

## EFFECTS OF ALLOPURINOL ON IN VIVO SUPPRESSION OF ARTHRITIS IN MICE AND EX VIVO MODULATION OF PHAGOCYTTIC PRODUCTION OF OXYGEN RADICALS IN WHOLE HUMAN BLOOD

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**Abstract**—Recently, we demonstrated elevated levels of xanthine oxidase in serum of patients with various inflammatory and autoimmune rheumatic diseases. The present study reports the antiarthritic efficacy of the xanthine oxidase inhibitor and immunosuppressant allopurinol in DBA/1xB10A(4r) mice suffering from peroxochromate-induced arthritis. A profound dose-dependent suppression of arthritis was noted ( $P < 0.001$ ). The ED<sub>50</sub> was  $80 \pm 14 \mu\text{mol/kg/day}$ . The arthritis index correlated positively to the phagocytic production of oxygen radicals ( $r^2 > 0.672$ ) and negatively to the concentration of allopurinol ( $r^2 = 0.915$ ). Ex vivo, allopurinol and various conventional antirheumatic drugs were screened for the inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-stimulated whole human blood chemiluminescence. The concentrations of antirheumatic drugs required to inhibit the chemiluminescence by 50% were compared to the therapeutic doses administered to rheumatic patients. While D-penicillamine and cis-platinum(II) increased the phagocytic generation of superoxide, nonsteroidal antiinflammatory drugs (NSAIDs), steroids, and slow-acting antirheumatic drugs (SAARDs) inhibited the whole blood chemiluminescence in a dose-dependent manner. Therapeutic doses of NSAIDs, SAARDs, or steroids inhibited the phagocytic generation of reactive oxygen species by 10–50%. In addition to well-known mechanisms of action of NSAIDs and SAARDs, our results support the hypothesis that most common anti-rheumatic drugs act also by modulating the levels of reactive oxygen species, which serve important mediator and signal transduction functions in inflammatory and autoimmune diseases. Pharmacologically safe antioxidants like allopurinol, which simultaneously modify the oxidative burst of phago-

cytes, inhibit xanthine oxidase, and display immunosuppressive effects may well be suited to control the consequences of chronic phagocytic hyperreactivity in rheumatic patients.

## INTRODUCTION

Recently, we demonstrated the presence of elevated levels of xanthine oxidase (XOD) in serum of patients with various inflammatory and autoimmune rheumatic diseases (1). In these patients the status of antioxidative sulfhydryls (SH) was significantly diminished and correlated inversely to the levels of XOD in serum. The intracellular SH status is crucially involved in the regulation of T-cell function (2). Under physiological conditions xanthine oxidase is ubiquitously present in the form of a dehydrogenase (XDH), which uses  $\text{NAD}^+$  as electron acceptor and degrades purines to uric acid (3). Elevated levels of reactive oxygen species (ROS) convert XDH to XOD, mainly by oxidizing structurally important sulfhydryls (4). XOD now no longer uses  $\text{NAD}^+$  as an electron acceptor, but transfers electrons onto oxygen, thereby generating superoxide anion radicals ( $\text{O}_2 \cdot^-$ ), peroxides ( $\text{O}_2^{2-}$ ), hydroxyl radicals ( $\cdot\text{OH}$ ) and singlet oxygen ( $^1\Delta\text{gO}_2$ ) (5). ROS are crucially involved in the degradation of the extracellular matrix and cause DNA strand breakage (6, 7). DNA damage activates the chromatin-bound enzyme poly(ADP-ribose) synthetase. Poly(ADP-ribose) synthetase promotes the repair of DNA by transferring the ADP-ribose part of  $\text{NAD}^+$  on to nuclear proteins. However, excessive activation of the enzyme depletes the intracellular  $\text{NAD}^+$  pools and eventually causes cell death. The subsequent release of XOD into systemic circulation extends the intracellular oxidative stress to the extracellular space. The poly-ADPR-dependent "suicide program" has been associated with synovial hypertrophy and pannus formation in rheumatic patients (8).

Reactive oxygen species are also known to regulate the nuclear factor kappa B (NF- $\kappa$ B) -dependent expression of proinflammatory cytokines, adhesion molecules, and the expression of MHC class I and II genes (9). Oxidative attack on the inhibiting subunit I $\kappa$ B of the cytosolic I $\kappa$ B/NF- $\kappa$ B complex causes its dissociation, and NF- $\kappa$ B travels into the nucleus, where it mediates gene transcription by interaction with its specific DNA recognition sites (10). The subsequent expression of proinflammatory cytokines including TNF- $\alpha$ , IL-1, IL-3, IL-8, and GM-CSF prime the phagocytic NADPH oxidases to the 5- to 10-fold elevated production of ROS (11). Similar to traditional second messengers like cAMP and inositolpolyphosphates, NF- $\kappa$ B does not require new protein synthesis (12). The independence from de novo protein synthesis and the activation of NF- $\kappa$ B by reactive oxygen species embody an efficient autoregulatory loop for the rapid transduction of extracellular signals into specific patterns of gene expression in the nucleus. Due to the collapse of the antioxidant defense system

in rheumatic patients (13) the dysregulated NF- $\kappa$ B-dependent gene expression may contribute to the onset and progression of rheumatic diseases. XOD, pathologically present in rheumatic serum, imbalances the ROS/NF- $\kappa$ B/cytokine network by the additional production of ROS. The leak of XOD into systemic circulation from oxidant-injured tissue was demonstrated first by Yokoyama et al. (14). XOD was also monitored in the bloodstream of patients with adult respiratory distress syndrome (ARDS), extremity ischemia-reperfusion, and ischemia-reperfusion of the liver (15, 16). An increased substrate level of hypoxanthine was found during hypovolemic traumatic shock (5). Histamine, released from mast cells and basophils, during inflammatory processes, lowers the  $K_m$  values for XODs substrates, thereby enhancing the activity of XOD (17). 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 (18), which are known to rapidly degrade biopolymers like hyaluronic acid and collagen (1, 6). 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 (17, 19). 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 (20). The oxidizing activity of PMNs induces the circulatory interruption in the capillaries, which again leads to ischemia and reperfusion. During ischemic reperfusion processes, the degradation of purine nucleotides is markedly affected, giving rise to enhanced levels of hypoxanthine and xanthine, thereby fueling the substrate pools for XOD (15).

Whether this circulus vitiosus can be interrupted by the XOD inhibitor allopurinol, is investigated in this study. Peroxochromate-induced arthritis (6) in male DBA/1xB10A(4r) mice seemed most appropriate to monitor the antiarthritic efficacy of allopurinol *in vivo*. Parameters including the arthritis index (21) and lymphocytic and phagocytic responses in unseparated blood (6, 22, 23) were used to quantify the disease activity in the presence and absence of varying concentrations of allopurinol. Special attention was paid to the correlation of whole blood chemiluminescence to both the concentrations of allopurinol and the arthritis index. In addition, the antiinflammatory reactivity of standard antirheumatic drugs including NSAIDs and SAARDs, was evaluated *ex vivo* in human blood and compared to allopurinol.

## MATERIALS AND METHODS

*Chemicals.* Unless otherwise indicated all reagents were purchased from Sigma, Munich. Potassium peroxochromate ( $K_3CrO_8$ ) was synthesized from KOH,  $H_2CrO_4$ , and  $H_2O_2$  as described (24). During the aqueous decay of  $K_3CrO_8$  at physiological pH, the chromium(V)-bound oxygen is

released as superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, the same reactants that are produced by activated phagocytes (6).

*Induction of Peroxochromate-Induced Arthritis (PIA) (6).* Male DBA/1xB10A(4r) mice, weighing 25–30 g, were kept under specified pathogen-free (SPF) conditions on a standard laboratory diet ad libitum and a 12-h light/dark cycle. On day zero, 120 DBA/1xB10(4r) mice each were anesthetized with diethylether and 3  $\mu\text{mol/kg}$   $\text{K}_3\text{CrO}_8$  administered topically by intraplantar application into the left hindpaws. The animals were then subdivided randomly into groups of 20. Allopurinol was dissolved in a minimal volume of dimethylsulfoxide (DMSO) and diluted with sterile phosphate-buffered saline (PBS). One hour after the induction of arthritis, 20 mice each received allopurinol by intraperitoneal application at the concentrations stated in the legends to the figures. Ten mice received either PBS or PBS plus DMSO at the highest concentration, which was required to dissolve allopurinol. This dose of DMSO did not detectably affect the arthritis. The intraperitoneal injections of allopurinol or PBS were repeated daily and the arthritis index determined by an experimenter, who was unaware of the experimental design. The arthritis index is a grading system routinely used to assess the arthritis (21): 0, normal paws; 1, erythema of toes; 2, erythema and swelling of paws; 3, swelling of ankles; 4, complete swelling of the whole leg and inability to bend it. The maximum achievable score is 16. The time versus arthritis index curves were recorded for 20 days and integrated.

*Whole Blood Chemiluminescence (CL).* Blood from healthy volunteers was drawn by venipuncture into polystyrene tubes coated with 10 mM EDTA. Murine blood was obtained from the median tail artery of five mice each per group on alternate days. The generation of superoxide was determined by chemiluminescence in a Berthold LB 953 (Wildbad, Germany) chemiluminometer in the presence of lucigenin (6). One milliliter contained: 100  $\mu\text{l}$  EDTA-stabilized human or murine blood, 100  $\mu\text{M}$  lucigenin, 100  $\mu\text{M}$  diethyldithiocarbamate, and 0.5  $\mu\text{M}$  12-*O*-tetradecanoylphorbol-13-acetate (TPA) in RPMI 1640, pH 7.4 (without phenol red). The resulting chemiluminescence was recorded for 1 h at 37°C and integrated.

The inhibition of whole blood chemiluminescence by antirheumatic drugs was tested in EDTA-stabilized human blood at concentrations from  $1 \times 10^{-9}$  to  $2 \times 10^{-2}$  mol/liter of NSAIDs or SAARDs. The concentration of antirheumatic compound required to inhibit the whole blood chemiluminescence by 50% ( $\text{IC}_{50}$ ) was evaluated graphically from the recorded CL versus inhibitor curves. When solvents other than RPMI 1640 became necessary, the same concentrations of solvent were added to the controls.

*Statistical Analysis.* All data were analyzed using Student's *t* test and are presented as means  $\pm$  standard deviation (*SD*).  $P < 0.001$  was considered significant. The overall responses were compared by calculating the area under the curve for each inhibitor. The curves were fitted by linear and non-linear regression.

## RESULTS

### *Ex Vivo Experiments*

*Allopurinol and Oxypurinol Inhibit Oxidative Burst of Polymorphonuclear Leukocytes and Monocytes.* When TPA-activated human blood was incubated with increasing concentrations of allopurinol or oxypurinol, the NADPH oxidase-dependent generation of superoxide by polymorphonuclear leukocytes and monocytes was inhibited in a dose-dependent manner (Figure 1).

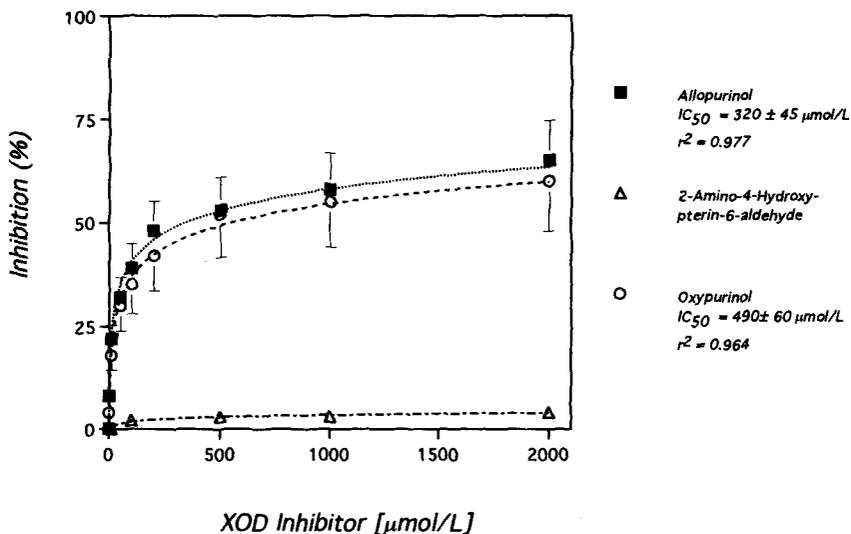


Fig. 1. Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-stimulated whole blood chemiluminescence by xanthine oxidase inhibitors. EDTA-stabilized human blood was incubated with increasing concentrations of XOD inhibitors and the phorbol-ester-activated, lucigenin-amplified chemiluminescence recorded for 60 min. The chemiluminescence scale indicates counts per hour (cph) over the 60-min integration period. The concentrations of inhibitors required to inhibit the chemiluminescence by 50% ( $IC_{50}$ ) were evaluated graphically from the dose-response curves. Mean  $\pm$  SD of triplicate experiments. The squared correlation coefficients ( $r^2$ ) were obtained by non-linear regression.

The experiments were repeated twice and the  $IC_{50}$  determined graphically as  $320 \pm 45 \mu\text{mol/liter}$  for allopurinol and  $490 \pm 60 \mu\text{mol/liter}$  for oxypurinol. Oxypurinol is the major metabolite of allopurinol and binds tightly to the active site of XOD (7). 2-Amino-4-hydroxypterin-6-aldehyde, an alternate inhibitor of xanthine oxidase and degradation product of folic acid in commercial vitamin preparations (7), did not detectably inhibit the TPA-activated generation of superoxide. The antiinflammatory reactivity of allopurinol and oxypurinol was compared to standard antirheumatic drugs including the NSAIDs aspirin, diclofenac, indomethacin, ibuprofen, sulfasalazine, thalidomide, and superoxide dismutase, the steroids dexamethasone and hydrocortisone, and the SAARDs auranofin, D-penicillamine, chloroquine, methotrexate, and *cis*-platinum(II). While D-penicillamine and *cis*-platinum(II) enhanced the release of superoxide and aspirin was inactive, all other tested drugs inhibited whole blood CL in a dose-dependent manner. The  $IC_{25}$  and  $IC_{50}$  values, listed in Table 1, were compared to the concentrations of drug administered to rheumatic patients.

In the cases of allopurinol, superoxide dismutase, hydrocortisone, ibuprofen, sulfasalazine, thalidomide, and auranofin, the therapeutically effective dose

**Table 1** Inhibition of Whole Blood Chemiluminescence by Antirheumatic Drugs<sup>a</sup>

Antirheumatic drugs	Whole Blood Chemiluminescence		Therapeutic Range (mol/kg/day)
	IC <sub>25</sub> (mol/liter)	IC <sub>50</sub> (mol/liter)	
Allopurinol	$1.20 \times 10^{-5}$	$3.20 \times 10^{-4}$	$1.0\text{--}9.5 \times 10^{-5}$
Oxypurinol <sup>b</sup>	$1.90 \times 10^{-5}$	$4.90 \times 10^{-4}$	—
Aspirin <sup>c</sup>	$> 1.00 \times 10^{-2}$	$> 1.00 \times 10^{-2}$	$3.3\text{--}7.9 \times 10^{-4}$
Diclofenac <sup>c</sup>	$1.25 \times 10^{-4}$	$3.25 \times 10^{-4}$	$4.5\text{--}9.0 \times 10^{-6}$
Indomethacin <sup>c</sup>	$2.20 \times 10^{-4}$	$3.50 \times 10^{-4}$	$3.0\text{--}8.0 \times 10^{-6}$
Ibuprofen <sup>c</sup>	$4.00 \times 10^{-3}$	$1.50 \times 10^{-2}$	$0.6\text{--}1.1 \times 10^{-4}$
Dexamethasone <sup>c</sup>	$2.00 \times 10^{-4}$	$1.40 \times 10^{-3}$	$0.4\text{--}2.9 \times 10^{-7}$
Hydrocortisone <sup>c</sup>	$2.00 \times 10^{-4}$	$4.50 \times 10^{-4}$	$0.8\text{--}6.3 \times 10^{-4}$
Chloroquine <sup>c</sup>	$2.50 \times 10^{-3}$	$6.20 \times 10^{-3}$	$0.7\text{--}1.4 \times 10^{-5}$
Methotrexate <sup>c</sup>	$1.10 \times 10^{-3}$	$3.00 \times 10^{-3}$	$0.8\text{--}1.6 \times 10^{-7}$
D-Penicillamine <sup>c</sup>		activation	$1.4\text{--}7.2 \times 10^{-5}$
cis-Platinum(II) <sup>c</sup>		activation	$1.0\text{--}1.2 \times 10^{-7}$
Cu <sub>2</sub> Zn <sub>2</sub> SOD <sup>c</sup>	$4.00 \times 10^{-8}$	$1.00 \times 10^{-7}$	$0.2\text{--}1.1 \times 10^{-8}$
Auranofin <sup>c</sup>	$4.00 \times 10^{-6}$	$6.00 \times 10^{-5}$	$0.5\text{--}1.5 \times 10^{-7}$
Sulfasalazine <sup>c</sup>	$1.10 \times 10^{-5}$	$2.50 \times 10^{-5}$	$0.4\text{--}1.1 \times 10^{-4}$
Thalidomide <sup>c</sup>	$1.50 \times 10^{-4}$	$1.80 \times 10^{-4}$	$0.5\text{--}1.5 \times 10^{-5}$

<sup>a</sup> Human blood was incubated with increasing concentrations of various antirheumatic drugs and the phorbol-ester-stimulated phagocytic generation of superoxide recorded over 60 min in a Berthold 953 luminometer and integrated. IC<sub>25</sub> and IC<sub>50</sub> were calculated planimetrically from the obtained dose-response curves. The therapeutic range was compiled from standard pharmacological literature (25).

<sup>b</sup> Active metabolite of allopurinol.

<sup>c</sup> Active metabolites not tested.

resembled the IC<sub>50</sub> required to inhibit CL *ex vivo*. The ED<sub>50</sub> and IC<sub>50</sub> of diclofenac, indomethacin, dexamethasone, chloroquine, and methotrexate differed by at least two orders of magnitude. However, when the therapeutic doses were related to 6 liters of blood instead to 70 kg of body weight, all tested antirheumatic drugs met the range of inhibition of whole blood chemiluminescence. Cumulation of antirheumatic drugs, which are usually given over months, may also contribute to their antiinflammatory efficacy. Cumulation depends on the kinetics of invasion and evasion, which is mainly reflected by the half-life and the number of daily doses (25). The half-life of methotrexate is 12 h and chloroquine's  $t_{1/2}$  is 214 h (25). Allopurinol itself has a half-life of 3 h, but is converted to oxypurinol with the half-life of 24 h (25). Figure 2 demonstrates the cumulation of oxypurinol. Up to five times higher concentrations are obtained within five days. At standard doses of allopurinol of 100–900 mg/day, a 70% inhibition of phagocytic responses (Figure 1) may be responsible for the antiinflammatory reactivity of allopurinol/oxypurinol.

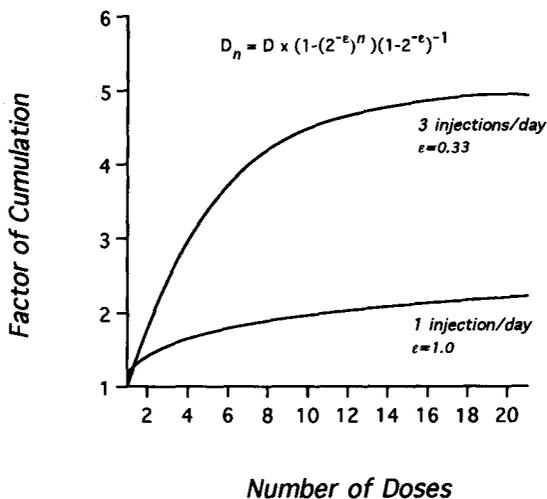


Fig. 2. Cumulation of oxypurinol. The theoretical cumulation of oxypurinol was calculated from the equation given in the figure (25).  $D_n$  represents the effective dose after  $n$  single applications.  $\epsilon$  is defined as quotient of interval of application (24 and 8 h, respectively) and half-life (24 h).

### *In Vivo Experiments*

*Allopurinol Inhibits Peroxochromate-Induced Arthritis (PIA).* When male DBA/1xB10A(4r) mice were subplantarily injected with 3  $\mu\text{mol/kg}$  of potassium peroxochromate, a profound inflammation developed (Figure 3) within 3 h and persisted at high levels for more than three weeks.

Within 24 h the inflammatory lesion was massively infiltrated by polymorphonuclear leukocytes followed by the invasion of monocytes/macrophages after 48 h. While the edema formation at the site of injection peaked at days 2–3 and slowly started to decline, a secondary swelling occurred at day 8 in noninjected hindpaws. In 40% of  $\text{K}_3\text{CrO}_8$ -treated animals, ankylosis of the knee joints caused the complete inability to bend the leg. After six days, the arthritis index began to wane, but stayed at high levels for more than 20 days. When 1–300  $\mu\text{mol/kg/day}$  allopurinol were injected intraperitoneally after the induction of arthritis with  $\text{K}_3\text{CrO}_8$ , a dose-dependent reduction of the arthritis index was observed (Figures 3 and 4).

The acute inflammatory response (day 1) was less affected at low doses of allopurinol (1–10  $\mu\text{mol/kg/day}$ ), but differed significantly from the disease controls at doses of 100 and 300  $\mu\text{mol/kg/day}$  ( $P < 0.001$ ) (Figure 3). Starting from the second application, the arthritis index declined readily and reached the levels of healthy controls on day 16 and 20 in the cases of 300 and 100  $\mu\text{mol/}$

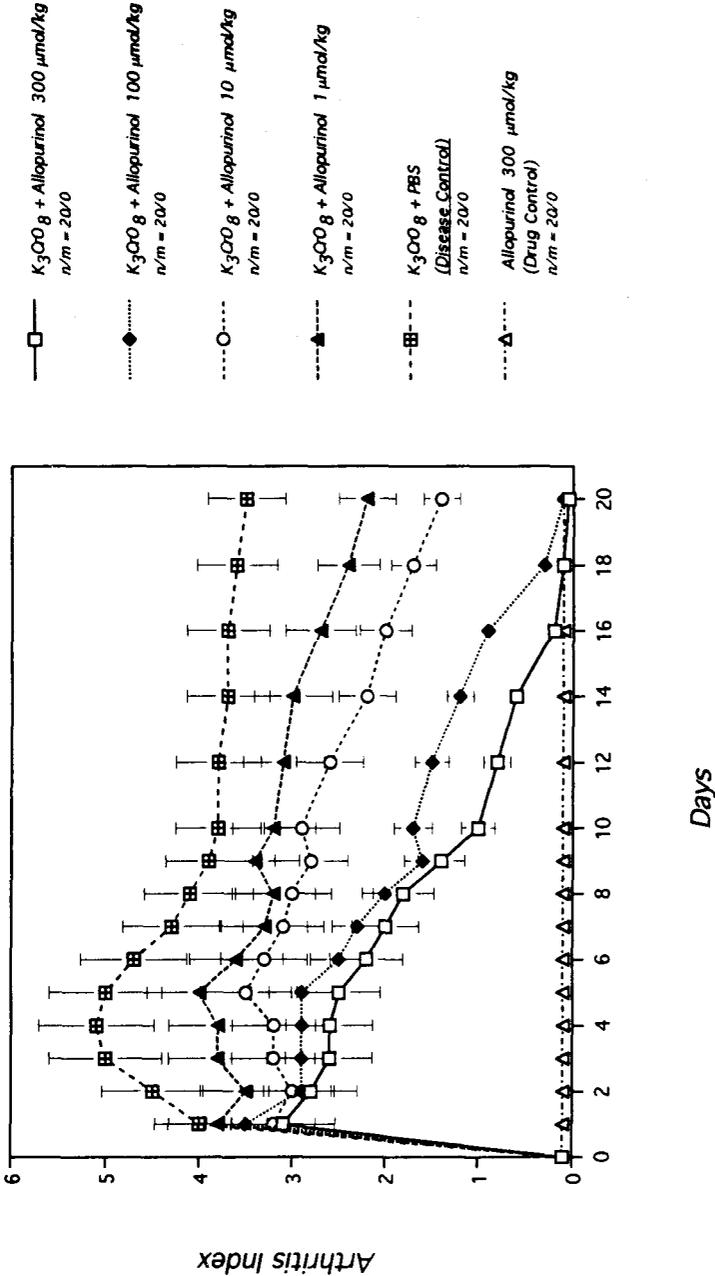


Fig. 3. Antiarthritic reactivity of allopurinol. Male DBA/1 × B10A(4r) mice were subplantarily injected with potassium peroxochromate (K<sub>3</sub>CrO<sub>8</sub>) and the arthritis index determined as stated in *Materials and Methods*. Daily intraperitoneal doses of allopurinol reduced the arthritis index in a dose-dependent manner. Mean ± SD of two independent experiments. The arthritis index of allopurinol-treated mice differed significantly from the disease controls ( $P < 0.001$ ).

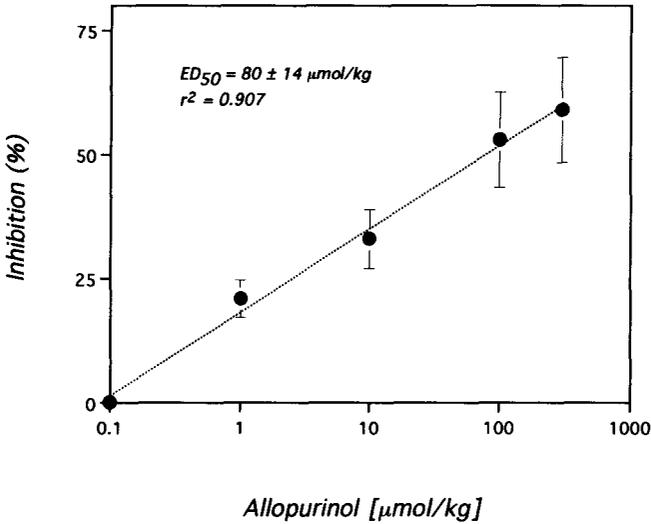


Fig. 4. Dose-dependent inhibition of peroxochromate arthritis in mice by allopurinol. The area under the dose-response curves of Figure 3 were integrated and the  $ED_{50}$  evaluated graphically. Mean  $\pm$  SD of two independent experiments.

kg/day allopurinol, respectively. Unlike the highest doses of allopurinol, concentrations below 10  $\mu\text{mol/kg/day}$  allopurinol did not cause the complete recovery, although a 20–35% inhibited arthritis index was calculated, when the curves were integrated (Figure 4). The effective daily dose of allopurinol, which caused a 50% reduction of arthritis ( $ED_{50}$ ), was estimated graphically as  $80 \pm 14 \mu\text{mol/kg/day}$  of allopurinol. Keeping in mind that allopurinol cumulates to twice the administered dose (Figure 2), the  $ED_{50}$  resembles the  $IC_{50}$  calculated for the *ex vivo* inhibition of the whole blood chemiluminescence (Figure 1).

*Whole Blood Chemiluminescence (CL) Correlates Negatively to Concentrations of Allopurinol.* Blood drawn by puncture of the median tail artery was checked for its capacity to generate superoxide upon stimulation with the protein kinase activator 12-*O*-tetradecanoylphorbol-13-acetate. Similar to the picture seen in rheumatic patients (11), a significant hyperreactivity of murine phagocytes was monitored in mice suffering from peroxochromate-induced arthritis. When compared to healthy mice, 5- to 10-fold elevated levels of superoxide were measured ( $P < 0.001$ ) (Figure 5).

The CL curves showed biphasic kinetics. At day 2 the acute inflammatory response peaked with an average of  $4 \times 10^6$  cph, which coincided with the swarming of neutrophils and monocytes into the inflamed area. The acute CL response then declined progressively until day 6. A secondary response followed, which peaked on day 12. The CL curve stayed at high levels until day

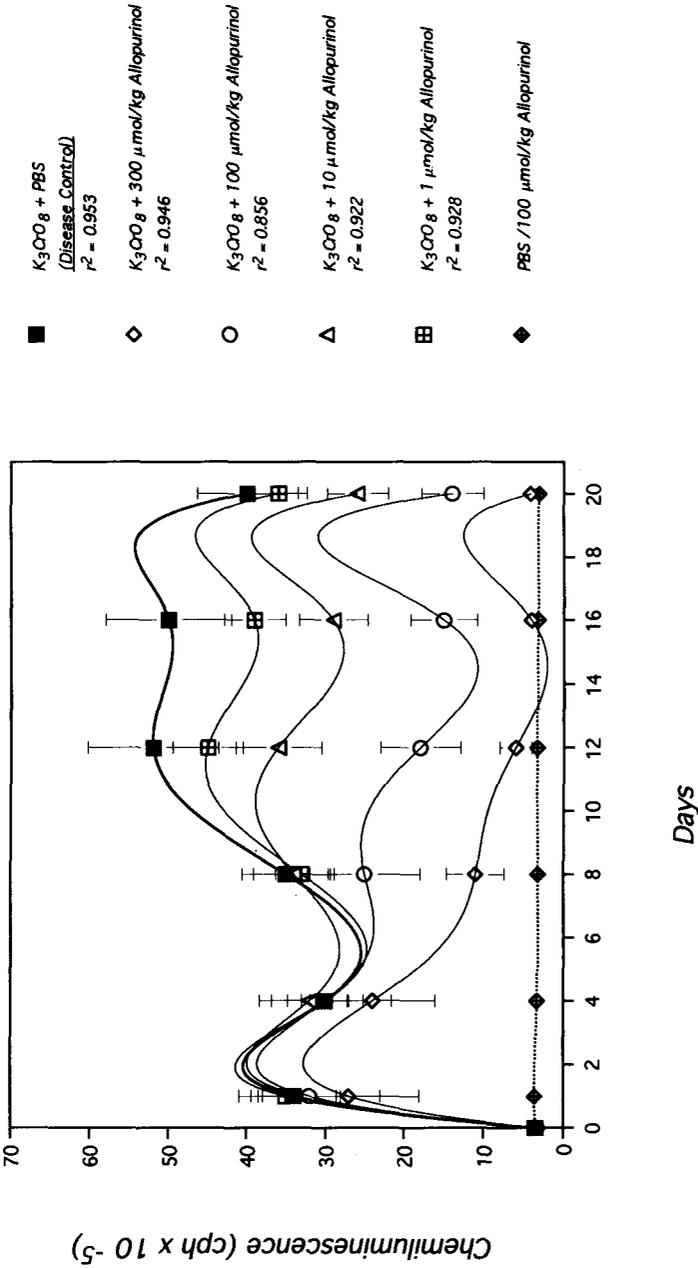


Fig. 5. Inhibition of whole blood chemiluminescence by allopurinol in mice suffering from peroxochromate-induced arthritis. Blood was drawn from the median tail artery, stimulated with 12-O-tetradecanoylphorbol-13-acetate, and the resulting generation of superoxide by granulocytes and monocytes monitored for 60 min and integrated. The curves were fitted by nonlinear regression.

17 and then began to wane slowly. This secondary CL response correlated with the development of arthritis in noninjected paws. A dose-dependent reduction of CL was monitored in allopurinol treated animals (Figure 6).

Doses of 300, 100, and 10  $\mu\text{mol/kg/day}$  of allopurinol caused 70, 40, and 20% reductions respectively, of CL ( $P < 0.001$ ). No significant reduction was obtained with 1  $\mu\text{mol/kg/day}$  of allopurinol. The concentration of allopurinol required to inhibit the CL response by 50% was calculated graphically as 110  $\mu\text{mol/kg/day}$ , resembling the daily dose needed to inhibit the arthritis index by 50% (80  $\mu\text{mol/kg/day}$ ; Figure 4). Interestingly, the secondary CL response of the disease controls (Figure 5; days 7–12) was almost completely suppressed in the presence of 300  $\mu\text{mol/kg/day}$  of allopurinol and dose-dependently inhibited by 1–100  $\mu\text{mol/kg/day}$  of allopurinol. Similar to the murine model of arthritis induced with Freund's complete adjuvant, where a swelling of noninjected hind-paws appears at days 9–12 (26), the secondary increase of CL in PIA mice may reflect an immunological response of proliferating lymphocytes, which have been shown to generate superoxide (27). Comparable to immunosuppressants such as methotrexate, this immunological response is blocked by allopurinol through the inhibition of de novo pyrimidine synthesis (28).

*Whole Blood Chemiluminescence Correlates Positively to Arthritis Index.* The arthritis index and whole blood CL of  $\text{K}_3\text{CrO}_8$ /allopurinol-treated mice were correlated positively in the cases of 10–100  $\mu\text{mol/kg/day}$  of allopurinol ( $r^2 = 0.856$ – $0.953$ ) (Figure 7).

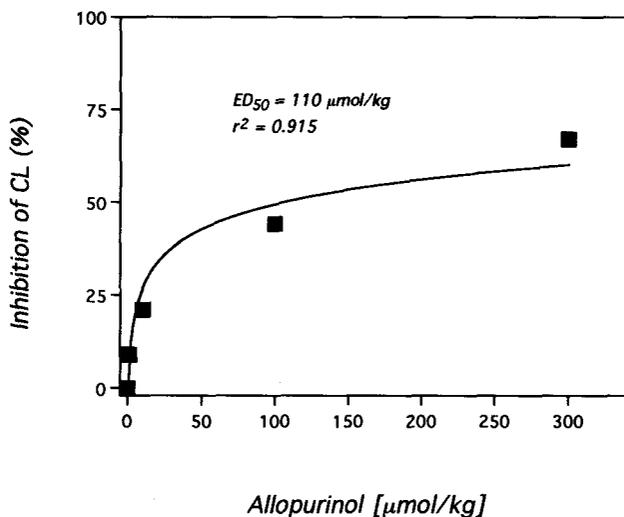


Fig. 6. Dose-dependent inhibition of whole blood chemiluminescence in allopurinol-treated mice suffering from peroxochromate arthritis.

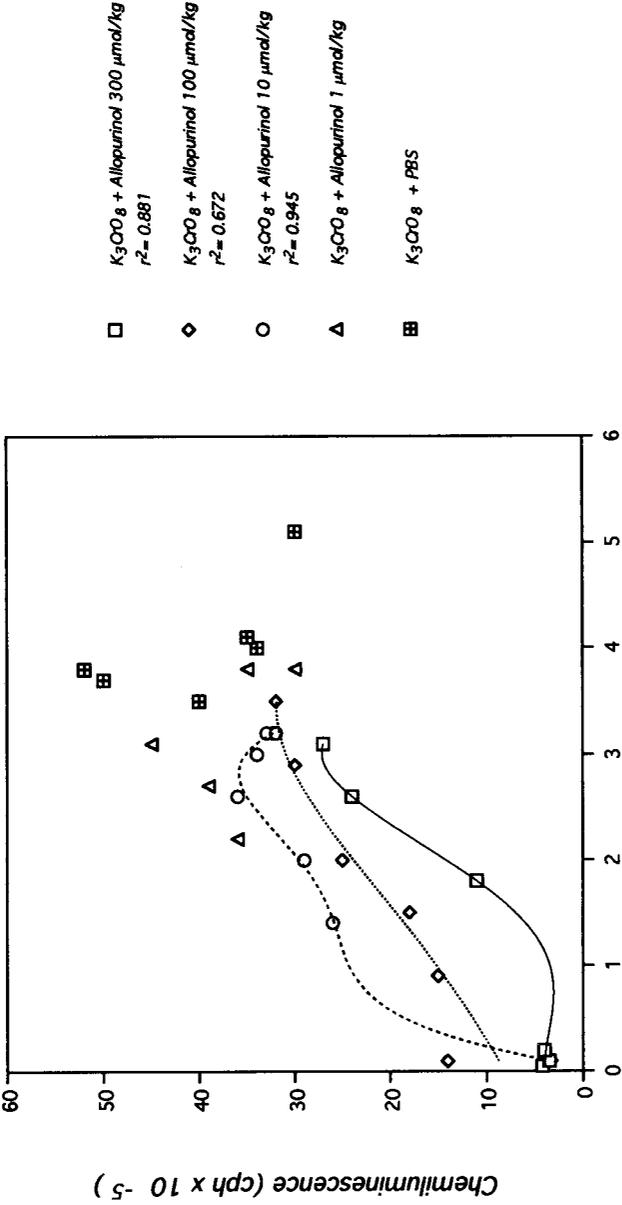


Fig. 7. Correlation between whole blood chemiluminescence and arthritis index in allopurinol-treated mice suffering from peroxochromate induced arthritis.

When the chemiluminescence was compared to the swelling in noninjected paws, the arthritis index and CL of both the disease controls and mice treated with 1  $\mu\text{mol/kg/day}$  of allopurinol correlated to the primary acute inflammatory phase (days 1–3) (Figures 3 and 5) and to the onset of the secondary immunological response (days 7–12).

## DISCUSSION

This study reports the profound antiarthritic reactivity of allopurinol in DBA/1xB10A(4r) mice suffering from peroxochromate-induced arthritis. Daily effective doses of 80  $\mu\text{mol/kg}$  of allopurinol suppressed the arthritis by 50%. The phagocytic generation of reactive oxygen species was dose-dependently inhibited. The onset, progression, and remission of arthritis correlated positively to TPA-activated phagocytic responses in whole blood. An inverse correlation between whole blood chemiluminescence and the concentrations of allopurinol was detected, confirming the *ex vivo* data obtained in unseparated human blood. The dose-dependent inhibition of phagocytic responses was also observed for most common antirheumatic drugs, suggesting the inhibition mechanism of antiinflammatory and antirheumatic reactivity.

Allopurinol is routinely used for the treatment of gout and other conditions of hyperuricemia (25). Low doses of allopurinol combined with mercaptopurine or azathioprin are also effective in antitumor therapy (25). Unlike conventional antirheumatic therapy with NSAIDs and SAARDs, which is often complicated by loss of effectiveness and severe side effects, daily doses of 100–900 mg allopurinol are well tolerated in most patients (25). The suppression of both lymphocytic and phagocytic responses combined with its immunosuppressive and XOD-inhibiting activity make allopurinol a promising candidate for the basic therapy of inflammatory and autoimmune rheumatic diseases. Our results add to the recent findings of Chocair et al. (29), which report a >90% reduced occurrence of rejection episodes in patients with renal transplantation treated with allopurinol plus conventional immunosuppressive therapy. Interestingly, the withdrawal of allopurinol from patients with tophaceous gout was reported to cause acute attacks of arthritis in 81% of patients (30). Recently, allopurinol was also shown to form stoichiometrical complexes with copper(II) (31). These Cu(II)–allopurinol complexes decreased markedly the oxyradical-induced DNA breakage. As the copper concentrations in plasma of rheumatic patients are commonly elevated by up to 300% (32), *in vivo* formation of Cu(II)–allopurinol can be responsible for the inhibition of NADPH oxidase-dependent superoxide production by mimicking superoxide dismutase-like activity (33).

The correlation of phagocytic responses and antiarthritic efficacy of anti-

rheumatic drugs make the chemiluminescent whole blood assay a convenient, quantitative, and economical method for the rapid screening of novel antirheumatic compounds and therapies (11, 34).

In light of the potential importance of antioxidants in controlling the consequences of chronic phagocytic hyperreactivity in rheumatic patients, further studies are warranted that address thoroughly the multitude of biochemical events that influence the autoregulatory loop between ROS and NF- $\kappa$ B-dependent gene expression of proinflammatory cytokines, adhesion molecules, and immunoregulatory cell surface receptors.

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