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EFFECT OF ALLOPURINOL ON NEUTROPHIL SUPEROXIDE PRODUCTION, CHEMOTAXIS, OR DEGRANULATION*

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Abstract—Recent studies examining the effect of allopurinol on bacteral killing by leukocytes [Tubaro *et al.*, *Biochem. Pharmac.* **29**, 3018 (1980); Tritsch and Neiswander, *Life Sci.* **32**, 1359 (1983)] have been interpreted by those authors as proof that xanthine oxidase is the major superoxide producing enzyme in activated leukocytes. To test the assertion that xanthine oxidase is involved in the production of superoxide by activated human neutrophils, the xanthine oxidase content of neutrophils was measured, and the effect of allopurinol on neutrophil functions, including superoxide production, was studied. Neutrophils were found to contain a level of xanthine oxidase insufficient to account for the flux of superoxide associated with neutrophil activation. Allopurinol did not inhibit superoxide production induced by opsonized zymosan, phorbol myristic acetate, or formylmethionylleucylphenylalanine. Furthermore, neither chemotaxis nor degranulation was affected by allopurinol. Allopurinol was also found ineffective in blocking superoxide-mediated carrageenan-induced foot edema in the rat. These studies are interpreted as evidence that xanthine oxidase is not a major superoxide-generating system in activated neutrophils as has been suggested by others.

The oxidative burst accompanying neutrophil activation is characterized by increased oxygen consumption, stimulation of the hexose monophosphate shunt, and the production of superoxide. Superoxide generated in this fashion is subsequently converted into additional active oxygen species including hydrogen peroxide, hydroxyl radical, hypochlorous acid, and perhaps singlet oxygen. These act in concert with other microbicidal agents to destroy the ingested microorganisms [1–3].

In humans, the production of superoxide by phagocytosing neutrophils has been generally ascribed to NADPH oxidase—a latent, plasma membrane bound enzyme which catalyzes the reaction: $NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$ [4, 5]. Recently, two groups have proposed that NADPH oxidase is not the major source of superoxide generated by activated neutrophils and macrophages. Instead, they have proposed that stimulation of xanthine oxidase is, in large part, responsible for the flux of superoxide observed [6, 7]. The basis for that conclusion were studies which showed that allopurinol, a potent inhibitor of xanthine oxidase, blocked bacterial killing by mouse neutrophils [6] and superoxide production by macrophages [7]. Because of the current interest in the roles of xanthine oxidase and superoxide in reperfusion injury and inflammation, we have further explored the effects of allopurinol on various human neutrophil functions, including superoxide production, and examined in more detail the possible role of xanthine oxidase in this superoxide production.

MATERIALS AND METHODS

Cell preparation. Human blood was collected from healthy human donors according to the principles of the Declaration of Helsinki. Informed consent was obtained, and all protocols were approved by the institutional Human Use Committee. For the determination of xanthine oxidase content and degranulation, highly purified neutrophils were utilized. Whole heparinized blood was layered over a Ficoll–Hypaque cushion and centrifuged. The pellet was resuspended in phosphate-buffered saline, and the erythrocytes were sedimented by addition of a 3% (w/v) dextran solution. The supernatant fraction was centrifuged, and the pellet was resuspended in hypotonic buffer to remove the residual erythrocytes. Neutrophils prepared in this manner were more than 95% pure upon microscopic examination [8]. In all other studies except those where xanthine oxidase content was determined, neutrophils were purified by layering fresh blood over a cushion of 9.8% sodium metrizoate and 5% Ficoll. Following gravity sedimentation of the erythrocytes, the upper layer containing plasma, mixed leukocytes, and platelets was removed, and the neurophils were sedimented by centrifugation at 250 *g* for 10 min. The neutrophils were then washed and resuspended in phosphate-buffered saline with 1% bovine serum albumin.

Assay of intracellular xanthine oxidase activity. High speed supernatant fractions were prepared from homogenates of highly purified resting and

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phorbol myristic acetate-activated neutrophils. Cells were suspended in a buffer containing 40 mM potassium phosphate, 27.5 mM sodium carbonate, 5 mM sodium bicarbonate, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride, pH 7.8. The suspended neutrophils were disrupted in a Broeck tissue homogenizer (100 strokes). An aliquot of the broken cell suspension was counted to quantify the extent of lysis, and the remainder was centrifuged for 10 min at 27,000 *g* at 4°. The resultant supernatant fraction was chromatographed on a Sephadex G-25 column equilibrated with 50 mM potassium phosphate, pH 7.8, to remove endogenous substrates. The eluate was assayed for xanthine oxidase activity. Xanthine oxidase activity was assayed spectrophotometrically in a buffer containing 50 mM potassium phosphate and 0.05 mM xanthine, pH 7.8, by measuring the rate of uric acid formation utilizing a molar extinction coefficient difference of 9500 M^{-1} cm^{-1} at 295 nm between uric acid and xanthine. The maximum theoretical yield of superoxide was calculated by assuming quantitative univalent transfer of two electrons from xanthine to oxygen.

Neutrophil function studies. Neutrophils were activated at 37° with either phorbol myristic acetate (250 ng/ml), formylmethionylleucylphenylalanine (1 μM) or opsonized zymosan (0.5 mg/ml). Superoxide production by intact cells was measured continuously by assessing superoxide dismutase-sensitive cytochrome *c* reduction [8]. Rates of cytochrome reduction were converted to superoxide production using a molar extinction coefficient difference of 21,000 M^{-1} cm^{-1} at 550 nm between the reduced and oxidized forms.

Neutrophil chemotaxis was assessed by the method of Boyden [9]. In each experiment, approximately 20,000 cells and an incubation time of 20 min were used. Filters were stained with hemotoxylin, and the lower surfaces were examined microscopically. Twelve fields were counted and averaged. Results are given as means ± S.E.M. for replicate chambers.

Azurophil degranulation was measured as previously described by quantifying release of myeloperoxidase into the extracellular medium. Following a 60-min incubation period, cells were centrifuged and myeloperoxidase activity in the supernatant fractions was calculated from the rate of H_2O_2-dependent oxidation of 1.4 mM tetramethylbenzidine [10]. One unit of myeloperoxidase activity is defined as the amount of myeloperoxidase causing a change in *A* at 655 nm of 1.0/min.

Carrageenan foot edema studies. Carrageenan foot edema was monitored as previously reported [11]. Carrageenan was administered by subplantar injection [12]. Edema formation was assessed by estimating foot volume by a water displacement method [13] which allowed multiple measurement with time for kinetic analysis. Each foot served as its own control.

Chemicals. Sodium metrizoate was purchased from the Accurate Chemical & Scientific Co. All other chemicals were purchased from the Sigma Chemical Co.

RESULTS

Xanthine oxidase levels in neutrophils. The quantities of superoxide generated by intact cells in response to opsonized zymosan A, phorbol myristic acetate, or formylated tripeptide were compared to the maximum theoretical generating capacity of the xanthine oxidase in preparations from both resting and activated cells (Table 1). Levels of superoxide production in intact cells (when calculated as nmoles superoxide produced/min/10^7 cells) were consistent with those reported by numerous other authors [14–17] utilizing similar concentrations of the various activators. Zymosan A induced less superoxide production than other stimulants, but this is also consistent with a previous report [14]. However, the maximum theoretical superoxide generating capacity of the xanthine oxidase in the cells was well below

Table 1. Superoxide production from activated neutrophils and by the xanthine oxidase system of activated and resting neutrophils*

Experimental system	XO activity (I.U./10^7 cells)	Superoxide produced (nmoles/min/10^7 cells) − Allopurinol	Superoxide produced (nmoles/min/10^7 cells) + Allopurinol
Xanthine oxidase activity (resting cells)	0.54 ± 0.15	1.07 ± 0.30†	0
Xanthine oxidase activity (PMA-activated)	0.23 ± 0.03	0.46 ± 0.60†	0
Xanthine oxidase activity (zymosan A-activated)	0.35 ± 0.19	0.70 ± 0.38†	0
Neutrophils activated by PMA (250 ng/ml)		53.3	61.9
Neutrophils activated by FMLP (1 μM)		52.0	50.3
Neutrophils activated by zymosan A (0.5 mg/ml)		28.6	30.5

* Xanthine oxidase (XO) activity was measured in homogenates prepared from resting cells and cells activated by phorbol myristic acetate (PMA). I.U. = micromoles of uric acid produced per minute at 25°. Values represent the mean ± S.D. for three cell preparations. Superoxide production from neutrophils activated with PMA, zymosan A, or *N*-formylmethionylleucylphenylalanine (FMLP) were measured. Values are the mean of duplicate measurements.

† Superoxide production is the calculated theoretical maximum assuming two moles of superoxide are generated per mole of xanthine converted to uric acid.

Table 2. Effects of allopurinol on neutrophil chemotaxis*

Experimental system		Chemotaxis
Upper chamber	Lower chamber	(cells/field)
Neutrophils	Buffer alone	7.9 ± 1.6
Neutrophils	FMLP (10^{-7} M)	61.5 ± 6.1
Neutrophils + allopurinol	FMLP (10^{-7} M)	70.6 ± 4.2

* Neutrophil chemotaxis toward *N*-formylmethionyl-leucylphenylalanine (FMLP) was measured in the presence and absence of 1.2 mM allopurinol. Values are the average of twelve fields ± S.E.M. All solutions contained 0.1% bovine serum albumin in Hanks' Balanced Salt Solution.

that observed by stimulus activation of the cells. This indicates that soluble xanthine oxidase is not a major source of this superoxide.

Effect of allopurinol on neutrophil functions. As demonstrated in Table 1, allopurinol at a final concentration of 0.9 mM had no significant effect on neutrophil superoxide production in response to phorbol myristic acetate, opsonized zymosan, or *N*-formylmethionylleucylphenylalanine, indicating that xanthine oxidase is not the source of the evolved superoxide. Likewise, allopurinol in the 1 mM range had no effect upon neutrophil chemotaxis toward chemotactic tripeptide nor upon degranulation of azurophilic granules as measured by myeloperoxidase release in response to the phorbol ester or opsonized zymosan (Tables 2 and 3). In addition, 1 mM allopurinol had no effect on the superoxide-dependent chlorination of exogenous taurine by phorbol myristic acetate which suggests that allopurinol also has no effect on the myeloperoxidase system (data not shown).

Effect of allopurinol on carrageenan-induced edema. If allopurinol were effective in blocking the expression of superoxide by neutrophils, then administration of the drug should attenuate those inflammatory processes mediated through neutrophil superoxide production. To test this, we examined the effect of allopurinol in the carrageenan-induced foot edema model. This neutrophil-mediated inflammatory process has been demonstrated to be superoxide dependent [11, 18].

Control rats and rats that had been injected intraperitoneally with 30 mg/kg of allopurinol 24 hr earlier were injected in the foot with carrageenan, and periodic measurements of edema were made. The results of that experiment are shown in Fig. 1. Allopurinol had no anti-inflammatory effect, indicating that the drug was ineffective in preventing superoxide production by neutrophils *in vivo*. (Superoxide dismutase derivates are potently anti-inflammatory in this model [11].) Similar results were also obtained when rats were pretreated for 3 consecutive days with the same dose of allopurinol prior to carageenan injection.

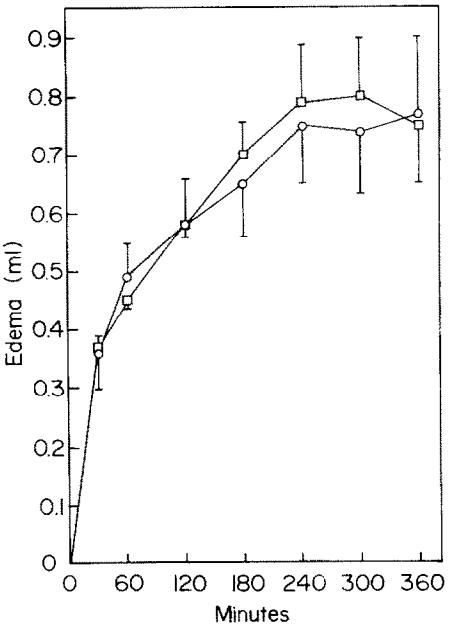

Fig. 1. Effect of allopurinol on carrageenan-induced foot edema. Control rats (○) and rats pretreated with 30 mg/kg of allopurinol 24 hr prior to the experiment (□) were injected with 0.1 ml of carrageenan in the foot pad. Edema was determined at the indicated times following carrageenan administration. Results are plotted as points with S.E.M. indicated (N = 3).

DISCUSSION

Previous studies have demonstrated that allopurinol inhibits both bacterial killing by neutrophils

Table 3. Effects of allopurinol on neutrophil degranulation*

Experimental system	MPO released (units/min/4 × 10^6 cells)	% Control
Control (no activator)	0.67	100
Control + allopurinol (1 mM)	0.51	76
PMA (250 ng/ml)	0.97	145
PMA + allopurinol	1.08	161
Opsonized zymosan	0.90	134
Opsonized zymosan + allopurinol	0.98	146

* Control neutrophils and those activated with either phorbol myristic acetate (PMA) or opsonized zymosan A in the presence or absence of allopurinol were incubated for 60 min at 37° with continuous shaking and then assayed for release of myeloperoxidase (MPO). Experiment values represent the mean of duplicate measurements and are compared to control with no stimulus.

and superoxide production by macrophages. This information led some investigators to conclude that xanthine oxidase, and not NADPH oxidase, is the enzyme responsible for stimulus-induced superoxide production [1, 2]. If correct, those observations would be of particular importance because of the relationship between tissue damage, xanthine oxidase-generated O_2^- and subsequent neutrophil-mediated inflammation. The results of our studies contradict that conclusion.

First, in neither resting nor activated neutrophils are xanthine oxidase levels sufficient to account for the flux of superoxide observed upon neutrophil activation, even if oxygen reduction by the xanthine oxidase were totally via the univalent pathway. This is probably not the case, however, since at pH 7.0 only 20% of the electrons entering xanthine oxidase exit via the univalent pathway. Instead, the majority of the electrons are used to directly form hydrogen peroxide [19]. Therefore, our estimates of the superoxide-generating capacity of xanthine oxidase are, if anything, higher than they should be. Our measurements indicate levels of xanthine oxidase in the human system to be significantly less than those in the murine system [6]. This may be due to differing assay systems since measurements in the murine system were done using an indirect assay system, while our studies directly measured xanthine oxidase activity by quantifying the conversion of xanthine to urate.

Second, allopurinol at millimolar concentration does not inhibit neutrophil superoxide production in response to a variety of stimuli including opsonized zymosan. This concentration of allopurinol exceeds by two orders of magnitude the K_I reported for allopurinol toward xanthine oxidase [20, 21]. Therefore, if xanthine oxidase were directly involved in neutrophil superoxide production, these concentrations of allopurinol should have drastically diminished superoxide generation and they did not. Allopurinol, at millimolar concentrations, also had no inhibitory effects upon neutrophil chemotaxis or degranulation, ruling out those systems as possible explanations of the reported antibactericidal action of allopurinol.

And finally, allopurinol proved ineffectual in blocking a neutrophil-mediated, superoxide-dependent inflammatory process *in vivo*. Allopurinol, at a concentration sufficiently high to block xanthine oxidase activity, was unable to block the generation of superoxide by neutrophils *in vivo*.

While the reported antibactericidal action of allopurinol [6, 7] remains unexplained, these results taken together refute the hypothesis that xanthine oxidase represents a major source of superoxide production in activated human neutrophils. Further studies will be needed to explain the observations of Tubaro and coworkers [6]. Those studies should be carefully conducted, however, to demonstrate that the action of allopurinol is achieved at a drug concentration consistent with the K_I for xanthine oxidase before attempts are made to implicate xanthine oxidase in the bactericidal process.

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