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ELEVATED LEVELS OF XANTHINE OXIDASE IN SERUM OF PATIENTS WITH INFLAMMATORY AND AUTOIMMUNE RHEUMATIC DISEASES

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Abstract—Sera of patients with various inflammatory and autoimmune rheumatic diseases were screened for the presence of xanthine oxidase (XOD) and compared to sera from healthy donors and patients with nonrheumatic diseases including AIDS, internal diseases, and different carcinomas. Up to 50-fold higher levels of XOD were detected in rheumatic sera ($P < 0.001$). In addition, serum sulfhydryls (SH) were determined as sensitive markers of oxidative stress. The SH status in rheumatic patients was diminished by 45–75% ($P < 0.001$) and inversely correlated to the concentration of serum XOD ($R = 0.73$), suggesting a causal interrelation. The depletion of serum sulfhydryls by the oxyradical-producing XOD/acetaldehyde system was mimicked successfully ex vivo in human serum from healthy donors. Cortisone treatment of patients suffering from systemic lupus erythematosus and rheumatoid arthritis impressively normalized elevated XOD concentrations in rheumatic sera to those of healthy controls. The participation of xanthine oxidase in the depletion of serum antioxidants in rheumatic patients is discussed in the light of substrate availability and K_m values.

INTRODUCTION

It is increasingly recognized that reactive oxygen species (ROS) released from hyperactivated monocytes and neutrophils during acute inflammatory episodes and chronic autoimmune processes severely imbalance the pro- and antioxidant defense system in serum (1). This impairment in antiradical defenses is commonly defined as oxidative stress and plays a crucial role in more than 50 human diseases including rheumatic diseases, arteriosclerosis, cancer, diabetes, and AIDS (2).

Serum sulfhydryls (SH) are the most critical targets for free radical attack

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(3). This vulnerability of cysteine residues is due to radical tunneling from neighboring radical centers. Once a free radical hits specific amino acyl sites within a protein, it will be rapidly transferred to cysteine residues via methionines, tryptophans, and tyrosines, subsequently forming cystine radicals (3). The consequences of oxidative modification of proteins are altered enzyme activities and increased proteolysis, eventually leading to pathological tissue degradation (4).

Sulfhydryls are also intrinsically involved in the regulation of the intra- and extracellular redox potential, serve as cofactor for several enzymes, are required for the synthesis of DNA precursors, and regulate T-cell function both in vitro and in vivo (5).

The catalytic activity of many enzymes depends on the redox status of sulfhydryls. A prominent example for this SH-dependency is xanthine dehydrogenase, a ubiquitous cytosolic enzyme that is involved in the metabolism of purine nucleotides to xanthine and hypoxanthine. It is well known that enhanced fluxes of oxyradicals convert the dehydrogenase form (XDH) to an oxidase (XOD), mainly by oxidizing structurally important sulfhydryls (6). XOD now no longer uses NAD^+ as an electron acceptor but transfers electrons onto oxygen, thereby generating significant amounts of superoxide and hydrogen peroxide. This conversion of XDH to XOD was shown in many diseases and is causally involved in clinical complications observed with shock and ischemia (7, 8). During ischemia, the degradation of purine nucleotides is markedly affected, giving rise to enhanced levels of xanthine and hypoxanthine (9, 10). Upon reperfusion of hypoxic areas, the catalysis of excess xanthine and hypoxanthine by XOD will cause a transient oxidative stress, which eventually may overcharge the antioxidant defense system. Subsequently, the collapse in antioxidant protection causes metabolic disregulations by oxidative modification of proteins, membrane peroxidations, DNA mutations, and cell lysis (1). The release of xanthine oxidase/xanthine/hypoxanthine into the circulation extends the intracellular stress to the extracellular space. Oxyradicals are powerful mediators for inflammatory cells (4). XOD, pathologically present in serum, would mimic the oxidative burst of polymorphs and monocytes. The production of superoxide by serum XOD induces the generation of chemoattractants for phagocytes and activates the inflammatory cascade (11). Inadequate hyperreactivity of neutrophils and macrophages enhances the extracellular oxidative stress and contributes significantly to the degradation of the extracellular matrix (12).

Despite increasing evidence for reperfusion injury in patients with acute and chronic inflammatory disorders (13–15), nothing is known about the presence of the xanthine oxidase/xanthine system in serum of patients suffering from autoimmune or inflammatory rheumatic diseases. It was of interest, therefore, to quantify rheumatic serum XOD activities, compare it to both healthy and

disease controls, and correlate it to the serum sulfhydryl status. Mimicking the XOD-dependent sulfhydryl oxidation ex vivo in serum from healthy donors, special attention was paid to the aldehyde oxidase function of XOD, as the catalysis of aldehydes by XOD produces high energetic oxygen radicals such as singlet oxygen and hydroxyl radicals, which are most effective in the degradation of biopolymers (16–18). XOD-dependent pathomechanisms in the etiology of rheumatic diseases are discussed.

MATERIALS AND METHODS

Sera. Blood from healthy volunteers and patients suffering from systemic lupus erythematosus (SLE), mixed connective tissue diseases (MCTD), scleroderma, rheumatoid arthritis (RA), reactive arthritis (RcA), internal diseases (IntD), carcinoma (CA), and AIDS was drawn by venipuncture after informed consent.

Study Cohort. The 193 patients were recruited from an in- and outpatient clinic at the University Hospital Steglitz, Berlin. Fifteen age- and sex-matched healthy colleagues of the laboratory and hospital staff served as normal controls. All patients were diagnosed according established criteria (19). Sixty-five patients had inflammatory rheumatic diseases including 15 with RcA and 25 each with rheumatoid factor positive and negative RA (RA+, RA−). Sixty-eight patients with SLE met at least four of the American College of Rheumatology revised criteria of SLE (20). Ten patients each were diagnosed for MCTD and scleroderma. Forty patients with nonrheumatic diseases served as disease controls. Of those 15 had AIDS (Walter Reed classification 5) (21), 10 patients had carcinoma of different organs, and 15 suffered from various internal diseases including bacterial infections.

Determination of Xanthine Oxidase. Unless otherwise indicated all reagents were purchased from Sigma, Munich.

Xanthine oxidase was determined by chemiluminescence (22, 23). 1 ml contained: 100 μl serum, 100 μM xanthine, 200 μM lucigenin, 10 μM diethylenetriaminepentaacetic acid (DTPA) in HEPES 100 mM, pH 7.4. The generation of superoxide was monitored in a Berthold LB 953 C chemiluminometer at 37°C over 15 min and integrated. Chromatographically purified XOD from buttermilk was used for calibration. One unit of XOD converts 1 μmol of xanthine to urate per minute.

Determination of Serum Sulfhydryls. The thiol redox status in serum is a sensitive marker of oxidative stress (1, 3, 4). Both nonprotein and protein sulfhydryls were quantified by disulfide exchange with 5,5′-dithio(bis-2-nitrobenzoic acid) (DTNB) at pH 8 (4). Serum, 100 μl, was added to 100 μl sodium dodecyl sulfate (10% w/v) and mixed thoroughly. Then 800 μl phosphate buffer 5 mM, pH 8.0, was added and the background absorption read at 412 nm. The solution was then incubated for 1 h at 37°C in the presence of 100 μl DTNB (0.4 mg/ml). The resulting thioquinone was measured at 412 nm (E = 13,600 $M^{-1}cm^{-1}$). Reduced glutathione (GSH) was used for calibration.

Statistical Analysis. All data were analyzed by Student's t test and expressed as mean ± *SD*. $P < 0.001$ was considered significant.

RESULTS

Clinical Data

Presence of XOD in Rheumatic Sera. When sera from rheumatic patients suffering from systemic lupus erythematosus, mixed connective tissue disease, scleroderma, reactive arthritis, and rheumatoid arthritis were screened for the presence of xanthine oxidase, significant concentrations of this oxyradical-producing enzyme were detected. Thus, SLE serum contained 15.1 ± 5.8 units/liter and serum from scleroderma patients had 12.3 ± 3.1 units/liter (Figure 1).

The highest level was measured with MCTD (24.3 ± 5.0 units/liter). The XOD levels in patients with inflammatory rheumatic diseases were slightly lower in general. Patients with reactive arthritis had 14.9 ± 3.9 units/liter serum XOD, whereas those with seronegative or seropositive rheumatoid arthritis ranged between 7.3 ± 1.8 and 10.3 ± 2.2 units/liter. The average value measured for healthy control sera was 0.5 ± 0.2 units/liter. Patients with nonrheumatic inflammatory and noninflammatory diseases served as disease controls. Levels of 7.9 ± 3.1 units/liter serum XOD were measured for AIDS, 3.9 ± 1.2 units/liter for internal diseases, and 6.1 ± 2.3 units/liter for various carcinoma, thus clearly distinguishable from both healthy and disease controls. In all cases, the

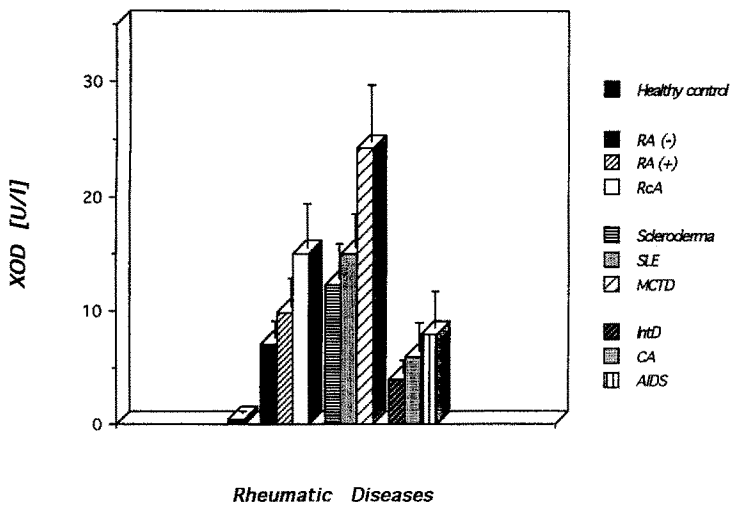

Fig. 1. Xanthine oxidase in rheumatic sera. Sera from patients with inflammatory rheumatic diseases and connective tissue diseases were screened for the presence of xanthine oxidase and compared to normal and disease controls. Mean ± *SD*. $P < 0.001$. For experimental details, see Materials and Methods.

XOD activity was inhibited completely in the presence of 0.5 mM allopurinol, a well-known inhibitor of XOD commonly used in the treatment of gout (24). The superoxide generation of serum XOD was totally abolished by 200 nM superoxide dismutase or $CuPu(Py)_2$, an active center analogue of SOD (25, 26). As the chemiluminogenic amplifier lucigenin predominantly measures superoxide, catalase showed no significant inhibition in this assay but was effective when lucigenin was substituted by luminol, a hydrogen peroxide detecting agent (23, 25, 27).

Depletion of Serum Sulfhydryls in Rheumatic Sera. The concentrations of sulfhydryls in serum from patients with SLE, scleroderma, MCTD, RA, and RcA were determined as DTNB reactive substance and compared to levels in healthy subjects and patients with non-rheumatic diseases (Figure 2).

The SH content in serum from healthy individuals was 570 ± 140 μM. Values of 305 ± 84 and 273 ± 75 μM SH were determined for RA− and RA+ and 126 ± 43 μM SH for patients with reactive arthritis, 228 ± 60 μM SH was monitored for scleroderma, 190 ± 65 μM for SLE, and 162 ± 46 μM SH for MCTD patients. The disease control group ranged between 252 ± 81 μM SH for AIDS patients, 385 ± 89 μM for patients with various carcinoma, and 408 ± 90 μM SH for patients suffering from internal diseases.

Correlating XOD and SH. The XOD levels and SH concentrations in serum of rheumatic patients were inversely correlated. High XOD levels par-

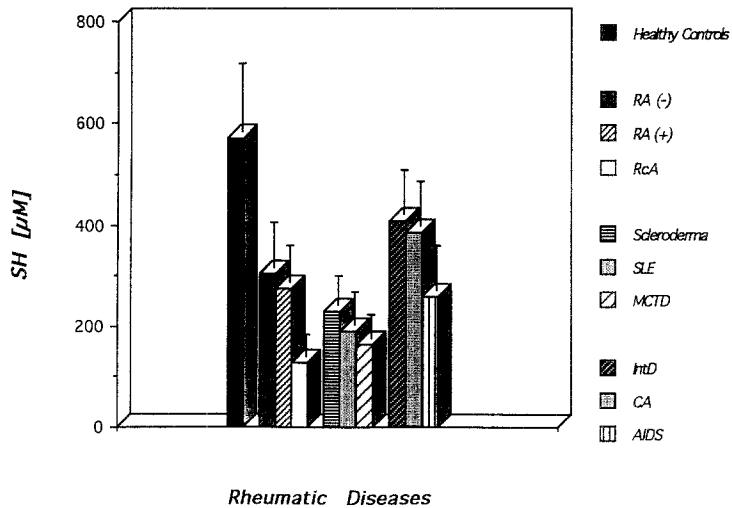

Fig. 2. Serum sulfhydryl concentrations in rheumatic diseases. The sulfhydryl concentrations in rheumatic sera were quantified by disulfide exchange with DTNB and compared to healthy and disease controls. Mean ± *SD*. $P < 0.001$. The experimental details are described in Materials and Methods.

alleled low SH concentrations in serum (Figures 1 and 2). The correlation coefficient for SLE patients was $R = 0.73$ (Figure 3).

Cortisone Effects on Serum Level of Xanthine Oxidase in SLE and RA Patients. Cortisone exerts manifold antiinflammatory reactivities. Of interest was whether the antioxidative properties of glucocorticoids influence the levels of XOD in plasma of rheumatic patients in vivo. Indeed, when the XOD levels of individuals suffering from SLE and rheumatoid arthritis were measured over a period of tapered cortisone treatment (initial dose 50 mg/day), a significant reduction of serum XOD activities was observed (Figure 4). The reduced levels correlated well with the clinical improvements.

Ex vivo Experiments

Mimicking Sulfhydryl Depletion with XOD/Acetaldehyde in Human Serum from Healthy Donors. The average SH concentration in healthy serum is 570 μM. A dose-dependent decline in DTNB-detectable serum sulfhydryls was observed in the presence of 20 units/liter XOD and increasing concentrations of acetaldehyde (Figure 5).

SOD and $CuPu(Py)_2$ at 80 nM inhibited the SH oxidation by 50%. A 90% inhibition was obtained with 1 μM Cu-thionein or 0.5 mM allopurinol.

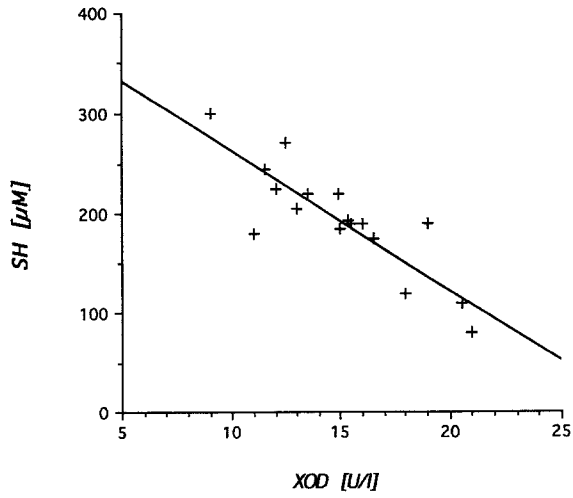

Fig. 3. Inverse correlation between serum sulfhydryl concentration and xanthine oxidase levels in patients with systemic lupus erythematosus.

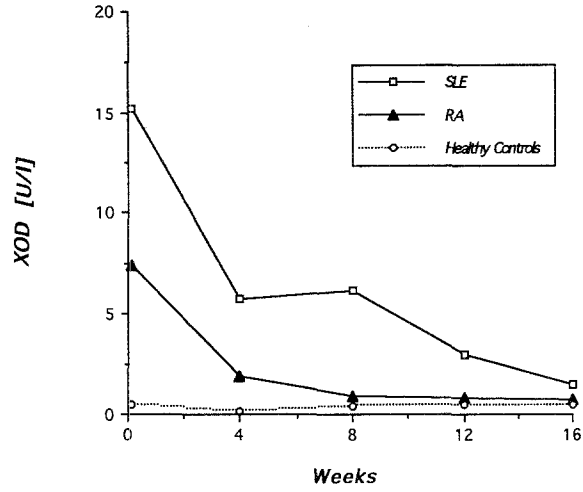

Fig. 4. Effects of cortisone treatment of SLE and RA patients on serum XOD levels.

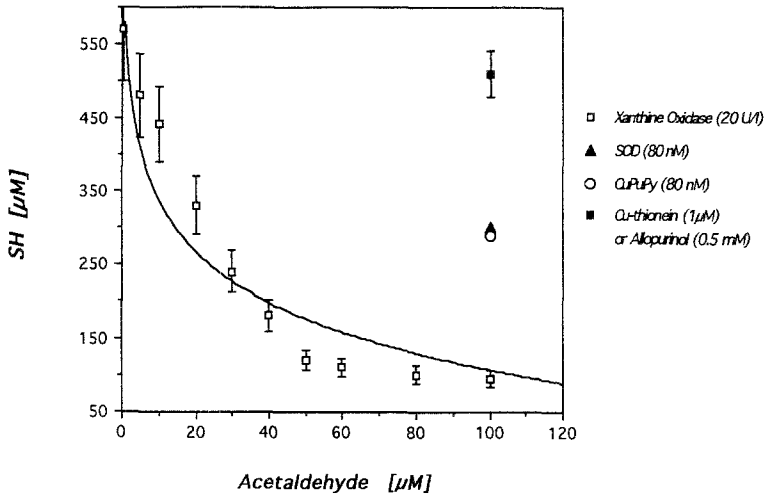

Fig. 5. Mimicking sulfhydryl depletion of healthy serum with XOD/acetaldehyde.

DISCUSSION

In this study we report the presence of xanthine oxidase in serum of patients with autoimmune and inflammatory rheumatic diseases and its absence in serum of healthy subjects. At the same time, serum sulfhydryls, which are the most critical targets for free radical attack (3, 4), were found depleted in rheumatic

sera. The XOD levels were inversely correlated to the serum sulfhydryl concentrations.

Under physiological conditions XOD is present in the form of a dehydrogenase that uses NAD^+ as electron acceptor and is mainly involved in the degradation of purines (8). Whereas uric acid is the end-product of purine metabolism in humans, in most other species the copper enzyme urate oxidase converts uric acid to allantoin, the excretory product in most mammals other than primates (24). Ischemic–reperfusion syndromes, observed in rheumatic patients, as well as hyperactive polymorphs and monocytes generate significant amounts of oxyradicals, which are likely candidates for the conversion of XDH to XOD. Enhanced intracellular fluxes of reactive oxygen species cause severe cell damage, subsequently releasing XOD into the systemic circulation. In addition to its natural substrates, xanthine and hypoxanthine, XOD uses an array of other substrates, e.g., acetaldehyde originating from ethanol metabolism, gut bacteria, or various enzyme reactions. The oxidation of acetaldehyde to acetic acid by XOD is accompanied by the generation of high energetic oxidants such as singlet oxygen and hydroxyl radicals (6, 18, 28), which are known to rapidly degrade the extracellular matrix (10). The leak of XOD into systemic circulation from oxidant-injured tissue was demonstrated recently by Yokoyama et al. (29). XOD was also monitored in the bloodstream of patients with adult respiratory distress syndrome (ARDS), extremity ischemia–reperfusion, and ischemia–reperfusion of the liver (10, 29, 30). An increased substrate level of hypoxanthine was found during hypovolemic traumatic shock (31). Histamine, released from mast cells and basophils during inflammatory processes, lowers the K_m values for XOD's substrates and is therefore a potent enhancer of the activity of XOD (32). Oxidants produced by XOD also activate inflammatory cells by ROS-derived chemoattractants formed from plasma compounds and increase the adhesiveness of the endothelium for those cells (11, 32). At the same time, H_2O_2 induces platelet-activating factor (PAF) production by the endothelium. PAF causes adherence via the PAF receptor on leukocytes (33). The oxidizing activity of PMNs induces the circulatory interruption in the capillaries, which again leads to ischemia. This circulus vitiosus may only be interrupted by antioxidants or inhibitors of XOD, e.g., allopurinol. In vivo the inflammatory crisis is controlled by glucocorticoids, which exert pronounced antiinflammatory reactivity by inhibiting the oxidative burst of monocytes and neutrophils and inducing the synthesis of antioxidant metallothioneins (34). Cortisones therefore limit the oxidant-induced tissue injuries and diminish the release of XOD and its substrates into the circulation.

An effective downregulation of acute inflammatory episodes can be expected from enzymatic antioxidants in serum. The major intracellular antioxidases (SOD, GSH-peroxidase, GSH-S-transferase, catalase), however, are virtually absent in serum (1, 35). We recently demonstrated the presence of Cu-thionein

in serum of healthy subjects, but its absence in serum of rheumatic patients (35). Cu-thionein belongs to the family of metallothioneins and is a powerful antioxidase (35–37). It scavenges ·OH radicals with an exceptionally high second-order rate constant of $k_2 = 1 \times 10^{12}\ M^{-1}sec^{-1}$, effectively dismutes superoxide, and consumes singlet oxygen (37, 38). Cu-thionein exerts pronounced antiinflammatory reactivities both in vitro and in vivo (35, 39). During long-term inflammatory processes and autoimmune diseases, however, enhanced fluxes of ROS cause the partial inactivation of the SOD activity of Cu-thionein, thereby rendering the plasma to uncontrolled oxidative stress (40). The exogenous supplementation of Cu-thionein to animals suffering from K_3CrO_8- or collagen(II)-induced arthritis inhibited the inflammatory process successfully (36).

The protection of nonenzymatic antioxidant protein thiolates by serum antioxidases appears important, as sulfhydryls are the main targets for free radical attack and are critically involved in a number of physiological functions. Thus, T-cell function is directly correlated with SH levels, both in vitro and in vivo (9). Sulfhydryls are also involved in the regulation of the intra- and extracellular redox potential, serve as cofactor for numerous enzymes, and are required for the synthesis of DNA precursors. Sulfhydryl depletion in HIV-positives was recently shown to activate viral transcription by activating nuclear factor kappa B (NFκB) from its inactive cytosolic form (9). NFκB regulates the expression of an array of genes. These include genes encoding for immunomodulatory cytokines, adhesion molecules, immunoregulatory cell surface receptors, and acute-phase proteins (41). As rheumatic sera are markedly depleted of sulfhydryls, it is tempting to speculate that NFκB-dependent variations in gene usage might be involved in the pathogenesis of rheumatic diseases. The oxyradical-dependent depletion of sulfhydryl pools and concomitant activation of NFκB, resulting in the production of proinflammatory cytokines that modulate the activities of macrophages, polymorphs, and T and B cells, might coordinate unspecific phagocytic responses and cellular and humoral immunity on a molecular level. The control of the primary trigger, the generation of superoxide by the phagocyte mimicking activity of XOD in plasma of rheumatic patients by enzymatic antioxidants might therefore represent a rational concept for the therapeutic intervention of autoimmune processes. In light of the potential importance of plasma antioxidants and antioxidases in protecting against inflammatory and autoimmune diseases, arteriosclerosis, cancer, and bacterial and viral infections, further studies are warranted that thoroughly address the multitude of biochemical events that regulate their status.

The inhibitory efficacy of enzymatic antioxidants in animal models of autoimmunity is awaited with great interest.

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