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Xanthine oxidase: an enzyme playing a role in the killing mechanism of polymorphonuclear leucocytes

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We have reported a marked increase in the xanthine oxidase (XO) activity of polymorphonuclear leucocytes (PMN) and macrophages obtained from infected animals compared with those obtained from normal mice [1]. An analogous, but more marked, effect occurred in the liver of similarly treated animals [2]. In the different pathological models, the time-dependence of the XO activity follows a bell-shaped curve, in which the XO increase does not exceed 2 weeks, despite the persistence of the infection. We therefore postulated that the increase in XO activity in PMN may represent a prompt defence mechanism, probably acting via the production of superoxide.

A crucial step in defining the role of XO in the defence activity of PMN is to assess the effect of a specific inhibitor, such as allopurinol or oxypurinol [3], on the killing activity from PMN of normal and infected animals. To ensure that possible effects of these inhibitors on the killing properties of PMN are related to their action on XO, we also performed experiments using adenine which, although structurally different, has inhibitory effects on XO comparable to those of allopurinol [4]. A further experiment was carried out to evaluate the effect of the XO substrate xanthine on the killing properties of PMN from normal animals.

Materials and Methods. Preliminary *in vitro* experiments were carried out in order to determine the inhibitory effect of allopurinol and adenine on the XO activity of PMN from normal mice. PMN (5×10^6) were disrupted by freezing and thawing, and using the Nachlas method [5] the concentrations of allopurinol and adenine required to give a 50 per cent reduction of XO activity were determined.

In vivo experiments were carried out using Charles River CD_1 male albino mice, body weight 20 ± 1 g, infected i.v. with 0.25 ml/10 g of *S. aureus* CN 6538 culture in tryptic soy broth (Difco). A control group was similarly treated with the same volume of saline. Four days later, PMN were elicited in both groups of animals by i.p. injection of 0.75 ml/10 g of a 12% (w/v) sodium caseinate (Difco cod. 0187/7) sterile solution in saline. After a further 16 hr, for each experiment PMN were obtained from 10 mice (either normal or infected) and harvested by double washing with 2 ml/mouse sterile Hank's balanced salt solution (HBSS) at pH 7.6. Among the infected animals, only those presenting kidney abscesses, as proof of continuing infection, and showing the expected increase in XO activity [1], were allowed to contribute to the pool. Elicited PMN were centrifuged twice at 450 rev/min for 3 min, washed each time with cold sterile HBSS and counted using a Thoma–Zeiss haemocytometer.

HBSS solution (1 ml) containing 2×10^6 PMN and 2×10^7 staphylococci (from *S. aureus* CN 6538 18 hr culture in tryptic soy broth, Difco) was added either to 1 ml of purine or pyrimidine base sterile solution in HBSS (at concentrations varying from 0.1 to 5 mM), or to 1 ml of HBSS (controls), both at pH 7.6. After 20 min, 0.5 ml of lysostaphin (Schwarz-Mann, Orangeburg, NY) solution in HBSS containing 0.2% (w/v) was added and the PMN solution allowed to stand for a further 10, 40, 100 and 220 min.

After these time intervals, aliquots of the PMN suspension were centrifuged at 2500 rev/min for 10 min, disrupted by resuspension in 2 ml of 0.5% trypsin (Merck, 3.5 U/mg) in distilled water, allowed to stand for 20 min at 37°, centrifuged again at 2500 rev/min and finally resuspended in 2 ml of HBSS. Cell counts were carried out by the pour plate technique in trypticase soy agar (Difco). The numbers of viable bacteria/ml were determined as colony-forming units (C.F.U.) and plotted as a function of incubation time.

To examine the possibility of a direct effect of the XO inhibitors on the bacteria, 1 ml of HBSS containing 2×10^7 staphylococci, but no PMN, was added either to 1 ml of the purine or pyrimidine base solution in HBSS or to 1 ml HBSS (controls). The numbers of viable bacteria/ml were determined, as for the PMN solution, at 30 min, 1, 2 and 4 hr, and plotted as C.F.U. against incubation time.

Viability of the PMN during incubation in the presence of XO inhibitors was determined as the phagocytic index, according to the method of Weinstein and Young [6]. After 20 min incubation, samples of the bacteria–leucocyte suspension were exposed to 1% (w/v) trypan blue for 1 min on a glass slide under a mineral oil-sealed cover slip. Failure to observe nuclear staining was considered evidence for lack of viability [7].

Results and discussion. In the preliminary *in vitro* experiments, 50 per cent reduction of the XO activity of PMN from normal mice was obtained with 0.0265 mM allopurinol or 0.140 mM adenine, giving a concentration ratio of 1:5.28.

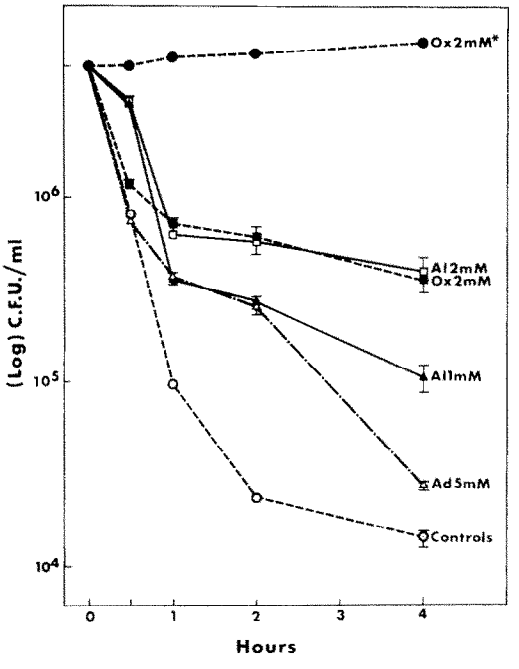

Fig. 1. Effect of allopurinol, oxypurinol and adenine on the bactericidal activity of phagocytes from normal mice. Each point is the mean of three different experiments (five for controls at 2 and 4 hr) each employing triplicate determinations on pools of cells from ten animals in each group. Ox, oxypurinol; Al, allopurinol; Ad, adenine; *, without phagocytes. When standard errors are omitted, they are smaller than symbols.

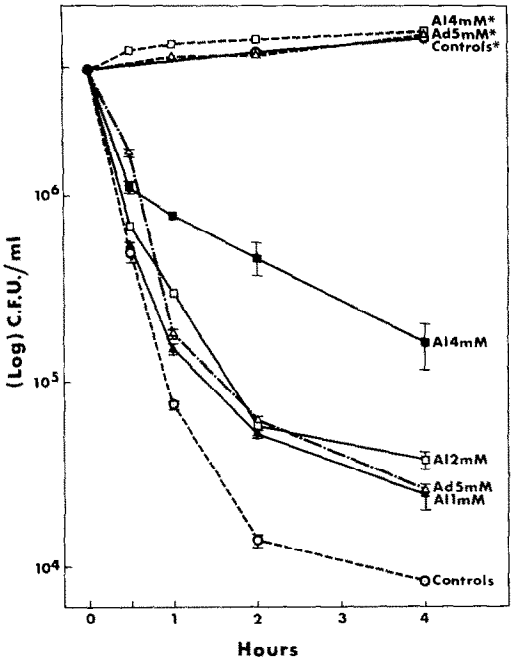

Fig. 2. Effect of allopurinol and adenine on the bactericidal activity of phagocytes from mice infected i.v. with *S. aureus* CN 6538 5 days before. Each point is the mean of three different experiments (four for allopurinol 2 mM at 2 and 4 hr) each employing triplicate determinations on pools of cells from ten animals in each group. Al, allopurinol; Ad, adenine; *, without phagocytes. When standard errors are omitted, they are smaller than symbols.

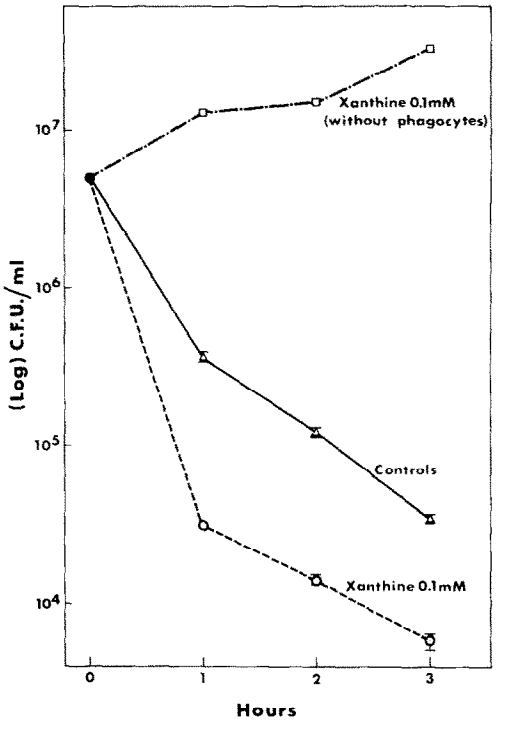

Fig. 3. Enhancement of the phagocytic bactericidal activity by xanthine using PMN from normal mice. Each point is the mean of three different experiments each employing triplicate determinations on pools of cells from ten animals in each group.

The results of the *in vivo* experiments are given in Figs. 1–3. In the absence of PMN, the XO inhibitors shows that allopurinol (1 mM, 2 mM) and oxypurinol (2 mM) decrease the bactericidal activity of PMN from normal mice, and that this decrease appears to be dose-dependent. Adenine (5 mM) has a similar effect at the earlier incubation times, but by 4 hr the killing activity of the PMN is tending towards control levels.

Figure 2 shows the effects of allopurinol (1 mM, 2 mM, 4mM) and adenine (5 mM) on PMN from infected mice. The bactericidal activity is again decreased, but to obtain an effect comparable to that observed in Fig. 1 with normal mice, approximately twice the concentration of allopurinol is required. Adenine also has an effect on the killing properties of the PMN that is less pronounced than that shown in Fig. 1, particularly at the early incubation times. At this concentration (5 mM), adenine exhibits an effect similar to that of allopurinol at 1 mM. The relative efficacies of allopurinol and adenine in the *in vivo* experiments therefore approximate the concentration ratio (1:5.28) which *in vitro* inhibited 50 per cent of XO activity, indicating that the effects of the XO inhibitors on the killing properties of PMN are indeed related to their action on XO. Together, these results support the concept that increased levels of endogenous XO are involved in the killing activity of PMN.

Figure 3 illustrates that addition of exogenous xanthine, even at low concentrations (0.1 mM), enhances the bactericidal activity of PMN obtained from normal mice, providing further evidence of a role for XO in this activity.

Identification of the enzyme responsible for superoxide–peroxide production in granulocytes is a major biochemical problem in the elucidation of the phagocytic function and its role in resisting infection [8]. Takanaka and Usui [9] found a deficiency of a plasma membrane NADPH-oxidase in patients affected by chronic granulomatous disease, and Babior and Kipnes [10] found in human neutrophils evidence for an FAD-requiring NADPH-oxidase that would be responsible for the superoxide-forming activity of these cells. Our results suggest that XO plays a role in the bactericidal activity of PMN and therefore may, at least in part, be involved in the superoxide production by these cells.

In summary, allopurinol decreased the killing properties of PMN, but twice the dose was required to elicit an effect on PMN from infected mice equal to that observed with PMN from normal mice. Adenine also decreased the bactericidal activity of PMN, but the effect was less pronounced. The relative efficacy of allopurinol and adenine in *in vivo* experiments approximated the concentration ratio required *in vitro* to achieve 50 per cent reduction of XO activity. Addition of xanthine to PMN from normal mice increased the killing activity of the PMN. The results are consistent with the proposed hypothesis that increased levels of endogenous XO activity are involved in the killing properties of PMN.

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A specific and sensitive microassay for hydroxyindoles in biological material—application for cerebral microvessels, brain, and pineal organ of the rat

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Serotonin (5-hydroxytryptamine; 5-HT) is an important neurotransmitter in the mammalian central nervous system. One of the most effective methods for measuring small amounts of serotonin involves conversion of serotonin to [^{3}H]melatonin [1]. In our experience, however, this radioenzymatic microassay for serotonin does not provide the sensitivity initially reported (50 pg, approximately 280 fmoles), mainly because the radioactive product ([^{3}H]melatonin) is not adequately resolved from other isotopic products, so that higher blanks and diminished sensitivity result. We have revised this assay to improve its sensitivity and reliability, by including unidimensional multiple-development thin-layer chromatography (UMD-TLC).

Materials and methods

Reagents were of analytical grade and were obtained commercially (Sigma Chemical Co., St. Louis, MO). Rat liver *N*-acetyltransferase (NAT: EC 2.3.1.5) and bovine hydroxyindole *O*-methyltransferase (HIOMT; EC 2.1.1.4) were prepared as described by Saavedra *et al* [1]; each microliter of NAT acylated 0.056 nmole of tryptamine in 15 min at 37°, and each microliter of HIOMT methylated 0.14 nmole of *N*-acetyl serotonin (NAS) in 15 min at 37°.

Precoated silica gel TLC plates (LK5D; Whatman, Inc., Clifton, NJ) were activated at 200° for 10 min immediately before use. Adenosyl-L-methionine-*S*-[methyl-^{3}H] (SAM; 5–11 Ci/mmole) and [^{3}H]melatonin (25 Ci/mmole) were purchased from New England Nuclear (Boston, MA).

Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained at an ambient temperature of 23 ± 2°, on a 12-hr light–dark cycle, and allowed access to food and water *ad lib*. Brain microvessels, free of contaminating blood elements, were prepared from individual rat brains by a previously described sucrose density-gradient method [2].

Serotonin assay. Tissue samples of microvessels (2–3 mg), brain (5–20 mg) and pineal (1–1.5 mg) were homogenized in 100, 200 or 400 μl of 4 M formic acid, respectively, with a 1-ml ground-glass homogenizer (Kontes Glass Co., Vineland, NJ). Ten microliters were removed for determination of protein content [3], while the remainder, along with an ethanol wash of equal volume, was collected in 1.5-ml plastic centrifuge tubes and centrifuged at 30,000 *g* for 30 min at 4°. Duplicate aliquots of the clear supernatant fluid (75 μl for microvessels, 150 μl for brain tissue, and 5–10 μl for pineal) were transferred to 16 × 100 mm tubes and dried *in vacuo*. The residue was dissolved in 50 μl of phosphate buffer (pH 7.9, 0.5 M) containing 5×10^{-6} M *m*-hydroxybenzylhydrazine and subjected to the following reactions.

Ten microliters of a 1.24-mM *S*-acetyl coenzyme A (AcCoA) solution was added to each of the tubes, which were then placed in a 37° water bath. The enzymatic reactions were begun by adding 10 μl NAT. After 30 min, each sample received 19 μl phosphate buffer, 2 μl (1 μCi, 0.147 nmole) [^{3}H]SAM, and 4 μl HIOMT. After an additional 15 min, the reaction was stopped by adding 0.5 ml of a boric acid buffer (pH 10.0, 0.5 M) containing 1 μg of unlabeled melatonin.

The melatonin was extracted quantitatively from the reaction mixture by vortexing each sample for 30 sec with 3 ml $CHCl_3$. Samples were spun for 10 min at 500 *g*, and the aqueous phase was aspirated and discarded. The organic phase was vortexed briefly with an equal volume of 1 mM HCl, which was removed similarly. Two milliliters of the organic phase was transferred to a fresh set of 13- × 100-mm test tubes and dried under a stream of nitrogen gas. The dried samples were dissolved in 75 μl of absolute ethanol and spotted on TLC plates. The TLC plates were developed by UMD ascending chromatography: the plates were developed to a height of 10 cm, allowed to dry for 5 min (maintained in the vertical position), and developed again a total of eight times with pure chloroform as the developing solvent [4]. The spots corresponding to authentic melatonin were visualized with short-wave ultraviolet light or with Ehrlich's reagent [5], scraped into 5-ml scintillation vials, eluted with 300 μl of absolute ethanol for 10 min, and counted in the presence of 3 ml toluene phosphor at an efficiency of approximately 32 per cent.

NAS assay. Samples assayed for NAS were treated similarly to those assayed for serotonin, except that NAT and AcCoA were omitted from the reaction mixture. Inasmuch as the brain contains minute amounts of NAS, this compound does not normally add to the amount of [^{3}H]melatonin formed. In the pineal, however, the radioactivity generated by endogenous NAS may be considerable and therefore must be subtracted from the total.

Results and discussion

Using conditions similar to those of Saavedra *et al*. [1], we found this newly developed assay to be linear between 125 fmoles and 4 pmoles of added serotonin or NAS in aqueous media (Fig. 1) or in the presence of tissue. When increasing amounts of serotonin were added to equal amounts of a microvessel homogenate, the isotopic melatonin formed remained linear over the range of concentrations tested (Fig. 1). Under these conditions, the recovery of exogenous serotonin was always more than 80 per cent, and the intra-assay coefficient of variation was less than 5 per cent.

To demonstrate the advantages of UMD-TLC, we compared samples chromatographed by UMD-TLC with those