ADJUVANT INDUCED ARTHRITIS IN THE RAT

A Thesis Presented for the Degree of
Master of Science
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Declaration for the Degree of M.Sc.

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Abbreviations

The following abbreviations are used throughout the text.

CyP450	: Cytochrome P450
DMB	: 11β-17α-dihydroxy-21-methyl-pregna-4-ene-3-20-dione-17-n-butyrate.
EAE	: Experimental allergic encephalitis.
FCA	: Freund's complete adjuvant.
FICA	: Freund's incomplete adjuvant.
HCG	: Human chorionic gonadotrophin.
HPETE	: Hydroxy eicosatetraenoic acid.
i.p.	: Intra-peritoneal.
i.v.	: Intra-venous.
LT	: Leukotriene.
NSAIDS	: Non-steroidal anti-inflammatory drugs.
PG	: Prostaglandin.
PMN	: Polymorphonuclear leucocyte.
PPD	: Purified protein derivative of tuberculin.
s.c.	: Subcutaneous.
SRSA	: Slow reacting substance of anaphylaxis.

Abstract

There is no cure for rheumatoid arthritis despite the use in drug development programmes of the rat adjuvant arthritis model over at least three decades. The histopathology, inflammatory mechanisms and immunological aspects of the model are reviewed. It is concluded that there is no immediately apparent explanation for the evident failure of the model, so far as its relevance to the human disease is concerned.

The marked response of the model to treatment with anti-inflammatory steroids and the non-steroidal anti-inflammatory agents has led to an undue emphasis on such drugs. There is a progressive change in the drug metabolising capability of affected animals, which restricts the introduction of drug administration in established phases of the disease. The chronicity of the model, and the value of taking repeated measurements over a period of time, introduces special problems of data analysis. These three aspects of the model have not been adequately addressed. This suggests that the use of the model has been deficient, not the model itself.

Cyclophosphamide given as a single dose can apparently arrest the disease. Changes in cell mediated immunity as a result of such treatment, tentatively related to changes in levels of circulating lymphocytes and polymorphonuclear leucocytes, suggest that more effective use of the model can be achieved by monitoring these and other aspects of levels of immune competence in adjuvant arthitis.

I

INTRODUCTION

General

In 1954, a disease was first described in the rat which has many similarities to human rheumatoid arthritis (Stoerk, Bielinski et al, 1954). This disease, now almost universally known as "rat adjuvant arthritis", has been extensively used as a model of human rheumatoid arthritis for the development of anti-rheumatic drugs. There is still no cure for the human disease, however, nor is there any really satisfactory treatment. There is only a belief that drug modification of the disease process is possible (Littler, 1981). This thesis explores possible reasons for the apparent failure of the rat model in this context.

There is no historical or archaeological evidence of human rheumatoid arthritis prior to the beginning of the 17th century, whilst there is abundant evidence of osteoarthritis and such inflammatory joint diseases as ankylosing spondylitis, from very early times (Goodwin and Goodwin, 1981). Despite its recent appearance, rheumatoid arthritis now ranks as one of the major diseases of the Western World.

There is a widespread belief that rheumatoid arthritis generally affects persons of late middle-age and old age, and that it is not life threatening. It seems probable that such a view has affected drug development, by establishing a perception of an acceptable risk-benefit ratio, and influencing the research effort expended in relation to the more obviously life-threatening cardiovascular diseases, for example. However, rheumatoid arthritis frequently affects young adults, and has an average age of onset of 40 years (Reynolds, 1978). Being a chronic, crippling disease, it may therefore persist throughout the major part of an individual's adult life. The view that the disease is not life threatening is also a misconception, in so far as the life expectation of a patient with rheumatoid arthritis is considerably less than for a similar healthy individual (Reynolds, 1978).

Rheumatoid arthritis, therefore, has enormous social and economic consequences, which have probably been underestimated, and which might have provided a more powerful spur to anti-rheumatic drug development. Adjuvant arthritis in the rat would appear to provide a useful pharmacological model to aid in the development of new drugs, and to extend basic understanding of the pathogenesis of the human disease. It is replete with relevant mechanisms of the human disease (vide infra), even if it is not an exact analogue, and is produced (almost uniquely) in an ideal laboratory animal for experimental drug development. Progress in

the development of effective drugs has nonetheless been slow. The use of the rat model is therefore reviewed here, in an effort to establish why this should have been so, despite more than thirty years research.

Much of what follows has the admitted advantage of hindsight. There seem, however, to have been two approaches to the development of anti-arthritic drugs. One is based on the assumption that there is a quick, simple and cheap pharmacological model which will lead to an effective therapy, and that the major problem is to find the model rather than the drug. This approach is exemplified by reports of arthritis induced in rabbits (Rawson and Torralba, 1967), pigs (Mansson, Norberg et al, 1971), birds (Brune, Walz et al, 1974), etc., all purporting to offer advantages as models for anti-rheumatic drug development. The second, somewhat neglected alternative, has been to question whether models enough exist already, and that it is their use which has been deficient, not the models themselves. An argument is developed here to support the view that this is particularly true of rat adjuvant arthritis.

Those drugs which have so far been developed for the treatment of human arthritis can be divided into three main categories. The first comprises the anti-inflammatory steroids. First introduced in 1949, these exert quite profound inhibitory effects on the acute manifestations of rheumatoid arthritis

(Littler, 1981). However, it has been known since at least 1970 that "....the corticosteroids are used for their palliative effect", and "the....underlying cause remains" (Goodman and Gilman, 1970). The effects of these compounds suggest therefore that suppression of the acute symptoms of rheumatoid arthritis does not imply that a fundamentally useful treatment has been found, that is, one which inhibits or suppresses tissue and joint damage. Nevertheless, drug development strategies continued for many years to rely on models of acute inflammation.

The second class consists of the non-steroidal anti-inflammatory drugs, the NSAIDs. The development of these agents was based on an elegant and logical biochemical approach (Vane, 1971). The obvious, acute disease manifestations were indeed improved by the NSAIDs (Littler, 1981), which provided fertile grounds for optimism, and led to the development of large numbers of such drugs. What might be criticised was the failure to pay due regard to the fact that other drugs, particularly the anti-inflammatory steroids, were also known to be effective on the acute symptoms, without demonstrably affecting the disease in the longer term (Littler, 1981).

The third class comprises a heterogeneous mixture of compounds grouped under the presumptive heading of "second line anti-rheumatic drugs" (Littler, 1981). These are substances which often have little or no immediate effect on acute

inflammation. Beneficial effects on joint pain, morning stiffness, X-ray indications of joint damage, and biochemical markers such as the erythrocyte sedimentation rate, tend to become apparent after longer periods of use (3 to 6 months for example), which suggests that the disease process may have been modified. Rheumatoid arthritis shows characteristic cycles of exacerbation and remission (Arendt-Racine, Atkinson et al, 1978) such that clear evidence of drug efficacy of this kind requires the study of many patients for periods of at least a year. Consequently, there seem to be no studies yet reported which unambiguously prove the efficacy of these drugs. The consensus of opinion from the available evidence is, however, that drugs such as d-penicillamine, the gold complexes or levamisole do inhibit joint damage and modify the disease process (Littler, 1981). Such long periods of treatment as are required for the study of these compounds exacerbate the toxic side effects which these drugs frequently induce. It may even be argued that the beneficial effects observed may prove partly a consequence of drug toxicity. A degree of debility may be induced in the patient such that there is insufficient metabolic energy available to fully support a chronic inflammation, or the processes responsible for the joint and tissue damage. Drug induced metabolic defects of this kind have been proposed as a therapeutic approach to chronic inflammation (Whitehouse, 1965).

Adjuvant induced arthritis in the rat

In an attempt to produce an animal model of autoimmunity, Stoerk (Stoerk, Bielinski et al, 1954) injected rats with a homogenate of homologous splenic tissue in Freund's Complete Adjuvant (FCA), an oil in water emulsion containing heat killed M. tuberculosum. This material was known to elevate the immune response of the recipient to co-administered antigen(s), the so-called "adjuvant effect". The intention was to trigger an immune reaction to the splenic tissue, which would, by cross-reacting with the host tissue, initiate an autoimmune disease process. Stoerk did in fact observe the development of a generalised chronic inflammation affecting primarily the joints of the hind legs, but spreading to include the fore-limbs and other sites. At about the same time, Pearson, in a similar attempt to create autoimmunity to muscle tissue, produced an almost identical disease to that reported by Stoerk by injecting rats with homogenised muscle tissue in FCA (Pearson, 1956). Stoerk appears not to have investigated the significance of his observations further. Pearson, however, reported that the inclusion of the muscle tissue in the inoculum was irrelevant, and that FCA alone induced the disease when injected into the sub-plantar tissue of one hind paw, or subcutaneously into the tail (Pearson and Wood,

1959). Other routes of administration were not effective (Pearson, Waksman et al, 1961), nor is the injection of M. tuberculosum in saline, or other hydrophilic base, nor injection of the oil base alone (generally referred to as Freund's Incomplete Adjuvant, FICA).

The general features of the disease may be described by reference to the sequence of events observed with Lewis and Wistar rats. Following subplantar injection into one hind paw, the signs of the disease follow a biphasic progression. In the primary phase, immediately following the injection, an acute inflammation appears at the site of injection. This becomes evident within the first hour. There are the classic signs of erythema, swelling and pain. The initial inflammation generally reaches a maximum 4 days after injection, maintains this level until about day 9, and thereafter diminishes slightly. At approximately day 14 in most, but not all, of the animals injected, inflammation at the site of injection increases, accompanied by the appearance of erythema and swelling in the contralateral, uninjected paw, and at both ankle joints. This is the secondary phase, throughout which there is characteristic progressive destruction of the diarthrodial joints. Inflammatory nodules may also appear in the tail, and there is often evidence of involvement of the fore-limbs. There is a loss of body weight within this period, the animals' coats show typical signs of poor condition, and there is often marked convex spinal curvature (Pearson, Waksman et al, 1961). The animals are

sensitive to the touch, and require careful handling. The joint swelling and other signs begin to subside from about day 20 following the initial injection.

The signs described here, it must be stressed, generally relate to strains of rats which are susceptible to the disease. Non-susceptible, or weakly susceptible, strains exist, for which the signs of the disease differ in various respects, or are absent altogether. The important feature is the appearance of systemic disease in responding strains after about 14 days.

The systemic nature of the disease, and the many analogies with the human disease which are discussed later, make this a model of immediate interest to the pharmacologist concerned with drug development. The accessibility of the outward signs of the disease, primarily the marked swelling of the hind limbs, add to the attraction. As is discussed in more detail below, such swelling is very dependent on mediators of acute inflammation, primarily the prostaglandins. Substances which inhibit prostaglandin synthesis are dramatically effective in this model, which largely explains the numbers of such drugs which have come into widespread use in the last two decades. Such drugs do not, however, affect the basic disease in either the rat or the human, although joint damage in the rat is generally retarded. There is no evidence that such is the case for the human disease. This is one important pharmacological aspect where the relevance of the

rat model to the human disease becomes questionable, in the context of the NSAIDs and steroids.

Emphasis on the easily measurable paw swelling is even less justifiable, given the extensive literature relating to virtually all aspects of the disease. Following Stoerk's brief initial account, Pearson in particular undertook a lengthy and detailed study of the disease which has spanned almost 30 years. Other authors have contributed to the point that now the pathology, immunology, biochemistry and pharmacology of this model have all received extensive attention. A problem which remains is that responses differ between strains of rats, and this was not appreciated in the early literature. Otherwise careful studies are compromised by the failure of the authors to specify the rat strain in which the study was performed. In the work reported in this thesis, the Wistar and Lewis strains, which behave very similarly, were used exclusively. These are generally regarded as "good responders", in the sense that there is a high incidence of the disease in appropriately injected animals, and the subsequent disease progression is severe. In contrast, the Wistar A.G. strain shows virtually no evidence of disease following adjuvant injection (Platier, Mach et al, 1982). It is important, therefore, that such a heterogeneity of strain responsiveness is borne in mind when considering comparisons (Pearson, Waksman et al, 1961) between human rheumatoid arthritis and the rat adjuvant disease. With that reservation, however, broad characteristics of

the major lesions in the majority of susceptible animals can be delineated as discussed in the following sections.

Histopathology of the articular and other lesions

The most detailed report of the histopathology of the majority of the induced lesions was provided by Pearson from studies of the disease in Sprague-Dawley rats (Pearson and Wood, 1959). Macroscopically, in the primary phase, swollen, oedematous, hyperaemic tissue appears around the injected foot and ankle, giving way to a dryer, more firm, proliferative tissue in the secondary phase. Microscopically, Pearson observed little or no inflammatory exudate within the joint in the acute phase and a relatively insignificant increase in synovial fluid. There was extensive peri-articular oedema. Infiltrating cells surrounded the tendinous sheath, and were occasionally seen in the subcutaneous tissue. In this phase mononuclear cells predominated. As the secondary phase became established, there was an increasing, diffuse, lymphocytic and histiocytic cell infiltration. Proliferation of connective tissue cells, including fibroblasts, osteoblasts, osteoclasts and chondroblasts occurred, and there was hypertrophy of the synovial lining cells. Hyperplastic tissue encroached into the synovial cavity, subchondral bone, articular cartilage and sometimes to the cortical shaft, condensing in the

later stages to form scar granulation tissue. This bound together adjacent bones, which in some cases fused together.

Radiological investigation has shown a progressive osteoporosis of the tarsal and metatarsal bones of the hind feet. This was detectable by day 10, and became increasingly severe over the subsequent 10 to 20 days (Pearson, Waksman et al, 1961). Similarly detailed autoradiographic investigations have shown evidence of cell proliferation in the joint capsule and synovium on the injected side from day 9 (Mohr and Wild, 1977). These proliferating cells developed and extended over the cartilage surface to form a layer initially one or two cells in thickness. Eventually however the entire cartilage surface was covered, the cell layer increasing progressively in thickness with invasion and destruction of the cartilage (Mohr and Wild, 1977).

Pearson has commented that involvement of polymorphonuclear cells (PMNs) occurs infrequently (Pearson and Wood, 1959, Pearson, Waksman et al, 1961, Hadler, 1976). This is an important point to clarify, since PMNs have seemed to play a central role in the thinking of many pharmacologists seeking treatments for inflammatory diseases. PMNs have been shown to accumulate at the pannus border, and in areas of cartilage loss (Mohr, Wild et al, 1981). Some authors regard PMNs as being of decisive importance in the destruction of cartilage. Certainly, PMN production as measured by the numbers appearing in the circulation is elevated

several-fold in this disease (Glenn, Bowman et al, 1977). However, despite the emphasis on the role played in the human and rat disease by the PMN, it is unnecessary to invoke this cell as a prime mediator of tissue and cartilage destruction. Cells with many of the characteristics of macrophages and fibroblasts are predominant in normal synovium (Fassbender, 1975), and there is infiltration of macrophages and fibroblasts as the disease progresses. These cells can act independently and co-operatively to induce major collagen resorption (Vaes, Huybrechts-Godin et al, 1980), are capable of cell division and multiplication, and can continuously synthesise the enzymes required to maintain the tissue damage observed (Keller, 1980).

In summary, therefore, the major feature of the disease is a progressive destruction of the diarthrodial joints, following proliferation and infiltration of PMNs, macrophages, fibroblasts and lymphocytes.

In human rheumatoid arthritis, involvement of tissues other than those of the joints is frequently observed. Rheumatoid nodules occur, usually singly, over bony prominences, and persist for months or years (Pearson, Waksman et al, 1961). Typically, such nodules show one or more central foci of necrosis, each surrounded by a palisade of large mononuclear cells, histiocyte or fibroblast in origin, and by a zone of dense and relatively avascular fibrous tissue diffusely infiltrated with lymphocytes

and plasma cells (Pearson, Waksman et al, 1961). In the rat, nodules also appear, most frequently on the tail. The lesions are less persistent, however, and the three zones observable in the human rheumatoid nodule are not so clearly defined in the case of the rat. They seem to exhibit greater similarity with the nodules of human erythema nodosum and rheumatic fever (Pearson, Waksman et al, 1961).

Chronic skin lesions are also observed in the later stages of the rat disease, which arise from changes which begin early in the disease, with a mononuclear cell infiltrate of the upper corium (Pearson, Waksman et al, 1961). The epidermis is invaded by PMNs, with formation of spongiform vesicles and pustules in the upper dermis. There is prominent acanthosis with patches of para- and hyperkeratosis.

Areas of caseation in the lungs have often been described (Pearson, Waksman et al, 1961, Silverstein and Sokoloff, 1960, Colombo, Steele et al, 1979). Iritis is frequently seen (Metzke and Tilgner, 1977), as is diarrhoea associated with changes in the gastrointestinal tract, although the connection between the two phenomena remains unclear (Pearson, Waksman et al, 1961). Mucocutaneous lesions have also been described (Pearson, Waksman et al, 1961).

Mediators of inflammation in the arthritic rat

The rationale for the development of the majority of drugs, in particular the NSAIDs, which have resulted from the use of the rat adjuvant model, has been based on an increasing understanding of the metabolism of arachidonic acid, and its role in inflammation. The pathways involved are shown in outline in Figure I-1. Arachidonic acid is present as a structural component of phospholipids in all tissues of the body (Ramwell, Leovey et al, 1977). It is the precursor of all bis-enoic prostaglandins (PGs). The initiating stage of arachidonic acid metabolism, which triggers the "arachidonic acid cascade", is the liberation of free arachidonic acid by phospholipases. The major subsequent metabolic routes are controlled by two classes of enzymes. The cyclo-oxygenases, which have received most attention in the past, are the enzymes leading eventually to the synthesis of the prostaglandins and thromboxanes. The biological effects of these substances are complex, and in some cases conflicting. Table I-1 lists the major effects attributed to specific chemical species along this pathway. Some are pro-inflammatory, causing increased vascular permeability, hyperalgia, platelet aggregation etc. Others, PGE2 for example, can potentiate the cardinal signs of inflammation. The discovery of this pathway, and the increasing

awareness of the role played by the products formed as mediators of inflammation, must be regarded as a major advance.

Figure I-1. Oxidative metabolism of arachidonic acid

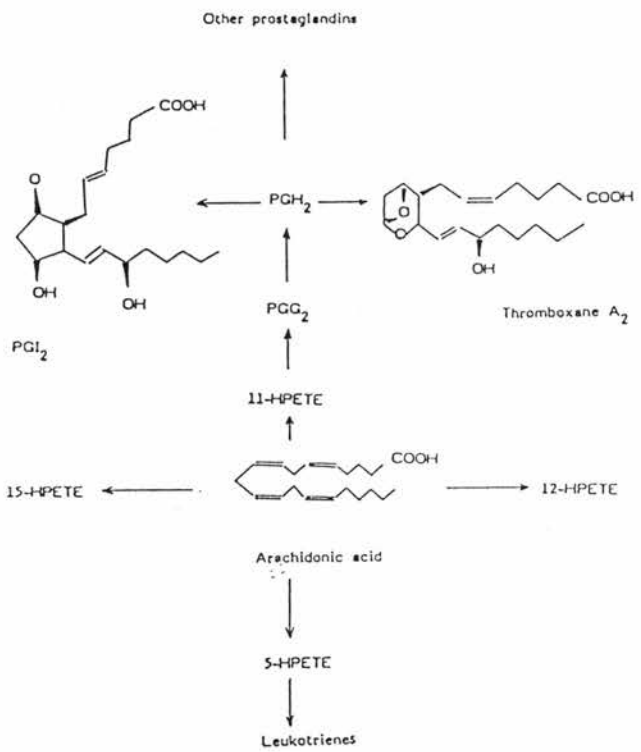


Table I-1. Selected activities of products of the cyclo-oxygenase/pathway of metabolism of arachidonic acid

PGEs generally	hyperalgesic, enhance pain producing properties of bradykinin, for example.
PGE_2	vasodilator, contributes to erythema production, potentiates the effects of several inflammatory mediators.
PGD_2	general pro-inflammatory agent.
PGI_2	stimulates adenyl cyclase, inhibits leucocyte migration (immobilises cells in inflammatory loci), protects blood vessel walls against leucocyte adherence and platelet aggregation.
PGF_2	general inhibitor of inflammation.
Thromboxane A_2	induces platelet aggregation and vasoconstriction, inhibits haemorrhage and inflammation.
Thromboxane B_2	PMN chemotaxin in mouse (but not man), involved in cell accumulation at sites of inflammation.

Drugs such as aspirin and indomethacin effectively inhibit or block the activity of the cyclo-oxygenase enzymes, preventing prostaglandin and thromboxane synthesis. They are apparently effective in treating rat adjuvant arthritis, and reduce the acute manifestations of inflammation in humans. The initial enthusiasm for the development of such drugs based on this approach, using rat adjuvant arthritis as one of the prime models, was therefore justifiable. As a class, such drugs have come to be known as "non-steroidal anti-inflammatory drugs" or NSAIDs. There can be little surprise that there has been a steady stream of such compounds developed by the pharmaceutical industry over the past 20 years or so, and it would be foolish to suggest that they have not provided symptomatic relief in many indications, including rheumatoid arthritis. In retrospect, however, it seems to have been naive to suppose that such an obviously complex disease, still with no known aetiology, should be so easily cured, simply by blocking one enzyme along a single metabolic pathway. There is, it has to be said, at least one precedent for such a belief. Hereditary angioneuroedema, apparently equally complex, can be almost totally controlled by treatment with androgens by affecting the production of a single component of the complement pathway (Fellner, 1980). However, such examples are rare, and hindsight suggests that the early enthusiasm should have been tempered with caution.

Caution would have been justified. It has become evident that cyclo-oxygenase inhibition may provide temporary symptomatic relief of the acute inflammatory symptoms, but it does not ameliorate the underlying disease, nor does it inhibit the progressive joint destruction (Littler, 1981, Hart and Huskisson, 1984). Because of the difficulty of assessing therapeutic responses in arthritis, this conclusion has been reached slowly, and is a general conclusion from the accumulated experience of treatment with NSAIDs. Perhaps, due to the extent of the early enthusiasm for this approach, there has now arisen an over-reaction in the opposite direction. Since NSAIDs generally are ineffective, then cyclo-oxygenase inhibition is ineffective, and the conclusion is that all drugs which have the ability to inhibit cyclo-oxygenase are therefore of no value in rheumatoid arthritis. Most NSAIDs have, however, a spectrum of pharmacological activities (Hart and Huskisson, 1984). For example, most NSAIDs are reversible uncouplers of oxidative phosphorylation (Tokumitso, Lee et al, 1977), and thereby inhibit general metabolic processes. This has itself been advocated as a viable alternative approach to the treatment of chronic inflammation (Whitehouse, 1965). Rather, therefore, than abandon all NSAIDs because the cyclo-oxygenase approach has failed, it would seem more sensible to determine rather carefully whether or not any specific "NSAID" can be shown to affect the disease progression, and if so, to discover what aspect of its pharmacological profile is responsible.

With reference again to Figure I-1, products formed via 12- and 15-HPETE, (15-hydroperoxy-6,8,11,14-eicosatetraenoic acid) have received little interest. Much more research has been generated by the discovery of the pathway via 5-HPETE to the leukotrienes (LTs). This shift of interest has partly occurred as a consequence of the growing realisation that the 11-HPETE derived PGs and thromboxanes are potentiators rather than direct mediators of inflammation.

Figure I-2 shows the biosynthetic pathway to the major leukotrienes. Arachidonic acid is oxidised via lipoxygenase(s) to the hydroxy acid 5-HPETE. This in turn is converted to the reactive epoxide LTA_4, and thereby to LTB_4 or LTC_4. The latter is linked by a thioether group to glutathione and was the first of the leukotrienes to be chemically synthesised. LTC_4 is further metabolised to LTD_4 and LTE_4. Table I-2 gives an indication of the biological activities currently attributed to the leukotrienes.

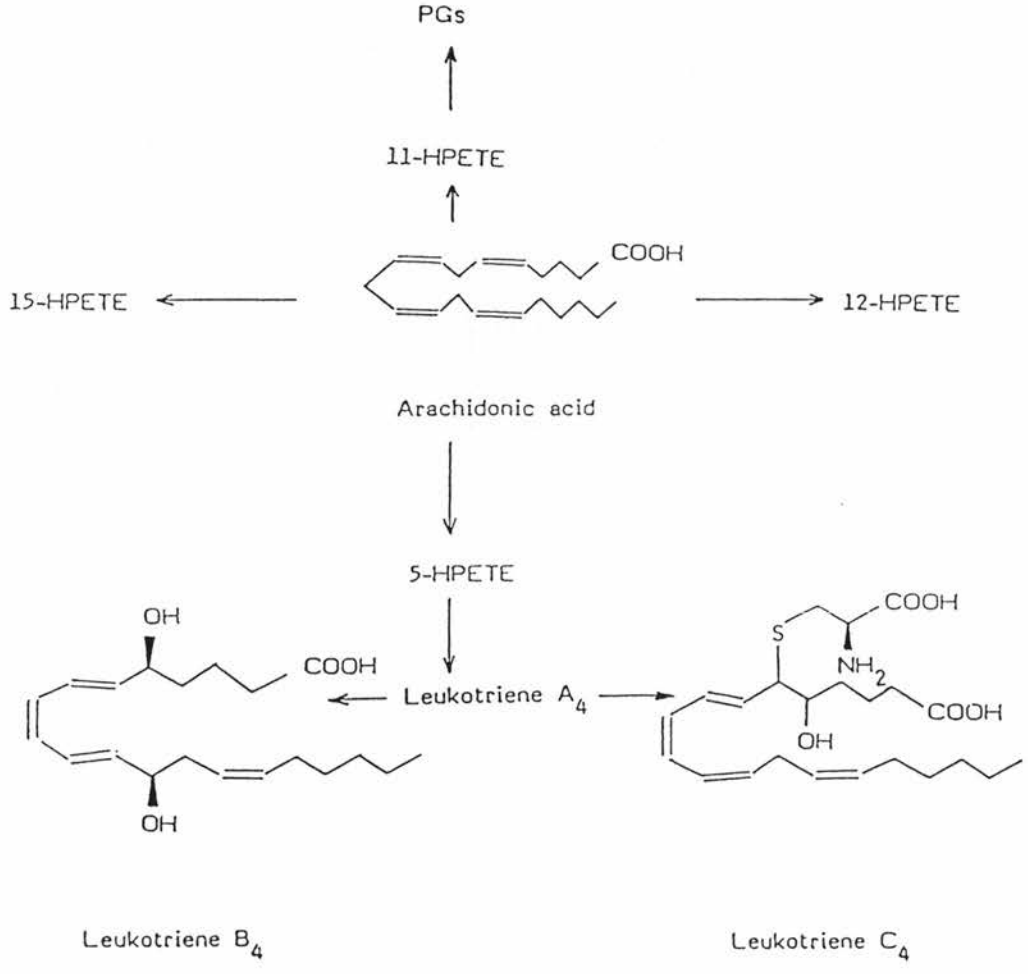
The discovery that LTC_4 is identical to the "slow reacting substance of anaphylaxis" (SRSA) has stimulated much research into the leukotrienes (Parker, 1982). LTB_4, in particular, was found to be a very potent chemotaxin for PMN's. By stimulating PMN migration into inflammatory loci, a battery of inflammatory mediators, and lytic enzymes, becomes available from this cell. As a result of the interest in PMNs and their inflammatory role, much attention has been centred on LTB_4. This substance also has the

property of increasing vascular permeability in the presence of vasodilatory PGs (Smith, Samuelsson et al, 1982), exhibiting a co-operative role in enhancing the inflammatory response. Evidence of LTB_4 in human synovial fluid in patients with RA has been reported (Davidson, Rae et al, 1982). The low levels generally seen have been attributed to rapid metabolism of LTB_4 to more polar compounds, which have much weaker biological activities than the parent (Davidson, Rae et al, 1982).

Table I-2. Selected activities of products of the lipoxygenase pathway of metabolism of arachidonic acid.

Leukotriene B_4	potent chemotaxin for PMNs, at higher concentrations it may stimulate release of lysozyme from leucocyte granules.
Leukotriene C_4	constrictor of coronary arteries and airway passages, increases vascular permeability.
Leukotriene A_4	constricts airway passages, increases vascular permeability.

Figure I-2. Oxidative metabolism of arachidonic acid



Surprisingly, in view of the lesson to be learned from the failure of the cyclo-oxygenase approach, there seems now to be a similar excess of enthusiasm for the leukotriene approach. A cure, or at least a major advance in symptomatic treatment, is now expected from drugs which block the leukotriene pathway. Apart from the warning inherent in the failure of the cyclo-oxygenase inhibitors, there are other very good reasons to doubt this. The first is that there is already available a class of compounds, widely used over a long period of time, which block the leukotriene pathway. These are the anti-inflammatory steroids (Moore, Hoult et al, 1980). They do not offer a cure, although, as with the NSAIDs, they do offer dramatic relief of the symptoms of the acute inflammation (Littler, 1981). The underlying joint damage continues unabated (Littler, 1981). The steroids have a particularly wide range of pharmacological action, but one major effect is to inhibit the synthesis of the phospholipase enzymes (Gryglewski, 1979). They prevent thereby the release of free arachidonic acid, and block all pathways of arachidonic acid metabolism. In the light of this it has been argued that selective inhibition is important, and that the objective should be to inhibit the lipoxygenases responsible for the initial stage of leukotriene synthesis, whilst leaving cyclo-oxygenase activity, and therefore the synthesis of the prostaglandins and thromboxanes, unchanged. So long as the steroids remain ineffective, this would seem to be an attempt to defend the

indefensible. A possible argument for inhibiting both cyclo-oxygenase and lipoxygenase activity, but at a stage beyond that of the phospholipases, is that this might remove the "rebound exacerbation" seen with the steroids (Littler, 1981). This cannot, however, be viewed as a major advance in efficacy.

There is yet a further reason for approaching lipoxygenase inhibition with caution. The major effect likely to be achieved by blocking the biosynthesis of the leukotrienes in the context of rheumatoid arthritis would seem to be the inhibition of PMN migration into the synovial lining layer, preventing PMN mediated joint damage. Given what has been said above, that cells fully capable of initiating and maintaining joint destruction are already resident within the diarthrodial joint, there seems no reason at all to believe that joint damage will be prevented by this means, or that the discovery of the lipoxygenase pathway will have any significant effect on the successful development of anti-arthritic drugs. It is therefore to be hoped that the pharmaceutical industry will resist the temptation to produce leukotriene inhibitors on the scale of the NSAIDs.

The role of the immune system

Further discussion of inflammatory processes requires consideration of the various types of cell involved, in particular those of the immune system. Broadly speaking, arachidonic acid metabolism is mostly important in the early, acute, phase of the inflammatory response, and the predominant cell involved is the PMN. As these cells enter the inflammatory locus and undertake scavenging operations to remove the stimulus, lytic enzymes of various kinds may be released which cause a degree of local tissue damage. If the stimulus persists macrophages are recruited to the inflammatory site and other processes more concerned with chronic inflammation are initiated. These processes are discussed in more detail below in the context of the acute and chronic phases of rat adjuvant arthritis. Inevitably, in such a chronic phase, tissue damage caused by enzymes released by PMNs and macrophages can become widespread and severe.

If the inflammatory stimulus is an antigen, an immune reaction becomes established. In one specific circumstance, where the antigen has sufficient repeating sub-units, direct stimulation of a specific set of cells, the B-lymphocytes, to produce antibody, can occur. This is, however, the exception, and it is generally the case that a number of different cell types become

involved in a cooperative manner. Macrophages become involved in "opsonising" antigen for presentation to B- and T-lymphocytes which act to produce lymphokines and antibody (Unanue, 1978). Certain of the lymphokines recruit further macrophages and lymphocytes to the site. Others activate macrophages and lymphocytes to function more aggressively. This minimal picture of the process illustrates to some degree the cellular cooperation, and the potential amplication factors which can accelerate the process. The scavenging activities of macrophages and the PMNs can, as before, cause localised tissue damage, which is likely to be more severe than in non-immune processes, reflecting the ability of the lymphokines inter alia to accelerate PMN and macrophage recruitment and to elevate their level of activity.

The net result is the initiation of a process with complex amplification and inhibitory pathways (Jerne, 1974), which, once established, is extremely difficult to modify by therapeutic intervention, other than by gross inhibition of the activity of almost all of the cooperating cells, that is by overt immunosuppression. In normal circumstances, however, the antigen is removed from the locus, feedback inhibitory processes become dominant, and the reaction effectively ceases. If, on the other hand, the antigen is not removed, a severe and chronic inflammation can become established. If the antigen is a locus on the tissue of the host, such a circumstance is particularly serious, there being a direct inflammatory assault on the host

tissue in addition to the peripheral tissue damage which occurs.

Normally host tissue does not appear to stimulate an immune reaction. This concept of self tolerance was first described by Ehrlich (Ehrlich, 1900). Burnett subsequently postulated that lymphocytes recognising "self-antigens" were erased or deleted in the embryonic stage (Burnett, 1959). Subsequent studies have however demonstrated that immunity against self antigens can be artificially induced. Injection of autologous thyroglobulin with FCA into rabbits induces antibody production, an inflammatory infiltrate into the thyroid, and the development of a form of chronic thyroiditis (Witebsky, Rose et al, 1957). Clearly, then, an immune reaction to "self" antigens is possible.

Present understanding considers the immune system to consist of a complex co-operating cellular network (Jerne, 1974). Self unresponsiveness is maintained by lymphocytes which suppress responsiveness to autologous antigens. Providing such cells are in the majority, there is no apparent reaction. However, experiments with anti-thyroglobulin in mice have demonstrated that, whilst thyroglobulin injected alone stimulated proliferation of the suppressor cell population, thyroglobulin injected with an adjuvant brought about a major humoral immune reaction, unless the adjuvant and thyroglobulin were injected into mice in which the suppressor population had previously been enhanced. In that case no reaction was detected (Witebsky, Rose et al, 1957).

Such experiments as these have established that an immunocompetent animal has in its circulation T- and B-lymphocytes responsive to self antigens. The outcome of any encounter with antigen depends entirely on the balance of helper/effector cells (which promote a "positive" immune reaction) and suppressor cells (which inhibit it).

The kind of aggressive, chronic, inflammatory response to self antigen which was suggested above is in principle possible, therefore. Although there are conflicting views, the consensus now is that, in rat adjuvant arthritis, the effector/suppressor ratio has in fact been shifted in favour of the effector cells for certain autologous tissues. There is then an immune reaction against these tissues, in a similar manner to that described above for experimentally induced thyroiditis. According to this view, rat adjuvant arthritis is an "autoimmune disease".

A point to be stressed is the role of phagocytic antigen-presenting cells, such as macrophages, in the promotion of an immune response. Historically, macrophages are seen to be phagocytic cells, acting primarily as scavengers. This is altogether too narrow a view, and, whilst it is true so far as it goes, in this discussion the macrophage is regarded as an "immune cell" playing a more or less essential role in the generation of an immune response (Keller, 1980). The macrophage has been known for some time to have the ability to process antigen and to

present the antigen to T-cells (Unanue, 1978). In this sense the macrophage acts as a co-operative immune cell. Arguments about whether the macrophage is an "immune cell" with the highly developed specificity of the lymphocyte are here seen as largely a matter of irrelevant semantics. This cell plays a major role in developing an immune reaction, and is an important part of the co-operating cell network which defines the nature of that response (Unanue, 1978). In therapeutic terms, for both the human and rat disease, consideration of this cell has been neglected and it should now be seen as an important target.

In the context of rat adjuvant arthritis the initial aim of both Stoerk and Pearson was to trigger an autoimmune reaction to muscle and splenic tissue by administering the antigen with FCA, similar to that of the thyroglobulin experiments described above. However, Pearson very soon afterwards, whilst apparently pursuing the same aim, (the development of autoimmunity to muscle tissue), discovered that the muscle preparation was irrelevant to the process under investigation, the disease can in fact be induced by injection of FCA alone.

Freund first described the use of FCA, "Freund's Complete Adjuvant", in 1944 (Freund and Bonanto, 1944), and subsequently in more detail (Freund, 1956). The material described consisted of finely ground, heat killed M. tuberculosum in a mineral oil containing an emulsifying agent (which in fact soon proved

superfluous). The antigen in question was added, along with water, the whole being agitated constantly until a water in oil emulsion was produced. This was then injected into the animal. Use of FCA in this way elicits an elevated cell mediated and humoral immune response when compared to antigen injected alone. In some cases, the use of the mineral oil alone (Freund's Incomplete Adjuvant (FICA)) also has the ability to elevate the immune reaction to certain dissolved antigens, albeit to a lesser degree (Whitehouse, 1973). Autologous antigenic material will stimulate suppressor cell production when injected alone. When injected as an emulsion with FCA, an immune reaction against autologous tissue bearing the "self" antigen is elicited. Consequently the nature of FCA, and the mechanism by which these effects are produced has been a point of great immunological interest.

FCA may have non-specific effects in addition to those on the immune system. There is for example, an increase in lymphatic complexity in mouse ears injected with FCA which has been attributed to an irritant effect of the particulate material present (Dale, 1961).

The observation that autologous tissue antigens injected in FCA could induce an immune response against tissue bearing that antigen created considerable interest. It immediately raised the possibility that human autoimmune diseases could be modelled in animals by this means. One such model for thyroiditis has been

described already. A more striking example is the effect of injecting an extract of guinea pig spinal chord in FCA into guinea pigs. Antibodies to myelin, the protein component of the membranous sheath which surrounds and insulates nerve fibres, are rapidly produced, and major nerve damage results (Lassman and Wisniewski, 1979). The antigen responsible has been localised to amino acids 114 to 122 of myelin basic protein (Rauch and Einstein, 1974). This disease, Experimental Allergic Encephalitis (EAE), is characterised by progressive paralysis and death.

The injection of FCA alone into rats by an appropriate route, as has been described, initiates an arthritis. All that seems to be necessary for the induction of the disease is a water in oil emulsion containing heat killed M. tuberculosum. This material contains one or more agents which will subsequently be referred to as "arthritogens". There are also agents present which are not themselves arthritogenic, but whose presence is essential. These are referred to below as "co-arthritogens". They are generally adjuvants, but it should be remembered throughout the subsequent discussion that whilst arthritogens and co-arthritogens appear to be adjuvants, the converse is not true. Despite a great deal of investigation into the nature of both the adjuvant and arthritogenic activity in FCA, no conclusive explanation of the ability of FCA to induce rat arthritis has been obtained.

Perhaps the first complication is that the arthritogenic effect depends on the route of administration. Injection of FCA intradermally into the tail or subplantar tissue will initiate the disease in a susceptible rat strain. S.c., i.p. or i.v. injection will not (Waksman, Pearson et al, 1960, Glenn and Gray, 1965), and indeed prevents subsequent disease induction by FCA injected via one of the established arthritogenic routes (Waksman, Pearson et al, 1960). The obvious conclusion from this observation might seem to be that creation of a depot of FCA at the injected site is important. Several authors have observed however, that intradermal or subplantar injection of FCA, followed by removal of the injected limb within one half-hour of injection, does not prevent disease formation, and microscopic studies clearly show rapid dissemination of lipid and bacterial material via the lymphatics (Glenn and Gray, 1965).

The oil component of the mixture has been the subject of several investigations, both in the context of rat arthritis and EAE. Both mineral and vegetable oils are potentially co-arthritogenic (Ward and Jones, 1962), and in one study more than 100 such materials were screened for co-arthritogenic activity (Whitehouse, Orr et al, 1974). Straight chain saturated aliphatic hydrocarbons with chain lengths of 15 to 20 units seem to produce the strongest response (Shaw, Alvord Jr et al, 1964b), whilst a chain length of 10 units seems to be the minimum requirement. Cyclisation, presence of oxygen atoms or double bonds

reduces the efficacy of chains longer than C10. Esters and triglycerides of fatty acids with a carbon chain length greater than 12, retinol acetate (but not the palmitate), vitamins E and K, all exhibit co-arthritogenic and adjuvant activity (Ward and Jones, 1962). Squalane has been identified as a potent co-arthritogen. Human sebum also is a potent co-arthritogen and adjuvant. This raises the question of whether cutaneous hyper-responsiveness may result from skin damage presenting specific antigens admixed with sebum components, which act adventitiously as an adjuvant (Whitehouse, Orr et al, 1974). The inflammatory lesions of acne might be an example of such a possibility.

It is clear that the oil component is of considerable importance as a co-arthritogen. Evidence has been obtained to show that prior injection of the oil component can reduce the severity of, and in some cases totally inhibit, subsequent arthritis induction by FCA (Zahiri, Gagnon et al, 1969, Cozine Jr, Stanfield et al, 1973). However, arthritis induction cannot be achieved by the oil component alone. The major arthritogenic components reside, therefore, in the bacterial derived material.

The Mycobacteria are unique in having an extremely high proportion of lipid material within the cells, and it is almost exclusively Mycobacteria which provoke the rat arthritis. M. tuberculosum, M. phlei, and M. butyricum are all effective, as is N. asteroides (Shaw, Alvord Jr et al, 1964a). Gram-negative

bacteria, S. typhosa, S. typhimurium for example, are generally ineffective (Shaw, Alvord Jr et al, 1964a). The activity of the Mycobacteria is perhaps curious given that a water soluble immunosuppressive d-arabino-d-mannan can be extracted from the cell wall of M. tuberculosum (Ellner and Daniel, 1979). Certainly, as is shown later, specific sub-fractions of M. butyricum cell wall preparations are more potent inducers of rat arthritis than the whole organism.

The specific component(s) of the Mycobacteria responsible for adjuvant and arthritogenic activities have now largely been identified. Bacterial endotoxins have long been known to have adjuvant activity (Merritt and Johnson, 1963). However, the specifically arthritogenic activity may reside in two or possibly three components. There seems to be only one report of arthritogenicity residing in a hydrosoluble component (a peptidoglycan (Pearson et al, 1977)). There have been several reports of the arthritogenicity of "Wax D", a chloroform extractable, ether soluble, acetone insoluble glycolipid. The specific structure of this material seems to be important. Wax D from human strain M. tuberculosum reflects the arthritogenic activity of the whole organism, but bovine strain M. tuberculosum Wax D and other Wax D's are claimed to be ineffective (White, Jolles et al, 1964). This particular study established that human strain M. tuberculosum Wax D contains meso-αα diaminopimelic acid, d-glutamic acid and d-and l-alanine. Muramic acid is also

present. The parallel between this material and the cell wall composition of the gram-positive bacteria has been emphasised (White, Jolles et al, 1964). Other authors have identified arthritogens in the Wax D fraction, and it has been suggested that pre-inoculation with Wax D protects against the arthritogenicity of subsequent FCA injection (Wood and Pearson, 1962), although this may be purely non-specific (Cozine Jr, Stanfield et al, 1973). There seems to be no doubt, however, that the cell wall fraction, when all lipid and hydrosoluble materials have been removed, is a powerful arthritogen (Audibert, Parant et al, 1973, Nagao, Ota et al, 1981). Within the cell wall residue, muramyl dipeptide has been proposed as a minimal adjuvant structure (Nagao, Ota et al, 1981). The cell wall retains its arthritogenicity if treated with trypsin or chymotrypsin but not if digested with lysozyme. Such treatment produces a water soluble fraction which, if recombined with the insoluble residues, remains unable to induce arthritis (Audibert, Parant et al, 1973).

Whilst the specific components of FCA responsible for the arthritogenic and co-arthritogenic effects are now largely identified, the mechanisms by which the disease is induced are not. It has been suggested that, in the rat specifically, antigenic determinants in Mycobacterial cell walls cross react with antigenic determinants native to the rat, and, in certain genetically susceptible strains, break the innate self-tolerance and induce an autoimmune process directed apparently against

certain types of tissue collagen (Audibert, Parant et al, 1973, Nagao, Ota et al, 1981). Unfortunately, there have been no reported attempts to correlate the susceptibility of various rat strains to adjuvant arthritis, with the ease with which antigenic tolerance may be broken with FCA, which would seem to be one logical approach towards validating the point.

Although the above suggestion may be correct, a more complex picture is emerging. A morphological examination of the physical nature of FCA has shown that "thick" emulsions comprise highly stable three-dimensional networks with interconnecting strands of water droplets containing the added antigen, interspersed within the oil phase. Mycobacterial fragments were found to be confined to this meshwork, coated with an adherent surface layer of antigen containing water droplets. Even in thinner emulsions, there was a marked tendency for water droplets containing antigen to adhere to the surfaces of the Mycobacterial fragments. This suggested that in FCA, antigenic material is brought into close contact with the Mycobacterial cell fragments (Dvorak and Dvorak, 1974). In an earlier investigation, it was observed that gram positive bacteria such as C. rubrum are ineffective as promoters of EAE, unless pre-coated with silicone material. These observations led to the suggestion that such a coating protects the fragment against digestion by phagocytic cells, which are nevertheless activated (Davies and Bonney, 1980). Such a suggestion has been made by others who proposed that oily material coats the bacterial

fragment. This, with its adherent antigen containing water droplet coating, affords a degree of protection from phagocytic digestion, whilst establishing chronic stimulation of the phagocytic, antigen presenting cells (Shaw, Alvord Jr et al, 1964a, Shaw, Alvord Jr et al, 1964b). If true, this would be a clear illustration of the pivotal importance of the macrophage and related cells in the chronic inflammatory process. In adjuvant arthritis, Streptococcal cell wall induced arthritis, and Erysipelothrix insidiosa induced arthritis, it has been shown that apparently non-biodegradable cell wall components containing a peptidoglycan moiety persist in the phagocytic cells of the hypertrophic synovium (Mohr, Wild et al, 1981). In such a case it could be that there is chronic stimulation of histiocytes which constantly present antigen to the T-cell system, thereby maintaining chronic phagocytic cell stimulation and a chronic cell mediated immune response. However, in rat disease it could also be that the involvement of the cell mediated immune system under such circumstances is of lesser importance. The central issue being stimulation of the phagocytes which induces chronic tissue damage (Davies and Bonney, 1980).

Such an emphasis on the role of phagocytic cells is relatively novel. The majority of immunological studies have concentrated on the cell mediated immune system, although it has long been known that drug induced suppression of the secondary lesions is possible, without there being any apparent effect on the general level of cell mediated immunity. This emphasis is

mainly due to the knowledge that the disease may be passively transferred to naive animals by lymphocytes taken from arthritic rats (Waksman and Wernstein, 1963, Pearson, 1964), and partly to the apparent ease of monitoring the cell mediated response using the skin tuberculin reaction to "purified protein derivative of tuberculin" (PPD). PPD is a component of M. tuberculosum, but is not itself arthritogenic, however, and probably plays no direct role in the disease process.

Accurate measurement of cell mediated immunity in pharmacological studies is difficult, and much of the literature describing reactions to changing levels of cell mediated immunity may, by virtue of the looseness of the terminology used, be misleading. It is useful to review precisely what is being measured by the tuberculin skin test, and the various similar tests employed with the intention of measuring cell mediated immunity. The general procedure is to challenge the skin of the animal in question with an antigen to which the animal has been previously sensitised. An erythematous wheal appears at the site of challenge which, although immunologically initiated, is mediated by an acute inflammatory process. It will therefore respond to anti-inflammatory drugs given at the appropriate time. In such circumstances, it is important to ensure that the timing of drug administration in relation to skin sensitisation and challenge is such that any observed reaction may be attributed to a direct effect on the immune status of the animal.

There is a more fundamental problem, which concerns the meaning of "the level of the cell-mediated immune response". One interpretation might well be that this refers to the number of lymphocytes present within the organism which have been activated and which "recognise" the antigen in question. This is only loosely assessed by the tuberculin reaction, however. In all such skin challenge tests, a very small number of appropriately activated lymphocytes are involved, the reaction which is finally measured being greatly amplified by the accessory cells, primarily the PMNs, which secrete those factors into the tissues at the site of challenge which are more directly responsible for the magnitude of the reaction observed. There are therefore many ways in which an investigational new drug may affect the magnitude of the skin reaction, without it having the slightest effect on the immune system. There is a persisting problem of how best to measure cell mediated immunity in order to unambiguously identify drugs with a direct immunomodulatory effect.

As has been said, however, animals in which arthritis has been induced by FCA containing M. tuberculosum exhibit a skin tuberculin reaction, which may be used as a measure of cell mediated immunity. It should again be stressed however that PPD is unrelated to the process of disease induction and maintenance, and it has been known for some time that drug induced disease suppression can apparently occur in the absence of any change in

the magnitude of the tuberculin reaction (Newbould, 1965, Currey and Ziff, 1968). Specifically, in a study of the cell mediated immune response in arthritic rats to both PPD and to a peptidoglycan derived also from M. tuberculosum, there were no apparent correlations between the severity of the arthritis and the reaction to PPD, the peptidoglycan, or both (Kohashi, Pearson et al, 1977a). One alternative technique is to inoculate the animals with ^{31}Cr labelled EL_4 cells (Chan, 1977a). A more realistic assessment of the number of circulating T-lymphocytes sensitised to this antigen may then be obtained by measuring the level of complement independent T-cell lysis of EL_4 cells ex vivo, which is unlikely to be affected by any anti-inflammatory effects of the drugs being studied. The humoral immune response to these cells may also be easily measured at the same time (adjuvant arthritic rats appear not to produce antibodies to PPD). Using this type of approach it has been shown that arthritis induction slightly enhances the humoral immune response to these cells, and greatly enhances the cell mediated immune reaction (Chan, 1977a). At least one drug has been investigated using this technique. 6-Mercaptopurine has no effect on the primary lesions of the rat arthritis, but does inhibit the formation of the secondary lesions (Chan, 1977b). It also diminishes the humoral and cell mediated immune reactions to EL_4 cells, and therefore appears to be acting as an immunosuppressor.

A further alternative again involves the sensitisation of the experimental animals with a suitable antigen. They are then subsequently challenged with the same antigen in one ear, the other receiving excipient (usually saline) alone. The response may be assessed simply as the relative increase in ear thickness. A more precise technique involves pre-dosing the animals with 5-^{125}I-2'-deoxyuridine. Cell migration is then apparent as a relative increase in radioactivity detectable at the site of challenge. In a typical experiment (Gans, Heyner et al, 1980), animals treated with FCA containing M. butyricum in place of M. tuberculosum, were then challenged in one ear, again with M. butyricum. With an insoluble antigen preparation such as this there is inevitably a non-specific inflammatory component which will compromise the measurement. However, this study was particularly interesting in that it demonstrated that the cell mediated immune reaction was diminishing in intensity when the secondary lesions were becoming apparent (Gans, Heyner et al, 1980). This is consistent with the tumour growth experiments described below, and studies described in more detail in chapter 3.

It is a puzzling contradiction of human arthritis that, whilst the disease is almost certainly autoimmune in nature, those having the disease appear to be immunosuppressed (Waxman, Lockshin et al, 1973). This also seems to be true for the rat, in

the sense that, in studies of chemically induced primary sarcoma in rats, tumour growth was normal if the inoculum was administered at the same time as the FCA, but was greatly enhanced if administered during the chronic phase of the disease (Gorog and Kovacs, 1979). This tends to point to a generalised immune deficiency state during at least the chronic phase of the arthritis, not withstanding the elevated reaction (to EL_4 cells for example) in the initial stages.

Of more importance is the evaluation of the role of cell mediated immunity in both the induction and maintenance of the rat disease. Studies of the kind described above illustrate fairly clearly that cell mediated immunity does undergo considerable change over the course of the disease. This does not, of itself, prove a causal relationship. It has been reported that the course of the disease was not affected by thymectomy, splenectomy, bile duct ligation, castration or pregnancy, whilst adrenalectomy and hypophysectomy caused death shortly after injection of the FCA (Glenn and Gray, 1965). In this particular study also, passive transfer of the disease was not achieved by serum, plasma, whole blood, or cellular components of lymph nodes, thymus or spleen. On the other hand, animals treated with FCA by one of the ineffective routes (s.c., i.v., etc), do not develop arthritis if later treated with a second dose of FCA by an effective, arthritogenic, route, which seems to imply some form of immunological tolerance (Maes and Claverie, 1977). Passive transfer using lymphocytes

was however successfully reported by Pearson (Pearson, 1964) and by Waksman (Waksman and Wernstein, 1963). These successful transfer experiments, coupled with the apparent state of tolerance caused by administering the FCA by an ineffective route, do point strongly to a direct involvement of the immune system. Heterologous anti-lymphocyte serum given at the time of disease induction, greatly decreases the disease severity, whilst if given in the later stages of the disease the effect is much weaker (Currey and Ziff, 1968). This tends to qualify the role of the immune system, suggesting that it is more influential on the process of disease initiation. The later stages may then be more concerned with the dissemination and phagocytosis of antigens present in the mycobacterium.

Further support for the immunological basis for disease induction in rat arthritis is that curious effects arise if FCA is administered along with a second antigen. If administered with ovalbumin, for example, the arthritis induction is inhibited (Nagao, Ota et al, 1981). A similar effect occurs with crude human chorionic gonadotrophin (HCG), but not with purified material (Maes and Claverie, 1977). Consequently, where immune competence is monitored by administering a co-antigen along with FCA (EL_4 cells for example as described above), care must be taken to ensure that there is no interference with the disease process being measured. This is a further argument in support of

the use of PPD to assess the immune status of the animals, since it is a component at least of FCA containing M. tuberculosum.

The above investigations tend to indicate that the lymphocyte is important in the initiation of the disease, but do not resolve the question of what part of the immune system is primarily involved. More recent studies have delineated the particular lymphocyte subsets, and the roles they play. Lymphocytes taken from the draining lymph nodes of Lewis rats pre-inoculated with FCA have been used to develop and maintain a T-lymphocyte cell line designated A2, which, when injected i.v. into rats previously subjected to whole body irradiation, induces rat arthritis, and can also, under appropriate circumstances, induce a state of tolerance in recipient animals (Holoshitz, Naparstek et al, 1983). This cell line was originally selected because of its high in vitro proliferative response to Mycobacteria. It was subsequently found to respond in a similar manner to type-II collagen. These studies clearly implicate the T-lymphocyte in disease initiation. They also raise the further question:- do some antigens present on Mycobacteria cross-react with rat type-II collagen, and is the ability of these T-lymphocytes to induce the disease a general property of cells reactive to type-II collagen? A cloned subline of A2, designated A2b, has now been produced, which is more arthritogenic than the parent A2 cell line (Holoshitz, Matitiau et al, 1984). It does not have the ability to induce a state of tolerance in recipients, however, nor does it

respond to type-II collagen in vitro. This suggests that cross reactivity with type-II collagen is not a pre-requisite for arthritogenic activity, and that different T-cell subsets are responsible for disease induction and for production of tolerance (Holoshitz, Matitiau et al, 1984).

Until relatively recently, studies of humoral immunity in rat arthritis were considerably less strongly emphasised than those of cell mediated immunity, the belief being that the humoral response was of peripheral importance. This stemmed from the early observation that, whilst passive transfer of the disease was possible with lymphocyte preparations, it was not possible with serum. Most information in this area therefore tends to derive from drug studies in which measurements of humoral immunity were included (Pletsityi, Evseev et al, 1982). Vitamin D, for example, which is known to suppress the outward signs of the disease, has been found to depress the levels of circulating antibody and of antibody secreting cells. It also suppresses the cell mediated immune reaction (Pletsityi, Evseev et al, 1982). As a general summary of the situation from work of this kind, humoral immunity in arthritic rats appears to be elevated in the initial stages at least, but to a lesser degree than the cell mediated response.

Many such studies employ the technique of injecting sheep erythrocytes intraperitoneally, simultaneously with the FCA. The

level of antibody raised to the sheep erythrocytes may then be subsequently measured very conveniently by a haemagglutination assay (Walz, Dimartino et al, 1971). The caution expressed above, that the presence of this second antigen may affect the course of the disease should always be borne in mind, and it has been argued that a similar procedure employing EL_4 cells should rather be used for this reason (Chan, 1977a).

Three developments have indicated that the humoral immune system may play a role in at least the initiation of the disease. The first was the report that the disease can apparently be re-established by passive transfer of arthritic rat serum to FCA challenged rats which have subsequently been treated with cyclophosphamide and in which all outward signs of the disease have been suppressed thereby. These observations led to the suggestion that immune complexes are formed on injection of FCA which are initially soluble, and rapidly dissipate throughout the animal. When antigen is in excess, the solubility of the antigen/antibody complexes is reduced, and the complexes precipitate in the tissues, in particular the joints, initiating chronic, phagocyte mediated joint damage (MacKenzie, Pick et al, 1978). The second development, which bears on the question of what specific antigen is involved in such immune complexes, was the discovery of a further rat arthritis very similar to adjuvant arthritis. This is initiated simply by the injection of (usually bovine) type-II collagen (Trentham, Townes et al, 1977). In

this disease, there is both a cell-mediated reaction to type-II collagen, and a large circulating antibody titre against both the collagen and its denatured alpha chains. This cannot be a simple consequence of joint and tissue damage, since such antibodies are never seen in nonimmunologic, non-specific models of inflammation in the rat (Trentham, McCune et al, 1980). And finally, it has been found to be possible to induce what again appears to be a very similar type of rat arthritis using FICA containing synthetic arthritogens which are not themselves immunogenic (Trentham, Townes et al, 1978).

These developments have again raised the possibility that antigenic determinants on the mycobacterium cross-react with those on autologous rat collagen, and that the use of synthetic arthritogens in some way exposes collagen type-II so as to trigger a "collagen-induced" arthritis. If this were the case, then the three rat arthritis models would be essentially identical, differing only in the mode of induction. There have been attempts to resolve this point. Measurable levels of anti-collagen antibody have been found in adjuvant arthritic rats. Serum from these animals has been absorbed on to Mycobacteria. The serum antibody titres observed were not affected by this process, which provides no support for the cross-reaction hypothesis. It does not, however, resolve the question, since it remains possible that enzymic breakdown of the Mycobacterial components within the tissues may reveal otherwise hidden determinants which themselves

are the source of the collagen cross-reactivity. This particular study did, however, firmly establish that circulating anti-collagen antibodies are found in rat adjuvant arthritis, establishing a further similarity between the three models (Trentham, Townes et al, 1977, Trentham, McCune et al, 1980).

The discovery of circulating antibodies to type-II collagen in the adjuvant arthritic rat has produced some particularly interesting studies which bear on the question of the nature of the disease. This is especially true of studies of tolerance induction and the identification of lymphocyte subsets involved in its initiation. The Lewis rat is a strain particularly susceptible to arthritis induction. If such rats are pre-inoculated with type-II collagen in an alum flocculate, their susceptibility to a subsequent injection of FCA is greatly diminished (Welles and Battisto, 1981). Type-II collagen administered in an adjuvant can, therefore, induce a state of at least partial non-responsiveness to disease induction by FCA. A similar state of tolerance can also be induced by passive immunization with rat type-II collagen antibodies (Cremer, Stuart et al, 1980). There is therefore growing evidence that injection of rats with FCA induces a local, immunologically triggered, inflammatory response, which exposes type-II collagen to immune surveillance (type-II collagen is normally only found at "immunologically privileged" sites). This then initiates an autoantigen immune response, probably enhanced by cross reaction

with Mycobacterial antigens, although there is still no clear evidence to support this point, and by the adjuvant effect. It has in fact been shown that repeated injection of FCA into most species will induce IgM class autoantibody formation (Noeva, 1979). This initiating phase then produces a situation of chronic phagocytic cell activation primarily within the diarthrodial joints, which creates and maintains the progressive process of joint destruction.

II

DRUGS, THEIR METABOLISM, AND THE ASSESSMENT OF THEIR EFFECTS

Introduction

In this chapter, three problems and their consequences are discussed which relate particularly to the use of the rat adjuvant arthritis model in anti-rheumatic drug development. These are:-

i. that steroids and NSAIDS have dramatic effects on the outward signs of the disease, but do not fundamentally affect the disease progression,

ii. rats with adjuvant arthritis exhibit a changing ability to metabolise drugs as the disease progresses,

iii. in chronic models involving repeated measurements, specific problems of data analysis arise which make it difficult to assess drugs which may only marginally reduce the outward disease signs, but which may have more fundamental, underlying effects.

To illustrate the points raised in this chapter, a number of experiments are described which share a common methodology detailed below. Descriptions of the individual experiments are given in the appropriate sections.

Materials and Methods: General

All animals employed in the studies described in this chapter were male Wistar or Lewis (Bantin and Kingman) rats. The animals were housed 5 per cage in holding rooms maintained so far as possible at a temperature of 22 $\pm$ 2^{o}C, 60% relative humidity, and with a 14/10 hr light/dark cycle. Food and water were supplied ad libitum.

Arthritogenic material was prepared by prolonged manual grinding of heat killed, dried, M. butyricum (Difco) in liquid paraffin. A dose of 0.05 mls of this material at a concentration of 10 mg per 10 mls was injected into the subplantar tissue of one hind paw. Control animals received 0.05 mls liquid paraffin similarly administered.

Paw thicknesses were measured by placing the paw on a vertically mounted circular anvil. The tip of a pointer mounted on a swivel and connected by a cantilever to a scale

marker was placed on the centre of the paw. The thickness of the paw was then read from the scale. The apparatus was calibrated before use with metal discs of accurately known thickness.

The thickness of the knee joints were measured by holding the animal with the limb in a relaxed position, and measuring the thickness across the knee joint with Snelltaster calipers, calibrated before use as described above.

Drugs were generally administered by oral injection directly into the stomach. Such drugs were finely ground into 5% mulgophen in water at a concentration such that between 1 and 3 mls of the suspension were administered at any one time. Subcutaneous injection, where specified, was to the forward ventral surface of the animal, the drug being finely ground and suspended in physiological saline. A dose of one ml of the suspension was injected at any one time.

Statistical analysis of variance or covariance for repeated measures was carried out on an Apricot micro-computer. The programme was designed and written by Mr. D. J. Nelson and Mrs. R. Campbell, based on a description of the method by Winer (Winer, 1971). Results are quoted in Figures and Tables as the mean $\pm$ the standard error.

Steroidal and non-steroidal anti-inflammatory drugs

The elucidation of the pathways of arachidonic acid metabolism and their role in inflammation resulted in the proposition that inhibition of the pathway to the prostaglandins and thromboxanes would provide an effective approach to the treatment of arthritis. The theory appeared to be substantiated by the dramatic effects of such intervention on the inflammatory signs of erythema, pyrexia and pain in both non-immunologically and immunologically produced models of inflammation. Although this approach has resulted in drugs which provide temporary symptomatic relief, exactly as is the case with the anti-inflammatory steroids, it seems to be irrelevant to the fundamental treatment of the human disease. That anti-rheumatic drug research has been dominated by this approach is a likely consequence of the overt effects of such drugs on the acute signs in rat and human arthritis, coupled with the elegance of the rationale on which their development was based. Otherwise it is difficult to see why the emphasis on this approach was not modified by the same reserve which was rapidly afforded the steroids.

However, it must be acknowledged that the effect of the NSAIDs on the outward signs of rat adjuvant disease is very dramatic (Newbould, 1963, Pearson and Chang, 1977), being far

more rapid and clear cut than is the case for the so-called second line anti-rheumatic drugs (Toivonen, Tokola et al, 1982), and is therefore worth illustrating in some detail, using what is possibly the archetypal NSAID, indomethacin.

Method (Experiment EXA3)

Three groups of 10 male Wistar rats were employed. The control group received subplantar injection of liquid paraffin on day 0. Aqueous mulgophen was given orally on each subsequent day. The arthritic control group received a subplantar injection of the arthritogen on day 0, and daily aqueous mulgophen orally thereafter. The remaining group received subplantar injection of arthritogen on day 0, and indomethacin in aqueous mulgophen orally thereafter, at a dose of 3.0/mg/kg/d.

Results

Figure II-1 shows the swelling of the injected rat paw at times up to 22 days following challenge with FCA. The swelling of the uninjected paw is presented in Figure II-2. Indomethacin at this dose greatly reduced the swelling of the injected paw at every time point, and almost completely inhibited the appearance of swelling in the uninjected paw. That the treatment clearly had an effect on the animals' well

being may be seen from the body weight measurements shown in Figure II-3. Although body weight gain in the indomethacin treated animals was not as great as that of the non-arthritic control group, it was greater than for the arthritic animals throughout the whole of the secondary phase, beginning on day 11.

Figure II-1. The effect on the swelling of the FCA injected paw of arthritic male Wistar rats treated with 3.0 mg/kg/d oral indomethacin, commencing on the day of FCA injection (day 0). Experiment EXA3.

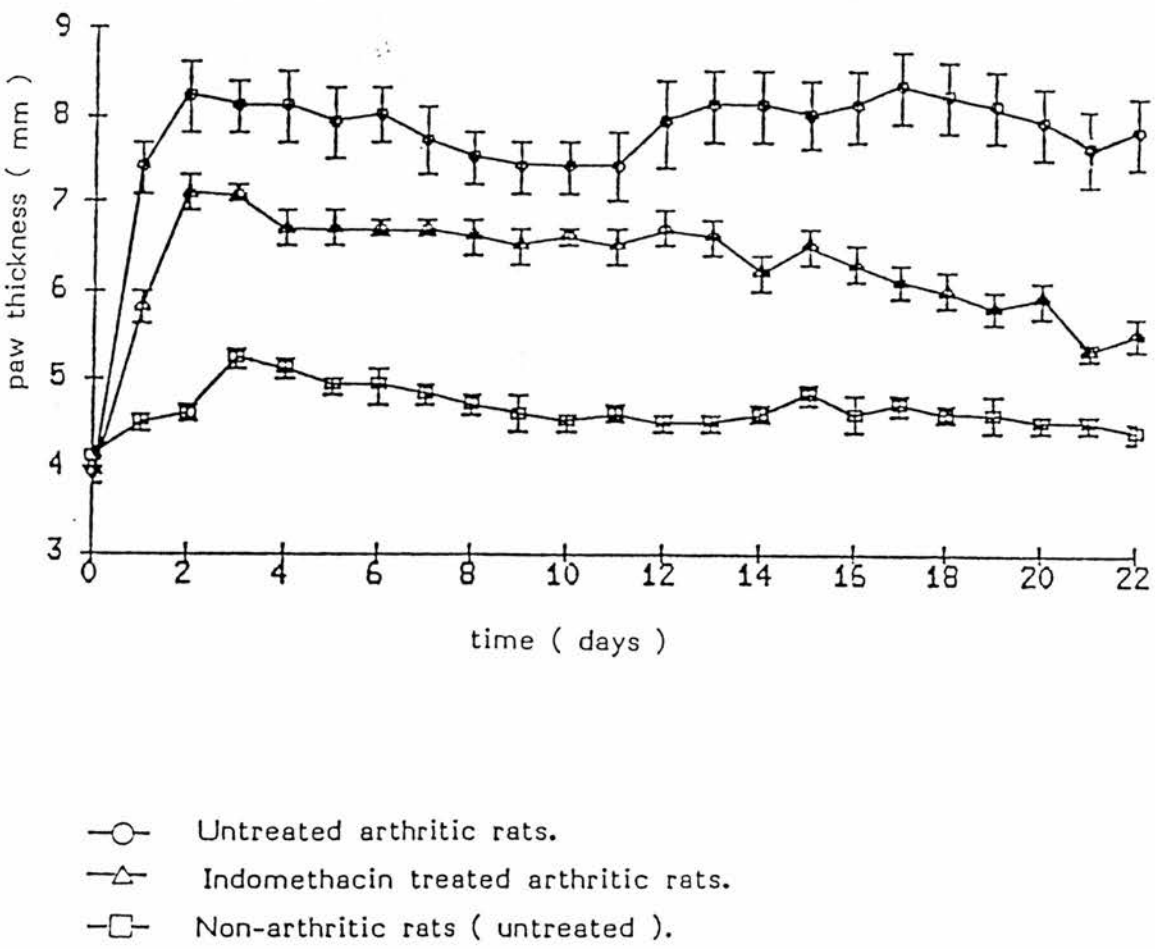
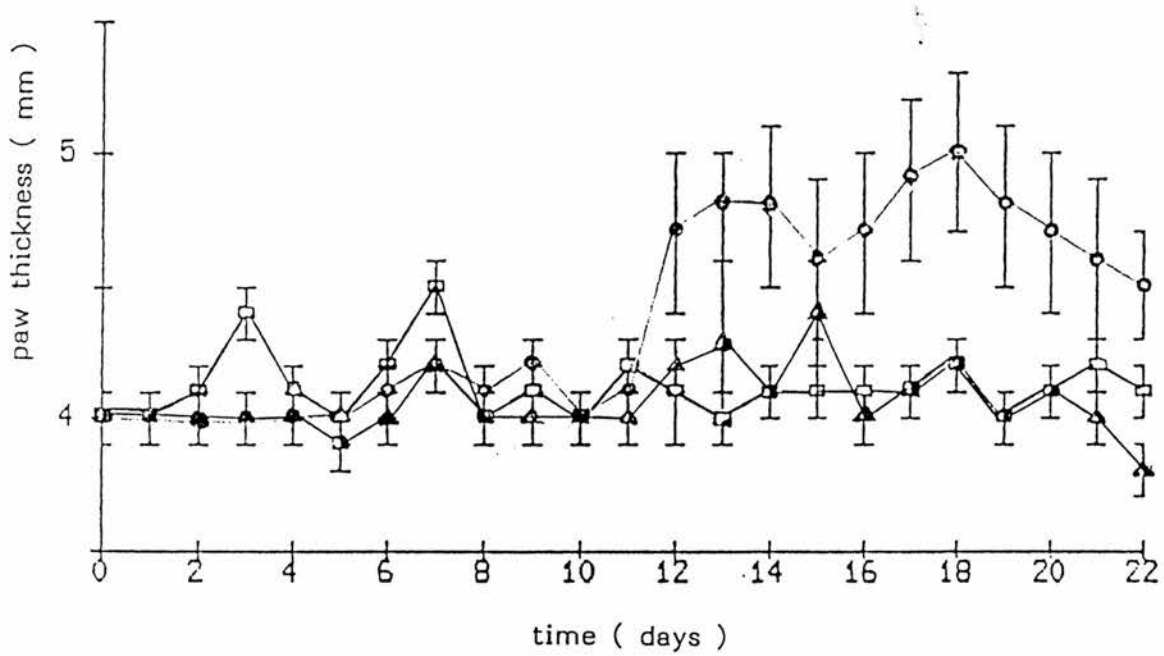
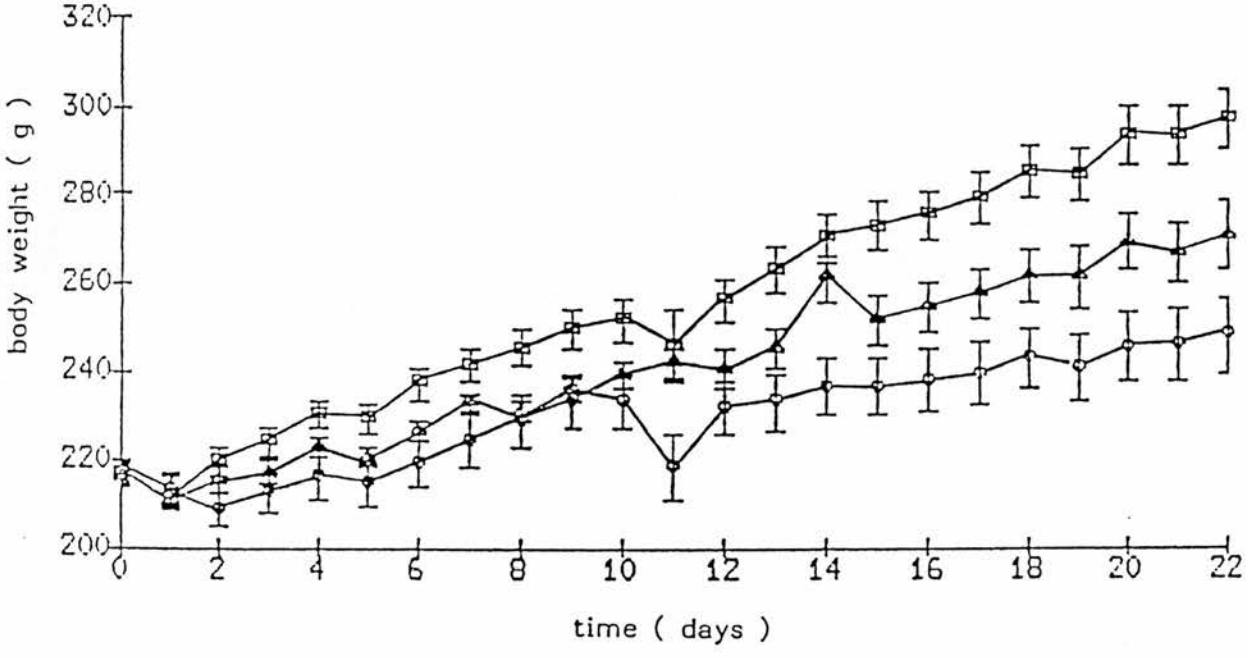


Figure II-2. The effect on the paw swelling in the uninjected limb of arthritic male Wistar rats treated with 3.0 mg/kg/d oral indomethacin, commencing on the day of FCA injection (day 0). Experiment EXA3.



-◎- Untreated arthritic rats.
-▲- Indomethacin treated arthritic rats.
-□- Non-arthritic rats (untreated).

Figure II-3. The body weights of untreated arthritic and non-arthritic Wistar rats in comparison to those of arthritic rats treated with 3.0 mg/kg/d oral indomethacin, commencing on the day of FCA injection (day 0). Experiment EXA3.



○ Untreated arthritic rats.

△ Indomethacin treated arthritic rats.

□ Non-arthritic rats (untreated).

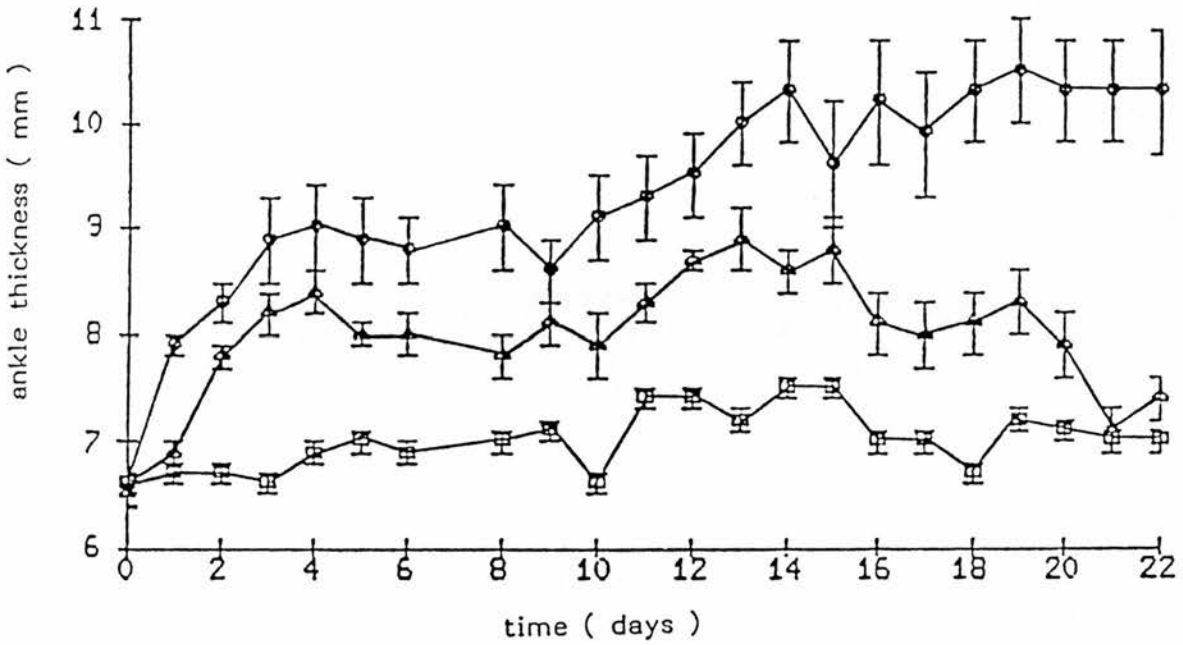
Assuming that the data are normally distributed, or at least that the t-test is sufficiently robust that any divergence from normality which may be encountered in these data sets will not appreciably affect the outcome, there was a significant difference between the group receiving indomethacin and that receiving placebo at Day 22 ($p < 0.05$) and between the indomethacin treated arthritic group and the non-arthritic control in respect of the injected paw ($p < 0.05$). This was also true for the uninjected paw swellings measured at Day 22, where the indomethacin treated animals in fact had paw thicknesses less than those of the non-arthritic controls at this time. Using the same approach, the body weights of the non-arthritic rats were significantly greater than those of the indomethacin treated arthritic rats ($p < 0.05$) which were in turn greater than those of the untreated arthritic animals.

The statistical approach used here whilst being correct, accepting the normality assumption and that the comparisons between indomethacin and placebo treated arthritic animals, and indomethacin treated arthritic and non-arthritic animals, were decided upon a priori, is clearly wasteful of data. This point is discussed in rather more detail below.

In the untreated animals, the speed with which the inflammation spread from the site of injection in the paw to the remaining parts of the limb was rapid, as is evident from the swelling of the ankle joint of the injected limb shown in Figure II-4. As a general observation, the appearance of the swelling in the upper limb was qualitatively different from that around the injection site in the paw, being dryer, firmer, with less oedema of the tissues. The thickness of the ankle joint may therefore also be a useful outward measure of the progress of the disease.

In terms of this metameter, the efficacy of indomethacin was slightly more apparent, the joint thickness in the injected limb returning almost to the level of the control animals 21 days after disease induction (Figure II-4). The increase in the ankle thickness of the uninjected limb during the secondary phase was almost completely suppressed by indomethacin (Figure II-5). Comparisons between the paw and ankle swelling in both injected and uninjected limbs are shown in Figures II-6 and II-7 respectively. Both metameters showed the same general response profile with time, and it would seem that either may be used to monitor the disease progression.

Figure II-4. The effect on the ankle swelling in the FCA injected limb of arthritic male Wistar rats treated with 3.0 mg/kg/d oral indomethacin, commencing on the day of FCA injection (day 0). Experiment EXA3.

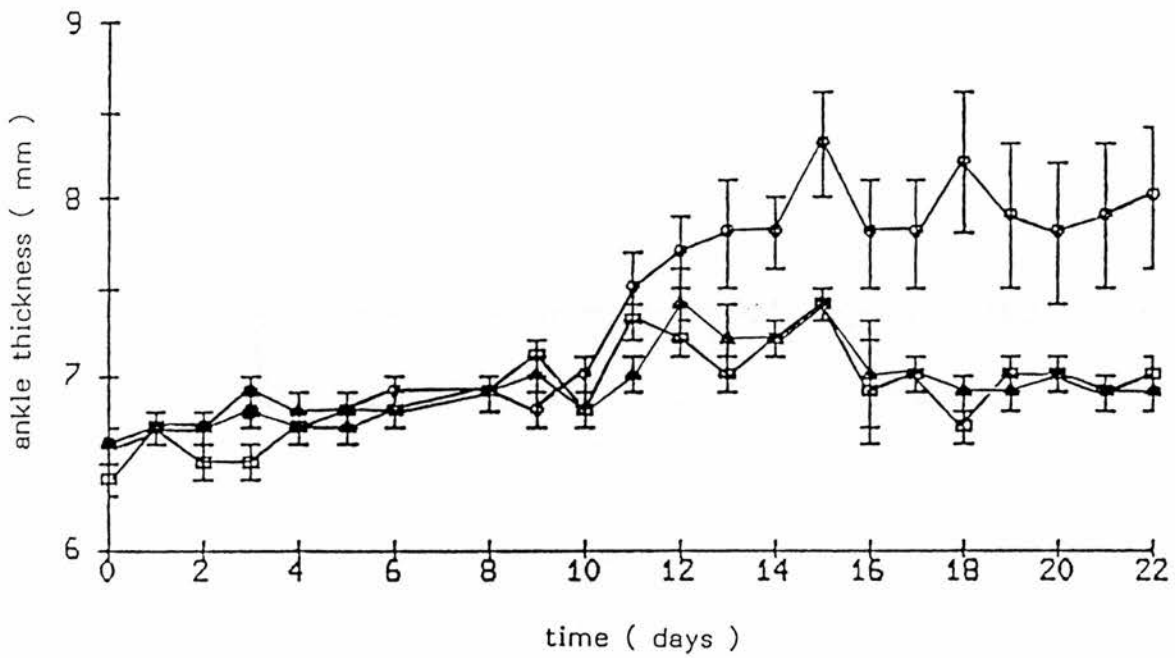


-○- Untreated arthritic rats.

-△- Indomethacin treated arthritic rats.

-□- Non-arthritic rats (untreated).

Figure II-5. The effect on the ankle swelling in the uninjected limb of arthritic male Wistar rats treated with 3.0 mg/kg/d oral indomethacin, commencing on the day of FCA injection (day 0). Experiment EXA3.

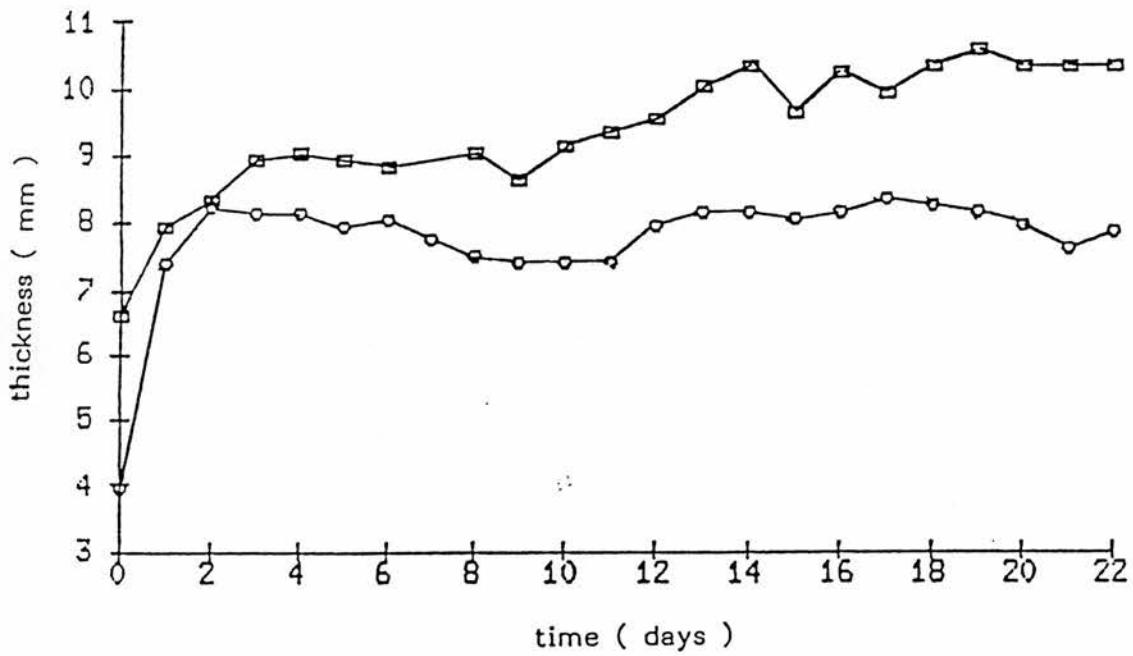


-○- Untreated arthritic rats.

-▲- Indomethacin treated arthritic rats.

-□- Non-arthritic rats (untreated).

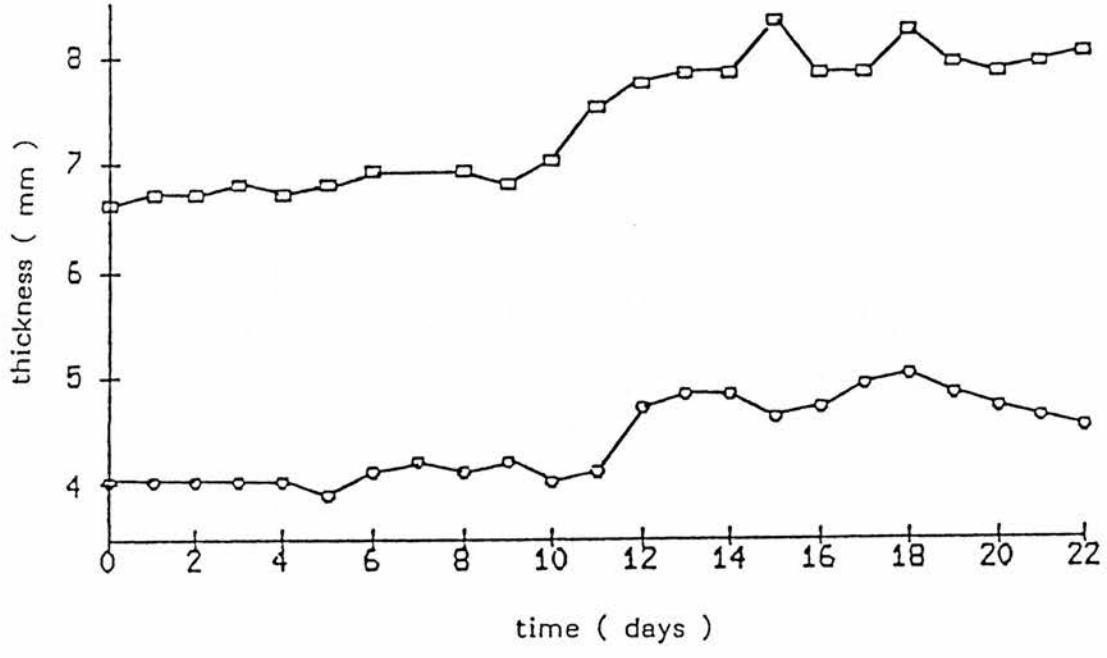
Figure II-6. The response-time profile of the mean ankle and paw swellings in the FCA injected limb of arthritic male Wistar rats. Experiment EXA3.



-○- FCA injected paw.

-□- Ankle swelling of FCA injected limb.

Figure II-7. The response-time profile of the ankle and paw swelling in the uninjected limb of arthritic male Wistar rats. Experiment EXA3.



From Figure II-1 it would appear that the increase in thickness of the injected paw in untreated animals was extremely rapid. The following experiment was conducted in order to illustrate the speed of this response.

Method (Experiment EXA50)

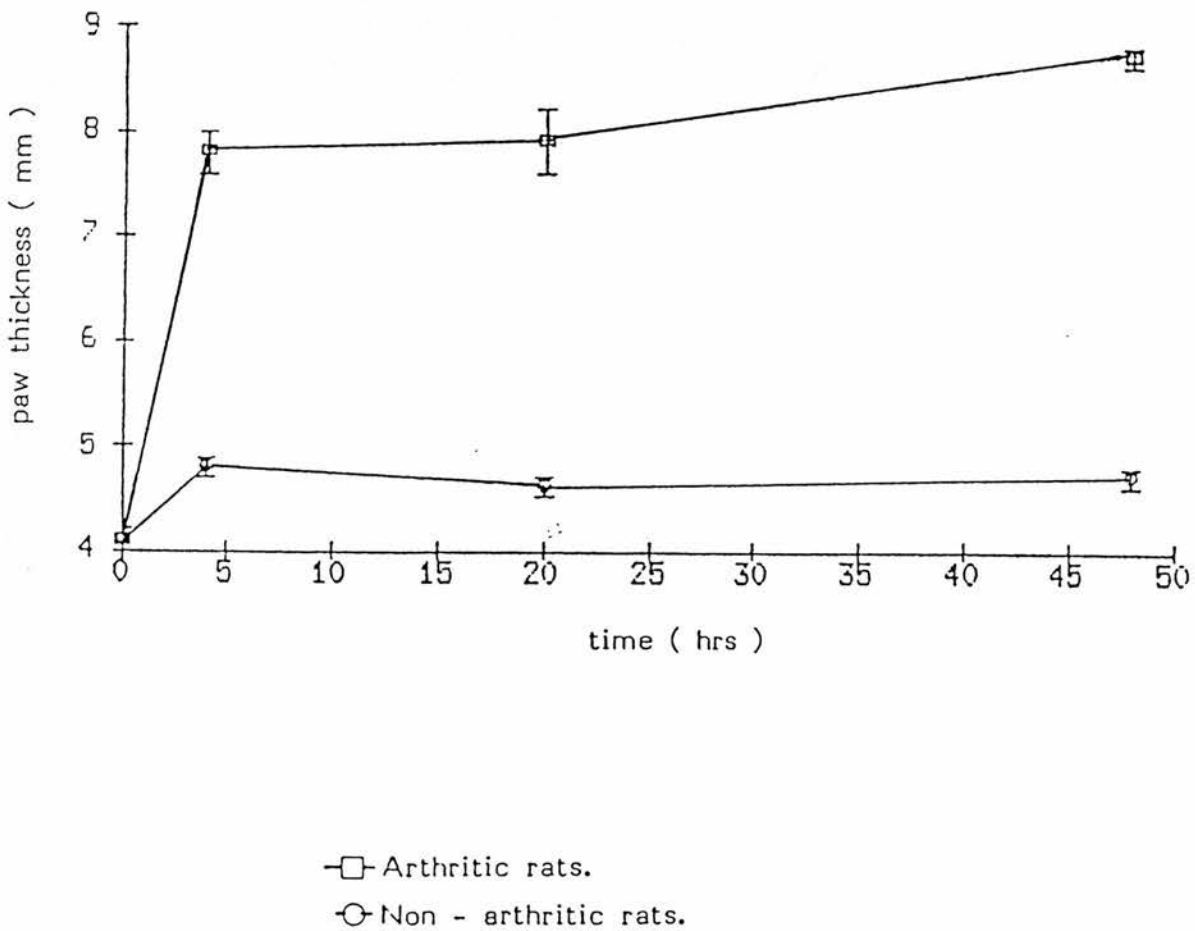
Two groups of 10 male Lewis rats were employed. The control group received subplantar injection of liquid paraffin, the arthritic group received arthritogen. Measurements of paw swelling were made at 4, 24 and 48 hrs only.

Results

Figure II-8 shows the swelling observed in the FCA injected limb at 4, 20 and 48 hours after the injection. By comparing this profile with that for the untreated animals over the initial period of Figure II-1 it is evident that the onset of the swelling was very rapid, being almost completely established within the first 4 hours, with little further change at 20 hours, and a slight further increase at 48 hours.

Again referring back to Figure II-1 therefore, treatment with indomethacin at this dose did not greatly inhibit the initial, extremely aggressive, phase of the response, although it was reduced to some degree. The effect became progressively more evident however from about Day 11 onward (Figure II-1).

Figure II-8. The rate of appearance of swelling in the FCA injected paw of male Wistar rats. Experiment EXA50.



Similar effects may be seen on established paw swelling if NSAID treatment commences in the secondary phase of the disease. For reasons discussed below however, the introduction of drug therapy in rats with established disease, and the interpretation of the results observed, requires particular care.

Given the clarity of the basic rationale, and such dramatic effects as illustrated above, it is hardly surprising that the arachidonic acid/NSAID approach was pursued with such vigour. This emphasis on drugs which could effectively intervene in the acute inflammatory process reached its zenith, or nadir, depending on the point of view, when it was suggested that study of the chronic phase of the rat disease could be dispensed with totally, and that drugs need only be screened for their effects in the initial 2 days of the disease (Bhargava, 1971).

So potent, in fact, are the NSAIDs in this model that their efficacy is evident when topically applied (Kyuki, 1982, Larson and Lombardino, 1980). Piroxicam is particularly effective when administered by this route (Larson and Lombardino, 1980), and it has been claimed that the induction of gastrointestinal ulceration by indomethacin (a major side effect of this drug, as with all NSAIDs) is significantly reduced in relation to its efficacy if given topically (Francis, Horn et al, 1983). This has naturally led to a search for topical delivery systems which will increase

the benefit/risk ratio for such compounds by reducing the level of systemic side effects. This approach has not so far produced any dramatic benefit.

The effects of the NSAIDs in rat arthritis are not restricted entirely to the overt lesions. Affected animals exhibit an increase in platelet aggregation, due apparently to a factor present in the plasma (Lassman, Kirby et al, 1974). This was thought for a time to be fibrinogen. It has however been shown that aspirin treated arthritic rats have normal levels of platelet aggregation, but retain the elevated levels of plasma fibrinogen characteristically seen in rat arthritis. Aspirin, and possibly NSAIDs generally, normalise platelet aggregation in arthritic rats, by mechanisms which do not involve fibrinogen (Lassman, Kirby et al, 1974). Platelet production is elevated in the arthritic rat, and the presence of circulating platelet aggregates has been demonstrated (Gorog and Kovacs, 1978). Aspirin also appears to protect arthritic rats against the formation of such aggregates and against thrombocytosis (Gorog and Kovacs, 1978).

Closer scrutiny does however indicate a number of deficiencies even when considering the effects of the NSAIDs on the outward signs of the acute inflammation in this model. For example, the pyrexia associated with the inflammation is not reduced quite in proportion to the decreased paw swelling, and the pain threshold is often reduced only by relatively high doses of

NSAIDs (Sofia, Vassar et al, 1973). Further, although there is clearly a major immunological component to rat arthritis, NSAID treatment has repeatedly been shown to have no effect on the immune response (Binderup, Bramm et al, 1982), with one possible exception. It has been reported that phenylbutazone given in high doses (> 100 mg/kg/d commencing just before and continuing for the few days following adjuvant injection) reduced the ability of spleen cells from adjuvant rats previous sensitised to EL_4 cells to lyse these cells in vitro (Chan, 1977a). The general consensus is that NSAIDs do not influence immune parameters. There have, for example, been no reports of their successful use in the treatment of experimental allergic encephalitis. In this disease, anti-myelin antibody is produced to host tissue. The immune component of the process has a more direct involvement in causing damage to the end organ. Rat adjuvant arthritis probably differs in that tissue damage is mediated more by PMN and macrophage activity which may be more responsive, in the rat at least, to the NSAIDS. Such a view is consistent with the premise that such drugs are unable to affect underlying immunologically mediated mechanisms of tissue destruction.

As an aside, there is also a form of rat arthritis which is induced by i.v. injection of a living culture of M. arthritides, and which is similar to adjuvant induced arthritis in respect of the progression of the signs of the disease. Here, the paw

swelling is in fact significantly exacerbated by treatment with salicylates (Eisen and Loveday, 1973).

More importantly however, whilst tissue swelling is dramatically reduced by NSAIDs, there is progressive bone and cartilage destruction (Ackerman, Rooks et al, 1979). X-ray analysis of indomethacin treated arthritic rats has established that bone damage is in fact reduced, but that the development of bone erosions and osteoporosis remain almost unchanged (Blackham, Burns et al, 1977). There is now general agreement that NSAIDs do not alter the long term disease progression in strains of rats susceptible to the disease (Littler, 1981), and in particular, all of the disease signs reappear if treatment is stopped (Newbould, 1965). The same pattern is seen in humans. There is a reduction in signs and symptoms of acute inflammation, but the underlying tissue damage continues (Semble, Metcalf et al, 1982). In making comparisons between the human and the rat disease therefore, there is a difference in so far as the bone and cartilage damage which occurs in the untreated arthritic rat appears with explosive rapidity, whilst this is not generally true of the human. The effect of the NSAIDs in the rats seems to be to reduce this rapidly progressive damage to a level more comparable to that in humans. It may be that, in rat arthritis, there is a mechanism for tissue damage which is disproportionately active when compared to the human, and which is mediated primarily by products of arachidonic acid metabolism. If both species have an

additional mechanism in common by which slower but more inexorable damage occurs, then the failure to exploit this aspect of the model would be one explanation for the present lack of progress in this field. In the subsequent chapter the role of the PMN in producing the explosive damage in the rat is explored, and possible approaches to establishing the nature of the more important mechanisms which may underlie both the human and rat diseases are discussed.

The anti-inflammatory steroids behave in a very similar manner to the NSAIDs. Their ability to reduce signs and symptoms of inflammation has long been known, and is at least as great as that of the NSAIDs (Kyuli, Shibuya et al, 1981). It was not realised until relatively recently, however, that the two classes of compound share a common pathway of activity. By preventing synthesis of phospholipases, the anti-inflammatory steroids effectively block all pathways of arachidonic acid metabolism, and thereby prevent the synthesis of prostaglandins and thromboxanes (Di Rosa, Persico et al, 1980). The lack, until recently, of a comprehensive rationale for the relevant mechanisms of action of the anti-inflammatory steroids may well explain the greater caution with which they have been approached. The benefit/risk ratios for the two classes of compound, are probably not greatly different. The anti-inflammatory steroids, however, have numerous activities in addition to their ability to inhibit prostaglandin and thromboxane synthesis. They can, for example, inhibit

mononuclear cell and PMN migration in vivo (Thieme, Mirkovich et al, 1982) which may be a consequence of their ability to inhibit leukotriene synthesis or to stabilise cell membranes. They also have generalised immunosuppressive effects (Littler, 1981).

As with the NSAIDs, it would be useful to illustrate the efficacy of this class of drugs at least on the outward signs of the disease, using in this instance the glucocorticoid 11β-17α-dihydroxy-21-methyl-pregna-4-ene-3,20-dione-17-n-butyrate (DMB) (Woods, Cairns et al). This steroid shows significant evidence of anti-inflammatory activity when administered transdermally to humans (Clanachan, Devitt et al, 1980).

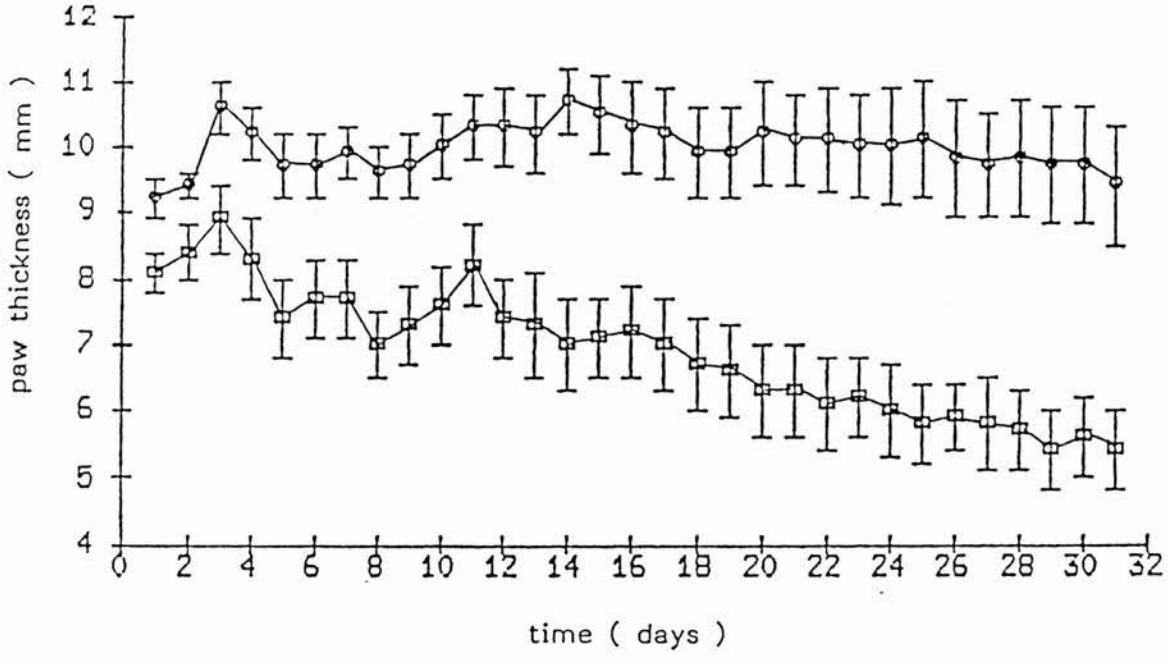
Method (Experiment EXA20)

Two groups of 10 male Wistar rats received subplantar injection of arthritogen on day 0. The arthritic control group received daily subcutaneous injection of saline, whilst the second group received the glucocorticoid DMB at a dose of 1.0 mg/kg/d in saline suspension by daily subcutaneous injection.

Results

Figure II-9 shows the effect on the FCA injected rat paw. The swelling in this paw was suppressed at every time point, and returned virtually to normal levels by about day 30. Dissemination of the disease to the uninjected paw was almost totally inhibited (Figure II-10). However, despite the apparent efficacy of the treatment, the body weight gain of the treated animals was consistently lower than for the arthritic animals (Figure II-11), and there was a considerable degree of splenic, adrenal and thymic atrophy (Table II-1).

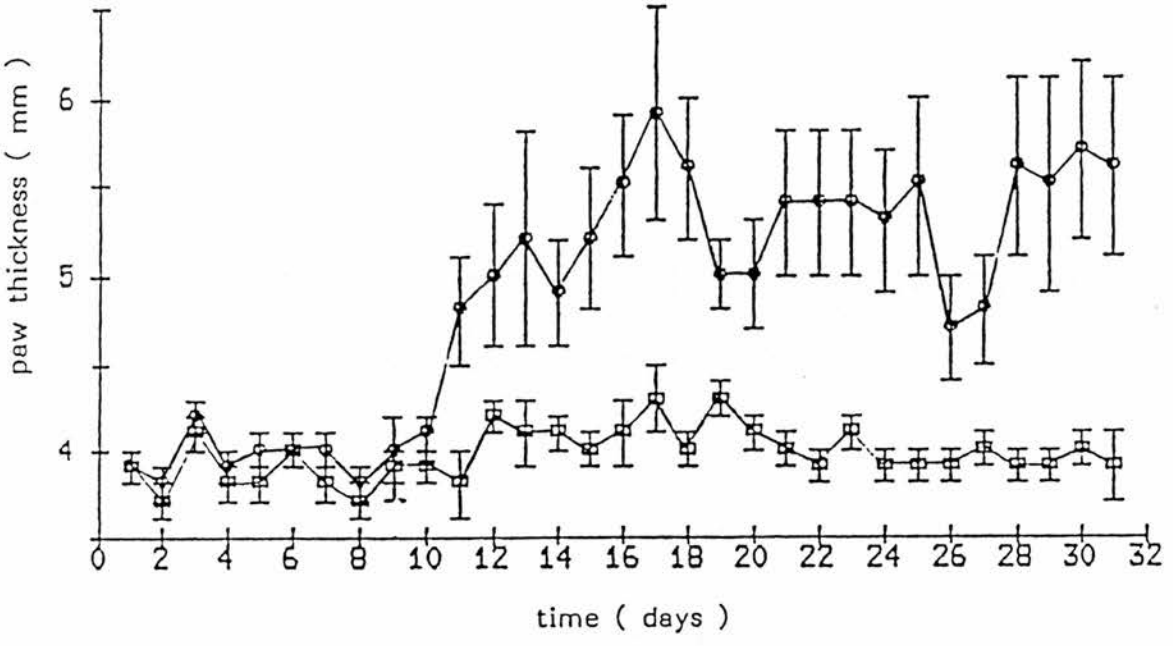
Figure II-9. The effect of the glucocorticoid DMB on the swelling of the FCA injected paw of male Wistar rats treated with daily subcutaneous injection of 1.0 mg/kg commencing on the day of FCA injection (day 0). Experiment EXA20.



-○- Untreated arthritic rats.

-□- DMB treated arthritic rats.

Figure II-10. The effect of the glucocorticoid DMB on the paw swelling in the uninjected limb of male Wistar rats treated with daily subcutaneous injection of 1.0 mg/kg commencing on the day of FCA injection (day 0). Experiment EXA20.



-O- Untreated arthritic rats.

-□- DMB treated arthritic rats.

Figure II-11. The body weights of arthritic male Wistar rats compared to those of arthritic rats treated with daily subcutaneous injection of 1.0 mg/kg of the glucocorticoid DMB commencing on the day of FCA injection (day 0). Experiment EXA20.

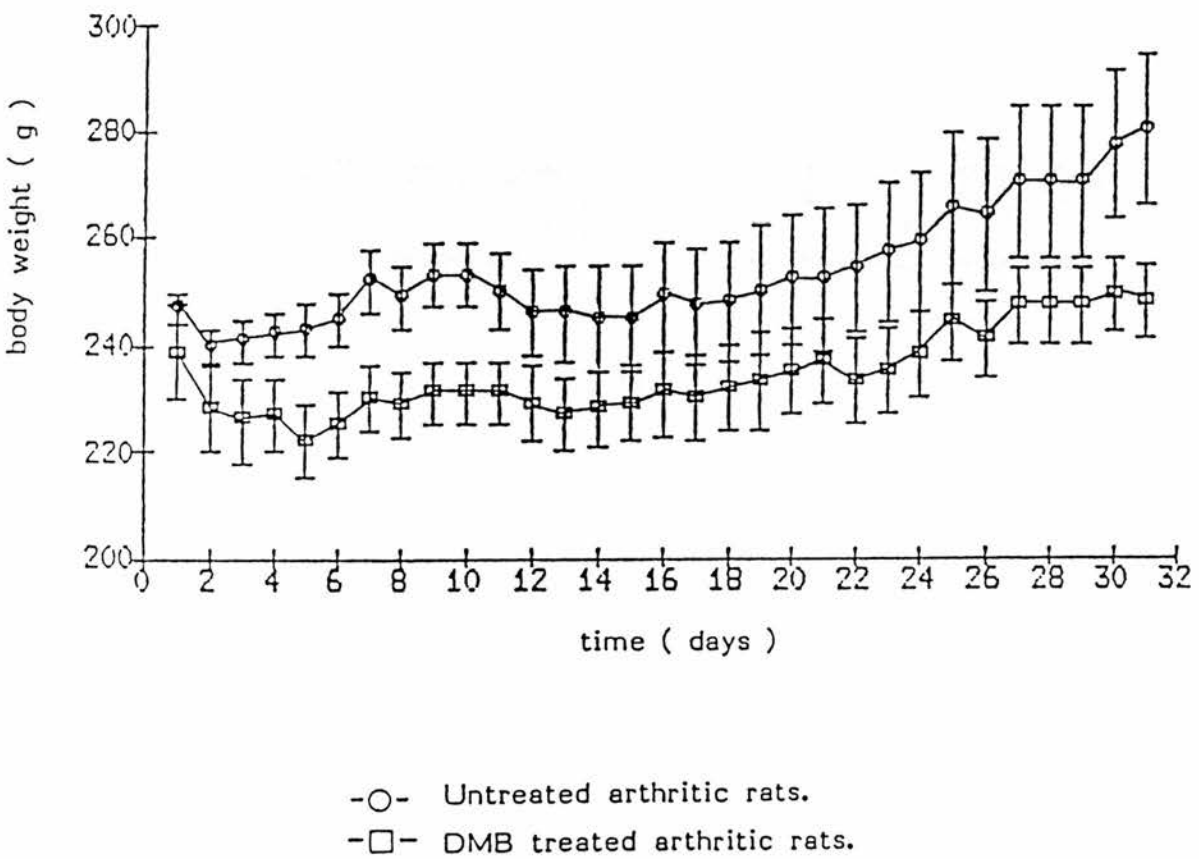


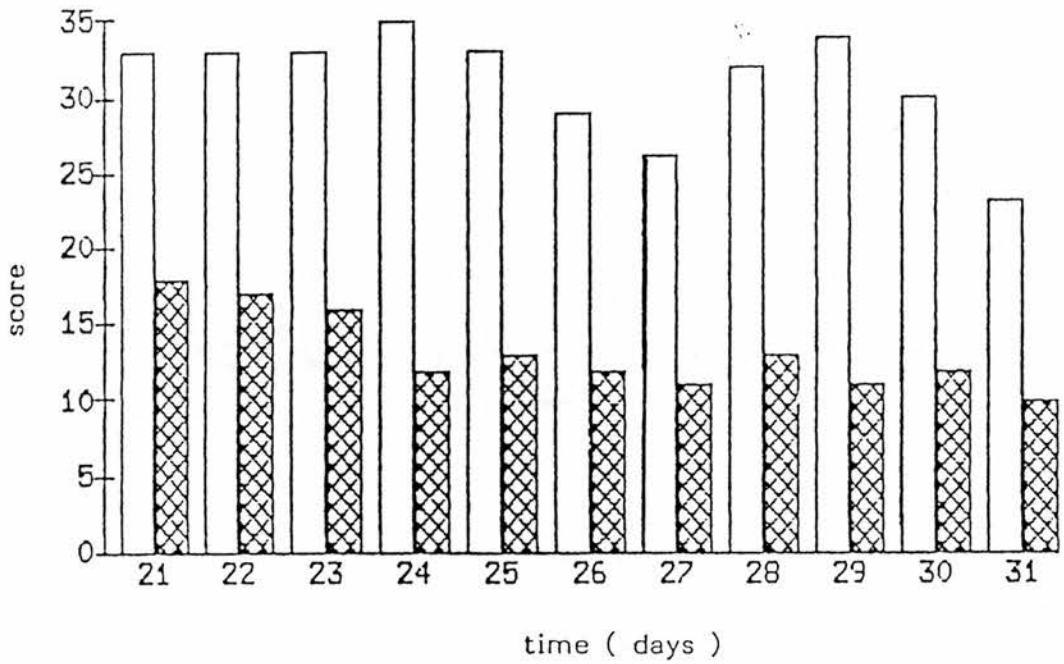
Table II-1. The weights of spleen, thymus and combined adrenals of male Wistar arthritic rats following treatment with DMB subcutaneously for 31 days at a dose of 1 mg/kg/d. Experiment EXA20.

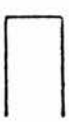
	Combined adrenal weight (mgs)	Thymus weight (mgs)	Spleen weight (mgs)
Untreated arthritic	56 ± 4	375 ± 33	967 ± 86
DMB treated arthritic	16 ± 1♦	197 ± 26♦	484 ± 26♦
Normal values	50 ± 4	592 ± 35	762 ± 32

♦ Significantly different from the untreated arthritic group, $P < 0.05$

Normal values quoted are taken from a separate experiment involving animals of a similar degree of maturity.

Figure II-12. The total arthritic score (see text) for untreated arthritic male Wistar rats compared to those treated with daily s.c. injection of 1.0 mg/kg DMB, commencing on the day of FCA injection (day 0). Experiment EXA20.





Untreated arthritic rats.

DMB treated arthritic rats.

An additional disease metameter was included in this particular experiment. An "arthritic score" may be defined, which is simply the total of the number of limbs, including the tail, which show visible evidence of disease progression, (erythema, oedema etc.). This quite deliberately takes no account of the severity of inflammation in the involved limbs, the intention being to attempt to identify those drugs which reduce the severity of inflammation, but which do not inhibit the spread of the disease. The effect of DMB on this metameter was impressive. In the primary phase all animals should exhibit an arthritic score of 1, whilst in the secondary phase the most severely affected animals may reach a score of 5. From Figure II-12 it is apparent that disease dissemination was apparently restricted by treatment with DMB. From approximately day 24 onwards only the injected limb appeared to be involved.

The results of the above experiment seem to indicate that there has been a real effect of the drug on the underlying disease, particularly in respect of the effect on the arthritic score. Due caution should be exercised in interpreting the results of such data, since very potent anti-inflammatory compounds may suppress the signs of disease to an undetectable level in all limbs, thereby giving the impression that the dissemination of the

arthritis has been prevented. It would then be necessary to determine the effect of terminating the treatment, in which case the signs of systemic disease should rapidly reappear, as is generally the case for the anti-inflammatory steroids. In this study, the effect of withholding the drug was not investigated. There have been sufficient studies to show that, exactly as with the NSAIDs, if treatment is stopped, the disease recurs, and, whilst chronic administration of steroids does reduce x-ray assessed bone damage, bone erosions and osteoporosis progress (Blackman, Burns et al, 1977).

The experiments described above serve to illustrate that NSAIDs and the corticosteroids have marked inhibitory effects on the apparent progression of the disease. There is however a growing body of evidence to show that the effect is superficial, and that no lasting benefit is gained. This reflects very well the growing experience with these drugs in human disease, and emphasises that, if the model is to prove useful, more fundamental parameters characteristic of the disease should be monitored than simply the outward manifestations of inflammation.

In the experiments described so far, and indeed in most published reports of studies involving rat adjuvant arthritis, drug administration has commenced on the day of disease induction. In the human situation, it has not so far been possible to anticipate the appearance of rheumatoid arthritis in susceptible

individuals. In every case therapy begins after the disease has become established. Logically, therefore, it would seem more relevant to investigate drug effects in established rat arthritis also. There are problems, however, the first being the rapidity with which tissue and bone damage occurs. This makes the timing of the first drug administration critical. It would be an unrealistically severe test of any drug to expect significant effects to be exhibited if administration were to begin after the stage where tissue and bone damage have become irreversible. A further difficulty is that there is a change in the pharmacokinetics of many drugs in the secondary phase of rat arthritis.

Drug pharmacokinetics in adjuvant arthritic rats

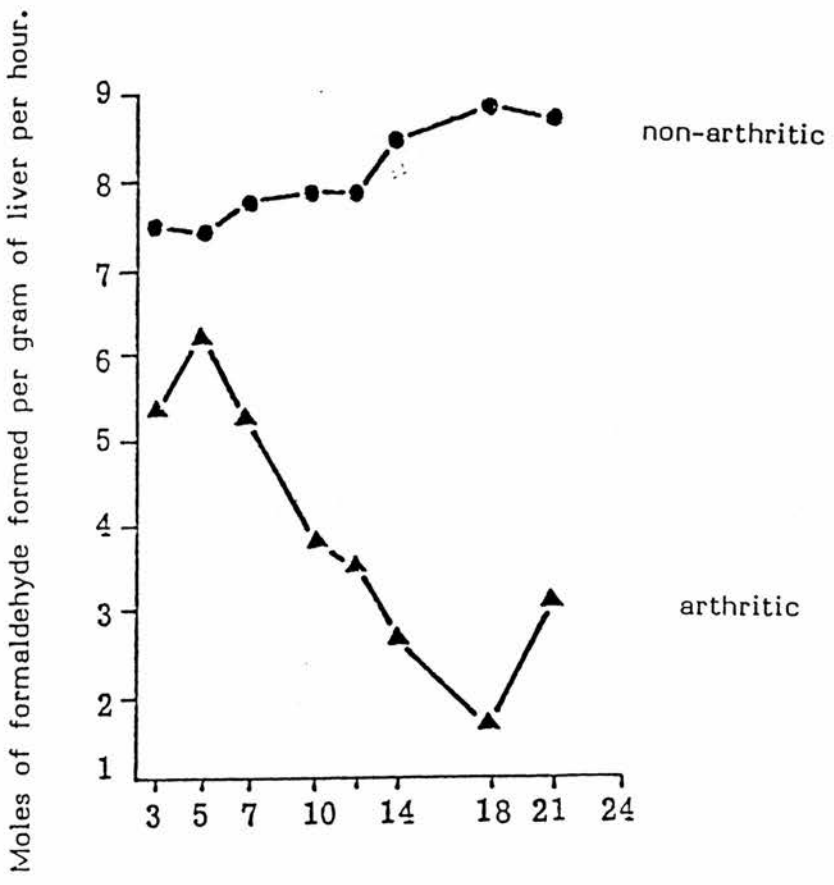
The development of rat arthritis is accompanied by a progressive alteration in the amounts and proportions of the serum proteins. The effect on serum albumin levels is particularly striking. There is a rapid fall immediately following FCA injection which reaches a minimum after about 2 days. Thereafter the levels again begin to rise. This is followed by a further more dramatic fall at the beginning of the secondary phase of the disease. Subsequent levels tend to remain low (Lowe, 1964). The total protein levels are generally unchanged, however, due to a

compensatory rise in α and β-globulins which may reflect increased hepatic synthesis of these proteins in response to the ongoing joint and tissue damage (Lowe, 1964, Billingham and Gordon, 1976). In this respect the changes in serum albumin are similar to those seen in human rheumatoid arthritis (Lowe, 1964). One consequence of these changes is that drug-albumin binding alters as the disease progresses, drug clearance is generally retarded, and there is an increase in the proportion of non-protein bound drug, which generally is the pharmacologically active form. Phenylbutazone plasma protein binding is known to be affected in this respect (Januki, Perlik et al, 1980), although there is a report that the number of phenylbutazone binding sites per albumin molecule appears to be increased in rat arthritis, possibly due to a disease associated change in the tertiary structure of the protein (Perry, Jonen et al 1976). Other drugs which have been shown to exhibit similarly reduced serum protein binding include the anti-inflammatory 2,3-dihydrobenzo-furan-2-ones, etodolac and furobufen (Closse, Haefliger et al, 1981). Benoxaprofen, which is normally 99% bound to serum protein similarly shows reduced binding in rat disease, in this case because there is weaker binding to the α and β-globulins which cannot therefore compensate for the reduced capacity for albumin binding (Chatfield, Green et al, 1978).

There is also an inability of arthritic rats in the secondary phase of the disease to metabolise the drugs administered, due

mainly to a reduced activity of the mixed function oxidase system. Perhaps the most striking example of this concerns the hexobarbitone induced sleeping times in arthritic rats (Morton and Chatfield, 1970). These are several-fold longer than in normal animals, showing a clear impairment of metabolic drug detoxification mechanisms. Specific enzyme systems have been shown to be impaired. As an example, Figure II-13 illustrates the progressive reduction in activity of hepatic aminopyrine demethylase as the disease progresses (Cawthorne, Palmer et al, 1976). Whether such an effect is specific to rat arthritis is not certain. Prolonged hexobarbitone sleeping times have been observed in a chemically induced "arthritis", produced by repeatedly injecting 6-sulphanilamidoindazole into aged rats (Muller and Hirschelmann, 1981). It may be that the changes observed are a general reaction to inflammation. However, there are conflicting reports concerning drug metabolic activity in experimental allergic encephalitis. There is evidence that the levels of the mixed function oxidases are normal (Beck and Whitehouse, 1973), whilst other studies appear to show a depression (Carlson and Ciaccio, 1975).

Figure II-13. The activity of hepatic aminopyrine demethylase as a function of time in adjuvant arthritic rats (From M. A. Cawthorne, E. D. Palmer and J. Green. Adjuvant induced arthritis and drug metabolising enzymes. Biochem. Pharmacol. 25, 2683-2688, 1976).



It is also the case that Buffalo rats, which show no overt evidence of inflammatory polyarthritis following FCA injection, nevertheless show an impaired ability to metabolise a range of drugs (Beck and Whitehouse, 1973). This tends to suggest that the effect is not merely due to the presence of chronic inflammation.

Although other factors are involved (Fernandini, Cayen et al, 1982), the major underlying metabolic lesion, whatever its aetiology, is a failure of the microsomal mixed function oxidase system. By day 14 of the disease, microsomal oxidative capacity is reduced some 80% (Carlson and Ciaccio, 1975) The effect is not apparently due to a loss of enzyme capability, however, in the sense that normal enzyme function in these rats can be restored by induction. Lengthened hexobarbitone induced sleeping times can be restored to normal by enzyme induction using phenobarbitone (Beck and Whitehouse, 1973). Similarly, impairment of the aryl hydrocarbon hydroxylase system can be reversed by enzyme induction with benzo(a)pyrene (Carlson and Ciaccio, 1975).

Deliberate induction of hepatic damage may alleviate the disease. Treatment of rats with dimethylnitrosamine induces a necrotising hepatitis which rapidly resolves when administration stops. When administered to arthritic rats the compound causes partial resolution of the disease (Pinals, 1973). Hepatic

disease has also been reported to ameliorate human arthritis (Hench, 1933). The liver can apparently synthesise an anti-inflammatory protein (Billingham, Gordon et al, 1971) and there is a possibility that this protein is liberated by necrotising hepatic tissue. The phenomenon of inhibited enzyme activity in rat arthritis is unlikely to be related to hepatic damage therefore.

The mixed function oxidase system in both man and many laboratory animals has been shown to consist of multiple forms of cytochrome P450 (CyP450) (Eichelbaum, 1981). As a consequence, whilst NSAID treatment in general suppresses overt disease signs and also repairs the metabolic lesion, in that the mixed function oxidase system is restored to normal, this is not true in every case. Phenylbutazone ameliorates the outward inflammatory signs of rat adjuvant arthritis, but does not restore hexobarbitone sleeping times to normal in the treated animals (Pasquale, Rassaert et al, 1975). It has been suggested, therefore, that some metabolic lesions are not corrected by phenylbutazone treatment, which may be a direct consequence of CyP450 polymorphism (Pasquale, Rassaert et al, 1975).

The mechanism(s) by which effects on the CyP450 system are produced remain ill-understood. In one study, interesting parallels have been observed between the situation in arthritic animals and those treated with honeybee venom, which consists largely of phospholipase-A and mellitin. In both cases CyP450

activity was found to be depressed without there being any apparent effect on haem biosynthesis, although levels of CyP450 (a haem protein) were greatly reduced (Billingham, Gordon et al, 1971), as were the associated mixed function oxidase activities. Both arthritic and bee venom treated animals exhibited increased microsomal haem oxidase activity. This enzyme catalyses the metabolism of haem proteins and may explain the reduced CyP450 levels (Eiseman, von Bredow et al, 1982). These authors develop the argument that increased levels of phospholipases, or of interferon induced by endotoxins or immune processes triggered by FCA injection, may be responsible for many of the effects observed (Eiseman, von Bredow et al, 1982).

Macroscopically, white deposits have been reported to form on the surface of the livers of rats shortly after FCA injection (Ishizuki, Furuhata et al, 1983). Hepatocytes from such animals showed a reduced ability to metabolise aminopyrine and aniline, and an impaired uptake of the dye indocyanine green (Ishizuki, Furuhata et al, 1983). Microscopically, these hepatocytes showed an absence of microvilli, protrusions were seen in the plasma membrane, and there was widespread distribution of the rough endoplasmic reticulum associated with a decrease in the area of smooth endoplasmic reticulum. The RNA/DNA ratio was found to be higher than in normal animals, and there is a suggestion that the changes seen may be a consequence of elevated acute phase protein

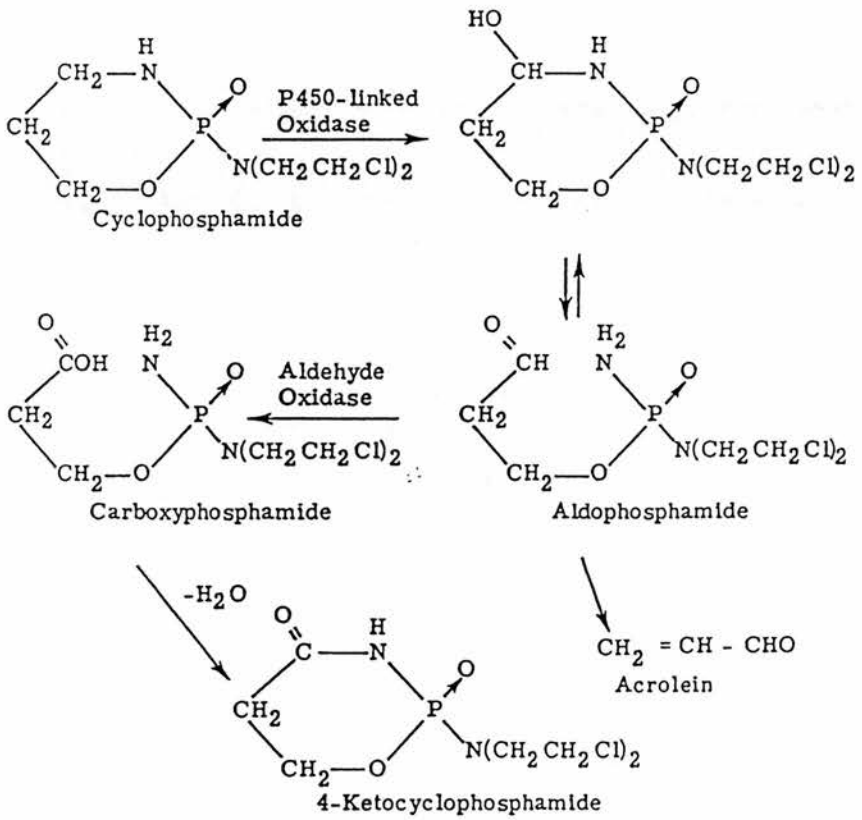
synthesis produced by the inflammatory stimulus (Ishizuki, Furuhata et al, 1983).

What is clear, therefore, is that induction of adjuvant disease is accompanied by serum protein changes which will affect drug distribution and end organ activity, and by a generalised reduction of CyP450 and therefore of at least the mixed function oxidases. Drug detoxification and clearance will be affected in ways which may be difficult to quantify or predict. These changes may be prevented by successful drug intervention. The situation where a specific drug is tested in the model by administering it from the day of adjuvant injection (which may prevent the development of the metabolic lesion) could well be very different from the situation where drug administration begins during the secondary phase, after the lesion has become established. The relevance to the human case is also problematical. It is not clear to what extent drug metabolism is altered in human rheumatoid arthritis, although there is some evidence of reduced drug handling capabilities (Ehrlich, Freeman-Narrod et al, 1979).

To emphasise the importance to drug testing, it is useful to consider how some of the currently available drugs are affected by these considerations. Phenylbutazone exhibits greatly increased clearance times consequent to changes in serum protein levels (Januki, Perlik et al, 1980), and increased toxicity is to be expected. Of the anti-rheumatic or second line drugs,

cyclophosphamide depends for its immunosuppressive effect on metabolic conversion to alkylating species according to the scheme shown in Figure II-14.

Figure II-14. The metabolic pathways of cyclophosphamide in normal rats.



This cannot be achieved by Cyp450 depleted hepatic microsomes (Kitchin, Schmid et al, 1981), and cyclophosphamide metabolic activation may not occur in arthritic rats which have entered the secondary phase (Beck and Whitehouse, 1973). Levamisole, a further example of a second line drug, under normal circumstances is extensively and rapidly metabolised by the liver, and accordingly has a very short plasma half-life (Symoens, Rosenthal et al, 1979). This compound's behaviour is also likely to be altered in established rat arthritis therefore. These two latter compounds illustrate the problem well. Cyclophosphamide is not metabolically activated in the secondary phase of rat arthritis, which may (at least partially) explain its evident lack of effect if administration begins during this phase (Kitchin, Schmid et al, 1981). Levamisole, being slow acting, if at all, does not inhibit the onset of the metabolic lesion, which leads to drug accumulation and heightened toxicity.

As a practical illustration of the problem, the effect of 3.0mg/kg/d of indomethacin in this model has been described earlier. In that experiment, indomethacin treatment commenced on the day of FCA injection, and no adverse reaction unambiguously attributable to the drug was observed. In a further experiment, the effect of 1.5mg/kg/bd given morning and evening was investigated in rats with established arthritis.

Method (Experiment EXA51)

Sixty male Lewis rats received subplantar injection of arthritogen on day 0. On day 20, the 45 most severely arthritic animals were selected and allocated to three groups of 15 animals. The groups were balanced with respect to the severity of the swelling of both paws. One group received oral mulgophen from day 21, the second received indomethacin orally at a dose of 1.5 mg/kg/bd. Treatment of this second group was terminated on day 26 due to the emergence of unacceptable side effects. Comparison of this group with the untreated control from day 21 to day 26 is presented as experiment EXA51. The remaining group received 30 mg/kg/d of an experimental indane-dione. The response of this group, which was continued to the completion of the study on day 37, is presented as experiment EXA51b.

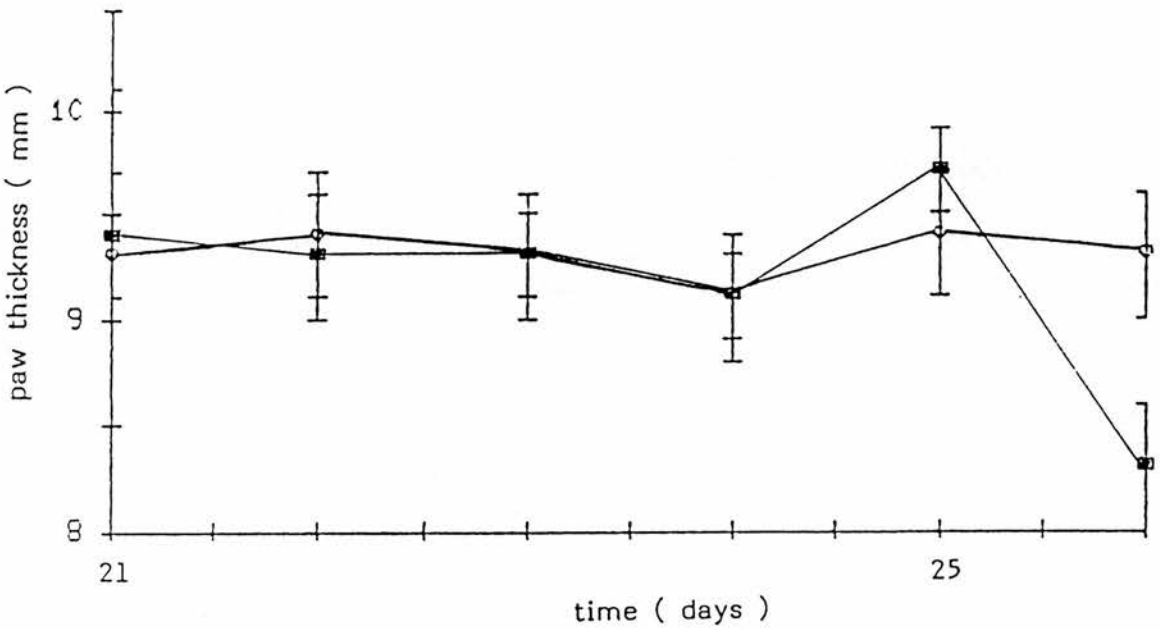
Results

As shown in Figures II-15 and II-16, the paw swelling began to respond to treatment after about four days. However, even when given in divided doses, the reduced metabolic clearance seems to be evident in the heightened toxicity. After 6 days of treatment, 3/15 animals were dead, after 7 days, 5/15, and

when the experiment was terminated, 8 out of 15 animals had died. There were no deaths in the untreated arthritic group.

The problem of altered drug handling with disease progression may lead to confusion when drug effects in this model are compared with those of the same drug in more acute models, or in humans. In any testing programme therefore, some attempt should be made to determine how the metabolism of specific drugs may change (Muller and Hirschelmann, 1981, Beck and Whitehouse, 1973), since the effects of the drug on established rat arthritis may be of most relevance to the human case. To some degree, the lack of emphasis on the ability of drugs to affect the disease when administration begins in the secondary phase, coupled with a tendency to ignore the effect of the disease on hepatic drug metabolism, has probably contributed to the failure to realise the full potential of the model.

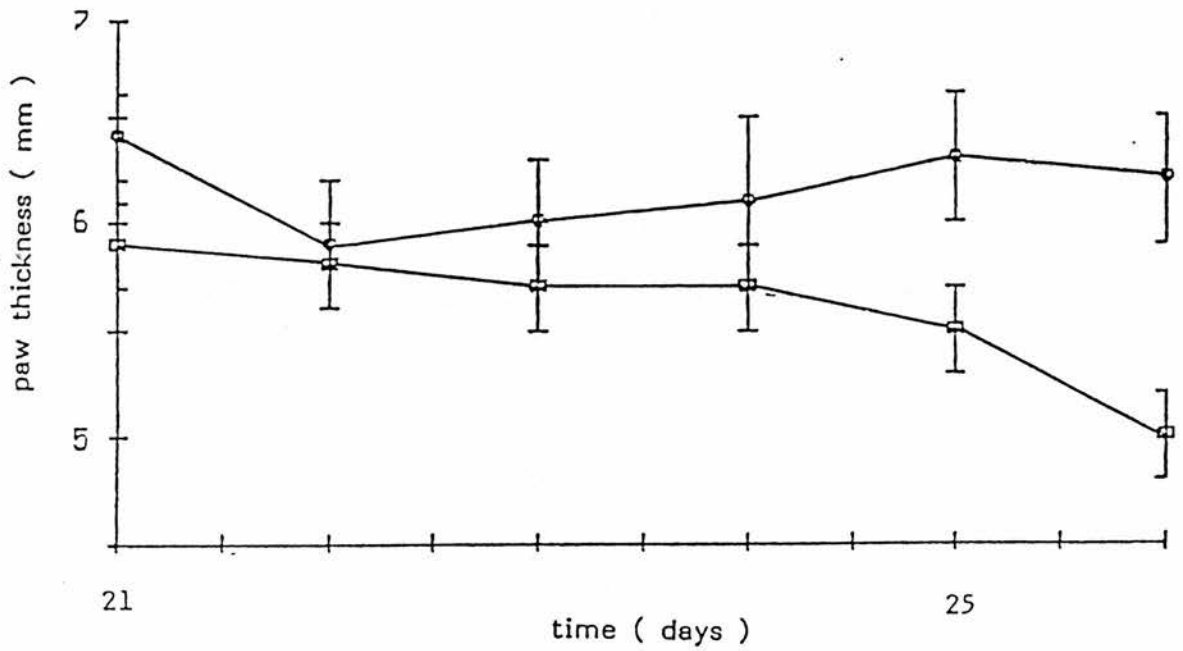
Figure II-15. The effect of 1.5 mg/kg/bd oral indomethacin on the swelling of the FCA injected paw in male Wistar rats with established arthritis. Dosing commenced on day 21 following FCA injection. Experiment EXA51.



-○- Untreated arthritic rats.

-■- Indomethacin treated arthritic rats.

Figure II-16. The effect of 1.5 mg/kg/bd oral indomethacin on the paw swelling in the uninjected limb in male Wistar rats with established arthritis. Dosing commenced on day 21 following FCA injection. Experiment EXA51.



-○- Untreated arthritic rats.

-□- Indomethacin treated arthritic rats.

The statistical analysis of repeated measures in chronic models

The third problem is not specific to rat arthritis, but is one which is encountered in most chronic situations, be it in animal pharmacology or investigational clinical studies of human disease. It is the problem of how to judge whether a treatment has been successful or not. In the case of the NSAIDs and the steroids, the effects observed are dramatic, and it is often possible to assert that a beneficial effect has been achieved simply by inspection of the data. In the example of Figure II-1, the paw and ankle swellings observed in the indomethacin treated group differ from the control group by rather more than the combined standard errors at all time points measured. It may therefore be asserted that the two groups differ, with an estimated probability of less than 0.05 that this assertion is incorrect ($P < 0.5$) without formal statistical analysis. However, with other probably more interesting drugs such as levamisole, d-penicillamine or the gold complexes, any effects are much less dramatic. In fact, in humans any putative effect of these drugs is slow to appear, and it may be that the same is true in rats. It is noteworthy that large numbers of patients had to be studied before statistically significant (according to current criteria) results could be quoted to support their efficacy in the human disease. If the rat disease is a good model of human arthritis in respect of those mechanisms which are ameliorated by

the second-line anti-rheumatics, (ie the chronic underlying disease progression is retarded in the long term, with perhaps only a marginal effect on the acute signs in the short term), it seems hardly surprising that these effects are not detected in the relatively small groups of animals usually employed in drug screening experiments. If this concept is true, the problem of how to detect marginal changes, and how to apply significance tests to these observations, becomes a matter of crucial interest. The problem is nicely illustrated by the following experiment, in which the effect of an investigational indane-dione on the paw and ankle swelling was assessed.

Method (Experiment EXA5)

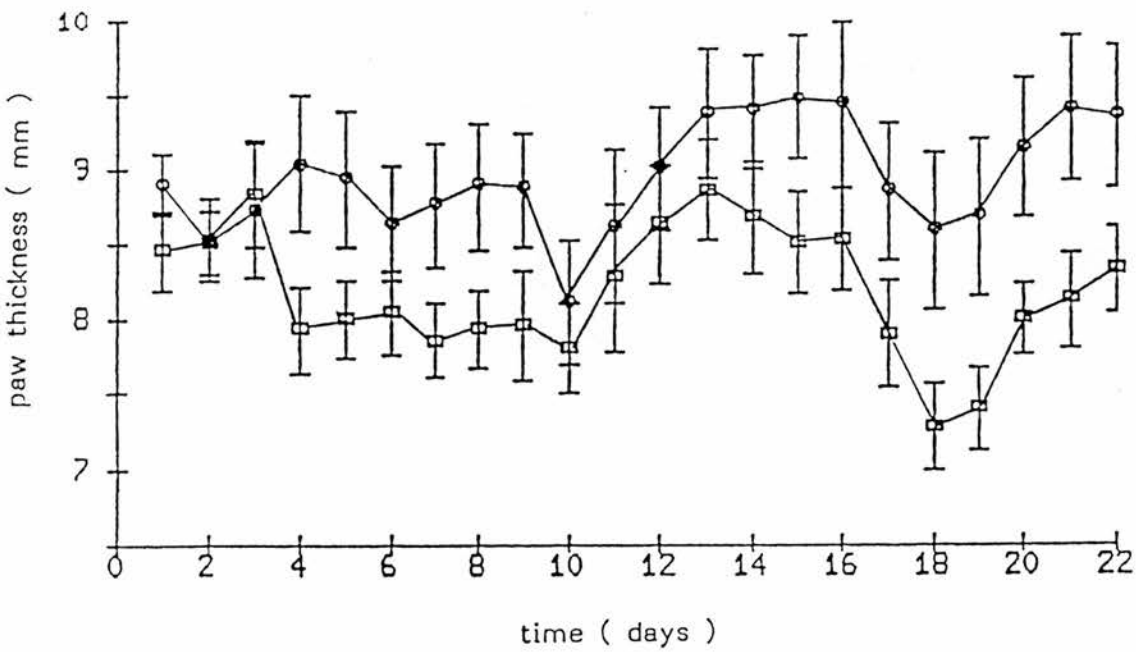
Male Wistar rats were divided into 2 groups of 10 animals. Both groups received injection of FCA into the left hind paw. One group received daily oral injection of an experimental indane-dione (30 mg/kg/d) in mulgophen. The second control arthritic group received daily oral dosing with mulgophen. Paw and ankle swellings were measured daily.

Results

The effects of the treatment on the injected and uninjected paw swellings are shown in Figures II-17 and II-18, and on the injected and uninjected ankle in Figures II-19 and II-20.

By inspection of the data, there is a suggestion that the swellings observed in the paws and the ankles are reduced slightly by the treatment.

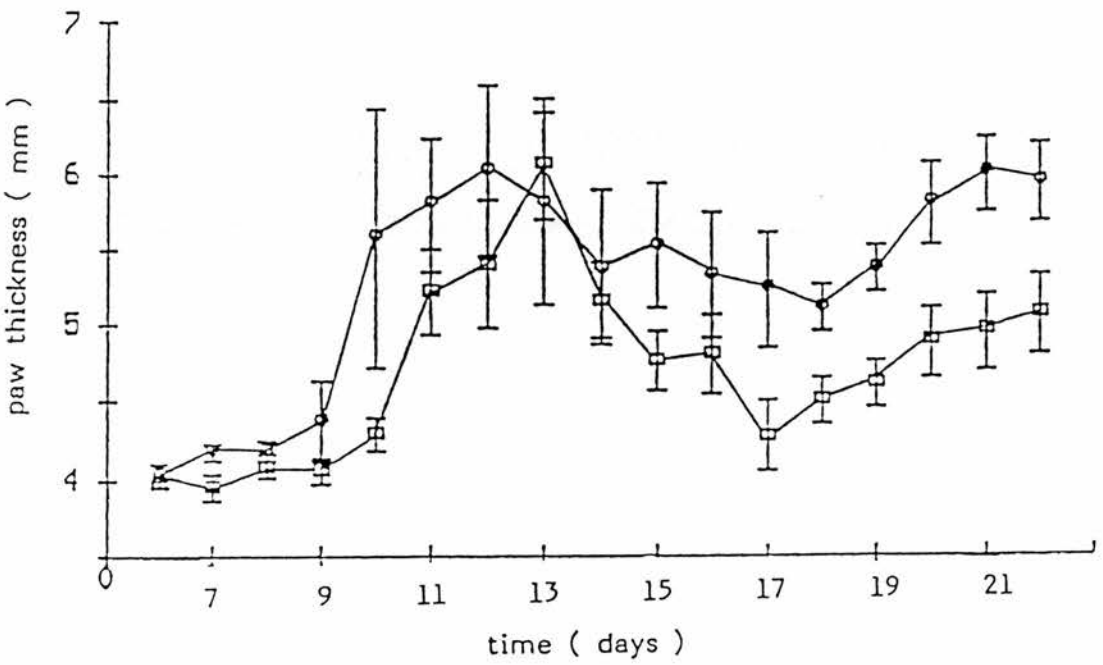
Figure II-17. The effect of 30 mg/kg/d of an orally administered experimental indane-dione on the paw swelling in the FCA injected limb of arthritic male Wistar rats. Dosing commenced on the day of FCA injection (day 0). Experiment EXA5.



-○- Untreated arthritic rats.

-□- Indane - dione treated arthritic rats.

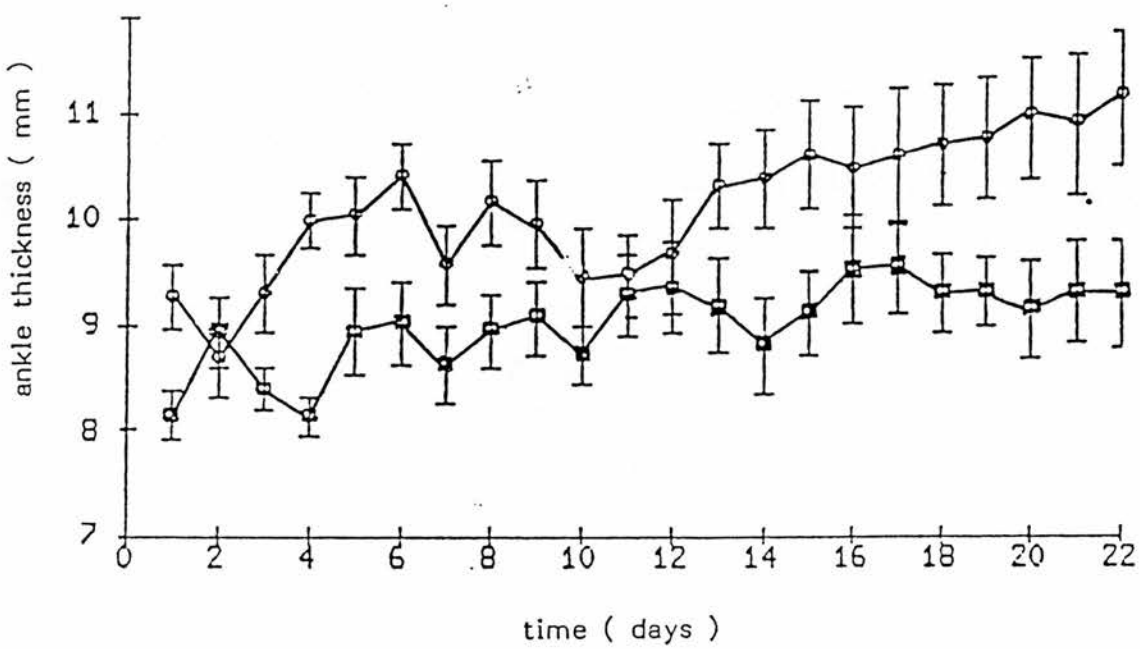
Figure II-18. The effect of 30 mg/kg/d of an orally administered experimental indane-dione on the paw swelling in the uninjected limb of arthritic male Wistar rats. Dosing commenced on the day of FCA injection (day 0). Experiment EXA5.



—○— Untreated arthritic rats.

—□— Indane - dione treated arthritic rats.

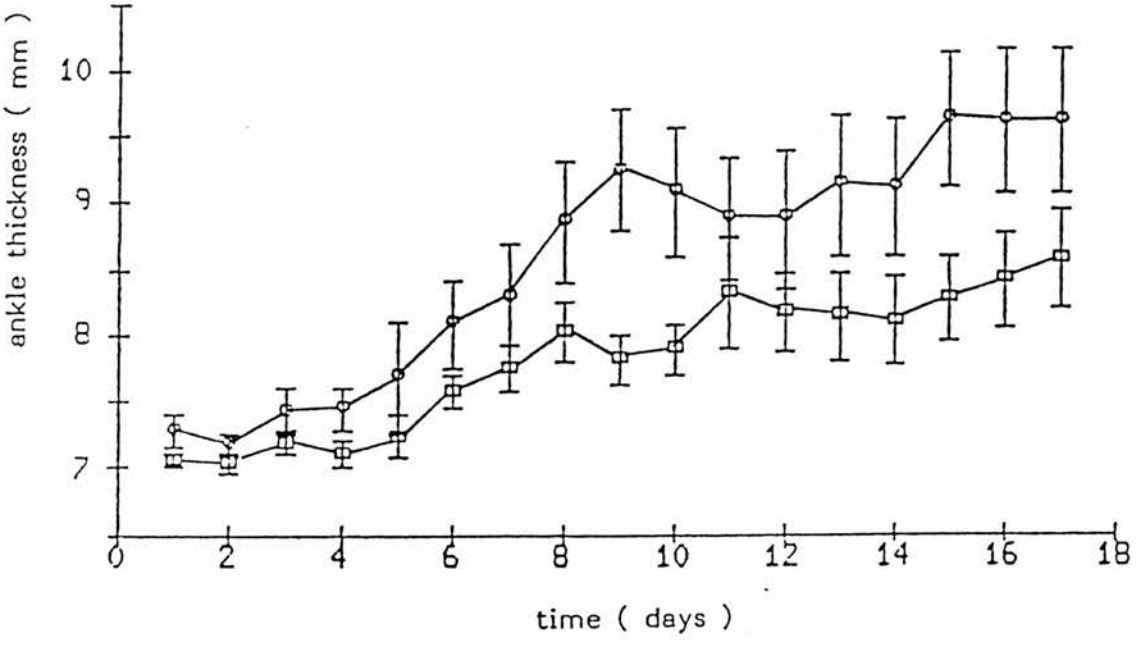
Figure II-19. The effect of 30 mg/kg/d of an orally administered experimental indane-dione on the ankle swelling in the FCA injected limb of arthritic male Wistar rats. Dosing commenced on the day of FCA injection (day 0). Experiment EXA5.



-○- Untreated arthritic rats.

-□- Indane - dione treated arthritic rats.

Figure II-20. The effect of 30 mg/kg/d of an orally administered experimental indane-dione on the ankle swelling in the uninjected limb of arthritic male Wistar rats. Dosing commenced on the day of FCA injection (day 0). Experiment EXA5.



-○- Untreated arthritic rats.

-□- Indane - dione treated arthritic rats.

In order to illustrate possible approaches to the statistical analysis of the data from the above experiment, taking first the injected paw as an example, over the period of the experiment, measurements were made on 20 consecutive days on each of two groups of animals, one group being the arthritic control, the other being treated with the test drug. By inspection of Figure II-17, on 12 out of 20 days the error bars do not overlap, on the remaining 10 days they do. Whilst it is very apparent that drug treatment was effective in the case of the experiment illustrated in Figure II-1, in this present example it is more difficult to decide whether the two treatments differ at the $P < 0.05$ level. In particular, by inspection of the data, the groups diverge "significantly" for the first time on day 4. It is tempting to assert, therefore, that a significant difference is achieved on this day. However, in a disease which may progress for another 50 days or more (as this does) a difference between the groups on one day only is of minimal interest, particularly since a cynic might observe that the "significant" difference appeared after repeated measurements on the same animals, and that, had we continued in this way "significant" differences were certain to appear (Siegel, 1956). (In general, if there were in fact no differences between the groups either in respect of treatment or time, results appearing to be "significantly" different at the level $P < 0.05$ will occur on average once in every 20 measurements made). Nor does it help to observe that "significantly" different

results appear on 4 of the subsequent 5 days. The same cynic would correctly observe that we are here dealing with dependent data sets (Krauth, 1980). That is, the paw swelling observed for any particular rat on day N will be dependent to a considerable degree on the swelling observed on day N-1. If we observe a significant difference on day N and on subsequent days, this is due initially to the fact that we are repeatedly measuring the same population, and the subsequent "significant" differences arise because of the dependence structure of the measurements. The problem then is, how to decide, with any degree of rigour, whether these groups differ or not.

The simplest approach is to sum the paw measurements for each rat over the whole period of the experiment, and to compare the groups in respect of this single metameter. As was described earlier, however, the progression of this disease fluctuates, and the measured values, for individual animals and for the group means, show cyclical exacerbations and (partial) remissions. These time dependent features are therefore lost by the summation procedure described, and this test tends to be very conservative, although in some simple situations it may prove useful. Applied to the data of Figures II-17 and II-18 this approach asserts that there is no significant difference between the groups.

An alternative is to attempt to ascertain whether or not the mean values of the two groups tend to diverge with time. (In all

of what follows it is assumed that paw swellings within each group are, in the main, normally distributed, or at least may be treated for the purposes of statistical testing as if they were. This will tend to be less valid in strains of animals which produce a significant proportion of non-responders, giving a biphasic distribution. Even in high responder strains such as those used here, the distributions tend to be skewed to the higher values. However, parametric tests in general do seem to be robust to the assumption of normality in this kind of population (Boneau, 1960)).

This can be done, for example, by using the Cox and Stuart test for trend (Conover, 1971) in the following way. Firstly, the differences between the group mean values are calculated for each day of the experiment. The problem then is to test whether these differences are tending to increase with time. To do this, the calculated differences are paired to produce data pairs (X_i, X_{i+c}) where each X_i is the difference between the group means at time i, and c equals one half of the number of time points measured. It is a requirement of this test that the elements of each data pair are independent. If c is small, and the elements of each pair of measurements were taken only a few days apart, the independence requirement will be violated. In this study the data elements are for measurements taken 11 days apart, by which time it is probable, but not certain, that sufficient random events

have affected the metameters that the data are effectively independent. The pairings obtained are shown in Table II-2.

Table II-2.

Cox and Stuart test for trend on the data for the injected paw of Experiment EXA5

Day of measurement	Difference between the mean paw swelling of the treated and untreated groups X_i (x0.1cm)	Day of measurement	Difference between the mean paw swelling of the treated and untreated groups X_{i+c} (x0.1cm)	Designation
1	5	12	4	-
2	0	13	6	+
3	-1	14	7	+
4	12	15	13	+
5	9	16	8	-
6	5	17	9	+
7	8	18	13	+
8	10	19	13	+
9	9	20	11	+
10	3	21	13	+
11	2	22	10	+

Nine positive and 2 negative designations shows a significantly diverging trend, $P < 0.05$.

Where, as is the case here, there is an odd number of data items, one item is discarded. By discarding the middle item, there is the greatest possible length of time between elements of each pair. The signs of the differences between the first and last element of each pair are shown in the last column of Table II-2. If there is a trend towards divergence these differences should show more positive than negative values. This may be tested using the Sign test (Conover, 1971). In the present case, for the injected paw there are 9 positive and 2 negative occurrences, which is significant ($P < 0.05$). It is not possible to perform a similar test of the uninjected paw unfortunately. It would only be reasonable to include in the analysis measurements made after about day 10, when secondary paw swelling becomes apparent, which leaves too few data points for this approach to be useful.

One advantage of the use of such a test of trend is that the temporal relationships are retained to some degree. A disadvantage is that the Sign test (Conover, 1971) on which the approach is based is a relatively weak test, since it takes account only of the signs of the changes observed, not their magnitude. Further, where a test drug has a rapid effect, achieving a plateau in the response very quickly, with the response curves running in parallel thereafter, the test is valueless.

A more powerful test is the one-way analysis of variance for repeated measures described by Winer (Winer, 1971). This is a parametric test appropriate for repeated measures designs of the kind encountered in many chronic studies. It detects gross differences in the mean tendency of each treatment group, and also detects differences in the shapes of the response curves. The underlying theory is relatively complex, and a detailed discussion is outwith the intended scope of this chapter. In outline, for each individual animal, the response measured at each time point is considered to be a linear combination of an effect due to the treatment, an effect due to time, and an effect which is due to a combination of time and treatment (the "treatment-time interaction"). Each effect in the linear combination has an appropriate error term. From these error terms, analysis of variance tables can be constructed. If there is an overall difference between the treatments, a significant "treatment effect" will be detected. This approach has the additional advantage that the temporal information is retained. If the response varies significantly as a function of time, there is a significant "time effect". Finally, if the response-time profile differs for the two groups, this becomes apparent as a significant "treatment-time interaction". A full mathematical description of the method has been given by Winer (Winer, 1971).

As an example, for the injected paw data illustrated in Figure II-17, the analysis of variance table (Table II-3)

indicates that the overall treatment difference fails to achieve significance (the actual level is approximately $P < 0.08$). There is no significant time effect, that is, the shape of the response curve does not vary significantly with time. The response-time profile is the same for both treatments and there is therefore no significant time/treatment interaction. For the uninjected paw (Figure II-18 and Table II-4), there is no significant treatment effect overall. There is now a significant time effect, which is clear from Figure II-18, but there is no difference between the treatments in respect of the response-time profiles.

Table II-3. One-way analysis of variance for repeated measures for the data of experiment EXA5, Figure II-17.

Source	Degrees of freedom	Sum of squares	Mean squares	F - ratio
Main treatment effects.	1	6376.0	6376.3	3.392
Error I.	18	33833.3	1879.6	
Time effects	21	4707.0	224.1	3.524
Treatment / time interaction	21	1662.5	79.1	1.244
Error II	378	24041.8	63.6	

Table II-4. One-way analysis of variance for repeated measures for the data of experiment EXA5, Figure II-18.

Source	Degrees of freedom	Sum of squares	Mean squares	F - ratio
Main treatment effects.	1	2676.8	2676.8	2.213
Error I.	18	21768.3	1209.4	
Time effects	16	11275.5	704.7	10.906 ♦
Treatment / time interaction	16	1276.5	79.8	1.234
Error II	288	18610.3	64.6	

♦ Significant, $P < 0.05$

Table II-5. One-way analysis of variance for repeated measures for the data of experiment EXA5, Figure II-19.

Source	Degrees of freedom	Sum of squares	Mean squares	F - ratio
Main treatment effects.	1	14467.0	14467.0	4.856 ♦
Error I.	18	53621.0	2978.9	
Time effects	21	9120.0	434.3	7.864 ♦
Treatment / time interaction	21	2452.5	116.8	2.1146
Error II	378	20876.0	55.2	

♦ Significant, $P < 0.05$

In the case of the injected ankle however (Figure II-19) there is a difference between treatments at the level $P < 0.05$, there is again a significant time effect, but no time/treatment interaction (Table II-5). Finally the measurements on the uninjected ankle (Figure II-20 and Table II-6) just fail to achieve a significant treatment difference. There is again a time effect, but no difference between the response-time profiles.

This example therefore proves particularly apt, with significant treatment effects observed for the injected ankle, the other measured parameters just failing to achieve the necessary significance level. This then is a case where statistical analysis suggests a treatment effect overall, but a repeat experiment would be advisable. In fact, in subsequent experiments not reported here, the compound shows consistent efficacy in this model. This particular compound also proves effective when administered in the secondary phase of the disease, which serves to illustrate a further statistical point. When comparing drugs administered from the day of FCA injection, as in the above example, the sensitivity or power of the experiment is reduced by the "between subject" variation. Covariance analysis is one method for minimising the effects of "between subject" variation. This technique cannot be easily employed in this instance, since the cyclical behaviour of the overt response metameters (paw swelling etc), and the dramatic response transients which occur within even the first few

hours of the experiment, make the paw or ankle swelling prior to adjuvant injection inappropriate as covariates. The situation is very different if drug therapy commences within the secondary phase. Under such circumstances it is usually possible to select a point in time at which the degree of paw swelling is fairly constant. The swelling may then be measured immediately before drug treatment commences. The values observed will reflect the inter-animal variation. The response to treatment is then followed as a function of time and represented as a linear combination of the same terms as described above, but with the addition of a value of the paw or ankle swelling observed before treatment commenced (the "covariate"). This approach compensates to a large measure for the inter-animal variation, and the test is more powerful in consequence. Not only, therefore, is it probably more valuable, scientifically, to determine whether or not drugs under test affect the established lesions, but the experiment can be conducted in such a way as to detect much smaller drug effects than would be the case if the drugs were administered from the day of FCA injection. Again, a full description of the method of covariance analysis for repeated measures is given by Winer (Winer, 1971).

Table II-6. One-way analysis of variance for repeated measures for the data of experiment EXA5, Figure II-20.

Source	Degrees of freedom	Sum of squares	Mean squares	F - ratio
Main treatment effects.	1	4743.8	4743.8	3.870
Error I.	18	22066.0	1225.9	
Time effects	16	14813.0	925.8	17.981 ♦
Treatment / time interaction	16	1333.5	83.3	1.619
Error II	288	14828.8	51.5	

♦ Significant, $P < 0.05$

The use of covariance analysis is illustrated by the following experiment.

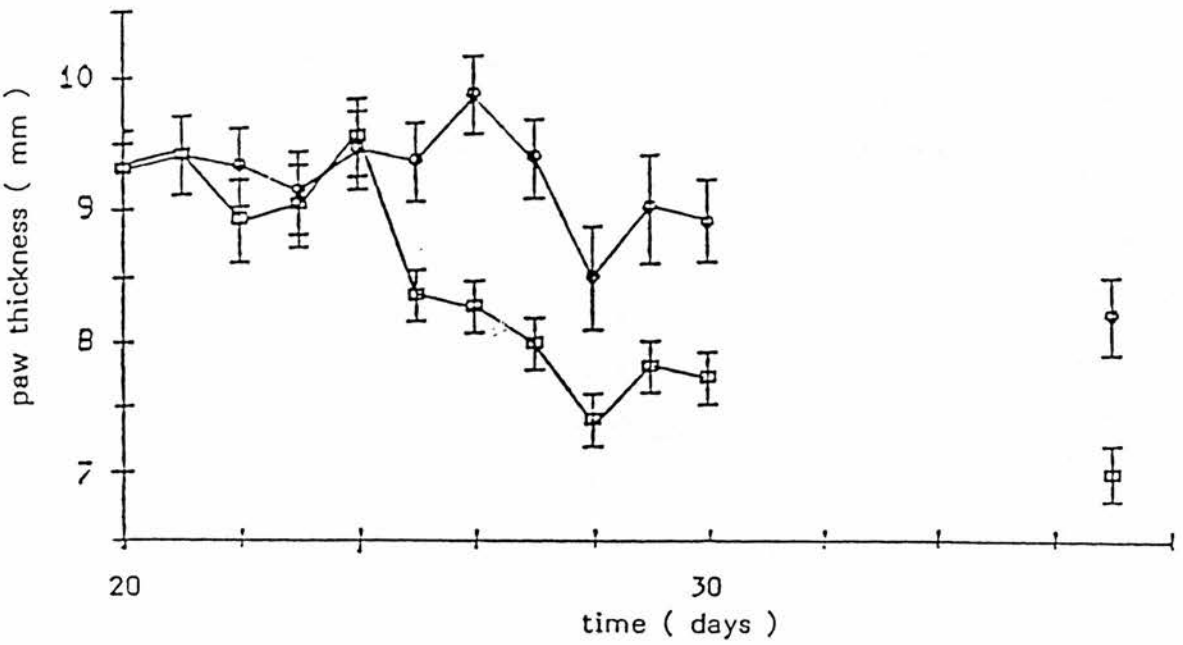
Method (Experiment EXA51b)

Sixty male Lewis rats received subplantar injection of arthritogen on day 0. On day 20, the 45 most severely arthritic animals were selected and allocated to three groups of 15 animals. The groups were balanced with respect to the severity of the swelling of both paws. One group received oral mulgophen from day 21, the second received indomethacin orally at a dose of 1.5 mg/kg/bd. Treatment of this second group was terminated on day 26 due to the emergence of unacceptable side effects. Comparison of this group with the untreated control from day 21 to day 26 is presented above as experiment EXA51. The remaining group received 30 mg/kg/d of an experimental indane-dione. The experiment was terminated on day 37.

Results

An apparent reduction of the swelling in the injected paw occurred from about Day 24 of the experiment, 3 days after treatment commenced. In the uninjected paw, similarly reduced swelling became evident also from about Day 24. The responses in both paws are shown in Figures II-21 and II-22.

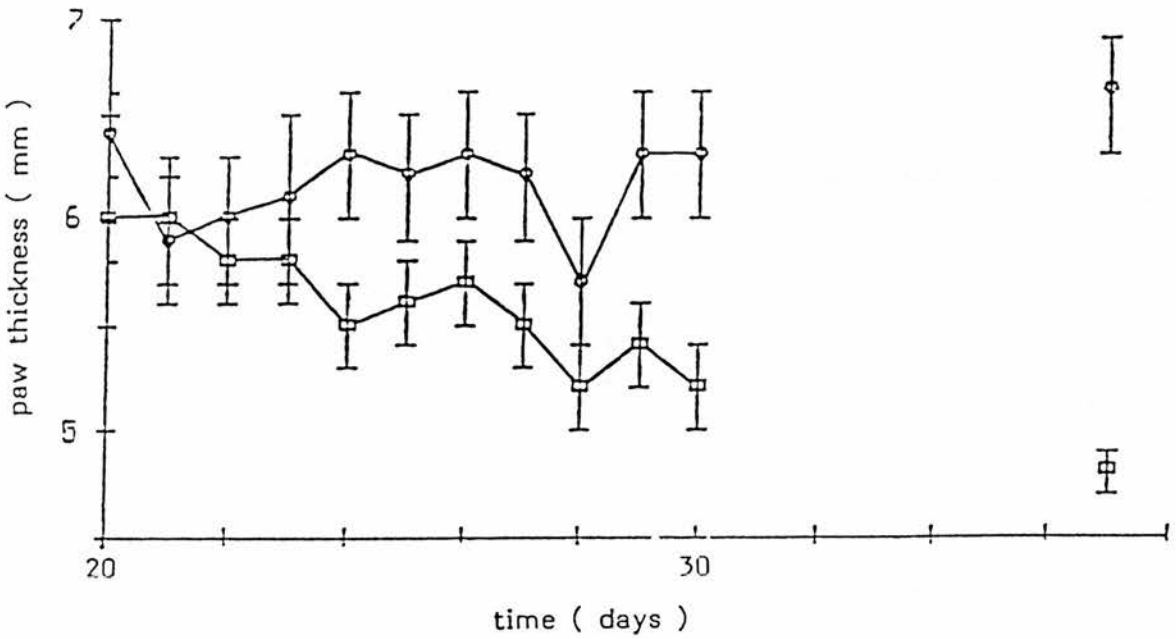
Figure II-21. The effect of 5 mg/kg/bd of an orally administered experimental indane-dione on the paw swelling in the FCA injected limb of male Lewis rats with established arthritis. Dosing commenced on day 21 following disease induction. Experiment EXA51b.



—○— Untreated arthritic rats.

—□— Indane - dione treated arthritic rats.

Figure II-22. The effect of 5 mg/kg/bd of an orally administered experimental indane-dione on the paw swelling in the uninjected limb of male Lewis rats with established arthritis. Dosing commenced on day 21 following disease induction. Experiment EXA51b.



-○- Untreated arthritic rats.

-□- Indane - dione treated arthritic rats.

From this experiment, it is interesting to note a possible consequence of altered drug handling. In the experiment where the indane dione was given from the day of FCA injection, a dose of 30 mg/kg/d produced a marginal effect. In this experiment animals were dosed with 5/mg/kg/bd only. Nevertheless there appears qualitatively to have been an effect of the drug at this dose. This would be consistent with reduced metabolic inactivation of the compound, which is probably hydroxylated to an inactive metabolite in rats with a normal mixed function oxidase system.

To show whether the drug has exerted a real effect it is possible here to carry out a one way analysis of covariance with repeated measures (Winer, 1971). Here, the covariate is the day 20 paw thickness, measured before drug therapy commenced. This compensates to a degree for the variation between the subjects before treatment commenced. Each subsequent measurement is then treated as a change relative to the baseline value for each individual rat. As may be seen from the appropriate F-values in Tables II-7 and II-8, the difference between treatments is highly significant according to this approach. The purpose of leaving a seven day hiatus between the final two measurements was to attempt to establish a data set independent of the measurements up to day 30. If this is valid, and such independence is in fact

achieved, then a clearly significant difference between the treatments is apparent at this time (t-test, $p < 0.05$).

Table II-7.

One - way analysis of covariance for repeated measures for the data of experiment EXA51b, Figure 21.

Source	Degrees of freedom	Sum of squares	Mean squares	F - ratio	Common slope
Main treatment effects.	1	4990.7	4990.7	15.072♦	
Covariate	1	20006.9	20006.9	60.419	0.762
Error I.	27	8940.6	331.1		
Time effects	9	5338.9	593.2	30.767♦	
Treatment / time interaction	9	1925.3	213.9	11.095♦	
Error II	252	4858.7	19.3		

♦ Significant, $P < 0.05$

Table II-8.

One - way analysis of covariance for repeated measures for the data of experiment EXA51b, Figure 22.

Source	Degrees of freedom	Sum of squares	Mean squares	F - ratio	Common slope
Main treatment effects.	1	2876.3	2876.3	15.533 ♦	
Covariate	1	21944.4	21944.4	118.511	0.762
Error I.	27	4999.5	185.2		
Time effects	9	678.4	75.4	5.357 ♦	
Treatment / time interaction	9	858.5	95.4	6.769 ♦	
Error II	252	3551.4	14.1		

♦ Significant, $P < 0.05$

In conclusion, therefore, whilst the precise relevance of the adjuvant rat arthritis model to the human disease remains an open question, it is true to state that the experimental application of the model in drug screening has been to a degree deficient. There has been an undue emphasis on NSAID and related drugs, followed by a too-ready willingness to search for other animal models when faced with the failure of this class of compound to fulfil its initial promise. There have also been descriptions of drug profiles in models of acute inflammation and rat arthritis which fail to acknowledge the changed pharmacokinetics which occur in the arthritis model. And finally, given that the more interesting, potentially anti-rheumatic, drugs may have less dramatic effects in this model when compared to the NSAIDs, and may also show effects which are slower in onset, there has been inadequate discussion of the problems of statistical analysis of data from this, and other, chronic models. There is yet no reason to believe that the failure of drug screening programmes to produce effective anti-rheumatic drugs is due to any inherent failure of the rat adjuvant arthritis model.

III

ALTERNATIVE MARKERS OF DISEASE PROGRESSION.

Two further, major, problems which relate to this model are the continuing lack of any clear understanding of the aetiology of the disease, and the lack of a metameter which may be used to unambiguously indicate that a fundamental change in the progression of the disease has occurred as a result of treatment. NSAIDs generally alleviate or abrogate signs of inflammation, but do not affect the underlying disease, and there is a rapid recurrence of inflammatory signs when such drugs are withdrawn (Newbould, 1965). Treatments do exist which have a more persistent effect (MacKenzie, Pick et al, 1978). The degree to which these exert a fundamental disease modifying effect remains to be seen. They do, however, offer alternative approaches towards identifying metameters more closely linked to the disease than the outward signs of inflammation.

In the experiments reported below the following general methods were employed.

Materials and Methods: General

The animals employed in these studies and the methods of paw and ankle measurement, were as described in Chapter 2. The preparation of arthritogenic material was also as described earlier, with the exception that M. butyricum was replaced by M. tuberculosum.

Cyclophosphamide (Ward Blenkinsop) was dissolved in physiological saline to a concentration of 250 mg per 100 mls just prior to use and administered by i.p. injection.

The cell mediated immune reaction to PPD was assessed as follows. The animals to be tested were carefully shaved on one flank 24 hours beforehand. On the day of challenge, a circular area approximately 3 cm in diameter was marked with a marking pen. The skinfold thickness at the centre of the area was measured at least 3 times with Snelltaster calipers, and the mean value taken. An intracutaneous injection of 0.1 ml of a solution of PPD at a concentration of 1 mg/ml was then made so that the solution was deposited in a circular depot in the centre of the marked area. Twenty four hours later the same site was again measured at least 3 times with Snelltaster calipers, and the mean value taken. The

difference between the skinfold thickness before and after challenge was taken as a measure of the level of cell mediated immunity to PPD.

Blood samples were in all cases taken from the a tail vein. A small incision was carefully made from which two drops of blood were taken. The first was discarded. The second was drawn into a 5 microlitre precision glass capillary tube (Brand micro-haematocrit, non-heparinised) and rapidly transferred to 10 ml of Isoton in prepared counting pots. Clotting was prevented by the speed of transfer and the degree of dilution. The remainder of the blood drop was used to make a smear on a microscope slide in the usual way. The smear was immediately fixed by immersion in 99% AR methanol for 10 minutes before staining with May-Grunwald/Giemsa.

Total red and white cell counts were obtained using a model ZB Coulter counter. Differential cell counts were obtained by microscopic inspection of at least 200 cells from complete longitudinal scans from each smear.

The effect of cyclophosphamide

This chapter investigates the possibility that immune aspects of the disease, and in particular cell mediated immunity, may be used to identify effective drug intervention in the basic disease process. Cyclophosphamide, given in a single dose of 100 mg/kg i.p. close to the time of arthritis induction, immediately suppresses most outward signs of the disease, with no apparent recurrence subsequently (MacKenzie, Pick et al, 1978).

Immunosuppressive drugs have been known for some time to suppress or prevent the outward signs of rat arthritis (MacKenzie, Pick et al, 1978). Azathioprine, 6-mercaptopurine, cytarabine and cyclophosphamide, given twice daily over the initial 14 days of the disease, suppress overt clinical signs in a dose related manner (Glenn, Bowman et al, 1977). The effects of cyclophosphamide, however, may not be due entirely to immunosuppression. At doses of 100 mg/kg or greater, cell mediated and humoral immunity to EL4 cells in arthritic rats are reduced by cyclophosphamide in concert with a reduction in the signs of the disease (Chang, 1977). At a lower range of doses, outward signs of the disease are again inhibited, but there is now no effect on the immune response to EL4 cells (Chang, 1977). There is also

evidence that cyclophosphamide can inhibit carrageenan induced oedema in the rat paw. This has therefore been taken to suggest an anti-inflammatory effect of the drug complementary to its immunosuppressive effect (Chang, 1977). It seems nevertheless probable that the major effect of cyclophosphamide is immunosuppressive. A detailed study of the effect of cyclophosphamide on cell mediated immunity in arthritic rats was therefore chosen as an approach to the question of whether drug intervention can fundamentally affect the disease, and whether measurement of cell mediated immunity can be a useful metameter of that effect.

For the following studies, arthritic animals were treated with a single intraperitoneal (i.p.) dose of 100 mg/kg of cyclophosphamide. It was thought that this dose was not so high as to be unduly toxic, but that any inherent anti-inflammatory effect was likely to be slight in comparison to effects arising from the degree of immunosuppression induced. The effect of this treatment was first investigated in arthritic Wistar rats in the following experiment.

Method (Experiment EXA6)

Thirty-five male Wistar rats with a mean body weight of 268 ± 2 g were divided into 7 groups of 5. Thirty animals received a subplantar injection of arthritogen (M. tuberculosum) into one hind paw on day 0. At the same time, 5 animals received an injection of liquid paraffin alone. This was the non-arthritic control. On day 3 of the experiment, 15 animals received an injection of 1 ml of physiological saline i.p., this constituted the arthritic control group. A further 15 received 100 mg/kg of cyclophosphamide i.p.

The cell mediated immune response to PPD was assessed in 5 animals from each of the two groups of arthritic animals on day 9, in a further 5 animals from each group on day 12, and in the remaining animals from each group on day 19. On this day also, the response in the non-arthritic control animals was measured. Paw and ankle thickness was measured for each animal throughout the study.

Results

Figure III-1 illustrates the effect on the injected paw. By day 3 of the experiment, swelling in this paw was well established. There was a marked reduction in swelling which occurred almost immediately after the cyclophosphamide was administered. The speed of this response suggests an appreciable anti-inflammatory effect. The swelling in this paw did not completely return to normal however, even by day 40 of the experiment. The effect on the injected ankle (Figure III-2) was particularly striking. There was a steady decrease in ankle thickness throughout the period of the experiment, returning by day 40 almost to the non-arthritic value. This treatment therefore had a marked inhibitory effect on the dissemination of the disease throughout the injected limb. In the uninjected limb, the paw swelling (shown in Figure III-3) was significantly less than for the arthritic group at almost every time point after the beginning of the secondary phase. Oddly, however, there was an increase in this metameter to the level of the arthritic group at day 40 of the experiment. This was not reflected in the uninjected ankle swelling, which remained significantly less than that of the arthritic group at all time points following onset of the secondary phase, with no

evidence of exacerbation at the later times (Figure III-4).

Total disease suppression was not achieved by this treatment. Figure III-5 shows the number of animals in each group which exhibited an arthritic score greater than 1, i.e. the number of arthritic animals in each group showing evidence of inflammation (erythema, swelling etc.) in at least one limb other than the injected limb. From this it is evident that approximately 50% of the cyclophosphamide treated animals developed signs of disseminated disease from about day 17 onwards. The maximum was reached by day 23, the secondary signs thereafter progressively disappeared until, by approximately day 32, no secondary signs were evident in this group. Cyclophosphamide treatment appeared to prevent the occurrence of secondary disease in about one-half of the animals. In the remainder, the disease appeared later than for untreated animals, was mild, transient, and disappeared by day 40. In contrast, almost 100% of the non-treated animals showed signs of secondary disease, a figure which is reached by day 13, almost 10 days earlier than for the cyclophosphamide group. There was then a slow disappearance of secondary signs, with rather more than one-third showing persisting disease up to day 40.

The effect of this treatment on the body weights of the animals was noteworthy. There was a progressive recovery of the body weights from the reduced levels observed in the untreated arthritic animals. By day 27 the mean body weight of the cyclophosphamide treated group was not appreciably different from that of the non-arthritic control group (Figure III-6). This was surprising in that the incidence of secondary disease was maximal at about this time in the treated group (Figure III-5).

Figure III-1. The effect on the swelling of the FCA injected paw of arthritic male Wistar rats treated with 100 mg/kg cyclophosphamide i.p. 3 days after FCA injection. Experiment EXA6.

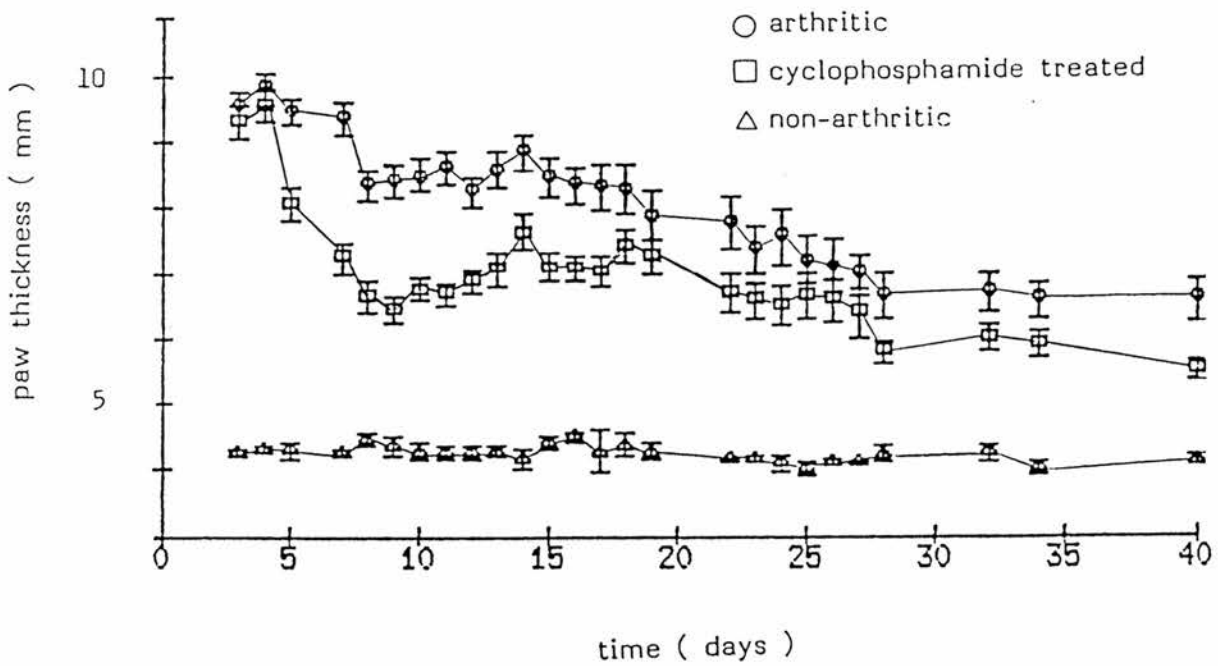


Figure III-2. The effect on the swelling of the FCA injected ankle of arthritic male Wistar rats treated with 100 mg/kg cyclophosphamide i.p. 3 days after FCA injection. Experiment EXA6.

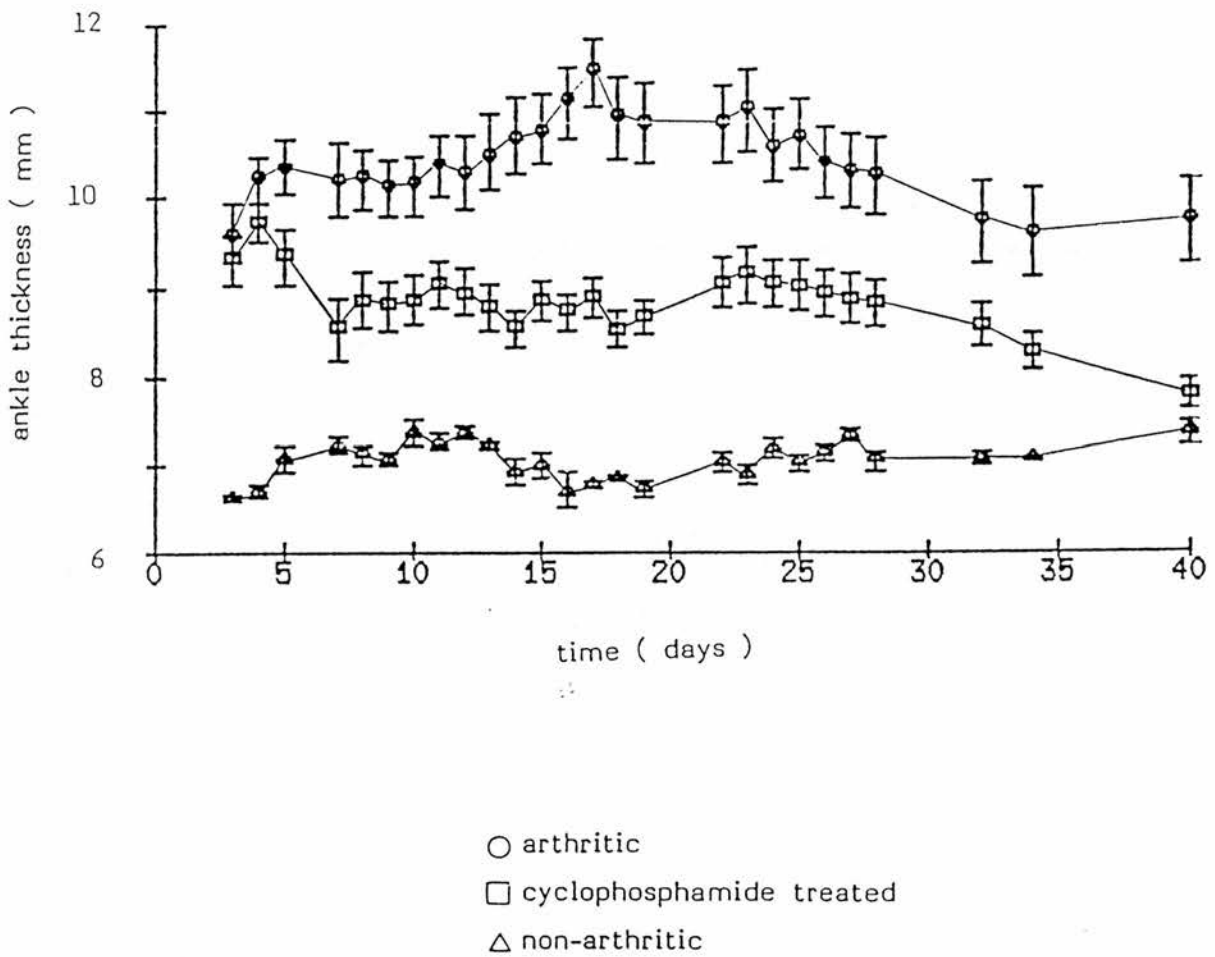


Figure III-3. The effect on the swelling of the uninjected paw of arthritic male Wistar rats treated with 100 mg/kg cyclophosphamide i.p. 3 days after FCA injection. Experiment EXA6.

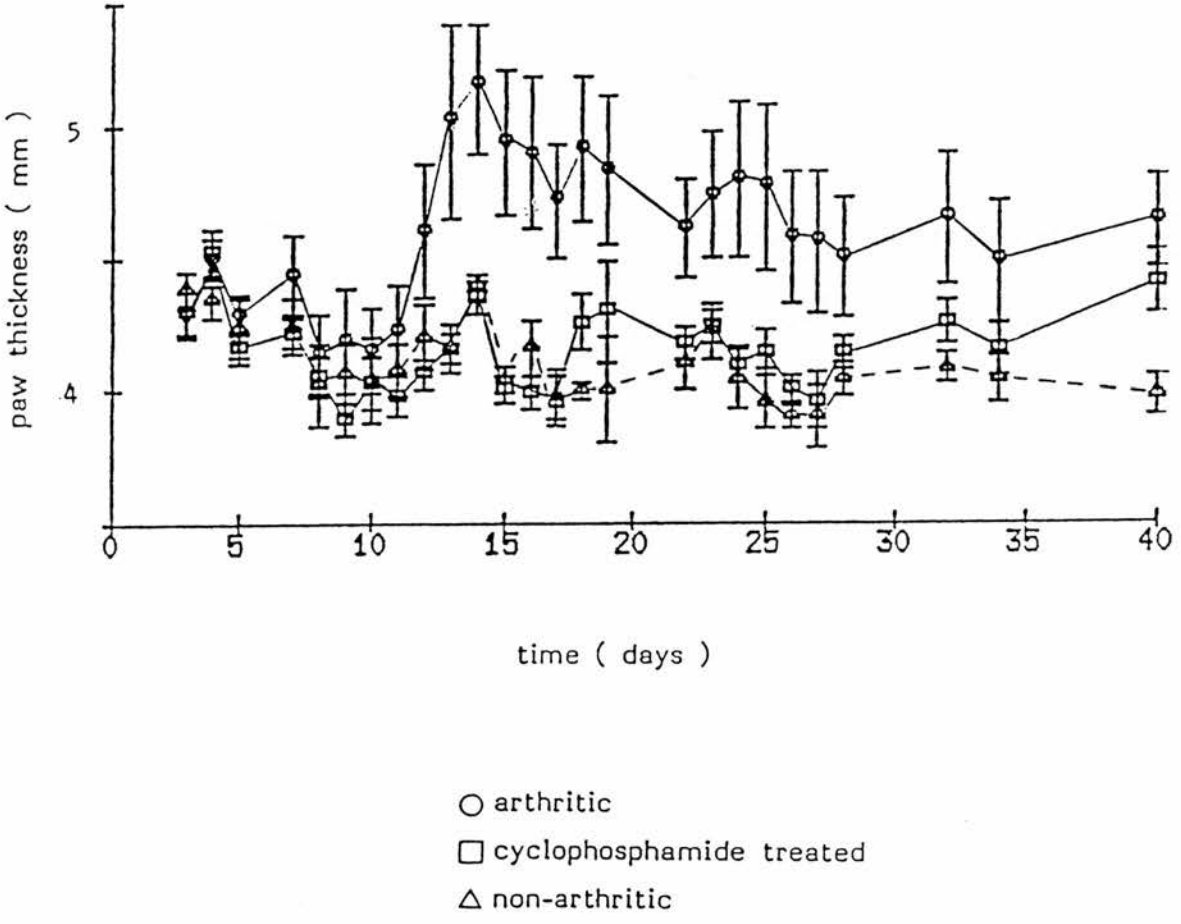


Figure III-4. The effect on the ankle swelling in the uninjected limb of arthritic male Wistar rats treated with 100 mg/kg cyclophosphamide i.p. 3 days after FCA injection. Experiment EXA6.

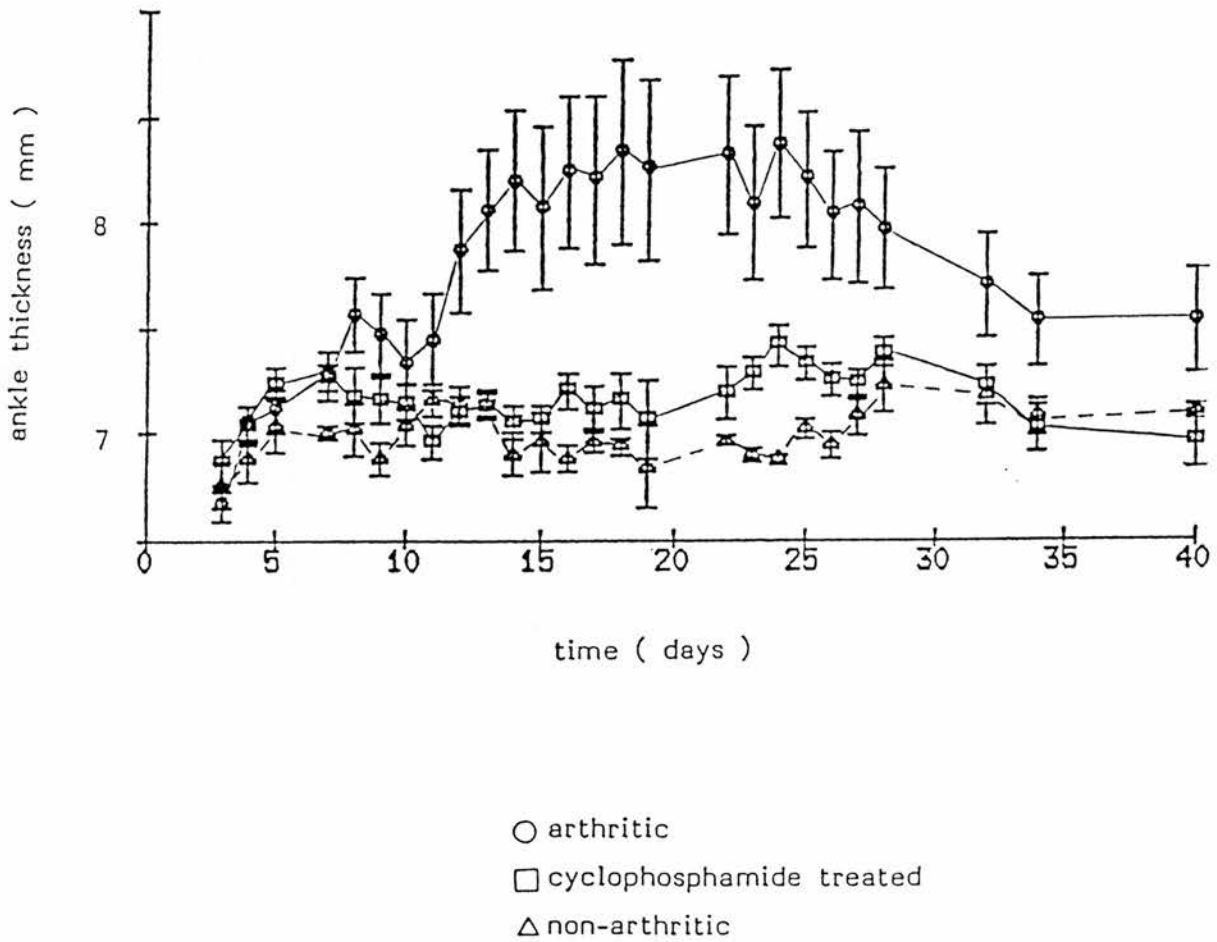


Figure III-5. The effect on disease dissemination in male Wistar rats treated with 100 mg/kg cyclophosphamide i.p. 3 days after FCA injection. Experiment EXA6.

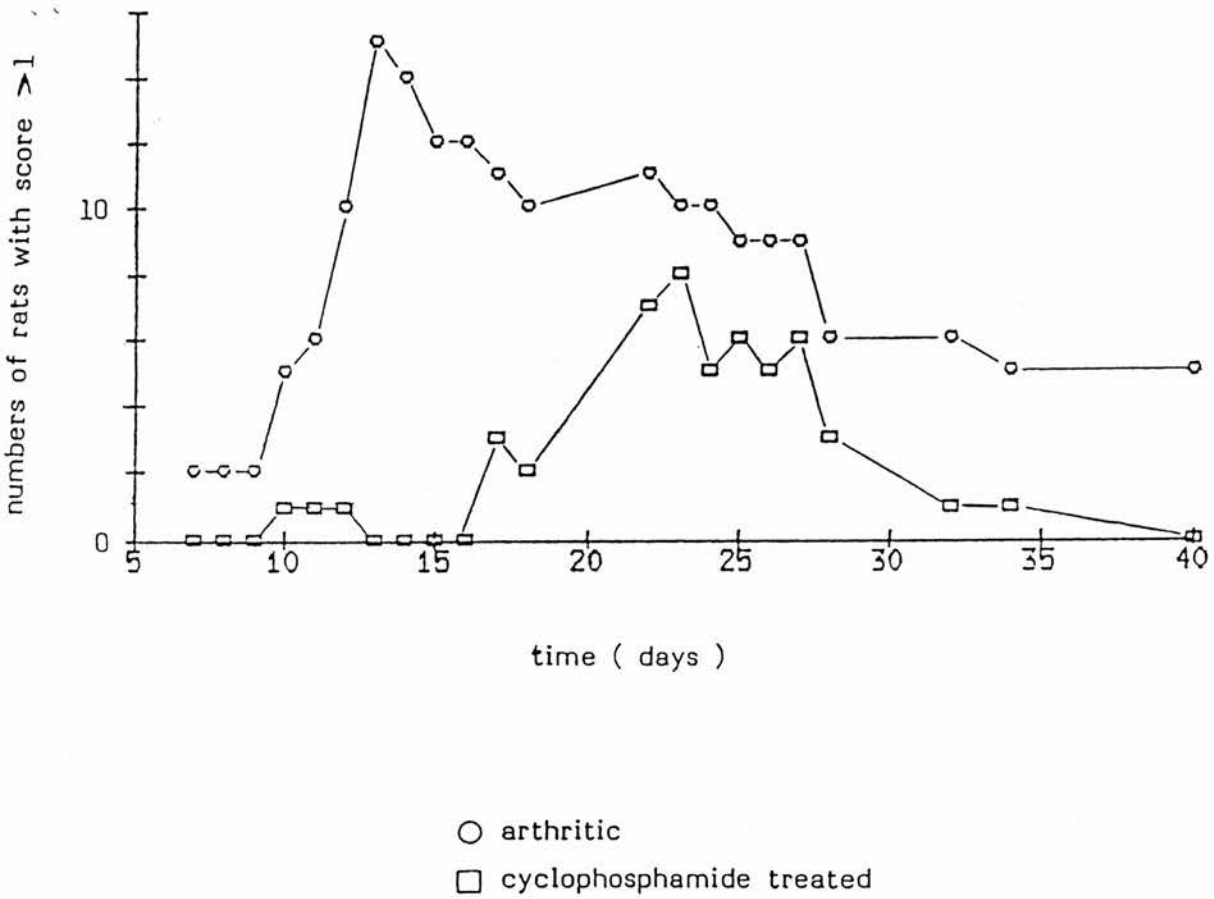
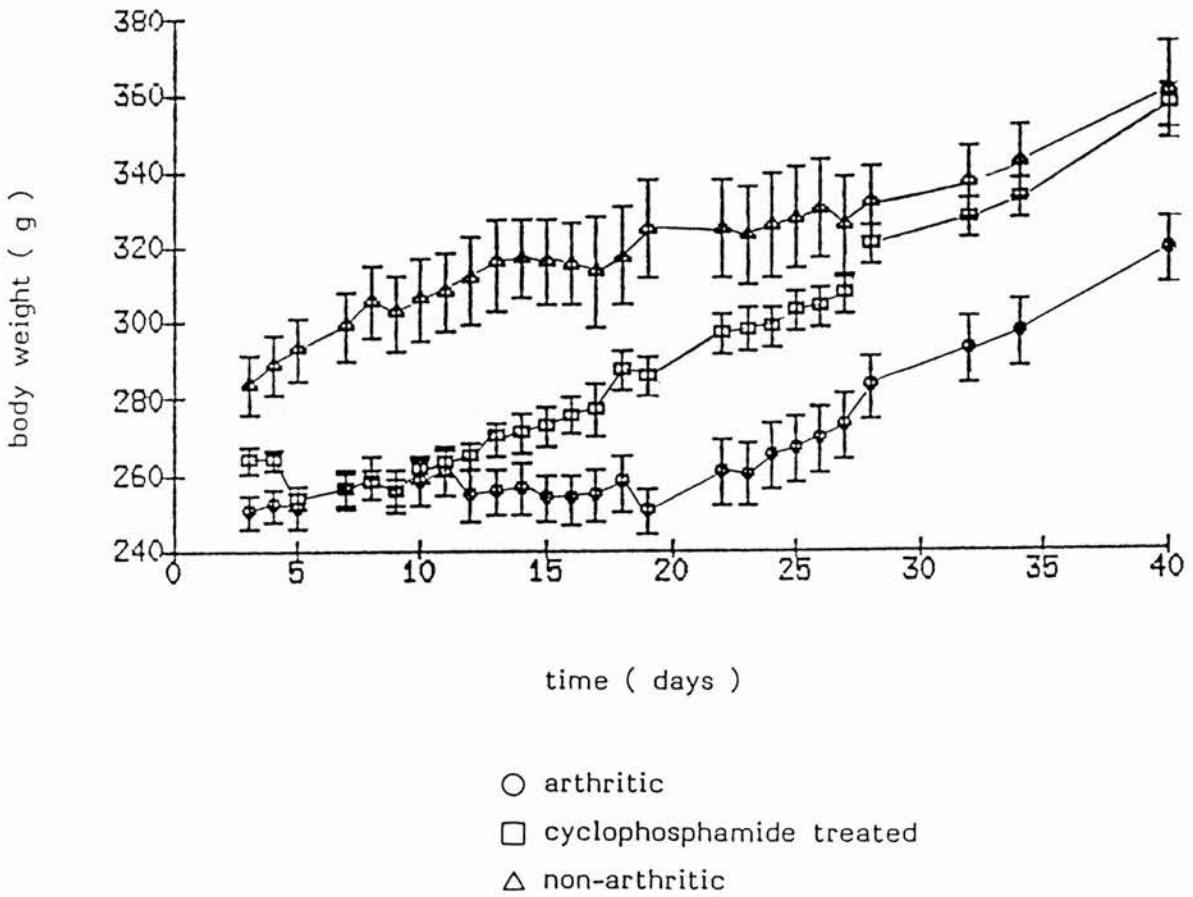


Figure III-6. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on body weight in male arthritic rats. Experiment EXA6.



It is evident from these data that it is possible to develop signs of secondary disease (albeit to a mild degree) without there necessarily being a concomitant reduction in body weight gain. A reduced body weight gain is not therefore a reliable indicator of the presence of the arthritis in these rats.

A single dose of cyclophosphamide can clearly abrogate development of the disease in most animals, measured in terms of the overt inflammatory signs, the effect persisting up to at least 40 days following FCA challenge. It is clearly of interest therefore to determine what other markers of the disease, if any, have been affected by this treatment.

Cell mediated immunity

A common and convenient means of assessing cell mediated immunity is by challenging the skin of the animal under study with an antigen to which it has previously been sensitised. Some methodological problems of this kind of approach have already been mentioned in Chapter 1.

In the context of rat adjuvant disease, the animals have been challenged with FCA in order to induce the disease, and they are therefore effectively sensitised to mycobacterial antigens. If M. tuberculosum is used in preparing FCA, PPD, being a soluble protein derivative of M. tuberculosum, offers many advantages for measuring the cell mediated immune response, as already indicated in Chapter 1.

PPD is not itself arthritogenic. The insoluble mycobacterial cell wall fraction has the advantage that it is arthritogenic (Waksman, Pearson et al, 1960). Attempts to cleave this fraction, to possibly produce a soluble material, generally result in a non-arthritogenic product (Audibert, Parant et al, 1973). The reaction to the cell-wall fraction in skin challenge experiments includes a non-specific component due presumably to the irritant effect of the particulate nature of the material. This is evident, for example, from an inspection of the data presented by Gans et al. (Gans, Heyner et al, 1980).

For the above reasons, although it seems more satisfactory to use an antigen for skin challenge experiments in the present context which is itself arthritogenic, PPD was employed to measure cell mediated immunity in these animals. For the experiments described here, the use of PPD was preferred and cell mediated immunity was assessed as the skin reaction to PPD. This is at least a reaction to a component of the arthritogen used (M.

tuberculosum), and avoids the need to sensitise the animal to an additional antigen. It does, however, prevent simultaneous measurement of the humoral immune reaction, without the introduction of a further antigen, since in a series of experiments not reported here, using erythrocytes coated with PPD (Mergenhagen, Notkins et al, 1966), no evidence of (agglutinating) antibody to PPD was ever detected.

The cell mediated immune response was selected for study as potentially a prime metameter of the disease state despite the fact that no clear correlation has been established between the intensity of the cell mediated immune reaction in individual animals and the severity of the disease signs, nor between the ability of different rat strains to maintain a cell mediated immune response to PPD or water soluble peptidoglycans, and their susceptibility to disease induction (Kohashi, Pearson et al, 1977).

Nevertheless there is a large body of evidence which establishes at least a loose connection between the strength of immune reactivity in these animals and the progression of the disease. Pearson and Wood were able to passively transfer the disease using spleen cells from syngenic donors (Pearson and Wood, 1964) and subsequently this was achieved with cloned T-cell subsets (Holoshitz, Matitiau et al, 1984). Treatment of arthritic rats with anti-lymphocyte globulin depletes the animals

of circulating lymphocytes, reduces their ability to mount an immune response, and reduces the severity of the arthritis (Gans, Heyner et al, 1980, Currey and Ziff, 1968, Kayashima, Koga et al, 1978). Similar lymphocyte depletion by whole body irradiation, or irradiation of lymphoid tissue locally, will cause remission of the disease (Schurman, Hirshman et al, 1981). The effects of X-irradiation on rat arthritis tend to suggest that the chronic inflammation depends both on processes occurring locally in the joint, and also centrally in the major lymphocyte producing organs (Schurman, Hirshman et al, 1981). Extracts of P. aeruginosa (Floersheim, Borel et al, 1972), vitamin D (Pletsityi, Evseev et al, 1982), oxonate, by induction of hyperuricemia (Lussier and De-Medicis, 1977, Lussier, De-Medicis et al, 1978), all suppress the cell mediated immune response, and have been shown to reduce the severity of the rat arthritis. Conversely, there is an early report that thymectomy does not influence the disease (Myles Glenn and Gray, 1964). There is also the view that rat arthritis is of viral origin (Chang and Hoffman, 1977). This is supported by the observation that administration of interferon (Garlinghouse Jr and Van Hoosier Jr, 1978) or interferon inducers such as tilorone (Kapusta, Young et al, 1979), alleviate the arthritis without there being any apparent change in cell mediated immune responsiveness. This alternative viewpoint is not discussed further here.

The consensus of opinion is that the immune system of the rat plays a crucial role in the induction and maintenance of the disease, and that the same is true for human rheumatoid arthritis (Ziff, 1979). In the rat, the cell mediated immune limb has received most attention. Studies of the involvement of humoral immunity are less extensive. This has largely been a consequence of the long held belief that passive transfer of the disease by arthritic rat serum was not possible. This view seems now to require qualification, however, given the evidence of MacKenzie et al. who successfully re-induced the disease in arthritic animals in which all outward signs of the disease had been suppressed by treatment with cyclophosphamide (MacKenzie, Pick et al, 1978). The dose of serum employed in these experiments seems to have been critical. The evidence of these studies points towards the involvement of an immunoglobulin fraction, but not apparently an anti-collagen antibody (MacKenzie, Pick et al, 1978).

Measurement of skin reactivity to PPD was included in the experiment described above in Figures III-1 to 6. Three time points were chosen for the measurement, with no animal receiving more than one challenge throughout the study, in order to obtain an indication of the time profile of the response. Many previously reported studies of this kind are restricted to a single time point. As will become apparent, single time point measurement of

the level of the cell mediated immune response can prove particularly misleading.

Method (Experiment EXA6 contd)

Animals were treated as described above (Experiment EXA6).

The cell mediated immune response to PPD was assessed in 5 animals from each of the two groups of arthritic animals on day 9, in a further 5 animals from each group on day 12, and in the remaining animals from each group on day 19. On this day also, the response in the non-arthritic control animals was measured. Paw and ankle thickness was measured for each animal throughout the study.

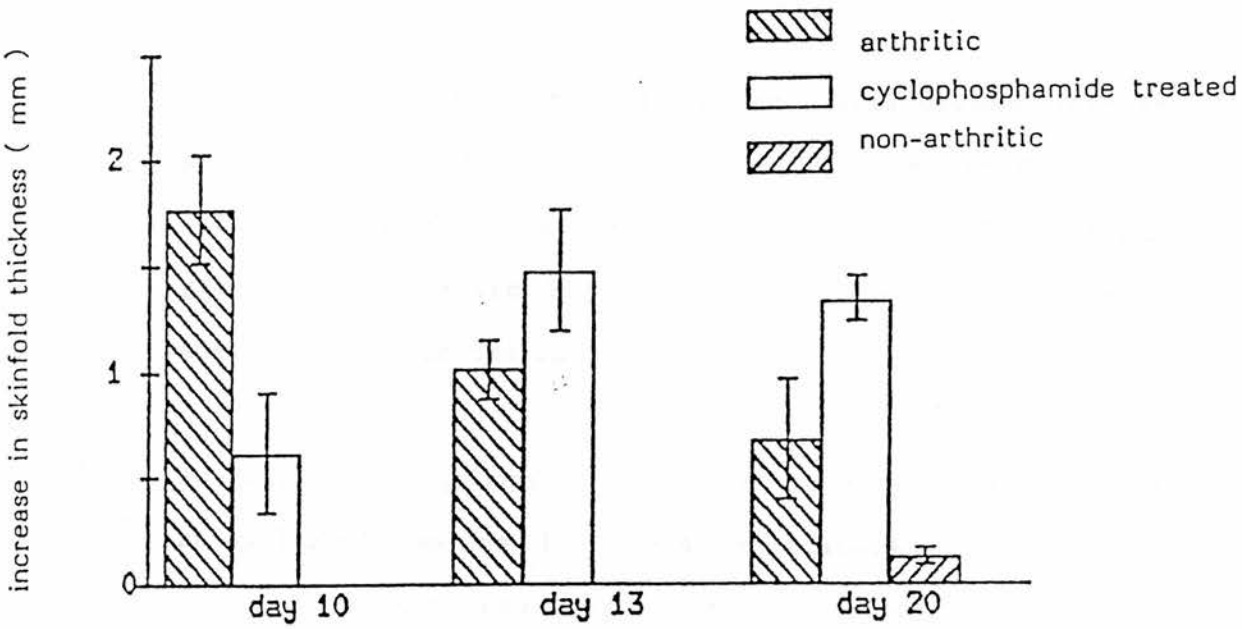
Results

The numbers of arthritic rats showing secondary signs according to their arthritic score, are shown in Figure III-5. The effect on the body weights is shown in Figure III-6.

The cell-mediated immune response to PPD is shown in Figure III-7. Clearly, treatment with cyclophosphamide changed the pattern of the response. At day 10, cell mediated immunity to PPD was significantly depressed in the treated group in relation to the untreated arthritic group. By day 13 the two groups showed an equal intensity of reaction, and by day 20 the situation was reversed.

Such a change is important to verify, particularly in relation to the response-time profile of other signs of the disease. For example, from day 10 to day 20, the CMI response (Figure III-7) and the numbers of arthritic rats showing secondary signs (Figure III-5) both fell. The cyclophosphamide treated group, on the other hand, showed a plateau in response from day 13 to day 20, whilst the number of affected animals increased (Figure III-5). There was unfortunately no information from this experiment to indicate how the response varies after the number of affected rats reaches its maximum on day 23.

Figure III-7. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on cell mediated immunity to PPD in male arthritic Wistar rats. Experiment EXA6.



Accordingly, a further experiment was carried out, again involving Wistar rats.

Method (Experiment EXA8A)

One hundred and sixty male Wistar rats were divided into 16 groups of 8 and 8 groups of 4. The 16 groups of 8 animals were injected with FCA (M. tuberculosum) on day 0. Eight groups of 4 received liquid paraffin alone and constituted the non-arthritic control.

On day 3, 8 groups of animals received injection of physiological saline i.p. These constituted the arthritic controls. At the same time a further 8 groups received 100 mg/kg of cyclophosphamide i.p. Cell mediated immunity to PPD was assessed on days 4, 7, 10, 15, 20, 24 and 42 in groups taken from the non-arthritic controls, the arthritic controls and the cyclophosphamide treated animals, such that no group of animals received more than one skin challenge during the course of the experiment.

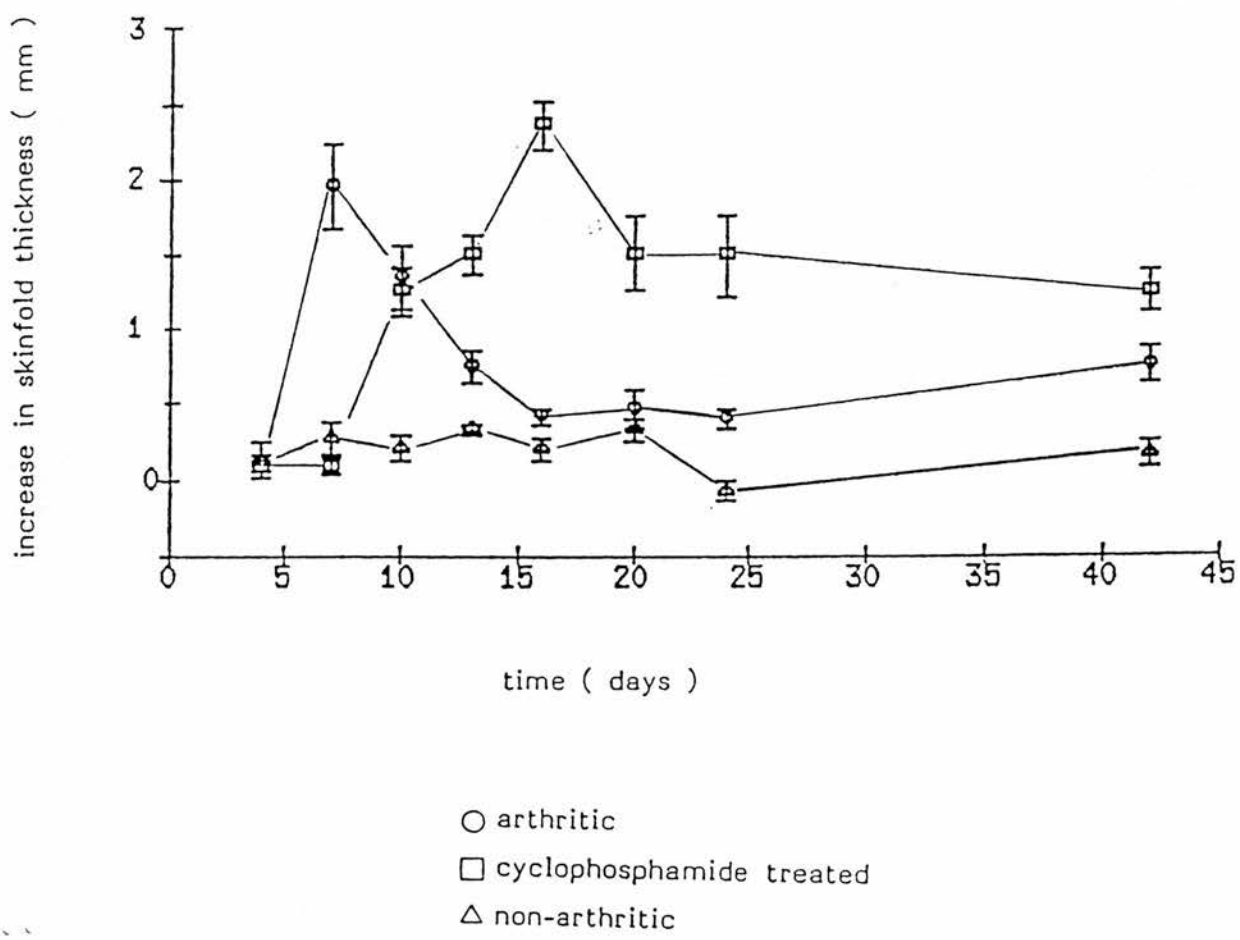
Results

The results are illustrated in Figure III-8. For the untreated arthritic animals, the cell mediated immune reaction had a rapid onset, with a maximum at about day 7. Thereafter there was a steady decrease, which nevertheless failed to return to the level of the control animals even by day 42 of the study. Onset of secondary signs generally occurs at about day 14, approximately 7 days after the maximum of the skin reaction therefore, at a time when this response has diminished to less than 25% of its maximum. In the cyclophosphamide group the maximum response was not attained until about day 17, the upward slope of the response curve being more shallow than for the untreated arthritic group. The response was also sustained at a higher level throughout. The height of the peak responses in both the treated and untreated animals appeared to be very similar, and in both cases the maximum number of animals with secondary signs of the disease occurred 7 to 9 days after the maximum skin reaction was observed.

There was again no correlation between the maximum cell mediated immune response in individual animals and the injected or uninjected paw swelling, measured at the time

when the maximum numbers of animals were affected with secondary disease, or indeed at any time. This is consistent with other reported work (Kohashi, Pearson et al, 1977, Otomo, Higuchi et al, 1981).

Figure III-8. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on cell mediated immunity to PPD in male arthritic Wistar rats. Experiment EXA8A.



This data well illustrates the need, in studies of this kind, to establish clearly the response-time profile over the whole range of the experiment. Comparisons at a single time point will be misleading if drug treatment alters the profile of the response. In this instance for example, comparison between the groups at day 7 would confirm an immunosuppressive effect of cyclophosphamide. Comparison at day 10 would indicate no significant effect of the drug, whilst a day 14 comparison, taken alone, would establish cyclophosphamide as an immunopotentiator, which it may well be, judged from the areas of the respective curves in Figure III-8.

Again, with reference to Figure III-8, the untreated arthritic group seemed to be unable to mount a significant cell mediated immune reaction to PPD after day 14. This may be specific to this antigen. It is perhaps worth recalling, however, that there is a generalised reduction in immune competence in these animals (Gorog and Kovacs, 1979) and that this is also true in human arthritis (Ziff, 1979). The profile shown in Figure III-8 for the arthritic group may then reflect a general inability to mount a significant cell mediated immune response to any antigen, a deficiency which was apparently not shared by the cyclophosphamide group.

To obtain further support for the effect of cyclophosphamide on cell mediated immunity in the adjuvant arthritic rat, the above

experiment was repeated using the more disease susceptible Lewis strain. Being inbred, the inter-animal variability in the response in these animals should be reduced. Cyclophosphamide was again administered i.p. at a dose of 100 mg/kg on day 3 of the experiment.

Method (Experiment EXA8)

Male Lewis rats were allocated to groups and treated exactly as described above in experiment EXA8A. Cell mediated immunity to PPD was assessed in this case on days 5, 8, 11, 14, 19 and 25. Paw and ankle measurements were made in one group from each of the arthritic and non-arthritic controls and from the cyclo-phosphamide treated animals. Assessment of cell mediated immunity was not performed in these groups.

Results

The body weight gain in these animals after treatment, shown in Figure III-9, strongly suggested a toxic effect of cyclo-phosphamide at this dose level in this strain of animals. Following dosing with cyclophosphamide there was a slight

decrease in body weight. The untreated arthritic animals initially gained weight at a similar rate to non-arthritic animals, the body weights then began to fall slightly, and tended to a constant value similar to that of the cyclophosphamide treated group. This profile seems rather different to that of the Wistar rats, for both the treated and untreated groups. It should be noted, however, that the body weights of the Wistar rats at the start of the experiment were slightly higher than was the case for the Lewis rats. It seems unlikely however that this would explain the difference observed.

The effect on the injected paw (Figure III-10) was more marked than in the Wistar strain. There was a rapid reduction in paw swelling which was progressive over the course of the experiment. By day 30 the swelling after cyclophosphamide treatment was approaching the non-arthritic control value, whereas in the Wistar rat at this time the thickness of the injected paw was barely different from the untreated arthritic animals.

Dissemination of the swelling to include the ankle in the injected limb was almost completely inhibited, the levels substantially returning to those of the non-arthritic control from about day 10 (Figure III-11). Dissemination to the non-injected limb was totally inhibited. Swelling in both the

non-injected paw (Figure III-12) and ankle (Figure III-13) did not diverge appreciably from non-arthritic control animals at any time. Secondary lesions occurred in almost 100% of the untreated arthritic animals judged from the mean arthritic score (Figure III-14). There was virtually no secondary involvement in the cyclophosphamide treated group.

The cell mediated immune profile to PPD in these animals is shown in Figure III-15. Despite the differences between the Wistar and Lewis strains in relation to overt signs of disease following cyclophosphamide treatment, the profiles of the cell mediated immune responses were rather similar. In the untreated animals, the response maximum occurred at about day 8 and declined steadily thereafter. Onset of the response was again slower in the cyclophosphamide treated group, the peak response occurring between day 14 and 20, the level of response remaining generally high throughout.

There has therefore been a more profound effect of the drug on the outward signs of the disease in Lewis rats. This is perhaps consistent with the effects on body weight, which appeared to reflect a higher degree of toxicity. Either the Lewis rats were more susceptible to the effects of cyclophosphamide, or possibly the metabolic activation of the drug, which was discussed in

chapter 2, occurred more efficiently in this strain. Whatever the explanation, the effect is most marked when considering the mean arthritic score of the groups at the various time points shown in Figure III-14. Secondary lesions occurred in almost 100% of the untreated arthritic animals. There was virtually no secondary involvement in the cyclophosphamide treated group.

Figure III-9. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on body weights in male arthritic Lewis rats. Experiment EXA8.

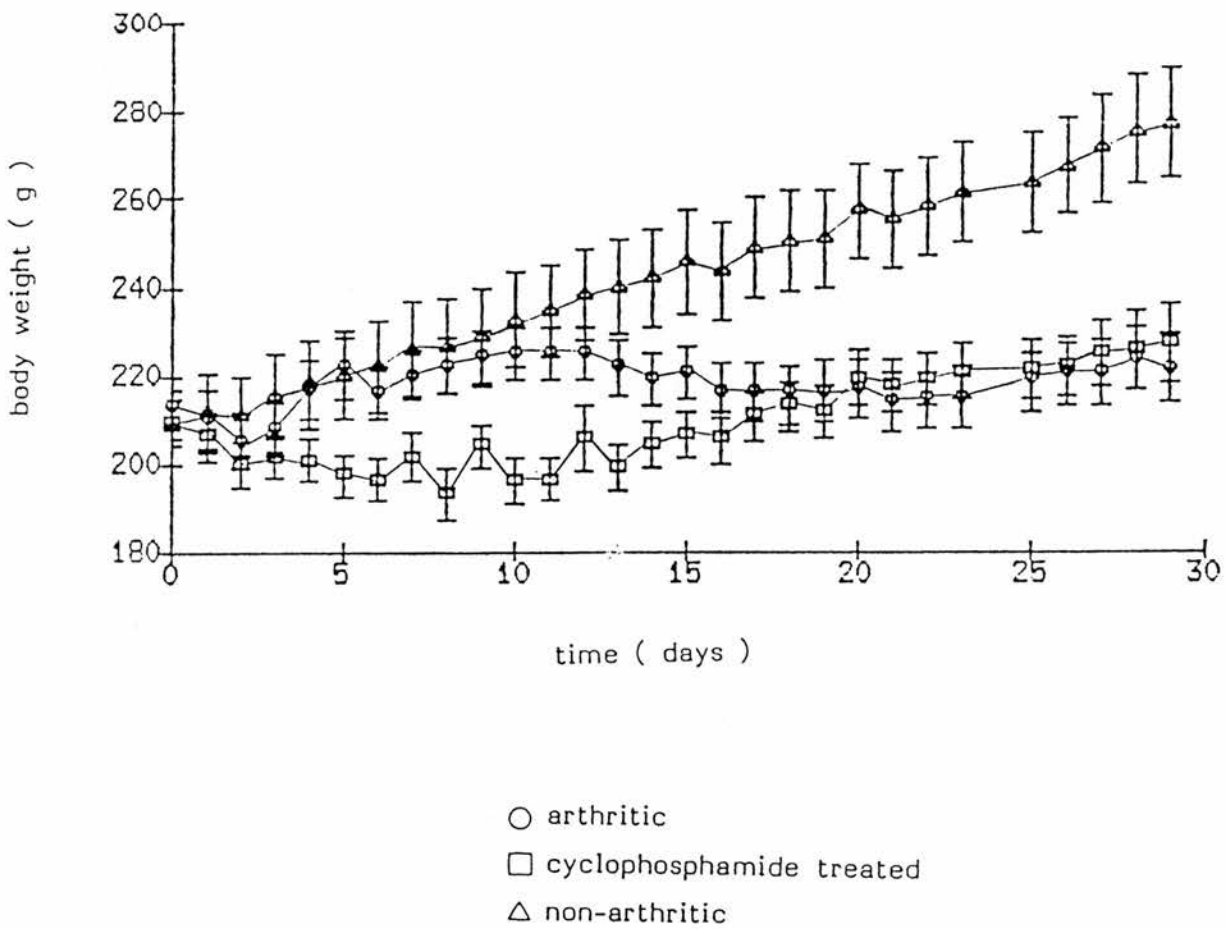


Figure III-10. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on the swelling in the injected paw of male arthritic Lewis rats. Experiment EXA8.

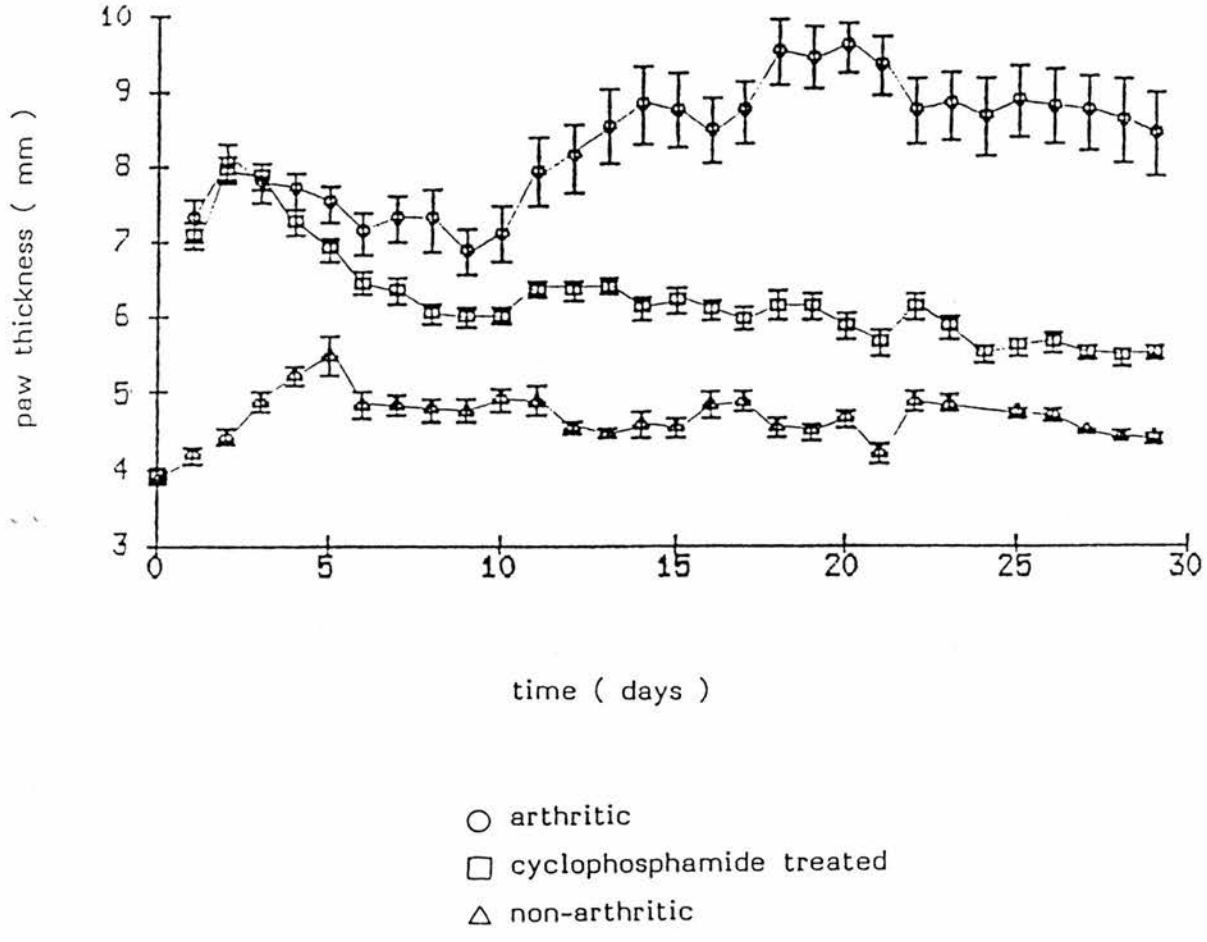


Figure III-11. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on the swelling in the ankle of the injected limb of male arthritic Lewis rats. Experiment EXA8.

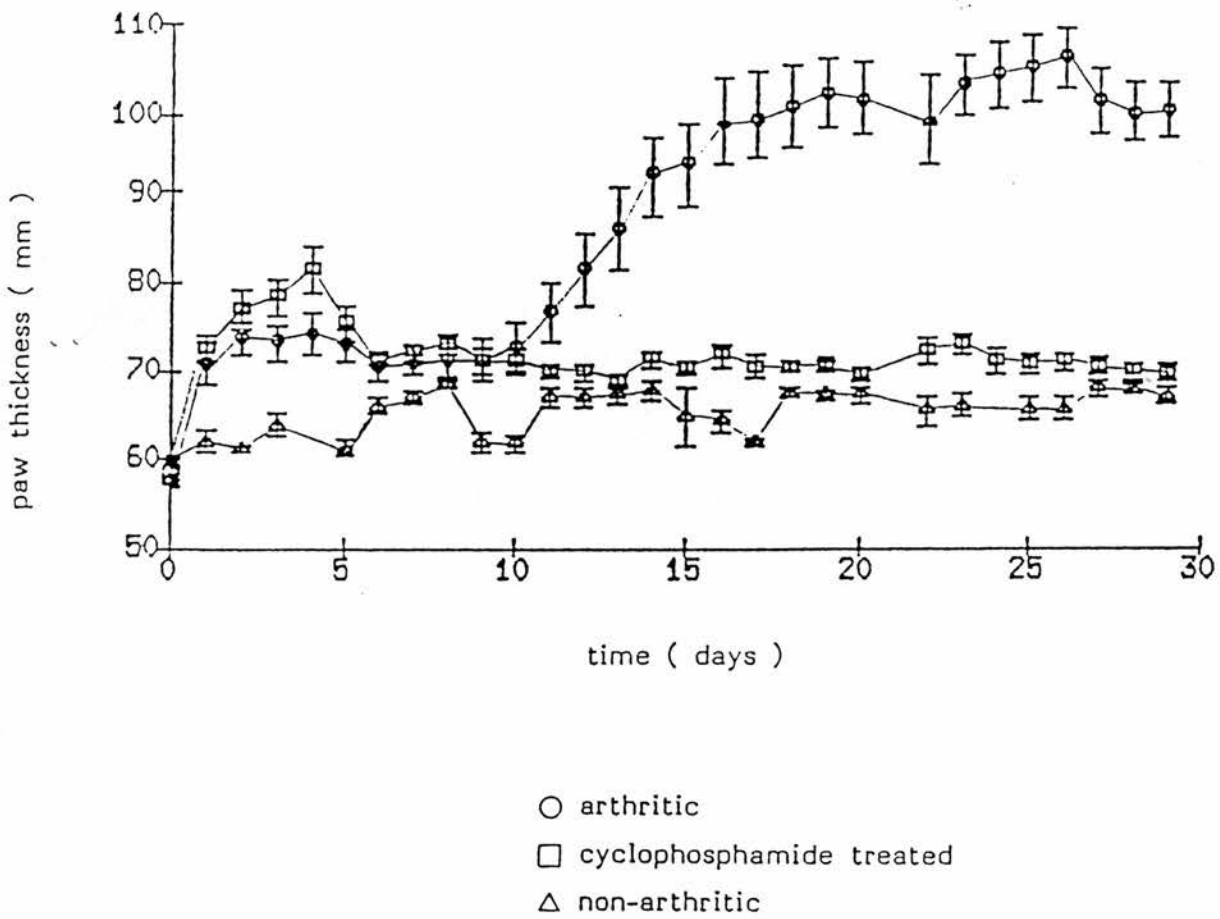


Figure III-12. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on the swelling in the paw of the uninjected limb of male arthritic Lewis rats. Experiment EXA8.

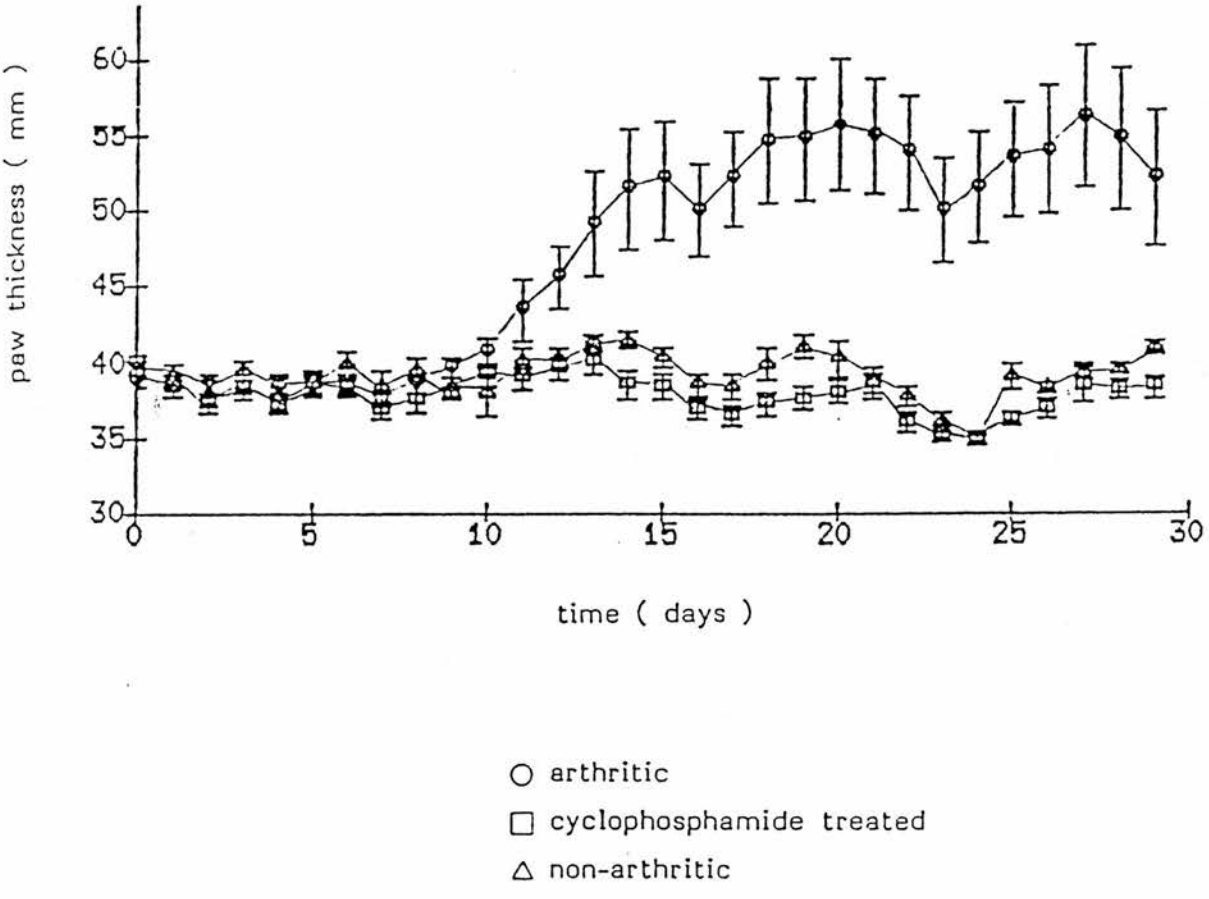


Figure III-13. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on the swelling in the ankle of the uninjected limb of male arthritic Lewis rats. Experiment EXA8.

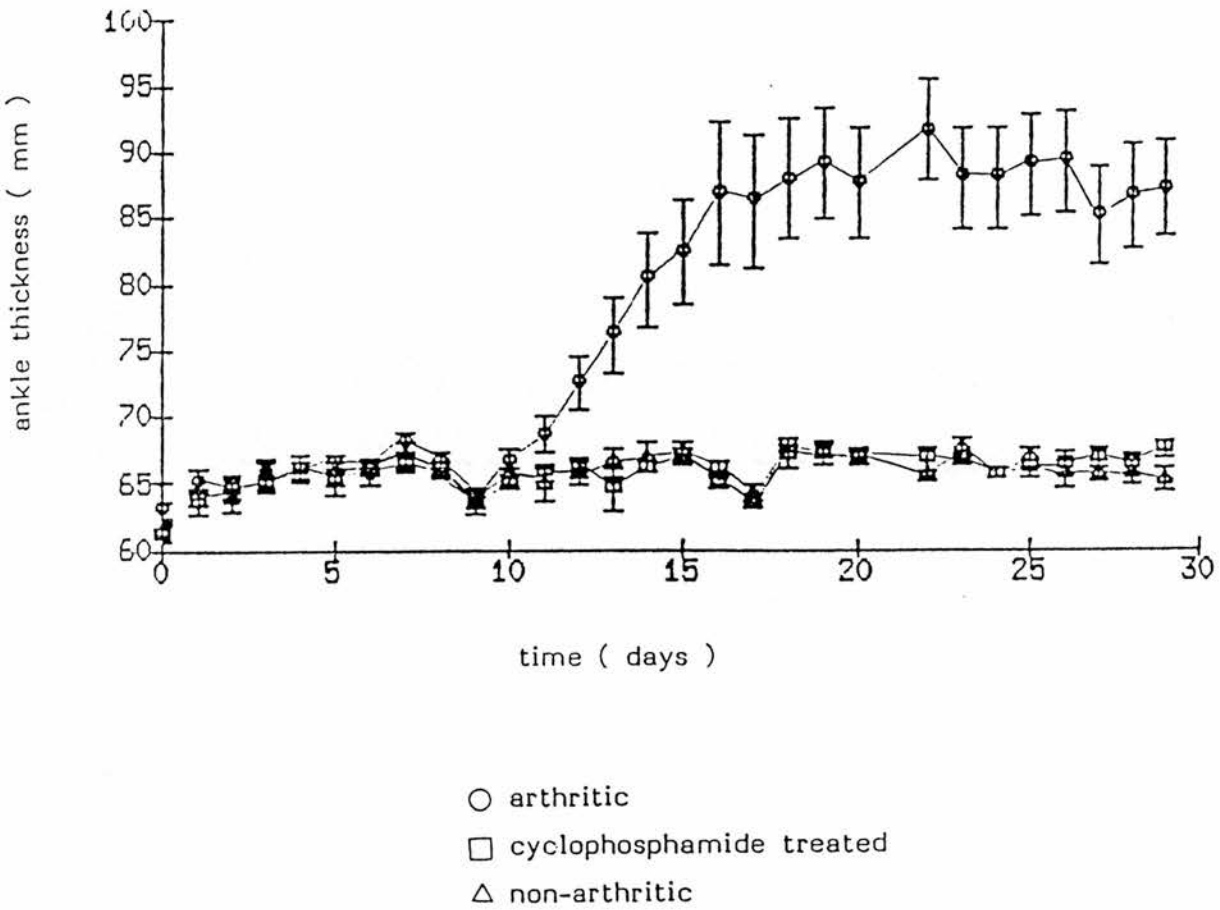


Figure III-14. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on disease dissemination in male arthritic Lewis rats. Experiment EXA8.

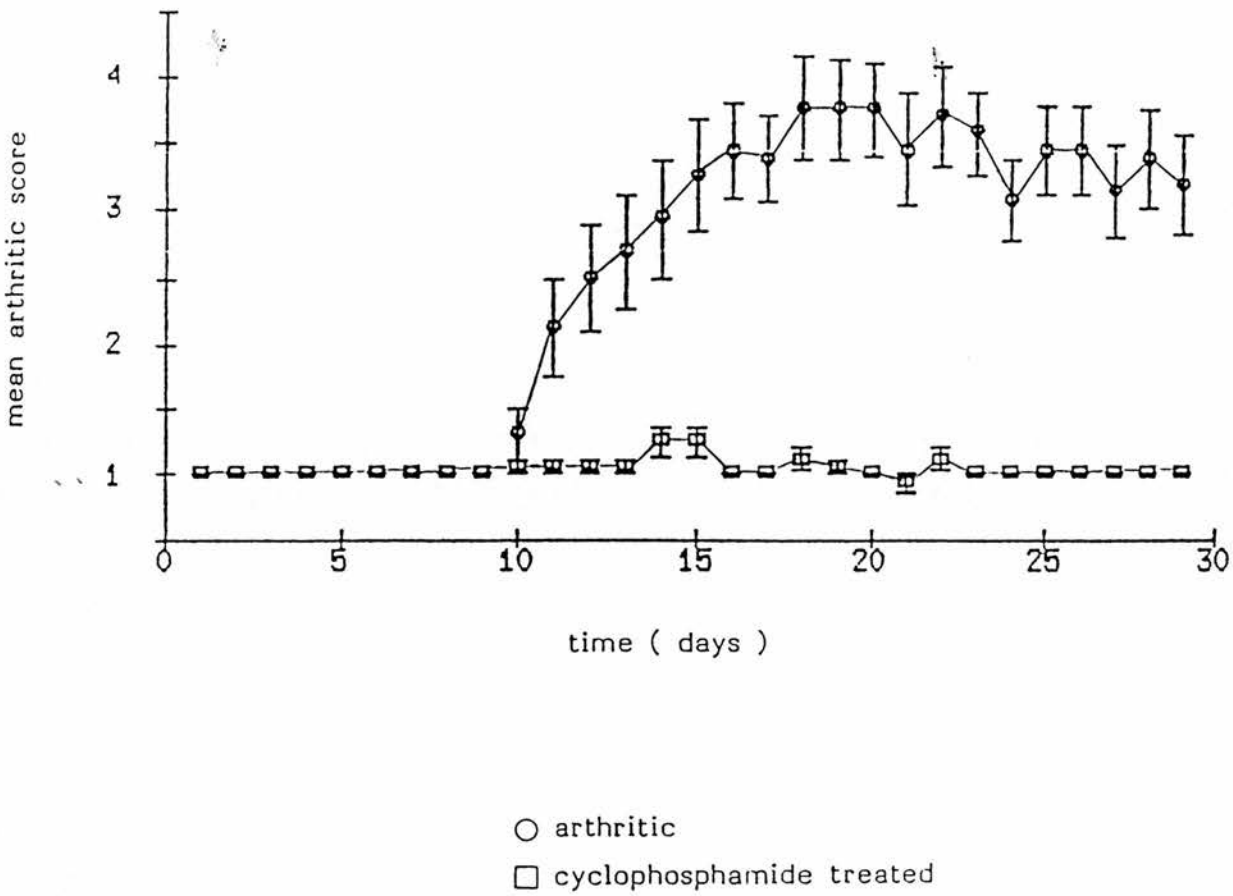
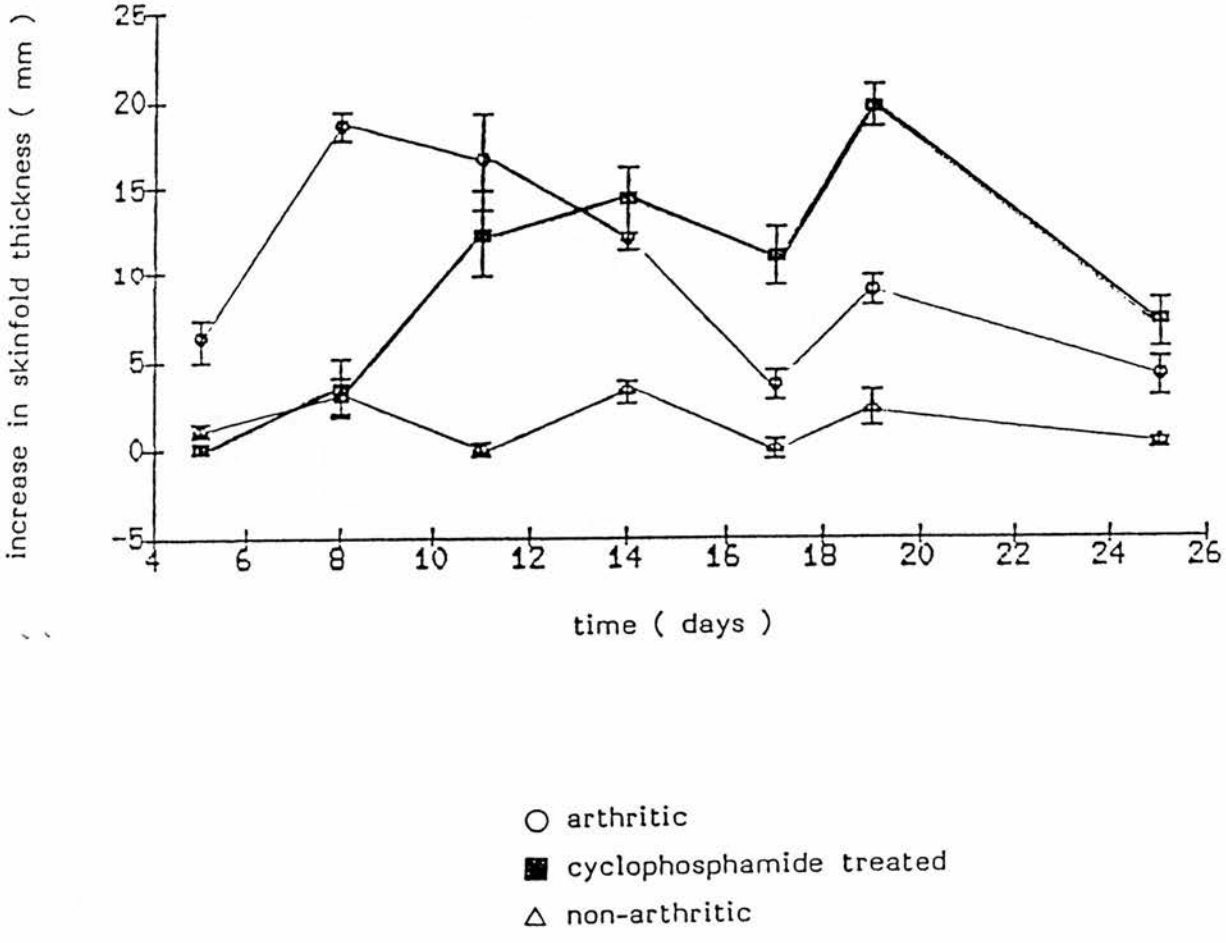


Figure III-15. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection on cell mediated immunity to PPD in male arthritic Lewis rats. Experiment EXA8.



The magnitude of the peak response in the treated and untreated groups again appeared to be very similar. It has been suggested that cyclophosphamide has an immunopotentiating effect under appropriate circumstances (MacKenzie, Pick et al, 1978, Cottney, Bruin et al, 1980, Turk, 1975). It seems important, therefore, to compare directly the magnitude of the response in both groups at its maximum value. To avoid complications of diurnal and seasonal changes, which may affect the level of immune responsiveness, a further experiment was conducted which allowed direct comparison of the response maxima at the same time on the same day.

Method (Experiment EXA54)

Three groups of 24 male Lewis rats were injected with arthritogen (M. tuberculosum) and treated with 100 mg/k of cyclo-phosphamide as specified in the diagram below. Cell mediated immunity to PPD was assessed in 8 animals from each group on days 15, 20 and 26. No animal received more than one skin challenge during the course of the experiment.

Treatment regimen for experiment EXA54.

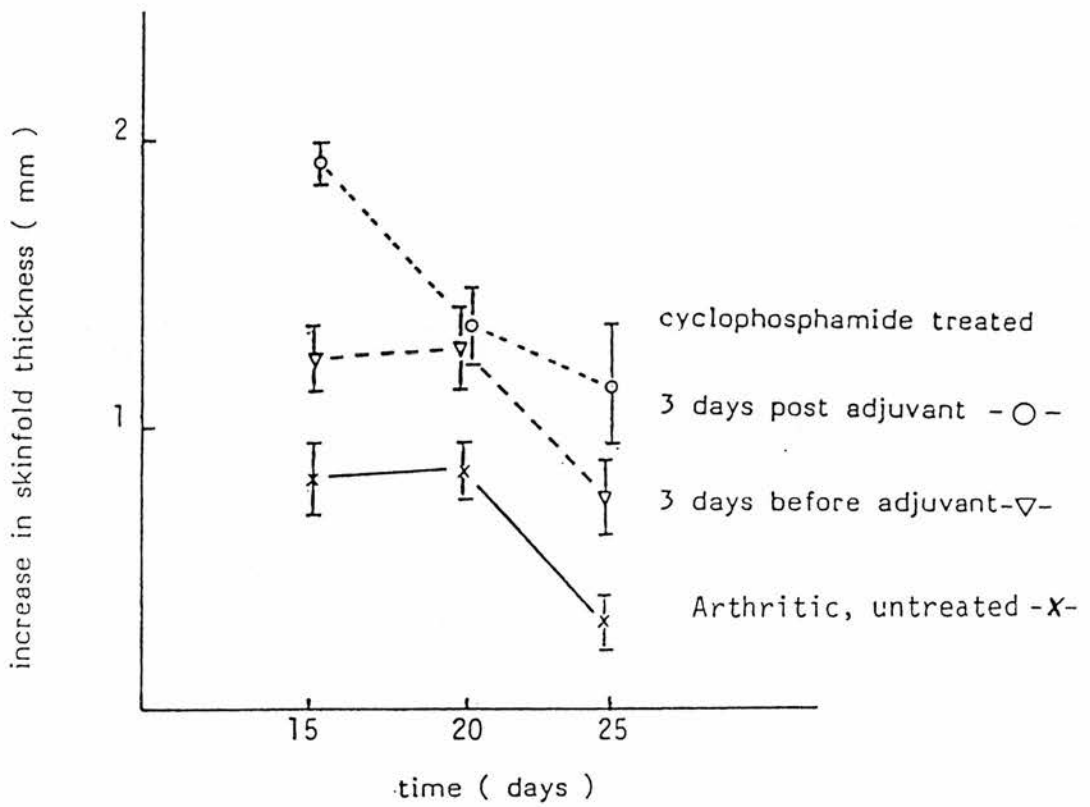
Group 1	cyclo-phosphamide	FCA			skin test	skin test	skin test
Group 2		FCA	cyclo-phosphamide				
Group 3				FCA			
Day	-3	0	+3	+7	+15	+20	+26

An additional group was included in order to determine to what degree the profile of the cell mediated immune response was dependent upon the timing of the cyclophosphamide dose in relation to the administration of FCA. Group 1 received 100 mg/kg cyclophosphamide i.p. 3 days before FCA injection. For the experimental design illustrated, the timing of the maximum of the cell mediated immune response was taken to be day 20 post FCA injection, from the data of Mackenzie et al. (MacKenzie, Pick et al, 1978), and again the treatment was so timed that the maximum response in this group occurred simultaneously with those of the other two.

Results

The profiles of the responses observed are shown in Figure III-16. The intention was to take measurements on the upward curve of the response, at the response maximum, and on the downward slope of the curve. From the data illustrated, cyclophosphamide given 3 days post adjuvant injection did induce a significantly greater cell mediated immune response to PPD at the peak maximum than occurred in the untreated arthritic animals, with an indication that the peak was in fact attained before day 20, probably closer to day 15. The timing of the dose in relation to the administration of FCA did seem to be important, in that the response maximum achieved when cyclophosphamide was given 3 days prior to FCA sensitisation was also greater than for the untreated group, but less than for these receiving the drug 3 days post adjuvant. These observations support the suggestion that cyclophosphamide, under appropriate circumstances, can exert an immunopotentiating effect.

Figure III-16. The effect of treatment with 100 mg/kg cyclophosphamide i.p. 3 days pre and post FCA injection on the peak cell mediated immune response to PPD in male arthritic Lewis rats. Experiment EXA54.



Circulating lymphocytes and PMNs

It has been reported that a leucocytosis accompanies the development of rat arthritis (Newbould, 1965), with a striking inversion of the proportion of PMNs to lymphocytes in the peripheral circulation (Myles Glenn, Bowman et al, 1977). In one example, a biphasic elevation of leucocyte counts was observed, the initial increase being detectable on the day immediately post FCA injection (Newbould, 1965). The increase was mainly due to a rise in the number of PMNs, with the level of lymphocytes remaining essentially constant. A further increase in the total leucocyte count occurred in the secondary phase of the disease, again due primarily to an increased PMN count (Newbould, 1965). The changes observed in this case were thought to be secondary phenomena, rather than the primary cause of the disease (Newbould, 1965). In healthy rats, the predominant cell type in the circulation is the lymphocyte, a situation which is the exact opposite of the case for humans (Myles Glenn, Bowman et al, 1977). With the onset of arthritis, in the rat, the PMN count increases 8- to 10-fold over the initial 14 day period, with lymphocytes increasing approximately 2-fold. As a consequence, the PMN becomes the predominant circulating cell in the arthritic rat.

It is accepted that measurement of circulating cells such as PMNs and lymphocytes, which can be sequestered into sites of tissue damage, and in particular into arthritic joints and inflamed tissue generally, may be misleading. However, an increased level of these cells in the circulation at least suggests an increased availability at sites of potential sequestration. The levels of these cells in arthritic rats in comparison to cyclophosphamide treated animals may offer an approach towards an understanding of the nature of the effect of cyclophosphamide. Reduced disease severity has been associated with a fall in the peripheral lymphocyte count, following treatment with anti-lymphocyte globulin (Currey and Ziff, 1968, Kayashima, Koga et al, 1978), for example. Cyclophosphamide induced disease remission has been associated with a reduction in circulating PMNs and lymphocytes (Myles Glenn, Bowman et al, 1977). Timegadine (Bramm, Binderup et al, 1981), which is unusual in that it appears effective even when dosing commences within the secondary phase of the disease, similarly induces remission concomitant with a reduced level of leucocytosis. Consequently, the effect of a single 100 mg/kg dose of cyclophosphamide given 3 days post FCA injection on the circulating cell population in arthritic Lewis rats was investigated in the series of experiments described below.

In addition, the erythrocyte count was monitored as potentially a further marker of the disease progression. In humans, a normochromic, normocytic anaemia is frequently associated with chronic disease (Hughes, 1978). There is generally no deficiency in the body iron stores. The source of the problem seems to be a failure of the reticuloendiothelial system to re-cycle iron from the erythrocytes at the end of their life span (Bluestone, 1979). A similar anaemia has been observed in rat arthritis. An 8% reduction in haematocrit has been reported (Currey and Ziff, 1968), and a 15% reduction in haemoglobin (Baumgartner, Obenaus et al, 1974). The anaemia has been described as normochromic and normocytic (Baumgartner, Obenaus et al, 1974) but further parallels with the human case have not been explored in great detail. Here, erythrocyte counts were measured to determine whether a similar anaemia is detectable in the Lewis strain. A point of interest would also be the response of such an anaemia to cyclophosphamide treatment. Cyclophosphamide induced bone marrow suppression is likely to occur initially, with a consequent fall in the circulating erythrocyte count. However, if cyclophosphamide treatment leads to a normal erythrocyte count in the later stages of the experiment, this might also act as an indicator of a more basic change in the disease progression. Such studies with cyclophosphamide are not reported here, however, although further research in this area as a marker of the possible interaction between the lymphocytic and reticulo-endothelial cell systems, may prove of value, for reasons mentioned later.

A pilot experiment was initially conducted to establish the response-time profile for white and red cell counts in untreated arthritic animals in relation to controls.

Method (Experiment EXA50)

Two groups of 10 male Lewis rats were employed. The control group received subplantar injection of liquid paraffin, the arthritic group received arthritogen. Measurements were made at 4, 24 and 48 hrs only.

Results

The data for the white cell count is shown in Figure III-17. There was a marked leucocytosis which persisted to at least day 30. A differential cell count conducted on day 21 showed a relative increase in the proportion of PMNs to lymphocytes in the circulation, and that the leucocytosis was primarily due to the increase in PMNs (Table III-1). Figure III-18 demonstrates that there was an anaemia which may have been biphasic, recovering at the end of the primary phase, but

reappearing in a more severe form as the secondary phase became established.

Two further experiments were therefore undertaken to establish the profile of the white cell response in arthritic Lewis rats, and in those receiving a single cyclophosphamide dose of 100 mg/kg i.p. 3 days after FCA injection.

Figure III-17. A comparison of the peripheral white cell counts in arthritic and non-arthritic male Lewis rats. Experiment EXA50.

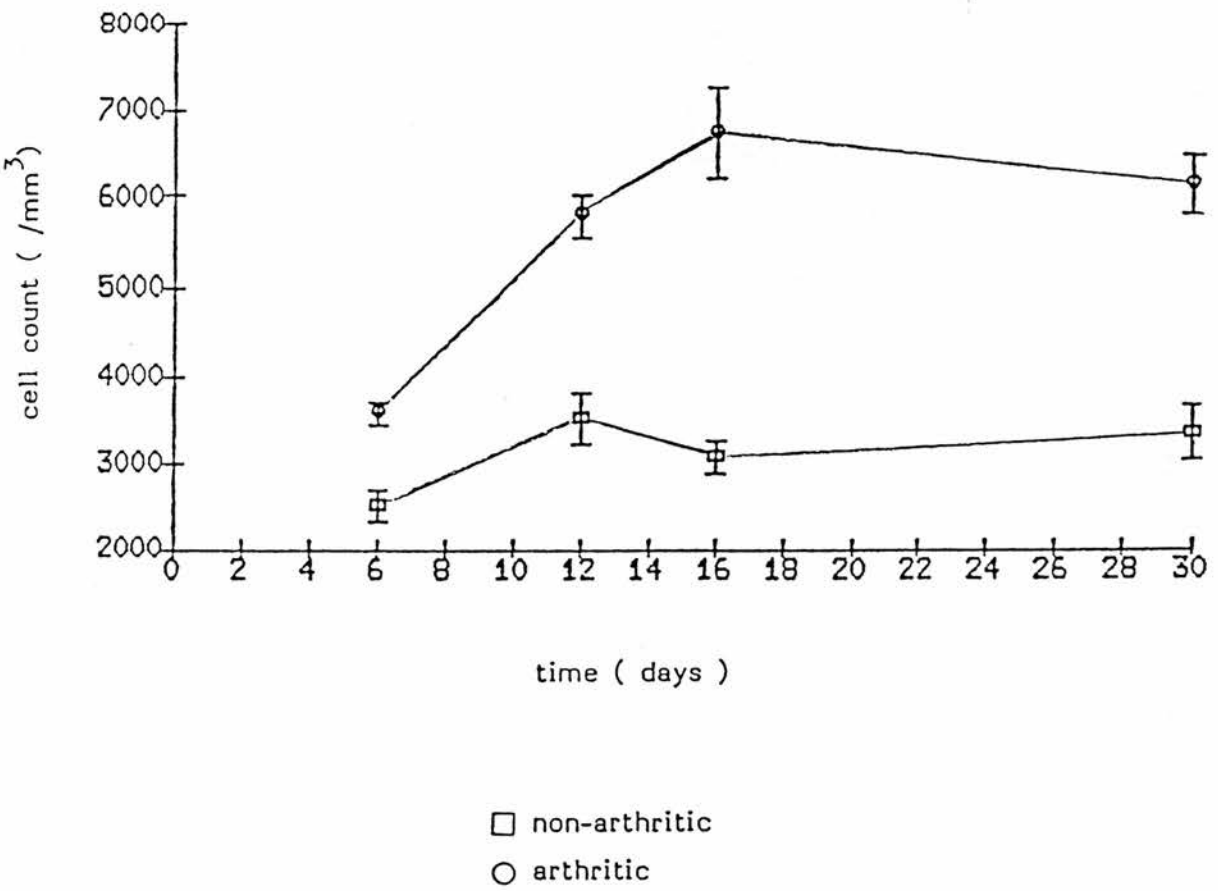


Figure III-18. A comparison of the erythrocyte counts in arthritic and non-arthritic male Lewis rats. Experiment EXA50.

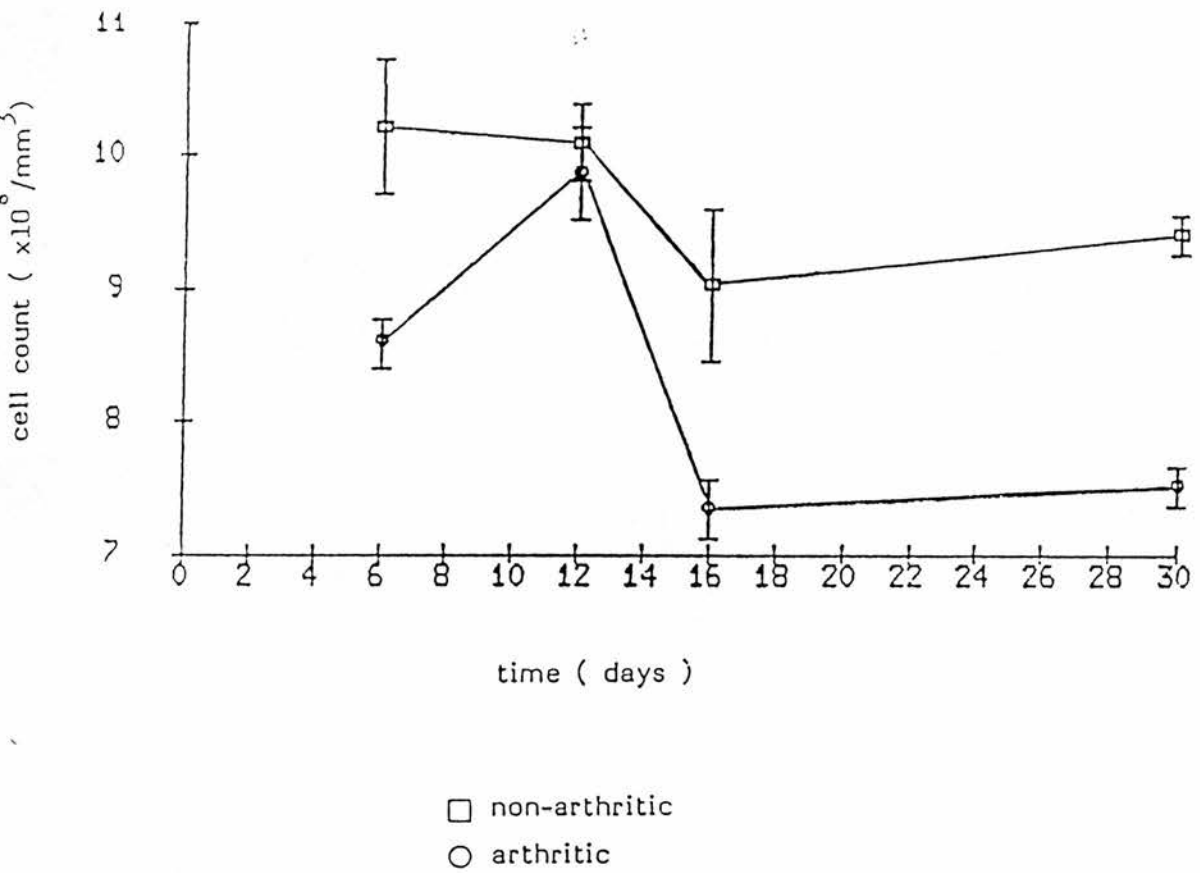


Table III-1. Differential white cell count at day 21 following disease induction in Lewis rats. Experiment EXA50.

	Lymphocytes(%)	PMNs(%)
Arthritic rats	36 ± 2	56 ± 2
Non-arthritic rats	78 ± 3	14 ± 2

Method (Experiment EXA60)

Eighteen male Lewis rats were divided into 2 groups of 9. All animals received injection of arthritogen on day 0. Blood samples were taken for total white cell and differential cell counts on days 1, 3, 6, 9, 14, 16, 21 and 30. The groups were taken in rotation so that samples were taken at intervals of not less than 6 days.

Results

The white cell count from day 1 to day 30 is shown in Figure III-19 for the arthritic animals. There was a prolonged leucocytosis, which tended to return to normal levels towards the end of the study. The cyclophosphamide treated animals exhibited a dramatic, transient, fall in the white cell count which returned to normal (non-arthritic) levels by day 11 (Figure III-20). By day 14 the values were very similar to those for the arthritic rats, and this was also true in the later stage of the experiment. Unfortunately, the values for the interval from day 14 to day 28 were lost due to an instrument fault, and it has not so far been possible

to repeat the experiment. It is not clear, therefore, whether the white cell counts are similar for both the cyclophosphamide treated and untreated arthritic rats within this period. It is worth noting, however, that the recovery of the white cell count occurred at almost precisely the same time as the onset of the cell mediated immune reaction to PPD in this strain of animals, shown in Figure III-15.

The time profile of the PMN count as a percentage of the total white cell count in the arthritic group is shown in Figure III-21 and is consistent with the view that the change in circulating numbers of PMNs was dominating the total white cell count. The ratio of PMNs to lymphocytes was found to tend towards the value for non-arthritic animals by day 30 of the experiment, although the total white cell count remained substantially higher.

The profile of the PMN percentage in the cyclophosphamide treated group is shown up to day 14 in Figure III-22. There was a very rapid drop from the initial elevated level observed during the primary phase. By day 8 there were virtually no PMNs in the circulation. There was then a rapid recovery to about day 14 of the disease, when the total circulating white cell count and the ratio of PMNs to lymphocytes returned to the levels of the untreated arthritic animals.

Figure III-19. The peripheral white cell count in arthritic rats as a function of time. Experiment EXA60.

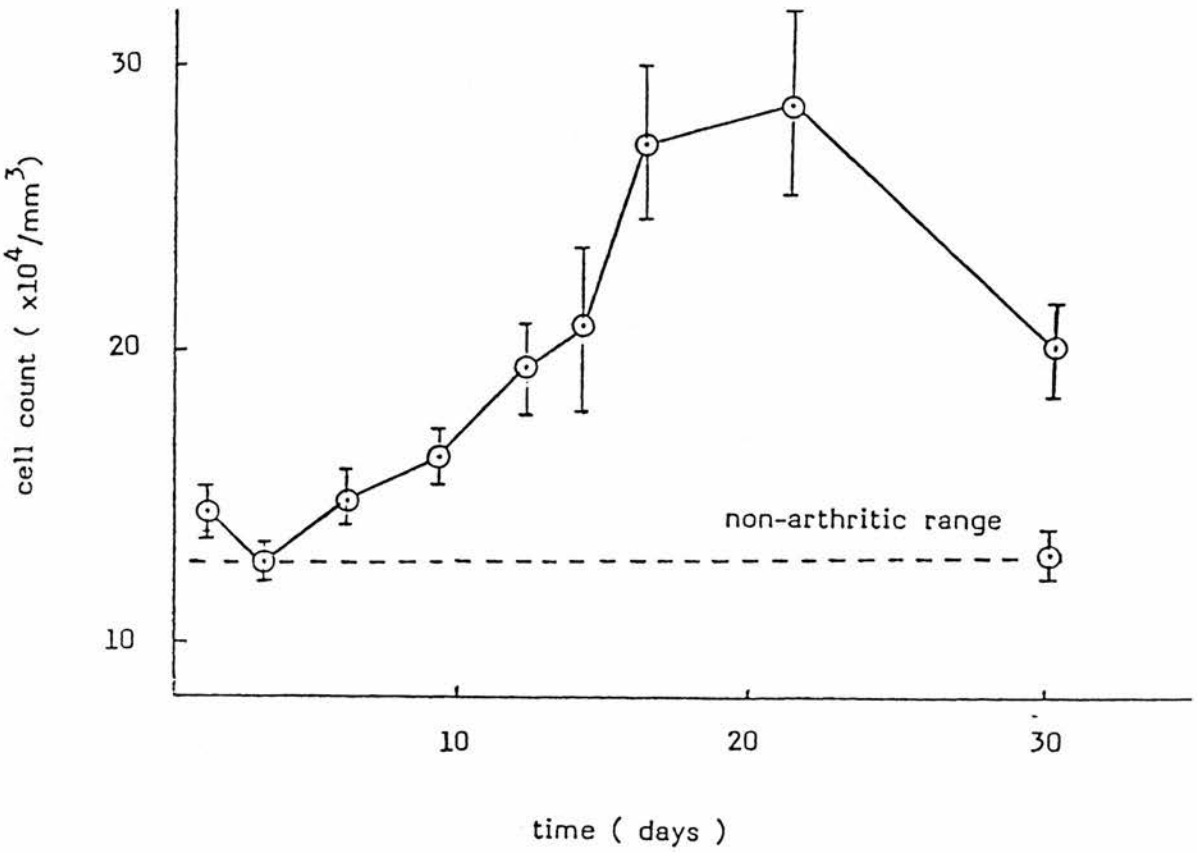


Figure III-20. The peripheral white cell count in arthritic rats treated with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection as a function of time. Experiment EXA61.

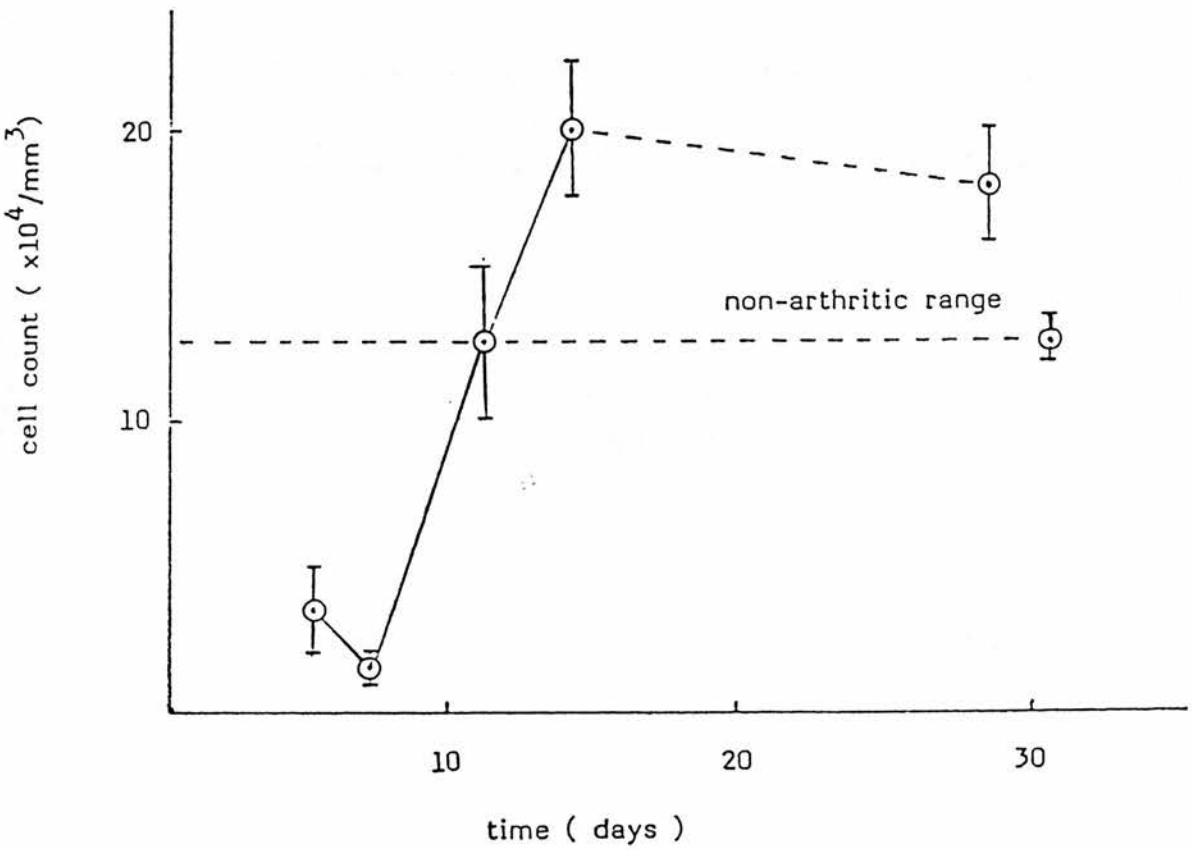


Figure III-21. PMNs as a percentage of the peripheral white cell count in arthritic rats as a function of time. Experiment EXA60.

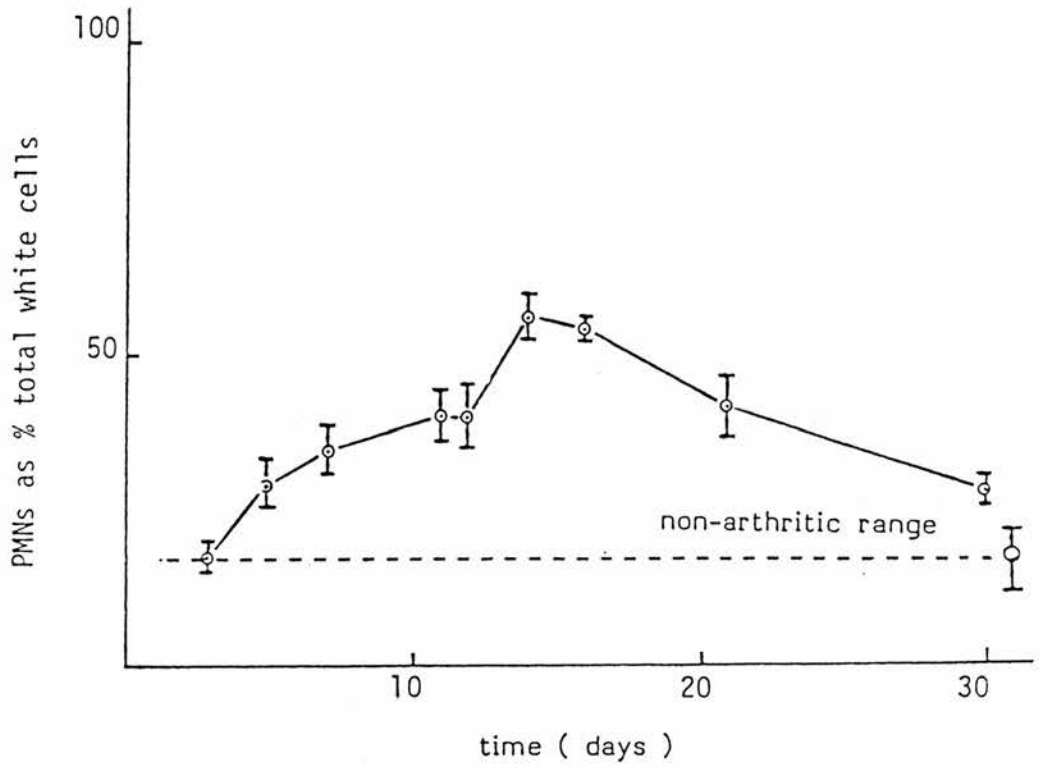
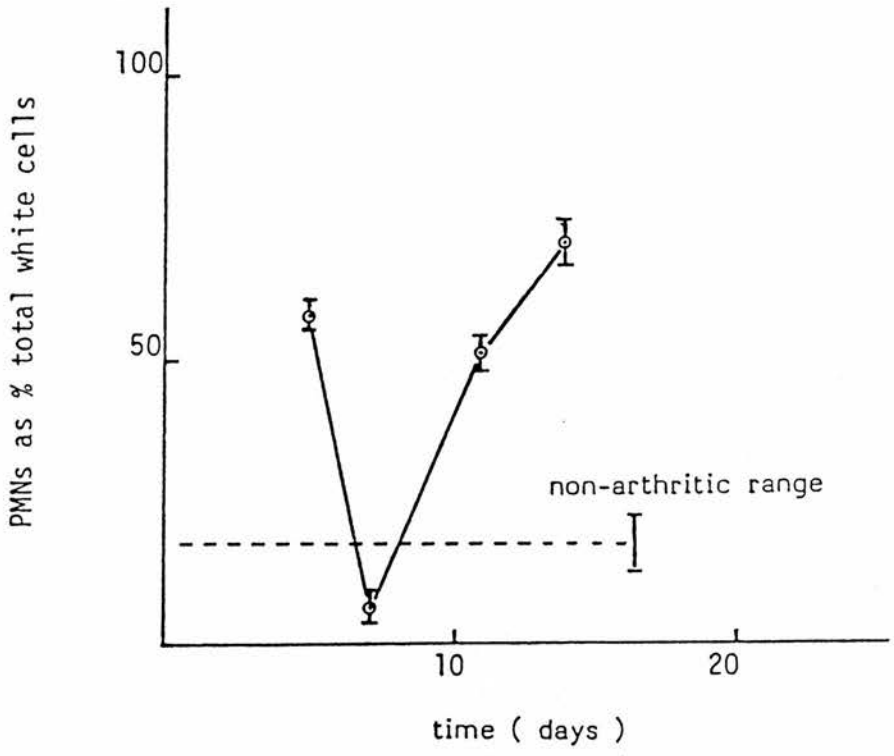


Figure III-22. PMNs as a percentage of the peripheral white cell count in arthritic rats treated with 100 mg/kg cyclophosphamide i.p. 3 days post FCA injection as a function of time. Experiment EXA61.



The point to be made here is that, whilst all outward disease signs have been suppressed in these animals by cyclophosphamide treatment, by day 14, the cell mediated immune response to PPD is increasing rapidly towards a value greater than the maximum level attained in the arthritic rats. Moreover, the blood picture at this time, in respect of lymphocyte and PMN counts, and their ratio, is almost identical for the two groups.

These observations seem to suggest the following. There is an enhanced cell mediated immune response in these animals following cyclophosphamide treatment. The initial suppression seems most likely to be due to the almost total absence of PMNs in the circulation, effectively preventing the expression of cell mediated immunity in the form of a skin reaction. In this phase, the level of cell mediated immunity, in the sense of the numbers of sensitised effector lymphocytes present, is not therefore known. As the PMN population recovers, the expression of cell mediated immunity shows heightened levels, which are maintained far longer than for the untreated animals. Cyclophosphamide at this dose appears to have preferentially depleted a cell population which would otherwise have acted to suppress the response to PPD.

There is evidence to suggest that a lymphocyte sub-set exists which moderates the severity of the disease, and that this sub-set is susceptible to cyclophosphamide and certain other treatments.

When moderate to low doses are given 2-3 days before disease induction, cyclophosphamide has been shown to exacerbate the outward inflammatory disease signs (Floersheim, Borel et al, 1972). The dose and timing in relation to the FCA injection are critical. Similar effects have been shown with other drugs, including d-penicillamine, where the timing and dose administered are again critical (Otomo, Higuchi et al, 1981, Otomo, Nakaike et al, 1981). This effect for both drugs seems to be related to thymus function. Thymectomised Wistar-King-Aptekman (WKA) rats show exacerbated disease signs if there is a space of about four weeks between thymectomy and FCA injection (Kayashima, Koga et al, 1976). The heightened disease activity is, however, restored to normal levels by transfer of syngeneic thymocytes (Kayashima, Koga et al, 1976). In this report, disease exacerbation was shown to occur if the rats received low dose whole body irradiation, but not with high doses (Kayashima, Koga et al, 1976). The implication seems to be that there exists a short lived, radiosensitive and cyclophosphamide sensitive T-lymphocyte which moderates the progress of the disease, possibly a suppressor T-cell (Kayashima, Koga et al, 1976).

In support of this view, experiments with the drug lobenzarit disodium (CCA) have shown that it appears to restore to normal the thymic involution seen in rat arthritis and inhibits the disease signs. Rats were treated with rabbit anti-rat lymphocyte serum, sufficient to reduce the peripheral lymphocyte count to 1/10 of

its normal value, whilst the PMN count stayed in the normal range. In these animals the beneficial effect of CCA was not apparent (Ohsugi, Nakano et al, 1983). (This study did not, unfortunately, refer to the ability, or otherwise, of the anti-lymphocyte serum to inhibit the disease of itself). However, the implication is that CCA depends for its effect on the presence of normal levels of lymphocytes. It would seem that drug manipulation of this particular T-suppressor subset is possible, and in particular that the numbers or activity of these cells may be elevated by CCA (Ohsugi, Nakano et al, 1983).

Such studies have led to the view that disease activity in arthritic rats is due to a balance between at least two T-lymphocyte subsets. One which is responsible for initiating the disease and perhaps also for maintaining it through the chronic phase, and one which moderates disease activity, the latter having the characteristics of a T-suppressor cell (Taurog, Sandberg et al, 1983).

There is, however, evidence that a further cell type plays a major role in this disease. In rat arthritis it has been shown that the in vitro response of splenic lymphocytes to both phytohaemagglutinin and to concanavelin-A is suppressed. The suppression does not occur in methotrexate or corticosteroid induced, or spontaneous, remissions (Kapusta, Young et al, 1979). It appears to be due to a serum borne factor produced in

proportion to the disease severity (Binderup, Bramm et al, 1978).

The same inhibitory effects are also demonstrated by adherent spleen cells from arthritic rats (Binderup, Bramm et al, 1982). Spleen cells from adjuvant arthritic rats may therefore be the source of the serum inhibitory factor. It seems likely that this cell population may also be an important therapeutic target in anti-rheumatic drug design (Binderup, Bramm et al, 1982).

The development of an adherent cell derived inhibitor of lymphocyte activation may explain the rapid disappearance of the cell mediated immune reaction to PPD, which accompanies the development of the secondary disease signs in both the Lewis and Wistar rat strains. It would seem necessary to conclude that the effect of cyclophosphamide at the dose employed here is to prevent the production of the serum borne factor which would otherwise have suppressed the CMI response. These results cannot be explained by selective suppression of the T-lymphocyte subset which moderates the disease, since in these studies the disease signs were reduced, not exacerbated. It would seem reasonable to suppose that the effect of cyclophosphamide in these experiments is to grossly suppress the lymphocyte population. Both lymphocyte populations implicated in controlling the progress of the disease are equally diminished, such that there are virtually no outward signs of the disease. The dose is presumably also high enough to

prevent release by the adherent cell population of the disease related serum inhibitory factor. Lymphocyte recovery then occurs in the absence of this control, reaching, and maintaining higher levels than in the arthritic animals. The failure of the disease to reappear under such circumstances is less explicable. One possibility is that the stimulus which initiated the disease is no longer present. This is inconsistent with the ability of cyclophosphamide given before FCA to suppress the disease (MacKenzie, Pick et al, 1978). A more likely possibility is that an adherent cell population is required for the initiation and maintenance of the disease, and that this population has also been depleted by cyclophosphamide.

These studies therefore begin to suggest a more productive approach to the use of the rat arthritis model in drug research. It is possible to establish by drug manipulation a preponderance of one or other identifiable T-cell subset. Either the moderating (suppressor) subset, or that which initiates and maintains the disease. The response of these cells to drug intervention may then be studied in a similar way to that described above for CCA. This offers a route to the investigation of truly "immuno-modulatory" drugs and their potential role in the treatment of rheumatoid arthritis. Further, there is an approach to the study of the functional role of the adherent cell (possibly macrophage) population described above, using cell-mediated immunity to PPD as a marker. Assuming that such cells play a vital role in the

initiation and maintenance of the disease by interacting with the lymphocyte population, diminution or prevention of such activity may have the kind of fundamental effect on the disease which is being sought. This would be particularly valuable if such an effect could be achieved without the need for such powerfully cytotoxic drugs as cyclophosphamide. If such cells release a factor which normally suppresses the cell mediated immune reaction, drug intervention which maintains the level of cell mediated immunity would suggest inhibition of the release of this factor and an effect on this specific cell population. Such an hypothesis would seem to justify further investigation. The functional role of the reticuloendothelial system as a whole might usefully be studied in parallel, using the anaemia of the disease as a marker.

Whilst at least consistent with the data, the above speculations require experimental validation. The intention of these experiments, to establish a metameter to reflect the progress of the disease in a more fundamental sense, has not been achieved, but there is an indication that a metameter which reflects the activity of a specific lymphocyte subset may satisfy this requirement. There is also the suggestion that it may be possible to relate lymphocyte activity to the presence of controlling factors derived from adherent cells, which may be used as a marker of effective drug intervention in the function of these cells.

IV

DISCUSSIONS AND CONCLUSIONS

There remains no cure for rheumatoid arthritis, nor is there any treatment which unambiguously delays the progression of the disease without presenting a concomitant burden of severe side-effects. This situation has arisen despite the extensive use of what is apparently an excellent animal model of the human disease over the past 30 years.

In Chapter I of this thesis, the general relevance to the human disease of this model, a polyarthritis induced in the rat by the injection of Freund's adjuvant, has been reviewed. Particular emphasis was given to mechanisms of acute inflammation in both diseases, and to the immunological processes which may be responsible for their chronicity. It was concluded from this initial review that there is no immediately evident lack of relevance to the human disease which explains its apparent failure to produce a satisfactory drug therapy.

In the subsequent Chapter, three aspects of the historical use of the model were discussed. The first concerned the dramatic response of the outward signs of the rat disease to r treatment with anti-inflammatory steroids and the non-steroidal anti-inflammatory drugs. The consequence of this seems to have been an undue emphasis on these drugs, and the disease signs most immediately affected by them. This being then followed by an equally strong reaction against the model as a whole when these two classes of drugs were found to exert no fundamental or lasting effect on the human disease.

Secondly, there are good scientific reasons for employing the model in circumstances where drug treatment commences after the disease has become established, in closer analogy to the human case. There is a progressive change in the drug metabolising capability of affected animals which seriously compromises this appoach however. Finally, in situations where measurements are repeatedly made of the same groups, animal or human, the detection of significant drug effects where these are not as dramatically apparent as with the anti-inflammatory steroids for example, requires rather sophisticated statistical techniques. Repeated use of the t-test at each measurement time, although frequently done, ignores the dependence structure of the data, is therefore wrong, and can lead to erroneous conclusions. these three problems have been illustrated, and the consequences of their general neglect presented as at least a partial explanation of the problem. It is concluded that the use of the model has been deficient, rather than the model itself.

In the final Chapter, the remaining major problem was addressed, namely the identification of some measureable parameter which can be used to unambiguously indicate a fundamental effect of a specific treatment in the model. Cyclophosphamide, given as a single dose at an appropriate stage of the disease, has been shown to apparently arrest the disease progression. An approach wich was proposed, therefore, is to explore various characteristics of the disease, and their response to cyclophosphamide treatment, to determine, if possible, which appear to be associated with a beneficial change in the condition of the animals.

Changes in the level of cell-mediated immunity was explored in some detail in this context, and tentatively related to changes in circulating lymphocyte and polymorphonuclear leukocyte counts. No definite conclusion as to the importance of these parameters is presently possible. Further lines of research have been suggested, however, and in particular the possibility that antigen presenting cells such as the macrophage, and their involvement with lymphocyte mediated processes, may be of importance in maintaining chronic inflammation of this type, seems to merit more detailed consideration.

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