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THE ACTIVATION OF GOLD COMPLEXES BY CYANIDE PRODUCED BY POLYMORPHONUCLEAR LEUKOCYTES—II

EVIDENCE FOR THE FORMATION AND BIOLOGICAL ACTIVITY OF AUROCYANIDE

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Abstract—Experiments have been conducted to investigate a possible mechanism which might explain why aurothiomalate (Autm), a gold complex used in the treatment of rheumatoid arthritis, is active *in vivo* but not *in vitro*, by testing the hypothesis that Autm is converted to aurocyanide by activated polymorphonuclear leukocytes (PMN) which generate cyanide from thiocyanate, an anion which is present in plasma at concentrations ranging from 20 to 200 μM. Two-stage experiments were conducted in which PMN, in the first stage, were activated by opsonized zymosan in the presence of Autm both with and without thiocyanate. Then, in the second stage, the effect of the drugs on superoxide (O_2^-) production stimulated by a further addition of zymosan was measured. Autm at concentrations of 10 and 100 μM decreased O_2^- production if thiocyanate was present, but not if it was absent. By contrast, preformed aurocyanide at 10 and 100 μM decreases O_2^- production by PMN stimulated by opsonized zymosan both in the presence and absence of thiocyanate. Changes in the ultraviolet spectra of the supernatants of PMN indicated that aurocyanide was formed by activated PMN in the presence of thiocyanate but not in its absence.

Gold complexes are useful in the long term treatment of rheumatoid arthritis. The most widely used complexes are the water soluble, polymeric complexes, such as aurothiomalate (Autm) and aurothioglucose, and the monomeric complex, auranofin. Auranofin shows activity in a variety of short term *in vitro* test systems in which Autm and aurothioglucose are generally inactive. Thus, Autm has little effect on the production of superoxide [1–3], the production of hydrogen peroxide and hydroxyl radical [3], and the release of lysosomal enzymes [4, 5], phagocytosis [5] and the antibody-dependent cellular cytotoxicity of polymorphonuclear leukocytes (PMN) of mixed leukocytes [6]. However, Autm has been shown to inhibit some functions of monocytes in long term experiments, such as the differentiation of monocytes into macrophage-like cells [7] and their ability to support the proliferation of lymphocytes [8] although these activities have been demonstrated at concentrations generally in excess of the plasma levels achieved during treatment with Autm.

There has been no explanation as to why Autm and aurothioglucose are active *in vivo* but not *in vitro* in short term experiments. This lack of activity *in vitro* has not only made it difficult to devise screening procedures for new gold complexes but has prevented an understanding of their mechanism of action. However, a possible lead to the cellular effects of these complexes is given by reports that activated PMN produce cyanide from thiocyanate, a ubiquitous anion in plasma [9] or from phagocytosed bacteria [10]. The significance of these observations is that cyanide is able to break down the polymeric Autm [11–14]. Several species are present at intermediate stages, but in the presence of excess cyanide, the very stable gold complex, aurocyanide, ($Au(CN)_2^-$), is formed:

$$\text{Autm} + 2\text{HCN} \rightarrow Au(CN)_2^- + \text{tmH} + H^+$$

where tmH is thiomalate.

Aurocyanide is an inhibitor of the oxidative burst of PMN and monocytes but is not a scavenger of superoxide [15]. Hence, it seemed likely that cyanide produced by activated PMN could activate Autm by its conversion to the inhibitory species, aurocyanide. Thus, the hypothesis was that the activated PMN would produce cyanide, that the cyanide would act on Autm to yield aurocyanide and that the aurocyanide would decrease the production of superoxide by PMN. Experimental evidence in support of this proposition has now been obtained and is presented in this paper.

MATERIALS AND METHODS

Isolation of PMN. Venous blood was collected from healthy donors who were not taking any drugs. The blood was mixed with an equal quantity of normal saline containing 4 mM sodium edetate and layered over a solution containing 10% mixed metrizoates (Isopaque, Nyegaard) and 6.3% Ficoll (Pharmacia) adjusted to a wt/mL of 1.080 g. After centrifuging for 30 min at 400 *g* at room temperature, the upper layer containing mononuclear cells [16] was discarded. PMN in the lower layer were then

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separated from the bulk of the red blood cells by sedimentation in a mixture of equal parts of calcium and magnesium free Hank's balanced salt solution (Gibco, Uxbridge, U.K.) and 3% dextran (Dextran T500, Pharmacia, Uppsala, Sweden) in normal saline. The cells in the supernatant were collected by centrifugation and the residual red blood cells lysed with ice-cold water for 30 sec. Tonicity was restored with Hank's balanced salt solution (10–12 mL), the cells washed once in Tyrode's solution containing 0.1% glucose and HEPES buffer (pH 7.4) and the PMN finally suspended in the same medium at a cell density of 10^7 PMN/mL.

Measurement of superoxide generation. Duplicate suspensions of PMN (2.5×10^6) were mixed, where appropriate, with solutions of Autm (1–100 μM; prepared from solid material supplied by May and Baker, Dagenham, U.K.), potassium aurocyanide (1–100 μM; Engelhard Ind., Sydney, Australia), sodium thiomalate (1–100 μM; prepared from thiomalic acid; Sigma Chemical Co., St Louis, MO), potassium thiocyanate (200 μM), ferricytochrome *c* (1 mg/mL; Sigma Type III) and superoxide dismutase (75 units, Sigma). After incubation for 8 min at 37°, calcium chloride was added to a final concentration of 3 mM and after a further 5 min, the PMN were stimulated with opsonized zymosan [17] at a final concentration of 1 mg/mL. The final volume of the cell suspensions was 0.5 mL. The generation of superoxide was stopped 60 min (t_{60}) after the first addition of zymosan by the addition of an ice-cold solution of *N*-ethylmaleimide (1 mM, 0.25 mL, Sigma). The incubations were conducted in polystyrene tubes (Sterilin). The amount of superoxide generated was calculated from the net amount of ferrocytochrome *c* formed using a value of E_{max} of 2.11×10^4 cm^2/mmol for the conversion of ferricytochrome *c* to ferrocytochrome *c* [18] and using cell suspensions containing superoxide dismutase as controls.

Three different protocols were used, in each case the cells being activated by zymosan in the absence and presence of Autm both with and without thiocyanate. The time of addition of zymosan was taken as time 0 (t_0).

Protocol A. Cytochrome *c* was added at 30 min after activation (t_{30}) by zymosan, and the oxidative burst stopped at t_{60}. In this protocol, cell activation with the production of cyanide, the putative conversion of Autm to aurocyanide, and the generation of O_2^-, all occur concurrently during the first 30 min, but the production of O_2^- was only measured between t_{30} and t_{60}.

Protocol B. Cytochrome *c* was added at t_{28}, a further addition of zymosan, 1 mg/mL, at t_{30}, and the oxidative burst stopped at t_{60}. In this protocol the events occur in two stages; the first comprises cell activation, the production of cyanide and the putative conversion of Autm to aurocyanide. In the second stage, further stimulation by the second dose of zymosan provides a further oxidative burst on which the effect of any generated aurocyanide can be measured. The effect of Autm in these incubations was compared with preformed aurocyanide and thiomalate.

Protocol C. Cytochrome *c* was added to PMN at the same time as the gold compounds, i.e. at t_{-13}, and the oxidative burst stopped at t_{60}. In this protocol, cell activation, the production of cyanide, the putative generation of aurocyanide, O_2^- and its measurement by cytochrome *c* reduction all occur concurrently. Again, the effect of Autm was compared with aurocyanide and thiomalate.

Ultraviolet spectroscopy. PMN were incubated as described for 30 min, at which time the reactions were terminated by cooling the tubes in ice. After centrifugation, the absorbance spectra of the supernatants were obtained from 220 to 320 nm. Background correction was made using identical cell suspensions and media apart from the exclusion of Autm. Spectra were examined from at least three independent experiments.

Statistics. Data is reported as mean ± SE in all cases and the significance levels of differences between treatments were determined by paired *t*-tests.

RESULTS

Effects of Autm, aurocyanide and thiomalate on the generation of O_2^-

With protocol A, in which the cytochrome *c* was added at 30 min (t_{30}) after the start of cell activation by zymosan (i.e. only during the later stages of the respiratory burst), the amount of O_2^- produced in the control incubations was, as might be expected, fairly low; 9.3 ± 0.9 nmol/5×10^6 PMN being recorded. Thiocyanate, 200 μM, markedly increased the amount of O_2^- generated to 17.3 ± 1.6 nmol/5×10^6 cells ($P < 0.01$). Autm (10 and 100 μM) if present with thiocyanate during cell activation decreased the production of O_2^- (Fig. 1). This result contrasts with the response to Autm in the absence of thiocyanate when Autm had no significant effect at 1 and 10 μM but increased the production of O_2^- at 100 μM. Thus, the presence of thiocyanate completely changes the effect of Autm and indicates that Autm is altered by its incubation with PMN in the presence of thiocyanate. Furthermore, the influence of thiocyanate indicates that aurocyanide may be the inhibitory species since the production of O_2^- was decreased under conditions when aurocyanide could be formed.

Because of the low rate of production of O_2^- with protocol A, a second procedure, protocol B, was developed. In this protocol, the addition of more zymosan at t_{30} caused a second activation of the PMN and a much greater production of O_2^-. The pattern of effects of thiocyanate and Autm were, however, similar to those seen with protocol A. The addition of thiocyanate, 200 μM, again increased the production of O_2^- from 27.0 ± 1.1 ($N = 13$) to 39.2 ± 1.7 ($N = 17$) nmol/5×10^6 PMN ($P < 0.001$). As in protocol A, Autm at 10 and 100 μM significantly decreased the production of O_2^- in the presence of thiocyanate while 100 μM Autm slightly stimulated the generation of O_2^- in the absence of thiocyanate (Fig. 1).

Preformed aurocyanide at 10 and 100 μM caused a concentration-dependent reduction in the production of O_2^- both in the presence and absence of thiocyanate (Fig. 1) in protocol B. This result indicates that the inhibition produced by Autm in the

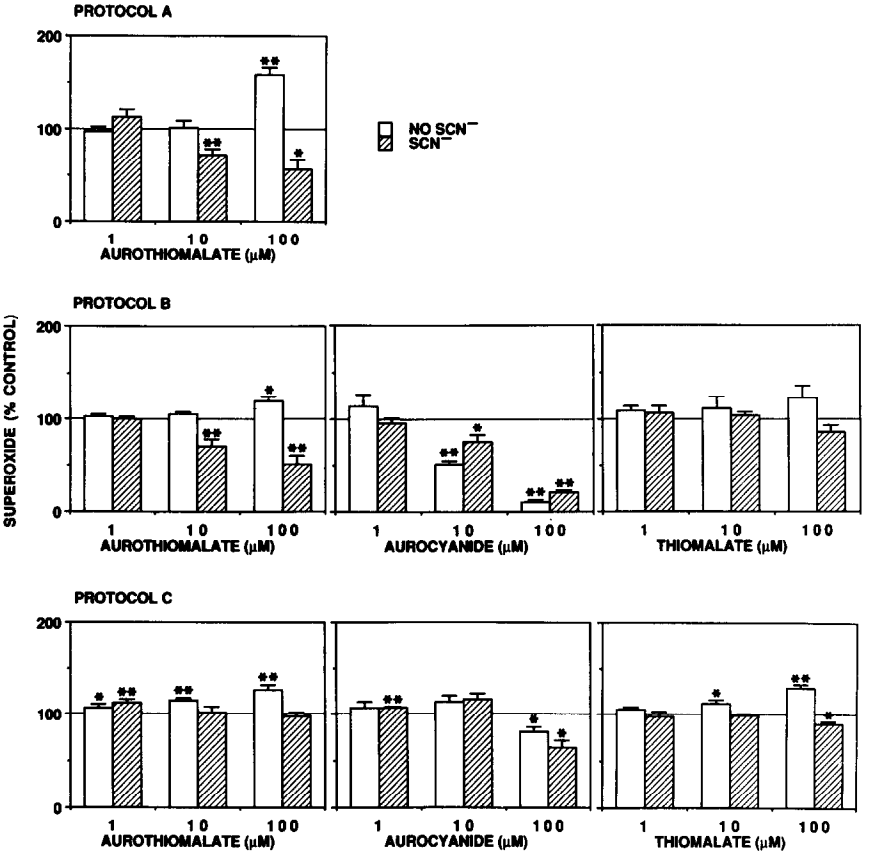

Fig. 1. The effect of aurothiomalate, aurocyanide and free thiomalate on the production of superoxide in the absence (open histograms) and presence of 200 μM thiocyanate (SCN^-; hatched histograms). Means and standard errors are shown. Significance levels of differences from controls are shown as $P < 0.05$ (*) and $P < 0.01$ (**). The control rates of superoxide in the presence and absence of thiocyanate, respectively were: protocol A 17.3 ± 4.4 (N = 8) and 9.3 ± 2.0 (N = 5); protocol B 39.2 ± 1.7 (N = 17) and 27 ± 1.1 (N = 13); protocol C 32.5 ± 1.9 (N = 16) and 26.4 ± 1.7 (N = 14).

presence of thiocyanate was due to the formation of aurocyanide and, furthermore, that the lack of inhibitory activity of Autm in the absence of thiocyanate was due to the absence of aurocyanide and not a consequence of the different baseline production of O_2^-. In the presence of thiocyanate, preformed aurocyanide at a concentration of 100 μM had a greater effect than the same concentration of Autm (Fig. 1) but this difference is to be expected since the effect of Autm must be limited by the availability of cyanide.

The activity of thiomalate which is liberated in the reaction between Autm and cyanide was examined. At concentrations up to 100 μM, it had no significant effect on the generation of O_2^- (Fig. 1), although there was a trend towards a decreased production in the presence of thiocyanate and an increased production of O_2^- in the absence of thiocyanate at the highest concentration of thiomalate (100 μM). Thiomalate contains a free sulphydryl group which can reduce cytochrome *c*. This reactivity was shown by the high background reduction of cytochrome *c* in the presence of 100 μM thiomalate. The background (reduction of cyctochrome *c* in the presence of superoxide dismutase) was increased by the equivalent of 13.5 ± 2.3 and 7.5 ± 1.3 nmol of $O_2^-/5 \times 10^6$ PMN in the absence and presence of thiocyanate, respectively. The reason for the effect of thiocyanate on the thiomalate background was not investigated.

In protocol C, the effects of Autm, aurocyanide and thiomalate were studied on the total production of O_2^- following a single activation of the PMN with opsonised zymosan. Again, the production of O_2^- was increased by thiocyanate, from 26.4 ± 1.7 (N = 14) to 32.5 ± 1.9 (N = 16) nmol/5 × 10^6 PMN ($P < 0.001$). The effects of Autm, aurocyanide and thiomalate were somewhat different from those observed with protocol B. Autm produced only slight stimulation of the production of O_2^- both in the presence and absence of thiocyanate. Preformed aurocyanide was less inhibitory than in protocol B and only the highest concentration (100 μM) inhibited the generation of O_2^-. The effect of free thiomalate followed the trends seen with protocol B, although with protocol C, the effects of thiomalate were statistically significant. At the higher concentrations, free thiomalate slightly inhibited the production of O_2^- in the presence of thiocyanate but

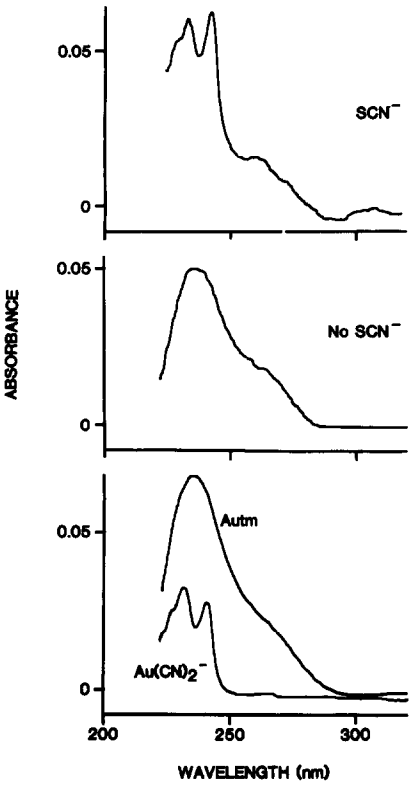

Fig. 2. Ultraviolet spectra of the supernatants of activated PMN incubated with aurothiomalate (Autm). Middle and upper spectra were obtained from incubations conducted in the absence of thiocyanate (SCN^-) and in the presence of 200 μM thiocyanate, respectively. Reference spectra of Autm and aurocyanide are shown in the lower section.

was slightly stimulatory in the absence of thiocyanate.

Formation of aurocyanide

The ultraviolet spectra of supernatants of incubations of PMN could be examined down to approximately 220 nm. Below this wavelength, the background absorbance exceeded 1.5 preventing accurate determination of absorbance. In the absence of thiocyanate or zymosan, the spectra of the supernatant solutions resembled that of Autm, but in the presence of both, the spectra changed markedly with peaks at 229 and 239 nm and a trough at 234 nm indicating the formation of aurocyanide (Fig. 2). These spectral changes were still present when calcium was deleted from the incubation medium and also when superoxide dismutase was added.

DISCUSSION

The results obtained in the present studies support the hypothesis that aurocyanide mediates the effects of Autm. The spectroscopic changes indicate directly that aurocyanide was formed by the activated PMN in the presence of thiocyanate while the marked influence of thiocyanate on the effects of Autm in protocols A and B is also consistent with the formation of aurocyanide. It is of interest that the activation of Autm could be demonstrated in protocols A and B but not in protocol C. Two reasons may be suggested for the lack of effect of Autm in protocol C. Firstly, in this latter protocol, the generation of O_2^- was measured at the same time as any aurocyanide could be formed and a lesser degree of inhibitory effect is reasonable. By contrast, protocols A and B allowed the oxidation of thiocyanate to cyanide with the consequent formation of aurocyanide before the generation of O_2^- was measured. Thus, any activation of Autm through its conversion to aurocyanide should be evident in these protocols.

A second reason for the lack of activity of Autm in protocol C is related to the influence of aurocyanide on the oxidative burst. In studies on the effects of preformed aurocyanide on the chemiluminescence of PMN, it has been observed that aurocyanide has little initial effect on chemiluminescence and its inhibitory effects develop only during the oxidative burst [15]. Thus, it was anticipated that inhibition by aurocyanide would be more marked in the later stages of an oxidative burst (protocol A) or during a second burst (protocol B) than throughout the first oxidative burst (protocol C). A lesser inhibitory effect was, in fact, observed in protocol C and the lowest concentration of aurocyanide tested, 1 μM, actually produced slight stimulation of the production of O_2^- when thiocyanate was present. Consequently, the small effect of Autm in protocol C is to be expected. It is probable that PMN within the synovial fluid of inflamed joints are in a state of continuous stimulation and, hence, the inhibitory effects of Autm and aurocyanide seen in protocol B may be more clinically relevant than the slight stimulatory effects seen with protocol C in which the PMN were stimulated only once with the production of O_2^- measured from the initiation of the oxidative burst. The overall conclusion is that Autm is activated by its conversion to aurocyanide which causes progressive inhibition of the generation of O_2^-, but careful experimental design is required to demonstrate such activation.

A clinically significant aspect of the formation of aurocyanide by PMN is that the aurocyanide is present in the supernatant of PMN suspensions. Synovial fluid in rheumatoid joints contains large numbers of PMN and the formation of aurocyanide by PMN at this site would make the aurocyanide available for interaction with other cells in the synovium. Another significant aspect of the results of the present experiments is that Autm and aurocyanide produced inhibitory effects at concentrations which could be achieved during treatment. The total concentration of gold in plasma during treatment with Autm is of the order of 5 to 30 μM [19] and, while there has been no detailed study of the interaction of cyanide with gold–protein complexes, the major species of gold in the circulation, it is of note that effects of Autm and aurocyanide can be observed at gold concentrations which are clinically relevant.

The activation of Autm through its conversion to aurocyanide allows an explanation of previously conflicting data on the effects of Autm on cellular

systems *in vitro*. As discussed previously, Autm generally has had little or no effect on PMN in published studies. An exception is in the marked effect of Autm on the iodination of PMN [20]. From the results of the present studies, the reason for the marked effect of Autm on iodination may be that iodination was measured in the presence of pooled serum which would have been a source of thiocyanate and thus allowed the formation of aurocyanide. By contrast, other short term studies on the effects of Autm on PMN *in vitro* have been conducted in the absence of serum. Thus, Autm has previously shown activity in short term incubations only under conditions allowing the formation of aurocyanide. Autm has been shown repeatedly to inhibit the proliferation of lymphocytes in suspensions of mixed mononuclear leukocytes but the incubations have always contained serum. Interestingly, the activity of Autm in such preparations is increased by increasing proportions of serum [21]. It was concluded that this effect of serum on the activity of Autm was due to the conversion of Autm to protein complexes of gold but the present work indicates that, rather, it may be active after conversion to aurocyanide.

Gold complexes other than aurocyanide and gold–protein complexes are not the only species which have been suggested as the active metabolites of Autm. It has previously been reported that myeloperoxidase oxidizes Autm to a trivalent gold complex, $AuCl_4^-$ whose formation was detected by the development of peaks at 228 and 311 nm in the ultraviolet spectrum [22]. Aurocyanide has a peak at 229 nm and hence cannot be distinguished from $AuCl_4^-$ in this region. However, no significant absorbance was detected at 311 nm where a peak for $AuCl_4^-$ should occur. Furthermore, gold (III) complexes are powerful oxidizing agents which should be reduced rapidly to gold (I) complexes in biological media [23]. By contrast, aurocyanide is an extremely stable gold (I) complex which should be resistant to breakdown in complex biological fluids.

The liberation of free thiomalate has also been proposed as mechanism for activation of Autm [24]. Thiomalate is present in plasma during treatment with Autm although the concentrations are low, with peak concentrations less than 0.6 μM following a 20 mg dose of Autm [25]. The bulk of the thiomalate liberated from Autm *in vivo* probably results from the reaction of Autm with proteins [26] and other endogeneous compounds containing thiol groups but the displacement of thiomalate from bonding to gold is also associated with the formation of aurocyanide from Autm. In protocol B, free thiomalate had a lesser effect than either preformed aurocyanide or Autm incubated with PMN and thiocyanate. From these comparative results, it is more likely that the activation of Autm by PMN is due to the formation of aurocyanide than to concurrent release of thiomalate. Nevertheless, thiocyanate did influence the response to free thiomalate and the release of thiomalate should be considered in further examination of the aurocyanide hypothesis.

A consistent observation in the present studies was the stimulatory effect of thiocyanate on the production of superoxide. This interaction may be of considerable biological interest since it is well known that smoking increases the plasma levels of thiocyanate, although thiocyanate is still a significant anion even in non-smokers. The levels of thiocyanate in non-smokers are approximately 40 μM and are elevated by cigarette smoking to between 50 and 200 μM depending on the number of cigarettes smoked per day [27]. The interactions of thiocyanate and PMN may be a significant aspect of the biological effects of smoking and should be studied further.

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