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Effects of antirheumatic drugs on protein sulphydryl reactivity of human serum

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The effect of a number of antirheumatic drugs on sulphydryl group reactivity in human serum has been determined using the thiol reagent, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). Drug effects in vivo have been measured using sera from patients with rheumatoid arthritis receiving these compounds and the drugs have also been added to healthy human serum in vitro. An increased rate of sulphydryl-disulphide exchange between serum sulphydryl groups and DTNB was noted in rheumatoid disease. This finding was not associated with anti-inflammatory drug therapy. In vivo, non-steroidal anti-inflammatory drugs did not affect this reaction, but aurothiomalate, D-penicillamine and levamisole all significantly increased the rate of sulphydryl-disulphide exchange. When the drugs were added to healthy human serum in vitro, indomethacin, aurothiomalate and D-penicillamine stimulated sulphydryl reactivity with DTNB but levamisole had no effect. Aurothiomalate appeared to act in this system as a mixture of gold atoms and thiomalate molecules, the thiomalate stimulating and the gold inhibiting thiol reactivity. This study demonstrates that the stimulation of sulphydryl-disulphide exchange reactions in vivo is a property of three long-acting drugs used to treat rheumatoid arthritis, but not of non-steroidal anti-inflammatory agents. This reactivity may therefore differentiate the second-line drugs showing true antirheumatic activity from 'simple' anti-inflammatory compounds. Comparative studies have shown that the addition of drugs to serum in vitro may give different results from those obtained with sera from rheumatoid patients receiving these agents. This may be due to in vivo metabolism of the compound, drug concentration effects or the parameters of the in vitro assay system. In view of this, caution is required in the interpretation of data obtained from in vitro studies of these compounds.

Protein sulphydryl groups may play an important role in a number of biological processes (Jensen 1959). More recently, the immune system has been identified as one such process and it has been demonstrated that thiols are involved in lymphoid cell proliferation (Broome & Jeng 1973; Bevan et al 1974), the production of antibody (Click et al 1972) and the function of complement receptors on the cell surface (Dierich et al 1974). It is generally accepted that immune mechanisms make a significant contribution to the pathogenesis of rheumatoid arthritis (Zvaifler 1973). Low concentrations of free sulphydryl groups have been found in sera from patients with Waldentrom's macroglobulinaemia and a number of connective tissue diseases including rheumatoid arthritis (RA), (Lorber et al 1964). These authors suggested that, in RA, low thiol (SH) levels may be associated with a disturbance of the sulphydryl-disulphide (SH-SS) exchange reaction. This could lead to the aggregation of serum proteins, particularly IgG, with the exposure of new antigenic sites leading to autoantibody production (rheumatoid factor). As a model for this hypothesis, heat-aggregation of IgG has been shown to be partly thiol dependent (Gerber 1964) and may be inhibited by SH-containing antirheumatic drugs such as sodium aurothiomalate and D-penicillamine (Gerber 1974).

Sulphydryl groups have been implicated in the inflammatory process (Oronsky et al 1969), and a number of anti-inflammatory drugs have been assessed for their ability to affect protein sulphydryl reactions both in vitro and in experimental animals. Such studies have generally involved measuring the reaction between serum protein thiol groups and an aromatic disulphide, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (Ellman 1959). Several non-steroidal anti-inflammatory drugs (NSAID) stimulate the rate of this reaction in human serum in vitro (Gerber et al 1967) whereas, using rat serum, aurothiomalate has been shown to be inhibitory (Walz & DiMartino 1972). Rats with adjuvant arthritis have decreased sulphydryl reactivity with DTNB which may be restored to normal by the administration of NSAID (Butler et al 1969).

In man, large doses of aspirin in vivo have also been shown to stimulate protein thiol reactivity with

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DTNB (Gerber et al 1967). Few data seem to be available concerning the effect of antirheumatic drugs on protein sulphydryl reactions in patients suffering from rheumatoid arthritis. This study set out therefore to elucidate the effects of NSAIDs and the second-line antirheumatic agents, aurothiomalate, D-penicillamine and levamisole on such reactions in vivo. These results have been compared with in vitro studies of drug interactions with healthy serum.

MATERIALS AND METHODS

Sodium aurothiomalate, D-penicillamine, levamisole and indomethacin were generously provided by May and Baker Ltd., Dista Products Ltd., Janssen Pharmaceutical Ltd. and Merck, Sharp and Dohme Ltd., respectively.

Peripheral blood was obtained from patients with classical or definite rheumatoid arthritis attending the Royal National Hospital for Rheumatic Diseases, Bath, and from healthy volunteers. The blood was allowed to clot at 37 °C and the serum was obtained by centrifugation. Serum was prepared similarly from blood obtained from male Wistar rats (200–300 g) by cardiac puncture under ether anaesthesia.

The exchange reaction between serum protein sulphydryl groups and DTNB (Sigma) may be represented by the reaction: Protein-SH + DTNB→ Protein-S-S-TNB + TNB–SH. The deep yellow product of this reaction, 5-thio-2-nitrobenzoic acid (TNB-SH), may be determined spectrophotometrically at 440 nm (Butler et al 1969). Human serum (1 ml) was mixed with 0·1 M phosphate buffer pH 7·4 (1 ml) and DTNB solution (0·5 ml) was added to a final concentration of 400 μM. The rate of appearance of TNB-SH was measured at 440 nm in a spectrophotometer. Control tubes were prepared containing 1 ml serum and 1·5 ml buffer. The DTNB solution was prepared freshly each day. For in vitro studies, drugs were dissolved in 0·1 M phosphate buffer, pH 7·4 just before use and were incubated with equal volumes of fresh healthy human serum for 30 min at room temperature (21 ± 1 °C) before the addition of DTNB. The rate of reaction between serum and DTNB was measured over 1–4 min at room temperature. The change in absorbance at 440 nm during the first minute of the reaction varied considerably between individual samples and so was not used to calculate the reaction rate. This initial rapid reactivity represents the interaction of low molecular weight (non-protein) thiols with DTNB (Jocelyn 1962). Known concentrations of reduced glutathione were reacted with DTNB in order to calibrate absorbance at 440 nm with molar thiol concentrations. From this, the rate of reaction of protein sulphydryl groups with DTNB was calculated as μmole thiol reacting per litre serum per minute. Groups of data were compared using the Student's *t*-test.

RESULTS

Serum was obtained from healthy volunteers and from RA patients on a variety of therapeutic regimes and was assayed for protein thiol reactivity with DTNB on the day of collection (Fig. 1). The results

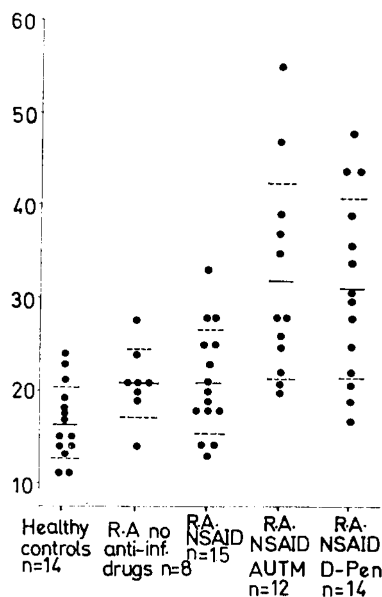

FIG. 1. Effect of drugs in vivo on serum protein sulphy dryl reactivity. Sera from healthy and rheumatoid subjects receiving various drug regimes were assayed for protein sylphydryl reactivity with DTNB. The rate of reaction was calculated from a glutathione standard. Horizontal bars mark the mean reaction rate for each group and the dotted bars represent one standard deviation from the mean. Groups of data were compared using Student's *t*-test: RA (no drugs) v healthy $P < 0{\cdot}02$; RA (AUTM) v RA (NSAID) $P < 0{\cdot}005$; RA (D-pen) v RA (NSAID) $P < 0{\cdot}0025$. Ordinate: rate of SH-SS reaction (μmol SH litre^{-1} min^{-1}).

show that rheumatoid serum proteins undergo a significantly increased rate of reaction with DTNB compared with healthy control serum ($P < 0{\cdot}02$). This is not associated with anti-inflammatory drug therapy. Patients receiving NSAIDs did not differ in their overall sulphydryl reactivity from patients not receiving such compounds. However, the administration of aurothiomalate or D-penicillamine to RA patients led to a significant increase in the rate

of reaction between serum and DTNB. Patients in this study were receiving either 10–50 mg aurothiomalate (Myocrisin, May and Baker Ltd.) per week or 250–750 mg D-penicillamine (Distamine, Dista Products Ltd.) daily and had been receiving these drugs for at least three months. The increase in reaction rate was highly significant when compared with the NSAID data for both aurothiomalate ($P < 0{\cdot}005$) and D-penicillamine ($P < 0{\cdot}0025$). As these results were obtained from random, single-point measurements, experiments were performed to determine the reproducibility of the assay system. Serum was obtained from 8 healthy volunteers at times 0, 3, 24 h, 7 and 14 days and the rate of SH reaction with DTNB measured. This did not vary significantly over the two-week period (unpublished data). Experiments were also carried out to compare the reactivity of matched serum and heparinized plasma samples both on the day of collection and following storage at −20 °C for 7 days. Serum and plasma gave identical results, but a fall in the rate of reaction was seen following storage of frozen material (unpublished data). The effect of another second-line antirheumatic drug, levamisole, on protein sulphydryl reactions was measured in plasma obtained during a double-blind clinical study of levamisole, 150 mg day^{-1} (levamisole, Janssen Pharmaceutical Ltd.) versus placebo. Plasma samples from both groups of patients were stored at −20 °C and assayed with DTNB without knowledge of the individual treatment (Fig. 2). Plasma from levamisole-treated patients showed a significantly increased rate of reaction with DTNB compared with the placebo control group ($P < 0{\cdot}001$).

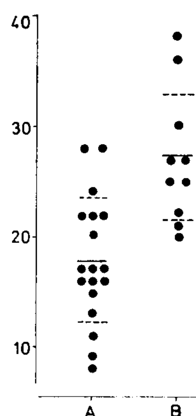

FIG. 2. Effect of levamisole therapy on protein sulphydryl reactivity. Plasma samples from patients receiving levamisole (150 mg day^{-1}) or placebo were assayed for protein sulphydryl reactivity with DTNB. The rate of reaction was calculated from a glutathione standard. Horizontal bars mark the mean reaction rate for each group and the dotted bars represent one standard deviation from the mean. Groups of data were compared using Student's t-test: RA (levamisole: B) v RA (placebo: A) $P < 0{\cdot}001$. Ordinate: rate of SH-SS reaction (μmol SH litre^{-1} min^{-1}).

The second-line antirheumatic drugs studied in vivo were also assessed for their ability to affect protein reactions with DTNB in vitro using healthy human serum. The results are presented in Fig. 3.

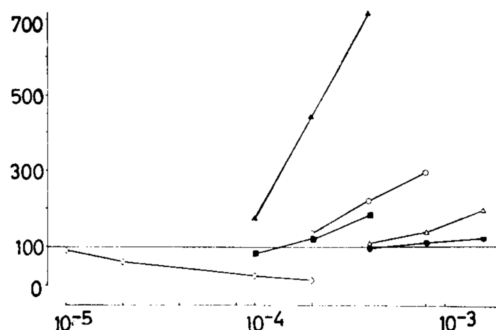

FIG. 3. Effect of drugs in vitro on serum protein sulphydryl reactivity. Drugs were incubated with fresh healthy serum for 30 min at room temperature and the SH reaction with DTNB was measured. The rate observed in the drug-treated sample was compared with the rate (=100) in an untreated sample tested in parallel. Each point represents the mean of 4 separate experiments. Key: ◇, sodium chloroaurate; ▲, thiomalate; ■, D-penicillamine; ○, Indomethacin; △, sodium aurothiomalate; ●, levamisole. Ordinate: relative rate of SH-SS reactions. Abscissa: drug concentration (mol litre^{-1}).

D-Penicillamine and aurothiomalate stimulated the rate of reaction in a dose-dependent manner but levamisole was not active in this system. D-penicillamine was 2–3 times more reactive than aurothiomalate and showed in vitro activity at concentrations ($2–4 \times 10^{-4}$ M) likely to occur in the sera of rheumatoid patients. As gold rapidly becomes bound to protein in vivo with the release of free thiomalate (Jellum & Munthe 1977), it was decided to determine the effect of gold, as sodium chloroaurate (BDH) and thiomalate (Sigma) separately on the reaction of serum with DTNB. As shown in Fig. 3, thiomalate greatly increased the rate of this reaction whereas gold strongly inhibited it. Indeed, aurothiomalate in vitro appeared to act as a mixture of gold and thiomalate. A typical NSAID, indomethacin, was tested in the in vitro system and showed a stimulatory effect that approximately equalled that of D-penicillamine (Fig. 3). The data obtained for indo-

methacin are in good agreement with that published by Gerber et al (1967).

DISCUSSION

Three second-line antirheumatic drugs—aurothiomalate, D-penicillamine and levamisole—have all been shown to stimulate serum protein sulphydryl reactions in vivo as measured by exchange with DTNB in vitro. This ability differentiates these compounds from NSAIDs which, in this study, did not affect this reaction. However, none of the patients was receiving high dose aspirin, which other workers have shown to stimulate the rate of SH reaction with DTNB (Gerber et al 1967).

There are several differences between the in vivo and in vitro effects of the second-line drugs used in these experiments. Levamisole showed no activity when added to serum in vitro but led to a significant increase in the rate of SH reactions in vivo. It seems likely that levamisole acts on protein sulphydryl groups following metabolism in vivo with the formation of a free thiol from the thiazole ring. The in vivo stimulation of sulphydryl reactions by aurothiomalate seems from in vitro studies to be due to the thiomalate component of the drug. Indeed aurothiomalate contains two active components with opposing effect on thiol reactivity. The thiomalate molecules will increase the rate of protein SH reaction with DTNB but the gold atoms inhibit this. This inhibitory activity on serum proteins is masked in vivo by excess thiomalate which arises following the sequestration of gold within macrophages in the inflamed synovium and other tissues (Vernon-Roberts et al 1976). In addition, serum gold levels in RA patients receiving chrysotherapy are of the order of 10^{-5} M (Rubinstein and Dietz 1973) at which concentration little reactivity was seen in our in vitro assays. Differences in in vivo and in vitro drug levels also account for the results obtained with indomethacin in this study. In vitro, indomethacin stimulated the rate of reaction between protein SH groups and DTNB at concentrations above 2×10^{-4} M. However, steady state plasma concentrations of indomethacin in rheumatoid patients are in the range $2–8 \times 10^{-6}$ M (Hvidberg et al 1972), two orders of magnitude below the minimum effective dose in vitro. It seems probable that the mechanism whereby indomethacin stimulates protein thiol reactivity towards DTNB differs from that of aurothiomalate, D-penicillamine and levamisole. These three compounds share the ability to interact directly with protein SH groups. Indomethacin and other NSAIDs do not possess active thiol groups and so may only stimulate SH reactivity by an indirect or 'allosteric' effect on protein conformation.

Difficulties arise in the interpretation of results obtained in in vitro experiments and in gauging the relevance of such data to in vivo situations. Thus, in this study, aurothiomalate stimulated protein sulphydryl reactivity both in vivo and in vitro, but Walz & DiMartino (1972) obtained the opposite result by adding this compound to rat serum in vitro. We have repeated their experiments and have confirmed their results. Several differences in the two assay systems may explain this discrepancy. Rat serum exhibits a much more rapid rate of reaction with DTNB than human serum (Butler et al 1969). This allows an accurate estimation of this reaction rate to be made using low concentrations of DTNB (65 μM) compared with the present study (400 μM). This in turn influences the various interactions between the SH-reactive components of the incubation mixture—serum proteins, DTNB, gold and thiomalate—and leads to different effects being observed. The in vitro assay system therefore is dependent on a number of artificial parameters. A more detailed study of factors affecting the assay of protein sulphydryl reactivity is in progress.

The mode of action of aurothiomalate, D-penicillamine and levamisole in RA is still unknown. We have shown that the first two of these drugs, with their similar clinical and toxic profiles, share the ability to enhance serum protein sulphydryl reactivity in vivo. This study demonstrates that levamisole also stimulates thiol reactivity in vivo and may therefore mediate some of its effects via sulphydryl groups. It is not known whether these three compounds influence SH groups directly or whether some other factor induced by the drugs is responsible. Similarly, it has not been established that the serum protein sulphydryl reactions are those relevant to the drugs' action (s). Cellular thiol reactions associated with lymphocytes or macrophages may be more important targets for these agents. All three drugs have been shown to affect lymphocyte and macrophage function either in vitro or in vivo. Aurothiomalate inhibits both cell types (Jessop et al 1973; Lies et al 1977), whereas levamisole has been termed an immunotherapeutic compound with anti-anergic properties (Symoens & Rosenthal 1977). The published literature on the influence of D-penicillamine on lymphocytes is conflicting, as discussed in a recent article by Lipsky & Ziff (1978) but the drug may lead to increased macrophage activity (Hunneyball et al 1978). Further studies are required to investigate the effects of thiol-reactive

antirheumatic drugs on both serum protein function and mononuclear cell activity in vivo. These experimental data must then be correlated with assessments of disease activity in patients with rheumatoid arthritis.

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