Early and late changes in sulphhydryl group and copper protein concentrations and activities during drug treatment with aurothiomalate and auranofin

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SUMMARY Superoxide dismutase activity (SOD), plasma and lysate thiol concentrations (PSH and LSH), and caeruloplasmin oxidase activity (CP) reflect the underlying reduction-oxidation imbalance associated with rheumatoid arthritis (RA), and are believed to be involved in the protection of the cell against free radical activity. The early and late changes in these parameters have been observed and compared with standard clinical and biochemical assessments of disease activity in 90 patients with active RA, randomly assigned to receive either sodium aurothiomalate, auranofin, or auranofin placebo. An index based on clinical criteria was used to identify patients as responders or non-responders after 24 weeks of therapy. In the first six weeks of treatment a change in SOD activity and LSH concentration in a direction away from controls was followed by a return towards control levels in responders only. This suggests that in RA evidence of clinical improvement induced by gold drugs is preceded by an initial biochemical response in an inflammatory direction. The extracellular parameters PSH and CP did not show the same early response, but PSH levels in responders showed a slower change towards normal values, though at no time were values obtained that might suggest a complete remission. Thus the intracellular parameters appear to reflect an early effect of the drugs on cells which may possibly be of use in predicting the outcome of therapy, whereas the extracellular parameters provide confirmatory evidence for an eventual improvement.

Key words: rheumatoid arthritis, redox status, superoxide dismutase, caeruloplasmin.

The gold compound auranofin (1-thio- β -D-glucopyranosato) (triethylphosphine) gold 2, 3, 4, 6-tetra-acetate) (AF) is orally absorbed and appears to be effective in the treatment of rheumatoid arthritis.^{1,2} It is at present being comprehensively assessed in controlled clinical trials and compared with the injectable drug, sodium aurothiomalate (GST or Myocrisin); it produces a different profile of clinical and biochemical activities.³ Both GST and AF metabolise rapidly in vivo to produce a number of new gold compounds distributed in serum, cells, and most tissues. In the main, gold in vivo is complexed as gold(I) through the formation

of gold-sulphur bonds with accessible sulphhydryl groups of proteins, peptides, and amino acids.^{3,4}

In rheumatoid arthritis the plasma sulphhydryl group concentration is low and the erythrocyte lysate sulphhydryl group concentration high compared with those of normal controls. This indicates a change in the degree of oxidation of the fluids on each side of the cell membrane.⁵ The change in the reduction-oxidation (redox) status of the sulphhydryl system is reinforced by the fact that the concentration of the main plasma oxidase, the copper containing protein caeruloplasmin, is raised in the disease state^{6,7} and the activity of superoxide dismutase, an intracellular copper protein involved in the control of the concentration of superoxide ion⁸ (a reduced form of oxygen), is decreased.^{9,10}

The activities of the copper proteins and the

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sulphydryl group concentrations are believed to be linked. Caeruloplasmin is a specific oxidase for small molecules containing the sulphydryl group, such as cysteine and glutathione, producing the corresponding disulphides.¹¹ By exchange with protein sulphydryl groups these disulphides are believed to influence plasma sulphydryl group concentrations.⁵ A close correlation between superoxide dismutase activity and glutathione concentration has also been shown in vivo and ex vivo using haemolysate.¹²

As part of the phagocytic process a burst of free radical activity is produced by the phagocytosing cell. Among other effects this activity causes the release of oxygen derived species such as superoxide ions, hydroxyl radicals, and hydrogen peroxide.¹³ Protection against these active oxygen species is afforded by glutathione¹⁴ and possibly caeruloplasmin¹⁵ as well as superoxide dismutase. It has been suggested that the ability of patients with RA to metabolise these oxygen species is impaired and that the extent to which this occurs is related to the severity of the disease. Furthermore, SOD activity in polymorphonuclear leucocytes of children with juvenile rheumatoid arthritis is low⁹ and the levels in leucocytes of adults with rheumatoid arthritis is high.¹⁶

Thus both because of the possible chemical action of the gold drugs on sulphydryl group activities and because of the role of these groups and copper proteins in defining redox status and protecting the cell against free radical attack it seemed of value to study and compare the changes in the sulphydryl group concentration and copper enzyme activity caused by AF and GST.

Patients and methods

Ninety patients (28 male, 62 female) with classical or definite RA according to the criteria of the American Rheumatism Association were studied during 24 weeks of chrysotherapy. All patients were receiving optimal doses of non-steroidal anti-inflammatory drugs (NSAIDs) and none were taking steroids or had previously been treated with gold in any form. In addition, none had received penicillamine, levamisole, or immunosuppressive drugs in the six months preceding the trial.

Initially, patients were randomly allocated to one of three treatment groups. Thirty patients (nine male, 21 female, median age 47 years, range 25–69 years, median duration of disease six years) were given an initial 10 mg injection of GST followed by 50 mg weekly until clinical response. Thereafter, the frequency of injection was reduced to fortnightly, three weekly, and, ultimately, four weekly. Thirty

patients (eight male, 22 female, median age 55 years, range 22–71 years, median duration of disease 6.5 years) received auranofin tablets (3 mg twice daily) throughout the study. Thirty patients (11 male, 19 female, median age 55 years, range 31–72 years, median duration of disease 5.3 years) were given placebo tablets identical with auranofin twice daily.

Patients were initially monitored weekly, and assessments of disease activity were performed at weeks 0, 3, 6, 12, and 24 of treatment. The parameters studied were: duration of morning stiffness recorded in minutes, pain score measured in centimetres on a 10 cm visual analogue scale, grip strength measured with an aneroid manometer attached to a small cuff and inflated to 20 mm of mercury, Ritchie articular index,¹⁷ haemoglobin concentration (Hb), and Westergren erythrocyte sedimentation rate (ESR).

Blood samples from 22 healthy volunteers (17 male, five female) were used to determine control values for comparison with values found for the patient population. None were taking NSAIDs, steroids, or had previously been treated with gold in any form.

The clinical indices of disease activity were compared with laboratory measurements of extracellular redox status—namely, caeruloplasmin oxidase activity and plasma thiol and intracellular redox status in erythrocytes—namely, superoxide dismutase activity and lysate thiol concentrations. All examinations were made on fresh blood samples, all were separated within four hours of sampling, and all estimations were carried out at standard times after separation and within 48 hours of sampling.

The methods of estimation have been described previously.^{5–10} Thiol concentrations in lysate and in plasma were estimated by the method of Ellman¹⁸ using the thiol-disulphide interchange reaction between 5,5'-dithiobis(2-nitrobenzoic acid) and biological thiols. Caeruloplasmin oxidase activity was measured by a modification of the method of Menden *et al*¹⁹ based on the caeruloplasmin catalysed oxidation of *p*-phenylenediamine to Bandrowski's base. Superoxide dismutase activity was measured by the method of Misra and Fridovich²⁰ based on the increase in the rate of photo-oxidation of *o*-dianisidine. The overall disease activity was assessed with the method of Mallya and Mace.²¹ This index defined four grades of disease activity from inactive (grade 1) to very active (grade 4) using a combination of morning stiffness, pain score, grip strength, articular index, Hb concentration, and ESR. Clinical improvement was defined as a decrease in activity grade by at least one grade after 24

weeks of treatment. The significance of the difference between normal values and patient populations was calculated by the Mann-Whitney U test and the difference between the treatment groups by the Kruskal-Wallis test. The significance of the change in all parameters at various stages of treatment was obtained with the Wilcoxon matched pairs signed rank test and the time dependent changes in SOD and LSH by a Spearman rank correlation.²²

Results

The three treatment groups were similar in terms of age, duration of disease, sex ratio, and initial clinical and laboratory variables. The method of assessing disease activity as described by Mallya and Mace showed that the majority of patients (70/90) had

moderately active disease, 14/90 had slightly active, and 6/90 very active disease.

After 24 weeks of therapy 23/30 patients (77%) continued GST, 26/30 (87%) continued AF, and 17/30 (57%) continued placebo. Ten of the withdrawals from the placebo group were for persistent disease activity and lack of drug effect. Adverse effects or intercurrent illness accounted for all other patients who discontinued.

CLINICAL ASSESSMENT

Comparison of 0 to 24 week results (Wilcoxon) showed significant improvements in duration of morning stiffness, pain score, grip strength, Ritchie articular index, and ESR for both GST and AF groups. Patients who remained on placebo showed no significant improvement in any parameter. Table 1 shows the proportion of patients in each group who either improved or deteriorated by at least one grade of the activity index. When an improvement of one grade was used as a definition of change in disease activity there were 25 responders (R) and 41 non-responders (NR) in the first six months of therapy.

REDOX ASSESSMENT

Redox parameters were all significantly different from normal at the start of the trial ($p < 0.001$ Mann-Whitney). Table 2 shows the median and interquartile range of redox parameters for all

Table 1 Change in disease activity grade during the first 24 weeks of therapy with GST, AF, or placebo in patients with rheumatoid arthritis

Treatment group	Responders			Non-responders		
	Number still on therapy	Improved one grade n %		Deteriorated one grade n %	Unchanged n %	
GST	23	14 61		0 0	9 39	
AF	26	8 31		2 8	16 62	
Placebo	17	3 18		3 18	11 65	

Table 2 Medians and interquartile (IQ) ranges of redox parameters for patients with rheumatoid arthritis and normal controls at the beginning of the trial

	Extracellular		Intracellular	
	CP (mg/100 ml)*	PSH ($\mu\text{mol/l}$)	SOD ($\mu\text{g/ml}$)	LSH ($\mu\text{mol/l}$)
Median	60.1	311	62.0	423
IQ range	47.0-74.3	266.5-361	44.0-85.0	264.5-564.5
Normal range	38.0-52.8	457-543	78.2-92.5	123-363

*SI conversion: caeruloplasmin mg/100 ml $\times 10 = \text{mg/l}$.

Table 3 Medians (M) and interquartile ranges (IQ) for the redox parameters during the first 24 weeks of therapy for each of the treatment groups*

	GST		AF		Placebo	
	Week 0 (n=29)	Week 24 (n=21)	Week 0 (n=29)	Week 24 (n=25)	Week 0 (n=29)	Week 24 (n=17)
CP	M 64.0	50.5	54.8	45.1	62.1	52.2
	IQ 51.1-73.8	41.9-67.7	46.0-75.0	32.3-64.4	48.9-74.9	38.6-67.4
PSH	M 320	357	326	338	287	330
	IQ 272-362	325-416	269-372	296-396	250-349	288-380
SOD	M 50.3	71.4	66.9	67.7	66.9	74.4
	IQ 36.7-69.9	43.5-91.1	39.7-85.0	51.1-85.0	50.3-92.6	63.9-96.3
LSH	M 465	460	507	462	340	395
	IQ 339-638	307-618	257-584	280-533	232-442	310-417

*Differences in numbers between Tables 1 and 2 and Table 3 are due to instrumental problems with one batch of samples.

patients compared with those for normal controls. It can be seen that CP and LSH are raised and SOD and PSH are low. In addition, there were no significant differences in any of these parameters between the three treatment groups (Kruskal-Wallis) and none which correlated with age, sex, or duration of disease.

Table 3 shows the changes in redox parameters between 0 and 24 weeks for the three treatments. The wide interquartile ranges in these groups mean that many apparent differences were not significant even at the 5% level, although if the direction of change in all groups was the same, a significant change could be observed if the groups were combined. CP activity decreased towards normal control values in all groups but did not reach significance at the 5% level in any. PSH rose towards normal control values in all groups, reaching significance in the GST and AF responder groups ($p<0.02$ and $p<0.025$ respectively). SOD activity also appeared to rise towards control levels in all groups but was significant only in the GST responder group ($p<0.01$). LSH rose away from normal control values in the placebo group ($p<0.02$) and also in the GST responder and AF non-responder groups; LSH fell in both the AF responder ($p<0.01$) and GST non-responder groups. With the exception of LSH all the changes were as expected from the analysis of complete treatment groups, with the largest changes being in the GST and AF improvers groups. The significant changes are listed in Table 4. Neither the GST nor AF non-responder groups showed any significant change and the only significant change in the placebo group was in the opposite sense, i.e. away from normal control values.

It was noted that the intracellular redox parameters (LSH and SOD) altered in the first few weeks of therapy, and consequently, all four parameters were examined graphically and the results for SOD and LSH are illustrated for the GST group (Figs 1a and 1b). The significance of these changes was examined (Wilcoxon) by comparing the results

at each time point with those at week 0 (Table 5). The improvement in PSH at 12 and 24 weeks in the responder group and the changes in SOD and LSH are the most significant changes, together with an unexpected and unexplained observation of a significant change in CP in the non-responder group at six weeks. As can be seen in Figs 1a and 1b the changes in SOD and LSH at about six weeks are in

Fig. 1 Changes in redox parameters for GST (a) responders and (b) non-responders. The figure shows median and interquartile ranges at time points from zero to 24 weeks.

Table 4 Significant changes in redox parameters between 0 and 24 weeks. All changes are towards normal values unless marked*

	GST		AF		Placebo	
	R† (n=14)	NR† (n=8)	R (n=8)	NR (n=17)	R (n=4)	NR (n=13)
CP	—	—	<0.10	—	—	—
PSH	<0.02	—	<0.025	—	—	—
SOD	<0.01	—	—	—	—	—
LSH	—	—	<0.01	—	—	<0.02*

†R=responder; NR=non-responder.

opposite senses for the responder and non-responder groups, with the responders showing a change away from the control values. Similar changes in the auranofin group, though consistent with these findings, were not statistically significant, and no trend was observed in the placebo group, except that LSH rose continually.

We have previously shown a relation between SOD and LSH. This relation was found to be present at both 0 and 24 weeks (Table 6), but graphical presentation of the results indicated some change in slope, which suggested that both gold drugs did affect the nature of the correlation. The change in SOD ν the change in LSH was plotted (Fig. 2), and it was evident that a Δ SOD ν a Δ LSH

correlation was present in the placebo group ($p<0.005$). This correlation, however, was not as clearly defined in the AF or GST groups.

Discussion

The criterion for inclusion of a patient in the trial was a clinical assessment of active disease and, as indicated by the large variation at the start of the trial, there was no attempt to preselect specific groups of patients on the basis of any individual parameter. The Mallya and Mace index proved a useful measure of the clinical efficacy of the drugs, which could then be used to assess the copper protein and sulphydryl group measurements. It

Table 5 Probability values from an analysis using a Wilcoxon test of the change in CP, PSH, SOD, and LSH between 0 and 3, 6, 12, and 24 weeks for responders and non-responders after treatment with GST

Week	Responders (n=14)				Non-responders (n=9)			
	CP	PSH	SOD	LSH	CP	PSH	SOD	LSH
0-3	0.3	0.1	0.8	0.5	0.4	0.2	0.2	0.7
0-6	0.7	0.1	0.04	0.08	0.05	0.6	0.06	0.04
0-12	0.6	0.06	0.7	0.3	0.8	0.4	0.5	0.4
0-24	0.5	0.03	0.1	0.1	0.2	0.1	0.7	0.9

Table 6 Correlation between SOD and LSH for each of the three treatment groups

Week	GST			AF			Placebo		
	n	Corr	Sig	n	Corr	Sig	n	Corr	Sig
0	27	-0.705	0.0005	26	-0.762	0.0005	27	-0.697	0.0005
24	20	-0.827	0.005	23	-0.598	0.0025	17	-0.758	0.0005

Fig. 2 Plot of the change in SOD against the change in LSH over 24 weeks. The gold drugs appear to affect the correlation between SOD and LSH.

should be stressed that the patients in the placebo treated group who remained on the therapy for six months are atypical of the group as a whole, as those with the most active disease had dropped out and are therefore not included in any statistical analysis of change with time. In addition, the high drop out rate of patients receiving placebo was largely because of worsening disease, and the tendency for some improvement to occur in the remaining placebo group is not unexpected. The deterioration in LSH even in this selected group, however, indicates that there is a difference in the biochemistry of this improvement compared with that produced by GST and AF. All patients in this study continued their regular daily intake of non-steroidal anti-inflammatory drugs and as far as possible this was not varied during the trial, though it is possible that those who improved may have reduced the dosage. It is also possible that some non-responders are patients whom without treatment with GST or AF would have deteriorated rather than not improved. Both these points would reduce the significance of differences observed during therapy and may mean that the changes discussed are more conservatively estimated than should be the case. Neither possibility should produce any fake positive result. Furthermore, there was more flexibility in the GST regimen than with the new drug, in that at a later stage in therapy the dosage was where possible reduced. Since GST still shows the larger effects on the time scale studied the comparisons made remain valid, particularly since there is no accurate way of comparing dosage schedules between such different drugs.

Division of patients on each treatment into responders and non-responders allowed assessment of the biochemical parameters, not only longitudinally on the basis of the assigned treatment group but also on the basis of the response to treatment. In the three treatment groups the significant changes in GST and AF groups after 24 weeks were confined to the responder groups and were in a direction towards normal control values, whereas the only significant change in the placebo group was away from normal (Table 4). Despite clinical improvement in some patients, however, the inorganic biochemical parameters did not return to normal levels. This suggests that the effect of therapy on that aspect of the underlying biochemistry investigated is to create changes in the *in vivo* activity which produce a more quiescent state without returning the underlying biochemistry to a normal non-rheumatoid situation or producing a complete remission.

During the early weeks of therapy and before any clinical response could be discerned the intracellular

parameters SOD and LSH showed a definite pattern of deterioration, particularly in the GST group, in those patients who later showed a clinical response. The pattern was most clearly seen when the GST group was subdivided into responders and non-responders. No other clinical or biochemical parameters showed a clear pattern of early deterioration in the responder group. This result, if borne out in future studies, might mean that the likely response of a patient to a particular therapy could be predicted early in the course of therapy, even before the stage at which clinically assessed improvement becomes apparent.

Sulphydryl group concentrations on both sides of the cell membrane are higher than CP and SOD (three groups per molecule of CP and 10^3 groups per molecule of SOD respectively). Consequently, in an assessment of the action of these reactive materials it seems better to consider the sulphydryl groups first and to view the action of CP and SOD as they affect the sulphydryl group-disulphide equilibrium. There has recently been increasing interest in the role of oxygen derived free radicals in inflammatory disease and tissue destruction, and a large amount of circumstantial evidence now exists which implicates these species in the pathogenesis of rheumatoid arthritis. One method of assessing the free radical activity is to monitor changes occurring in parameters relatively stable with time involved in the defence of the cell against free radical attack. Two such parameters are the intracellular compounds superoxide dismutase and glutathione. Both have been separately discussed and postulated as the prime defence mechanism, but there is no consensus as to their relative importance. *In vitro* there is no doubt that, molecule for molecule, SOD is more effective at removing superoxide ions than are thiols, but we have found that in the relative concentrations of SOD and thiol found in either red or white cells it is the thiol concentration which is predominant.¹³ Furthermore, the superoxide ion is short lived, lasting approximately 10^{-5} s⁸ and thus will react close to the environment in which it is generated. This suggests that in many cases the more ubiquitous sulphydryl system will be present at the reactive site in higher relative concentration than that suggested above. In addition, the superoxide ion is only one of the free radicals believed to be produced by the phagocytosing cell. For the others the specificity of SOD for superoxide ion would suggest that it would be less effective in these cases, whereas the more general radical scavenging activity of sulphydryl groups would indicate a role for them in these processes also.

The method of analysis used for thiol estimation does not detect haemoglobin thiol and consequently,

glutathione is the largest single component of LSH. As well as being a key element in the defence of the cell against attack by radicals in general, it is a key species in protein synthesis, a major source of non-protein bound sulphydryl groups within the cell, and, through the glutathione to diglutathione ratio, is used to control key enzyme processes in cellular metabolism.²³ During inflammation cellular degradation of erythrocytes in the joint will release glutathione into the extracellular environment and hence into the plasma. The major individual component of the plasma sulphydryl group concentration (PSH) is the sulphydryl group on albumin. This group is protected from reaction with other proteins by being situated in a pocket in the protein structure. Exchange reactions between other proteins and small molecule disulphides are likely, however, and consequently an indirect equilibrium between each plasma protein and membrane sulphydryl groups is to be expected (Fig. 3). In this way sulphydryl reacting compounds, including small molecule disulphides (RSSR'), can act to potentiate the various protein-SH concentrations in plasma. Caeruloplasmin is a specific oxidase for small molecule sulphydryl groups (RSH), producing small molecule disulphides (RSSR'). It would, therefore, be expected to have a direct effect on this equilibrium. Thus the effect of additional extracellular glutathione produced by inflammation would be to increase the concentration of plasma protein present in the disulphide form and cause a corresponding reduction in PSH.

The inorganic biochemical parameters chosen are thus one measure of the oxidative status across the cell membrane and of the free radical protection system in erythrocytes. Since both erythrocytes and leucocytes are derived from the same stem cells, and since the mature erythrocyte can synthesise glutathione in much the same way as the leucocyte, it may be that the intracellular parameters—although determined in this study by erythrocyte

concentration—also reflect analogous changes in leucocytes, though the exact nature of such changes seems to depend on the disease and cellular type.

One practical point arises from this work—namely, that sulphydryl groups interfere with most SOD assays *in vitro*, so that determination of SOD in lysates from red or white cells, for which the sulphydryl group concentration is not known, must be regarded as suspect. In the present study, however, the lysate sulphydryl group concentrations are low since the sulphydryl groups have been allowed to oxidise and consequently, with the exception of one or two of the highest results, are below the level at which added glutathione interferes with the assay. Thus the correlation between SOD activity and lysate thiol concentration appears to have a biochemical rather than an analytical meaning. In all cases at 0 and 24 weeks a significant correlation between SOD and LSH at the 0.1% level is obtained (Table 6). When the change in SOD over the 24 week period is plotted against the change in the sulphydryl group concentration over the 24 week period, however, a very different pattern emerges (Fig. 2). The placebo group shows a significant correlation, but for auranofin this correlation is less obvious, and no correlation is evident for GST. Thus although the drugs over 24 weeks produce little change in mean concentration of lysate thiol or SOD, there is evidence that they do potentiate the free radical defence system in a rather more subtle way.

In conclusion, in agreement with previous studies^{12 24 25} this work has further confirmed that plasma thiol levels are an indicator, albeit a rather slow one, of eventual therapeutic outcome. The intracellular parameters give an early indication of therapeutic response analogous to that found for penicillamine.²⁶ For penicillamine Munthe suggested that a rise of about 20% was required if the patient was to show response, and it is clear from our choice of responders that a similar rise is observed here (Fig. 1a). It is too soon to recommend this approach for use in clinical assessment but, taken together with other studies on the thiol system and bearing in mind the confirmatory value of two correlated analyses, it would seem to offer potential in this direction if confirmed in further studies. In any event the fundamental nature of the process and the nature of the relation between sulphydryl groups and superoxide dismutase activity would seem to be good subjects for further investigation.

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Fig. 3 Exchange reactions between plasma protein, small molecule disulphide, and cell membrane. CP is caeruloplasmin, an oxidase with many postulated functions, one of which is the oxidation of the sulphydryl group in small molecules.

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