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STUDIES OF THE EFFECT OF METAL CONTAINING DRUGS ON ACUTE AND CHRONIC INFLAMMATION

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SUMMARY

Metal ions are involved in the functional activity of enzymes and cells of the inflammatory response. The effect of these in controlling or regulating inflammation has been the subject of considerable interest. This is particularly so for metal ion therapies such as Au(I) complexes which have been used to treat chronic unresolving inflammatory disorders such as rheumatoid arthritis.

One of the most perplexing problems of chrysotherapy is exactly how gold elicits its therapeutic activity and induces an apparent remission of the inflammatory disease process. The use of the adjuvant induced polyarthritis in rats has led to some interesting although ambivalent findings of the effects of gold. These findings may reflect, however the variable use and the limitations of this animal model of inflammation.

The second chapter describes investigations concerned with the in vivo biological effects of gold compounds on the adjuvant induced polyarthritis model of inflammation in various rat strains. The aims of this work were to (i) to investigate the antiarthritic activity of GST in adjuvant arthritis in rats and to establish the effect of varying the routes of administration and differing the time of administration, (ii) to investigate the comparative antiarthritic efficacy of a number of gold complexes, (iii) to determine the antiarthritic activity of GST and AF in adjuvant polyarthritis using differing rat strains using differing adjuvants, to standardise the assay and to clarify why previous work has such ambivalent findings

using gold salts, (iv) to investigate the effect of GST therapy on endogenous copper distribution.

Previous experimental studies have indicated a number of biological effects of monovalent gold complexes on macrophages, although their mechanism of action in suppressing or remitting arthritis is not clear. The third chapter describes the effect of gold salts on two aspects of macrophage functions that have not been widely studied, Fc receptor binding activity (FcBA) and the production of $^{\bullet}O_2^-$ radicals by a PMA stimulus. The effects of chrysotherapy on the presentation of Fc receptors on the cell surface of adherent macrophages and $^{\bullet}O_2^-$ radical production by macrophages from both adjuvantised and non-adjuvantised rats treated with and without gold sodium thiomalate has been studied. Moreover the effect of GST on monocytes in vitro has been studied, and the possibility that gold may mediate some of its antiarthritic activities through its effects on Fc receptor expression has been considered.

Some copper complexes have been shown to possess anti-inflammatory properties. However, many that have been categorised as anti-inflammatory also demonstrate irritant properties. The fourth chapter is concerned with the investigation of the biological activity of copper complexes with particular reference to the complex of copper and D-penicillamine $[Cu(I)_8\ Cu(II)_6\ (\text{D-penicillamine})_{12}]\ Cl^{5-}$ which has been shown to have substantial superoxide dismutating activity as well as antiulcerogenic activity. The aims of this study were (i) to determine the relative irritancy of a number of copper complexes; (ii) to study the effect of administration of these complexes on the liver metabolism of

pentobarbitone measured by induced sleep times; (iii) to investigate the comparative anti-inflammatory activity of these complexes against a number of standard animal models of inflammation including carrageenan paw oedema, TBC impregnated sponge implants and adjuvant induced arthritis in rats; and (iv) to study the relative biodistribution of the mixed valency copper-penicillamine complex.

The mixed valency copper-penicillamine complex appears to be unique amongst the copper complexes investigated since it is essentially non-irritant although it does possess substantial anti-inflammatory activity and results in marked accumulation of copper at inflammatory sites. The systemic biodistribution of copper from this complex shows a particular preference for the reticuloendothelial system.

DECLARATION

This thesis contains no material which has been accepted or summitted for the award of any other degree or diploma in any University. Furthermore, to the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

The work for this thesis herein has been the subject of the following publications:

1. GARRETT,I.R. and WHITEHOUSE,M.W.
   Heavy metal (Au,Pt) nephropathy:
   Studies in normal and inflamed rats.
   Adv. in Inflammation Res. 6, 291, 1983.

2. GARRETT,I.R.,WHITEHOUSE,M.W. and VERNON-ROBERTS,B.
   The anti-inflammatory/anti-arthritic effect of some copper-D-penicillamine complexes.
   Clin.Exp.Pharmacol.Physiol. 10, 42, 1983.

3. GARRETT,I.R., WHITEHOUSE,M.W., VERNON-ROBERTS,B. and BROOKS,P.M.
   Ambivalent properties of gold drugs in adjuvant induced polyarthritis in rats.
   J.Rheumatol. 12(6), 1, 1985.

4. GARRETT,I.R., BEVERIDGE,S.J. and WHITEHOUSE,M.W.
   Biodistribution of ^{64}Cu in inflamed rats following administration of two anti-inflammatory copper complexes.
   Agents and Actions 17(1), 104, 1985.

5. BETTS,W.H.,GARRETT,I.R.and WHITEHOUSE,M.W.
   Therapy with metal complexes.
   in Anti-rheumatic and Anti-inflammatory Drugs
   Ed. Rainsford,K.D., C.R.C.Press, Boca Raton, California, U.S.A. 1985.

6. GARRETT,I.R. and WHITEHOUSE,M.W.
   Copper and Inflammation.
   in The Metabolism of Copper in Animals and Man,
   Ed. McC.Howell,J. and Gawthorne,J.M.
   C.R.C.Press, Boca Raton, California, U.S.A. (in press).

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I.R.Garrett
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CHAPTER 1

REVIEW OF CURRENT LITERATURE

I - INTRODUCTION TO METAL ION THERAPIES

A. Essential Elements

Of the 90 naturally occurring elements in the periodic table, almost all are present in the human body. However, fewer than half are known to have any biological role[1], the rest being fortuitous reminders of our geochemical origins. The biologically essential elements are divided into two classes according to the amounts required to maintain metabolic balance, namely, the macrominerals and the microminerals (trace elements). Over the last 10 years there has been an increasing amount of interest and research into the role of metal ions in biological systems. The significance of metals in vivo has been recently expressed by Williams[2] "Although the human body is but 3% metals, life depends upon these elements far more than this figure suggests".

1. Macrominerals

The macrominerals sodium, calcium, magnesium, potassium, phosphorus and chlorine are present in the body in gram per kilogram concentrations and required in the diet daily in gram amounts. These elements serve as structural components of tissue, or as constituents of body fluids and are essential for the function of all cells[3]. These elements however will not be discussed further in this thesis.

2. Microminerals (Trace elements)

The microminerals are in substantially lower concentrations in the body (milligrams per kilogram). In general a trace element is one which constitutes less than 0.01% of the body mass[4,5]. 14 trace elements are now considered to be essential in animals (TABLE 1). In order to describe an element as essential it is important to show that its absence will cause the organism to develop characteristic pathological deficiency symptoms. By the use of this criteria, it has become obvious that the presence of certain metal ions are essential. However it has become increasingly apparent that not all the essential elements are necessary to all organisims. Claims of essentiality have been made for several other trace elements: cadmium, lead, bromine and tin but these have not been confirmed[6,7].

TABLE 1 Essential Trace Elements

Element	Function
Arsenic	Iron metabolism.
Chromium	Maintenance of normal glucose tolerance.
Cobalt	Erythropoiesis.
Copper	Caeruloplasmin, cartilage synthetic enzymes Bone,myelin,immune system,superoxide dismutase.
Chlorine	Structure of bones and teeth.
Iodine	Thyroid hormones.
Iron	Haem respiratory carrier,enzymes, Immune system.
Manganese	Enzymes in protein and energy metabolism, Mucopolysaccaride synthesis.
Molybdenum	Enzymes in the metabolism of xanthine, sulfites, thiol containing amino acids.
Nickel	Nucleic acid,lipid metabolism,iron absorption.
Selenium	Glutathione peroxidase.
Silicon	Structural component of connective tissues.
Vanadium	Lipid metabolism, regulation of cholesterol synthesis, ATPases.
Zinc	Enzymes in most major metabolic pathways, nucleic and protein synthesis, immune system.

There are some elements widely distributed in living material such as lithium and rubidium, which seem to be beneficial to organisms but their absence produces no apparent ill effects. These elements may well be essential although the requirements may be satisfied by almost vanishingly small amounts.

3. Toxic elements

Another group of elements exists which has neither essential or beneficial effects but are in fact toxic to normal metabolic processes, even in small amounts. The pathological effects and dose levels required in metal ion poisoning are reasonably complex and variable, depending upon factors which modify the uptake of the metal as well as those controlling its subsequent metabolism (detoxification processes).

It may be surprising that some elements have been listed as toxic and although they appear to have essential biological roles (TABLE 1). Indeed for selenium, deficiency has turned out to be a much greater problem than its toxicity[8]. These situations emphasise that no element is inherently beneficial or toxic, but rather their biological effect depends on the amount of element present in the organism. Therefore, we have the extremes of too little or too much causing illness and possibly death, with a range of intake/tissue concentration associated with optimum functioning of the organism.

B. Bio-metals

1. Classification

The classification as a metal is largely based on a consideration of physical properties and implies the element has certain characteristics such as lustre, high melting point or considerable mechanical strength. All these characteristics are recognizable attributes of familiar metals such as iron, but it is often the case that this division of the elements into metals and non-metals is somewhat arbitrary.

One disadvantage of this is that it focuses attention on physical characteristics while ignoring chemical properties. However, with such a large number of metallic elements, each with their own rich and varied chemistry, it would be difficult to arrive at a comprehensive yet useful chemical classification. Nevertheless, such a perspective is needed, since in biochemical studies it is seldom that either physical properties or even the elemental state itself are considered. With few exceptions the metallic elements do not occur in their native state but are found in salts or complexes of the positively charged metal ion[9]. Perhaps then, a limited but useful definition of a bio-metal is an element which under certain biological conditions may react by losing one or more electrons to form a cation. This does not imply however that metals may not react in a variety of other ways.

2. Chemistry

The essential trace metals are involved in a variety of biochemical functions in the body, but most act primarily in enzyme systems such as metalloenzymes which have a very wide range of activity[10-13]. Several essential elements do have functions in host defence mechanisims and immune systems[14,15]. The bioavailability of the metal ion depends on the metal ligand bond and certain metals prefer certain ligands. The hard soft acid base (HSAB) theory[16] has been developed to explain this. In a metal complex M--L or [M:L] the metal (M) is the electron acceptor (Lewis acid) and the ligand (L) is the electron donor (Lewis base). According to the HSAB theory, a strong bond is formed either by hard acid / hard base or soft acid / soft base combinations. Hard / soft bonds are weak and do not occur. This theory enables us to consider the behaviour of heavy metals such as Pb, Hg, and Cd in terms of their preference for soft bases, such as sulphydral groups in enzymes (TABLE 2). Once heavy metals attach they tend to bind irreversibly and block enzyme functions. Treatment of heavy metal poisoning often involves chelation of the metal by another soft base (e.g.D-penicillamine[17]) to solublise and remove the metal.

TABLE 2 Some natural ligands

Ligand species	Metal ions
Halide	Cu(II), Pt(II)
Oxy(carboxylate,phenolic,hydroxyl	Cu(II), Ca, Zn, Fe(II), Fe(III)
Oxy(phosphate)	Ca, Zn, Pb, Fe
Nitrogen(amine)	Cu(II), Zn, Pt(II), Pt(IV)
Nitrogen(peptide)	Cu(II), Cu(III)
Nitrogen(imidazole, haem)	Cu(II), Fe(II)
Sulphur(thiol)	Au(I), Cu(I), Pt(I), Pt(IV)
Sulphur(sulphide/disulphide)	Cu(I), Fe(II), Pt(II)
Cyanide/Nitrile/Thiocyanide	Cu(I), Fe(III), Co(II), Pt(II)
Olefin (C=C)	Cu(I), Ag(I), Au(I), Pt(II)

3. Biological factors affecting metal ion interactions

Free metal ions exist in vivo in exceedingly small quantities. The ions are either complexed with water or bound to biological molecules. Thus, the biological function and reactivity of these metal complexes must be understood in terms of metal-ligand interactions and the exchange of ligands likely to occur in vivo[18].

The ligands form co-ordinate bonds by donating lone pairs of electrons to positively charged metal ions. Metal ions such as gold(I) and copper(I) can be stabilised in vivo by ligands of limited natural abundance such as thiol, -SR, -CN groups.

The chemistry of these ligands that constitute metal complexes is just as important as that of the metals but often receives less attention. Metal ligands are, in effect, metal buffers which can greatly influence the redox characteristics of metal ions. Pka or acidity of these ligands may vary considerably with their environment, which in turn governs their ability to form stable metal complexes. When considering the transport of metal ions through the biological system the probable formation of complexes between proteins and metals is most significant. Some small ligands may generate surprisingly large metal complexes in vivo. For example, Cu(II) and penicillamine (an amino acid thiol) form a very stable, highly charged, hydrated purple complex, which contains copper in two valency states $[Cu(I)_8\ Cu(II)_6\ Pen_{12}]^{-5}$ [19]. This complex has been shown to have superoxide dismutase-like activity in vitro[20]. Moreover, gold(I) sodium thiomalate (Myocrisin) exists as $S-(AuS)_n-Au$

polyacidic-oligomers with a degree of polymerization greater than 6 [21]. Exogenous metal complexes will tend to donate metals to those bioligands which turn over less rapidly *in vivo*. Ligands may either inactivate (detoxify) or activate metal ions. Inactivation of metal ions *in vivo* is exemplified by the sequestration of iron by the protein ferritin and of intoxicant heavy metals (Cd,Pb,Hg) by thiol-rich protein such as the metallothioneins. Paradoxically, EDTA used therapeutically will inactivate lead but will also activate other metal ions (e.g. by promoting the destructive autoxidation of Fe(II)). Metal ions are essentially metabolised by successive interactions with ligands of varying size, reactivity and distribution. Metal ion/complex induced pharmacology is in fact the consequence of metal-ligand interactions on or at the actual loci *in vivo* [22].

C. The Involvement of Metals in Inflammation

Metal ions are involved in the functional activity of enzymes and cells of the inflammatory response [23]. The effect of these in controlling or regulating inflammation has been the subject of considerable interest. This is particularly so for metal ion therapies such as Au(I) complexes which have been used to treat chronic unresolving inflammatory disorders such as rheumatoid arthritis [24].

1. Inflammatory disease

The inflammatory response is responsible for the removal of noxious agents which have penetrated an organisms defence or possibly for anti-neoplastic surveillance of the organism. Inflammation is a multi-mediated phenomenon in which all mediators come and go at the appropriate moment. They play their roles in increasing vascular permeability, attracting leucocytes, producing pain, and necrosis. The extent of involvement of any single mediator would be incidental or dependent upon its specific properties in producing symptoms - some directly, some indirectly, some by potentiating or releasing other agents. Inflammation has acute and chronic phases which often occur together. There are many diseases involving these responses, most of which resolve following a period of inflammation and repair. Some disease states exist, however in which unresolved chronic inflammation is associated with major destruction of normal adjacent tissues.

One such disease is rheumatoid arthritis[25]. This disease is a chronic inflammatory disease, in which nonsuppurative inflammation of the joints is frequently combined with a variety of extra-articular manifestations. The disease processes within the joint begins as an inflammation of the synovium in which there is oedema, vascular congestion, fibrin exudation, and cellular infiltration. The course of the disease varies greatly from patient to patient and is characterised by a striking tendency toward periodic remission and exacerbation. In many instances the arthritis

is of a mild degree and clears completely or remains confined to a few joints, causing little or no impairment in function. Commonly however, there is a tendency toward relapse or continued inflammation, leading to a thickening of the synovium and permanent joint damage.

The degree of articular disability which occurs in this disease depends largely on the amount of damage to the cartilage. If severe, large areas of cartilage and bone may be destroyed. Eventually, the formation of fibrous connective tissue ultimately interferes with joint function and may cause ankylosis.

When confronted by such diseases of this destructive nature, questions arise as to whether these inflammatory responses are either normal, excessive, insufficient or aberrant. Successful treatment of this type of disease with drugs or metal complexes therefore may involve inhibition of the unwarranted destructive processes without compromising the inflammatory component, perhaps by down-regulating an excessive, or stimulating an insufficient, inflammatory response. A number of aberrant inflammatory responses result from incomplete or inappropriate cell function. An example of this, Chronic granulomatus disease (CGD) in which phagocytes appear to be unable to generate $^{\bullet}O_2^-$ radicals to effect proper antibacterial activity[26].

One of the main cells involved in the inflammatory process is the macrophage. The cell has been characterised as a cell dedicated to the ingestion of debris and foreign material, and the role of macrophage activation in chronic inflammation has been extensively reviewed[27]. The monocyte is now acknowledged to be a multi-potential cell capable of a vast number of functions depending on its distribution within the body and has been shown to have inflammatory modulating activity.

2. Macrophages, lymphocytes and inflammation

The macrophage was first described by Metchinkoff (1892)[28] when he observed the endocytosis of very fine particles, now termed pinocytosis. He pointed out that the phagocytic cells of the body fall into two groups according to size, the larger of which are known as macrophages. Expansion of knowledge in this field has been rapid and has been adequately summarised in several reviews.[29-32]

The term activated macrophage was introduced into the literature and employed by Mackaness in the 1960's to describe enhanced microbicidal activity of macrophages. A number of functional aspects of macrophages are either increased or changed when the cell becomes activated including, increased phagocytic activity, increased expression of Fc receptors, expression of Ia antigens, increased microbicidal activity and an increase in superoxide radical production[30]. Activated macrophages produce, store and release several proteinases and appear to be well equipped for the

degradation of the structural elements of connective tissues, such as proteoglycan, collagen and elastin.

In the diseased synovium of rheumatoid arthritis, local antibody synthesis and immune complex formation occur, and in this situation the phagocytic cells of the lining layer and mononuclear phagocytes in the tissues may be important sources of hydrolytic enzymes[33]. It has been shown in vitro that macrophages secrete lysosomal enzymes when exposed to preformed immune complexes[34].

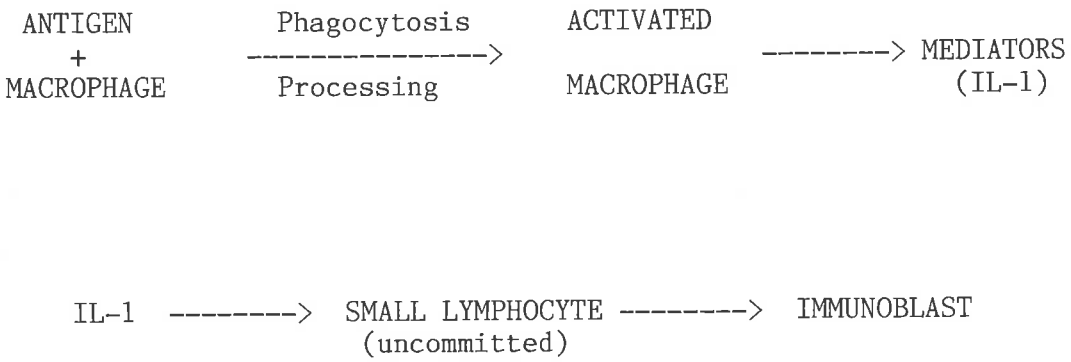
As phagocytic cells, macrophages play an important part in immune-complex clearance (involving binding to surface receptors for immunoglobulin and complement), and defects in this mechanism could be an important factor in the evolution of circulating immune complex disease.

Macrophages are intimately involved in interactions of T and B cells with antigens, and appear to have an important role in the production of some antibodies. It has been suggested that they may process antigen and present it in a highly immunogenic form to B lymphocytes[34]; that they may co-operate with T cells in generating a chemical which activates complement, thus providing an important signal for B cell activation[35]; that T cell activation by antigen releases a factor which attaches itself to the surface of macrophages, and regulates B cell function[36]; or, as suggested by Ada and Parish[37], that they are involved in immune stimulation or

tolerance depending on the concentration of antigen on the surface of the macrophages and their proximity to B cells in lymphoid organs.

Both normal and aberrant immune responses represent the end result of a complex series of cellular events in which specialised lymphocytes are stimulated to respond immunologically to an antigen[38,39]. The sequence of cellular events in many immune responses is depicted in FIGURE 1. It would appear that a preparative step may be required in which certain antigens are initially phagocytosed and processed by macrophages. Following this reaction, the macrophage produce mediators (eg Interleukins) which serves to stimulate a receptive population of uncommitted lymphocytes. Subsequently stimulated cells undergo morphologic transformation into large immunoblasts and ultimately mature into immune effector cells[40].

FIGURE 1



3. Macrophages and metals

The role of metal ions in the function or dysfunction of macrophages has recently received much attention especially with the pivotal role of macrophages in both non-immune and immune components of the inflammatory response. A large proportion of this work relates to the effect of environmental contaminants upon macrophage function (eg. Lead, Vanadium, mineral dusts) using in vitro studies. Most of these studies survey the cellular toxicity of metals such as cadmium, nickel, chromium and vanadium[41-44] which are lethal to cultured macrophages. In other studies I have considered the effects of metals such as cobalt and chromium and their effects on cultured macrophages in relation to wear particles from failed Co-Cr joint prostheses, and have found that these metals had pronounced effects upon macrophage functions[45]. However, one of the important factors when dealing with metal ion effects on cell populations especially in vitro is their inherent toxicity when in an uncomplexed form. In vivo effects may therefore bear little if any similarity to effects seen in vitro. Other metals such as zinc, copper and gold have been shown to affect macrophage function and some of these will be discussed further in later chapters.

D. Bio-metal Therapies

1. Bio-metal pharmacology

Consideration of metal ion therapies and inorganic pharmacology of inflammatory conditions have recently been published[18,46-47]. The chemistry of inflammation includes metal (Cu, Fe, Zn, Mo) - regulated oxy radical formation by reactive leucocytes[48], tissue redistribution of trace metals[49-51], and enhanced synthesis of metal binding proteins (metallothionein[52], caeruloplasmin[53], haptoglobin[54] and ferritin[55]) which serve as transport or detoxicant systems for both endogenous and exogenous metals. It is difficult to assign one particular functional role to these metalloproteins as most appear to have a multifunctional role. For example, caeruloplasmin may transfer copper to extra-hepatic tissues and also act as an antoxidant by oxidising catalytic/pro-oxidant Fe(II) to Fe(III), or promote other oxidation reactions involving hydrogen peroxide. Haptoglobin (Hp) inactivates haemoglobin (Hb) iron by forming an Hb-Hp complex, which is endowed however with intrinsic peroxidative activity. Metallothioneins are ubiquitous intracellular proteins which detoxify heavy metals such as cadmium, mercury and gold and can sequester essential metals such as zinc and copper[52]. Metallothioneins undoubtly determine the success or otherwise of a potential metal therapy, by preserving the liver, kidney and intestine from nonspecific intoxication by those metal ions which bind to thiols. One consequence is that certain organs at risk, such as the kidney, may resist intoxication by metal drugs

(e.g. those based on Au(I) or Pt(II)) more effectively when the host animal is inflamed[56].

2. Metals in medicine

The use of metal ions for medicinal purposes has attracted attention ever since one of the first drug compendia known to the western world (The Ebers papyrus). This papyrus refers to the use of metallic copper and copper salts to treat occular inflammation[57]. The subsequent development of metal-containing drugs has been particularly chequered due to a variety of reasons such as (i) the instability of metal complex solutions; (ii) limited solubility of metal complexes; (iii) uncertain composition of metal complexes; (iv) low therapeutic ratio with many metal complexes; (v) non-absorption from the gastrointestinal tract by some metal complexes; and, (vi) local irritation by metal complexes.

The introduction of metal-complexing agents (ligands) into medicine has been more readily accepted. This is probably because their mode of action as metal-ligands was more comprehensible even though, at the time when many of these bioactive compounds were introduced as drugs, their interaction with metal ions was poorly understood. Metal complexing drugs that may complex metals (drug ligands) include representatives of (i) conventional anti-inflammatory drugs (e.g. salicylate) and (ii) slow acting drugs that offer a second line of therapy for treating rheumatoid arthritis (e.g. D-penicillamine). Any detailed description of the in vivo

effects of such drugs should include their potential role as pro-drugs, with the possibility of their transformation to more active agents in the gut, or after absorption, by forming complexes with endogenous metal ions, particularly copper, zinc and iron.

With the involvement of metal ions in the functioning of enzymes and regulation of ~~activity~~ cell activity, manipulation of biologically active metal ions appears to affect the inflammatory response. This has been shown to occur with deprivation and repletion studies of the two bio-metals copper and zinc[58-60]. In addition, a large number of copper complexes have been shown to be anti-inflammatory in some animal models[61]. Zinc has been shown to possess antiarthritic activity against rheumatoid arthritis[62], and gold has been used for many years to successfully treat severe disease[63].

The stability of any given metal complex, whether administered as such or formed *in vivo*, must be considered against a background of competing endogenous ligands which usually transform a reactive complex into a less reactive one. Saturation of certain endogenous ligands with exogenous metal ions induces the symptoms of intoxication. Tolerable intoxication may indeed be the goal in introducing (i) metal complexes as anti-cancer drugs[64] and antiviral agents[65] (ii) or metal ligands with biometals *in vivo* to generate reactive chemotherapeutic agents *in situ*. One day such agents may be used to selectively intoxicate inflammatory cells and aberrant tissue

growth in inflamed tissue (e.g. pannus formation at the expense of normal articular cartilage). Likely candidates must include agents where the metal ion is the prime intoxicant. Paradoxically, an equally promising strategy to regulate chronic inflammation and attendant tissue damage includes the detoxicant effect of certain metal-regulating agents (e.g. caeruloplasmin, desferrioxamine) for regulating iron catalysed lipid oxidation and/or production of destructive free radicals.

This thesis is concerned therefore with the application and study of metal-containing drugs based on gold and copper to treat arthritis and inflammatory conditions.

II - REVIEW OF GOLD COMPLEXES

A. INTRODUCTION

No treatment for rheumatoid arthritis is curative, although a few drugs exist which induce an apparent "remission". Of these, gold(I) compounds have been used the most extensively during treatment by "chrysotherapy"[24,63,66-70]. Despite extensive clinical and laboratory research, there is still no clear unified concept to account for the mode of action of any of the gold drugs, and it is probable that their actions are multifactorial.

The following question must be asked: how is it that this rare element, so prized because of its non-reactivity as a metal, is able to confer such obvious therapeutic benefit and biological reactivity as a metal ion? There is certainly no evidence for any natural function for gold in living organisms, so it must be considered a xenobiotic metal ion. The scope of this review is restricted to some of the more interesting aspects of chrysotherapy as the vast amount of literature on gold therapy has already been surveyed with by a number of authors[70-73].

B. CHEMISTRY

Gold is a Group 1B element that may exist in a number of oxidation states (e.g. -I,0,I,II,III,V)[74-80]. The oxidation states -I,II, and V are neither important nor stable in biological systems.

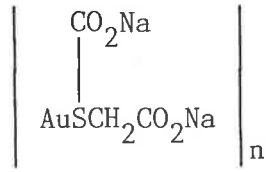
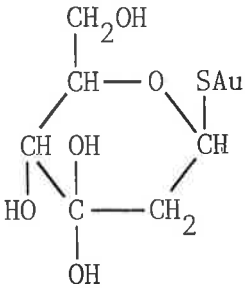
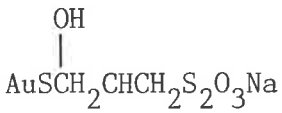
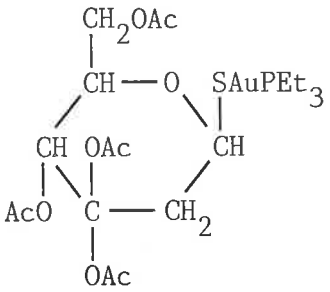
In vitro, the principal oxidation states appear to be 0,I and III. Therapeutically, it may be presented as stabilised gold(I) complexes (e.g. gold sodium thiomalate (GST) and Auranofin (AF)),or as a colloid (i.e. gold(0))[81]. Colloidal gold has had limited success as a clinical agent.

C.Gold(I) Complexes

The aqueous chemistry of Au(I), like that of Au(III), is mainly that of complex anions. Gold(I) halides are unstable and readily dissociate to gold(0) and gold(III), so gold(I) must be stabilised by complexation with "soft" or "class(b)" ligands[46] (e.g. thiolates, thioethers and phosphine[82-84]. Such stable complexes include a number of therapeutically active gold(I) drugs.

The chemical formulations of the more commonly used water and lipid soluble drugs, most of which have a high ratio of therapeutic effectiveness to toxicity[71], are shown in TABLE 3. The frequent depiction of these complexes as monomeric coordinated structures is over-simplistic, conveying only some of their chemical characteristics. These monomeric formulations (with the exception of A.F.) are rather implausible, since physico-chemical studies indicate that a number of therapeutic gold complexes really exist as oligomers containing bridging sulphur and gold atoms[21].

TABLE 3 Biologically active gold compounds

Generic name	Trade name	%Au	Formula
Gold sodium thiomalate (GST) disodium aurothiomalate	Myochrysin Myocrisin Tauredon	50.5	
Gold thioglucose Aurothioglucose	Solganol	50.3	
Gold sodium thiosulphate	Sanochrysine Sanocrisin Aurothion Crisalbine Thiochrysine	40.2	$Na_3Au(S_2O_3)_2$
Gold sodium 3-thio-2pro panol-1-sulphonate, sodium 3-aurothio-2-hydroxyproane sulphonate	Allochrysine	52.9	
S-Triethylphosphine gold 2,3,4,6-tetra-O-acyl-1 thio-B-D-glycopyranoside	Auranofin (AF) (SK&F D-39164) Ridaura	29.1	
Chloro(triethyphosphine) gold	SK&F 36914	56.2	Et_3PAuCl

Some of the salient physical properties of these gold complexes are listed below:-

(i) - Au(I) forms both polymeric and discrete thiolate complexes in vitro. Polymer formation may well influence therapeutic activity (e.g. it has been reported that commercial formulations of GST may contain both active and inactive components of the drug[85]).

(ii) - The relative proportions of monomeric and polymeric forms in solution may be altered by chemical and physical manipulations (e.g.sterilisation, aging and preservatives) and this may affect both efficacy and potential toxicity.

(iii) - The different solubilities of various commercial gold(I) formulations in both water and lipid phases determines not only their biological reactivities but also their different modes of administration.

The reactivity of gold gives rise to a biochemistry that is potentially very complicated. However, one dominant feature of gold(I) chemistry is the pivotal role of thiol (mercaptan,sulphydryl) groups. Gold is one of several heavy metals known to react avidly with these groups to form mercaptides. Shaw has proposed a model for this in vitro reactivity of gold(I) complexes, using GST as an example, whereby it reacts with a protein (RSH) to form mixed thiolate complex [21].

$$RSH + AuTM \longrightarrow RSAuTM + H^+$$

Displacement of the thiomalate from gold occurs with the involvement of normal amounts of endogenous thiols:

$$RSAuTM + R'SH \longrightarrow RSAuSR' + TMSH$$

Essentially, gold(I) is transformed from one thioligand (thiomalate) to another (RS), liberating free thiomalate (TMSH). Because there are many biological thiols (e.g. cysteine, glutathione, metallothioneins), gold(I) has a large number of potential binding sites in vitro through which it might act. This is reflected in the multiplicity of effects attributed to gold therapy[21,67,71-73].

Gold may potentially affect proteins and enzymatic reactions by binding to (a) functional thiol groups at the active site; (b) a non-functional thiol group within the active site; (c) a thiol near an active site but close enough to modify reactions sterically; (d) remote thiol groups but modifying the overall enzyme structure; or (e) a thiol group on the substrate.

D. Gold (III) Complexes

Gold(III) is invariably complexed in aqueous solutions as an anionic species (e.g. $(Au(III)Cl_3OH)^-$). Ligand exchange reactions for the formation of gold(III) complexes occur much more slowly than those with gold(I) complexes. Weak complexes of Au(III) (e.g. $(AuCl_4)$ are quite powerful oxidants and have been used as protein

precipitants. Pharmacological and toxicological data indicate that gold(III) is more toxic than the gold(I) thiolates that are used clinically. There are probably a number of inter-related reasons for this, including its greater oxidant potential, wider inhibition of enzymes and greater retention in tissues. For these reasons, its therapeutic value remains doubtful.

E. Pharmacokinetics and Distribution

Gold is normally present in the body in trace amounts as an environmental contaminant. It is "recognised" and metabolised by the body as a potentially toxic metal ion along with other thiophilic metals (e.g.Pt(II) and Cd).

The circulating levels of gold rise slowly during the initial phase of gold therapy, with plateau levels being attained after six to eight weeks of continuous therapy[86,87]. This slow rise in serum gold levels may be coincident with therapeutic responses. The serum gold concentrations correlate directly with the administered dose but not with clinical evidence of disease modulation. Following intramuscular administration of 50mg GST, serum concentrations of 20-50uM can be attained over the next one to two days, with levels of 10-15 uM being maintained during uninterrupted therapy[88,89]. Serum levels in patients treated with AF are much lower, reaching only 4-6 uM.

In serum, gold is primarily protein-bound (>90%), particularly to the thiol group of albumin (80-90%)[90-93]. If administered in greater doses, there are increases in non-albumin bound gold (e.g. immunoglobulins, complement and lymphocytes). Some recent studies have indicated that, in patients with rheumatoid arthritis undergoing chrysotherapy, there may be a significant uptake of gold by erythrocytes (RBC),[94-97] which could precede the development of toxic reactions[97]. This has not been substantiated in another study[98]. RBC from patients treated with AF bind significantly more gold (up to 40% of the blood level) than those treated with GST[99,100]. The implications of this increased RBC binding are not known.

Tissue binding of gold will depend on levels of metallothionein and other thiol proteins, some of which can be induced locally or systemically by a number of endogenous mediators, including heavy metals and inflammatory products. The distribution of gold into superficial and deep compartments has been proposed[101]. In this model, gold is transported from a superficial compartment (e.g.blood) to the deep compartments (e.g.kidneys, liver and spleen), from where it is slowly released. The delay in the therapeutic effects of chrysotherapy may be due to preferential storage of gold(I) in the deep compartments, which would effectively withdraw it from the therapeutic sites until the deeper compartments become saturated.

Gold(I) is widely dispersed throughout the body following its interaction with natural thiols and other ligands, although preferentially concentrated in the tissues that constitute the reticuloendothelial system (liver, spleen and lymph nodes)[102,103]. The water-soluble complexes such as GST and the hydrophobic complexes such as AF, differ in their bio-distribution and pattern of elimination[104]. This may well reflect the differences in both the solubility and targeting abilities of the two drugs in the biological milieu. Of particular interest, is the high concentration of gold found within joints, particularly in the synovium, the cartilage, cortical bone and muscle[105]. The synovial accumulation of gold may underlie its therapeutic action, and levels as high as 30uM have been detected in synovial fluid after GST administration[105]. Greater concentrations of gold are found in inflamed joints[103] indicating possible sequestration by inflammatory cells.

Serum thiol levels are reduced in patients with infections or inflammatory diseases (including rheumatoid arthritis)[106]. This may affect the gold-retaining capacity of serum, and thus the overall distribution of this metal. By contrast, levels of thiols in RBC of patients with arthritis are increased. Therefore, an increase in the binding of hydrophobic gold drugs (e.g. AF) may be possible, which could explain why AF is therapeutically active at lower levels than GST[107,108].

F. Roles of gold or ligand as the pharmaco-active species.

The biological fate of gold drugs is still not fully understood. There is an increasing amount of evidence that gold complexes dissociate soon after entry into the body[108,109]. Gold may then be complexed with endogenous ligands (e.g.albumin), with a major proportion being either detoxified, stored, and/or bound to proteins, with a very minor proportion available for therapeutic activity. It has been suggested that the liberated exogenous thioligand (thiomalate) may be partly responsible for the efficacy of gold complexes. Molecules containing reactive thiol groups (e.g. penicillamine) have several biological effects[110]. Although sodium thiomalate, thioglucose and D-penicillamine are inactive in most anti-arthritic assays in laboratory animals, this may only reflect the limitation of our assay systems. Further evidence is still needed to determine whether thiolates such as thiomalate, thiosulphate or thioglucose can mediate some of the anti-arthritic activity of their respective Au(I) complexes.

Paradoxically, the stabilisation of these thioligands by complexation with metal ions, the metal serving as a delivery system for a reactive thiol, may be of value. The idea of Au(I) formulations being "prodrugs" may then be highly relevant when discussing the pharmacology of GST.

G. Modes of Action

Gold(I) complexes possess some unique chemical and physical properties. Their major sites of biological action include (i)enzymes, (ii) other proteins, (iii) cells and (iv) interactions with other metals. That gold drugs probably inhibit inflammation by several distinct mechanisms, is now increasingly recognised.

1.Proteins

There has been a substantial amount of work investigating the effects of gold(I) on proteins, in particular, the inhibition of enzymes and their subsequent release[21,71]. Gold(I) was shown to inhibit lysosomal and non-lysosomal enzymes in a majority of cases, depending on the amount of drug used and the origin of the enzyme (TABLE 4). This is particularly interesting because of the key role of hydrolytic enzymes that are released from polymorphonuclear leucocytes and macrophages during inflammation. Neutral extracellular proteases degrade collagen and proteoglycans, the two major components of articular cartilage. Other acid hydrolases, while playing an important role in intracellular digestion, may have a limited activity extracellularly. Although most studies to date have centred on the acid hydrolases, only a few have examined the effects of gold(I) on neutral proteases[111-113]. While in vitro studies of the effects of gold on functional enzymes do give some insight into how gold(I) might inhibit inflammatory processes, they fail to explain the delayed onset of therapeutic benefit that is repeatedly seen with chrysotherapy.

TABLE 4 Effect of some gold(I) drugs on hydrolytic enzymes

Enzyme	Gold complex (concentration mM)	Effect on Enzymatic Activity
B-N-acetylglucosaminidase	GST(2-5)	- (27-88%)
	ATG(2)	0
	SKF 36914 (5)	- (94%)
	AF (1)	0
Acid phosphatase	GST(0.1-5)	- (40-64%)
Cathepsins	GST (0.001)	- (0-38%)
	ATS (1)	- (30%)
	SKF 36914 (0.002)	- (25%)
	AF (0.05)	- (25%)
Collagenase	ATS (5)	- (30%)
Elastase	GST (0.02-2.5)	- (40-63%)
B-Glucuronidase	GST (1-10)	- (0-40%)
	ATG (1-2)	0
	AF (1)	0

One important factor affecting the degradative activities in the synovial fluid or tissues of inflamed joints, is the co-existence of enzyme inhibitors. Perhaps the relevant areas for investigation of enzyme activity in the joint therefore lie in the status of the enzymic microenvironment, both surrounding and inside the inflammatory cells. It should also be pointed out, that in vitro assays may not reflect the in vivo situation, where other competitive ligands exist. Gold concentrations used to show enzyme inhibition are often much higher than that found in serum and in the synovial tissue of patients undergoing chrysotherapy. In addition, the presentation of gold in vitro may well be different to that in vivo. For these reasons, the relevance of in vitro results to the in vivo situation remains doubtful.

2.Collagen

A number of papers have described interactions of gold with collagen[114-118]. These have shown abnormalities in banding of collagen (after at least eight weeks of therapy)[114] an effect which has been assumed to be secondary to gold deposition. Others have shown that gold reverses the decrease in cross-linking induced by lathyrogenic chemicals (e.g.B-aminopropionyl nitrile). Gold also may reverse the decreased intramolecular binding induced by copper deficiency[115]. It was suggested that extensive cross-linking of collagen molecules would make the collagen less vulnerable to enzyme digestion, so reducing the likelihood of forming auto-antigens[115]. The relevance of these effects of gold on collagen to the efficacy of

gold in patients with rheumatoid arthritis remains conjectural. However, the delayed onset of this collagen binding does correlate reasonably well with the delayed onset of clinical responses, and may explain the delayed therapeutic activity of gold(I) complexes.

3.Cellular function

Mononuclear cells (macrophages and lymphocytes) play important roles in chronic inflammation. Both types of cells are present in the inflammatory lesions of patients with rheumatoid arthritis. The idea that gold can mediate its in vivo antiarthritic effects by altering the functional activity of these cells was initiated by the finding of localised gold in "aurosomes" of synovial tissue macrophages following chrysotherapy.

The effects of gold on cells involved in inflammation have been studied extensively both in non-immunological (TABLE 5) and immunological assays. The results obtained from these in vitro studies may not be representative of the situation in vivo, for reasons outlined in discussing the enzyme studies. Many in vitro immunological studies demonstrate inhibitory effects of gold. However, in vivo the finding has been ambivalent, with both enhancement and suppression of immunological responses being observed[71].

TABLE 5 Effects of gold(I) drugs on non-immune cell functions

Cell migration	Gold reduces the numbers of neutrophils and macrophages migrating into subcutaneous cotton pellets or polyurethane implants.[119,120]
Vascular permeability	Gold reduces fluid exudate in subcutaneous cotton pellets but not in polyurethane sponges.[120,121]
Phagocytosis	Decreased in neutrophils and macrophages isolated from gold treated patients.[122]
Prostaglandin	Gold inhibits *in vitro* synthesis of PGE_1 and PGE_2.[71]
Enzyme release	Gold inhibits release of enzymes from cells possibly by stabilisation of lysosomal membranes.[71]

These findings have led to the use of the terms "immunomodulatory" and "immunoregulatory" to describe some of the actions of gold. Perhaps more relevant are the studies directed towards the in vivo changes of cell functions following chrysotherapy using for example, methods such as a skin window technique to investigate macrophage function in gold-treated patients[122]. With this technique, it was shown that phagocytosis by macrophages from patients with rheumatoid arthritis is increased compared to controls, and that gold treatment reduces it to control (non-rheumatoid) levels. This highlights the need for similar in vivo studies on the effect of gold in different states of inflammation.

4.Animal models

Since the major manifestations of rheumatoid arthritis are related to chronic inflammation, it would be hoped that animal models used to assess gold activity in vivo would relate to this type of inflammation. Drugs such as GST and D-penicillamine can take six weeks to three months to produce measurable improvements in patients with rheumatoid arthritis[70,110]. Consequently the most acceptable animal model should be one in which a response to chronic inflammation rather than acute inflammation is assessed. For this reason the adjuvant-induced polyarthritis in rats is generally regarded as the most acceptable model since it may be measured over a period of at least three weeks. Because of this, there has been a large amount of work investigating the effect of gold on this model disease in rats. However, as with the in vivo immunological studies, the results appear to be ambivalent.

TABLE 6 Previous investigations on the effects of gold drugs on adjuvant induced polyarthritis in rats.

YEAR	RAT[a] STRAIN	ADJUVANT[b] LIPID	GOLD DRUG	DOSE (mg/kg)	TIME OF DOSING	EFFECT[c]	REF
1963	WIS	MIN	GST	12.5	-1-+14	0	(123)
1968	SD	MIN	GST	5-25	-12-+18	0/-	(124)
1971	LEW[d]	PAR	GST	5-40	-7-+16	+	(125)
					0-+16	+	
1973	SD	MIN	GST	2.5-10	0-+21	+	(126)
					21-+42	+	
1975	SD	MIN	GST	5.0	-5-+15	+	(127)
1976	LEW[d]	PAR	AF	5-20	0-+16	+	(128)
1978	WIS	PAR	GST	5.0	0-+20	0	(129)
1979	LEW[d]	PAR	GST	5.0	0-+20	+	(130)
			SKF36914	5.0	0-+20	+	
			AF	5.0	0-+20	+	
1980	WIS	PAR	GST	5-10	0-+21	0	(130)
			SKF36914	5-10	0-+21	0	
			AF	5-10	0-+21	0	
1980	LEW[d]	PAR	GST	5-10	0-+21	+	(131)
			SKF36914	5-10	0-+21	+	
			AF	5-10	0-+21	+	
1984	SD	MIN	GST	0.7-35	-90-+21	(-)	(132)

a SD - Sprague Dawley rats, WIS - Unspecified Wistar, LEW - Lewis (USA).

b TBC/in lipid; MIN - Mineral oil, PAR - Paraffin oil

c 0 = NO EFFECT, + = SUPPRESSION, (-) = EXACERBATION

d Clearly distinct from the Lewis rats available in Australia (see ref 134)

The conflicting results reported certainly have clouded and even possibly hindered the analysis of how gold sodium thiomalate (GST) and other gold drugs might act in either rats or man.[123-133] (TABLE 6)

GST had no effect on the type 11-collagen arthritis in Wistar rats[135]. These negative results may only reflect the nature of the rat strain used, as Wistar rats do not appear to provide a gold-sensitive model using bacterial adjuvants either[123,129,131]. This emphasises one of the inherent problems in using such animal models of arthritis.

5.Interactions with other metal ions.

Gold(I) injected as GST into experimental animals significantly alters the uptake of zinc and copper into the cytosol proteins of liver and kidney[136]. Gold may displace certain essential trace elements from their natural binding sites, and this may contribute to the beneficial effects seen in some patients. Attention is now being paid to the possible role of metallothioneins as the sites of zinc and copper interaction with gold[136,137].

H.Toxicity

The major factor limiting the role of chrysotherapy for the management of rheumatoid arthritis is the significant incidence of side effects[138]. These include marrow aplasia, potentially the most serious side effect, with a poor prognosis. Gold nephropathy and thrombocytopenia, although more common, usually have good prognoses

as both can be satisfactorily treated. Mucocutaneous reactions including pruritis, lichen planus, and dermatitis have been reported. Although they are common, they are rarely of serious concern. Encouragingly, a recent evaluation of gold toxicity has indicated that the incidence of these latter reactions has decreased even though the use of gold has actually increased[139]. This may reflect the increasing awareness of such side effects and recognition that by lowering doses, it may be possible to reduce the untoward effects without diminishing effectiveness. Nowadays, the availability of purer preparations of gold complexes may also contribute to reducing the frequency and magnitude of these side effects.

The toxicity of gold complexes in experimental systems has been reviewed by Payne[140-145]. The long term toxic effects of Auranofin are still unclear, but there are recent reports indicating gastrointestinal complications[146]. An interesting prospect is the possibility of predicting impending toxic reactions to gold. Unfortunately, the individual side effects to chrysotherapy do not appear to correlate with whole blood or plasma gold levels[147-153]. Although at least one study has shown that patients with toxic reactions had significantly higher gold concentrations in RBC, suggesting that this may be a method of predicting impending toxic reactions[97], a later study was unable to confirm these results[98]. Furthermore, as significantly higher levels of gold has been found in RBC of smokers compared to non-smokers, RBC gold levels may be of only limited use.

A number of detoxifying agents such as 2,3 dimercaptopropanol (BAL), D-penicillamine[154] or N-acetylcysteine[155] have been reported to be useful in mobilising and facilitating the excretion of gold from patients exhibiting gold toxicity. However, the effectiveness of D-penicillamine in this regard has recently been questioned[156]. An alternative method of reducing toxic side effects may well be to stimulate the body's own detoxifying mechanisms, such as inducing further glutathione or metallothionein synthesis.

I. Clinical Practice and Prospects for the Future

Gold complexes have been used largely for the treatment of classical seropositive rheumatoid arthritis. However, these drugs have also been used to treat other diseases. Successful treatment with gold has been reported for juvenile rheumatoid arthritis, palindromic rheumatism and intermittent hydrarthrosis[157]. It is less effective in psoriatic arthritis[158]. Fifty years ago, reports indicated that chrysotherapy had a beneficial effect in asthma. However, only the Japanese seen to be currently pursuing the use of gold to treat this disease[159].

With the discovery that platinum complexes, especially cisplatin, are effective antitumor agents, it has been suggested that gold(I) complexes may have some potential use as anti-neoplastic agents. Research in this area has indicated that gold triphenylphosphines may demonstrate both pro- and anti-tumor activity[160].

The wide range of actions of gold on proteins and cells involved in inflammatory processes leads to systemic effects which include both therapeutic benefit and, unfortunately, toxicity. Further research should be directed towards obtaining preparations with reduced toxicity and/or that simulate systemic detoxifying mechanisms. Perhaps "gold-mimics" may be discovered, with similar anti-inflammatory properties to gold complexes, but without the untoward toxic side effects. Until that time, gold complexes will remain as important "curative" drugs for inflammatory arthropathies.

III - REVIEW OF COPPER AND INFLAMMATION

A. HISTORICAL

It is easy to trace the history of well-known and widely used analgesic anti-inflammatory agents such as aspirin[161], ibuprofen[162] or piroxicam[163] because they were designed to perform certain tasks, most notably reducing fever in man (aspirin) or experimental inflammation in guinea pigs (ibuprofen) or rats (piroxicam). By contrast, the advent of copper in medicine is almost lost in history and to this day we cannot be sure what it does to control inflammation.

Allusions to treating inflammatory disorders with copper and its salts can be traced back to the Ebers Papyrus written in Upper Egypt about 1500 B.C.[57]. The copper bracelet has long been used as a folk remedy for arthritis. Its scientific scrutiny[164] led to the observations that copper(II) applied to the skin with a suitable carrier (cupriphore) is indeed systemically distributed[165] and therapeutically active in controlling experimental inflammation[166]. Copper drugs were used as alternatives to the well-known gold-thiolates for treating tuberculosis and arthritic disorders during the 1930's particularly in France and Germany. These clinical reports received little credibility until they were reviewed by Sorenson and Hangarter[167] largely in the light of experimental studies in animals with copper salts in the late 1960's and early 1970's.

It is worth noting the accidental nature of some of these "modern" studies. Sorenson has related[168] how copper acetate came to be tested in rats in 1966 at the Searle Drug Co. following the "lead" that anti-inflammatory activity of a serum fraction was associated with the natural blue copper-protein, caeruloplasmin. Meanwhile Bonta at the Organon Laboratories in Holland had found that basic copper carbonate (Malachite) inhibited inflammation in guinea pigs when given orally and in rats when given subcutaneously[169]. These activities were attributed to the gastro-irritability (guinea pig) and local irritancy (rat) of the almost insoluble malachite, releasing Cu(II) and triggering natural anti-inflammatory responses.

Thus we see that copper is both a very ancient remedy and a relatively modern drug. It is also both pro- and anti- inflammatory and presents other paradoxes in bio-systems, to which I shall refer later.

B. Brief overview of inflammation

Inflammation is the normal healthy response of a vascularised tissue to injury (physical, chemical, microbial invasion etc.). Its function is to limit the extent of injury, prepare the body for immunological defence and trigger wound healing. It becomes a clinical problem only when the original inflammatory stimulus is not terminated or the inflammatory response is inappropriate, either too little or too much, due to malregulation.

The clinical signs of inflammation have long been recognised as heat and erythema from increased blood flow within local vessels, as swelling from increased vascular permeability and/or cellular infiltration and as pain caused by locally released inflammatory and algesic mediators resulting in an associated loss of function. The magnitude of these events is influenced by the nutritional and hormonal status of the individual as well as by genetic factors.

A number of inflammatory mediators are listed in TABLE 7. Copper complexes can affect (i) the formation of several of those mediators eg. kinins, PGE_2, (ii) accelerate their destruction eg. superoxide or (iii) minimise the tissue responses to other mediators once formed eg. histamine, serotonin. Furthermore they can moderate the reactivity of many different types of cells participating in the inflammatory/repair response including platelets, granulocytes, basophils, mast cells, monocytes and fibroblasts[170]. The problem is to separate those effects of copper complexes that are physiologically significant and attainable in vivo, without serious toxicity, from all other effects, measured or putative, that are likely to affect the course of inflammation with concurrent host toxicity.

TABLE 7 Representative mediators of inflammation.

Mediator	Source	Effects
Histamine	Mast cells,basophils	Increase vascular permeability, chemokinesis,mucus production, smooth muscle contraction.
Serotonin	Mast cells,platelets cells of the endoch-romaffin system	Increase vascular permeability, smooth muscle contraction
Bradykinin	Kininogen (by proteo-lytic cleavage)	Vasodilation,increase in vascular permeability,production of pain, smooth muscle contraction
C3a	C3 complement protein	Degranulation of mast cells, smooth muscle contraction
C5 fragments C5a	C5 complement protein	Degranulation of mast cells, chemotaxis of inflamatory cells, oxygen radical production, neutrophil secretion
PGE_2	Arachidonic acid (cyclo-oxygenase pathway)	Vasodilation,potentiate permeability effects of histamine and bradykinin, increase permeability when acting with chemotactic agent
LTB_4	Arachidonic acid (lipoxygenase pathway)	Chemotaxis of neutrophils, increase vascular permeability in the presence of PGE_2
LTD_4	Arachidonic acid (lipoxygenase pathway)	Smooth muscle contraction, increase vascular permeability
Platelet-activating factor	Basophils,neutrophils monocytes,macrophages	Release of mediators from platelets,neutrophil aggregation, neutrophil secretion, superoxide production by neutrophils, increase vascular permeability, smooth muscle contraction
Reactive oxygen species	Neutrophils macrophages	From lipid peroxides cross linked tyrosyl proteins Oxidase protein SH-> -S-S- Depolymerise polysaccharides Liberate histamine(mast cells)
Gamma-Interferon	T-lymphocytes	Activation of macrophages, modulation of immune reactions
Interleukin 1	Macrophages	Fever,fibroblast proliferation induction of collagenase and prostaglandin production lymphocyte proliferation.

Many forms of stress, including trauma and pregnancy, disease and most noxia, including inflammagens, mobilise copper from its body stores raising the level of circulating caeruloplasmin and dissociable copper (II) bound to serum albumin and amino acids. Paradoxically one sign of successful treatment of inflammation with exogenous copper complexes is reduction of serum copper levels that were formerly raised in inflammation. This is a little difficult to reconcile with the simplistic view that treating an inflammation with exogenous copper merely amplifies the, as yet unproven, anti-inflammatory role of the hypercupraemia, naturally elicited by inflammation etc. The pro-inflammatory character of metallic copper and Cu(II) must also be recognised.

C. Dietary copper status and experimental inflammation

Some of the most commonly used experimental models of inflammation include (i) acute irritation of shaved skin on guinea pigs with UV light causing an erythema, (ii) of rat paws with various irritants eg. soluble carrageenan or kaolin suspensions eliciting an exudate, (iii) in the pleural cavity of rats and guinea pigs with crystals or soluble inflammagens, and (iv) of synovial cavities of rabbits and dogs injected with irritants such as sodium urate crystals. Inflammation is then measured by exudate production, erythema (UV), local hyperthermia and/or cellular ingress into cavities or implanted sponges.

Models of chronic inflammation include granulomata development around or within implanted irritants eg plastic sponges or an auto-allergic polyarthritis in rats induced with immunological adjuvants.

1. Acute models of inflammation

Diet may be one of the major determinants of the effects of copper on the inflammatory process. Dietary insufficiency of copper is due to either (i) low or reduced availability or (ii) malabsorption of copper from the gastrointestinal tract. The effect of low copper diets on acute and chronic inflammatory processes has been the subject of several studies. Severe restriction of dietary copper intake in adult rats caused a significant enhancement of the acute inflammatory response to a number of irritants[59,171]. It appears that minute changes in copper levels of the diet, or subsequently in the tissues, can have significant effects on the development of the acute inflammatory reaction. Moreover the length of copper deprivation appears to play a crucial role in this effect possibly because of effects on tissue copper levels[171]. Subsequent work has shown that 0.6ppm of copper in the diet over a 2 month period was sufficient to enhance the inflammatory response in male rats although not in female rats[172,173]. Furthermore, copper deprivation at 0.6ppm for up to 5 months in female rats still had no effect on the inflammatory reaction.

Copper restriction in rats, induced by administering 0.4ppm of copper in the diet, for 1 and 3 months produced a measurable difference in the inflammatory response to carrageenan injection[58,174]. However, serum copper levels determined immediately before the irritant injection were very similar (14.4ug/100ml for the 1 month deprivation, 14.6ug/100ml for the 3 month deprivation and 180ug/100ml for the control group). These observations indicate that the pro-inflammatory effect of copper deficiency may be critically dependent upon the actual tissue levels of copper and that serum estimations of copper deficiencies following dietary copper deprivation may not necessarily predict the likely response of an animal to an inflammagen.

2. Models of chronic inflammation

Only as recently as 1978 was it reported that copper deficiency inhibited the development of experimental arthritis in young female rats[175], and this was subsequently confirmed[176]. This effect of copper deficiency was just the opposite to that observed with acute inflammation[58] and it was suggested that this may be due to lower immuno-competence of the young rat[175,177]. Kishore and his colleagues therefore designed experiments to determine if the reduction in inflammation previously observed in adjuvant-challenged copper deficient rats was indeed an epiphenomenon of the inactivity of the immune system. Their results indicated that copper deficient rats were actually in a state of apparent immuno suppression as demonstrated by their impaired responses to the T cell-dependent contact sensitizer, oxazolone. However, they could not confirm the

reduction in the adjuvant polyarthritis with copper deficiency[59]. These discrepancies may be due to differences between rat strains, or in arthritogenicity of the adjuvants used in different laboratories[134].

Rainsford[178] recently proposed that marginal copper deficiency in the population may be a contributing factor in the etiology of rheumatoid arthritis, possibly due to dietary and environmentally induced perturbations in the levels of certain trace elements which influence copper ion status. This hypothesis is supported by the observations that patients suffering from rheumatoid arthritis in the "non-acute" phase have serum copper levels which tend to be lower than normal[179]. Limited copper deficiency may occur in man, and may be a result[180] of and a contributing factor[181] to, rheumatoid disease.

Unfortunately most of the information we have on this topic is derived from laboratory animals, rather than man. Most pre-formulated animal diets tend to be low in some metals (Cu, Fe), or to contain added metal chelators to increase their storage life.

D. Changes in copper metabolism in inflammation

1. With acute inflammation

The acute inflammatory reaction causes among other events a remarkable increase in the total copper and caeruloplasmin concentration in the serum[182]. Total serum copper correlates with caeruloplasmin levels equally well in normal and inflamed animals[183]. The identity of non-caeruloplasmin-bound (NCB) copper, notably the presence of low molecular weight complexes, are not known. However a large fraction of the NCB copper present in inflamed sera is likely to be carried in the form of Cu(II) complexes with histidine and albumin formed after absorption of the ion (probably as Cu-aminoacid complexes)from the gastrointestinal tract[184]. Rather more is known of the movements of copper which occur in other tissue compartments during the acute inflammatory process. Total copper content of the liver did not change significantly during the acute and recovery phases of carrageenan pleurisy in the rat[185], indicating that in this form of acute inflammation serum copper can increase without depleting existing liver copper stores. Another report indicates that the copper content of the kidneys is not affected by the onset and remission of acute inflammation[186].

2. With chronic inflammation

In patients with rheumatoid arthritis there is nearly always a significant increase in total serum copper during the active phase of the disease[187-189] although there were some contradictory earlier reports[190-193]. Accompanying these elevated serum copper levels are

increases in plasma caeruloplasmin[187,189]. Both copper and caeruloplasmin have been shown to be present in appreciable quantities in the synovial fluid of rheumatoid arthritic patients[194,195]. In many other types of chronic inflammatory conditions, both in man and animals, there are also significant rises in serum copper and/or caeruloplasmin concentrations[182]. In rats with adjuvant arthritis, there are increases in total plasma copper levels and in caeruloplasmin activity of the blood[196]. These changes precede the appearance of the arthritic disease with secondary inflammation but probably reflect the inflammatory stress and primary inflammation at the site of the adjuvant injection.

One of the major drawbacks in determining the role of copper in the inflammatory process is that so little is yet known about the overall movement of copper, both as Cu(II) and as Cu(I) between different compartments of the body during the inflammatory process. Some attempts have been made recently to clarify whether copper stores are directly involved in the changes of copper metabolism in chronic inflammation. Karabelas found a dramatic increase of liver copper concentration in rats (180%) 21 days after an adjuvant injection[196]. Others[59] found a smaller increase in liver copper (30%), while another group reported over a 50% decrease in liver copper following injection of Freunds complete adjuvant into dogs[197].

E. ANTI-INFLAMMATORY PROPERTIES OF COPPER PROTEINS

There are a number of copper-containing enzymes that might play a role in the natural anti-inflammatory response. Some of these enzymes have been tested as copper delivering anti-inflammatory agents. Those with anti-inflammatory activity in the mouse paw oedema model, include ascorbate oxidase, lactase, diamine oxidase, as well as superoxide dismutase and caeruloplasmin[198]. All produce significant anti-inflammatory activity in this model compared to an albumin-copper complex, which was without effect.

1. Caeruloplasmin

The rise in serum caeruloplasmin levels, along with other glycoproteins and fibrinogen, the so-called "acute phase reactants", is a characteristic systemic response to trauma or inflammation. This may reflect a need to boost a delivery of copper to the peripheral tissues for local complexation in the forms that are pharmacologically active.

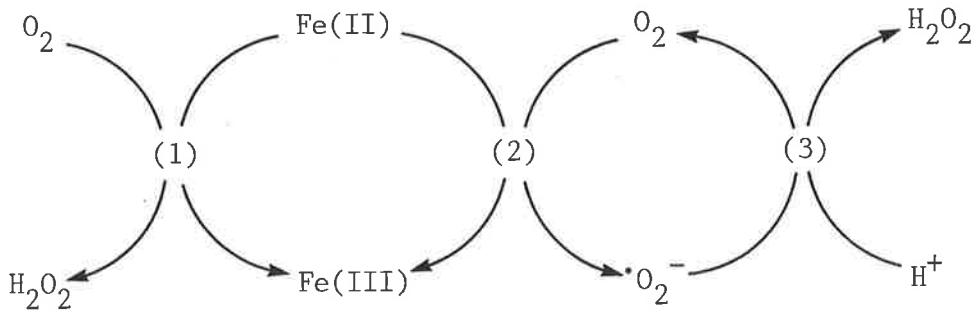
Indirect evidence that caeruloplasmin possesses anti-inflammatory activity was provided recently.[171,172]. It was found that acute inflammatory reactions provoked by injecting monosodium urate crystals into the footpad of rats could be reduced significantly if the crystals were injected simultaneously with caeruloplasmin. This was not seen when the crystals were injected simultaneously with albumin[172]. It was suggested that the putative anti-inflammatory activity of caeruloplasmin could explain the spontaneous remission of rheumatoid arthritis during pregnancy when

caeruloplasmin levels rise appreciably.

Although, in the mouse paw oedema assay, caeruloplasmin given (i.v.) had both a pro-inflammatory and an anti-inflammatory effect depending upon its source, the anti-inflammatory activity was potentiated by pretreating the protein with ascorbate[198].

Caeruloplasmin possesses significant oxidase activity directed towards a number of substrates, of which ferrous ions, phenols, and some aromatic amines such as epinephrine and hydroxyindoles are the most important physiologically[199,200]. It is quite possible that caeruloplasmin regulates the levels in the circulation of certain biologically active molecules. Caeruloplasmin is thought to represent the major ferrioxidase in plasma[201]. Studies have shown that intravenous administration of caeruloplasmin to copper-deficient rats results in increased serum iron levels[202]. It has been proposed that caeruloplasmin may act <u>in vivo</u> as a molecular link between copper and iron. Several investigators have noted that auto-oxidation of lipids could be inhibited <u>in vitro</u> by the $alpha_2$-globulin fraction of human plasma[203,204]. Caeruloplasmin has been shown to be a major antioxidant in plasma[205] and subsequent studies have confirmed that caeruloplasmin can inhibit autoxidation of lipids[206-208]. Here the caeruloplasmin, by catalysing and removing decompartmentalised Fe(II), prevents its spontaneous autoxidation with concomitant reductions of dioxygen to superoxide ($^{\bullet}O_2^-$) (see FIGURE 2). Caeruloplasmin was found to inhibit a number of superoxide mediated reactions[209].

FIGURE 2. Detoxification of unbound or decompartmentalised ferrous iron by caeruloplasmin.



Caeruloplasmin catalyses reaction 1. Reaction 2 = spontaneous autoxidation generating Superoxide ($\cdot O_2^-$) which, with H_2O_2, may then generate the highly reactive local toxin, the hydroxyl radical ($OH\cdot$). When reaction 3 is catalysed by superoxide dismutase, [$\cdot O_2^-$] is kept so low that $OH\cdot$ formation is minimal.

Inflammation and tissue injury may occur to some extent as a result of an imbalance between the actions of oxygen-derived free radicals (eg $\cdot O_2^-$) and endogenous free radical scavengers. Under conditions where levels of caeruloplasmin are markedly elevated, as during pregnancy and acute infections with inflammatory diseases such as rheumatoid arthritis, this copper protein may play a major role as a circulating antioxidant and scavenger of oxygen-derived free radicals thereby protecting the host from the potential ill-effects of local tissue injury. By virtue of its antioxidant and superoxide-scavenging activities, caeruloplasmin probably can be considered the most "beneficial" of the acute phase reactants.

2. Superoxide dismutase

The mammalian liver enzyme, (Zn-Cu) superoxide dismutase has been studied as an anti-inflammatory agent in both animal models of inflammation and in human subjects[210-213]. For example, it has been shown to decrease (i) carrageenan-induced pleurisy in rats e.g. reduce exudate volume and neutrophil counts[212], (ii) the rat polyarthritis following injection of Mycobact. tuberculosis (e.g. reduce paw swelling)[212] or injection of Mycobact. butyricum in the tail of rats[214], (iii) nystatin-induced paw oedema, but not a passive cutaneous anaphylaxis and Arthus reaction in rats[214] and (iv) fetlock swelling in horses when injected with a mixture of irritants[215].

In contrast, using the carrageenan rat paw oedema assay, it was shown that native superoxide dismutase does not exhibit anti-inflammatory activity unless it is cross-linked either with albumin or self-crosslinked to form much larger soluble protein conjugates which are not rapidly cleared from the circulation ($t_{1/2}$ > 5 hours)[216,217]. The native enzyme was cleared rapidly from rat plasma with a half life of less than 30 minutes. Recognising this, Huber has argued that because native superoxide dismutase was shown to be anti-inflammatory in some studies, its efficacy does not rely solely on maintenance of significant superoxide dismutase reactivity in plasma[212].

While several clinical studies of the treatment of rheumatoid arthritis with bovine liver superoxide dismutase (Orgotein^{R}) have been commenced[214], only a few of these were completed. Most progress reports have appeared only in abstract form.

For, example superoxide dismutase therapy (i.v.) in patients with rheumatoid arthritis compared favourably with gold but this compound was not identified in a double blind study[218]. Similarly rheumatoid arthritis patients maintained on corticosteroids and aspirin and further treated with superoxide dismutase, showed improvement in disease indices which extended for four weeks after cessation of the superoxide dismutase treatment[219].

In order to overcome the problem of the rapid clearance of superoxide dismutase from the circulation and to potentiate its local effect, several clinical studies have used intra-articular injections of superoxide dismutase. Rheumatoid arthritis patients treated with superoxide dismutase showed marked improvement over salicylate- or corticoid-treated controls[220,221]. Osteoarthritis patients similarly showed improvement[220,222-224]. These results contrast with a study in experimentally-induced osteoarthritis in rabbits where intra-articular injections of superoxide dismutase not only failed to lead to an improvement, but actually induced severe synovitis[225].

Superoxide dismutase preparations have been used widely by the horse racing industry, and some reports of their activity have recently appeared in the literature[226-228].

F. ANTI-INFLAMMATORY PROPERTIES OF COPPER COMPLEXES

Over the past decade, the effects of various copper(I) and copper(II) complexes on a number of models of inflammation have been extensively studied in attempts to define and quantify the various pharmaco-activities of these copper complexes.

1. Copper(II) Peptides

There are a number of endogenous ligands that will complex copper(II), both as discrete entities and as constituents of larger molecules (protein, polynucleotides). Cu(II) binding to $-PO_3H_2^-$ groups is relatively weak compared to binding with peptide (CONH) and other natural protein ligands (alpha or omega, $-NH_2$, imidazole), especially when this is reinforced by chelation with adjacent COO^- groups.

Copper(II) binding to albumin involves (i) the terminal alpha-amino groups of this protein, (ii) the imidazole group of the histidine moiety at position 3 in human or rat albumins, and (iii) the amide N of two peptide bonds (1-2, 2-3). As models of this albumin "receptor", histidine (COOH) tripeptides such as Gly-Gly-His have been intensively investigated as quasi-natural Cu ligands[229,230]. The reverse peptide, His-Gly-Gly has a lesser affinity for Cu(II). This is reflected by the appreciable toxicity or irritancy of the Cu(II)-His-Gly-Gly (blue complex) vis a vis Cu(II)-Gly-Gly-His (purple complex) or Cu-albumin which are virtually non-irritant. ^{64}Cu-distribution studies of these two ^{64}Cu-tripeptides also indicates a wide variation in the biodistribution of ^{64}Cu. Both peptides show appreciable anti-inflammatory activity.

2. Copper (I) complexes

The monovalent state of copper is metastable, undergoing spontaneous dismutation to metallic copper and copper (II) in concentrated or neutral solutions. Ligands which stabilise Cu(I) include chloride or cyanide ions, pyridine and thiols. Several copper proteins, including caeruloplasmin, contains Cu(I) usually associated with Cu(II).

Copper(I)-thiolates show anti-inflammatory activity in rats (FIGURE 3). It is not known if their activity is due to the copper(I)-S complex as such. However, since these complexes are relatively labile and their aqueous solutions need to be stablised by excess thiols, it is likely that they decompose readily in vivo. The question then is what is the fate of the Cu(I): is it transferred to other endogenous Cu(I) ligands or is it oxidised to Cu(II) before exhibiting pharmaco-activity?

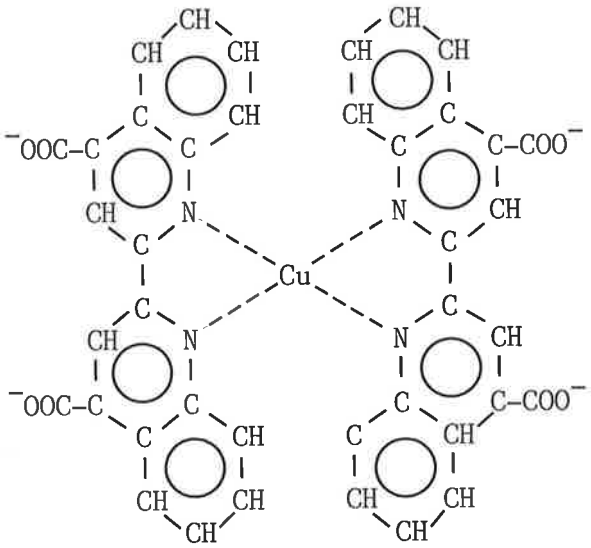
By contrast the highly coloured (red-purple) copper(I) bicischoninate tetraanion (FIGURE 3) is very stable in vitro and excreted largely unchanged in the urine after parenteral administration. Due to its very negative charge and consequent short half-life it manifests almost no anti-inflammatory activity in acute tests. It does however, show anti-arthritic activity in rats. Less soluble Cu(I)-bipyridyl complexes might be expected to show greater activity (and toxicity).

FIGURE 3. Some copper(I) complexes with anti-inflammatory or antiarthritic activity.

Thiolates Cu-SR where R= $-CHCOO^-$, 1-glucosyl

CH_2COO^-

Bicinchoninate:



D-penicillamine $(CH_3)_2C(SH)-CH(NH_2)CO_2H$

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3. Copper(I/II)-D-penicillamine

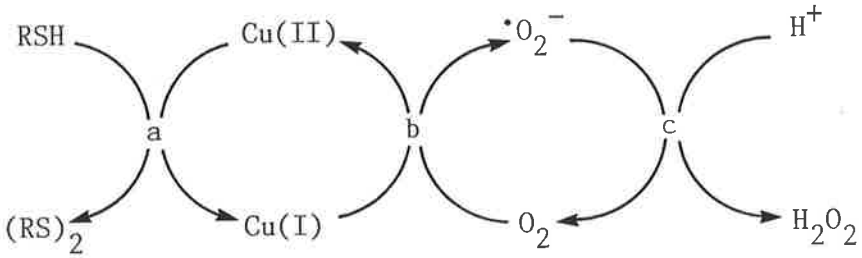
D-penicillamine (B,B-dimethylcysteine) an effective drug in the treatment of rheumatoid arthritis[231], has previously been used to accelerate the urinary excretion of copper, present in excess in hepatolenticular degeneration (Wilsons disease)[232]. It forms stable complexes with copper[233]. The interaction of copper(II) and D-penicillamine has been extensively studied, and the formation of a stable, red-violet mixed-valency copper-penicillamine complex reported[19,233,236]. Subsequent studies of the crystalline complex have shown it to be $[Cu(I)_8Cu(II)_6$ (D-Penicillamine)$_{12}]Cl$[19,235]. This mixed-valency copper-penicillamine complex is hydrophilic, stable in air and exposure to powerful chelators eg. Chelex-100 or EDTA. The complex may form in vivo after first forming a transient complex with Cu(II)-albumin (albumin-Cu-penicillamine)[237]. It is also excreted unchanged in the urine following intravenous infusion[238].

A number of biological activities have been attributed to this mixed valency complex including anti-ulcerogenic activity in the Shay rat[239] and ability to mimic superoxide dismutase[240]. In vitro studies have demonstrated that the complex itself is unable to catalyse the dismutation of $^{\bullet}O_2^-$, but it slowly decomposes to other copper(II) complexes which do so[241]. Substances that can scavenge oxygen-derived free radicals have some potential as pharmacological agents in diseases where superoxide anion generation may be involved particularly in keeping the $[^{\bullet}O_2^-]$ so low that it cannot react with H_2O_2 to give hydroxyl radicals. Apart from acting as an SOD "mimic", the other modes of anti-inflammatory activity of this copper complex are not known.

The inflammatory site is a probable target for copper based therapies. Copper levels may significantly increase at an inflammatory site, without therapy. Copper-containing drugs which deliver more copper to an inflammatory focus may then enhance the therapeutic (anti-inflammatory) activity of endogenous copper. Much of the copper contained at an inflammatory site (eg sponge granuloma) in rats dosed with ^{64}Cu-penicillamine complex appears to be exogenous copper derived from the complex itself[242].

These data show that this complex of copper and penicillamine may be fairly unique in (i) its ability to act as non-irritant injectable copper complex which is rapidly excreted from the body, yet (ii) still exhibit anti-inflammatory properties. Whether this may in part represent a mode of action of D-penicillamine, a widely used anti-rheumatic agent, remains uncertain. It has been shown however[237], that it is possible to form the mixed valency copper penicillamine complex in vivo. Furthermore copper and D-penicillamine, together, are able to inhibit some immune responses in model in vitro systems[243] which may involve either generating H_2O_2 (by combination reactions a-c in FIGURE 4) producing local cytotoxin or acting as a possible superoxide dimutase mimic.

FIGURE 4 Reduction of copper(II) by tissue thiols



a = reduction of Cu(II) by tissue thiols

b = spontaneous reoxidation of Cu(I) with concomitant reduction to superoxide

c = spontaneous of activity of superoxide dismutase.

4. Other Cu(II) complexes

The activity of low molecular weight complexes has been thoroughly reviewed by Sorenson [244-246]. A number of copper (II) complexes have been tested against a variety of animal models of inflammation including the carrageenan paw oedema, adjuvant-induced polyarthritis and the cotton wad granuloma. Many of these copper complexes were found to be more active than the parent ligands where these possessed anti-inflammatory activity eg. salicylate, phenylbutazone. It has been suggested[247] therefore that the therapeutic effects of these ligands (where these are anti-inflammatory agents) may result from the formation of copper complexes in vivo. The increased anti-inflammatory activity of these complexes may be due to the synergistic effects of both the parent ligand and copper(II), the parent compound acting as a carrier for the copper(II) analogous to thiomalate which appears to be a carrier for gold(I) in the antiarthritic drug gold sodium thiomalate.

Copper complexes, given orally, are very much less effective in rats as anti-inflammatory agents than when given parenterally. However the copper complexes of therapeutic ligands were considerably less ulcerogenic to the gastric mucosa than the parent ligands[168,248,249]. One major problem of many copper complexes is their irritancy to tissues, namely, subdermal tissues and skin when injected subcutaneously and gastric mucosa when given orally. It has been argued that the anti-inflammatory efficacy of copper complexes, in some cases at least, may be due to their irritancy of local tissues[302]. As gastric ulcerogenicity is one of the more consistent adverse reactions with a large number of anti-inflammatory drugs, this was considered a potentially important finding. The anti-ulcer activity of copper complexes has been further studied in rats using the Shay model of restraint-induced ulcers [168,239] and it was found that a complex of copper and D-penicillamine was strongly anti-ulcerant. The biological mechanism of copper irritancy to the tissues is not fully understood but may involve reductions of Cu(II) by tissue thiols, with consequent oxidation of Cu(I) to Cu(II) generating superoxide.

A number of methods have been employed to circumvent this problem : (i) by co-administering these complexes orally with sunflower oil[249] (ii) by administration of topical formulations containing copper(II), (iii) by using complexes of copper which are biologically stable where copper is not decoupled at the site of administration such as the mixed valency copper complex of D-penicillamine.

More work is certainly needed to resolve such questions:

(i) Does copper insufficiency really support inflammation?

(ii) To what extent do the apparently beneficial properties of copper supplementation reflect copper intoxications ie. is there real therapeutic benefit?

(iii) What are the "humoral" triggers which first switch on and subsequently switch off the changes in copper metabolism with inflammatory stress.

(iv) Possible changes in the intrinsic toxicity of copper with inflammation.

(v) The limitations of Sorenson's hypothesis that acidic anti-inflammatory drugs are cupriphores transporting copper(II) to sites of inflammation.

(vi) The mechanisms by which copper drugs suppress inflammation or facilitate healing.

The list of putative mechanisms given in TABLE 8 and will probably have to be extended quite considerably by giving consideration to yet other means by which changes in copper levels within certain compartments in the body have a profound influence on the chemistry of inflammation. Even this copper-centred approach to understanding how one metal can so profoundly influence the whole gamut of inflammatory responses is questionable, if it fails to look for compensating or synergistic effects of other natural metal bioregulants. Thus we end up trying to comprehend

- multiple effects of the one metal, copper and

- simultaneous multiple interactions with (i) other metals (ii) all metal ligands. It will probably require a very extensive

mathematical model to express so many bio-variables. Nevertheless, given a reasonable measure of good fortune, it may be possible to re-introduce copper pharmacology into modern medicine. The chief determinant of acceptability will of course be the long-term tolerance of a metal supplement by sick people, with various compromised organ functions. In this context metal drugs may exhibit an advantage over conventional organic drugs, since the inflammatory process seems to raise the level of metal-binding proteins such as metallothioneins that are able to "buffer" many metal ions and diminish their overt toxicity.

TABLE 8 Proposed anti-inflammatory actions of endogenous copper and/or applied copper complexes.

Mechanism	Evidence
Stabilisation of lysosomal enzymes	Copper decreased lysosome permeability and lowered free verses bound lysosomal enzyme levels.[251]
Modulation of the physiological effects of histamine	Histamine forms a hydroxy-bridged copper complex which is a reactive form of histamine. When injected, this complex is 50x more potent than histamine alone.[252,253]
Modulation of prostaglandin synthesis	Decrease the synthesis of PGE_2, and increases the synthesis of PGF_2.[254-258]
SOD mimics	In the various assays used for SOD activity copper complexes also have been shown to have SOD-like activity.[241,259-264]
Induction of lysyl oxidase	Lysyl oxidase (a copper-containing enzyme needed for collagen and elastin synthesis) can be induced in copper deficient chickens by $CuSO_4$ supplements, and other copper complexes.[265,266]
Stabilisation of gamma globulin	IgG is stabilised by a histidine-cysteine-copper complex, which has been shown to be depleted in rheumatoid arthritis. A copper complex of penicillamine can replace this His-Cys complex.[267,268]
Antimycoplasma effects	Several copper complexes were shown to be lethal to in vitro cultures of different mycoplasmas.[269,270]
Immunosuppression	Copper sulphate potentiates the inhibitory effect of penicillamine and other thiols on lymphocyte function[271-273]
Antoxidant effects	Ferroxidase activity of caeruloplasmin.[274] Superoxide dismutase - scavenges the superoxide radical[209]
Inhibition of kallikrein	Kallikrein (the enzyme which generates plasma kinins) irreversibly inhibited by 1 uM Cu(II).[275]

CHAPTER 2

GOLD THERAPY AND ADJUVANT ARTHRITIS

A. Summary

One of the most perplexing problems of chrysotherapy is exactly how gold elicits its therapeutic activity and induces an apparent remission of the inflammatory disease process. The use of the adjuvant induced polyarthritis in rats has led to some interesting although ambivalent findings of the effects of gold. These findings may reflect, however the variable use and the limitations of this animal model of inflammation.

This chapter describes investigations concerned with the in vivo biological effects of gold compounds on the adjuvant induced polyarthritis model of inflammation in various rat strains. The aims of this work were to (i) to investigate the antiarthritic activity of GST in adjuvant arthritis in rats and to establish the effect of varying the routes of administration and differing the time of administration, (ii) to investigate the comparative antiarthritic efficacy of a number of gold complexes, (iii) to determine the antiarthritic activity of GST and AF in adjuvant polyarthritis using differing rat strains using differing adjuvants, to standardise the assay and to clarify why previous work has such ambivalent findings using gold salts, (iv) to investigate the effect of GST therapy on endogenous copper distribution.

Results

In Dark Agouti rats GST was shown to have antiarthritic properties which appeared to be independent of the route of administration. Most gold drugs were effective against this model, however ambivalent properties were seen when either the rat strain, adjuvant or in fact the gold compound were varied which would help to explain previous conflicting findings. The organic ligands of GST (thiomalate) alone did not appear to have the same properties. While Auranofin (AF) appeared to reduce disease activity in either DA rats or JC Lewis rats independent of the variation in dosing schedule, GST reduced the severity of disease in DA rats when dosed from either Day0-14 or day6-14 but was unable to reduce disease activity when given from Day8-14. In JC Lewis rats GST when given from Day0-14 exacerbated disease activity, however when given either from day6-14 or day8-14 no effect on disease activity was seen. GST and AF therapy induced substantially different renal outputs, where GST induced a self limiting polyuria in DA rats but not in JC lewis rats. Moreover, there was high urinary gold in GST treated rats and low urinary gold in AF treated rats from both rat strains. GST therapy did substantially effect the endogenous copper distribution in both rats strains.

B. MATERIALS AND METHODS

1. Animals

Groups of 5 male Dark Agouti (DA) (150-250 g) and Lewis (JC) (200-250g) (Institute of Medical and Veterinary Science, Adelaide) and Ginger Hooded (GH) (180-240g) rats (The Queen Elizabeth Hospital, Adelaide) were used in these experiments.

2. Adjuvants

(a) Squalane/TBC

Heat-killed, delipidated human strain Mycobacterium tuberculosis (TBC) (Tuberculin Section, Ministry of Agriculture, Fisheries and Food, Weybridge, U.K.) was finely ground and dispersed in squalane (SQ) (Fluka) at a concentration of 10mg/ml. 50ul of this adjuvant was injected intradermally near the base of the tail of the rats on day 0.

(b) Triolein/TBC

Heat-killed, delipidated human strain Mycobacterium tuberculosis (TBC) was finely ground and dispersed in Triolein (TO) (Sigma) at a concentration of 10mg/ml. 50ul of this adjuvant was injected intradermally near the base of the tail of the rats on day0.

(c) Squalane/CP

On day 0 groups of rats were injected at the base of the tail with 150ul of the arthritogen constituted with CP-20961[226] (Avridine, Chas Phizer Ltd.) dispersed in squalane (50mg/ml).

3. Disease outline

From day +10 post adjuvant inoculation onwards, a polyarthritic disease becomes apparent. This disease was characterised by an increase in footpad thickness and the appearance of nodular lesions associated with peripheral joint swellings.

On Day 14 following adjuvant injection, the thickness of the hind paws was measured with a micrometer, and the weight loss of the animal was recorded. The overall severity of the arthritis was also assessed, with an arthritis score being assigned (maximum score = 7) after averaging the scores for front and rear paws (0-3 for each front paw; 0-4 for each rear paw; depending on the number of lesions and whether there was prominent ankle or wrist involvement). Experience showed that hind paw thickness was the most sensitive and reproducible indicator of arthritis up to Day +14 after adjuvant injection. The experiment was terminated on Day +14, the limit imposed by the Ethics Committee concerned.

4. Drug treatments

All drug preparations were made freshly every day when required, from pure drug preparations obtained from the manufacturers except for Myocrisin (Gold sodium thiomalate and a phenylmercurial preservative in aqueous solution). All drugs were given from day 0 until day 14 on alternate days unless otherwise specified.

(a) Gold sodium thiomalate (Myocrisin, May and Baker Ltd.)

MW = 390

This was prepared by diluting a 50mg ampule of drug with saline to a concentration of 5mg/ml. The drug was administered either subcutaneously of intraperitoneally at a dose of 0.5ml/200g body weight. This represents a dose level 12.5mg GST/kg body weight (32umoles of Au/kg body weight). Other doses regimens used included 1.56, 3.125, 6.25 and 25mg GST/kg. This manufacturers preparation contained an added preservative of 0.002% phenylmercuric nitrate being present in subsequent dilutions.

(b) Gold sodium thiomalate (Aldrich Co.Ltd.)

MW = 390

This was prepared by dissolving 50mg/ml of the pure powdered drug in 10mls of saline. This again was administered subcutaneously at a concentration of 0.5ml/200g body weight which was equivalent to 12.5mg GST/kg body weight or 32umoles of Au/kg body weight.

(c) Gold sodium thioglucose (Sigma Chemical Co.),

MW = 392

Was prepared by dissolving 50mg of the pure powdered drug in 10mls of saline (5mg/ml) and the dose administered was 0.5ml/200gm body weight or 32umole Au/kg body weight.

(d) Gold sodium thiosulphate (ICN Pharmaceuticals Inc)

MW = 490

Was prepared by dissolving 63mg of the pure powdered drug in 10ml of saline = 6.4mg/ml. With a dosage of 0.5ml/200gm body weight (32umole Au/kg body weight).

(e) Auranofin (Smith, Kline and French Laboratories)

MW = 678

The compound was suspended the in saline, by grinding 87mg of powdered auranofin in a mortar and pestle with a very small amount of dispersing agent (Tween 20) followed by dilution with 10ml of saline. The final concentration was 8.7mg/ml. 0.5ml was given orally per 200gm body weight (32umole/kg body weight) unless otherwise stated.

(f) Thiomalate (Mercaptosuccinic acid Sigma Chem. Co.)

MW = 150

This was prepared at equivalent dosing to that of aurothiomalate (32umole/kg). 19.2mg of thiomalic acid was dissolved in 10ml saline and the pH adjusted to 7.0. The drug preparation was then used immediately 0.5ml/200gm body weight.

(g) Phenylmercuric nitrate

MW = 62.5

This was prepared for a dosing of 2x that received in a 12.5mg/k dose of Myocrisin. 0.2mg of Phenylmercuric nitrate was disolved in 100ml of saline (0.002mg/ml). A dose of 0.5ml/200gm was administered which was equivalent to 0.005mg/kg (0.08umoles /kg).

5. Plasma gold levels (at Day +14)

Gold was determined by atomic absorption analysis[96]. To 0.3ml of heparinised plasma was added 1ml of saturated potassium permanganate solution and the mixture shaken in a fume cupboard for 30 minutes at room temperature. Two mls of 6M Hydrochloric acid (Aristar grade BDH) was then added and shaking continued for a further 30 minutes at room temperature.

The tubes then were placed in boiling water for 5 minutes. The resulting clear solutions were were cooled before adding 2mls of methyl isobutyl ketone (Spectrosol BDH) then vortexed mixed and centrifuged at 250xg for 5 minutes. After standing overnight, the supernatant was analysed for gold content using an atomic absorption spectrometer (Technicon Varian AA775).

6. Copper levels

The copper concentration of various tissues was estimated by flame atomic absorption spectrometry. Tissue samples were digested to dryness with 0.5ml concentrated nitric acid (HNO_3) and then with 0.25ml H_2O_2 and disolved in 5ml of 5% Hydrochloric acid (HCL) for analysis.

EXPERIMENT 1

AIM - To determine the antiarthritic activity of gold sodium thiomalate (GST) and the therapeutically effective dose level against adjuvant arthritis induced in Dark Agouti rats.

OUTLINE - Groups of 5 animals were injected with adjuvant on day 0. Each group received either 0, 1.56, 3.125, 6.25, 12.5, 25 mg/kg of GST intraperitoneally every 2 days until day 14.
At day 14 all animals were assessed for disease activity.

GROUPS	ADJUVANT	DRUG	AMOUNT OF GST	
			mg/kg	umole/kg
1	SAL	SAL	0	0
2	Mtb/TO	SAL	0	0
3	Mtb/TO	GST	1.56	4
4	Mtb/TO	GST	3.125	8
5	Mtb/TO	GST	6.25	16
6	Mtb/TO	GST	12.5	32
7	Mtb/TO	GST	25	64

RAT STRAIN - Dark Agouti

ADJUVANT - Mtb/Triolein (Mtb/TO)

DRUGS - Gold sodium thiomalate 0, 1.56, 3.12, 6.25, 12.5, 25mg/kg intraperitoneally every 2 days.

ASSESSMENTS - Weight change
Footpad thickness
Arthritic score
Serum gold
Mortality

TABLE 9 The effects of gold sodium thiomalate given at different concentrations to Dark Agouti rats with Mtb/Triolein induced polyarthritis.

TREATMENT mg/kg GST	WEIGHT LOSS (grms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM	uM
NORM/SAL	+15±3	5.4±0.05	0	0	0
ADJ/SAL	-27±9	6.9±0.5	4.0±1.1	0	0
1.56	-23±3	6.8±0.3	4.2±0.3	2.3±0.2	12
3.12	- 6±2*	5.8±0.1*	1.3±0.3*	2.2±0.2	11
6.25	- 2±2*	5.5±0.05*	0.7±0.2*	3.7±0.3	19
12.5	-10±2	5.5±0.03**	0.5±0.2*	5.1±0.7	26
25.0	-24±3	5.6±0.1*	0.3±0.1*	6.9±0.9	35

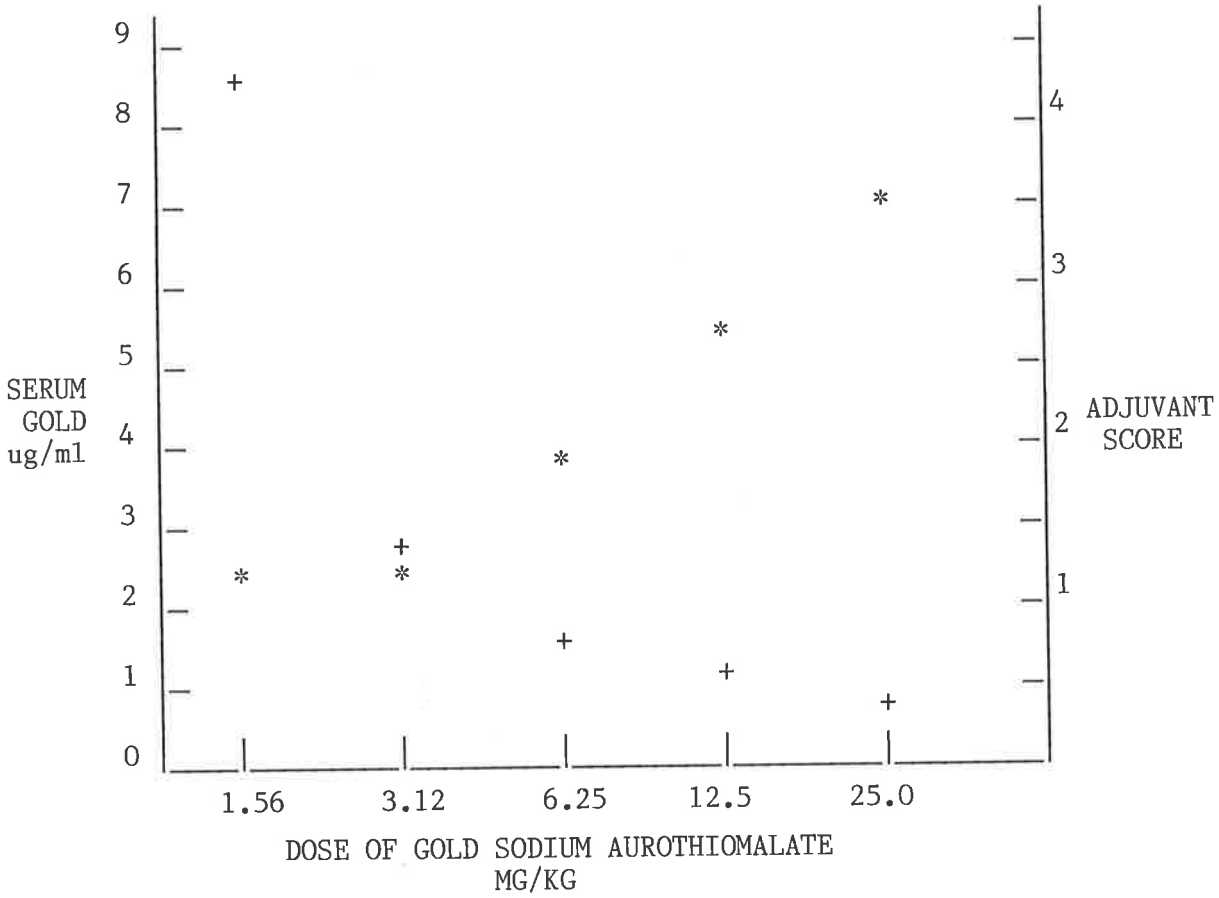
Significance values from control arthritic group (ADJ/SAL)

* - $p<0.05$
** - $p<0.01$
*** - $p<0.001$

DA/Mtb/TO Adjuvant made from the lipid Triolein and Myco.tuberculosis used in Dark Agouti rats.

NORM Non-adjuvant treated rats.
SAL Saline
ADJ Adjuvant
GST Gold sodium thiomalate

FIGURE 5 Serum gold level (ug/ml) and adjuvant score in rats with adjuvant polyarthritis induced with Mtb/Triolein plotted against dose of GST administered every two days. "+" = Arthritic Score, "*" = Serum Gold



RESULTS

Polyarthritic Disease

The Mtb/TO adjuvant induced a polyarthritic disease in DA rats (TABLE 8) resulting in substantial weight loss (-27±9gms.) and a moderate increase in footpad thickness (6.9±0.5mm). GST elicited a dose dependent suppression of this disease with the highest dose (25mg/kg) significantly reducing disease activity (footpad thickness 5.6±0.1mm, $p<0.05$) compared to the adjuvant control group. Strong suppressive activity was also observed for 12.5mg/kg and 6.25mg/kg dose levels, both significantly reduced footpad thickness 5.5±0.03mm, $p<0.01$ and 5.5±0.05mm, $p<0.05$ respectively and lowered the arthritic scores 0.5±0.2, $p<0.05$ and 0.7±0.2, $p<0.05$ respectively. The 1.56mg/kg dose had little effect on disease activity where the footpad thickness was 6.8±0.5, (not significantly different from the arthritic control).

Blood Gold Levels

Above a dose level of 3.12mg/kg serum gold concentrations showed a linear relationship when plotted against dose level (FIGURE 5). Therapeutic dose range of this drug was from 3.12 to 12.5mg/kg which resulted in serum gold levels of 2.2±0.2ug/ml and 6.9±0.9ug/ml respectively. As serum levels of gold rose above 3.12mg/kg, the drug was more effective at reducing the arthritic disease.

DISCUSSION

GST elicited dose dependent antiarthritic activity in the adjuvant (Mtb/TO) induced polyarthritis in DA rats. Therapeutic effectiveness of gold drugs against polyarthritic disease may represent a measure of tolerable toxicity and this would be dependent upon the detoxifying mechanisms available in the animal, the type of drug used, dosing regimen employed, and the type of disease induced in these animals.

A number of important factors may have contributed to the overall therapeutic value of GST against this disease, (1) The route of administration may have had some influence over the effectiveness of this drug against this polyarthritic disease. In previous work[123-132] the most widely used routes were by subcutaneous or intramuscular injections with variations in therapeutic activity of gold seen. (2) The effect of varying the time of dosing may be important due to varying detoxification mechanisms and elimination process in the animals. (3) Different gold preparations may elicit varied antiarthritic activities against adjuvant induced disease[123-132]. A number of gold preparations are available for use against rheumatic diseases, ie Gold sodium thiomalate (GST), Aurothioglucose (ATG), Aurothiosulphate (ATS) and the new oral gold formulation Auranofin (AF) (FIGURE 2). To date only extensive studies of GST and auranofin have been carried out, probably due to the wide clinical use of these drugs. (4) The adjuvant disease, used widely to investigate the antiarthritic properties of gold preparations, can now be more closely defined by the use of pure lipid instead of

highly variable mineral oil[134].

These avenues of investigation were studied in subsequent experiments, where the level of gold dosing for these experiments, based on the above findings, was equivalent to 12.5mg/kg of GST or 32umoles of gold as this provided maximum therapeutic efficacy with minimal toxicity and side effects.

EXPERIMENT 2

AIM - To investigate the effect of varying the route of administration of GST on its antiarthritic efficacy in adjuvantised rats.

OUTLINE - Groups of 5 rats received adjuvant on day 0. The control group was treated with saline over the next 14 days whereas other groups received GST at 12.5 mg/kg every two days either intraperitoneally, subcutaneously, intramuscularly or oral administration.

GROUP	ADJUVANT	DRUG	AMOUNT mg/kg	ROUTE
1	TO/Mtb	SAL	0	s/c
2	TO/Mtb	GST	12.5	s/c
3	TO/Mtb	GST	12.5	i/p
4	TO/Mtb	GST	12.5	i/m
5	TO/Mtb	GST	12.5	oral
6	TO/Mtb	SAL	0	Alzet
7	TO/Mtb	GST	12.5	Alzet

RAT STRAIN - Dark Agouti

ADJUVANTS - Mtb/Triolein (Mtb/TO)

DRUGS - Gold sodium thiomalate 12.5mg/kg either s/c, i/p, i/m, oral, slow release alzet capsules.

ASSESSMENTS - Footpad thickness
Arthritic score
Serum gold
Weight change

TABLE 10 The effects of gold sodium thiomalate administered by either the subcutaneous, intramuscular, intraperitoneal or oral routes on adjuvant polyarthritis induced in Dark Agouti rats using the Mtb/TO adjuvant.

GROUP ADJ	TREATMENT GST (mg/kg) ±SEM	WEIGHT LOSS (grms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM
NORMAL	SAL	+15±3	5.4±0.05	0	0
1	SAL	-34±6	6.4±0.3	3.6±0.7	0
2	12.5 i/p	- 5±1***	5.5±0.1**	0.5±0.2***	4.3±0.5
3	12.5 s/c	-10±1*	5.5±0.1*	0.3±0.2***	4.8±0.5
4	12.5 i/m	- 5±1*	5.5±0.1*	0.7±0.4*	4.2±0.3
5	12.5 oral	-14±10*	6.1±0.2	3.3±0.4	0.5±0.2
6	SAL Alz	-32±7	6.5±0.2	3.4±0.8	0
7	12.5 Alz.	-15±8*	5.6±0.1*	0.8±0.3*	3.9±0.6

Significance values from control arthritic ADJ/SAL group

* - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$

DA/Mtb/TO Adjuvant made from the lipid Triolein and Myco.tuberculosis used in Dark Agouti rats.

GST Gold sodium thiomalate
SAL Saline
i/p Intraperitoneal
s/c Subcutaneous
i/m Intramuscularly
oral Orally
Alz. Subcutaneously implanted Alzet delivery capsule

RESULTS

Gold sodium thiomalate (GST) administered either subcutaneously, intraperitoneally or intramuscularly, was equally effective at preventing the expression of adjuvant arthritis evidenced by the reduction in footpad thickness to near normal (5.4 ± 0.05) levels i/p: 5.5 ± 0.1 $p<0.05$, s/c 5.5 ± 0.1 $p<0.01$, i/m 5.5 ± 0.1 $p<0.05$. However orally dosed GST remained singularly ineffective (TABLE 10). Delivery of GST by the slow release capsule resulted in substantial reduction in disease severity having similar therapeutic activity as subcutaneous administration. Comparable serum gold levels were obtained from animals dosed with GST either subcutaneously, intraperitoneally, intramuscularly or by alzet routes of administration (4.3 ± 0.5ug/ml, 4.8 ± 0.5ug/ml, 4.2 ± 0.3ug/ml and 3.9 ± 0.6ug/ml respectively). Only low serum gold levels were detected in the serum from animals treated orally with GST (0.5 ± 0.2ug/ml).

DISCUSSION

These findings provided evidence that administration of GST by either subcutaneous, intraperitoneal, intramuscular routes or by alzet slow release capsules were equally effective. These modes of administration (except oral) all appeared to deliver therapeutically active forms of gold which elicited beneficial activity against the induced polyarthritis. A number of previous investigations used various routes of administration of gold complexes (mainly i/m, s/c) but have shown conflicting results (TABLE 6). The results here, indicated that the ambivalent results seen previously were probably

not the result of the differing routes of administration.

Oral dosing with GST elicited little if any antiarthritic activity. This agreed with previous findings that this drug has never been shown to be orally active, probably due to either drug breakdown in the gut or the non-absorptive properties of this gold complex. A new oral gold complex Auranofin, has been manufactured by Smith, Kline and French and has been investigated in subsequent experiments.

Alzet slow release capsules have been used for a number of years to deliver drugs over an extended periods for various applications[234]. They have provided slow release dosing of the drug of choice, relying on osmotic pressure to release the compound[250]. Compared to a control group of animals with saline-loaded alzet capsules, the animals with gold-loaded alzet capsules had substantially reduced disease. This indicated that the reduction in disease severity was not due to surgical manipulation of implantation but to GST from the alzet capsules. With the efficacy of this mode of administration when compared with s/c administration the idea of slow release formulations may well be preferable to pulsed dosing, by providing a method of administering the drug without the need for repeated injections. These findings imply that there appeared little therapeutic variation of GST when dosed either subcutaneously, intraperitoneally, intramuscularly or by slow release of GST. And slow release therapy may provide better alternative mode of administration than by conventional routes.

EXPERIMENT 3

AIM - To investigate the antiarthritic activity of the oral gold complex Auranofin against adjuvant arthritis induced in Dark Agouti rats and determine a therapeutically active dose level.

OUTLINE - Groups of 5 animals were injected with adjuvant on day 0. Each group received either 0, 2.7, 5.4, 10.9, or 21.8 mg/kg of Auranofin orally every 2 days until day14. At day 14 the animals were assessed for disease activity.

GROUP	ADJUVANT	DRUG	AMOUNT OF DRUG mg/kg	umole/kg
1	Mtb/TO	SAL	0	0
2	Mtb/TO	AF	2.6	4
3	Mtb/TO	AF	5.2	8
4	Mtb/TO	AF	10.4	16
5	Mtb/TO	AF	20.8	32

RAT STRAIN - Dark Agouti

ADJUVANTS - Mtb/Triolein (Mtb/TO)

DRUGS - Auranofin 0, 2.6, 5.2, 10.4 and 20.8 mg AF/kg orally every 2 days.

ASSESSMENTS - Footpad thickness
Arthritic score
Serum gold
Weight change
RBC gold/ml of blood

TABLE 11 The effects of auranofin given therapeutically at various concentrations to Dark Agouti rats with polyarthritis induced with Triolein/Mtb adjuvant (DA/Mtb/TO).

GROUP	TREATMENT mg/kg AF	WEIGHT LOSS (grm.) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	MORTALITY %
1	SAL	-16±4	7.3±0.3	4.1±0.5	0
2	2.7	-34±11*	6.0±0.4*	2.7±1.1	0
3	5.4	-42±7**	6.3±0.4	3.5±1.0	0
4	10.9	-34±11*	5.3±0.1***	0.1±0.1***	25
5	21.8	-25±3	5.3±0.2***	0.8±0.7***	25

Significance values from control arthritic group (Group 1)

* - $p<0.05$
** - $p<0.01$
*** - $p<0.001$

DA/TO/Mtb Adjuvant made from the lipid Triolein and Myco.tuberculosis used in Dark Agouti rats.

AF Auranofin
SAL Saline

TABLE 12 Serum and red blood cell gold levels following administration of Auranofin at different concentrations in to Triolein/Mtb adjuvant (DA/TO/Mtb) induced polyarthritic Dark Agouti rats.

GROUP	TREATMENT AURANOFIN (mg/kg)	SERUM GOLD (ug/ml) ±SEM	RED BLOOD CELL GOLD (ug/ml of blood) ±SEM
1	SAL	0	0
2	2.7	0.53±0.04	0.1 ±0.08
3	5.4	1.07±0.03	0.73±0.13
4	10.9	1.51±0.03	0.91±0.04
5	21.8	2.19±0.08	3.05±0.21

DA/TO/Mtb Adjuvant made from the lipid Triolein and Myco.tuberculosis used in Dark Agouti rats.

AF Auranofin
SAL Saline

RESULTS

Auranofin, given orally, had strong antiarthritic activity when dosed at 10.9 and 21.8mg/kg (ie 16 and 32umole of AF/kg) and this was emphasised by the decrease in footpad thickness (5.3±0.1mm, $p<0.001$ and 5.3±0.2mm, $p<0.001$ respectively) and arthritic score (0.1±0.1, $p<0.001$ and 0.8±0.7, $p<0.001$ respectively). Below these doses there appeared little significant antiarthritic activity.

Blood gold levels

TABLE 12 shows serum gold levels following auranofin dosing. They range from 0.53±0.04ug/ml for the low dose group to 2.19±0.08ug/ml for the highest dosed group. The red blood cells did contain substantial amounts of gold, and the ratio of red blood cell gold to serum gold increased with the dose administered. At the highest dose level (21.8mg/kg) serum gold was 2.19ug Au/ml and red blood cell gold content was 3.05ug Au/ml of blood.

DISCUSSION

These results confirm previous findings[128,131] where auranofin, an orally active gold formulation, possessed antiarthritic properties in this model of rat polyarthritis. However only two of the doses used induced a significant reduction in disease activity (10.4mg/kg and 20.8mg/kg of gold). The mortality of the drug at these dosing levels was 25% and the weight loss in the surviving animals was quite substantial indicating inherent toxicity of this oral gold formulation. Although this drug was shown previously to be effective

against this inflammatory model the papers did omit to include mortality figures[131].

As has been reported previously, serum gold levels were substantially lower in auranofin treated animals than in animals treated with parenterally administered GST. In comparison to the results in experiment 1 the serum gold level in auranofin treated animals was found to be approximately 50% of that found in GST treated animals. It has been postulated previously that this reduced level may result in a decrease in toxic side effects with auranofin treatment[97].

These results represented different compartmentalisation of gold by auranofin compared to GST since red blood cells from AF treated animals contained substantial, if not equal, amounts of gold to that seem in the serum. Conversely, the level of gold in red blood cells from animals treated with GST was found to be undetectable. The implications of high red cell gold levels are still unknown although it was proposed that having a large proportion of the circulating gold incorporated into red blood cells would increase toxic side effects, but no evidence has yet been found to substantiate this[98]. Because of the differing compartmentalisation of gold by lipophilic auranofin and hydrophilic GST, it would be expected that their sites of therapeutic activity, if not their modes of action, may be different.

These findings have shown that auranofin was an orally

active gold preparation having similar if not greater therapeutic activity than GST in this animal model of arthritis and it appeared to produce differing gold biodistribution in the blood than did GST. As a result, the site(s) of action and the therapeutic activities of these gold complexes may differ.

EXPERIMENT 4

AIM - To investigate the comparative antiarthritic activity of a number of gold complexes including the effect of thiomalate as well as the preservative phenylmercuric nitrate (used in the commercial preparation of Myocrisin).

METHOD - Groups of 5 animals received adjuvant on day 0. Each group received either saline, GST (Myocrisin or the pure drug from Merck), SATG, SATS, allochrysine, AF, thiomalate or phenylmercuric nitrate subcutaneously over the next 14 days.

GROUP	DRUG	AMOUNT OF DRUG (mg/kg)	umoles Au/kg
1	SAL	0	0
2	GST May & Baker	12.5	32
3	GST Merck	12.5	32
4	SATG	12.0	32
5	SATS	16.0	32
6	Allochrysine	12.0	32
7	Auranofin	14.0	32
8	TM	4.6	32
9	OHg	0.005	0.08

RAT STRAIN - Dark Agouti, J.C.Lewis

ADJUVANTS - Mtb/Triolein (Mtb/TO) in DA rats.
Mtb/Squalane (Mtb/TO) in J.C.Lewis rats

DRUGS - Gold sodium thiomalate with phenylmercuric nitrate (GST May & Baker)
Gold sodium thiomalate pure (GST Merck)
Sodium Aurothioglucose (SATG),
Sodium Aurothiosulphate (SATS)
Allochrysine (ALL)
Auranofin (AF)
Thiomalate (TM).
Phenylmercuric nitrate (OHg)

ASSESSMENTS - Footpad thickness
Tail diameter
Arthritic score
Serum gold
Weight change
Mortality

TABLE 13 The comparative effects of gold complexes on adjuvant arthritis induced in D.A. rats using Mtb/Triolein adjuvant.

GROUP	TREATMENT	WEIGHT LOSS (grms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM
1	SAL	-16±2	7.3±0.3	4.1±0.5	0
2	GST May & Baker	- 4±1**	5.5±0.1***	0.2±0.1**	4.1±0.9
3	GST Merck	- 4±2*	5.7±0.1***	0.6±0.4**	3.6±0.9
4	SATG	- 2±1*	5.6±0.1***	0.6±0.2**	3.9±0.7
5	SATS	- 1±1**	5.6±0.1***	0.2±0.1**	4.2±0.6
6	ALL	- 1±1**	5.9±0.1***	1.6±0.6*	3.8±0.7
7	AF	-25±3	5.3±0.2***	0.8±0.7***	2.3±0.5
8	TM	-14±5	6.8±0.3	4.0±0.6	0
9	OHg	-21±6	7.5±0.6	4.5±1.3	0

Significance values from Control arthritic group

* - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$

DA/Mtb/TO Adjuvant made from Triolein and Myco.tuberculosis

DRUGS
SAL Saline
GST May & Baker - Gold sodium thiomalate (+ mercurial preservative)
GST Merck - Gold sodium thiomalate (pure)
SATG - Sodium aurothioglucose,
SATS - Sodium aurothiosulphate
ALL - Allochrysine,
AF - Auranofin
TM - Thiomalate,
OHg - Phenylmercuric nitrate

TABLE 14 The comparative effects of gold complexes on adjuvant arthritis induced in J.C.Lewis rats using Mtb/Squalane adjuvant.

GROUP	TREATMENT	WEIGHT LOSS (grms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM
1	SAL	-19±4	6.6±0.2	2.2±0.5	0
2	GST May & Baker	-36±5*	7.3±0.2*	3.6±0.5*	4.5±1.2
3	GST Merck	-29±8	7.2±0.2*	4.1±0.6*	5.1±0.7
4	SATG	-13±5	7.8±0.4*	4.0±0.9*	4.8±0.6
5	SATS	-20±5	7.5±0.5*	4.2±0.4*	5.2±0.8
6	ALL	-17±8	7.2±0.4*	3.9±0.5*	4.3±0.6
7	AF	-32±3	5.8±0.1**	0.12±0.1**	1.8±0.15
8	TM	-20±6	6.6±0.2	1.9±0.5	0
9	OHg	-16±4	6.6±0.3	2.4±0.6	0

Significance values from Control arthritic group

* - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$

JC/Mtb/SQ - Adjuvant made from Squalane and Myco.tuberculosis

DRUGS
SAL Saline
GST May & Baker - Gold sodium thiomalate (+ mercurial preservative)
GST Merck - Gold sodium thiomalate (pure)
SATG - Sodium aurothioglucose,
SATS - Sodium aurothiosulphate
ALL - Allochrysine,
AF - Auranofin
TM - Thiomalate,
OHg - Phenylmercuric nitrate

RESULTS

DA/Mtb/TO

All parenterally administered gold formulations gave similar levels of antiarthritic activity, evidenced by a decrease in footpad thickness when compared with the adjuvant control group (GST-May and Baker 5.5±0.1 $p<0.001$, GST-Merck 5.7±0.1 $p<0.001$, SATG 5.6±0.1 $p<0.001$, SATS 5.6±0.2 $p<0.001$, ALL 5.9±0.1 $p<0.001$,). Auranofin given orally at 32umoles/kg also significantly reduced disease activity with a decrease in footpad thickness to 5.3±0.2 $p<0.001$). Subcutaneous administration of thiomalate (4.6mg/kg) or phenylmercuric nitrate (a preservative used in the commercial preparation of Myocrisin (May and Baker)) elicited no antiarthritic activity, although it was given at twice the level which the animals would normally have received.

JC/Mtb/SQ

The adjuvant Mtb/SQ did induce a mild disease in JC Lewis rats. The effect of parenteral gold administration in these animals was to exacerbate the disease activity as seen by the increase in paw thickness GST-May & Baker 7.3±0.2 $p<0.05$, GST-Merck 7.2±0.2 $p<0.05$, SATG 7.8±0.4 $p<0.05$, SATS 7.5±0.5,$p<0.05$, ALL 7.2±0.4 $p<0.05$,. Only auranofin reduced disease activity with reduced footpad thickness to 5.8±0.1 $p<0.01$ and reduced the arthritic score to 0.12±0.1 $p<0.01$. The use of thiomalate or the preservative phenylmercuric nitrate appeared not to effect disease activity in these animals.

DISCUSSION

DA/Mtb/TO

The antiarthritic efficacy of parenteral gold preparations was not confined to sodium aurothiomalate alone since sodium aurothiosulphate and sodium aurothioglucose both had equipotent activity against the polyarthritis induced by the Mtb/TO adjuvant. The results indicate that some gold preparations may have significant although limited activity (eg Allochrysine (5.9 ± 0.1 $p<0.001$)). Auranofin was as effective at suppressing the induced disease as were the parenteral drugs. One would, at this stage, regard auranofin as having similar activity, however its obvious inherent toxicity problems are of concern.

Although phenylmercuric nitrate was not thought to have antiarthritic properties it is, in itself, a heavy metal and it was felt worth investigating. As expected, it did not elicit any significant antiarthritic activity in this model of polyarthritis although a level twice that which the animals would normally have received was used. Pure GST produced similar antiarthritic activity to that of Myocrisin (GST + Preservative). It can be concluded that the antiarthritic activity of sodium aurothiomalate or myocrisin was primarily due to the gold complex itself and not due to the preservative.

These experiments indicated parenteral gold formulations were active against the Mtb/TO induced polyarthritis in DA rats. The antiarthritic activity of GST was shown to result from the gold complex alone and not to the presence of a mercurial preservative.

JC/Mtb/SQ

The stronger adjuvant Mtb/SQ was used in these animals as Mtb/TO did not induce any disease activity in JC Lewis rats. In contrast to DA rats parenteral gold drugs appear to exacerbate disease activity in JC Lewis rats. A stimulatory effect of GST therapy in adjuvant disease has been reported by only one group[132]. Previous to this, parenteral gold was shown either to reduce or to have little effect on adjuvant induced arthritis.

The only drug used to have had any substantial effect in suppressing disease activity was auranofin. It appeared therefore, unlike the parenteral gold formulation, that auranofin had antiarthritic effects in both rat strains.

It can be concluded from these experiments that parenteral gold drugs (GST, SATG, SATS, ALL) showed antiarthritic activity in the DA rat. In contrast, however they appeared to stimulate disease activity in the JC Lewis rat. This implied that the effectiveness of parenteral gold drugs against adjuvant induced arthritis was dependent on the particular strain of rat which was employed. Orally administered AF showed substantial antiarthritic efficacy in both rat strains. This again emphasises some of the finding of previous experiments where GST and AF appeared to have substantially different behaviour in their biological activity in these arthritic rats and this may indicate basic differing mechanisms of action. An investigation into to the effect of rat strain, adjuvant and gold drug was the subject of subsequent studies.

EXPERIMENT 5

AIMS - To investigate the effects of varying the rat strain, the adjuvant and gold drugs used.

OUTLINE - This investigation compares the differing effects of using three different rat strains, three different comparing the antiarthritic effect of GST and AF.

GROUPS	ADJUVANT	DRUG	#animals/group
1	Mtb/SQ	SAL	5
2	Mtb/SQ	ATM	5
3	Mtb/SQ	AF	5
4	Mtb/TO	SAL	5
5	Mtb/TO	ATM	5
6	Mtb/TO	AF	5
7	CP/SQ	SAL	5
8	CP/SQ	ATM	5
9	CP/SQ	AF	5

RAT STRAIN - Dark Agouti, J.C.Lewis and Ginger Hooded

ADJUVANTS - Mtb/Squalane (Mtb/SQ), Mtb/Triolein (Mtb/TO)
Avridine/Squalane (CP/SQ)

DRUGS - Gold sodium thiomalate 32umole/kg every 2 days
- Auranofin 32umole/kg orally every 2 days

ASSESSMENTS - Footpad thickness
Arthritic score
Serum gold
Weight change

TABLE 15 The effects of gold sodium thiomalate and auranofin on adjuvant polyarthritis induced in Dark Agouti, JC Lewis and Ginger hooded rats by the Squalane/Mtb adjuvant.

RAT STRAIN /ADJUVANT	TREATMENT	WEIGHT LOSS (grms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM
DA/Mtb/SQ	SAL	-25±4	7.3±0.3	5.1±0.3	0
	GST	-20±4	6.2±0.1**	2.3±0.2***	4.3±0.9
	AF	-18±2*	5.8±0.2***	0.7±0.5***	2.2±0.3
JC/Mtb/SQ	SAL	-15±3	6.2±0.2	1.7±0.9	0
	GST	-17±4	7.4±0.2***	4.5±0.7**	4.5±0.6
	AF	-14±2	5.5±0.4	0	1.5±0.2
GH/Mtb/SQ	SAL	- 2±1	7.5±0.4	2.9±0.7	0
	GST	- 8±3**	7.6±0.3	2.9±0.4	5.3±0.4
	AF	- 1±1	6.3±0.2**	0	1.2±0.4

SAL - Saline; GST - Gold Sodium Thiomalate; AF- Auranofin

* $p<0.05$ From control arthritic saline group.
** $p<0.01$
*** $p<0.001$

All measurements were taken at day 14 after adjuvant inoculation

TABLE 16 The effects of gold sodium thiomalate and auranofin on adjuvant polyarthritis induced in Dark Agouti, JC Lewis and Ginger hooded rats by the Triolein/Mtb adjuvant.

RAT STRAIN /ADJUVANT	TREATMENT	WEIGHT LOSS (grms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM
DA/Mtb/TO	SAL	−15±4	7.3±0.4	4.0±0.5	0
	GST	− 4±1*	5.4±0.1***	0.5±0.01***	3.9±0.5
	AF	−18±2	5.1±0.2***	0.7±0.5***	1.8±0.3
JC/Mtb/TO	SAL	− 4±2	5.6±0.1	0	0
	GST	−10±3	6.1±0.2	1.0±0.6	4.2±0.7
	AF	− 5±1	5.5±0.3	0	1.6±0.2
GH/Mtb/TO	SAL	+16±3	5.6±0.3	0	0
	GST	+25±2*	5.6±0.2	0	4.8±0.3
	AF	+15±2	5.7±0.2	0	2.1±0.2

SAL - Saline; GST - Gold Sodium Thiomalate; AF- Auranofin

* $p<0.05$ From control arthritic saline group
** $p<0.01$
*** $p<0.001$

All measurements were taken at day 14 after adjuvant inoculation

TABLE 17 The effects of gold sodium thiomalate and auranofin on adjuvant polyarthritis induced in Dark Agouti, JC Lewis and Ginger hooded rats by the Squalane/Avridine adjuvant

RAT STRAIN /ADJUVANT	TREATMENT	WEIGHT LOSS (grms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM
DA/CP/SQ	SAL	-39±3	7.7±0.3	4.7±0.6	0
	GST	-43±5	8.1±0.5	4.5±1.0	4.0±0.7
	AF	-15±2***	6.7±0.4*	1.9±0.9**	1.8±0.5
JC/CP/SQ	SAL	- 7±2	5.4±0.4	2.0±1.3	0
	GST	-20±10	5.7±0.5	2.9±1.5	3.3±0.6
	AF	-25±15	5.6±0.8	2.3±1.9	1.2±0.3
GH/CP/SQ	SAL	- 5±3	7.6±0.7	2.6±1.3	0
	GST	-18±1*	7.4±0.6	1.5±0.7	3.2±0.3
	AF	- 2±1	5.9±0.1***	0	2.1±0.4

SAL - Saline; GST - Gold Sodium Thiomalate; AF- Auranofin
CP - Avridine (CP 20961 - Chaz Phizer).

* $p<0.05$
** $p<0.01$
*** $p<0.001$

All measurements were taken at day 14 after adjuvant inoculation

Results

Arthritogen - Mtb/SQ adjuvant(TABLE 15)

In DA rats, the severe arthritis was partly suppressed by GST and was inhibited almost totally by AF. Weight loss associated with arthritis development was reduced by AF, but not by GST. In GH rats, the severe arthritis was not altered by GST and weight loss was significantly increased. By contrast, AF significantly reduced the severity of the arthritis and prevented weight loss. In JC rats, the mild arthritis was made worse by GST, whereas AF had no effect.

Arthritogen - Mtb/TO adjuvant(TABLE 16)

In DA rats, the severe arthritis and weight loss was inhibited almost totally by both GST and AF. In GH rats, few arthritic lesions developed and were unaffected by GST or AF. However, the animals treated with GST showed a significant increase in weight gain compared with the untreated and AF-treated groups. In JC rats, arthritis did not develop in untreated or AF-treated animals, but some lesions and increased mean footpad thickness ($p<0.1$) were noted in the groups treated with GST.

Arthritogen - CP/SQ adjuvant(TABLE 17)

In DA rats, a severe arthritis developed that was not affected by treatment with GST, but was reduced significantly by AF which also reduced weight loss. In GH rats, the severe arthritis was unchanged by treatment with GST, and weight loss was significantly

increased. Treatment with AF markedly suppressed both the arthritis and weight loss. In JC rats, few arthritic lesions developed and no significant changes were noted after treatment with GST or AF.

Serum gold

In all three rat strains, the serum gold levels were similar and were consistently 2-3 orders of magnitude higher after treatment with GST when compared with AF treatment. Moreover, there were no significant differences in serum gold levels between the groups receiving different adjuvants.

Discussion

Utilizing three rats strains, three adjuvants and two gold drugs, this study has shown (a) that the arthritogenic potential of adjuvants was dependent upon the arthritogen and the oil used consistent with previous findings[134], and (b) the response to an adjuvant differs between different strains of rats. Moreover, depending upon the combination of adjuvant and strain, treatment with gold drugs may (i) totally or partially suppress the arthritis; (ii) have no discernible effect; or (iii) augment the disease process. AF was more effective than GST in suppressing the arthritis induced by the Mtb/SQ or CP/SQ adjuvants. GST,but not AF, induced a more severe arthritis in the JC strain when given the Mtb/SQ and Mtb/TO adjuvants. These varying responses to gold drugs were not associated with significant variations in the serum gold levels induced by GST and of AF which were similar irrespective of adjuvant, strain or

arthritis.

While, of necessity, the experiments described were terminated 14 days after adjuvant injection, they provide ample evidence for the need for caution in studying gold (and possibly other) drugs using the adjuvant polyarthritis model in the rat. They also show that results obtained with each strain/adjuvant/gold drug combination may not be extrapolated to circumstances when the combinations are varied.

The repeatability of observations would be dependent also upon strain variations, the purity and preparation of materials constituting adjuvants, and drug vehicles, routes and regimens. Notwithstanding these constraints, these findings support the concept that GST and AF have rather different mechanisms of action. The variations in the different experimental combinations offer the possibility now of studying differences in immunological and other parameters which may underlie the responses to chrysotherapy.

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EXPERIMENT 6

AIM - To investigate the effect of delayed administration of GST and AF on adjuvant disease induced in both Dark Agouti and J.C.Lewis rats.

OUTLINE - Groups of 5 animals were injected with adjuvant on day 0. Each group received either saline, GST or AF from day 0 until day 14 or day 6 until day 14. (1) Dark Agouti (DA) and (2) J.C Lewis rats.

GROUPS	ADJUVANT	DRUG	AMOUNT mg/kg	DOSING PERIOD
1	Mtb/SQ	SAL	0	0-14
2	Mtb/SQ	GST	12.5	0-14
3	Mtb/SQ	GST	12.5	6-14
4	Mtb/SQ	GST	12.5	8-14
5	Mtb/SQ	AF	20.8	0-14
6	Mtb/SQ	AF	20.8	6-14
7	Mtb/SQ	AF	20.4	8-14

RAT STRAIN - Dark Agouti, J.C Lewis.

ADJUVANTS - Mtb/Squalane (Mtb/SQ)

DRUGS - Gold sodium thiomalate 12.5mg/kg s/c.
Auranofin 20.8 mg/kg orally.

ASSESSMENTS - Weight change
Footpad thickness
Arthritic score
Serum gold

TABLE 18 The effects of Gold sodium thiomalate and Auranofin when administered either from day 0 until day 14 or day 6 until day 14 on the adjuvant disease induced in Dark Agouti rats.

RAT STRAIN /ADJUVANT	TREATMENT	WEIGHT LOSS (gms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM
DA/Mtb/SQ	SAL	27±3	7.3±0.3	4.5±0.3	0
DA/Mtb/SQ	GST0-14	22±3	6.2±0.2**	1.8±0.3***	4.3±0.9
DA/Mtb/SQ	GST6-14	38±1	6.5±0.3*	2.7±0.6**	7.1±3.9
DA/Mtb/SQ	GST8-14	38±4	6.8±0.5	3.4±0.9	11.5±0.7
DA/Mtb/SQ	AF0-14	33±6	5.8±0.2**	1.1±0.5***	2.2±0.3
DA/Mtb/SQ	AF6-14	49±5**	5.6±0.3*	1.2±0.3***	1.9±0.8
DA/Mtb/SQ	AF8-14	43±3**	5.3±0.1***	0.9±0.4***	2.8±0.1

Significance values from Control arthritic group (DA/Mtb/SQ/SAL)

* - $p<0.05$
** - $p<0.01$
*** - $p<0.001$

DA/Mtb/SQ Adjuvant made from the lipid Squalane and Myco.tuberculosis used in Dark Agouti rats.

GST Gold sodium thiomalate
AF Auranofin
SAL Saline

TABLE 19 The effects of Gold sodium thiomalate and Auranofin when administered either from day 0 until day 14 or day 6 until day 14 on the adjuvant disease induced in J.C.Lewis rats.

RAT STRAIN /ADJUVANT	TREATMENT	WEIGHT LOSS (gms) ±SEM	FOOTPAD THICKNESS (mm) ±SEM	ARTHRITIC SCORE ±SEM	SERUM GOLD (ug/ml) ±SEM
JC/Mtb/SQ	SAL	10±3	6.2±0.3	1.7±0.6	0
JC/Mtb/SQ	GST0-14	22±4*	7.4±0.2***	3.6±0.3**	4.5±1.0
JC/Mtb/SQ	GST6-14	22±1*	6.2±0.3	1.9±0.3	9.1±1.2
JC/Mtb/SQ	GST8-14	20±3*	5.8±0.1	1.1±0.3	11.3±0.3
JC/Mtb/SQ	AF0-14	27±9*	5.5±0.4	0.1±0.1**	1.5±0.2
JC/Mtb/SQ	AF6-14	35±9**	5.7±0.5	0.2±0.1*	1.6±0.3
JC/Mtb/SQ	AF8-14	43±3**	5.8±0.2	0.2±0.1*	2.3±0.3

Significance values from Control arthritic group (JC/Mtb/SQ/SAL)

* - $p<0.05$
** - $p<0.01$
*** - $p<0.001$

JC/Mtb/SQ Adjuvant made from the lipid Squalane and Myco.tuberculosis used in J.C. Lewis rats.

GST Gold sodium thiomalate
AF Auranofin
SAL Saline

Results

DA/Mtb/SQ/GST

GST was an effective antiarthritic drug in previous experiments when given from day 0 until day 14 against adjuvant induced arthritis in DA rats. These experiments confirmed the previous findings where GST significantly reduced the severity of the polyarthritis disease evidenced by reduced paw swelling and lower arthritic score (6.2±0.2mm $p<0.01$, 1.8±0.3 $p<0.001$ respectively). GST given on this regimen resulted in a serum gold concentration of 4.3±0.9ug/ml. When GST therapy was withheld until day 6 it appeared that gold was still able to significantly reduce the severity of the disease (paw thickness 6.5±0.3mm $p<0.05$, arthritic score 2.7±0.6 $p<0.0.01$). Serum gold levels were greater, than animals treated from day 0 -14 (7.1±3.9ug/ml). When GST was withheld until day8, the development of the polyarthritic disease was not significantly inhibited compared to controls (paw swelling 6.8±0.5mm and arthritic score 3.4±0.9). Serum gold levels in these animals was markedly greater (x2.5) than seen in animals dosed with GST over the full 14day period (11.5±0.7ug/ml).

DA/Mtb/SQ/AF

Auranofin induced a significant decrease in the severity of the adjuvant polyarthritis in DA rats when given either from day0, day6 or day8 onwards (paw swelling 5.8±0.2mm $p<0.01$, 5.6±0.3mm $p<0.05$ and 5.3±0.1mm $p<0.001$ respectively). An increased weight loss was observed when in the delayed dosing groups only. Gold serum

concentrations in these animals appeared independent of the dosing regimen used (2.2±0.3ug/ml, 1.9±0.8ug/ml and 2.8±0.1 respectively).

JC/Mtb/SQ/GST

As seen previously J.C.Lewis produce only a very mild disease with the Mtb/Squalane adjuvant. When these animals were dosed with GST from day0 to day14 there was an exacerbation of the severity of the induced polyarthritis with increased footpad thickness and arthritic score (7.4±0.2mm $p<0.001$ and 3.6±0.3 respectively). Dosing of GST over this period produces a serum gold level similar to that obtained in DA rats (4.5±1.0ug/ml). When GST therapy was withheld until day 6 or day 8 there was no significant effect on the polyarthritic disease (paw swelling 6.2±0.3mm and 5.8±0.1mm respectively). Significant increases in the gold content of serum from both delayed GST treated groups were seen compared to that of continuously treated animals (day0 - day 14) (9.1±1.2ug/ml and 11.3±0.3 respectively).

JC/Mtb/SQ/AF

Auranofin treatment given from day0, day6 or day8 reduced the severity of adjuvant induced arthritis to near normal control levels, although this was not significantly different to the arthritic controls (footpad thickness 5.5±0.4mm, 5.7±0.5mm and 5.8±0.2mm respectively). Serum gold concentrations from rats treated with auranofin on a delayed regimen or dosed therapeutically were not significantly different, however they were less than the levels in GST treated animals (1.5±0.2ug/ml, 1.6±0.3ug/ml, 2.3±0.3ug/ml).

Discussion

GST was shown to be an effective antiarthritic agent when used against polyarthritis in DA rats dosed from day 0-14. By allowing the primary lesion (tail lesion) to establish until day6 and then initiating GST therapy antiarthritic activity was still seen. No significant antiarthritic activity was observed if GST dosing was further restricted until day 8. The therapeutic activity of GST appeared restricted to a time period before and up to day 6 and not after day8. This period coincided with the presentation of the polyarthritic disease in the DA rats which occurred around day 8-10 onwards. Suppression of disease activity therefore required predosing before expression of the arthritic condition to elicit therapeutic activity.

In comparison to GST, AF therapy reduced disease activity independent of the dosing regimen used, even when given as late as day 8. AF therefore either appeared to be a more potent antiarthritic agent than GST or it possesses an entirely different mode of action in this experimental model of arthritic disease. Weight loss in the delayed dosing groups was significantly greater than in the control arthritic group, this possibly indicated a toxicity problem when dosed as a late therapy.

Use of the J.C.Lewis rat strain indicated that GST therapy when given over the full 14 day period was able to exacerbate the polyarthritic disease. In contrast, when GST therapy was delayed until either day6 or day8 no significant difference in the disease activity from the control arthritic group could be detected. Conversely, auranofin therapy caused a significant decreases in the severity of the induced polyarthritis when dosed either from day0, day6 or day8 until day 14.

One important aspect of of these gold therapies was the serum gold levels achieved in these animals. With GST similar serum gold levels were achieved in both DA and J.C.Lewis rats. With delayed dosing both strains had significantly higher levels of serum gold. However, the therapeutic activity of GST did not appear to correlate with gold serum levels. The reason for lower gold levels in the 0-14day dosing groups in both rat strains may have been due to the induction of detoxifying mechanisms in these groups with the subsequent loss of gold from the serum by binding to metallothioneins[52] in the liver and kidneys. Auranofin, a lipophilic drug, produced substantially lower levels of serum gold in all AF treated groups when compared to the GST treated groups, although there was little difference between the differing AF treated groups.

Treatment (0-14days) by GST appeared effective at (i) reducing the polyarthritic disease in DA rats; (ii) exacerbating the polyarthritic disease in J.C.Lewis rats. Delayed administration of GST (i) in DA rats was only effective at reducing the disease

activity if given at day 6 (or before); (ii) in JC Lewis rats appeared not to effect the severity of the disease. Auranofin appeared to reduce the severity of the polyarthritic disease in both rat strains independent of the timing of administration.

These results gave further evidence that the therapeutic mode of action of GST and AF may be different and that their serum biodistribution as well as their systemic distribution may be vastly dissimilar.

EXPERIMENT 7

AIM - To investigate the effect of gold therapy on the water consumption and renal function in rats with adjuvant arthritis induced in Dark Agouti and J.C.Lewis rats.

OUTLINE - Groups of 5 animals were injected with adjuvant on day 0. Each group received either saline, GST or AF from day 0 until day 14. The animals were housed in metabolic cages overnight following each injection of gold (each alternate days). Urinary protein was estimated by using albustix. Gold content was measured as shown previously. Over the 14 day period (a)The volume of water consumed was measured, (b) the renal output was measured, protein in the urine was estimated urine gold content was determined over this period.
(1) Dark Agouti (DA) and (2) J.C Lewis rats.

GROUPS	ADJUVANT	DRUG	AMOUNT mg/kg	TIME PERIOD OF DOSING.
1	Mtb/SQ	SAL	0	0-14
2	Mtb/SQ	GST	12.5	0-14
3	Mtb/SQ	AF	20.8	0-14

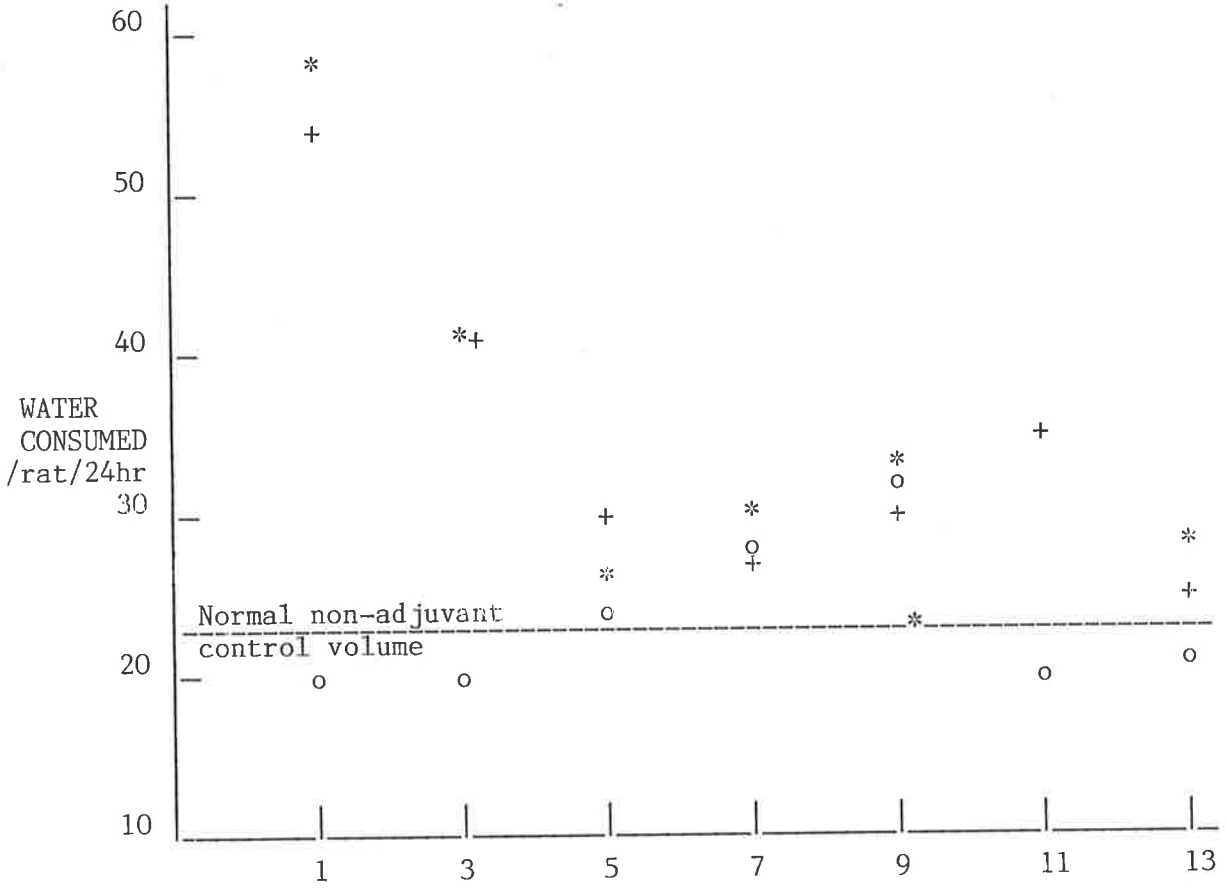
RAT STRAIN - Dark Agouti, J.C Lewis.

ADJUVANTS - Mtb/Squalane (Mtb/SQ)

DRUGS - Gold sodium thiomalate 12.5mg/kg s/c.
Auranofin 20.8mg/kg orally.

ASSESSMENTS - Water consumption.
Urine output
Proteinuria
Urine gold
Kidney histology

FIGURE 6 The water consumption /rat/24hours, urinary volume and protein, ug/ml of gold and total amount of gold/24hrs/24hrs following administration of GST and AF to adjuvant arthritic DA rats over a 14 day period.
"o" = SAL, "*"= GST, "+"= AF.



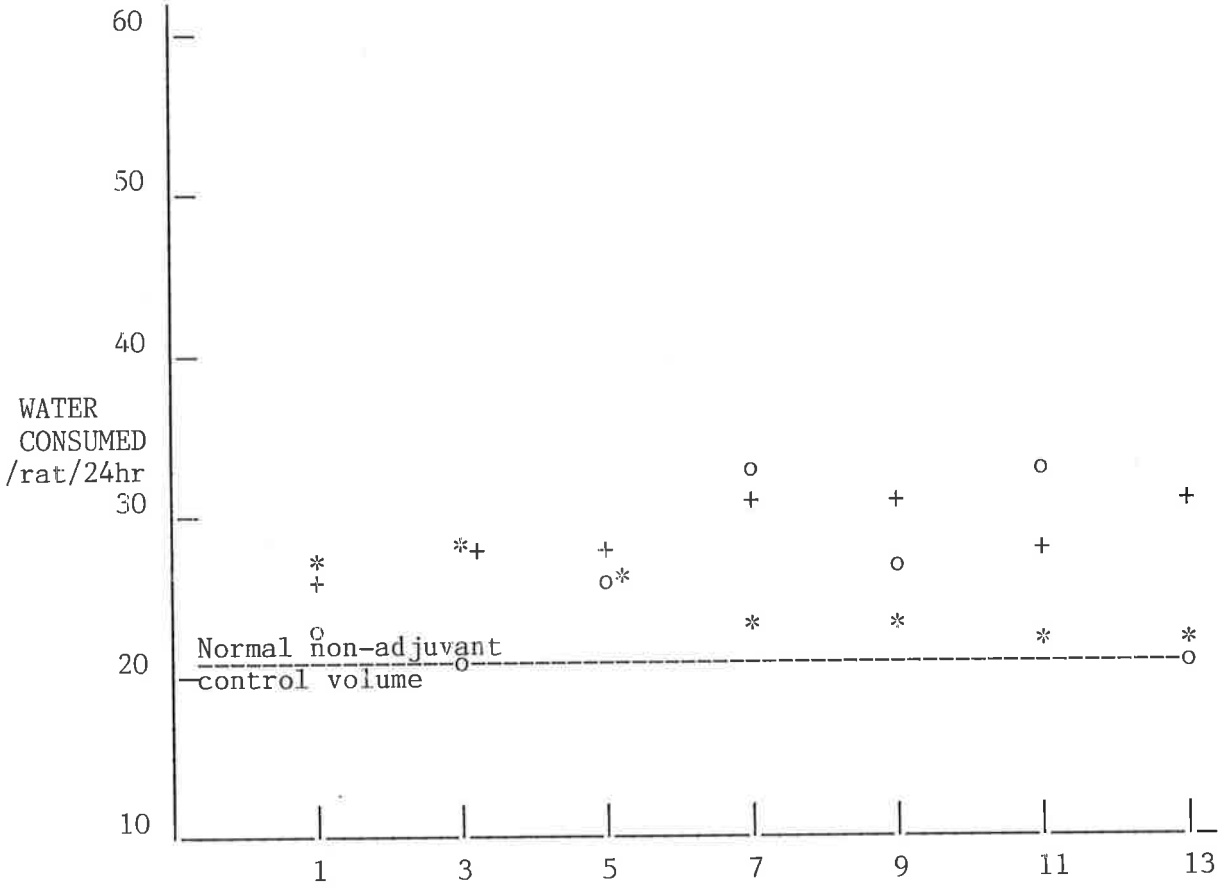
URINARY ASSESSMENT (Normal non-adjuvant urinary volume 17.2ml/24hrs)

SAL only							
ml/rat/24hrs	20	17	18	16	17	19	17
Protein#	+	+	–	–	–	–	–
ug Au/ml urine	–	–	–	–	–	–	–
ug Au/rat/24hr	–	–	–	–	–	–	–
GST							
ml/rat/24hrs	36	38	16	15	17	15	13
Protein#	+++	+	+	–	–	–	–
ug Au/ml urine	19	3	6	5	4	6	6
ug Au/rat/24hr	684	114	96	75	68	90	78
AF							
ml/rat/24hrs	19	12	14	18	16	15	13
Protein#	+	+	–	–	–	–	–
ug Au/ml urine	0.14	0.14	0.08	0.15	0.12	0.14	0.2
ug Au/rat/24hr	3	2	2	3	2	2	3

: +++ = 3mg/ml, ++ = 1-2mg/ml, + = trace amounts

: Days of gold dosing

FIGURE 7 The water consumption /rat/24hours, urinary volume and protein, ug/ml of gold and total amount of gold/24hrs/24hrs following administration of GST and AF to adjuvant arthritic JC Lewis rats over a 14 day period.
"o" = SAL, "*"= GST, "+"= AF.



URINE ASSESSMENT - (Normal non-adjuvant urinary volume 15.5ml/24hrs)

SAL only							
ml/rat/24hrs	18	16	15	17	16	17	16
Protein#	+	+	-	-	-	-	-
ug Au/ml urine	-	-	-	-	-	-	-
ug Au/rat/24hr	-	-	-	-	-	-	-
GST							
ml/rat/24hrs	26	18	16	6	10	12	12
Protein#	+++	++	++	+	-	-	-
Au/ml	11	26	42	69	52	41	38
Au/rat/24hrs	286	684	672	414	520	492	456
AF							
ml/rat/24hrs	10	22	14	13	15	12	13
Protein#	+	+	-	-	-	-	-
ug Au/ml urine	0.6	0.7	1.4	2.0	1.6	1.8	1.5
ug Au/rat/24hr	10	16	20	26	24	22	19

: +++ = 3mg/ml, ++ = 1-2mg/ml, + = trace amounts
: Days of gold dosing

RESULTS

ADJ/DA (FIGURE 6)

The water consumption for a normal rat was 23ml/rat/24hr. The water consumed by DA rats following an injection of adjuvant increased from 23ml/rat/24hrs during days 1-3 to over 30ml/rat/24hr by day 9. After day 9, when adjuvant polyarthritis became apparent, the level of consumption decreased. Urine output over this period did not change significantly and no urinary protein was present.

ADJ/DA/GST

When DA rats were dosed concurrently with GST and adjuvant a marked increase in water consumption occurred at day 1 (57ml/rat/24hrs). An increase in urine volume was detected (36ml/rat/24hr), and a high level of urinary protein (+++) was also measured. A urinary gold concentration of 19ugAu/ml/24hs was measured which meant the total output of gold/rat/24hrs was 684ug, which was 46% of the administered gold dose. Water consumption decreased over the following 4 days until day 5 when it was similar to both the normal control and the untreated adjuvant arthritic group. Urinary protein content decreased over this time and by day 7 was undetectable. The urinary gold content also decreased markedly by day3 and remained comparatively low (3-6ug/ml). Total urinary gold content/ 24hrs also decreased by day 5 and remained at a level of 68-96ug/rat/24hrs (approx 12% of the dose administered).

ADJ/DA/AF

AF administration to adjuvantised DA rats induced a water consumption similar to that induced by GST at day 1 (53mls/rat/24hrs). This decreased over the next 4 days to 29mls/rat/24hrs. In contrast, the urinary volume following AF administration was not significantly changed throughout the experiment and only trace amounts of urinary protein could be detected. Unlike GST therapy, AF resulted in only low levels of urinary gold (0.08ug/ml - 0.15ug/ml). Total gold output was no greater than 3ug/rat/24hr (approximately 0.24% of the administered dose).

ADJ/JC LEWIS (FIGURE 7)

Adjuvant induced an increase in water consumption by JC Lewis rats from 20ml/rat/24hr to approx 30ml/rat/24hrs by day 7 after which it returned to 20ml/rat/24hrs by day 13. There appeared to be no rise in urine output or urinary protein over this period. The normal water consumption for this strain of rats was 21ml/rat/24hr.

ADJ/JC/GST

When JC Lewis rats were dosed with both adjuvant and GST the levels of water consumed was only slightly higher than that seen in adjuvantised animals. There was a rise in the volume of urinary output on day 1 but this returned to control levels by day 5. Urinary protein content was highest at day 1 and continued to be present in measurable amounts until day 7. Urinary gold content rose over the

first 7 days from 11ug/ml to 69ug/ml and then decreased to 38ug/ml by day 13. The total gold content of the urine was 286ug/rat/24hr at day 1 (approx 23% of the dose given) and rose to between 414-684ug/rat/24hr over the rest of the study (33%-55% of the dose administered).

ADJ/JC/AF

Auranofin treatment did not change water consumed by these animals over the experimental period. The urinary output of these animals remained similar to the control group with only trace amounts of urinary protein being detected on day 1 and 3. The amount of urinary gold detected from these rats was higher than that seen in the urine of DA rats treated under the same conditions. The total gold output over the 24 hr period following dosing was 10-26ug/rat/24hr (0.8-2.1% of the administered dose).

DISCUSSION

DA

Adjuvant increased water consumption, above that seen in normal rats, from day 1 until day 7. This could have been due to the presence of fever with associated increased respiration which resulted in increased water requirement. The apparent decrease in water consumption after day 9 may be explained by the onset of systemic arthritic disease due to adjuvant which rendered the animals less mobile. Increased water consumption following the first doses of

GST compared to either the normal control or the adjuvantised groups may have resulted from renal damage (tubular necrosis) reported previously[143]. This damage to the renal tissues was further evidenced by the high renal output, substantial proteinuria and high levels of urinary gold over this time. Following this initial period of gold induced polyuria the renal output and water consumption returned to control levels by day 5 although the gold induced renal tubular necrosis would not repair with in this time. Further, the renal gold levels were dramatically reduced from Day5-day13 to 4ug/ml-6ug/ml. These results indicated that the gold induced polyuria which appeared to be transitory and self-remitting after day 5 and suggested either there may be some form of conservation of gold in these animals, or that there was loss of gold by some other route (ie liver). Detoxification of heavy metals by metalloproteins is an important consideration of metal therapies. The induction of metallothionein synthesis has been shown to occur in the kidneys of rats treated with GST[137]. This may be a detoxification process to protect the kidney from gold induced damage and may explain the self remitting effect.

In comparison to the effects of GST, AF therapy did not induce any increase in urinary output or urinary protein content above that seen in normal and control adjuvant animals and may have been due to very low levels of gold in the urine of these animals. This indicates that the excretion of AF derived gold may not be via the urine but possibly by another route such as the liver.

Although there appeared not to be any increase in the urinary output from AF treated animals there was however marked water consumption. This apparent contradiction between water consumption and urine output may have resulted from loss of water through respiration due to fever and subsequent increase in water requirement. This would not, however, appear to be a reasonable explanation. It has been reported that AF does causes diarrhea in patients and this side effect was noted in these animals over the first 3 days of treatment with AF. An alternative explanation to account for the increased water consumption by these animals therefore would be water loss via the GI tract due to AF induced diarrhea with accompanying water excretion. This increased water intake decreased by day 5 and may in fact suggest that there is some form of detoxification of AF in the GI tract possibly similar to that induced in the kidneys of DA rats treated with GST.

JC LEWIS

In comparison to DA rats JC Lewis rats did not increase their water consumption following GST therapy and although the urine output volume was slightly increased on day 1, little difference was seen from then on. Urinary protein was present up until day 7 and whereas GST in DA rats produced a high urinary gold level on day 1 which decreased on subsequent days, GST treated JC Lewis rats produced high urinary gold which did not decrease on subsequent days. JC Lewis appeared to be unable to inhibit or reduce the amount of gold excreted in the urine and they continued to excrete gold at

between 30-50% of the Au dose administered. These levels were 4-5 orders of magnitude greater than that seen in DA rats. GST in these animals was not therapeutically active in reducing the polyarthritic disease (although the disease is mild) and in fact GST increased the disease severity. The inability of JC Lewis rats to prevent renal gold "leakage" after day 5 showed that this side effect is not self limiting and may reflect the fact that these rats do not respond to gold by increased metallothionein production to detoxify gold.

AF therapy in JC lewis rats also indicated a contrasting effect compared to DA rats where there appeared to be no increase in water consumption. Although only trace amounts of urinary protein were detected there were measurable levels of gold in the urine from these rats (approximately 8x that seen in DA rats).

There were ambivalent findings between these two rat strains in response to gold therapy where GST suppressed adjuvant polyarthritic disease in DA rats and exacerbated it in JC Lewis rats. Further, GST therapy showed different effects on the renal tissues from both rat strains. These results may suggest that endogenous conservation of gold by some means may itself induce therapeutic activity possibly by induction of metallothionein or metal binding proteins by gold.

EXPERIMENT 8

AIM - To investigate the effect of GST therapy on endogenous copper distribution in animals with and without adjuvant induced arthritis.

OUTLINE - Groups of 5 animals were injected with adjuvant on day 0. Each group received either saline or GST at 24 or 48 hours before removal of organs from the animals following exangination on day 14.
Two strains of rat were used in these investigations (1) Dark Agouti (DA) and (2) J.C Lewis rats.

GROUPS	ADJUVANT	DRUG	AMOUNT mg/kg	TIME BEFORE ORGAN REMOVAL
1	NORMAL	SAL	0	24
2	Mtb/SQ	SAL	0	24
3	Mtb/SQ	GST	12.5	24

RAT STRAIN - Dark Agouti, J.C Lewis.

ADJUVANTS - Mtb/Squalane (Mtb/SQ)

DRUGS - Gold sodium thiomalate 12.5mg/kg s/c. given at 48hrs before the end of the experiment

ASSESSMENTS - Tissue copper concentrations

TABLE 20 The effects of gold sodium thiomalate administered subcutaneously on the biodistribution of copper in adjuvantised Dark Agouti and rats.

TISSUE DA RAT	NORMAL ug Cu/gm ±SEM	GROUP 1 SAL ug Cu/gm ±SEM	GROUP 2 GST 48hrs ug Cu/gm ±SEM
KIDNEY	14.7±1.9	15.7±2.8	58.6±5.7$^{\#\#\#}$
LIVER	5.1±0.5	11.4±0.5***	9.3±0.5
THYMUS	4.9±1.7	4.9±2.2	8.7±6.6
ADRENAL	14.1±5.1	18.8±7.5	0.9±0.8$^{\#\#\#}$
SPLEEN	1.3±0.1	4.7±0.3***	4.2±0.2
HEART	7.4±1.4	9.2±2.0	9.0±1.1
BRAIN	3.7±0.3	4.4±0.7	3.9±0.3
SERUM /ml	0.9±0.1	1.8±0.2*	1.5±0.2

Significance values from control normal group (Normal)
* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$

Significance values from control arthritic group (Group 1)
- $p<0.05$, ## - $p<0.01$, ### - $p<0.001$

NORMAL - Non-adjuvantised

GROUP 1 - Adjuvantised non-gold treated

GROUP 2 - Adjuvantised gold treated 48hrs before organs removed.

TABLE 21 The effects of gold sodium thiomalate administered subcutaneously on the biodistribution of copper in adjuvantised JC Lewis rats.

TISSUE J.C.RAT	NORMAL ug Cu/gm ±SEM	GROUP 1 SAL ug Cu/gm ±SEM	GROUP 2 GST 48hrs ug Cu/gm ±SEM
KIDNEY	8.4±0.8	7.7±0.6	65.0±4.9$^{###}$
LIVER	4.3±0.9	11.3±0.6***	9.3±0.9
THYMUS	2.3±0.7	12.8±0.7***	2.9±1.3$^{###}$
ADRENAL	7.0±2.8	8.4±3.3	92.2±24$^{###}$
SPLEEN	1.4±0.2	2.8±0.9	5.4±0.8
HEART	4.2±0.4	6.2±0.5	6.9±0.3
BRAIN	3.2±0.4	3.1±0.3	3.6±0.3
SERUM /ml	1.2±0.1	2.1±0.2*	1.9±0.1

Significance values from control normal group (Normal)
* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$

Significance values from control arthritic group (Group 1)
- $p<0.05$, ## - $p<0.01$, ### - $p<0.001$

NORMAL - Non-adjuvantised

GROUP 1 - Adjuvantised non-gold treated

Group 2 - Adjuvantised gold treated 48hrs before organs removed.

RESULTS

In both rat strains (DA and JC Lewis) the induction of adjuvant polyarthritis results in an increase in serum, liver, and spleen tissue copper concentration (DA; 1.8±0.2ug/gm $p<0.01$, 11.4±0.5ug/gm $p<0.001$, 4.7±0.3ug/gm $p<0.001$ respectively) (LEW; 2.1±0.2ug/gm $p<0.05$, 11.3±0.6ug/gm $p<0.001$, 2.8±0.9ug/gm N.S. respectively), when compared to normal rats. As well, adjuvant also increased the copper concentration in the thymus of JC.Lewis rats (12.8±0.7ug/gm $p<0.001$).

When gold sodium thiomalate was injected once at a concentration of 12.5mg/kg into both adjuvantised DA and JC lewis rats 48 hrs before the end of the experiment, marked increases in kidney copper concentration were seen compared to adjuvantised controls (DA; 58.6±5.7ug/gm $p<0.001$) (LEW; 65.0±4.9ug/gm $p<0.001$). GST dosing also caused changes in copper concentrations in the adrenals and thymus of both rat strains. In the DA rat the adrenal copper concentration was significantly reduced compared to adjuvant control animals 0.9±0.8ug/gm $p<0.001$ and the thymus copper concentration was increased 8.7±6.6ug/gm NS. In contrast GST therapy in the JCLewis rats resulted in a significant increase in the adrenal copper concentration 92.2±24ug/gm $p<0.001$, while significantly decreasing thymic copper concentration compared to adjuvant animals 2.9±1.3ug/ml $p<0.001$.

Discussion

The rise in serum copper in adjuvant arthritis has been shown to result from the acute phase response by increasing the circulating copper protein, caeruloplasmin. It is thought that the rise in serum copper derives from copper liver stores and it appeared that this was so. However in these experiments the concentration in the liver markedly increased as result of adjuvant although this probably was as a result of the increased serum copper within the liver.

One interesting observation was that adjuvant significantly increased thymic copper concentrations in JC Lewis rats when compared to normals and this could not be explained by an increase in serum copper. It is known that the thymus atrophies when under an inflammatory stress and this may have resulted in an increase the measurable copper concentration per gram of this tissue. It is difficult, at this stage, to comment on the adjuvant induced increases in spleen copper concentrations. There may be an increased requirement for copper by the spleen tissues possibly due to the copper dependent synthetic processes, although these increases can be explained by an increase in serum copper.

The marked change in kidney copper concentration induced by GST therapy may be in response to a direct effect of gold on kidney tissue. Heavy metals are known to induce metallothioneins in tissues such as kidneys and previous work[137] has shown that gold did induce

metallothioneins and increasing kidney copper and zinc concentrations. This may be due to an injury by gold to renal tissues and previous experiments have shown that kidney leakage (proteinuria) does occur at least in the first few days. Another possible explanation is that gold displaces endogenous copper from tissue binding sites, and displaced copper is then bound by kidney tissue where there is an increase in metallothionein.

There were contrasting changes seen in the adrenal and thymic tissues in both rat strains. GST therapy causes a marked decrease in adrenal copper concentration in DA rats and an increase in JC Lewis rats. The requirement of the adrenal tissue for copper lies in the glutathione pathway and the production of corticosteroids. Therefore any change in the copper concentration in this organ may affect the production of these compounds with possible consequences for inflammatory reactions. It is not clear at this stage if the increase in copper concentration in J.C.Lewis rats and the decrease in DA rats with gold treatment is due a direct effect of gold on the adrenal tissues or whether it is due to the effect of gold on the expression of the adjuvant disease activity (ie by increasing the severity of the disease in J.C.Lewis or decreasing the disease severity in DA rats).

The effects of gold therapy on the thymus copper concentration may affect the involvement of the thymus in the immunological function of the animals and therefore the expression of

the adjuvant polyarthritis however this is doubtful as the thymus plays little if any role in the inflammatory process in adult animals. One may question the importance of copper in the disease process, however the overwhelming evidence of copper being important as an anti-inflammatory agent suggests that an understanding of the role of, or mechanisms of action of, heavy metal drugs must include their effect on the endogenous copper levels. Gold may well mediate some of its anti-arthritic activity through its influence on endogenous copper and its biodistribution.

CONCLUSIONS

The use of the adjuvant induced arthritis in rats to investigate the therapeutic activity and mode of action of gold in remitting progressive inflammatory conditions, such as rheumatoid arthritis, has been understandably extensive. However the model has been criticised for its lack of similarity to rheumatoid arthritis. However, there are no other biologically more appropriate animal models of inflammation and for this reason its use, although with questionable relevance, remains widespread.

With the use of any assay, standardisation is of prime importance. However with the evolution of the adjuvant-induced arthritis assay this has rarely been adhered to. For instance, previous work using this model (TABLE 6), shows variations in assay procedures. For example there have been differing rats strains used, some inbred, some outbred. Moreover, there have been variations in the adjuvant consistency due to differences in the supply, age and quality of the mineral oil. Further, taking into account variations in housing, age, stress, and infection of the animals, it could be predicted that there would be differences in the outcome in efficacy of gold salts against this disease. A further objective of this thesis, besides investigating the antiarthritic activity of gold salts was to standardise the assay system in hope of understanding the ambivalent findings of previous work.

The use of the DA/Mtb/TO assay provided a system in which GST showed consistent therapeutic efficacy between the dose range of 3.125mg/kg and 25mg/kg and resulted in serum gold levels of 2.3ug/ml - 6.9ug/ml. These results compare well with previous findings on the suppressive activity of GST which used 5-10mg/kg doses of GST. The albumin content of normal serum is 4% (0.67mM) and serum gold from GST administration binds to albumin (>90%)[90-93]. If it is assumed that 90% binding of serum gold to albumin occurred, then from experiment 1 the amount of albumin bound gold in serum containing 0.012-0.035mM of Au is 0.011mM - 0.032mM, and therefore the ratio of gold to albumin was 0.011/0.67 - 0.032/0.67 $\underline{=}$ 1/60 - 1/20. This indicated that at any one time there was only 1 in 60 albumin molecules aurated at the low dose (1.56mg/kg) and 1/20 molecules at the higher dose (25mg/kg). It may be possible to argue that a auration ratio of >1/60 may provide therapeutic efficacy and >1/20 may cause toxic side effects. However, it is important to note that in inflamed animals and humans the serum albumin as well as serum thiol levels fall[106] and therefore the Au binding capacity of serum from inflamed animals may be reduced. This may affect the overall gold-retaining capacity of serum and may possibly lead to increased toxicity. The peak doses immediately following administration are usually much higher[89]. Using this argument as an example it could be of great benefit to study Au/Albumin binding ratios in patients showing therapeutic benefit and also presenting with toxic side effects. Moreover, the use of <u>in vitro</u> prepared aurated albumin (Au-albumin) as an alternative to i/m administered GST therapy may

prove to be of value as the levels of serum gold may be more easily maintained, and possibly will be the subject of further study.

From previous literature[123-132] it can be seen that a number of differing rat strains have been used in the adjuvant arthritic model. This probably reflects the previous use or the local availability of certain rat strains. TABLE 6 shows that adjuvant disease in both Sprague Dawley and Lewis (from America) rats was sensitive to gold therapy, whereas gold was unable to affect disease activity in Wistar rats. From this appraisal of previous literature it was felt that the rat strain used would be an important factor in this assay. When considering these findings, the following points were addressed (i) route of administration of gold drugs, (ii) variation of the drug regimens (iii) different gold preparations (iv) different adjuvants on the efficacy of gold drugs (v) different rat strains.

The results from experiment 2 showed that apart from orally dosed GST all parenteral routes were equally effective at reducing the induced arthritis when dosed therapeutically. Gold loaded-Alzet slow release capsules implanted in these animals provided a therapeutically effective mode of administration of GST similar to subcutaneous dosing. One of the major problems with current dosage regimens has been a peaking of serum gold levels seen in patients and experimental animals following repeated injections of GST[89]. It is, at this stage, not known if this effect is responsible for toxicity

sometimes seen with gold therapy, however high serum gold even transitory should be preferentially avoided. Although sequential gold serum levels were not obtained in these experiments it would appear from other studies using this method of drug administration, that serum peaking of drugs does not occur and the capsules resulted in "trickle feeding" of the administered drug. This would clearly be of great benefit in contrast to repeated injection therapies. Although only limited investigations were carried using this novel drug system the use of these capsules has been widespread and future use of this method of gold administration or of other antiarthritic agents may prove rewarding.

Because of its recent use in the clinic, Auranofin (AF) was the first of the alternative gold complexes to be studied. AF appeared to be therapeutically active in this assay, however problems such as high mortality and diarrhea, have been evident. Lower serum gold levels were found in AF treated rats when compared to GST therapy and this has been used as an argument for the preference of AF to GST. However, this author considers that a number of important points have been overlooked. GST therapy results in >90% of blood borne gold being located in the serum compartment and little if any resides in the cells. Blood gold levels from GST treatment were expressed as Au/ml of serum. The use of this measurement is highly relevant when discussing the blood levels of gold resulting from hydrophilic gold therapy. However, unlike GST, lipophilic AF results in substantial red cell gold levels and so one may then question the

use of blood gold levels expressed as Au/ml serum.

An alternative way of expressing blood levels of gold would be by determining the amount of gold /ml of whole blood. If it is assumed that 54-59% (av.57%) of the blood volume is serum volume alone, then from the results of experiments 1 & 3 the blood gold content /ml of the whole blood [component due to serum (57% of serum concentration of gold) + the component due to rbc gold (rbc gold /ml of blood)] resulting from both GST and AF therapy can be determined and are outlined below in TABLE 22. It can be seen, that the relative blood concentrations of Au /ml of blood are similar for both AF and GST therapies. Moreover, for the high dose of gold (ie 21.8umole dose) the total gold content of the blood /ml is higher in the AF treated group than the GST treated group. These findings are in marked contrast to the concept that AF results in lower blood levels of gold since it is only when the serum levels of gold are compared this is true. In a discussion of the therapeutic efficacy of these two drugs, the nature of the active gold complex and its biodistribution in the body is uncertain and therefore the appropriate expression of blood gold levels is unclear, however blood gold levels expressed as ug/ml of blood may be a more pertinent way of comparing these two widely varying gold complexes.

TABLE 22 Serum, red blood cell and total blood gold levels (ug/ml blood) following administration of Auranofin and GST at different concentrations to arthritic rats. Results are taken from experiment 1 and 3.

GROUP	TREATMENT GOLD umoles/kg	AURANOFIN SERUM GOLD (ug/ml)	AURANOFIN RBC GOLD (ug/ml) (blood)	GST SERUM GOLD (ug/ml)	AF* TOTAL GOLD (ug/ml) (blood)	GST+ TOTAL GOLD (ug/ml) (blood)
1	SAL	0	0	0	0	0
2	2.7	0.53	0.1	2.3	0.4	1.3
3	5.4	1.07	0.73	2.2	1.33	1.25
4	10.9	1.51	0.91	3.7	1.77	2.11
5	21.8	2.19	3.05	5.1	4.29	2.91

GST Gold sodium thiomalate
AF Auranofin
SAL Saline

* Rbc gold/ml of blood + 57% serum gold level

\+ GST - 57% of the serum gold level

Further study of the other gold complexes (experiment 4) showed they had similar efficacy to GST reducing adjuvant arthritis in rats, although the complex Allochrysine was not as effective. These results also confirmed that administration of either thiomalate or phenylmercuric nitrate had no effect on disease activity. Although thiomalate gave no therapeutic efficacy when dosed in this form this does not rule out the possibility that if administered in some other form (ie bound to another metal such as copper), it may have

beneficial activity and these results could not determine if it was partly responsible for the therapeutic activity seen with GST. This idea was further augmented by the fact that D-penicillamine, a thiol containing compound which has been in wide use as an antiarthritic agent, also has never been shown to be active in this assay. This indicated that this assay has limited ability to detect or screen for potential antiarthritic agents.

By the use of an alternative rat strain (JC.Lewis) it was seen that variation in the rat strain used led to marked variation in the therapeutic activity of different gold complexes. In fact, the parenteral gold complexes exacerbated the severity of the induced arthritis in some animals (JC Lewis), whereas Auranofin showed suppressive activity. These findings were in direct contrast to those seen with the DA rat strain and indicated the need for further studies investigating the effects in alternative rat strains, varying adjuvants, and different gold preparations. These detailed studies seen in experiment 5 provide evidence for the need for caution in studying gold (and possibly other drugs) using the adjuvant polyarthritis model in rats. They also indicate why previous findings on the effect of gold in this model have been ambivalent and suggest that future work using this assay system must involve careful monitoring of the assay variables. The genetic variation in susceptibility to adjuvants is of great interest and would suggest that factors exist in some strains that help prevent the induction of systemic polyarthritic disease. Clearly the future study of these

factors may help in the understanding and control of progressive joint diseases. The role in which gold plays in either suppressing or increasing disease activity may be crucial in understanding their mode of action in conditions such as rheumatoid arthritis.

In previous work a number of different dosage regimens have been used with predosing of up to -90 days to post adjuvant injection up to +21days. However, most dosage regimens were therapeutic dosing initiated from day0 (ie day of adjuvant injection). Results from experiment 6 showed that GST was therapeutically active in the DA rat when dosed before day 6 and exacerbated disease activity in JC Lewis rats only when dosed from day 0. It appeared then that GST failed to elicit any effect when dosed at or around the time when the polyarthritic disease was expressed. There are a number of reasons why this contrasting effect may be seen. Thus, predosing is required before any therapeutic effect is elicited and dosing after day 6 does not provide enough systemic storage of gold. Moreover, there exists some GST sensitive component or event, possibly immunological, that is expressed at or around day6-8 after which GST therapy effect has little or no effect. These ideas have some support from the delayed antiarthritic activity seen with chrysotherapy in rheumatoid patients. In regard to RA these possible GST sensitive events may occur in the sequential evolution of the slow progressive destructive processes associated with this disease, however the outward expression of efficacy of GST therapy on this event may only be seen some weeks later.

One interesting aspect of the variation in dosage regimens was that the delayed GST dosage schedules induce substantially higher serum gold levels in both animal strains. Why this happens is at this stage unclear, but it may imply induction of detoxifying mechanisms by prolonged dosing with GST which then act to reduce serum gold levels. The induction of metalloproteins (metallothioneins) is one way the body detoxifies heavy metal insults and is thought to occur in cells such as hepatocytes in the liver and possibly in macrophages. This is further supported by the finding of the major proportion of gold following chrysotherapy inside macrophages of the inflamed synovia[103]. Whether the induction of metallothioneins has any effect on the inflammatory disease process (particularly the adjuvant induced arthritis) remains uncertain, however the stimulation of inflammatory events by gold may in fact be an alternative explanation to the accepted idea of the suppressive effects of GST. This concept is supported by the finding of stimulatory effect of GST on adjuvant induced arthritis in JCLewis rats.

Again in contrast to GST, AF shows therapeutic activity and serum gold levels which are independent of either the dosing regimen or rat strain. The ability of AF to reduce disease activity in both rat strains when dosed beginning as late as day8 indicates antiarthritic activity in this assay possibly by a mechanism different from that of GST.

It was noted that GST treated animals were consuming a larger volume of water than were non-gold treated animals, and a study was undertaken to determine the effect of gold therapy on consumption and how it affected renal function. Experiment 7 showed that GST markedly increased water consumption and renal output in DA rats and did not appear to affect these in JC Lewis rats. Proteinuria occurred in both strains up until day 7, however a major difference between the two strains was in the amount of gold excreted in the urine. JCLewis were unable to reduce the level of gold excreted in the urine where 30-40% of the administered dose /24hrs was apparent in the urine and this was at least fourfold greater than that seen in the DA rat. It is difficult, from these results, to determine why there is this variation between the different rat strains. However, once again these results highlight the difference between rat strains that may be encountered in this assay. The much higher levels of gold in the urine of JC Lewis rats may indicate their inability to either detoxify gold or retain gold for therapeutic benefit. These results are also reflected in animals dosed with AF where although the levels of gold excreted in the urine of JC Lewis rats is much lower than with GST therapy it is still 10x greater that the level seen to be excreted by DA rats treated with AF.

It is difficult to understand why GST dosing increases kidney copper concentrations (experiment 8) but does not appear to increase copper in other tissues such as liver. Metallothionein binding of copper in the kidneys may help to explain some of the

increase and it would appear from previous work by Sharmra that the metal binding proteins are induced in the kidney of rats[136,137]. These results may reflect displacement of endogenous copper by gold from tissue storage sites. Since copper is toxic when uncomplexed, the kidneys may be a site of detoxification so increasing copper in this tissue. Alternatively, gold induces a marked effect on renal tissues and results in proteinuria, increased urinary output, and has been cited as causing renal tubular necrosis[140-145]. The role of the adrenals and thymus in inflammation has been outlined, however the role copper may play in this process in these tissues remains doubtful.

The importance of copper in the disease process may be questioned, however the overwhelming evidence of copper being important as an anti-inflammatory agent suggests that an understanding of the role of, or mechanisms of action of, heavy metal drugs must include their effect on the endogenous copper levels and gold may well mediate some of its anti-arthritic activity through its influence on endogenous copper and its biodistribution.

While of necessity the experiments were terminated 14 days after adjuvant injection the assay had provided valuable information as to why ambivalent results have been seen previously. They have also provided evidence that GST and AF appear to have rather different mechanisms of action.

CHAPTER 3

GOLD THERAPY AND MACROPHAGE FUNCTION

A. SUMMARY

Macrophages do play a vital role in the pathology of diseases such as rheumatoid arthritis (outlined in Chapter 1 of this thesis). Since the introduction of injectable gold derivatives for the treatment of rheumatoid arthritis (chrysotherapy) over four decades ago, there have been many studies of the effects of stabilised gold complexes on biological systems (Tables 4 & 5) including the effect of gold salts on cell functions such as phagocytosis, enzyme release and migration. These experimental studies have indicated a number of biological effects of monovalent gold complexes on macrophages, although their mechanism of action in suppressing or remitting arthritis is not clear. A study has been carried out of the effect of gold salts on two aspects of macrophage functions that have not been widely studied, Fc receptor binding activity (FcBA) and the production of $\cdot O_2^-$ radicals by a PMA stimulus.

Fc receptors are found on a wide variety of cells including macrophages[276-279], polymorphonuclear leucocytes(PMN)[280], B cells and some classes of T cells[281]. The role receptors play in the physiology of these cells has not been well defined. However, for the macrophage, these receptors function, in part, in the recognition and ingestion of immune complexes.

Immunological reactions play a major role in the perpetration of chronic diseases such as rheumatoid arthritis evidenced by lymphoid cell infiltration of synovium, local synthesis of IgG and rheumatoid factor by plasma cells, and the presence of antigen-antibody complexes, IgG-IgG complexes, IgG-IgM(RF) complexes. The recognition and removal of the complexes is thought to be mediated by macrophages, and these cells may be an important source of tissue damaging hydrolytic enzymes[33]. It has been shown in vitro that macrophages secrete lysosomal enzymes when exposed to preformed immune complexes[34].

Through the work of Fridovich and others, it has become apparent that biological systems are able to convert oxygen into a compound of great reactivity[282]. This compound, the superoxide anion ($\cdot O_2^-$) is an extremely powerful oxidation-reduction reagent capable of undergoing either oxidation to O_2 or reduction to H_2O_2 with the liberation of large amounts of energy. The production of this compound by the one electron reduction of oxygen at inflammatory sites has been found responsible for, or at least capable of, damage to extracellular components such as accompanies chronic inflammatory conditions.

The mechanisms of action of agents used to control the inflammatory processes in the clinical situation are only vaguely understood and may mediate some of their anti-inflammatory effects through the inflammatory cells such as macrophages. These cells are capable of producing substantial levels of $\cdot O_2^-$ and they appear to play a major role in the destructive aspects of chronic inflammation.

Modulation of the production of the superoxide radical by these cells may be a mechanism of action of some slow acting anti-rheumatic drugs such as aurothiomalate.

The effects of chrysotherapy on the presentation of Fc receptors on the cell surface of adherent macrophages and $\cdot O_2^-$ radical production by macrophages from both adjuvantised and non-adjuvantised rats treated with and without gold sodium thiomalate has been studied. Moreover the effect of GST on monocytes in vitro has been studied, and the possibility that gold may mediate some of its antiarthritic activities through its effects on Fc receptor expression has been considered.

The results indicate that: (i) parenteral GST produced increases in Fc binding capacity of macrophages isolated from the peritoneal cavity of rats, (ii) inflammation affects the levels of $\cdot O_2^-$ production (iii) gold does have an effect on the production of $\cdot O_2^-$ by macrophages isolated from inflamed and non-inflamed rats. GST may elicit some antiarthritic activity by reducing the Fc binding capacity of blood monocytes and increasing this activity of cells from other tissue compartments.

B. Materials and Methods

1. Adjuvant Disease

On Day 0, groups of 8 male Dark Agouti (DA) (150-250 g) and J.C.Lewis (JC) (200-250g) (Institute of Medical and Veterinary Science, Adelaide) and were injected intradermally near the tail base with 50ul of finely ground, heat-killed, delipidated human strain Mycobacterium tuberculosis (TBC) (Tuberculin Section, Ministry of Agriculture, Fisheries and Food, Weybridge, U.K.) dispersed in squalane (SQ) (Fluka) at a concentration of 10mg/ml. On Day 14 after adjuvant injection, the polyarthritic disease was assessed, by measurement of footpad thickness with a micrometer. Peritoneal exudate cells and peripheral blood monocytes were removed and assessed for Fc binding activity and ability to produce $\cdot O_2^-$ radicals.

2. Drug Treatments

Gold sodium thiomalate was given to the animals over a 14 day period by subcutaneous injection on alternate days commencing on Day 0 at 12.5mg/kg body weight (32 umoles Au/kg).

3. Preparation of purified monocytes

Rats were killed by cervical dislocation and peritoneal cells were harvested by washing out the peritoneal cavity with Hanks balanced salt solution (HBSS) (Flow Laboratories) and exudate cells were then washed three times in HBSS. Peripheral blood was removed before death by cardiac puncture, withdrawing approx 10ml blood /

rat. Peritoneal exudate cells and peripheral blood was then overlayed onto a Ficoll-Hypaque density gradient. This was then centrifuged spun at 400 x g for 45 min and the interface cells removed. Following 3 washes with HBSS. >95% of these isolated cells were found to be esterase-positive (monocytes). The purified cells were then assayed for their Fc binding activity and their ability to produce oxygen radicals as determined by cytochrome C reduction.

4. Fc Assay

Harvested monocyte/macrophage cells were resuspended in RPMI 1640 media (Flow laboratories) at a concentration of 4 x 10^6/mL. 0.5mL of the cell suspension (2 x 10^6 cells) was cultured onto 13mm sterile glass coverslips and then incubated at 37°C in 5% CO_2 incubator for 1 hour. Cells were then washed with HBSS and non-adherent cells removed.

Red blood cells (r.b.c.) from healthy normal rats were washed three times in saline. They were then incubated with purified anti-rat red blood cell IgG (Cappel) for 1 hour at 37°C in Hanks balanced salt solution (HBSS). These IgG coated r.b.c. were then diluted to 4 x 10^7/mL. The concentration of IgG used was lower than that needed for haemagglutination.

0.5mL (2 x 10^6) of IgG (anti-rat r.b.c) coated r.b.c. were overlayed on each macrophage monolayer and incubated for a further 1 hour. Cultures were washed and the non-bound IgG coated r.b.c. were removed. Monolayers were then fixed in 3% Gluteraldehyde and stained then mounted on coverslips and were viewed under a microscope. With

the aid of a graticule the number of adherent r.b.c. / monocyte were determined on each coverslip after counting 200 monocytes per coverslip. Fc binding capacity of normal rats was normalised to 1.00 rbc/ macrophage with the other groups normalised to this.

5. Cytochrome C assay

The ability of monocytes isolated from both the peripheral blood and peritoneal cavity of treated rats were assessed for their ability to reduce cytochrome C as a measure of the production of $\cdot O_2^-$. 100ul (2500 cells) of purified monocyte cell preparations from the peripheral blood or the peritoneal cavity were incubated with 20ul of a 14ug/ml stock solution of Phorbyl Myristrate Acetate (PMA) (Sigma) and 20ul of a 1mM stock solution of cytochrome C (Sigma) in microtitre wells (at least 3 wells / point / experiment). This mixture was incubated at 37°C for 30min. Following the 30min incubation a 100ul sample from each well was taken diluted 1/10 in Hanks buffer and centrifuged at 3000rpm to remove cells. The resultant supernatant was then placed in 1ml cuvettes and the absorption at 550nm was determined. A standard volume 10ul of 0.5M FeCN solution was added to the supernatant and the absorption at 550nm was determined (a measure of the absorption of oxidised cytochrome C). Results were expressed as umoles of cytochrome C reduced /10^6 monocytes / 30min using the following equation taking into account the extinction coefficient of cytochrome C (21.1) and dilution factors of the assay.

$$\text{umoles of cytochrome (reduced) / } 10^6 \text{ cells} = \frac{A-B \times 3317.5}{\text{cell conc.} \times 10^{-6}} / 10^6 \text{macrophages}$$

A - Absorption at 550nm of supernatant
B - Absorption at 550nm of supernatant following the addition of FeCN

EXPERIMENT 9

AIM - To study the effect of GST on the severity of adjuvant-induced polyarthritis in Dark Agouti and JC Lewis rats in relation to studies of Fc binding and superoxide anion production by mononuclear phagocytes.

OUTLINE - Groups of 5 animals were injected with or without adjuvant on day 0. Each group received either saline or GST from day 0 until day 14. Disease severity was assessed on day 14 by measuring footpad thickness. Two rat strains were used in these experiments. (1) Dark Agouti (DA) and (2) J.C Lewis rats.

GROUP	ADJUVANT	DRUG	AMOUNT mg/kg
1	Saline	SAL	0
2	SQ/Mtb	SAL	0
3	Saline	GST	12.5
4	SQ/Mtb	GST	12.5

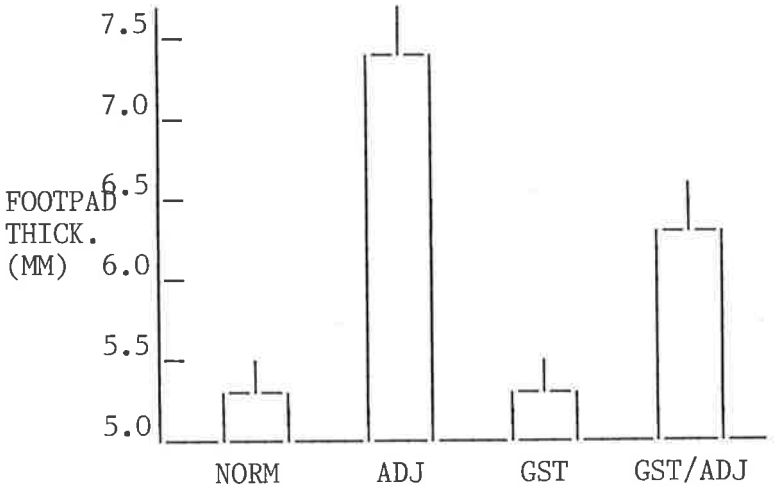
RAT STRAIN - Dark Agouti, J.C Lewis.

ADJUVANTS - Squalane/Mtb (SQ/Mtb)

DRUGS - Gold Sodium thiomalate 12.5mg/kg s/c on alternate days.

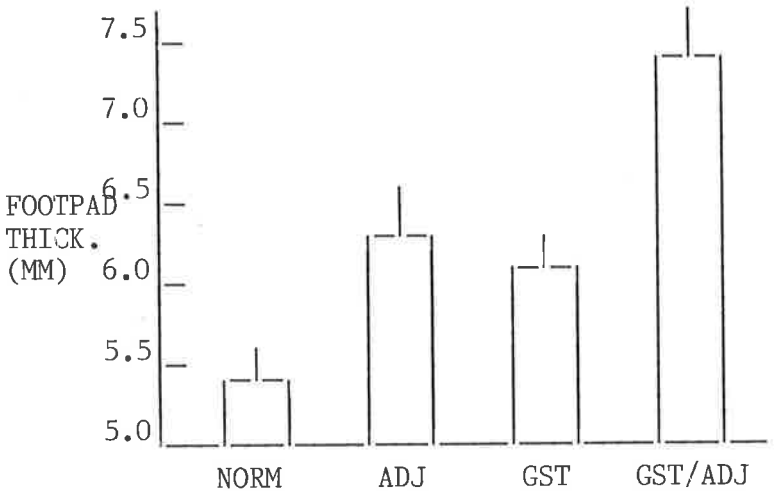
ASSESSMENTS - Disease Severity (footpad thickness)

FIGURE 8 Effects of GST therapy on the footpad thickness of adjuvant induced arthritis in DA rats.



NORM - Non-adjuvantised animals
ADJ - Adjuvant treated animals
GST - Gold sodium thiomalate

FIGURE 9 Effects of GST therapy on the footpad thickness of adjuvant induced arthritis in JC Lewis rats.



NORM - Non-adjuvantised animals
ADJ - Adjuvant treated animals
GST - Gold sodium thiomalate

RESULTS

As seen in earlier experiments presented in this thesis adjuvant induces a severe polyarthritis in Dark Agouti rats (ADJ Footpad thickness = 7.4±0.4mm $p<0.001$). GST therapy markedly reduces the severity of this disease (GST/ADJ footpad thickness = 6.3±0.2mm $p<0.01$) whereas GST therapy alone does not have any effect on footpad thickness in non-adjuvantised animals (GST). In JC Lewis animals only a mild disease was seen in response to adjuvant injection (ADJ footpad thickness = 6.3±0.3mm $p<0.01$) whereas unlike DA rats GST increased the disease severity in these animals GST/ADJ footpad thickness = 7.4±0.2mm $p<0.001$). GST therapy alone increased the footpad thickness, although not significantly, in non-adjuvantised rats (GST).

DISCUSSION

These results confirm earlier findings of the ambivalent nature of GST against adjuvant polyarthritis in rats. These findings will be discussed in subsequent experiments where macrophages/monocytes from the peripheral blood and peritoneal cavity of these animals were harvested and assessed for functional activities.

EXPERIMENT 10

AIM - To investigate the Fc binding capacity of monocytes isolated from peripheral blood and peritoneal cavity of adjuvantised rats treated with and without GST.

OUTLINE - Groups of 5 animals were injected with or without adjuvant on day 0. Each group received either saline or GST from day 0 until day 14. Purified mononuclear cells from either the peripheral blood or the peritoneal cavity were assessed for their Fc binding activity as assessed by the binding of IgG coated rbc.
Two rat strains were used in these experiments.
(1) Dark Agouti (DA) and (2) J.C Lewis rats.

GROUP	ADJUVANT	DRUGS	AMOUNT mg/kg
1	Saline	SAL	0
2	SQ/Mtb	SAL	0
3	Saline	GST	12.5
4	SQ/Mtb	GST	12.5

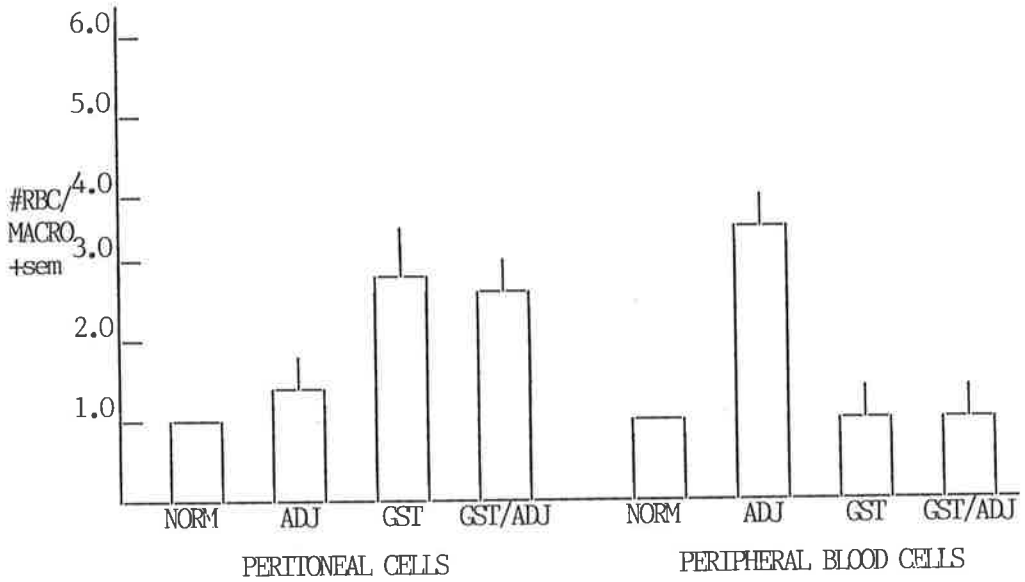
RAT STRAIN - Dark Agouti, J.C Lewis.

ADJUVANTS - Squalane/Mtb (SQ/Mtb)

DRUGS - Gold sodium thiomalate 12.5mg/kg s/c on alternate days.

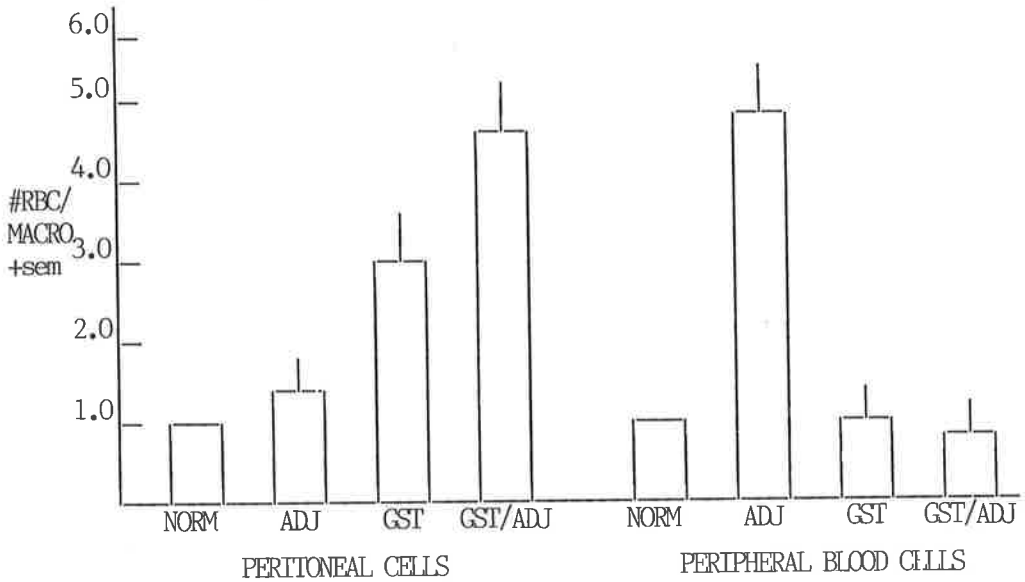
ASSESSMENTS - Fc Binding Activity (FcBA)

FIGURE 10 Changes in Fc binding activity (FcBA) of peritoneal and peripheral blood monocytes from DA rats.



NORM - Non-adjuvantised animals
ADJ - Adjuvant treated animals
GST - Gold sodium thiomalate

FIGURE 11 Changes in Fc binding activity (FcBA) of peritoneal and peripheral blood monocytes from JC Lewis rats.



NORM - Non-adjuvantised animals
ADJ - Adjuvant treated animals
GST - Gold sodium thiomalate

RESULTS

1. D.A. Rats

Figure 8 shows that following adjuvant injection Dark Agouti rats (DA/ADJ) developed a marked polyarthritis by day 14 with a significant increase in footpad thickness. Gold therapy (DA/ADJ/GST) reduced the severity of this adjuvant induced swelling, whereas GST therapy alone (DA/GST) did not affect the paw size in these animals.

Adherent macrophages were isolated from both the peritoneal exudate (PE) and the peripheral blood (PB) of these animals and their ability to bind IgG coated rbc (Fc Binding Activity (FcBA)) was determined. There was no change in the FcBA of PE cells from DA/ADJ animals compared to normal (1.1±0.1) whereas cells isolated from the PB showed a markedly enhanced FcBC (3.3±0.4). The FcBC of cells isolated from the PE of DA/GST and DA/ADJ/GST animals showed a marked increase in FcBA (2.8±0.6, 2.6±0.3) whereas those isolated from the PB did not (1.02±0.1, 0.96±0.2).

2. J.C.Lewis Rats

J.C.Lewis rats appear to be relatively insensitive to the induction of adjuvant polyarthritis using this adjuvant (Figure 9). Gold therapy however, over this period exacerbated the severity of the induced polyarthritis in these rats (JC/ADJ/GST). GST therapy alone in JC lewis rats (JC/GST) caused a significant increase in the footpad thickness of these animals and the presentation of this "disease" was similar to that of the JC/ADJ group seen in the adjuvantised group. The effect of GST therapy in adjuvantised JCLewis

rats (JC/ADJ/GST) was to exacerbate disease activity evidenced by a marked increase in footpad thickness (FIGURE 9).

Although the FcBA of adherent cells isolated from the PE of JC/ADJ rats (1.4±0.2) was not increased compared to normal, cells isolated from the PB of these animals exhibited a marked increase in FcBA (4.83±0.3). GST therapy alone (JC/GST) increased FcBA of adherent PE cells (3.04±0.8) although not affecting the binding of PB adherent macrophages (1.0±0.2). A similar pattern was seen in cells from JC/ADJ/GST animals where GST therapy markedly increased FcBA by PE (4.5±0.7 and did not appear to effect PE cells (0.72±0.4).

Discussion

Gold has been shown to have a number of biological activities both *in vitro* and *in vivo*. It has been shown to inhibit enzymes important in the destructive aspects of the inflammatory process[21,71] as well as affecting a number of functional aspects of inflammatory cells such as macrophages[71,119-122]. Adjuvant induced polyarthritis has been used widely to investigate the mode of action of gold in the inflammatory process as it is generally regarded as the most acceptable model for the study of chronic inflammation rather than acute inflammation. There has been a large amount of work investigating the effects of gold on this model of disease, however the results appear to be ambivalent[123-133]. Recently it has been shown that the effects of gold in this model appear to be dependent not only the adjuvant and strain of animal used but also on the type of gold complex used[133].

Previously it has been reported that gold may both enhance or suppress some immunological responses. These findings have led to the use of the terms "immunomodulatory" and "immunoregulatory" to describe some of the actions of gold[71].

One important event in the development of adjuvant arthritis would be the role of macrophages and their presentation of antigen via Fc receptors to immune effector cells such as lymphocytes. Adjuvant clearly induces a severe polyarthritic disease in DA rats. This was reflected in the relative increases in the FcBA of adherent macrophages from the PB, although no increase in the FcBA by PE cells were noted. The increase in the PB FcBA may have been due to a systemic response to the adjuvant. GST therapy alone increased the FcBA of cells from the PE of both rat strains although not in the PE cells. GST therapy in adjuvant treated rats (DA/ADJ/GST and JC/ADJ/GST) also showed a marked increase FcBA in cells from the PE. However, even though these animals have received adjuvant there was no increase in the FcBA of cells isolated from the PB. These findings were in marked contrast to the effects seen in adjuvant only (ADJ) treated rats suggesting that GST therapy alters cell populations or affects the resident local populations of both these compartments.

A number of possible explanations exist which would explain these findings. GST therapy may have caused (a) altered trafficking of Fc-bearing monocytes so that more of these cells migrated to various compartments such as the peritoneal cavity; (b) affected local resident populations of monocytes which induced increases in FcBA; (c) increased loss of Fc-bearing monocytes in the PB due to

increased adherance; or (d) increased harvesting of Fc-bearing cell from the PE. While the reason for these changes are not known at this stage, these cells did appear to be affected by gold therapy. This study has investigated a functional capacity of cells isolated from inflamed (ADJ) and GST treated animals, whereas a number of previous studies which have only looked at the effect of gold therapy *in vitro*.

Thus, gold therapy in adjuvantised DA rats may stimulate or regulate the immune system thereby enabling it to cope with an insulting adjuvant and reducing disease severity. However gold in J.C.Lewis rats may have caused stimulation of the immune system to the point of exacerbating the disease activity. The idea that gold may act as an immune stimulant is not new, and it would appear that gold may possibly elicit some of its therapeutic activity against adjuvant polyarthritis by stimulation of at least one component of the immune system.

EXPERIMENT 11

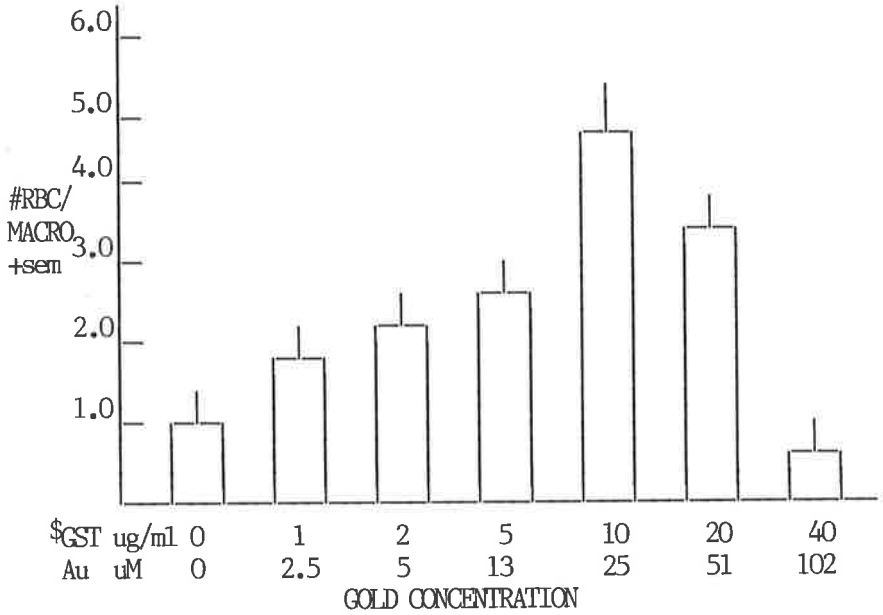
AIM - To investigate the effect of GST on Fc receptor expression of normal peritoneal macrophages isolated from Dark Agouti rats.

OUTLINE - Non-elicited peritoneal exudate cells were isolated from Dark Agouti rats. The cells were purified on a Ficoll gradient then cultured in 16mm wells. GST was added in varying concentrations from 1ug/ml-40ug/ml.

GROUP	DRUGS	AMOUNT GST ug/ml	uM
1	Saline	0	0
2	GST	1	2.5
3	GST	2	5
4	GST	5	12.2
5	GST	10	24.0
6	GST	20	49.0
7	GST	40	98.0

RAT STRAIN - Dark Agouti
DRUGS - Gold Sodium Thiomalate 1,2,5,10,20,40 ug/ml
ASSESSMENTS - Fc receptor binding assay

FIGURE 12 The in vitro effect of various concentrations of GST on Fc binding activity of normal peritoneal macrophages from rats.



$ - Note this is ug/ml of Gold sodium thiomalate (50.5% Au).

RESULTS

It can be seen from FIGURE 12 that GST increases FcBA of normal macrophages/monocytes harvested from the peritoneal cavity of normal untreated DA rats over the dose range 1-10ug/ml. Above this level the FcBA decreases until, at 40ug/ml, the FcBA is below the normal level.

DISCUSSION

It is difficult to equate functional aspects of monocytes *in vitro* at high drug levels from those seen in cell isolated from animals treated systemically with the same drug. However this experiment showed that at relatively low GST levels 0-10ug/ml (0-5uM Au) there is a marked increase in FcBA by PE cells. Previously it has shown that gold salts suppress a number of macrophage activities *in vitro*. However, the gold levels used to demonstrate this effect are much greater than the levels that have been determined in the serum of gold-treated patients and animals. The serum gold levels obtained in rats treated with GST have were measured in experiment 1. They show that the levels obtained by dosing with 1.56-25mg/kg of GST every second day produced stable serum levels of 2.3-6.9ug/ml (12-35uM). Administration of 12.5mg/kg produced a serum gold level of 5.1ug/ml (26uM). This amount of gold when used *in vitro* induced the highest Fc BA = 4.3 ± 0.4 (Figure 12).

GST induced the FcBA both *in vitro* as well as *in vivo* which indicated that this effect of GST on macrophages may have been a

direct of the drug and not as the result of some intermediary step *in vivo*. When higher levels of GST were used *in vitro* (102uM), FcBA activity was lower than normal. The results possibly indicated a suppressive or toxic effect of gold concentrations greater than 26uM. This clearly raises some doubts as to the results seen in previous work (TABLES 4 & 5) where *in vitro* studies used doses far in excess of those used in this experiment, and the levels seen *in vivo* following chrysotherapy. This highlights the difficulties of relating *in vitro* findings to *in vivo* effects.

EXPERIMENT 12

AIM - To investigate the $^{\bullet}O_2^-$ production by monocytes isolated from peripheral blood and peritoneal cavity of adjuvantised rats treated with and with out GST.

OUTLINE - Groups of 5 animals were injected with or without adjuvant on day 0. Each group received either saline or GST from day 0 until day 14. Purified mononuclear cells from either the peripheral blood or the peritoneal cavity were assessed for their ability to reduce cytochrome C Two rat strains were used in these experiments. (1) Dark Agouti (DA) and (2) J.C Lewis rats.

GROUP	ADJUVANT	DRUGS	AMOUNT mg/kg
1	Saline	SAL	0
2	SQ/Mtb	SAL	0
3	Saline	GST	12.5
4	SQ/Mtb	GST	12.5

RAT STRAIN - Dark Agouti, J.C Lewis.

ADJUVANTS - Squalane/Mtb (SQ/Mtb)

DRUGS - Gold Sodium Aurothiomalate 12.5mg/kg s/c on alternate days.

ASSESSMENTS - Cytochrome C reduction

FIGURE 13 $\cdot O_2^-$ production by macrophages/monocytes harvested from adjuvant and GST treated Dark Agouti rats measured by the cytochrome C reduction assay.

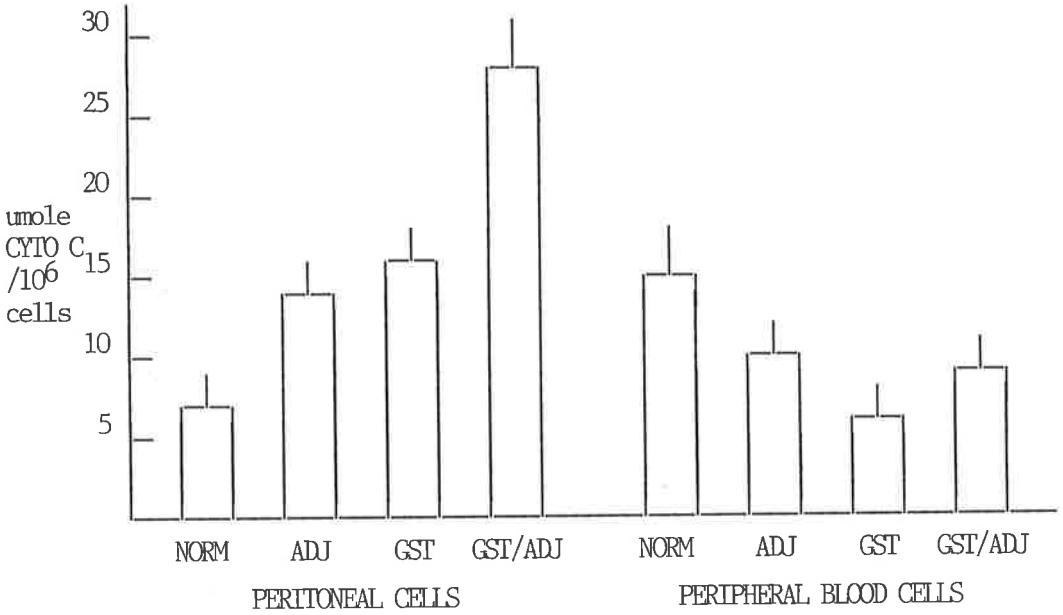
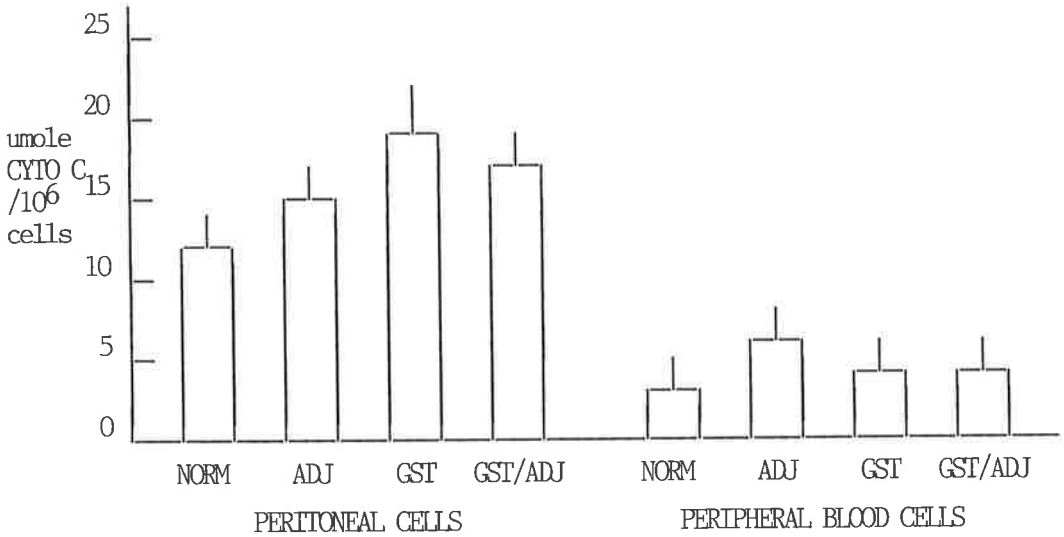


FIGURE 14 $\cdot O_2^-$ production by macrophages/monocytes harvested from adjuvant and GST treated JC Lewis rats measured by the cytochrome C reduction assay.



Results

It can be seen from FIGURE 13 that both adjuvant and GST therapy induced increased $^{\bullet}O_2^-$ radical production from macrophages isolated from the peritoneal cavity of DA rats. This effect was exacerbated when adjuvantised DA rats are treated with GST (GST/ADJ). However the effect of adjuvant and GST on peripheral blood monocytes was to reduce $^{\bullet}O_2^-$ radical production below normal levels. Although these findings were similar in JC Lewis rats the effect of adjuvant and GST was not as pronounced (FIGURE 14) with only GST significantly increasing $^{\bullet}O_2^-$ production above normal levels in cells isolated from the peritoneal cavity. No significant effect were seen in cells from the peripheral blood.

Discussion

These results support the concept that GST therapy acts as a stimulating agent on macrophages which have either migrated to or are resident in, the peritoneal cavity. This is in contrast to the peripheral blood cells which appeared not to be affected by GST (JC Lewis) or show reduced $^{\bullet}O_2^-$ radical production. Increased $^{\bullet}O_2^-$ radical production by macrophages indicates a higher state of activation of the cells. It would appear that GST therapy activated cells in the peritoneal cavity whereas it has the opposite effect in the peripheral blood. These results are similar to the FcBA seen in FIGURES 10 & 11, at least in the peritoneal cavity where GST and adjuvant increase FcBA.

$^{\bullet}O_2^-$ radical production by macrophages has been demonstrated in a number of inflammatory conditions and their ability to induce tissue degradation has been studied[195]. It can be seen from the present results that gold may increase $^{\bullet}O_2^-$ production in migrant cells without showing this effect in the peripheral blood. This stimulation of $^{\bullet}O_2^-$ radical production by macrophages may result in increased tissue degradation, and may result in a more competent cells (macrophages) that can augment the inflammatory reaction; may result from the migration of more mature (stimulated) monocytes to extravascular compartments such as the peritoneal cavity and inflammatory sites, and may represent the systemic activation of cells and immune machanisms.

Conclusions

The mechanism of action of gold in adjuvant polyarthritis is unclear and its effect on the disease is ambivalent depending upon the adjuvant, rat strain and gold drug used. However, gold at low doses does have the ability to stimulate or activate macrophages and one effect of gold may be to induce the production of activated cells which can take part in the inflammatory process. Macrophages are responsible for the production of a large number of pro-inflammatory mediators such as Interleukin-1 and Prostaglandins, and their increased release may lead to exacerbation of the inflammatory response. Whether this is a mode of action of gold is not known, but monocytes in the peripheral circulation would be subject to exposure to concentrations of gold (10-20uM) as would other tissue sites. The concentration of gold in differing tissue compartments varies greatly throughout the body, as does its potential activities (as gold is subject to detoxification or inactivation by metallothioneins). The levels of gold present at inflammatory sites, such as the synovia has been shown to be significantly higher than the serum levels obtained[103,105], although a major proportion of this gold would be bound to detoxifying metalloproteins.

These experiments suggested that there may be an increase in the number of Fc receptors per cell and increased $\cdot O_2^-$ radical production as a result of gold therapy. The process by which GST stimulated Fc binding activity or $\cdot O_2^-$ radical production probably is complex. This may have occurred either by stimulation or activation

of the macrophages by gold, possibly resulting from an increase in metallothionein production by the cells to detoxify gold. Alternatively, gold may alter the trafficking of cells so that more mature or activated monocytes in the peripheral blood selectively migrate into extravascular spaces (such as the peritoneal cavity). The concept of gold selectively affecting trafficking of some cells is supported by the findings of experiment 10 and 12 where there was a decrease in FcBA and $\cdot O_2^-$ radical production by cells from the PB whereas these parameters were increased in cells from the PE. Previous literature suggests that gold therapy reduces the migration of macrophages into an inflamed site[119,120]. If gold does induce the selective migration and retention of activated macrophages in extravascular spaces, this would reduce the number of such mature macrophages in the peripheral blood circulation. It could be argued that this would lead to a reduction in the number of activated macrophages available to effectively migrate to an inflammatory site. There are, of course, many other reasons why gold would reduce the number of macrophages at inflammatory sites[73].

There has been substantial evidence to suggest that Fc receptor appearance and increases in $\cdot O_2^-$ radical production by macrophages indicates that they are mature activated cells. As gold appears to increase these functional activities, then it would appear that gold was capable of stimulating the maturation of the macrophage (possibly by stimulation of metallothioneins) which would result in increased immunocompetence of the cells. This effect can be seen in

vitro (experiment 11) where GST induced an increase in FcBA in cells isolated from the peritoneal cavity of normal rats. Clearly, further work needs to be carried out to clarify these findings.

From these results it can be seen that the study of macrophage functional activities should not be limited to cells isolated from the peripheral blood alone. Although blood is an easily accessible compartment from which to harvest monocytes, studies should encompass cells isolated from other tissues compartments such as the peritoneal cavity or synovial cavity as these cells may respond in differing ways. Further, if diseases such as RA result from an immunodeficiency induced by genetic, viral, or biochemical mechanisms, then it might be possible for this to be reversed by using immunostimulants (such as GST) to bring about a remission of disease activity.

CHAPTER 4

INVESTIGATION OF THE BIOLOGICAL ACTIVITY OF COPPER COMPLEXES PARTICULARLY COPPER-PENICILLAMINE IN ANIMALS.

A. SUMMARY

Some copper complexes have been shown to possess anti-inflammatory properties[245]. However, many that have been categorised as anti-inflammatory also demonstrate irritant properties. This chapter is concerned with the investigation of the biological activity of copper complexes with particular reference to the complex of copper and D-penicillamine $[Cu(I)_8\ Cu(II)_6\ (D\text{-penicillamine})_{12}]\ Cl^{5-}$ which has been shown to have substantial superoxide dismutating activity as well as antiulcerogenic activity[239-241]. The aims of this study were (i) to determine the relative irritancy of a number of copper complexes; (ii) to study the effect of administration of these complexes on the liver metabolism of pentobarbitone measured by induced sleep times; (iii) to investigate the comparative anti-inflammatory activity of these complexes against a number of standard animal models of inflammation including carrageenan paw oedema, TBC impregnated sponge implants and adjuvant induced arthritis in rats; and (iv) to study the relative biodistribution of the mixed valency copper-penicillamine complex.

Results

Copper complexes of Glycine, Salicylate and $CuCl_2$ were all found to be highly irritant when injected into the rear paws of rats, whereas the copper complex of penicillamine disulphide was less irritant. The mixed valency copper complex of D-penicillamine (Cu pen) did not appear to cause any identifiable irritancy, and in contrast with other copper complexes did not affect the pentobarbitone sleep times in rats. The Cu-pen complex was anti-inflammatory when assayed in the TBC impregnated implant assay and produced high levels of copper at the inflammatory site. The complex was also found to have substantial anti-inflammatory activity when assayed in either the carrageenan paw oedema assay or in adjuvant induced arthritis in rats.

The mixed valency copper-penicillamine complex appears to be unique amongst the copper complexes investigated since it is essentially non-irritant although it does possess substantial anti-inflammatory activity and results in marked accumulation ~~increases~~ of copper at inflammatory sites. The systemic biodistribution of copper from this complex shows a particular preference for the reticuloendothelial system.

B. MATERIALS AND METHODS

1. The preparation of copper complexes

Copper-D-penicillamine complex was prepared in a 4:3 molar ratio[233] of D-penicillamine:Cu, by adding 1 volume of D-penicillamine (Sigma) (67mM) in 0.15M NaCl slowly to an equal volume of $CuCl_2$ (Analar BDH) (50mM). The pH was subsequently adjusted to 7.4. After neutralisation, the solution was stable in air and a deep-red to purple in colour. The copper D-penicillamine solution was administered subcutaneously at a dose of 0.5mL/200gms (equivalent to 63 umoles copper/kg.

Other copper complexes of $Cu(Gly)_2$, Cu-penicillamine disulphide (Cuox pen), $Cu(Sal)_2$ were made up in saline (0.15M NaCl) in the ratios Cu:ligand 1:2, 1:1, 1:2 respectively together with $CuCl_2$ at a final copper concentration of 25mM. All copper formulations investigated were administered at 0.5mL/200gms (63umoles of copper/kg) by a subcutaneous injection.

2. The irritancy of copper complexes

0.1 ml of each copper formulation (2.5umole of copper) was injected into the rear paws of J.C.Lewis rats (250-300g) while control animals received saline alone. Footpad oedema (thickness) was assessed at 3 hours and at 8 hours following the injection of copper complexes.

3. Effect on liver metabolism by copper complexes

Groups of four J.C.Lewis rats (250-300g) were injected with the different copper complexes once per day for three days. Weight loss, serum copper and mortality were recorded. "Sleep times" induced by a standard dose of pentobarbitone (25mg/kg) as a measure of liver metabolising ability was measured and pentobarbital sleep times (PST) extended over 1.4 x that of normal indicated significant depression of hepatic pentobarbitone detoxification.

4. Anti-inflammatory assays

(a) Carrageenan Paw Oedema

0.05ml of a 1% sodium carrageenan (Marine Colloids) in 150mM NaCl was injected into the subplantar region of the rear paws of J.C.Lewis rats. The subsequent oedema was assessed by measuring the footpad thickness 3hours after injection. Animals were dosed subcutaneously with the red-violet copper-D-penicillamine complex at different times up to 48hrs prior to and 60min following the carrageenan injection while control animals received saline alone.

(b) T.B.C. impregnated sponge

Male Dark Agouti rats (200-300g) were used in these experiments. Experimental granulomas were produced using 15x15x7mm polyurethane sponges (Olympic A31-80), impregnated with 0.5mg/ml Mycobact.tuberculosis. On day 0, while under anaesthesia, each rat received two sponge implants, one on each flank through a dorsal

midline incision. The sponges were washed with ethanol before TBC impregnation to remove any possible contaminants, either chemical or bacterial.

Animals were dosed with either saline (control) or with different copper complexes each day for the last three days of the experiment. After either 5, 7 or 14 days the sponges were surgically excised without the surrounding inflammatory capsule, dried in an oven to a constant weight and the dry weight gain for each determined. In addition, both serum and sponge copper contents were determined using a Technicon atomic absorption spectrometer as previously outlined (page 71).

(c) Adjuvant Induced Polyarthritis

On Day 0, groups of 8 male Dark Agouti (DA) rats (150-250 g) (Institute of Medical and Veterinary Science, Adelaide) were injected intradermally near the tail base with 50ul of finely ground, heat-killed, delipidated human strain Mycobacterium tuberculosis (TBC) (Tuberculin Section, Ministry of Agriculture, Fisheries and Food, Weybridge, U.K.) dispersed in squalane (SQ) (Fluka) at a concentration of 10mg/ml. The animals received the mixed valency copper penicillamine complex subcutaneously on alternate days for a 14 day period, while control animals were dosed with saline alone. On Day 14 following adjuvant injection, the thickness of the hind paws was ascertained and the animal weight loss recorded. The overall severity of the arthritis was assessed, with an arthritis score being

assigned (maximum score = 7) after averaging the scores for front and rear paws (0-3 for each front paw; 0-4 for each rear paw) depending on the number of lesions and whether there was prominent ankle or wrist involvement.

5. Copper distribution study

(a)Radiochemical

^{64}Copper-D-penicillamine complex was prepared as above using ^{64}Cu labelled $CuCl_2$ (50mM) in 0.15M NaCl

(b)Biological

Male J.C. Lewis rats (average weight 250±30g ;Gilles Plains Field Station, Adelaide, South Australia) were housed in metabolic cages. Carrageenan paw oedema was induced by injecting 0.1mL of a 1% solution of sodium carrageenan (Marine Colloids) in 150mM NaCl sub-plantar in both rear paws 1 hour before administering the ^{64}Cu-labelled compound. The control group for this experiment consisted of non-inflamed animals. In the first experiment, groups of both control and experimental animals (n=4) were sacrificed at intervals of 2.5, 5, 24 and 48 hours following drug administration. Organs were excised and specimens taken.

In the second experiment animals were implanted with TBC impregnated sponge implants as detailed above. Animals were treated with the copper drug only once 5 days after the sponge implantation, then sacrificed 24 hours later and tissue samples taken. The control group for this experiment consisted of non-implanted animals.

(c) Analytical

Radioactivity was determined using an LKB Model 1260 Multigamma counter. Tissue copper levels were measured by using a Varian Technicon atomic absorption spectrometer following digestion of the tissues with nitric acid[283] and expressed as ug of copper/gram of wet wt. tissue.

% Relative specific activity of each tissue (% R.S.A.) was determined by:

$$\%\ \text{R.S.A.} = \frac{[\text{COUNTS/GRAM OF TISSUE}]}{[\text{ug COPPER/GRAM OF TISSUE}]} \times \frac{100}{[\text{SPECIFIC ACTIVITY OF THE LABELLED COMPLEX}]}$$

EXPERIMENT 13

AIM - To investigate the irritancy of some copper complexes injected into the rear paws of rats.

OUTLINE - Groups of 5 rats were injected in the rear paws with a either saline, or copper complexes of glycine, salicylate, penicillamine disulphide or D-penicillamine.
Irritancy of these complexes was determined by measuring paw oedema at 3 and 8 hours following the administration of these complexes.

GROUP	COPPER COMPLEX	AMOUNT umoles	ROUTE
1	Saline	0	s/p
2	$Cu(Gly)_2$	2.5	s/p
3	$CuoxPen^2$	2.5	s/p
4	$Cu(Sal)_2$	2.5	s/p
5	$Cu\text{-}Pen)^2$	2.5	s/p
6	$CuCl_2$	2.5	s/p

RAT STRAIN - J.C.Lewis

COPPER COMPLEXES - $Cu(Gly)_2$, $Cu(Sal)_2$, $CuCl_2$
CuoxPen: Cu(Penicillamine disulphide)$_2$
Cu-Pen: $[Cu(I)_8Cu(II)_6$ (D-Penicillamine)$_{12}]Cl^{5-}$
Route of administration sub-plantar (s/p)

ASSESSMENTS - Footpad thickness

TABLE 23 The irritancy of different copper complexes injected into the rear paws of rats measured by paw oedema at 3hrs and 8hrs following injection.

COPPER COMPLEX	FOOTPAD THICKNESS(mm.) 3 HOURS	8 HOURS
Saline	5.19±0.03	5.12±0.03
$Cu(GLY)_2$	6.29±0.04***	6.44±0.09***
CuoxPen	5.67±0.04**	5.65±0.07**
$Cu(Sal)_2$	6.48±0.06***	6.47±0.14***
CuPen	5.17±0.02	5.15±0.03
$CuCl_2$	6.64±0.05***	6.84±0.06***

Significance values from saline-injected control

* - $p<0.05$
** - $p<0.01$
*** - $p<0.001$

$Cu(Gly)_2$: complex of copper and glycine
$Cu(Sal)_2$: complex of copper and salicylate
CuoxPen : Cu(Penicillamine disulphide)$_2$
Cu-Pen : $[Cu(I)_8Cu(II)_6$ (D-Penicillamine)$_{12}]Cl^{5-}$

Results

When saline was injected into the rear paws it did not produce any measurable oedema at 3 or 8 hours following injection (the normal uninjected paw measurement was 5.15±0.03mm). $CuCl_2$ and $Cu(Gly)_2$ both induced severe oedema at 3 hours ($CuCl_2$ 6.64 ±0.05mm, $p<0.001$ and $Cu(Gly)_2$ 6.29±0.04mm $p<0.001$) which was increased at 8 hours (6.84±0.06mm $p<0.001$ and 6.44±0.04mm $p<0.001$ respectively). An injection of $Cu(Sal)_2$ or Cuoxpen caused significant increases in the paw size at 3 hours ($Cu(Sal)_2$ 6.48 ±0.06mm $p<0.001$ Cuoxpen 5.67±0.04mm $p<0.01$), and the swelling remained unchanged at 8hours (6.47±0.14mm and 5.65±0.07 respectively). CuPen was the only copper complex used in this experiment which did not induce any measurable oedema (5.17 ±0.02mm at 3hours and 5.15±0.03mm at 8hours).

Discussion

A number of copper complexes are highly irritant when administered either subcutaneously, intradermally or orally[248,302,304]. Oedema formation appears to be due to the reaction of copper from these complexes with tissue components resulting in the release of oedema inducing mediators. Results in TABLE 23 show that $Cu(Gly)_2$, $Cu(Sal)_2$ and $CuCl_2$ when injected into the rear paws of rats induced a marked increase of paw thickness.

A problem which occurs when trying to assess the relative anti-inflammatory activity of such complexes is the effect of irritancy on the inflammatory assay used to assess its therapeutic efficacy. It has been suggested that the anti-inflammatory

effectiveness of some copper complexes may be due in part to their counter irritation (induction of endogenous anti-inflammatory proteins or mediators)[302]. If these copper complexes were to be used as therapeutic agents then a major problem would exist in the form of their local irritation at the site of administration. While Cu-salicylate has been used as a therapeutic agent in horses for the treatment of shin sores, large amounts of salicylate must be used to reduce the irritation of this drug.

Cuox Pen was irritant when injected into the paws of rats, although producing only moderate oedema. This may have been due to the higher affinity for copper by penicillamine disulphide compared with the other ligands resulting in less copper being available to become uncoupled from the ligand to react which tissue components.

When the mixed valency complex of copper and D-penicillamine is injected into the paws of rats it did not induce any measurable irritant effect, indicating that copper is probably tightly bound into the complex and not released at the site of administration. The stability of this complex has already been investigated previously and it has been found that the complex was excreted in the urine intact following i/v administration[238]. This indicated that this complex is comparatively biological stable. The non-irritant properties of this complex clearly has important implications in providing a non-irritant form of injectable copper.

EXPERIMENT 14

AIM - To investigate the hepatotoxicity of some copper complexes injected into rats.

OUTLINE - Groups of 5 rats were injected with either saline, or copper complexes of glycine, salicylate, penicillamine disulphide or D-penicillamine once per day for three days, after which these rats were challenged with pentobarbitone 25mg/kg (male) given i.p.. Sleeping times were compared to saline treated (normal) groups. Extended pentobarbitone sleep times (PST), >1.4 x that of normal, indicated significant depression of hepatic pentobarbitone detoxication.

GROUP	COPPER COMPLEX	AMOUNT umoles	ROUTE
1	Saline	0	s/c
2	$Cu(Gly)_2$	63	s/c
3	$CuoxPen^2$	63	s/c
4	$Cu(Sal)_2$	63	s/c
5	$Cu-Pen)^2$	63	s/c
6	$CuCl_2$	63	s/c

RAT STRAIN - J.C.Lewis

COPPER COMPLEXES - $Cu(Gly)_2$, $Cu(Sal)_2$, $CuCl_2$
CuoxPen: Cu(Penicillamine disulphide)$_2$
Cu-Pen: $[Cu(I)_8Cu(II)_6$ (D-Penicillamine)$_{12}]Cl^{5-}$
Route of administration sub-plantar (s/p)

ASSESSMENTS - Pentobarbitone induced sleep times
Serum copper
Weight loss
Mortality

TABLE 24 - The toxicity of different copper complexes assessed by measured by weight change, sleep time serum copper and mortality.

COPPER COMPLEX	WEIGHT LOSS (gms)	SLEEP TIME (minutes)	SERUM COPPER (ug/ml)	MORTALITY %
Saline	+3±2	69±5.3	1.0±0.1	0
$Cu(Gly)_2$	7±3	140±3.5***	2.7±0.5**	0
CuoxPen	25±3	149±9.4***	2.4±0.3**	0
$Cu(Sal)_2$	10±4	91±4.1*	1.9±0.2**	0
CuPen	18±3	68±2.5	1.9±0.2**	0
$CuCl_2$	5±2	84±3.5	1.5±0.1*	0

* - $p < 0.05$
** - $p < 0.01$
*** - $p < 0.001$

$Cu(Gly)_2$: complex of copper and glycine
$Cu(Sal)_2$: complex of copper and salicylate
$CuoxPen^2$: Cu(Penicillamine disulphide)$_2$
Cu-Pen : $[Cu(I)_8Cu(II)_6$ (D-Penicillamine)$_{12}]Cl^{5-}$

Results

The saline treated animals added 3gms in weight over the experimental period and a serum copper of 1.0ug/ml was measured. A standard dose of pentobarbitone (25mg/kg) produced a sleeping time of 69 minutes in these control animals.

$Cu(Gly)_2$ dosing in rats caused a 7gm weight loss and resulted in a serum copper level of 2.7ug/ml. The pentobarbitone induced time for $Cu(Gly)_2$ dosed animals was increased to 2x the saline treated group (140 min. $p<0.001$).

Cu-pen disulphide complex caused a 25gm weight loss, a rise in serum copper levels to 2.4ug/ml and an extended sleep time to 2.1x the saline group (149min. $p<0.001$).

$Cu(Sal)_2$ induced a weight loss of 10gms, a rise in serum copper to 1.9ug/ml and an extended sleep time of 1.3x the saline group (91min. $p<0.05$).

$CuCl_2$ caused only a 5gm weight loss, a rise in the serum copper to only 1.5ug/ml and a sleep time of 1.2x saline group (84min not significant).

Cupen was the only copper complex investigated which did not increase the pentobarbitone induced sleep time (68min). However, it caused a weight loss of 18gms and raised the serum copper level to 1.9ug/ml.

Discussion

Untreated inflamed rats lose some of their capacity to metabolise xenobiotics[309,310], probably due to the effect of endogenous anti-inflammatory agents on the liver which augments fibrinogen, haptoglobin and ceruloplasmin synthesis and inhibits the synthesis of albumin (and drug-metabolising enzymes). This loss of hepatic function also may occur with the toxic effects of metal therapies. Both Cu-penicillamine disulphide and $Cu(Gly)_2$ caused a marked depression of the hepatic pentobarbitone detoxification (2x the control level). Moreover, both these copper complexes increased the serum copper levels to greater than 2.0ug/ml. This rise in serum copper may result either (a) by the copper complexes ~~were~~ circulating in the blood not being removed, or, (b) the complexes caused inflammatory stress of their own initiating a rise in the endogenous copper levels (ie ceruloplasmin). $Cu(Sal)_2$ and $CuCl_2$ caused a rise in PST of greater than 1.2x controls which indicates that there was some effect on the hepatic metabolising ability. However, Cupen was the only copper complex studied that did not increase the PST suggesting that the liver was spared from any copper toxicity by this complex probably due to its biological stability <u>in vivo</u>.

EXPERIMENT 15

AIM - To investigate the anti-inflammatory effect of some copper complexes assessed by the TBC-impregnated sponge assay.

OUTLINE - Groups of 5 rats received two sponges in each flank and given saline or copper complexes of glycine, salicylate, penicillamine disulphide or D-penicillamine.
Anti-inflammatory effects were measured by determining the dry weight gain. The copper content was determined over either (i) a 5 day period were the animals were dosed with the copper complexes for the last 3 days or (ii) over 7 and 14 days where the animals were dosed with copper-Pen over this period.

GROUP	COPPER COMPLEX	AMOUNT umoles	ROUTE
1	Saline	0	s/c
2	$Cu(Gly)_2$	63	s/c
3	$CuoxPen^2$	63	s/c
4	$Cu(Sal)_2$	63	s/c
5	$Cu\text{-}Pen)^2$	63	s/c
6	$CuCl_2$	63	s/c

RAT STRAIN - J.C.Lewis

COPPER COMPLEXES - $Cu(Gly)_2$, $Cu(Sal)_2$, $CuCl_2$
CuoxPen: Cu(Penicillamine disulphide)$_2$
Cu-Pen: $[Cu(I)_8Cu(II)_6(\text{D-Penicillamine})_{12}]Cl^{5-}$
Route of administration subcutaneous.(s/c)

ASSESSMENTS - Dry weight gain of excised sponges
Total sponge copper content

TABLE 25 The effect of some copper complexes on sponge dry weight gain and copper content of TBC impregnated sponges over a 5 day period animals dosed with the copper complexes for the last 3 days.

COPPER COMPLEX	DRY WT. GAIN (mgs)	SPONGE COPPER (ug/gm sponge)
Saline	141±0.5	1.52±0.01
$Cu(Gly)_2$	112±1.2***	1.83±0.1
CuoxPen	120±3.0***	2.53±0.1***
$Cu(Sal)_2$	114±2.6**	1.72±0.1
CuPen	130±2.0*	2.43±0.06***
$CuCl_2$	119±3.0***	2.11±0.25*

TABLE 26 The effect of the mixed valency copper-penicillamine complex on sponge dry weight gain and copper content of TBC impregnated sponges over a 7 and 14 day period (animals were dosed for the 7 or 14 period with copper complexes).

DRUG	DRY WT. GAIN (mgms)	SPONGE COPPER (ug/gm sponge)
Saline(0-7)	132±0.92	1.9±0.05
CuPen(0-7)	128±2.1	5.3±0.1***
Saline(0-14)	135±1.2	1.6±0.1
Cupen(0-14)	125±2.5*	7.8±0.1***

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

$Cu(Gly)_2$: complex of copper and glycine
$Cu(Sal)_2$: complex of copper and salicylate
CuoxPen : Cu(Penicillamine disulphide)$_2$
Cu-Pen : $[Cu(I)_8Cu(II)_6$ (D-Penicillamine)$_{12}]Cl^{5-}$

Results

5 Day Sponge Assay

The dry weight gain of the sponges removed from saline treated animals following a 5 day period was 141±0.5mg with a copper content of 1.52±0.01ug/gm of sponge. The effect of dosing animals with $Cu(Gly)_2$ was to significantly reduce the dry weight gain of the sponges to 112±1.2mg $p<0.001$. Copper content of these sponges was however not significantly increased at 1.83±0.1ug/mg. Cu-penicillamine disulphide significantly, reduced the sponge dry wt. gain to 120±3.0mg $p<0.001$ and increased copper content of the sponge at 2.53±0.1ug/gm $p<0.001$. $Cu(Sal)_2$ administration significantly reduced the sponge dry wt. weight of 114±2.6mg, $p<0.01$ but there was no significant effect on the copper content at 1.72±0.1ug/gm. Cupen significantly reduced the dry weight gain of the sponges to 130±2.0mg, $p<0.05$ and significantly increased the copper content to 2.43±0.06ug/gm, $p<0.001$. $CuCl_2$ significantly reduced the sponge dry wt. weight to 119±3.0mg, $p<0.01$ and produced a significant increase in copper concentration to 2.11±0.25ug/gm, $p<0.05$.

7 and 14 Day Assay

The anti-inflammatory effect of Cupen was investigated over both a 7 day period and 14 day period in the sponge assay. It was found that there was a non-significant reduction in the dry weight gain in the sponges excised from Cupen treated rats at 7 days, (128±2.1mg NS), and a significant reduction at 14 days (125±2.5mg,

$p<0.05$). The sponge copper levels from saline treated animals had concentrations of 1.9 ± 0.05ug/gm of sponge at day7, and 1.6 ± 0.1ug/gm of sponge at day14. Sponges from the CuPen treated animals had significantly increased copper concentrations of 5.3 ± 0.1ug/gm ($p<0.001$) of sponge at day7 and 7.8 ± 0.1ug/gm ($p<0.001$) at day14.

Discussion

Many copper complexes have been shown to have anti-inflammatory properties in a number of assays[168]. Dry weight gain of TBC impregnated sponges following implantation into rats represents the the ingress of protein-rich fluid exudate into the sponge matrix and very little of this weight is due to the influx of inflammatory cells[120].

All the copper complexes including $Cu(Gly)_2$, Cuox pen, $Cu(Sal)_2$, Cupen and $CuCl_2$ show significant anti-inflammatory activity in the TBC sponge assay. It would appear that the most irritant copper complexes appear to be the complexes which provide the greatest anti-inflammatory efficacy ie $Cu(Gly)_2$ $Cu(sal)_2$ and $CuCl_2$ in this assay. By contrast Cupen, which is comparatively non-irritant, appears to have the least anti-inflammatory property.

One problem of interpreting the results of this type of assay to estimate anti-inflammatory efficacy arises from the fact that these complexes induce the production of anti-inflammatory proteins due to their irritancy, and this may not reflect the possible role of copper in the inflammatory process or as anti-inflammatory agents. The anti-inflammatory activities assigned

to some previously investigated copper complexes would, therefore, seem to be questionable.

As Cu-pen is a non-irritant form of copper, and because of its ability to substantially increase copper levels at the site of inflammation, this complex may have some value as an injectable non-irritant source of copper. The role of copper at the inflammatory site is not fully understood although it is well known that the copper concentration does increase both in the serum of inflamed animals[177] and at inflammatory sites.

Because of the role of oxygen radicals as prime intoxicants of inflamed tissues the role of copper in mediating or controlling the production of these radicals is of major interest. Given the role of the copper containing protein Superoxide dismutase (SOD) in dismutating these essentially harmful radicals, copper may mediate some of its anti-inflammatory activity at the inflammatory site via SOD activity.

When animals were dosed with Cupen the copper concentration at the inflammatory site was increased markedly and became more pronounced by 14 days. Although this copper complex showed only moderate anti-inflammatory effect in this assay it must be remembered that this assay only measured one component of the inflammatory process (the exudation of inflammatory protein). The anti-inflammatory activity of this complex and its ability to effect copper levels at the inflammatory site is the subject of subsequent experiments.

EXPERIMENT 16

AIM - To investigate the anti-inflammatory activity of the mixed valency copper complex of copper and D-penicillamine assayed in the carrageenan paw oedema assay.

OUTLINE - Groups of 5 rats received 0.5ml of carrageenan injected intradermally into their paws. The animals where dosed with Copper-penicillamine at various times before and after the carrageenan injection. The induced oedema in the paws of the animals were measured by using water displacement technique measured in mls.

GROUP	COPPER COMPLEX	AMOUNT umoles	TIME OF Cu-Pen INJECTION BEFORE CARRAGEENAN.
1	Saline	0	0
2	Cu-Pen	63	-48hrs
3	Cu-Pen	63	-24hrs
4	Cu-Pen	63	-10hrs
5	Cu-Pen	63	- 3hrs
6	Cu-Pen	63	- 2hrs
7	Cu-pen	63	-30min
8	Cu-Pen	63	+30min
9	Cu-Pen	63	+60min

RAT STRAIN - J.C.Lewis

COPPER COMPLEX - Cupen : $[Cu(I)_8Cu(II)_6\ (D\text{-Penicillamine})_{12}]Cl^{5-}$

ASSESSMENT - Footpad oedema assessed by water displacement.

TABLE 27 The effect of copper-penicillamine given at different times on the development of a carrageenan paw oedema in rats measured by water displacement.

GROUP	DRUG	TIME OF Cu-PEN INJECTION	INCREASE IN PAW OEDEMA (mls.)
1	Saline	-	1.02±0.12
2	Cu-Pen	-48hrs	1.04±0.02
3	Cu-Pen	-24hrs	0.86±0.04
4	Cu-Pen	-10hrs	0.62±0.02*
5	Cu-Pen	- 3hrs	0.72±0.07*
6	Cu-pen	- 2hrs	0.65±0.06*
7	Cu-pen	-30min	0.60±0.05*
8	Cu-Pen	+30min	0.35±0.04***
9	Cu-Pen	+60min	0.73±0.13

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Cu-Pen: $[Cu(I)_8Cu(II)_6(D\text{-Penicillamine})_{12}]Cl^{5-}$

RESULTS

Carrageenan injection into the paws of the rats induced a paw size increase due to oedema of 1.02±0.12ml after 3hrs. It can be seen from TABLE 27 that the mixed valency copper D-penicillamine complex $[Cu(I)_8\ Cu(II)_6\ (D\text{-Penicillamine})_{12}]Cl^{5-}$ showed potent anti-inflammatory activity in this assay. Significant reduction of the induced oedema occured when the drug was administered between 10hrs prior to and 30min following the carrageenan injection. Maximum reduction of the paw oedema occurred when the animals were dosed at 30min following the carrageenan injection.

DISCUSSION

Carrageenan-induced paw oedema has been used extensively to assay anti-inflammatory drugs for their in vivo activity. However a number of drugs which exhibit clinical therapeutic activity appear to exert little effect in this assay (gold sodium aurothiomalate, D-penicillamine). However, after complexing copper with D-penicillamine the resulting stable copper complex appeared to exhibit marked anti-inflammatory activity in the carrageenan paw oedema assay. The assay itself is a measurement of acute oedema formation in response to the irritant (carrageenan). The formation of oxygen radicals is thought to be one of the main mechanisms responsible for the primary generation and progression of oedema[285]. Any drug or agent that will either reduce the level of the generated radicals by scavenging or reducing their production may show activity in this model. The $[Cu(I)_8\ Cu(II)_6\ (D\text{-Penicillamine})_{12}]Cl^{5-}$ copper

complex has previously been shown to have substantial oxygen radical scavenging ability *in vitro*[240]. Recent literature, however, has suggested that the complex itself was unable to catalyse the dismutation of $^{\bullet}O_2^-$, but that it slowly decomposes to other copper(II) complexes which possess this property[241]. The results presented here indicate that this complex may also be active *in vivo*.

The inflammatory site is a possible target for copper based therapies and, viewed in conjunction with the results from these experiments and from the results of Experiment 15, the complex appeared to act as an anti-inflammatory agent and was able to increase the copper at inflammatory sites. Whether this indicated that the complex may have acted as an SOD mimic at the site of inflammation is not clear but the complex is biologically stable and has been shown to act as a oxygen radical scavenger *in vitro*. Further experiments will aim to investigate the anti-inflammatory nature of this unique copper complex and to ascertain that the amount of copper entering the inflammatory site was due to the complex itself.

EXPERIMENT 17

AIM - To investigate the possible anti-inflammatory / antiarthritic activity of the copper and D-penicillamine $[Cu(I)_8\ Cu(II)_6\ (D\text{-penicillamine})_{12}]Cl^{5-}$ complex. Determine a dose response against this assay and investigate its activity in relation to D-Penicillamine.

OUTLINE - Groups of 5 rats received an intradermal injection of adjuvant into the base of the tail on day 0. Animals were dosed every two days from day 0 until day 14 with either saline or the copper-penicillamine complex at various doses and the severity of the induced polyarthritis determined.

GROUP	COPPER COMPLEX	AMOUNT CU umoles/kg	ROUTE
1	Saline	0	s/c
2	Cu-Pen	63	s/c
3	Cu-Pen	31.5	s/c
4	Cu-Pen	15.7	s/c
5	Cu-Pen	7.8	s/c
6	Pen	0	s/c

RAT STRAIN - Dark Agouti

ADJUVANT - Mtb/Squalane (Mtb/SQ)

COPPER COMPLEX - $[Cu(I)_8\ Cu(II)_6\ (D\text{-Penicillamine})_{12}]Cl^{5-}$

ASSESSMENTS - Footpad thickness
Arthritic Score
Weight loss
Tail diameter

TABLE 28 The dose response of the copper-penicillamine complex in adjuvant induced arthritis in DA rats.

DRUG/ AMOUNT umoles/kg	WEIGHT LOSS (gms)	FOOTPAD THICKNESS (mm.)	TAIL DIA. (mm.)	A.A. SCORE
NORM	+08±3	5.3±0.1	9.5±0.1	0
SAL	-19±5	6.9±0.3	10.5±0.2	4.1±0.1
63	-20±4	5.4±0.3***	10.1±0.4	0.7±0.2***
31.5	-28±7	5.6±0.4**	10.3±0.2	1.4±0.4**
15.7	-24±2	5.4±0.2**	10.3±0.2	1.1±0.5**
7.8	-28±10	6.2±0.4	10.6±0.3	3.3±0.4
Pen	-23±7	6.8±0.2	10.8±0.5	3.1±1.0

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Pen - D-Penicillamine

Results

The Mtb/squalane adjuvant induced a relatively severe polyarthritis in DA rats with a weight loss of 19±5gms, an increased footpad thickness of 6.9±0.3mm, and arthritic score of 4.1±0.1 by day 14. The copper-penicillamine complex showed a dose response related reduction in the severity of the induced polyarthritic disease. The most therapeutically active dose was 63umoles/kg which significantlly reduced the disease activity to near control levels (footpad thickness 5.4±0.3mm $p<0.001$, arthritic score 0.7±0.2 $p<0.001$). However, the lowest dose used (7.8umoles/kg) had no significant effect on disease activity (footpad thickness 6.2±0.4mm NS, arthritic score 3.3±0.4 NS). The parent compound of Cupen, D-penicillamine, failed to induce any significant antiarthritic activity.

Discussion

Previous work presented in this thesis has shown that the Dark Agouti rat was susceptible to the induction of polyarthritis induced by Mtb/squalane adjuvant over a 14 day period. The copper-penicillamine complex shows a dose dependent suppression of this disease in this animal strain, and the complex was active at the 15.7umole/kg dose level.

It has been shown previously that gold sodium aurothiomalate was active in suppressing disease in this model, whereas D-penicillamine appeared to be inactive. Inactivity of D-penicillamine in this model has been reported previously[231], and this drug appears inactive in most animal models of inflammation. By

contrast the complexing of copper to this thiol-containing ligand resulted in the formation of a complex which was quite strongly antiarthritic against adjuvant induced polyarthritis in rats.

The concept of the complexation of copper to known anti-inflammatory and antiarthritic agents has been discussed by Sorenson who suggested that this may be a mechanism of action of at least some agents for which copper complexation enhances the therapeutic activity of the parent compounds[244]. D-penicillamine was first used for this purpose to remove excess copper in Wilsons disease[232]. It has been suggested that the $[Cu(I)_8\ Cu(II)_6\ (D\text{-Penicillamine})_{12}]Cl^{5-}$ complex may form <u>in vivo</u> [237] and the complex has also been shown to have marked anti-ulcerogenic activity in the Shay rat[239].

It would be unrealistic, at this stage, to suggest that the mechanism of action of D-penicillamine is by the formation of the copper complex $[Cu(I)_8\ Cu(II)_6\ (D\text{-Penicillamine})_{12}]Cl^{5-}$ <u>in vivo</u> which then functions as the active agent. However, the results presented here indicate that this complex may be a potent anti-inflammatory agent in its own right. One possible mode of action of this complex may be to deliver copper to a targeted site where it will be anti-inflammatory or antiarthritic, possibly by $\cdot O_2^-$ radical scavenging activity (as an SOD mimic). Since the biodistribution of this complex may help to determine the site of action of this copper complex, this was studied in the following experiment.

EXPERIMENT 18

AIM - To study the biodistribution of copper following dosing with the ^{64}Cu-penicillamine complex in rats with either carrageenan paw oedema or with implanted TBC-impregnated sponges.

OUTLINE - (i) Carrageenan paw oedema:
Groups of 5 rats were dosed with ^{64}Cu-penicillamine at 3hrs following carrageenan injection. Tissue samples were taken 24hrs following ^{64}Cu-penicillamine injections
(ii) TBC sponge implants:
Groups of 5 rats received two sponges in each flank and were dosed with ^{64}Cu-penicillamine on day5 after sponge implantation. Sponges and tissues were removed 24hr following ^{64}Cu-penicillamine dosing.

GROUP	INFLAMMATORY STRESS	COPPER COMPLEX	TIME REMOVAL OF TISSUES
1	NON-INFLAMED	^{64}Cu-pen	24hrs
2	CARRAGEENAN	^{64}Cu-pen	24hrs
3	TBC SPONGE	^{64}Cu-pen	24hrs

RAT STRAIN - J.C.Lewis

COPPER COMPLEXES - ^{64}Cu labelled copper-penicillamine complex ie. $[Cu(I)_8Cu(II)_6\ (D\text{-Penicillamine})_{12}]Cl^{5-}$

ASSESSMENTS - Tissue samples - (i) copper concentration
(ii) amount of ^{64}Cu

Tissue samples taken:
kidney, liver, spleen, adrenal, brain, blood
thymus, heart, serum,
Sponge and sponge capsule (TBC sponge only)

TABLE 29 Tissue copper concentration and relative specific activity of ^{64}Cu in the carrageenen and sponge granuloma models of inflammation, 24 hours after dosing with [^{64}Cu]-D-Penicillamine.

TISSUE	CONTROL[a]		CARRAGEENAN		SPONGE	
	ug/g +S.E.	% R.S.A.*	ug/g +S.E.	% R.S.A.	ug/g +S.E.	% R.S.A.
KIDNEY	17.5±0.7	86	19.1±1.6	76	13.4±0.9	100
LIVER	24.3±1.2	100	28.2±1.2	100	23.1±0.4	100
SPLEEN	4.1±1.2	01	2.9±0.3	19	2.5±0.1	80
ADRENAL	49.8±19.9	02	34.5±7.2	07	7.1±1.2	21
BRAIN	3.3±0.4	01	3.9±0.4	12	2.5±0.3	06
BLOOD	1.75±0.3	31	2.3±0.4	31	2.5±0.4	51
THYMUS	11.9±4.3	16	28.6±8.7	11	6.2±1.1	25
HEART	5.9±0.4	07	5.7±0.8	03	5.9±0.3	50
SERUM	1.1±0.1	90	1.6±0.2	60	1.8±0.1	100
CAPSULE	-	-	-	-	1.6±0.2	100
SPONGE	-	-	-	-	2.5±0.3	47

a The control group consisted of normal, non-inflamed animals, dosed once subcutaneously with ^{64}Cu-D-penicillamine at 12.5mg/kg.

* A measure of exchangeability of endogenous copper with applied ^{64}Cu

TABLE 30 Amount of copper complex excreted in the urine of treated animals over 24hrs following dosing with either CuPen or CuGly measured by (i) excretion of ^{64}Cu (ii) excretion of coloured complex (Optical Density) (iii) Urinary copper. Results expressed as % of initial dose.

Copper Complex	Amount of ^{64}Cu	Optical Density	Copper Concentration
CUPEN	31%	53%	47%
CuGLY	3%	-	7%

RESULTS

A comparison of ^{64}Cu and copper levels in the carrageenan and sponge granuloma models of inflammation after 24 hours.

Selection of a 24 hour sampling time enabled a valid comparison of ^{64}Cu distribution in the various organs and thus between the two models of inflammation. The carrageenan inflammation is short term (4-6 hours) and mostly oedemic. The sponge granuloma model was chosen as an alternative inflammatory stress because of its substantially longer duration (6 days) and proliferative character. TABLE 29 provides comparative data on both total and radioactive copper per gram of wet weight tissue or body fluid in both these inflammatory models.

(i)In the carrageenan inflamed animals: there was no apparent difference in copper concentrations or % R.S.A. in kidney, liver, adrenals, thymus and heart tissue between either the non-inflamed or inflamed groups. Although there was no difference in copper content of brain or spleen tissue, the two organs contained more ^{64}Cu in the inflamed group than in the non-inflamed group. In the inflamed group, serum showed an increase in copper content with an overall decrease in the % R.S.A. compared to the non-inflamed group.

(ii) In sponge inflamed animals: while the renal copper concentration decreased in the sponge group compared to both non-inflamed and carrageenan groups, the % R.S.A. rose. A similar trend was also seen for adrenals and thymus tissue. Spleen copper concentrations were not significantly different in the three groups; however, the % R.S.A. rapidly rose to a level well above that of the carrageenan group. Copper levels in liver tissue were the same in both inflamed groups and the controls, with 100% R.S.A. indicating a possible saturation of liver binding sites. It would appear that the brain is not a target organ for this copper formulation, as both the copper concentration and the % R.S.A. remained very low for all groups of animals.

Serum copper levels were lowest in the non-inflamed animals raised in the carrageenan group and highest in the sponge granuloma group. The percentage exchangeable copper was very high in the serum of both non-inflamed and granuloma groups but much lower in the

carrageenan group. The copper concentrations in the sponge implants were greater than those of the surrounding tissue capsule.

Discussion

Carrageenan inflammation caused little change in the biodistribution of ^{64}Cu in animals given subcutaneous ^{64}Cu-D-penicillamine, except for minor changes in the blood, the thymus and the adrenal gland. One difficulty in computing changes in radioactivity of the adrenals per standard mass was the variable weights of these organs, ranging from 20 to 80 mg. When the radioactivity was converted to counts per gram of wet tissue, small variations in weight make a substantial difference to the final value (cpm/g).

These results show that the sponge granuloma model of inflammation can substantially alter the distribution of endogenous copper. It is possible, therefore, to propose models of dynamic change in copper biodistribution as a result of inflammatory stress, rather than simply considering total copper or labelled levels alone. The following three types of change in copper status could be considered.

Type I . No change in copper concentration but an increase in the percentage R.S.A. This would suggest that the bio-exchangeability of the tissue-associated copper increases as a result of inflammation. Another possible inference is that the tissue-associated copper is mobilised during inflammation and replaced by exogenous copper from the copper-containing drugs.

Type II. A decrease in concentration of copper with a concomitant increase in the percentage R.S.A. This could occur when the rate of mobilisation from the tissue exceeds the rate of replacement by exogenous copper following inflammatory stress. An example of this mode of copper redistribution is provided by the adrenals from copper-treated animals with sponge granulomae.

Type III. No change in copper concentration or the percentage R.S.A. This may mean that either the organ is not a target tissue for exogenous copper or that the endogenous copper is not readily exchanged during the inflammatory stress. Alternatively, the organ has been saturated with labelled copper, such as occurred in the liver tissue of all three experimental groups treated with ^{64}Cu-D-penicillamine.

The longer-term, more chronic, inflammation associated with the sponge granuloma shows a great tendancy towards Type I and Type II changes in organs such as kidney, liver, spleen, adrenals, thymus and serum. In contrast, the short-term acute inflammatory stress of the carrageenan paw oedema, after 24 hours, showed predominantly Type III changes. Thus, significant differences in biodistribution patterns may only be triggered by long standing inflammatory stress such as accompanies the implantation of irritant impregnated sponges.

With ^{64}Cu-D-penicillamine, the concentration of copper in the connective tissue capsule surrounding the sponge was similar to that of the serum, reflecting a possible equilibrium. However the very high % R.S.A. of the capsule (100%) for ^{64}Cu-D-penicillamine indicated that all of the copper in this tissue was probably due to

copper from the drugs alone. These results suggest that copper moved to inflammatory sites, and that the copper in situ was readily exchangeable.

The copper concentrations in the sponges from animals treated with ^{64}Cu-D-penicillamine were higher than in the corresponding samples of blood or sponge capsules. With the relatively high percentage R.S.A (47%) found in these sponges, these results would indicate that exogenous ^{64}Cu from the copper penicillamine formulation was sequestered at inflammatory sites. This may partly explain how administered copper complexes elicit their anti-inflammatory effect when administered parenterally.

Conclusions

Copper is involved in many biochemical processes[5] some of which are clearly related to inflammation. It is difficult, therefore, to speculate on the possible mechanisms through which the proposed "anti-inflammatory" role of endogenous copper is achieved. However, the requirement for copper in free radical metabolism may provide one explanation of the regulatory action of endogenous copper on the inflammatory response.

During the last decade considerable evidence has been adduced to establish the importance of free radicals as mediators of the inflammatory response[284-286]. Ever since free-radical reactions were first implicated in this process, their relevance to the development of associated pathological changes has been sought. McCord[287] was the first to suggest that such reactions may mediate joint injury occurring in rheumatoid arthritis (RA) and this has led to use of superoxide dismutase (SOD) and drugs with SOD-like activity in the treatment of RA and related disorders[222,288].

Apart from SOD, there are two other aspects of copper metabolism which implicate free-radical reactions in the pathogenesis of RA. First, there is the proposed antioxidant effect of elevated levels of caeruloplasmin[204,205] and, second, it has become increasingly clear that a wide range of copper-amino acid complexes can also act as free radical scavengers. This may be of particular significance in RA since a non-caeruloplasmin bound fraction of serum copper is also reported to be elevated in this disease[190,289].

In vivo copper binding ligands are present in large excess over the copper concentration and consequently virtually all copper is present in a complexed form, either as Cu(I) or Cu(II). The chemistry of copper in these oxidation states is quite different. For example, Cu(II) is associated preferentially but not exclusively with "hard" ligands such as carboxylates or amine groups, whereas Cu(I) is associated mainly with sulphydryl or phosphine groups. Both oxidation states are present in caeruloplasmin (CP), the major transport protein and one which possess anti-inflammatory activity. Cu(II) alone binds to albumin. Metallothionein, which is involved in absorption of copper in the intestine, binds copper as Cu(I).

It has been suggested that it is not the tissue concentration alone of copper which determines its therapeutic usefulness, but the form of copper and its interaction with other proteins and trace elements such as zinc may be important. It is possible that synthetic copper containing molecules mimicking the copper proteins such as CP may prove useful, therefore, as therapeutically active exogenous forms of copper therapy.

It has been shown that CP is able to transport copper to both malignant and normal cells[290] and the role of CP as a copper transport protein is now widely accepted (TABLE 30). CP binds about 95% of the copper in normal serum, and the remaining 5%, which exists primarily as copper complexes of albumin and histidine, may provide a necessary auxiliary transport mechanism.

TABLE 31 Functions of caeruloplasmin

(1) Transport of Cu: Cu -- Cp -- Cu-proteins
(2) Mobilisation of iron into serum: Ferroxidase activity Fe(II) receptor sites
(3) Catalytic oxidation of plasma reductants: Effects on -SH compounds, ascorbate Regulation of oxidation of biogenic amines catecholamines, hydroxyindoles, neuro drugs.
(4) Serum anti-oxidant: Inhibits peroxidation of polyeonates Scavenges superoxide ion.
(5) Endogenous modulator of inflammatory response: Stimulated by leucocyte Endogenous Mediator (Interleukin 1) Acute phase reactant.

There is some evidence to suggest that CP may, at times, not contain a full complement of copper, reflected in the discord between serum oxidase activity and copper levels. Copper serum levels reveal little of this oxidase activity of CP. The potential transfer of copper to active sites and subsequent rise in oxidase activity may be only visualised as a small rise in the serum copper levels. Measurement of serum copper therefore may not be truely indicative of the CP response and activity. Moreover, single serum measurements tell little of the dynamic movement of copper from stores to active sites in the blood.

Previous studies have shown that serum copper increases with increases in liver copper, demonstrated by bile duct ligation resulting in an increase in hepatic copper levels with subsequent increases in serum copper[291]. Moreover, copper injections appeared to increase hepatic copper, and this appears to eventuate from increases in CP synthesis to maintain hepatic copper levels[292-294]. Holtzman and Gaumnitz presented evidence that plasma CP levels are not regulated solely by hepatic copper[295,296], and they found that copper deficient rats release CP at similar rate of release to that seen in normals. In adrenalectamised rats serum copper and CP levels are increased[297-300] whereas copper excretion via the bile duct is reduced[297,300]. Furthermore, corticosteroids have been shown to increase the excretion of copper in the bile[301].

Long term inflammatory stresses such as chronic inflammation increase corticosteroid production. This may increase copper excretion and possibly lead to copper depletion, resulting in loss of copper mediated anti-inflammatory activity such as that which occurs in copper deficient rats[182]. Therefore, copper supplementation therapy may play a crucial role in restoring anti-inflammatory efficacy in either copper deficient or chronically inflamed animals. Copper containing drugs would therefore have a beneficial role in the treatment of chronic inflammatory diseases.

While the therapeutic efficacy of a number of complexes has previously been demonstrated, controversy exists concerning the possible role of their irritancy. Uncomplexed or loosely bound copper reacts with local tissue components resulting in irritancy and oedema formation with the possible induction of endogenous anti-inflammatory mediators. It has been suggested that the anti-inflammatory activity of copper complexes via the parenteral routes of administration may be due to counter-irritation caused by free copper ions, since many of the complexes possessed local irritancy in the absence of oral activity in the rat[302-304].

In Experiment 13, the mixed valency Cu(I/II) D-pen complex showed no irritant effect whereas other copper complexes induced oedema responses. This indicated that any subsequent anti-inflammatory activity shown by this complex probably would not be due to counter-irritation effects and this clearly has important implications when considering this complex as a potential therapeutic agent.

The mixed valency copper(I/II)-D-penicillamine complex has been shown to have a number of biological activities. While its similarity to the endogenous copper containing proteins, caeruloplasmin or superoxide dismutase, may only be coincidental, both contain a number of copper molecules and both have $^{\bullet}O_2^-$ scavenging activity. The non-irritant property of the complex may be reflected in its biological stability.

The complex itself has been shown to be excreted unchanged in the urine of animals[238] and this was noted in the experimental animals used in this thesis. In fact, approximately approximately 50% of the injected copper complex was excreted in the urine of these animals within 24hrs (TABLE 30)

Although a major proportion of the copper, and probably the complex itself, was excreted in the urine of animals treated with Cupen, CuGly induced comparatively small amounts of urinary copper excretion. This suggested the Cupen complex has a urinary excretion pattern which is different from some other copper complexes. This may be of benefit in reducing possible copper toxicity.

The effect of copper on hepatic toxicity is of major concern when investigating heavy metal therapies for inflammatory disorders. The liver is a major site of metabolism and detoxification of drugs and toxic or noxious agent. The acute phase proteins are synthesised in response to substances released from the inflammatory site (eg Interleukin-1) at an enhanced rate and released into the blood. The hepatocyte is so occupied by the synthesis of acute phase reactants and metal binding proteins that it apparently fails in other functions. This leads decreased production of catalase[305], glutathione-S-transferases[306], B-galactosidase and B-N-acetylglucosaminidase[307] and possibly glutathione synthetase and reductase. The resulting decrease of hepatic glutathione is sufficient to account for the occurrence of lipid peroxidation[308].

Lipid peroxides are metabolised by the cytochrome p-450 system, which is subsequently inactivated[309] and decreased metabolism of drugs will be a consequence[310]. Decreased hepatic drug metabolism may then lead to longer half lives of drugs during inflammation.

Enhanced lipid peroxidation may therefore be one of the underlying mechanisms in the development of liver abnormalities which have been reported during rheumatoid arthritis[311], and possibly may occur during therapy with heavy metals, especially copper. Lipid peroxidation is counteracted by increased serum levels of the copper containing antioxidant protein, caeruloplasmin. Inhibition of superoxide mediated lipid peroxidation at the inflammatory site may lead, to decreased amounts of chemotactic substances and of proteolytic enzymes derived from granulocytes and macrophages. This may explain the anti-inflammatory effects of some copper chelates, which are effective superoxide scavengers.

Copper also has the ability to catalyse lipid peroxidation. Thus, the introduction of uncoupled or loosely bound copper molecules in the liver may result in an increase of hepatic lipid peroxidation which would subsequently lead to a decrease in drug metabolism. A convenient way to test depression of metabolic activity is by the use of the sleep inducing drug pentobarbitone. The properties of this drug can be standardised and it is dependent on the the cytochrome p-450 metabolic pathway for its inactivation. With depressed hepatic metabolism of this drug increased sleeping times result.

It can be seen from Experiment 14 that many of the copper complexes tested were able to markedly increased the pentobarbitone sleep time (PBT) probably resulting from induction of lipid peroxidation in the liver with possible induction of anti-inflammatory proteins such as CP and acute phase reactants. CuPen was the only copper complex which did not have any effect on PBT. The paw oedema irritancy of the copper complexes shown in Experiment 13 correlated well with the hepatic effects seen in Experiment 14. In both assays Cupen failed to have any substantial effect, and any anti-inflammatory efficacy of this complex could be due to an anti-inflammatory mechanism which is not dependent upon counter-irritation.

In contrast to other copper complexes $CuCl_2$ did not significantly increase PBT. This is due probably to local precipitation of uncomplexed copper at the injection site reducing the level transported to the liver.

These results emphasise the problems of interpreting data associated with investigating the anti-inflammatory nature of copper drugs. The local tissue irritancy of copper complexes are not the only effect of copper and in fact these results suggest that liver toxicity may also be a problem. However, lipid peroxidation may be an hepatic signal of systemic inflammatory stress, and some of the anti-inflammatory nature of copper complexes may be due to their mimicking the role of inflammatory mediators, inducing lipid peroxidation, and stimulating the endogenous anti-inflammatory

response (ie counter-irritation). This may be an alternative explanation to the therapeutic efficacy of copper complexes. Whether this mode of action is detrimental or not is unclear. Furthermore, it may explain the enhanced therapeutic activity seen with complexes of known anti-inflammatory drugs and copper. Useful forms of copper therapy should elicit anti-inflammatory activity without unwarranted toxic or irritant side effects, but it is still unclear if the effects on the liver metabolism are undesirable.

The successful use of copper complexes for the treatment of inflammatory conditions was reported long before these substances were shown to possess anti-inflammatory activity in animal models of inflammation[167,312]. The clinical observations coupled to animal studies prompted Sorenson[288] to suggest that copper complexes of non-steroidal anti-inflammatory ligands were formed in vivo and that these complexes were responsible for the benifical activity of these drugs. Indeed, Sorenson showed that copper complexes of several non anti-inflammatory complexing agents possessed anti-inflammatory effects in rats, and that copper complexes of established anti-inflammatory drugs were more effective than the parent in this species. Other workers have also demonstrated anti-inflammatory activity for a number of copper complexes in models of acute and chronic hind paw oedema in rats[57,169,302,303,313-318]. As to whether the copper complex of an anti-inflammatory drug is more effective than the parent drug alone has been questioned[302]. In recent studies that anti-inflammatory activity produced by exogenous copper is

usually associated with increased in serum copper[319] which may be due to detoxification of copper by binding to CP in the liver.

With the proposed role of $^{\bullet}O_2^-$ radicals in the mediation of inflammation, the presence of oxygen radical scavenging complexes at the inflammatory site may be of significant value in ameliorating disease activity. The TBC sponge implantation assay was employed in this study to determine whether Cupen could act in this way. The results showed that all copper complexes examined demonstrate some anti-inflammatory activity, and Cuoxpen and Cupen both significantly increased the amount of copper at the inflammatory site. Cupen is known to have $^{\bullet}O_2^-$ radical scavenging properties and may therefore be acting as a mimic of SOD or CP at the site of inflammation. Cupen substantially increased the amount of copper at the inflamed site, but its role at the site remains unclear. Cupen may serve as a copper-based therapeutic anti-inflammatory molecule. It is important to note again that other copper complexes are able to increase copper at inflammatory sites but their local irritancy presents problems of interpreting the effects of their administration.

The classical carrageenan paw oedema assay in which many anti-inflammatory drugs have been tested was used to assess the potential anti-inflammatory activity of Cupen. The formation of oxygen radicals is thought to be one of the principal mechanisms responsible for the primary generation of oedema in the carrageenan assay[285]. Cupen elicited marked anti-inflammatory activity when administered between 10hr before, and 30min after, carrageenan

injection, with the maximum effect seen at 30min before carrageenan injection. These findings indicate that Cupen has a relatively short half life in the body, emphasised by the relatively high urinary excretion levels and biodistribution pattern in the serum. These results suggest that the anti-inflammatory activity of this molecule may not be due to copper loading, but possibly due to the activity of the complex itself.

One of the most widely used animal models of inflammation to assess anti-rheumatic drugs has been the adjuvant induced polyarthritis in rats. In Chapter 2 of this thesis this model was used to investigate the anti-arthritic activity of a number of gold drugs, especially gold sodium aurothiomalate. As Cupen has shown substantial anti-inflammatory activity in previous assay systems it was felt that its efficacy against the adjuvant arthritic model should be tested. Previously the complex has been shown to have anti-ulcerogenic effects in the Shay rats[239]. It was found that the complex does show marked anti-inflammatory properties against adjuvant-induced polyarthritis when dosed between 16-63umole/kg.

This anti-inflammatory activity of Cupen is in direct contrast to the lack of efficacy of D-penicillamine[320,321] in this assay system and raises the following questions as to the mode of action of D-penicillamine: (i) Does D-penicillamine act as a delivery molecule for copper to target tissues as it is known that D-penicillamine binds and transports copper <u>in vivo</u>? (ii) Does copper stabilise D-penicillamine <u>in vivo</u> (ie preventing its oxidation)

thereby increasing its therapeutic activity? (iii) Does the $[Cu(I)_8Cu(II)_6$ (D-Penicillamine)$_{12}]Cl^{5-}$ complex itself, once formed, act directly on target tissues acting as an oxygen radical scavenger? A reason for the delayed effect of D-penicillamine in rheumatoid arthritis may be the very small amounts of Cupen formed *in vivo*. The results presented do not directly answer these questions but suggest a possible role for Cupen as an endogenously produced anti-inflammatory agent during D-penicillamine therapy. In addition to the oxygen radical scavenging properties of Cupen, it has been shown that a combination of copper and D-penicillamine has anti-proliferative effects on T-lymphocytes[243,272] and may act as an immunosuppressive agent, at least *in vitro*. Since T-cells probably play an improtant role in the pathogenesis of rheumatoid arthritis and possibly in adjuvant arthritis, then this may be a mode of action of Cupen against adjuvant disease. This may further suggest that the mode of action of D-penicillamine, and probably Cupen, may be multifactorial and similar to gold drugs.

The final set of experiments have investigated the biodistribution and relative turnover of copper in tissues, inflamed and non-inflamed animals given Cupen using a hitherto unused technique. By determining ^{64}Cu levels and copper concentrations in a number of tissues it was seen that CuCpen has marked effects on copper metabolism of a number of tissues and was able to enter inflammatory sites.

This non-irritant copper complex of D-penicillamine and copper exhibits substantial anti-inflammatory activity in a number of experimental models of inflammation. However, this anti-inflammatory activity appears not to be due to counter-irritation. The molecule is readily excreted and is relatively biologically stable, and increases the copper content in a number of tissues. Although the exact mechanism of action remains unclear, the complex has been implicated as an oxygen radical scavenger and possibly a mimic of superoxide dismutase and caeruloplasmin. It would clearly be presumptuous to assume that D-penicillamine may act against rheumatoid arthritis in this way, but the Cupen complex does offer an agent that possesses marked anti-inflammatory activity of potential clinical use.

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