Gold binding to blood cells and serum proteins during chrysotherapy

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SUMMARY The binding of gold to peripheral erythrocytes and serum protein fractions was studied during chrysotherapy of 1 to 2 years' duration in 43 patients with rheumatoid arthritis. In 45% of the patients more than 10% of the gold was found to be strongly bound to blood cells. 5–15% of the metal is bound to non-albumin protein fractions at serum gold concentrations larger than 2 µg/ml. In contrast to the cellular binding of gold the relative binding of gold to non-albumin proteins was inversely proportional to the serum concentrations. Binding to neither blood cells nor to non-albumin protein fractions was found to be correlated with clinical parameters.

Gold salts have been used in the treatment of rheumatoid arthritis (RA) for more than 40 years. Although chrysotherapy has proved to be fairly beneficial in the management of rheumatoid arthritis, controversy still exists on the optimal dosage schedule (Krusius *et al.*, 1970; Freyberg, 1972; Lorber, *et al.*, 1973, 1975; Cats, 1976; Rothermich *et al.*, 1976; Sharp *et al.*, 1977). Most reports indicate a lack of correlation between pharmacological and clinical parameters, including the occurrence of adverse reactions (Krusius *et al.*, 1970; Freyberg, 1972; Gerber *et al.*, 1972; Mascarenhas *et al.*, 1972; Rubinstein and Dietz, 1973; Jessop and Johns, 1973; Lorber, *et al.*, 1973, 1975; Gottlieb *et al.*, 1974; Sharp *et al.*, 1977).

In vitro experiments suggest that most of the gold in the circulation is bound to albumin (Lawrence, 1961; McQueen, and Dykes, 1969; Mascarenhas *et al.*, 1972; Campion *et al.*, 1974; Danpure, 1976; Ward *et al.*, 1977), whereas a minor fraction seems to be bound to immunoglobulins, complement (Lorber *et al.*, 1972; Schultz *et al.*, 1973; Ward *et al.*, 1977), or to low molecular weight materials (Campion *et al.*, 1974; Ward *et al.*, 1977). Almost all of these studies have been performed in vitro. In only one study (Ward *et al.*, 1977) was the gold distribution measured in the blood of patients with rheumatoid arthritis receiving chrysotherapy. In the study presented here the binding of gold to erythrocytes and the distribution of gold among the

serum proteins was studied in relationship to clinical parameters.

Material and methods

PATIENTS

Forty-two patients comprising 18 men (age range 22–75 years) and 24 women (age range 22–76 years) with definite or classical rheumatoid arthritis lasting for more than 6 months and fulfilling the criteria of the American Rheumatism Association (Ropes *et al.*, 1959) were admitted. None of the patients had previously received chrysotherapy, corticosteroids, immunosuppressive drugs, or D-penicillamine. Only medication with nonsteroidal anti-inflammatory drugs was allowed simultaneously with the gold therapy. During the trial all patients received aurothioglucose (20% oily suspension, Noury Pharma, Oss) by intramuscular injections according to the following standard schedule: The first week 5 mg twice a week, the second week 10 mg twice a week, the third week 20 mg once, after which the dose was increased to 30, 40, and 50 mg a week subsequently in most of the cases. All patients were followed up for 1 to 2 years in this study.

CLINICAL TESTING PROCEDURES

Before admission to the trial and subsequently at 3-month intervals a complete physical examination was made of all patients. On each occasion the following parameters were assessed; number of ARA criteria, subjective judgment of clinician and patient, pain, morning stiffness, weariness, grip strength, walking time over 10 metres, circumference of large

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and proximal interphalangeal joints, joint index (Ritchie *et al.*, 1968), number of swollen joints, ability to perform activities of daily life (questionnaire), haemoglobin concentration, percentage serum albumin, and erythrocyte sedimentation rate (ESR). On admission to the trial these parameters were assessed on the following 4-point scale; hardly affected (diminished or enhanced), mildly, moderately, or severely affected. By summation of the scores of these variables a total score was obtained for each patient indicating the overall activity of the disease at onset of therapy. On the basis of this total score 3 patients were classified as mildly affected, 14 as mildly to moderately, 22 as moderately, and 3 as severely.

The response of each parameter after a year of therapy was estimated on a 7-point scale, ranging from markedly improved to markedly deteriorated. A change in the joint index, number of swollen joints, pain, and ESR received a weight of 2. By summation of these scores for each patient a total response score was obtained, ranging from 0 (maximal deterioration) to 102 (maximal improvement). Based on this total response score the clinical response of each patient was classified as (a) improved (69 or more), or (b) no significant change or deteriorated (68 or less).

To test the validity of this kind of evaluation, the total response score was compared with the overall judgment of the clinician and with the joint index score separately. The latter 2 parameters correlated well with the total response score ($r=0.71$, $P<0.01$; and $r=0.70$, $P<0.01$, respectively.)

It should be pointed out that in assessment of the clinical response, the occurrence of adverse reactions was excluded as a parameter from the total response score. Adverse reactions were scored separately; mucocutaneous symptoms were confirmed by a dermatologist. In 22 patients mild adverse reactions were noted (Table 1). Usually therapy was discontinued for 1–3 months. Nine patients developed adverse reactions which were classified as serious (Table 1). In these cases gold therapy was permanently stopped. In no patients did serious bone marrow depression or nephrotic syndrome appear.

Table 1 Occurrence of adverse reactions

	Mild	More serious
Dermatitis	4	5
Stomatitis	1	4
Conjunctivitis	2	1
Itching, rash	13	1
Proteinuria	2	—
Vasculitis	—	1
Total	22 (in 13 patients)	12 (in 9 patients)

At the onset of therapy and subsequently once a year radiographs of hands, feet, and afflicted joints were analysed by a roentgenologist. This was done serially. The point scale hardly, mildly, moderately, and severely affected was used to classify the patients radiographically at the onset of therapy. A 7-point scale ranging from markedly improved to markedly deteriorated was applied to estimate the radiographical response to therapy so far as the afflicted joints were concerned.

BLOOD SAMPLING

Serum and blood samples, with heparin or EDTA as anticoagulant, were obtained from the patients just before they received their next injection of aurothioglucose. After centrifugation of the blood, plasma was carefully separated, and the packed erythrocytes were suspended in Hijmans's washing medium (Steffelaar *et al.*, 1976, 1977). After a second centrifugation the cells were suspended in saline up to the original blood volume. 25 μ l serum was subjected to electrophoresis on cellulose acetate paper of appropriate thickness immediately after collection. Albumin, α_1 , α_2 , β , and γ fractions were cut out and dissolved in 80% acetic acid (1.0 ml final volume).

All samples were kept frozen in closed vials at -20°C until measurement.

GOLD DETERMINATIONS

Blood, serum, plasma, and erythrocyte samples were diluted 5 times in water. 5 μ l was injected directly into a pyrolytically coated graphite cup contained in a carbon rod atomiser (type 63, Varian) mounted in a Varian type AA-5 atomic absorption spectrophotometer (single channel) equipped with a Par lock-in amplifier type 120, a Biomation transient recorder model 802, and a Varian recorder type A-25. 10 μ l of undiluted electrophoresis fractions was injected directly. Since quantitatively the gold content of the albumin fraction greatly exceeds that of the α_1 fraction, and occasionally the α_1 fraction did not separate sharply from the albumin fraction, the gold content of the α_1 fraction may be overestimated. Therefore the percentage of gold found in the albumin plus α_1 fractions were calculated. The remainder of the metal was bound to and usually evenly distributed over the α_2 , β , and γ fractions.

Signals were compared with aurothiomalate (Merck, Sharpe and Dohme) standards (0.10, 0.20, 0.50, and 1.00 $\mu\text{g Au/ml}$) in exactly corresponding matrices. Pure nitrogen was employed as sheathing gas, cooling was forced by running tap water (cooling time for the electrodermal part between each sample application was 60 s), and the atomiser was

used in the rampmode with a suitable dry-ash-atomise cycle. The gold resonance line at 242.8 nm was used. Interference of nonatomic or spectral nature was estimated with a deuterium lamp and subtracted if necessary. This was always done for the measurement of gold in the serum protein fractions. After every 10 samples a standard was included to monitor the small drift. The standard deviation of the signal responses of standard samples (0.20 and 1.00 $\mu\text{g Au/ml}$) was 5% of the mean response. Standards were made in pooled human blood, serum, plasma, erythrocyte suspension, and 80% acetic acid supplemented with appropriate amounts of cellulose-acetate paper and human serum by addition of aurothiomalate. They were kept frozen at -20°C in small closed vials and used only once.

Results

CELLULAR GOLD BINDING

In preliminary investigations it has been found that measuring the gold concentration in EDTA plasma gave unreproducible results, whereas determination in heparin plasma or serum gave good reproducibility. This discrepancy could be completely attributed to unreproducible effects of EDTA in the matrices during the atomisation process. The heparin plasma concentrations of gold did not differ from those in the serum, which indicates that no appreciable quantities of gold were bound to fibrinogen. However, in many patients with rheumatoid arthritis under chrysotherapy the calculated blood levels of gold (the plasma concentration multiplied by (1-haematocrit)) were significantly lower than

the concentrations measured directly in the blood. As shown in Fig. 1 the difference could be attributed to gold bound to isolated erythrocytes. We were unable to remove the bound gold from the erythrocytes by repeated washings in media containing 4.5% bovine serum albumin or in human AB serum, nor in media used for fixation of cells for electron microscopy. In the latter case the metal was not seen in electron micrographs of the blood cells. Attempts to remove the metal from the erythrocytes by incubation in isotonic 0.05 M cysteine, pH 7.4, were also unsuccessful.

Isolated buffy coat cells also bind comparable quantities of gold. Since the number of white cells is much smaller than the number of red cells the buffy coat cells do not contribute quantitatively to cellular gold binding.

As shown in Fig. 1 cellular gold binding develops from onset of therapy simultaneously with a rise of the gold blood level. After some months of therapy cellular gold binding may vary for individual patients from less than 10% (arbitrarily assigned as no cellular gold binding) up to 35% of the gold in the blood. If the blood gold level decreases during therapy, for instance due to interruption of therapy, half of the gold can become bound cellularly (Fig. 1). Binding of gold to erythrocytes is a constant parameter throughout therapy for each individual patient. There was no clear correlation between the blood gold levels (ranging from 0.6 to 3.2 $\mu\text{g Au/ml}$ in 42 patients) and the occurrence of cellular gold binding—that is, more than 10% of the gold in the blood bound to red cells—which suggests that this property is predominantly determined by the

Fig. 1 Cellular gold binding in a patient with rheumatoid arthritis receiving chrysotherapy. ●—● Gold concentration measured directly in the heparinised-blood. ○—○ Gold concentration in the blood calculated by multiplying the plasma values by (1-haematocrit). □—□ Gold concentration measured directly in the isolated blood cell suspension.

erythrocytes. The degree of cellular gold binding was dependent on the blood gold level only at higher blood gold concentrations. For example, Fig. 2 shows results in a patient who received 1000 mg of gold accidentally. Two months elapsed before

the blood level became normal ($T_{1/2}=25$ days). Only at very high gold concentrations in the blood was cellular gold binding induced, but as soon as the blood gold level became normal less than 10% of the gold was bound cellularly. In this patient the decay in the renal gold excretion was less rapid than that of the blood concentration.

In-vitro incubation of heparinised blood from healthy volunteers with aurothioglucose (1.0, 3.0, and 5.0 $\mu\text{g Au/ml}$) for 10 or 60 min resulted in cellular binding of gold, the degree of which increased with the gold concentration and the incubation time. There was also a considerable individual variation in the degree of cellular gold binding.

Correlations of cellular gold binding in the patients with other red cell properties such as blood group and rhesus type were lacking.

Fig. 2 Gold distribution and renal excretion in a patient with rheumatoid arthritis who by accident received 1000 mg of gold at once. Standard therapy was discontinued for 3 months, during which the patient developed no side effects. After this period mild complaints concerning hair losses and itching soles of the feet were noted for 6 months.

DISTRIBUTION OF GOLD OVER SERUM PROTEINS

At the serum gold concentrations usually reached in standard therapy 85–95% of the gold is bound to albumin (Fig. 3). At lower serum gold concentrations the relative proportion of the gold bound to non-albumin fractions increased significantly ($P<0.001$ for comparison between the percentage bound at 1.0 $\mu\text{g Au/ml}$ and at 2.0 $\mu\text{g Au/ml}$ serum).

CLINICAL RESULTS

No correlation was found between the clinical and radiographical classification of the patients at onset of therapy. Six patients, however, also showed the main radiographical features of degenerative joint disease. These features were not included in the item

Fig. 3 The relationship between the percentage of gold bound to albumin plus α_1 and the gold concentration in the serum. Drawn lines give results of at least 5 determinations per patient during their course of therapy. The figure gives the results of 12 representative patients.

list for radiographical evaluation of patients with rheumatoid arthritis. When these 6 patients were omitted a correlation was found between the clinical and radiographical classification of the patients at onset of therapy ($r=0.61$, $P<0.01$).

After 1–2 years of investigation 35 of the 42 ambulant patients (83%) were classified as clinically improved so far as the total response score was concerned, 4 patients showed no therapeutic effect, and 3 patients deteriorated slightly. As already mentioned, this classification does not take into account the occurrence of side effects, that is, a patient may improve markedly but still show serious side effects. Radiographically only 2 patients improved slightly, 50% of the patients remained unchanged, 40% deteriorated slightly, and 2 patients moderately. Obviously, there was no correlation between the clinical and radiographic results after therapy.

Mild side effects occurred in 13 patients. In 9 other patients therapy was stopped because of more serious adverse reactions occurring after 4–29 months of therapy. It should be pointed out that more serious side effects (Table 1) all occurred in patients who were classified finally as clinically improved. The occurrence of eosinophilia ($>0.4 \times 10^9/l$) and assessed once every 3 weeks) and/or raised IgE levels (>500 IE/ml and measured once every 3 months) in these patients under chrysotherapy was uncorrelated with manifestations of the side effects listed in Table 1.

GOLD DISTRIBUTION AND CLINICAL RESULTS

The blood gold levels were correlated neither with therapeutic results (total response score) nor with the occurrence of side effects. The patient who had received 1000 mg of gold (Fig. 2) developed only mild side effects at a time the blood level had already become normal.

Nineteen out of 42 patients (45%) showed cellular gold binding, in 6 patients only to a minor degree (10–20%); in 13 patients more than 20% of the gold in the blood was bound to erythrocytes. We were unable to establish a relationship between this parameter and the therapeutic results (total response score). Cellular gold binding seemed negatively correlated with the occurrence of more serious side effects ($P=0.08$). The relationship, however, is not statistically significant. The degree of gold binding to non-albumin protein fractions at serum gold concentrations of 1.0 and 2.0 $\mu\text{g/ml}$ was correlated neither with therapeutic results nor with the occurrence of side effects nor with cellular gold binding in the patients.

Discussion

In this study it was found that in 45% of patients with rheumatoid arthritis receiving chrysotherapy an appreciable quantity of the gold in the circulation (up to 35%) is bound to blood cells. The metal is not removed from the cells by washing with albumin containing media including human AB serum, which indicates a strong binding. It has been suggested that aurothiomalate interacts with thiol groups of proteins (Gerber *et al.*, 1974). Interaction of the loaded cells with free thiol groups of cysteine did not release any of the metal from the cells. The bound metal was not detectable under the electron microscope, which suggests a diffuse binding of gold over or in the blood cells. Cellular gold binding proved to be a constant parameter for each patient, predominantly determined by the properties of the patients' blood cells and to a less extent by the blood gold levels, and could be induced in vitro.

In-vitro most of the radioactive labelled gold binds to albumin (McQueen and Dykes, 1969; Mascarenhas *et al.*, 1972; Campion *et al.*, 1974; Danpure, 1976). Ward *et al.* (1977) measured the plasma gold distribution in vivo in 3 patients with rheumatoid arthritis. They separated albumin from globulin and low molecular weight materials by gel fractionation over Sephadex G-200. At relatively high peak plasma levels (6–8 $\mu\text{g Au/ml}$) 88–93% of the gold was bound to albumin, 6–10% was bound to globulins, and less than 3% was bound to low molecular weight materials. Our degree of gold binding to albumin at high plasma gold levels in vivo agrees well with the above-mentioned results obtained by different techniques. Ward *et al.* (1977) did not report the occurrence of unbound metal in vivo as did Campion *et al.* (1974). The latter group found 10% 'unbound' metal, that is, metal which passed an ultrafiltration line, in some sera of patients with rheumatoid arthritis receiving gold therapy irrespective of the plasma gold levels. However, the techniques they used are not precisely stated. Our study showed that at lower serum gold levels relatively more gold was bound to non-albumin fractions. The results of this study suggest that the number of binding places for gold on non-albumin protein fractions is small compared to the number of binding places on albumin, whereas the strength of the binding to non-albumin proteins is larger.

None of the available pharmacological parameters of gold metabolism in man are believed to correlate with therapeutic results or the occurrence of side effects. This study shows that 2 new parameters, the cellular binding of gold and the distribution of gold among the serum proteins in vivo, are not correlated with clinical results either. Possibly cellular gold

binding correlates inversely with the occurrence of serious side effects.

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