

## PROTECTIVE FACTORS AGAINST OXYGEN FREE RADICALS AND HYDROGEN PEROXIDE IN RHEUMATOID ARTHRITIS SYNOVIAL FLUID

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Oxygen free radicals are probably involved in the pathogenesis of rheumatoid arthritis (RA). The enzymes involved in protection against oxygen free radicals and  $H_2O_2$  (superoxide dismutase, catalase, and glutathione peroxidase) were measured. Superoxide dismutase was not increased, glutathione peroxidase was slightly and catalase was strongly elevated in RA synovial fluid (SF) compared with control SF. Although these enzymes are present in SF, the activities are insufficient to protect against oxygen free radicals and  $H_2O_2$ . In contrast to transferrin, ferritin was increased in RA synovial fluid. Ceruloplasmin was also elevated. When rat liver microsomes were used as a target for oxygen free radicals, serum and SF were both protective. Gel filtration experiments showed that the fraction pattern in which there was maximal protective potential against lipid peroxidation corresponded closely to the level of ceruloplasmin. After removal of ceruloplasmin from serum or SF, about 70% of the protective capacity disappeared. It is concluded that ceruloplasmin is an important protector against oxygen free radicals.

Evidence is accumulating which suggests that oxygen free radicals such as superoxide ( $O_2^-$ ) and

hydroxyl radical ( $OH^\cdot$ ), and related oxygen species such as hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen, are involved in the pathogenesis of rheumatoid arthritis (RA). During phagocytosis granulocytes and macrophages produce large amounts of  $O_2^-$  and  $H_2O_2$  (1). Oxygen free radicals destroy lipids by a process called lipid peroxidation. In synovial fluid (SF) from RA patients, degradation products of lipid peroxidation can be detected (2,3). In RA patients and in experimentally-induced arthritis in animals, superoxide dismutase (SOD), an enzyme destroying  $O_2^-$ , given systemically or locally, induces a decrease of inflammation (4,5). In RA hyaluronic acid is depolymerized, although no hyaluronidase is present in SF and the proteases in SF are unable to degrade hyaluronic acid. Oxygen free radicals have been shown to cause depolymerization of hyaluronic acid (6,7).

The deleterious effect of oxygen free radicals increases when iron is present. Although there is much debate about the exact mechanism, it is generally accepted that iron stimulates the formation of  $OH^\cdot$ , possibly by the reaction:  $O_2^- + H_2O_2 \xrightarrow{Fe^{3+}} OH^\cdot + OH^- + O_2$ . In most systems which have been investigated, this catalytic function of iron is blocked when it is bound to its specific binding proteins.

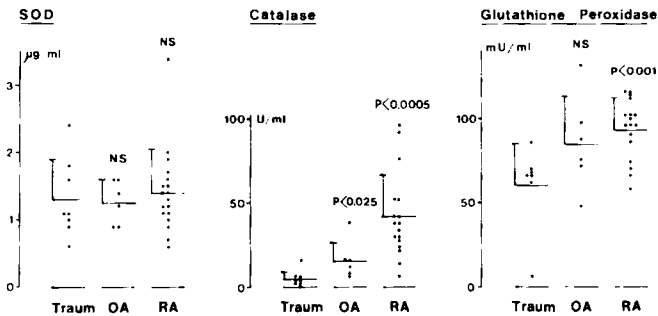
The large number of phagocytosing granulocytes present in RA SF will produce a considerable amount of oxygen free radicals and  $H_2O_2$ . The aim of this study was to investigate the protection in SF against oxygen free radicals and  $H_2O_2$ . The following proteins, known to protect in vitro, were measured in SF: 1) SOD, which catalyses the reaction  $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ ; 2) catalase and glutathione peroxidase, which enzymatically reduce  $H_2O_2$  to  $H_2O$ ; 3) ceruloplasmin, which scavenges  $O_2^-$ ; 4) transferrin and ferritin, which are iron binding proteins.

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**Figure 1.** Superoxide dismutase (SOD), catalase, and glutathione peroxidase activities in synovial fluid (mean  $\pm$  SD) of patients with traumatic knee lesions (Traum), osteoarthritis (OA), and rheumatoid arthritis (RA). *P* values for OA and RA versus Traum are shown; NS = not significant.

Lipid peroxidation of rat liver microsomes induced by oxygen free radicals was used as a model. The effect of oxygen free radicals in this system can be inhibited by serum and SF. Factors responsible for the inhibition were investigated.

## MATERIALS AND METHODS

**Synovial fluid and sera.** SF was obtained from 7 patients with traumatic knee lesions, 6 patients with osteoarthritis (OA) of the knee, and 17 patients with classic or definite RA as defined by the American Rheumatism Association criteria (8). Sera were also obtained from the last 2 groups. Heparin was added to the SF samples. They were then centrifuged for 10 minutes at 3,000 revolutions per minute and the supernatants were stored at  $-70^{\circ}\text{C}$ . Samples containing more than  $15\ \mu\text{g}$  hemoglobin per ml were excluded.

**Superoxide dismutase.** SOD activity was determined using the cytochrome C reduction inhibition method of McCord (9). The SOD concentration was expressed in  $\mu\text{g}$  using bovine erythrocyte SOD (Boehringer, Mannheim, FRG) as a standard (lot 1071101, 5,000 units/mg). Under the applied conditions, SF produced no spontaneous cytochrome C reduction. Ceruloplasmin at the concentration present in SF had no significant effect on the assay. SOD added to SF was determined to have a recovery of 89%, so no significant inhibition was present in SF.

**Catalase.** Catalase was measured by tracing the degradation of H<sub>2</sub>O<sub>2</sub> spectrophotometrically, according to the method of Aebi (10). The catalase activity in SF could be blocked effectively by  $10\ \mu\text{M}$  NaN<sub>3</sub>. Catalase added to SF was determined to have a recovery of 90%.

**Glutathione peroxidase.** Glutathione peroxidase content was determined according to the method of Paglia and Valentine (11). One unit corresponds to 1  $\mu\text{mole}$  NADPH oxidation per minute. The recovery of the enzyme in SF was 100%. Ceruloplasmin and transferrin levels were estimated by single radial immunodiffusion (12). Ferritin content was determined using an enzyme immunoassay (Ferrizyme, Abbott Laboratories, Chicago, IL).

**Measurement of inhibition of O<sub>2</sub><sup>-</sup> induced lipid peroxidation.** Lipid peroxidation of rat liver microsomes isolated from Wistar rats (13) was performed as follows: the incubation mixture contained (in final concentrations) 33 mM Tris-HCl (pH 6.8), 0.33 mM xanthine, 0.133 mg/ml dialyzed xanthine oxidase, 120  $\mu\text{M}$  FeCl<sub>3</sub>, 2 mM ADP, and 1 mg protein/ml microsomes. The final volume was 3 ml. The incubation was performed at  $37^{\circ}\text{C}$  and started by the addition of xanthine oxidase. At indicated times, samples were drawn and thiobarbituric acid-reacting products were measured (14).

Serum or SF was applied to a  $100 \times 1.6\ \text{cm}$  column of Sephadex G-200 and eluted at a flow rate of 6.5 ml/hour with 50 mM Tris-HCl (pH 7.4) with 0.1M NaCl. Fractions of the eluate were assayed for the capacity to inhibit lipid peroxidation of rat liver microsomes and for the concentration of transferrin and ceruloplasmin.

Serum and SF free from ceruloplasmin were prepared with a column of human ceruloplasmin antibody linked to CNBr-activated Sepharose 4B. Before chromatography SF was pretreated with hyaluronidase to decrease the viscosity. The eluate was dialyzed against 2 mM sodium phosphate (pH 7.0) and concentrated to the original protein concentration. The final preparations were checked for the presence of ceruloplasmin. Human ceruloplasmin type X was from Sigma Chemical Co., St. Louis, MO.

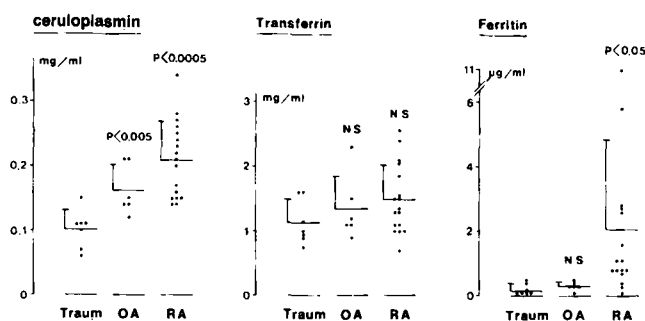
**Statistical analysis.** Significance of differences in concentrations was determined by Student's *t*-test.

## RESULTS

**Total protein.** SF from patients with traumatic knee lesions contained  $42 \pm 8\ \text{mg}$  protein/ml (mean  $\pm$  SD), SF from patients with OA contained  $49 \pm 13\ \text{mg}$  protein/ml, and RA SF contained  $56 \pm 7\ \text{mg}$  protein/ml.

**Superoxide dismutase, catalase, and glutathione peroxidase.** SOD activities were found to be as follows:  $1.2 \pm 0.6\ \mu\text{g/ml}$  in SF from patients with traumatic knee lesions,  $1.3 \pm 0.3\ \mu\text{g/ml}$  in OA SF, and  $1.4 \pm 0.6\ \mu\text{g/ml}$  in RA SF. No significant differences were found between these groups (Figure 1).

The catalase activity in SF from patients with traumatic knee lesions was low:  $3 \pm 2\ \text{units/ml}$ . In OA SF it was significantly increased to  $16 \pm 11\ \text{units/ml}$  ( $P < 0.025$ ), and in RA SF the highest activity was found:  $42 \pm 25\ \text{units/ml}$  (Figure 1). The concentration in RA patients was significantly higher than in OA ( $P < 0.025$ ) and trauma patients ( $P < 0.0005$ ). Four of the RA patients had received aurothioglucose; they had very high catalase levels:  $78 \pm 20\ \text{units/ml}$ . The increase of catalase in OA and RA cannot be explained by lysis of small amounts of erythrocytes. The accepted maximal amount of hemoglobin for used SF was  $15\ \mu\text{g/ml}$ . Lysis of erythrocytes resulting in  $15\ \mu\text{g/ml}$



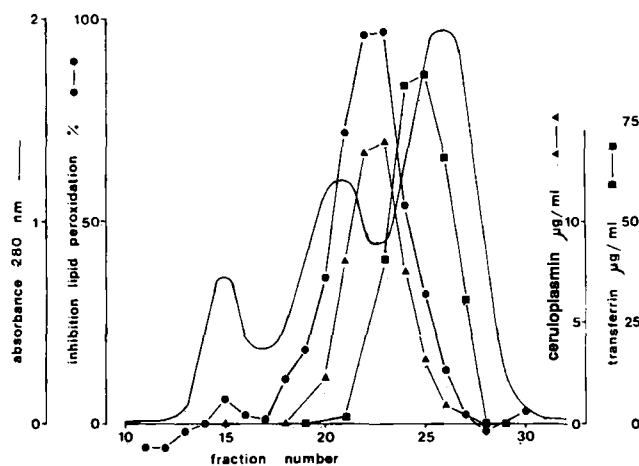
**Figure 2.** Ceruloplasmin, transferrin, and ferritin concentrations in synovial fluid in patients with traumatic knee lesions (Traum), osteoarthritis (OA), and rheumatoid arthritis (RA). *P* values for OA and RA versus Traum are shown; NS = not significant.

hemoglobin in SF increased catalase activity by only 3 units/ml.

Glutathione peroxidase in SF from patients with traumatic knee lesions was  $60 \pm 25$  mU/ml, in OA SF it was  $85 \pm 29$  mU/ml, and in RA SF the activity increased significantly to  $93 \pm 18$  mU/ml ( $P < 0.001$  versus trauma patients) (Figure 1).

**Transferrin, ferritin, and ceruloplasmin.** The concentrations of transferrin, ferritin, and ceruloplasmin in SF from the 3 groups are shown in Figure 2. Transferrin was not significantly elevated in OA SF or in RA SF. The ferritin concentration in RA SF was strongly increased. In RA SF the average ceruloplasmin concentration was twice as high as in SF from trauma patients, and in OA SF the amount was also elevated.

The distribution of these 3 proteins between serum and SF from the patients is shown in Table 1. Both transferrin and ceruloplasmin were present in SF in a concentration below the serum concentration. The SF/serum concentration ratio was slightly, but not significantly, higher in RA than in OA. Ferritin was



**Figure 3.** Sephadex G-200 gel filtration of synovial fluid from a patient with rheumatoid arthritis.

present in SF from RA patients in a concentration 15 times higher than in the corresponding serum.

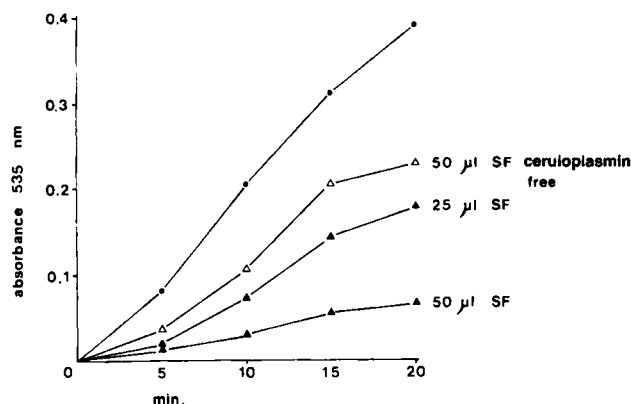
**Inhibition by serum and SF of  $O_2^-$  induced lipid peroxidation.** Peroxidation of rat liver microsomes, induced by  $O_2^-$  generated by xanthine and xanthine oxidase in the presence of  $Fe^{3+}$ , could be blocked by addition of serum and SF from both RA patients and controls. The inhibition was concentration-dependent, and about 3% serum or about 6% SF resulted in complete inhibition. To determine which fraction was responsible for this inhibition, G-200 gel filtration was performed. In the eluate the capacity to inhibit the lipid peroxidation was measured. The G-200 gel filtration pattern of RA SF (Figure 3) is representative of the SF and sera both of RA patients and controls.

For all samples, maximal inhibition of the lipid peroxidation was found between the second and third peaks of absorption at 280 nm. Occasionally a small peak at the void volume was found; this was not further investigated. Ceruloplasmin and transferrin

**Table 1.** Distribution of ceruloplasmin, transferrin, and ferritin between serum and synovial fluid of patients with osteoarthritis and rheumatoid arthritis\*

	SF	Serum	SF/serum ratio	<i>P</i>
Ceruloplasmin, mg/ml				
OA (n = 6)	$0.162 \pm 0.039$	$0.350 \pm 0.063$	$0.46 \pm 0.09$	NS (OA vs. RA)
RA (n = 17)	$0.209 \pm 0.059$	$0.423 \pm 0.148$	$0.50 \pm 0.14$	
Transferrin, mg/ml				
OA (n = 6)	$1.35 \pm 0.51$	$2.10 \pm 0.81$	$0.64 \pm 0.08$	NS (OA vs. RA)
RA (n = 17)	$1.49 \pm 0.53$	$2.18 \pm 0.77$	$0.71 \pm 0.22$	
Ferritin, µg/ml				
RA (n = 17)	$2.09 \pm 2.77$	$0.15 \pm 0.24$	$14.8 \pm 13.6$	

\* Values are mean  $\pm$  SD. SF = synovial fluid; OA = osteoarthritis; RA = rheumatoid arthritis; NS = not significant.



**Figure 4.** Superoxide-induced lipid peroxidation of rat liver microsomes, followed by measurement of thiobarbituric acid-reacting products. Complete system (●—●); final volume 3 ml. Effect of synovial fluid (SF) from a patient with rheumatoid arthritis (▲—▲), and the same fluid after removal of ceruloplasmin (△—△).

levels were determined in the fractions obtained. The ceruloplasmin concentration and the inhibition of the lipid peroxidation in individual fractions conformed closely to the same pattern. From the elution pattern it can be concluded that under these conditions, transferrin offers almost no protection against oxygen free radicals.

To investigate the possibility that ceruloplasmin was able to inhibit lipid peroxidation in the described system, the inhibition of purified human ceruloplasmin was measured. Ceruloplasmin was able to inhibit peroxidation of rat liver microsomes in a concentration-dependent manner. The necessary concentration was on the same order as found in serum and SF.

Serum and SF from control and RA patients were treated with human anti-ceruloplasmin to obtain fluids free of ceruloplasmin. The capacity of this serum and SF to inhibit lipid peroxidation was compared with that of the original serum or SF. Figure 4 shows the effect of removing ceruloplasmin on the inhibition of lipid peroxidation of rat liver microsomes. In all groups the capacity to inhibit lipid peroxidation was decreased by 70% with removal of ceruloplasmin.

## DISCUSSION

There is much evidence to suggest that oxygen free radicals and H<sub>2</sub>O<sub>2</sub> are closely involved in the pathogenesis of RA. Granulocytes are strongly increased in number in RA SF and produce large amounts of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> during the phagocytosis of immune complexes and other materials (1). Oxygen free radicals and other secondarily formed radicals are

possibly responsible for at least part of the joint destruction. Several substances are known to protect against oxygen free radicals *in vitro*, but only a small number of investigations have been performed on detecting these protectors in RA.

Conflicting data are presented in the literature about the concentration of superoxide dismutase in RA SF. Blake et al found no SOD at all in RA SF (15). Igari et al (16), however, showed a low concentration in OA SF but a 4 times higher level in RA SF. The concentration of SOD in granulocytes was decreased by 40% in juvenile RA patients (17).

Banford et al (18) measured SOD in erythrocytes in patients with RA. The SOD concentration of hemolysate was decreased in RA; however, a decrease in hemoglobin concentration was also found. The low concentration of SOD in hemolysate found in that study can be explained by the anemia commonly found in RA.

In the present study a low SOD concentration in SF from patients with traumatic knee lesions and with OA was found, in agreement with the results reported by Igari et al for OA. In contrast with Igari's findings, the SOD concentration in RA was not elevated in our study. It is very doubtful that this low SOD concentration can protect against oxygen free radicals. In various cell types and tissues, SOD activity is 18–440 times higher, and in *in vitro* systems higher concentrations are needed to protect against oxygen free radicals.

Catalase and glutathione peroxidase are capable of detoxifying H<sub>2</sub>O<sub>2</sub>. Blake et al (15) found low concentrations of catalase in RA SF, but no reference group was investigated. We estimated almost no catalase activity in SF from trauma patients. In OA SF the concentration was elevated, and in RA SF a further increase occurred. Within the RA group 4 patients who received aurothioglucose had very high catalase levels. Although catalase is significantly increased in RA, we agree with Blake et al that the concentration is too low to expect considerable protection against H<sub>2</sub>O<sub>2</sub> in SF.

Glutathione peroxidase was elevated in RA SF compared with SF from trauma patients. This increase was nearly as great as the increase of total protein found in RA SF. No increase in transferrin concentration was found in RA SF. The concentration is, however, high enough to bind about 4 times the amount of iron known to be present in SF (19). In most systems transferrin-bound iron is not able to catalyze the formation of OH<sup>·</sup>. Thus, before iron can have a

deleterious effect, it must be separated from transferrin. Another possibility is that iron is bound very tightly in a small complex which prevents binding to transferrin.

Ferritin was strongly increased in RA SF compared with both OA and traumatic SF. This is in agreement with the results of Blake et al (20). The ferritin concentration in RA SF was on the average 15 times higher than in the corresponding serum. It is unlikely that ferritin in RA SF is derived from serum, which is the case for most other serum proteins, such as ceruloplasmin and transferrin. The concentration of these proteins in SF is less than the corresponding serum value. We favor the hypothesis that ferritin is synthesized by synovial membrane cells and enters SF by secretion or by release from dying cells. The high production of ferritin by synovial cells, as shown by Muirden et al (21), is a protective reaction to the continuous iron release in SF, due to blood loss and the resulting breakdown of hemoglobin. Ferritin and transferrin can both play a dual role: 1) protection against oxygen free radicals by binding free iron, or 2) stimulation of OH<sup>·</sup> production by release of free iron from the binding protein. In this context the percentage of iron saturation could be very important.

Ceruloplasmin has been shown to possess O<sub>2</sub><sup>-</sup> scavenging activity (22). The present investigations confirm that this acute phase reactant is elevated in RA SF (23,24).

There are several potential protectors against oxygen free radicals in SF, but the relative importance of these proteins is unknown. Stocks et al (25) have investigated inhibition of lipid peroxidation by serum. They used brain homogenate as a lipid peroxidating system, which could be blocked by small amounts of serum. They concluded that the protection by serum against oxygen free radical induced lipid peroxidation was based on the presence of transferrin and another protein, most likely ceruloplasmin. In the present study O<sub>2</sub><sup>-</sup> induced lipid peroxidation of rat liver microsomes was used. Serum and SF from both controls and RA patients blocked this process.

Gel filtration of serum or SF from both groups showed a peak at which lipid peroxidation was inhibited. The position of this peak did not correspond with transferrin levels, but did correspond with ceruloplasmin levels. The postulated role of ceruloplasmin was strengthened by the fact that purified human ceruloplasmin was able to inhibit lipid peroxidation in the system used on the same order of concentrations as were present in serum or SF. Furthermore, the inhibition of lipid peroxidation by ceruloplasmin-free serum

or SF was diminished by about 70% compared with the corresponding complete serum or SF.

Ceruloplasmin is definitely shown to be an important protector against oxygen free radicals in serum and synovial fluid both in controls and rheumatoid arthritis patients. Our system is not appropriate for detecting a possible inhibitory effect of transferrin or other iron binding proteins, since rather high concentrations of iron were added.

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