflammation. Our experiments showed that inhibition of the paw edema formation and the arthritic alterations in the joint tissues are a result of reduced secretion of proinflammatory cytokines.
flamatory cytokines and oxygen and nitrogen inflammatory mediators.

Chronic inflammation in rheumatoid arthritis is accompanied by an enhanced production of reactive oxygen species by infiltrating monocytes/macrophages and polymorphonuclear leukocytes. They damage lipids and hyaluronic acid, increasing the viscosity of synovial fluid thus making the movements in the affected joints difficult (Halliwell et al., 1988). The observed decrease in secretion of both superoxide radicals and $\text{H}_2\text{O}_2$ after yeast SOD treatment of mice with adjuvant arthritis correlates with an improved histology thus showing that this treatment directly reduces the joint tissue damage. Yeast SOD administration breaks the oxidation cascade in the affected joints. Besides that, it apparently substantially limits the activity of the existing positive feedback between the secretion of reactive oxygen metabolites and production of proinflammatory cytokines in different inflammatory conditions (Liu et al., 2001; Anrather et al., 2006).

Another important molecule, taking part in the pathogenesis of arthritis, is NO. Numerous data exist about increased NO levels in the serum and synovial fluid of patients with rheumatoid arthritis. The interrelationship between SOD and NO in vitro and in vivo has been poorly elucidated and the existing data are contradictory. Cu/Zn SOD in vitro enhances the production of NO by macrophages eliminating the superoxide radical which is an inhibitor of its synthesis (Liew and Cox, 1991). From the other side, overexpression of human Cu/Zn SOD in transgenic mice results in a decrease not only in superoxide radical secretion but in NO production as well (Mirochnitchenko and Inouye, 1996). Our results showed that yeast SOD treatment significantly decreases the NO production in the late stage of inflammation.

As a potent inducer of IFN-$\gamma$, IL-12 participates in the induction phase of arthritis. Its blockade...
by anti-IL-12 monoclonal antibodies has not lowered the incidence of disease in experimental murine models, but has attenuated the severity of arthritis, both clinically and histopathologically (Mafait et al., 1998). In our experiments, attenuated arthritic inflammation after yeast SOD treatment was associated with decreased serum IL-12 levels.

There are contradictory data about the role of IFN-γ in different murine models of arthritis. Several investigators have reported that IFN-γ injection increases the incidence and accelerates the onset of arthritis (Mauritz et al., 1988; Nakajima et al., 1990; Weisenberg et al., 1989), whereas another group has reduced the severity of the disease by systemic administration of IFN-γ (Boissier et al., 1995). In our experiment, decreased serum levels of IFN-γ after yeast SOD application correlated with attenuated inflammation only in the late stage of arthritic development (14th day).

IL-6 is found in large quantities in synovial fluid and serum of RA patients (Uson et al., 1997). Furthermore, antibodies to the IL-6 receptor have been successfully used in the treatment of RA patients (Nishimoto et al., 2009). Our results showed that yeast SOD treatment of AA mice leads to a decrease in the serum IL-6 levels which is associated with attenuation of inflammation.

TNF-α is considered as a key mediator in the pathogenesis of rheumatoid arthritis and experimental models of arthritis in rodents. It is capable to induce other cytokines (for example, IL-1β and IL-6) that ultimately leads to cartilage and bone destruction. Anti-RA therapy by soluble TNFR or anti-TNF-α monoclonal antibodies has been reported to be effective in reducing symptoms of joint inflammation in humans (Lorenz et al., 1996; Choy and Panayi, 2001; Feldmann et al., 1996b). The serum levels of TNF-α as a cytokine important for the arthritic development were also reduced by yeast SOD treatment.

Our experiments showed that treatment of adjuvant-induced arthritis in mice with yeast SOD substantially decreases the levels of proinflammatory cytokines and this mechanism can explain the effect of SOD in reducing arthritic inflammation. The results presented prove the effectiveness of yeast SOD in inhibition of inflammatory processes in mice with adjuvant arthritis demonstrating its effect on some key factors in arthritic development – superoxide radicals, H₂O₂, NO, and proinflammatory cytokines.


