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AURANOFIN INHIBITS THE ACTIVATION PATHWAYS OF POLYMORPHONUCLEAR LEUKOCYTES AT MULTIPLE SITES

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Abstract—In order to characterize the mechanism by which the anti-rheumatic gold complex auranofin (AF) affects the functions of resting and activated polymorphonuclear leukocytes (PMN) the following studies were performed: (1) The effect of AF on the major processes involved in the respiratory burst of PMN: glucose transport and phosphorylation; hexose monophosphate (HMP) shunt activity in intact cells and in a cell-free system; superoxide production by particulate fractions and intact PMN measured as lucigenin-dependent chemiluminescence. (2) A comparison of the effects of AF added to the PMN before, at the time of, or subsequent to the stimulants [*N*-formyl-methionyl-leucyl phenylalanine (FMLP), concanavalin A (ConA), calcium ionophore (A23187) and phorbol myristate acetate (PMA)]. (3) The effect of AF on PMN activated by two stimulates (PMA, ConA) added sequentially. AF (0.1–10 μM) caused a dose-dependent inhibition of lucigenin-dependent chemiluminescence regardless of the activator (FMLP, ConA, A23187, PMA) when AF was added before the activator. In contrast, when AF was added to PMN after stimulation, it inhibited only the chemiluminescence of PMN stimulated by PMA. Furthermore, the chemiluminescence was largely unaffected by AF in sequentially activated PMN. The relative sensitivity to AF of the various processes studied indicates that blockade of the activation signal appears to be responsible for inhibition of the respiratory burst of PMN.

Auranofin (AF) is a gold complex which is widely used in the treatment of rheumatoid arthritis (RA) [1, 2] but its exact mechanism of action is unknown [3, 4]. Since the polymorphonuclear leukocyte (PMN) is an effector cell in the inflammatory process, the effect of AF on function of these cells has been widely investigated in an attempt to understand the clinical effects of AF. It has been shown that AF, *in vitro* at pharmacological concentrations, modulates many functions of stimulated PMN, some of which may be involved in the participation of PMN in inflammation. These include aggregation [5, 6], chemotaxis [5, 7], lysosomal enzyme release [4–10], phagocytosis [5, 7, 11], leukotriene B4 release [12], membrane potential changes [7], chemiluminescence [3, 5, 7, 11], superoxide production [7, 11, 13, 14] and glucose oxidation through the hexose monophosphate (HMP) shunt [6]. AF also affects properties and functions of unstimulated PMN, inhibiting spreading, causing a decrease in the negative surface charge of the resting cells, and gradually depolarizing the resting membrane potential [7]. Despite this observed suppression of multiple responses of PMN by AF, there is no evidence pointing to a key site of inhibition and the precise mechanism of action of AF is unknown.

The purpose of our study was to investigate the inhibitory effect of AF on the respiratory burst of PMN by comparing the degree of inhibition of the major processes involved. In particular we examined the effect of the drug on (a) the ability of PMN to maintain adequate NADPH concentration (glucose transport and HMP shunt activity), (b) NADPH-oxidase activity in a particulate fraction, and (c) lucigenin-dependent chemiluminescence as a measure of the oxidative burst which follows cell activation. Lucigenin is oxidized primarily by extracellular superoxide [15, 16] and its chemiluminescence is largely a measure of the release of this oxygen metabolite produced by activated PMN. To explore further the mechanism of action of AF we have studied its effects on different steps of the transduction by utilizing various compounds which are known to stimulate the cells by different pathways. The inhibition of the respiratory burst of PMN was also assessed by addition of AF to PMN prior to, at the time of, and subsequent to stimulation. PMN from patients with chronic granulomatous disease (CGD) incapable of superoxide production, were used to study the effects of AF on some processes in the absence of an oxidative burst.

MATERIALS AND METHODS

Materials. AF was obtained from Smith, Kline and French Laboratories (Sydney, Australia). Stimulants (PMA, FMLP, ConA, and A23187). $NADP^+$, NADPH, ATP and lucigenin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.); phosphate buffered saline (calcium and magnesium free-PBS), heat inactivated foetal calf serum from Flow Laboratories (Sydney, Australia) and Ficoll-Paque, Dextran T-500 from Pharmacia AB, (Uppsala, Sweden). All other chemicals were of

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reagent grade. Stock solutions of PMA (1 mg/mL), A23187 (5 mg/mL) and FMLP (1 mg/mL) were prepared in dimethyl sulphoxide and stored at −20°.

Subjects. Studies were conducted on the PMN of healthy volunteers apart from studies on the PMN of two CGD patients whose parents had given informed consent. These patients had been selected since their PMN had produced no detectable lucigenin-dependent chemiluminescence. The study was approved by the ethics committee of the Prince of Wales Hospital.

Preparation of PMN suspension. PMN were isolated from 10 mL samples of heparinized blood from normal volunteers by the method of Boyum [17]. Cells containing more than 98% of PMN were resuspended in protein free Krebs–Ringer phosphate buffer containing 5.5 mmol/L glucose (KRPG) at a cell concentration of 1×10^6/mL, kept on ice and used within 5 hr.

PMN viability. This was assessed by the exclusion of trypan blue. Cell suspensions were mixed with an equal volume of 0.25% trypan blue in normal saline and the proportion of blue staining cells were determined.

Uptake of deoxyglucose. 2-Deoxy-D-[^{3}H]glucose ([^{3}H]DOG) was employed to measure glucose uptake. [^{3}H]DOG enters cells at the same sites as glucose by carrier-facilitated transport and is then irreversibly phosphorylated, but is not further metabolized and thus accumulates within the cells [18]. Therefore, the total amounts of phosphorylated and nonphosphorylated [^{3}H]DOG in the PMN were determined in [^{3}H]DOG uptake assay. Concentrations of AF between 1 and 100 μM were used to study the effect of drug on deoxyglucose transport in PMN. [^{3}H]DOG uptake by 2×10^6 PMN was measured as described previously [19].

HMP shunt activity in intact PMN. The activity of the HMP shunt was measured by the oxidation of [^{14}C]glucose labelled in the C1 position to $^{14}CO_2$ as described previously [19]. [1-^{14}C]Glucose (0.2 μCi) was added to PMN together with either 2 mM methylene blue which stimulates the hexose monophosphate shunt without stimulating the respiratory burst [6] or 0.1 μg/mL of PMA which causes the activation of the HMP shunt due to activation of NADPH-oxidase in the plasma membrane. This method is dependent also on hexokinase activity.

HMP shunt activity in a cell free system. The activity of the enzymes involved in the HMP shunt was measured using a modification of the method described by DeChatelet *et al.* [20]. PMN were isolated as described above, resuspended in distilled water at a cell concentration 10^7/mL and disrupted by sonication. KRPG was added to the sonicate and cell debris removed by centrifugation at 500 *g* for 10 min. The supernatant at a concentration equivalent to 10^6 PMN/mL was supplemented with ATP (1 mM) and $NADP^+$ (0.24 μM). The radioactive [1-^{14}C]glucose (0.2 μCi) was used to measure the effect of AF on the generation of $^{14}CO_2$.

Chemiluminescence of PMN. In experiments on the effect of AF on resting cells, PMN (2×10^5/mL) were preincubated with AF (0.1–5 μM) and lucigenin (0.3 mg/mL), for 5 min at 37° in KRPG. The cells were then stimulated with PMA (0.1 μg/mL), FMLP (5 μM), ConA (30 μg/mL) and A23187 (5 μM). The chemiluminescence was measured at 37° in a Packard Picolite 6500 luminometer (modified to incorporate temperature control) over 5 sec intervals every 2 min for a total of 30 min after stimulation. This allowed up to 12 tubes to be run in parallel. In some experiments the effects of AF on the chemiluminescence of PMN, the chemiluminescence of single incubations was measured continuously for 5 min with the AF being added to cells before as well as 0.5, 1 and 1.5 min after the addition of PMA.

Isolation of a particulate fraction containing NADPH–oxidase activity. Human PMN were isolated from heparinized blood obtained from normal volunteers as described above, but the final pellet was resuspended at a cell concentration of 10^7/mL in KRPG buffer containing 1 mM sodium azide. The PMN were prewarmed for 5 min at 37° and activated by the addition of PMA at a final concentration of 1 μg/mL. After 3 min the activation was terminated by addition of 20 mL ice-cold PBS (Ca and Mg free). The cells were centrifuged at 200 *g* for 10 min at 4°, resuspended in 1 mL of distilled water and immediately disrupted by sonication (Branson Soniprobe) four times for 4 sec on melting ice. Then, 1 mL of ice-cold 0.68 M sucrose was added to the sonicate and the unbroken cells and cell debris were removed by centrifugation at 500 *g* for 10 min. The supernatant was centrifuged at 27,000 *g* for 30 min at 4°. The final pellet was resuspended at a concentration equivalent to 10^6 cells/mL in 0.34 M sucrose. The resuspended 27,000 *g* pellets are referred to as the particulate fraction. This fraction consists mainly of plasma membrane fragments, azurophil and specific granules [21]. All experiments were performed on freshly isolated particulate fractions, although they can be stored at −70° for several months [22].

NADPH–oxidase activity in the particulate fraction. The effect of AF on NADPH–oxidase activity was determined by the lucigenin-dependent chemiluminescence method [23]. Reaction mixtures were prepared in a total volume of 1 mL, using PBS (Ca and Mg free) with 1 mM sodium azide. The tubes containing particulate fraction (2×10^5 cell equivalent) and lucigenin (0.6 mg/mL) were prewarmed for 3 min at 37° and after addition of 100 μL of AF (0.1–10 μM) or buffer (controls) incubated for a further 5 min. The reaction was started by adding 100 μL NADPH (0.2 mM) and the chemiluminescence was measured as described above.

Statistics. The overall significance of differences between treatments were determined by one factor analysis of variance with repeated measures, while the Scheffe test was used to examine the significance level of specific contrasts. The Statview program (Brainpower, Inc., Calebases, CA, U.S.A.) was used.

RESULTS

The effect of AF on deoxyglucose transport

AF inhibited [^{3}H]DOG transport in resting and PMA activated PMN. Some of these data were published previously [19] but are presented again for comparison. Table 1 shows the dose-dependent effect of AF on [^{3}H]DOG transport in PMN from healthy subjects and patients with CGD both before

Table 1. Effect of AF on activity of PMN

	Control activity	% Control activity AF concentrations 1 μM	10 μM	100 μM
DOG transport (cpm)				
Resting PMN	60,777 ± 5806	91.4 ± 5.2	21.1 ± 6.4*	5.2 ± 1.3*
PMA-activated PMN	47,797 ± 5552	84.5 ± 3.6	11.9 ± 4.2*	3.9 ± 0.9*
Resting CGD PMN	109,052 ± 162	89.4 ± 15.6	14.2 ± 2.0*	3.9 ± 0.9*
PMA-treated CGD PMN	114,715 ± 5381	81.8 ± 0.8	11.3 ± 1.6*	2.0 ± 1.9*
HMP shunt activity (cpm)				
PMA-activated PMN	3239 ± 282	116.9 ± 12.1	24.8 ± 6.7*	0.3 ± 0.2*
MB-activated PMN	6185 ± 1200	107.8 ± 9.9	21.6 ± 3.4*	2.9 ± 0.8*
MB-activated CGD PMN	6215 ± 756	67.7 ± 7.1*	17.1 ± 3.1*	0.85 ± 0.8*
Cell-free system	305,799 ± 24,056	53.3 ± 3.5*	31.3 ± 4.3*	23.9 ± 6.4*
Chemiluminescence (counts × 1000)				
Particulate fraction	519.2 ± 192.4	75.8 ± 13.8	56.8 ± 13.7*	39.5 ± 7.9*
Intact PMN	18,822 ± 9055	13.6 ± 3.0*	2.3 ± 1.3*	0.8 ± 0.1*

Results are calculated as a percentage of control (without AF) and presented as a mean ± SD for 3–8 experiments (or mean and range for 2 experiments) on the PMN from CGD patients. Significant differences from control ($P < 0.05$) are shown by (*).

and after their stimulation with PMA. AF tended to be less inhibitory on unstimulated than PMA-stimulated normal PMN but the differences are not significant. The possibility that the effect of AF on [^{3}H]DOG uptake in normal PMN may be influenced by the stimulation of NADPH–oxidase was tested by using PMN from two CGD patients (whose PMN had been shown to be incapable of this activation). In PMN from these two patients with CGD, control uptake of [^{3}H]DOG was higher than in the PMN from healthy subjects, but as in the healthy subjects, the presence of PMA had no significant effect on degree of inhibition of glucose uptake caused by AF. Thus, the combined data suggest that AF has an inhibitory effect on the [^{3}H]DOG uptake by normal and CGD PMN and its effect is likely to be independent of the activity of NADPH–oxidase.

The effect of AF on HMP shunt activity

The activity of the HMP shunt was assessed by measurement of $^{14}CO_2$ released during glucose-6-phosphate oxidation generated from [1-^{14}C]glucose. The amounts of radioactivity CO_2 released by PMA and methylene blue activated normal and CGD PMN were expressed as percentage of activity of the resting cells. The stimulation of the HMP shunt by methylene blue in normal PMN or in PMN from CGD patients allowed the measurement of the direct effect of drug on the HMP cytosolic enzymes without the complicating interactions with other processes accompanying the activation of PMN. AF at a low concentration (1 μM) had no significant effect while at higher concentrations (10 and 100 μM) inhibited HMP shunt activity in both normal and CGD PMN stimulated by PMA or methylene blue (Table 1). These results provide evidence for a direct inhibitory effect of AF on the HMP shunt enzymes, consistent with results reported by Wolach *et al.* [6].

The direct effect of AF on enzymes involved in HMP shunt (or hexokinase) activity was further demonstrated by the measurements of [1-^{14}C]glucose oxidation in a cell free system in the presence of $NADP^+$ and ATP. HMP shunt activity in the cell free system was inhibited by AF although the dose–response relationship differs from that seen in intact cells (Table 1).

Effect of AF on NADPH–oxidase complex in particulate fractions from activated PMN

The particulate fractions from activated PMN are known to generate superoxide in the presence of NADPH [13, 23–25]. In this study we used lucigenin-dependent chemiluminescence to measure NADPH-dependent generation of superoxide by a particulate fraction. The results from six independent experiments are presented in Table 1. AF had no significant effect at 1 μM but there was inhibition at 10 and 100 μM. Overall, AF had much less effect on chemiluminescence generated by the particulate fraction of PMA activated PMN than on all intact cell functions measured (Table 1).

Effect of AF added prior to stimulation on the oxidative burst

The effect of AF on the lucigenin-dependent chemiluminescence of intact cells activated by PMA, A23187, ConA and FMLP was studied. The total chemiluminescence over 30 min was calculated and all data is presented as a percentage of control. Comparison of dose–response curves obtained with above stimulants, shows that AF had a potent, concentration-dependent, effect on total chemiluminescence of human PMN which had been exposed to the drug prior to stimulation (Fig. 1). The concentration of AF causing 50% inhibition of total chemiluminescence was less than 0.1 μM for ConA stimulated PMN and between 0.1 and 0.5 μM when FMLP, A23187 or PMA were used.

The effect of low concentrations of AF on the oxidative burst of PMN is dependent on the cell numbers [19]. Experiments on the chemiluminescence of PMN were generally conducted at

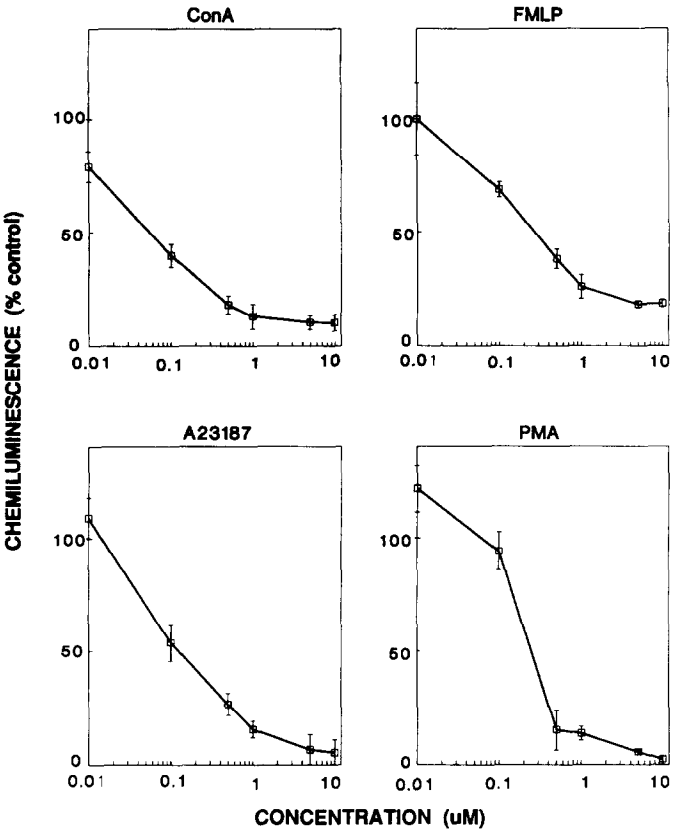

Fig. 1. Influence of varying concentrations of AF on lucigenin-dependent chemiluminescence of PMN. The four panels show the dose–response curves for PMN preincubated with AF for 10 min then activated by ConA, FMLP, A23187 and PMA. The total chemiluminescence over 30 min was calculated and results expressed as percentage of control, i.e. without AF. Data are presented as mean ± SD of 3–12 experiments.

2×10^5 PMN/mL while experiments on the HMP shunt and [^{3}H]DOG uptake were conducted at 1×10^6 and 2×10^6 PMN/mL, respectively. Therefore, in order to compare the effect of AF on lucigen-dependent chemiluminescence with its effect on the uptake and metabolism of glucose, additional chemiluminescence experiments were also conducted with 1×10^6 and 2×10^6 PMN/mL. AF at a concentration of 10 μM, inhibited chemiluminescence of 1×10^6 and 2×10^6 PMN to a much greater extent (% of control: 0.33 ± 0.1 and 0.4 ± 0.2, respectively) than the HMP shunt and [^{3}H]DOG uptake (Table 1).

Effect of AF added with or after stimulants of the oxidative burst

In all cases, AF inhibited the oxidative burst of PMN when the drug was added prior to the stimulant. However, it had variable effect when added at the same time as or after the stimulant (Fig. 2). Under these conditions, AF (1 μM) had no significant effect on the oxidative burst after activation by ConA (Figs 2 and 3A), FMLP or A23187 (Fig. 2). By contrast, AF (1 μM) added with or after the addition of PMA still inhibited the oxidative burst, the inhibition being produced within 5 to 10 sec of addition of AF. Similar to the effect when resting cells are preincubated with AF prior to stimulation with PMA, chemiluminescence reached the plateau immediately and after about 2 min started to decline (Figs 2 and 3B).

The effect of AF on heterogeneous sequential stimulation of PMN

The effect of AF on PMA and ConA stimulated PMN was further examined by the sequential use of two stimulants ConA and PMA. AF at the concentration of 1 μM was added 30 sec after initial activation (as in previous experiments), while the second stimulant was added a further 30 sec later. Prior activation of the PMN by ConA prevented the inhibitory effect of AF on the subsequent activation of the cells by PMA (added as a second stimulant at 1 min). In fact, PMA caused additional cell activation with a distinct second peak (Fig. 3C). When the stimulants were added in the reverse order, the inhibitory effect of AF on cells which had been activated by PMA, was largely overcome when ConA was subsequently added (Fig. 3D). Overall, the addition of the second heterologous stimulant to AF-treated PMN modulates the pharmacological effect of AF on the cells.

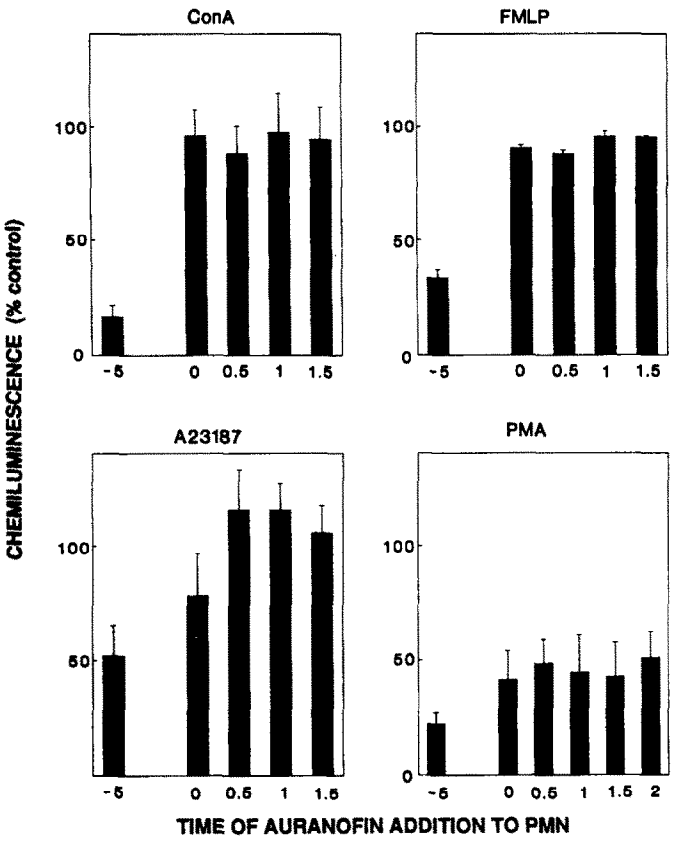

Fig. 2. The effect of AF on lucigenin-dependent chemiluminescence of PMN activated by ConA, FMLP, A23187 and PMA. AF (1 μM) was added to cells either 5 min prior to, at the time of, or 0.5, 1 and 1.5 min after stimulants. Results expressed as percentage of control are from three individual experiments. Data are shown as mean ± SD. Significant differences from control ($P < 0.05$) are shown by (*).

PMN viability

The effect of AF on the viability of PMN was examined. Trypan blue exclusion was at least 98% in the presence 100 μM AF.

DISCUSSION

In the present work, the effects of AF on various aspects of the oxidative burst were examined. One aspect studied concerned the supply of metabolites. An essential metabolite for the respiratory burst is NADPH. This is generated by the cytosolic enzymes involved in HMP shunt but also requires the transport of glucose into PMN and its phosphorylation by hexokinase. Therefore, the inhibitory effect of AF on the oxidative burst of PMN under certain conditions could be related to (a) the lack of substrate for the superoxide generating enzyme, NADPH–oxidase, (b) direct inhibition of NADPH–oxidase, or (c) inhibition of signal transduction. We studied the relative sensitivity of these processes to AF.

When [^{3}H]deoxyglucose is employed as a marker of hexose transport the assay actually measures both glucose transport, and phosphorylation. Our results indicate that the processes responsible for adequate cytosolic concentration of glucose-6-phosphate were inhibited by AF although the degree of inhibition was considerably lower than the degree of inhibition of the respiratory burst measured as lucigenin-dependent chemiluminescence. The present studies show also that AF had a greater inhibitory effect on lucigenin-dependent chemiluminescence than on the HMP shunt of PMA-activated PMN. This is a somewhat surprising finding since the HMP shunt functions only when $NADP^+$ is available, which is generated by NADPH–oxidase and the glutathione cycle [26]. One possible explanation for this finding is that, even in the presence of 10 μM AF, there is still some residual NADPH–oxidase activity but the generated superoxide is converted to further metabolites, such as hydrogen peroxide or hypochlorous acid [27–30] and is not released extracellularly to be detected by lucigenin. The peroxidase–glutathione reductase system could then generate additional $NADP^+$ [27, 28, 30] thus boosting the activity of the HMP shunt. A direct effect of AF on the enzymes involved in the HMP shunt is however apparent. AF still inhibits the HMP shunt in the absence of the oxidative burst; in PMN in the presence of methylene blue, and in PMN from CGD patients. Our results are thus in agreement with Wolach *et al.* [6] who found that AF had a direct toxic effect on the HMP shunt. However, the effect of AF on the HMP shunt

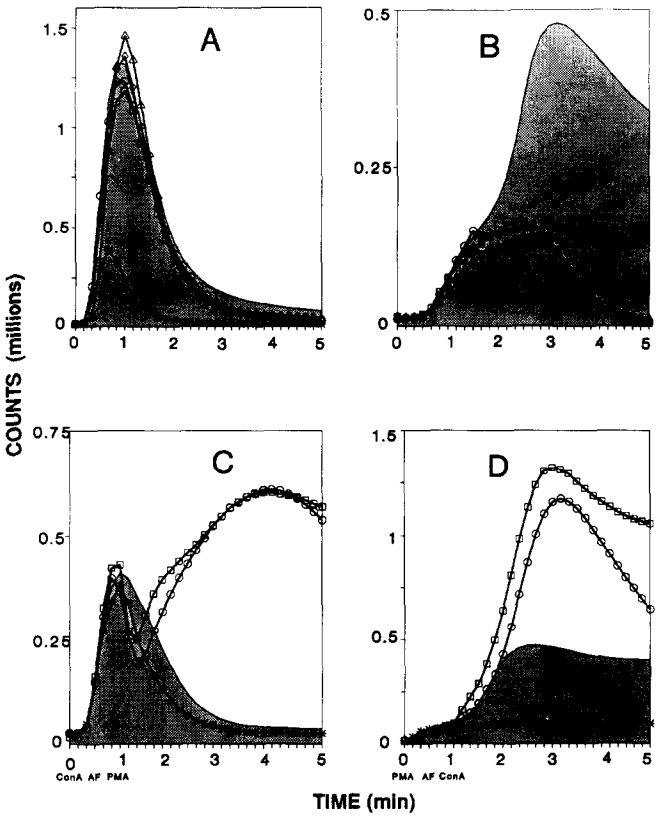

Fig. 3. Panels A and B: Effect of AF on time-course of lucigenin-dependent chemiluminescence of PMN activated by ConA (panel A) and PMA (panel B). AF (1 μM) was added to PMN either 5 min prior to, at the time of, or 0.5, 1 and 1.5 min after stimulants. Chemiluminescence was measured continuously for 5 min. Key: control (edge of shaded area), 5 min preincubation (*), simultaneous addition with stimulant (△), delayed addition to stimulated cells: at 0.5 min (□), 1 min (◇) and 1.5 min (○). Panels C and D: The effect of AF (1 μM) on time-course of lucigenin-dependent chemiluminescence of PMN stimulated by sequential addition of ConA and PMA (panel C) or PMA and ConA (panel D). PMN were stimulated with the first stimulant at zero time and AF was added 30 sec later. To some samples the second stimulant was added 1 min after the first stimulation. Chemiluminescence was measured continuously for 5 min. Key: control single stimulation (edge of shaded area), control sequential stimulation (□), AF added to single stimulated cells (*) and AF added between stimulants during sequential stimulation (○).

does not fully account for its very potent inhibition of the respiratory burst. Furthermore, when the direct effect of AF on HMP cytosolic enzymes and hexokinase activity was evaluated in a cell free system, the HMP shunt was inhibited by AF to an even lesser degree than observed during methylene blue stimulation of intact cells. On the basis of these results it appears that the inhibitory effect of AF on the respiratory burst of PMN is not caused by the inability of PMN to maintain adequate NADPH levels. Thus, glucose transport, hexokinase activity and HMP shunt enzymes are not key sites of the action of AF in the inhibition of oxidative metabolism of PMN.

The direct effect of AF on the NADPH–oxidase complex is another possible mode of AF action on the PMN respiratory burst. The particulate fraction was isolated from PMA-activated PMN to assess the likelihood that AF has a direct effect on NADPH–oxidase activity. It is apparent that AF is a weak inhibitor of NADPH–oxidase. A similar conclusion was drawn by Minta and Williams [13] that Parente *et al.* [25]. Furthermore, the translocation of a cytosolic component essential to form an electron transport chain [31, 32] is also not inhibited by AF [25]. On the basis of these published data and our results, it can be concluded that neither formation of the NADPH–oxidase complex nor its activity can be inhibited by AF sufficiently to account for the overall inhibition of the respiratory burst of PMN. Thus, the most sensitive components of the PMN respiratory burst to AF must exist in the steps prior to activation of NADPH–oxidase.

The effects of AF on the PMN respiratory burst when the drug was added at the time of, or subsequent to activation suggest that the mode of AF action relies on inhibition of multiple steps in the activation pathway rather than a single common step. AF, when added before stimulation, inhibited the initiation of the respiratory burst regardless of the

stimulant used. This implies that the steps which are inhibited by the drug in PMA pathways are permanently involved in NADPH–oxidase activation. On the other hand, when FMLP, ConA or A23187 are used, AF can prevent activation of resting cells, but cannot inhibit previously activated PMN. This implies that steps which are involved in the maintenance of the PMN respiratory burst after activation by these stimulants are not inhibited by AF, but the steps which are only transiently required for initiation of stimulation are inhibited. Thus, it can be implied that there are two types of steps in the activation pathways which can be inhibited by AF, one which is required temporarily for the initiation of the activation only and another which is permanently required to maintain superoxide production. Furthermore, the results indicate that the AF-inhibitable steps in the PMA transduction pathway does not appear to be involved in the continuation of the oxidative burst after stimulation by FMLP, ConA and calcium ionophore A23187. The lack of effect of AF on lucigenin-dependent chemiluminescence when added after FMLP, ConA or A23187, is consistent with the observation that the drug does not directly inhibit the NADPH–oxidase involved in the production of superoxide (present study and Ref. 25).

To explore further the mechanisms of action of AF on signal transduction we have studied its effect on sequentially activated PMN. It was found that the presence of a second stimulant (ConA) largely overcomes the inhibitory effect of AF on PMA-activated PMN. Furthermore, the inhibition of PMA-activated cells by AF can no longer be seen when PMA is added as a second stimulant to AF-treated and ConA-activated PMN. It is apparent that the presence of stimulants which can activate the cells after delayed addition of drug impaired the ability of AF to inhibit the steps in the PMA activation pathway. Alternatively, it is possible that the AF-sensitive steps are no longer in use during sequential stimulation. It can be postulated that during sequential stimulation, parallel activation pathways exist which allow the activation signals to be conveyed to NADPH–oxidase, despite the presence of an AF-inhibited step in some pathways. Further, the recent results of Parente *et al.* [33] and Friscio *et al.* [34] which indicate that the primary target of AF is PKC itself, seems to partially explain the stimulus-dependent effects of AF on PMN respiratory burst seen in our studies. Since the PKC is a receptor for PMA [35] and the major transducer of the activation signal, it can be expected that both activation and activity of PMN will be promptly inhibited by AF. In contrast, calcium ionophore A23187 activation of PMN is not mediated by PKC [33, 36, 37], thus the gold-inhibitable step in the A23187 activation pathway is distinct from PKC and is required only for initiation of the activation. This is consistent with our hypothesis that AF affects multiple steps in the transduction pathway of PMN. The mechanisms by which AF inhibits the respiratory burst of PMN activated by FMLP and ConA, which would include PKC inhibition, is unclear. Since it is widely accepted that the PKC step is included in FMLP and ConA activation pathways [38, 39], the inhibition of the respiratory burst by AF added before a stimulant would imply that PKC itself and/or other steps are inhibited by AF. However, the lack of effect of AF on PMN when AF was added after FMLP or ConA might indicate that PKC is not involved in the continuation of the respiratory burst or that an alternative pathway of signal transduction can be activated when the PKC step was inhibited.

The clinical relevance of the data obtained in the present study is not clear but two possibilities should be considered. Our finding of the restricted effect of AF on PMN which are already activated indicates that the potential *in vivo* effects of the drug may have been overestimated from most previous studies in which AF was added to the cells before their stimulation since the cells in synovial fluid are predominantly in the activated state [40]. Further work with other stimulants of the oxidative burst and other functions of both PMN and mononuclear leukocytes is required to determine the extent to which the lack of effect of AF is lost after its addition to cells which are already stimulated.

A further restriction of the effect of AF *in vivo* concerns the concentrations of pharmacologically active species circulating in plasma. During dosage with AF, the plasma concentration of gold is about 1 μM [41] with somewhat higher concentrations in synovial fluid [42] but much of this is protein bound, possibly as a complex with albumin [43]. Binding to the plasma proteins decreases both the association of gold to cells [44] and the effect of AF on isolated PMN [6, 7] but, in the present studies, we examined the effects of AF in a protein-free medium and found effects on the oxidative burst of PMN at concentrations below 0.1 μM, considerably below the total plasma and synovial fluid concentrations of gold. It is not known if intact AF occurs in plasma at this level during treatment but the high potency of AF on isolated PMN indicates that an effect on these cells *in vivo* cannot be disregarded. The active species *in vivo* in subjects receiving oral AF therapy is unknown but there is evidence of considerable levels of a gold complex which, like AF, binds to red blood cells in plasma of rats [45] and the existence of a glutathione–gold–albumin complex has recently been proposed [46]. Further work is required to define the concentrations of AF or related reactive gold species *in vivo* in order to determine fully the clinical significance of *in vitro* studies of the type discussed in this paper.

Our data can be interpreted to support the following. The inhibition of the activation signal appears to be responsible for termination of the respiratory burst of PMN, since the steps most sensitive to AF seem to be in the transduction pathway. There may be two or even more types of AF-inhibitable steps in the transduction pathway; one which is required only temporarily for initiation of activation and another which is required to maintain the respiratory burst. The AF-sensitive early steps in the transduction pathway may be included in the activation pathways of all stimulants tested but the maintenance step is not common to these pathways and consequently, AF has no effect on PMN already activated by certain stimulants. During heterogeneous stimulation of PMN, the steps inhibited by AF are excluded from the amalgamated activation pathway.

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