The effects of thiol compounds on mononuclear cell functions in arthritic rats.

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Award date:
1985

Awarding institution:
University of Bath

THE EFFECTS OF THIOL COMPOUNDS ON MONONUCLEAR CELL FUNCTIONS IN ARTHRITIC RATS

Submitted by

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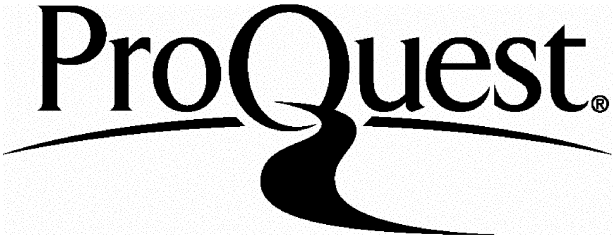
for the Degree of Doctor of Philosophy

at the University of Bath

1985



.... To my Father

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ACKNOWLEDGMENTS

I wish to thank Dr. N.D. Hall and Dr. L.J. Notarianni for the supervision, guidance and encouragement throughout the whole project and also during the preparation of this thesis.

I am grateful to Dr. M.J. McKeown for his invaluable assistance and practical advice.

I should like to acknowledge the helpfulness and extreme patience of those with whom I worked, and wish to thank the administration of the Royal National Hospital for Rheumatic Diseases, Bath, for giving me the opportunity to work in the research laboratory.

Acknowledgments should also be extended to all the nice people working in the University Animal House for their expert handling of animals and extreme patience and understanding: and to all the members of the technical staff of the School of Pharmacy and Pharmacology for all their willing help.

I also wish to express my indebtedness to the kind people of Lilly Research Centre, Windlesham, for the gift of IL-2 standard and heat-killed M. Tuberculosis.

I also owe grateful thanks to Maureen Walters who typed the thesis for me with much patience and excellent word-processing skill.

Finally, I would like to acknowledge the love and continuous encouragement bestowed upon me by my family, without which this work could not have been completed.

SUMMARY

Levels of free SH groups are depressed in the sera of patients with rheumatoid arthritis (RA) especially during active disease. However, the immunopathological significance of this effect is uncertain. Using adjuvant arthritis (AA) as a model of RA, the changes in serum protein SH level and reactivity during the course of AA were studied using a spectrophotometric method. A major depression of serum SH level and reactivity was found in arthritic rats and this was associated with persistent inflammatory phase of disease. This depression may be caused by oxidative stress due to production of oxidative species by activated phagocytes. In vivo D-penicillamine treatment of AA rats increased both serum SH level of reactivity towards normal values over days 14-21. This result suggests that D-pencillamine may act as a reducing agent, restoring the free SH groups that have been oxidised (blocked) in active AA.

Rat mononuclear cell proliferation to the mitogen ConA was stimulated by the addition of 2-mercaptoethanol (2-ME) to the cultures. This ConA response was completely inhibited by the cell surface SH blocking agent, PHMPSA. During the course of AA in Sprague-Dawley rats, spleen (SC) and lymph node cells (LNC) responded poorly in vitro to ConA. The addition of 2-ME was found to reverse and enhance this depressed ConA response. D-penicillamine in vivo also modified the pattern of ConA

stimulated proliferative response of AA rat SC and LNC, converting the markedly inhibited response to ConA at day 14 to an enhanced response when compared with cultures from untreated arthritic rats and nonarthritic rats. These results indicate that cell surface thiols are necessary for the proliferation of rat SC and LNC and that the altered cell function in AA may be due, at least in part, to blockade of the functional free cell surface SH groups. 2-ME *in vitro* and D-penicillamine *in vivo* could help to maintain cell surface SH groups in a reduced state.

Alternatively, the depressed response could be attributed to suppression from activated macrophages via a mechanism involving SH groups. The depressed immune response in AA was not due to prostaglandin synthesis by activated macrophages nor was it due to an impaired synthesis of interleukins. Increased amounts of IL-1 were produced by AA rat peritoneal macrophages and SC from these animals produced slightly reduced amounts of IL-2. D-penicillamine treatment decreased the synthesis of IL-1 but had no effect on IL-2 production. The synthesis of these interleukins was not SH dependent as 2-ME and PHMPSA had no effect.

Although D-penicillamine treatment *in vivo* leads to dramatic changes in certain mononuclear cell (MNC) functions, it has no effect on the clinical course of AA. The significance of altered MNC activity in the pathogenesis of AA is therefore

questionable and the usefulness of AA as a model for detecting novel antirheumatic drugs with immunoregulatory actions is limited.

CHAPTER ONE: INTRODUCTION

The introduction to this thesis is divided into three parts, PART (I) describes the cellular interactions involved in the immune response. PART (II) deals with rheumatoid arthritis (RA) and the various experimental animal models of RA. A brief discussion of the chemotherapy of RA is also included. PART (III) deals with the role of SH groups in RA and its involvement in the immune system.

PART (I) THE IMMUNE RESPONSE

1.1 Cellular interactions in the immune response

In a broad sense, there are two types of specific immune response to antigen: (a) humoral immunity which results in specific antibody synthesis and secretion and (b) cell-mediated immunity which is associated with sensitised lymphocytes and their lymphokines.

Cellular immunity is normally regarded as a function of thymus-derived lymphocytes although non-specific accessory cells, probably macrophages (møs) are also involved (Roitt et al., 1969). Humoral immunity to most antigens (T lymphocyte-dependent antigens) involves antigen specific T cell help being mediated to antigen specific B cells, by mø (Roitt et al., 1969) and their secreted immunoregulatory soluble factors

(Oppenheim and Gery, 1982).

It is now evident that a complex and intricate network of interactions occur between the two arms of immune response. These interactions can result in both amplification (enhancement) or suppression of immune reactivity.

This section will describe some of the interactions between T and B lymphocytes and macrophages resulting in humoral production of antibody and thus, without ignoring the importance of cell-mediated immunity, will concentrate on the humoral immune response to T-dependent antigens.

There are at least three types of cells involved in the immune response. Two of these cells are lymphocytic, morphologically indistinguishable and have been classified, on the basis of their site of differentiation, as thymus-derived (T cell) and bursa (birds) - or bone-marrow (mammals)-derived (B cell). The third cell type is phagocytic, often referred as an accessory cell and is thought to be mø (Herscowitz, 1979).

1.2 The cells involved in the humoral immune response

(a) B-lymphocytes

B-lymphocytes, the precursors of cells that synthesise antibody, are derived from the cloacal lymphoid organ, the

Bursa of Fabricius in birds (Moore and Owen, 1966). However, in mammals, B-lymphocytes are produced in a variety of major haemopoietic organs including foetal liver, foetal spleen and embryonic bone marrow (Owen, 1977). The definitive marker for B cells in both mice and humans is membrane surface immunoglobulin (sIg). All mature human and murine B cells have easily detectable sIg. IgM and IgD are the main Ig classes present in the plasma membranes of mature B cells (Moller, 1977). In addition, human and murine B cells also bear cell surface receptors for the Fc portion of IgG (Dicker, 1976) and for activated complement, C3 (Bianco and Nussenzweig, 1977) and express HLA-D and HLA-DR determinants in humans and H2, I-region (Ia) determinants in mice (Ferrone et al., 1978; McKenzie and Potter, 1979).

During the normal course of an immune response to most antigens, B cells produce specific antibody of the IgM class (the primary response) and on subsequent challenge with the same antigen, they will produce IgG class instead (the secondary response) (Roitt et al., 1981).

(b) T-lymphocytes

T lymphocytes are derived from the thymus. It is also at this site that the T cells become mature and "educated" to discriminate between "self" and "non-self" antigens (Zinkernagel et al., 1978). Those T cells which provide

"help" for B cells specific for "self" components (and thus lead to autoimmunity) will be eliminated (Howie, 1978).

Although T cells do not have readily detectable cell sIg, all murine T cell express the Thy-1 determinant (McKenzie and Potter, 1979) and all human T cells have a plasma membrane receptor for sheep erythrocyte (SRBC) and will form rosettes when incubated with SRBC under appropriate in vitro conditions (Jondal et al., 1973).

As will be discussed later, subpopulations of human and murine T cells show differential expression of cell surface receptors for the Fc portion of IgG or IgM respectively (Moretta et al., 1978). Subpopulations of murine T cells have also been defined by their differential expression of the Ly series of surface antigens coded by the major histocompatibility complex (MHC) (Cantor and Boyse, 1977; McKenzie and Potter, 1979) and the presence of specific membrane Ia-determinants (McKenzie and Potter, 1979).

(c) The functional subsets of T lymphocytes in mice

Helper T (TH) cells:

Helper T cells express $Lyl^{+}23^{-}$ surface phenotype, and generally lack I-associated (Ia) antigens. They are able to help B cells to proliferate and differentiate into antibody

secreting progeny (Miller, 1970). T cell help is most important in the IgG antibody response to low molecular weight protein antigens and to cell surface antigens. High molecular weight proteins or sugars with repeating antigenic determinants such as the LPS and bacterial flagella proteins are able to bypass the requirement for T cell help to induce antibody production directly (Coutinho and Moller, 1975). In humans, helper T cells possess an Fc receptor for IgM (Moretta et al., 1978) and react with certain monoclonal antibodies such as OK-T4 (Reinherz and Schlossman, 1980).

Also included in the T cell subset are the T cells (TD cells) which involve in delayed-type hypersensitivity. These cells bear the same general surface characteristics as the helper T cells, i.e. $Lyl^{+}23^{-}$ but they are usually Ia^{-}. Activated TD cells will secrete humoral factors (lymphokines) which are important in cell-mediated immunity (David and David, 1972; Waksman and Namby, 1976).

Cytotoxic T (Tc) cells

Tc cells express $Lyl^{-}23^{+}$ surface phenotype and are usually Ia^{+} when activated. They can directly lyse allogeneic target cells in the absence of specific antibody or complement (Cantor and Boyse, 1975). In humans, Tc cells react with monoclonal antibodies OK-T5 and 8 (Reinherz and Schlossman, 1980).

T suppressor (Ts) cells

Ts cells also express $Ly1^{-}23^{+}$ surface phenotype but bear an Ia-determinant coded by the I-J region of MHC. This antigen [Ia^{+} coded by (I-J)] is not expressed on TH, Tc or B cells and appears to be a unique marker for Ts cells (Miller, 1980).

Ts can regulate or suppress a variety of immunological reactions (Gershon, 1974; Moller, 1975). They have a function opposite that of TH cells. Ts cells can inhibit the triggering of B cells and other T cells functions - they serve to suppress the immune responses to specific antigens (Gershon, 1974).

There are also some T cells (Tf) which are responsible for feedback suppression and express both Ly1 and Ly23 antigens in addition to the surface component Qa1 coded by an MHC gene mapping between H2-D and Tla (Miller, 1980).

In humans, Ts cells bear an Fc receptor for IgG (Moretta et al., 1978) and react with antilymphocyte antibodies present in the sera of patients with active juvenile chronic polyarthritis (Strelkauskas et al., 1978 a,b) and monoclonal antibodies, OK-T5 and T8 (Strelkauskas et al., 1978 a,b).

(d) Macrophage (Mø) accessory cells

The møs are cells of the monocyte-leucocyte lineage with a generalised tissue distribution, e.g. they are present in the liver (Kuffler cells), lung (pulmonary alveolar møs), connective tissue (histiocytes), bone (osteoclasts), spleen, lymph node and bone-marrow (Nelson, 1976). The møs bear plasma membrane receptors for the Fc portion of IgG (IgG_1 and IgG_3), C3b, macrophage chemotactic factors, lymphokines such as macrophage inhibiting factor (MIF) and several hormones including insulin and calcitonin (Zuckerman and Douglas, 1979). Møs express cell surface HLA-D and HLA-DR antigens in humans and Ia determinants in the mouse (Ferrone et al., 1978).

Møs play a crucial role in antigen processing, antigen presentation and cell-cell interactions in the immune response. Most in vitro assays associated with the immune functions of T and/or B cells required the presence of møs (Roelants, 1977).

Møs are required to process antigen and present it in a highly immunologic form to B cells (Unanue, 1972), in the generation of an antibody response to T-dependent antigens and for induction of most T cell-mediated immune responses, including the in vitro generation of cytotoxic cells, in vitro production of certain lymphokines, and in vitro blastogenic

response to soluble antigens and mitogens such as PHA and ConA (Moller, 1978).

Although Møs play a crucial role in the complex series of cellular interactions leading to the generation and expression of immune responses their function is not antigen specific. Since mø is not part of the antigen-specific network of reactive lymphocytes it is regarded as an accessory cell (Lipsky and Kettman, 1982). Several members of the mononuclear phagocyte system have been shown to support mitogenic responses and function as antigen presenting cells. These include peripheral blood monocytes (Rosenberg and Lipsky, 1979), Kupffer cells of the liver (Lipsky and Rogoff, 1980), peritoneal møs (Rosenthal et al., 1976), splenic adherent cells (Cowing et al., 1978) and alveolar møs (Lipscomb et al., 1981). However, not all of these populations could express these accessory functions. Only cells which bear cell surface Ia antigens (Cowing et al., 1978; Lipsky and Rogoff, 1980) could effectively act as accessory cells (Yamashita and Shevach, 1977). More recently, other cell types which bear the Ia antigens were also found to have an accessory role in immune responses. These include the Langerhans cells of the skin (Braathen and Thorsby, 1982), dendritic cells of the lymphoid organs (Steinman et al., 1980) and also endothelial cells or fibroblasts (Lipsky and Kettman, 1982).

Mø may also modify lymphocyte reactivity through the action of secreted immunoregulatory molecules, particularly interleukin-1 (IL-1). IL-1 is a protein or a family of closely-related proteins with, as Oppenheim and Gery (1982) put it, "a multiplicity of pleomorphic amplifying effects on immunological and inflammatory reactions." It has been shown to promote lymphocyte differentiation (Rosenberg and Lipsky, 1981) and even to affect a variety of nonlymphocyte target cells (Oppenheim and Gery, 1982). IL-1 will also facilitate the production of interleukin-2 (IL-2) by presumably Ly^+ helper subpopulations (Smith et al., 1980). IL-2 is the mitogenic lymphokine that is ultimately responsible for the proliferation of T cells (Farrar et al., 1980b; Smith et al., 1980) in the presence of a specific antigen.

1.3 Cellular interactions in the humoral immune response

The induction and expression of immunity requires the effective presentation of antigen at the surface of møs or similar cells (Unanue, 1972). This is particularly true in the case of mø participation in the T cell-B cell collaboration in the production of antibodies to thymus (T)-dependent antigens.

The mechanisms by which møs enhance immune responses can be categorised into two parts. The first involves all the functions associated with antigens and antigen specificity

i.e. antigen uptake, processing and presentation. The second involves the release of antigen non-specific soluble factors (Rosenstreich, 1981).

When antigen is taken up by møs a great proportion of it is degraded by phagocytic digestion and rendered nonimmunogenic, while a small part of the antigen remains in the mø in a nondegraded, immunogenic form (Unanue and Cerottini, 1970) in some form of association with the Ia antigens of the MHC.

The uptake of antigen by mø is followed by the presentation of this antigen to the immune lymphocytes to induce their activation. This event involves the direct contact of T lymphocytes and møs and is dependent on the mø surface Ia antigens and lymphocyte receptors (Rosenstreich, 1981).

How does reactive T cell recognise mø Ia and antigen? One hypothesis is that T cells bear a dual receptor with separate recognition units for Ia and for antigens. When both receptors are complexed, T cell activation occurs. The second hypothesis is that a single T-cell receptor recognises a complex of mø Ia with antigen (Rosenstreich, 1981).

In order to achieve effective antigen presentation, the mø containing an antigen will physically interact and bind specific T lymphocytes to form mø-T cell clusters. This antigen-specific mø-T cell cluster formation is Ia dependent

(since antigen-specific clusters do not occur unless the T cells and mø̸s are Ia identical) (Ben-Sasson et al., 1977) and mutually stimulatory for both cell types (Moore et al., 1980; Powell et al., 1980).

Exposure to the appropriate combination of processed antigen and mø̸ surface Ia alone may not be sufficient to activate lymphocytes. Additional and essential signals in the form of soluble factors generated by mø̸s are required for the initiation of lymphocyte activation (Rosenstreich, 1981). Mø̸s are known to secrete several immunoregulatory molecules, one of which being interleukin-1 (IL-1) (Gery et al., 1972; Oppenheim et al., 1979). IL-1 production appears to be a characteristic of mø̸s that bear Ia antigens as well as those that do not (Lee et al., 1979) although it is not certain whether or not all mø̸s can secrete IL-1 (Lipsky and Kettman, 1982).

IL-1, originally termed Lymphocyte Activating Factor (LAF), is defined by its ability to enhance mitogen stimulated thymocyte proliferation (Gery et al., 1972). Although it is an antigen-nonspecific mediator, it serves as an essential activation signal in all T cell-dependent antigen-specific immune responses (Mizel and Ben-zu, 1980). IL-1 is able to induce the synthesis and secretion of the T cell-derived mitogenic lymphokine interleukin-2 (IL-2) which serves to amplify T cell blastogenesis (Farrar et al., 1980b; Smith et al., 1980). The

stimulation of IL-2 synthesis by IL-1 is dependent on the presence of another activating signal, either a specific antigen or a polyclonal T cell mitogen such as PHA or ConA (Mizel, 1982). Although IL-1 is required for T cell proliferation, it does not appear to be necessary for T cell recognition of the immunologic moiety presented by mø (Chu et al., 1984).

Besides having a range of lymphocyte-directed functions (Oppenheim and Gery, 1982) IL-1 also has effects on many cell functions associated with inflammatory responses. IL-1 appears to be similar if not identical to endogenous pyrogen affecting cells in the hypothalamus to cause fever via PG function (Murphy et al., 1980) and as serum amyloid (SAA)-inducer to stimulate hepatocytes to produce SAA and other acute phase proteins (McAdam and Dinarello, 1980). It also stimulates collagenase and prostaglandins production by rheumatoid synovial cells (Mizel et al., 1981) and promotes fibroblast proliferation in vitro (Schmidt et al., 1981). It may also induce degradation of matrix proteoglycan and collagen by activating chrondrocytes (Jasin and Dingle, 1981). Thus, IL-1 may permit mø to regulate, in a concerted fashion, the in vivo activities of a variety of cell types that are involved in immunological and inflammatory responses (Mizel, 1982).

As stated above, IL-2 functions to provide the stimulus (signal) necessary for T cell proliferation. Once the activating signal has been received, IL-2 responsive cells will proliferate indefinitely solely in the presence of IL-2 (Smith et al., 1979b). The acquisition of the IL-2 responsive state appears to be mediated by the appearance of IL-2-specific membrane binding sites (i.e. Tac) on appropriately activated cells (Smith et al., 1979b). The activated T cells and all the subsequent daughter cells continue to express IL-2-specific responsiveness. This unique feature of T cells has allowed the creation of continuous long-term T cell lines (Smith et al., 1979b).

T cells from all three functional T cell subsets (i.e. cytotoxic, helper and suppressor) have now been shown to be capable of proliferating in response to IL-2 (Baker et al., 1979; Schreirer et al., 1980; and MacDonald et al., 1980). However, of these three subsets, only T helper cells have been found to both produce and respond to IL-2 (Schreier, 1980).

In an antigen-induced antibody response, IL-2 will induce the proliferation of antigen-specific T helper cells by interacting with IL-2 receptor expressed on these cells (Lipkowitz et al., 1984). This interaction will spur the antigen-primed T helper lymphocytes to enter DNA synthesis and undergo clonal expansion (Oppenheim and Gery, 1982). The clonal replication of helper T cells will probably generate

certain helper factors (Tada and Hayakawa, 1980) to trigger B lymphocyte activation and antibody production.

Since IL-1 has been shown to play a role in the generation of helper T cells (Farrar and Koopman, 1979) it is quite likely that IL-1 is acting in part via induction of IL-2 to increase the frequency of helper T cells (Watson, 1979). However other studies have argued that IL-1 may also directly affect B cells by promoting the differentiation of B cells and thus increase the frequency of B cells that are capable of responding to helper T cells (Wood, 1979; Hoffman et al., 1980). Hence, it is possible that IL-1 drives the helper T cells (and perhaps the B cells) to a partially activated state that is moved to completion in the presence of IL-2 (Mizel, 1982).

The cellular interactions associated with the humoral antibody response are subject to various control mechanisms which will regulate or suppress the magnitude of the immune response and the level of antibody synthesis. These control mechanisms may be mediated by the suppressor T cells, the idiotypic network and the hyperactivated mø.

Suppressor T cells may exert negative feedback control on helper T cells (Cantor and Gershon, 1979) probably via, certain soluble antigen-specific I-J positive suppressor factors (Taniguchi and Miller, 1978). In humans, suppressor T cells have been found to depress T cell proliferation (Shou et

al., 1976); and block immunoglobulin synthesis by B cells (Gupta et al., 1979). Specific antibody, after binding to antigen, will switch off B cell activity via FC-mediated mechanisms (Taylor, 1982). These may affect the B cell directly (Kolsch et al., 1980) or interfere with the T-B cell co-operation (Hoffman, 1980). The clonal replication of antigen-specific T cells and the production of specific antibody will cause increases in the expression of certain idiotypes and will disturb the "network" leading to the suppression of the response (Roitt et al., 1981).

Mø suppression may involve several different mechanisms. Ptak et al. (1977) have demonstrated that mø membranes will block immune responses in vivo by binding the necessary growth factors required for lymphocyte activation. Activated mø may modulate immune responses via secreted prostaglandins. In vivo, exogenous prostaglandins will decrease antibody formation and in vitro they will depress antigen- and mitogen-induced lymphocyte stimulation (Rosenstreich, 1981).

Oxidative metabolites such as hydrogen peroxide, superoxide anion, and hydroxyl radical, produced by activated mø, may have immunoregulatory consequences. This is based on the finding that reduced glutathione (GSH) will remove the continually generated deleterious oxygen intermediates that are harmful to the survival of lymphocytes in vitro (Hoffeld and Oppenheim, 1980). Indeed, hydrogen peroxide, for

instance, has been found to suppress lymphocyte activity (Hoffeld et al., 1981) including the suppressor cells (Zoschke et al., 1982), but may, in other assay systems, be found necessary for lymphocyte activation (Novogrodsky et al., 1982).

Hence, cellular interactions are necessary for the activation and regulation of clonal expansion of an immune response. The immune system appears to be constantly adjusting through negative and positive feedback mechanisms to 'self' and 'non-self' antigens. Increasing appreciation of the complexity of immunoregulation has brought with it the awareness of the potential defects in this system.

Part (II) RHEUMATOID ARTHRITIS AND THE EXPERIMENTAL MODELS OF RHEUMATOID ARTHRITIS

(A) The aetiology and immunopathogenesis of rheumatoid arthritis

1.1 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is "a subacute or chronic, non-suppurative, inflammatory polyarthritis affecting mainly the peripheral synovial joints, normally in a symmetrical fashion, running a prolonged course of exacerbation and remission, and accompanied by signs of systemic disturbance such as anaemia, weight loss, and a raised erythrocyte sedimentation rate" (Duthie, 1969). Articular involvement is manifested clinically by pain, stiffness, limitation of motion, and signs of inflammation, i.e., swelling, heat, erythema and tenderness. Extraarticular features include rheumatoid nodules, arteritis, neuropathy, scleritis, pericarditis, lymphadenopathy and splenomegaly (Zvaifler, 1979a).

1.2 Aetiology

The aetiology of RA is unknown. Indeed, it is not clear whether RA is one disease with multiple aetiologies or a symptom complex produced by a single causative factor (Zvaifler, 1979a). To date, however, attention has been

focused on two hypotheses, the role of microbial infection and the genetic predisposition to the disease.

The occurrence of polyarthritis during many human bacterial and viral diseases and the resemblance of RA to certain illnesses in animals caused by microorganisms make infection an attractive hypothesis (Pearson, 1979). However, despite intensive study, evidence implicating a specific infective agent in RA is still lacking. For example, direct approaches to the problem involving viral identification by electron microscopy, attempts to isolate viruses and microbes by culture of synovial fluid and membrane, or a combination of both (Denman, 1975; Dourmashkin et al., 1976) have met with little success. Attempts to rescue viruses by co-cultivation or DNA or RNA hybridization have also failed (Zvaifler, 1979a). Recently, there has been increasing interest in the possible role of Epstein-Barr virus (EBV), a herpes virus, based on the high prevalence in RA sera of circulating antibodies to a nuclear antigen (RANA) present in EBV-transformed Wil2 human lymphoblastoid cell lines (Alspaugh et al., 1978). Whether this signifies a primary aetiological role for EBV infection in RA or a phenomenon secondary to the disease may be decided by investigation of patients with very early disease (Roitt, 1980).

The interest in the role of genetic factors in the aetiopathogenesis of rheumatic diseases is generated through

the work of Brewerton et al., (1973), who demonstrated a strong association between ankylosing spondylitis and the presence of histocompatibility antigen, HLA-B27. A relatively high risk of predisposition to RA has been found in HLA-DR4+ compared to HLA-DR4- individuals (Panayi et al., 1976; Stastny, 1978). However, Jones et al. (1983) suggested that HLA-DR4 is a marker of disease severity in RA rather than of susceptibility to arthritis.

Hence, it appears that RA may be due to the exposure of individuals with a particular genetic constitution to an infectious agent. The illness may result from an inappropriate immune response to a ubiquitous agent or conversely, the individual who develops RA may be less able to withstand infection by an uncommon pathogen (Zvaifler, 1979a).

1.3 The immunopathogenesis of RA

Although the underlying cause of RA remains obscure it is accepted that the debilitating damage that occurs in joints is directly connected with a local chronic immune associated inflammatory reaction (Zvaifler, 1973). An increasing number of observations suggest that both cellular and humoral immunological events mediate its pathogenesis. The presence in the synovial membrane of plasma cells, local production of immunoglobulins, the demonstration in the tissues and the circulation of rheumatoid factor and immune complexes together

with the associated complement consumption in synovial fluid imply a role for humoral immune mechanisms (Smiley et al., 1968; Zubler et al., 1976; Zvaifler, 1979a). In addition, the detection of lymphokines in rheumatoid synovial fluid (Stastny et al., 1975) and the high proportion of T lymphocytes in the synovial tissue (Duke et al., 1981) assert the importance of a hyperactive cell-mediated immune response. Also, the clinical observations of the therapeutic efficacy of cytotoxic (immunosuppressive) drugs in RA and lymphocyte depletion by thoracic duct drainage also support an immune pathogenesis of this disease (Pearson et al., 1979; Paulus et al., 1977).

Defects in the function of lymphocyte subpopulations may contribute to the pathogenesis of RA in any one of a few pathways involving the helper or suppressor role of T lymphocytes, leading to an inadequate response or to uncontrolled hyperresponsiveness respectively (Slavin and Strober, 1981). Indeed, cellular abnormalities in RA were shown to be predominantly within the T cell subpopulation (Bankhurst et al., 1976) as the lymphocytes extracted from the synovial fluids and synovial tissues of RA patients had been found to be predominantly of the T cell lineage (van Boxel and Paget, 1975; Abrahamsen et al., 1975). A mild degree of impaired T cell function in RA has been reported by many studies (reviewed by Hall and Bacon, 1981).

Lymphocyte activation by PHA and ConA, but not PWM, was found to be depressed in a subgroup of RA patients with erosive joint disease (Silverman et al., 1976). This suggests deficiency in T cell function. Chattapadhyay et al., (1979) demonstrated the lack of suppressor cell activity from synovial tissue lymphocytes. Abdou et al. (1981) also demonstrated the suppressor T cell dysfunction and the presence of anti-suppressor cell antibody in the peripheral blood of active early RA patients but not in inactive RA and late active RA patients. The association of RA and Juvenile Rheumatoid arthritis (JRA) with features consistent with a disturbance of immunoregulation such as hypergammaglobulinaemia (Zvaifler, 1972) may also suggest suppressor T cell hypofunction as common variable hypogammaglobulinaemia is often associated with increased suppressor cell function (Waldman et al., 1974).

It has been demonstrated that rheumatoid synovial tissue and fluid contain activated T lymphocytes (Galili et al., 1981, Burmester et al., 1981; Janossy et al., 1981). HLA-DR or Ia antigen (from Ia-bearing mø's or dendritic cells) appears to be the stimulus recognised by the T cells which subsequently becomes activated (Lattime et al., 1980). Surface marker studies of lymphocytes clustered around these HLA-DR+ mø-like (dendritic) cells in rheumatoid synovium indicate a preponderance of "helper" phenotype cells and a lack of "suppressor" cells (Duke et al., 1982; Klareskog et al.,

1982). This has led to a hypothesis that the pathogenesis of RA is the result of an imbalance of T lymphocyte/mø immunoregulatory mechanisms (Janossy et al., 1981).

Several lymphokines, including IL-2 macrophage migration inhibitory factor and mitogenic factor, have been detected in the fluid of inflamed joints from RA patients (Stastny et al., 1975; Crout et al., 1976; Wilkins et al., 1983). Since lymphokines are known to induce the release of IL-1 from møs (Meltzer et al., 1977; Oppenheim et al., 1980) and since the synovium normally contains cells of the mononuclear phagocytic lineage including infiltrating monocytes (Zvaifler, 1973) it seems likely that IL-1 might also be present in the synovium. Indeed, IL-1 like factor has been isolated from most inflamed joint fluids including RA (Wood et al., 1983). If this IL-1 like factor is indeed IL-1 then it might contribute to joint destruction. Potentially, IL-1 could stimulate the secretion of collagenase and prostaglandins (PG) from synoviocytes (Mizel et al., 1981). Collagenase would degrade the native collagen of the cartilage while PG would promote bone resorption and immediate events of inflammation (Wood, 1983).

Other alternative mechanisms that may contribute to the pathogenesis of RA include the immune response to collagen and the role of Epstein-Barr virus (EBV). The demonstration of antibodies directed to collagens in sera and synovial membranes of RA patients (Mestecky and Miller, 1975;

Adriopoulos et al., 1976) suggested that collagen, acting as an autoantigen, may be involved in the initiation and/or perpetuation of a chronic inflammatory process.

Collagen-anticollagen immune complexes have been found in synovial fluid cells, presumably after phagocytosis (Steffen et al., 1974). Menzel et al. (1976) also reported the presence of such complexes and antibodies to denaturised human collagen in the synovial fluids of some patients of RA. A study of rheumatoid synovial membrane using tetramethylrhodamine isothiocynate-labelled collagens revealed prominent staining of plasma cells only with Type II collagen, indicating the production of antibodies to cartilage collagen (Mestecky and Miller, 1975).

It is still not clear whether an immune response to collagen is a primary event in the inflammatory process or whether the formation of anti-type II collagen antibodies is secondary to the pathological collagen degradation and exposure of antigen site on collagen peptides (Wager, 1976). It may be that the production of antibodies against collagen molecules with subsequent formation and phagocytosis of collagen-anticollagen immune complexes could mediate the pathological mechanisms of RA (Wager, 1976). Collagen-induced arthritis in rats will also be discussed later.

Normal peripheral blood mononuclear cells when infected by EBV develop into lymphoblastoid cell lines which contain a "RA associated" nuclear antigen (RANA).

As mentioned earlier, most patients with RA have precipitating antibodies against this antigen (Alspaugh et al., 1978). It has been observed that RA lymphocytes form lymphoblast cell lines more readily than normal lymphocytes (Slaughter et al., 1978). Also, Depper et al. (1979) found that rheumatoid peripheral blood mononuclear cells developed B lymphoblastoid colonies in a much shorter median time than normal peripheral blood mononuclear cells. This may be due to a deficiency of suppressor T cells capable of regulating or suppressing lymphoblastoid cell line formation in RA.

B. The Experimental Models of Rheumatoid Arthritis

Arthritis occurring in animals are mostly inherited dysplasias or infectious or degenerative diseases. Many are analogous to human arthritis. There are no ideal animal models for rheumatoid arthritis and most of the other rheumatoid diseases of obscure aetiology (Pearson, 1979). The lack of suitable animal models has hindered the search and the devising of good screening methods for truly effective anti-rheumatic drugs. Hence, one must use inadequate models to select usually inadequate drugs (Swingle, 1974). However, not entirely unsuccessful attempts have been made to devise animal models

for the various rheumatoid diseases. In this part of the thesis only the experimental models of rheumatoid arthritis are discussed. An animal model may be considered to have two purposes:

(1) To verify or test the reasonableness of a theory concerning the aetiology of the disease under consideration.
(2) To allow manipulation of various factors which may exacerbate or ameliorate the pathological dysfunction under investigation (Whitehouse et al., 1974).

1.1 Adjuvant Arthritis

Adjuvant arthritis (AA) has characteristically been considered to be a unique response of the rat to the appropriate combination of oil, heat-killed mycobacteria, and route of injection. This experimental disease has perhaps been the most extensively studied of all of the models of arthritis.

AA was first described by Stoerk et al. (1954) who observed polyarthritis in rats injected with a mixture of spleen homogenates and adjuvants. Later Pearson (1956) showed that complete Freund's adjuvant (CFA) was the one agent needed to induce this disease. AA can be induced only in rats. Species other than the rat are resistant to this disease and different strains of rats vary considerably in their susceptibility

(Swingle, 1974). AA is commonly produced by a single injection of CFA containing mycobacteria into either the tail or the paw.

1.2 Method of induction

(a) Preparation of the adjuvant

Most commonly, heat-killed mycobacteria (e.g. M. butyricum, M. tuberculosis, or M. phei) are either suspended or emulsified in mineral oil or paraffin oil such that 0.2 - 1.0 mg. of the mycobacteria are contained in the injection volume (usually 0.05 or 0.10 ml.) (Swingle, 1974). As high as 95 - 100% successful induction of the disease is attainable in most strains of rats if adjuvant is properly prepared, i.e. by grinding the mycobacteria in a mortar and pestle before preparing the suspension (Swingle, 1974).

Some other bacteria, bacterial cell walls or bacterial peptidoglycans can substitute for mycobacteria. Bacterial sources include Nocardia, Streptomyces, Staphylococcus, Streptococcus and Corynebacterium (Paronetto, 1970; Koga et al., 1973; Koga et al., 1976; Kohasi et al., 1977). The essential component appears to be a polymer of two or more disaccharides containing N-acetylglucosamine and N-acetyl muramic acid with a basic peptide unit (Kohasi et al., 1976b; Koga et al., 1979).

Recently, Chang et al. (1980) observed that a synthetic, apparently non-immunogenic, low molecular weight compound, n,n-dioctadecyl-N^1,N^1-bis(2-hydroxyethyl) propanediamine (CP-20961) can effectively substitute for peptidoglycan in Freund's adjuvant to induce AA. CP-20961, an interferon-inducer with antiviral activity (Hoffmann et al., 1973) was also shown to have adjuvanticity by enhancing the development of cell-mediated and humoral immune responses to the tumour cell line, EL4 in rats (Chang et al., 1980).

(b) Site and route of injection of the adjuvant

The intradermal route of injection is commonly used and is most effective when given in the foot pad or tail (Ward and Jones, 1962; Glenn and Gray, 1965). The volume and concentration of injected material are important. At least 0.1 mg. whole mycobacteria in a volume of 0.05 - 0.1 ml. is necessary to induce AA by foot pad or tail administration (Glenn and Gray, 1965).

(c) Experimental animals

Various strains of rats differ in their response to CFA (Glenn and Gray, 1965; Swingle et al., 1969). Lewis rats, when immunised with conventional CFA, were highly susceptible to AA, while Fisher and Buffalo rats were less susceptible (Swingle et al., 1969).

The resistance to the disease in certain strains of rats is genetically dominant (Perlik and Zidek, 1973) and may prove to have an anatomical rather than a physiological basis. Whitehouse et al. (1973) have suggested that the apparent resistance of the Buffalo strain of rat is caused by the relatively poor lymphatic drainage of the site of injection of the adjuvant.

Both male and female rats develop AA (Swingle, 1974). However, AA is influenced by the age of the rat. Glenn and Gray (1965) found both young (< 21 days) and old (> 9 months) rats to be relatively resistant to the disease.

1.3 Clinical features and histopathology of adjuvant arthritis

Local injection of CFA results in an intense, transient, inflammatory response. Unlike RA, AA has a clear-cut starting point (the time of injection of CFA), followed by a well-defined latent period of 10 - 16 days (Pearson and Chang, 1977).

Usually, after about twelve days post-injection, arthritis and periarthritis suddenly appear in the extremities especially in the distal small joints of the paws. These are characterised by redness and swelling of varying severity and painfulness. The arthritis is often polyarticular and sometimes migratory

(Pearson, 1979). The arthritis persists and intensifies during the 7-14 days after onset, then recedes over the ensuing weeks to months (Glenn and Gray, 1965). Erythematous nodules also develop over the tail, the external ears and the tip of the nose. An acute balanitis and urethritis is seen in some male rats. Occasionally, granulomas appear in the liver, spleen and lungs. Death rarely occurs from the disease except in the most severely affected animals (Pearson, 1979).

Histologically, the predominant early lesion is oedema and sparse mononuclear cell infiltrate. Within 2 or 3 days, intense synovitis with focal and diffuse accumulation of lymphocytes and mononuclear cells develops. A fibroblastic response leads to vascular granulation tissue involving bone. Pannus-like formations occur, and, in an intense osteoblastic reaction with periosteal new bone formation, results in ankylosis of joints (Pearson, 1979). Low grade synovitis and periarthritis with lymphocytic cellular infiltrate may persist for months (Burstein and Waksman, 1964). The ear lesions, balanitis and periarthritis all appear to be associated with perivasculitis (Pearson, 1979).

Considering the specie differences there are some remarkable similarities between AA and RA and these are summarised in Table X.

Clinical	AA	RA
Acute and recurrent arthritis Peripheral joints	++	++
Chronic deforming arthritis	++	++
Eye lesions	+	+
Progressive and destructive joint disease	++	++
Pathological		
Acute and subacute synovitis	++	++
Primarily mononuclear cell response	++	++
Invasion of bone and joint space by pannus	++	++
Bursitis and tendinitis	++	++
Ankylosis		
Fibrous	++	+
Bony	+	+

+ = rarely

++ = commonly

Table X Comparison of AA and RA (from Pearson, 1979)

Other laboratory findings of AA which resemble RA include anaemia, decreased serum iron, decreased rate of incorporation of iron into red cells (Mikolajew et al., 1969), and elevation of erythrocyte sedimentation rate, serum glycoproteins and copper (Gralla and Wiseman, 1968; Weimer et al., 1968). Rheumatoid factor and antinuclear antibodies, detected in RA, however, have not been reported in AA. Depressed levels of hepatic aminopyrine demethylase and of cytochrome P450, observed in RA, have also been reported in AA (Cawthorne et al., 1976) and may be associated with altered drug metabolism by the liver.

1.4 The Immunopathogenesis of adjuvant arthritis

Although the aetiology and pathogenesis of AA have not been established, the disease is believed to be due to an immunological process, possibly involving a delayed hypersensitivity reaction to disseminated antigenic constituents of the mycobacteria (Waksman et al., 1960) and/or an autoimmune reaction to homologous cell-bound antigen (Ryzewsky, 1967). The conclusion drawn from the early immunological studies that AA is primarily a disease of cell-mediated immunity (Waksman et al., 1960) was subsequently strengthened by numerous pharmacological studies showing that immunosuppressive drugs can prevent the development of adjuvant disease (Billingham and Davis, 1979). The failure to demonstrate either passive transfer of AA with serum (Waksman

et al., 1960) or rheumatoid-factor production in AA rats (Katz and Piliero, 1969; Billingham and Davis, 1979) provided further evidence that in AA, unlike RA, antibody-mediated pathogenic mechanisms appear to play little or no role. Finally, passive transfer of the disease with lymph node (Waksman and Wennersten, 1963), spleen (Pearson and Wood, 1964), or thoracic duct (Whitehouse et al., 1969) cells has been widely accepted as fairly conclusive for cell-mediated immune mechanisms as the key to pathogenesis of the disease.

There is also evidence that a certain T-cell subpopulation is involved in regulating the disease. Kayashima et al. (1976, 1978) reported that procedures which remove suppressor cells, i.e., low doses of cyclophosphamide, low doses of whole body irradiation, and adult thymectomy, enhance the arthritic process of AA when given before the administration of CFA. Furthermore, normal syngeneic thymocytes could abolish the enhancing effect of both low doses of cyclophosphamide and adult thymectomy. Wells and Battisto (1983) have demonstrated that AA can be suppressed by pre-treatment with several forms of altered syngeneic cells and suppression of AA is accompanied by a specific alteration in cellular immunity as shown by a decrease in the proliferative response to M. butyricum but not to a non-specific mitogen like ConA. They suggested that the decrease in proliferative and arthritogenic responses was attributable to induced suppressor cells.

Studies on lymphocyte functions during AA by determining their ability to respond to various mitogens *in vitro* have suggested the involvement of T suppressor cells in AA. For example, the diminished responses of splenic lymphocytes to PHA and ConA in active AA are due to the presence of suppressor cells (Kourounakis and Kapusta, 1974).

Spleen cells from rat with active AA can also suppress the mitogenic responses of normal splenic lymphocytes implicating the existence of suppressor cells in AA (Kourounakis and Kapusta, 1974).

Gertzbein et al. (1979) also demonstrated a depressed response of adjuvant lymph node lymphocytes to PHA which the authors speculated to be due to inhibition by the suppressor factor released by a major subpopulation of pre-selected T suppressor cells by the arthritic process.

Piatier-Tonneau et al. (1982) found that there is a strain-dependent susceptibility to the induction of AA which is correlated with differences in T suppressor cells regulation. They showed that in the resistant Wistar A-G (WAG) rats, the *in vitro* response to the highly susceptible Lewis (LEW) rats' alloantigens was markedly inhibited 11 days after CFA injection, while LEW rats *in vitro* response to WAG alloantigens was increased slightly. Furthermore, WAG rats spleen cells 4 days post-inoculation exhibited T cell-mediated

active suppression of the WAG *in vitro* response to LEW alloantigens when they were co-cultured with WAG normal spleen cells. This suppression was abolished by removal of T cells on nylon wool column or by irradiation of T cells. In contrast, T cells from CFA-treated LEW rats did not produce any effect on LEW *in vitro* response to WAG alloantigens. This suggests that the severe arthritis induced in LEW rats could be associated with defective suppressor cell functions, while in WAG rats CFA activated suppressor T cells could control the disease (Piatier-Tonneau et al., 1982).

Study by Tsukano et al. (1983) revealed that unresponsiveness to arthritogenic dose of CFA could be adoptively transferred by draining lymph node cells from rats rendered unresponsive by pre-treatment with subarthritogenic dose of the same adjuvant. These lymph node cells were probably T suppressor cells. These workers found that rats primed with subarthritic dose of CFA could elicit severe arthritis after reinoculation with non-mycobacterial synthetic adjuvant, suggesting that low dose induced unresponsiveness is antigen specific. These workers also suggested that the majority of the suppressor cells involved in the pathogenesis of AA in the low responder strain are non-specific naturally existing T cells (Kayashima et al., 1976, 1978), whereas those in high responder strain are antigen specific T cells.

Binderup (1983a) showed that rats with AA have a decreased ConA-induced T suppressor cell activity in spleen cell suspensions 2 to 4 weeks after the induction of the disease. The development of defective T suppressor cell activity was positively correlated with the severity of the arthritic lesions. A marked impairment of T suppressor cell activity was observed in rats receiving a second booster injection of CFA, a treatment which may contribute to severe systemic changes of the disease. These results suggest that the rats with AA may fail to generate a normal level of T suppressor cell function on antigen stimulation and that this defect may give rise to the development and chronicity of the polyarthritic lesions (Binderup, 1983a). Several mechanisms were suggested by the same author to explain the decreased ConA-induced T suppressor cell activity in AA. Precursor T cells may be resistant to induction or decreased in relative number in the spleen cell suspensions. Other cell types (e.g. mø) may interfere with the normal induction of T-suppressor cells.

The function of møs was found to be altered during the course of AA (Binderup et al., 1980a,b). Suppressive møs were found in the spleen and lymph nodes shortly after the induction of disease; these cells could inhibit the lymphocyte response to both T- and B-cell mitogens. Mø depletion with carbonyl iron particles prior to induction of disease resulted in restoration of suppressor cell activity to normal.

Furthermore, lymphocytes from arthritic rats may release a soluble factor(s) that can activate macrophages to exert an inhibitory effect on the generation of T-suppressor cells, probably through the release of prostaglandins and/or other macrophage-derived mediators (Binderup, 1983b). Alternatively, the macrophages may exert a direct inhibitory effect on T precursor cells via cell-to-cell contact or via the release of soluble mediators (Binderup, 1983b).

Hence, the pathogenesis of AA seems to be not simply a cell-mediated response but rather the result of complex interaction of cell-mediated and humoral responses (MacKenzie et al., 1978), more than one type of suppressor cells may be involved in the regulation of AA.

There is also accumulating evidence for a role of viruses in the pathogenesis of AA. Interferon (an anti-viral agent) inducing agents Statalon (Kapusta and Mendenson, 1967) pyran copolymer (Kapusta and Mendenson, 1969) and tilorone (Chang and Hoffman, 1977) can inhibit AA. Virazole, an anti-viral agent which does not induce synthesis of interferon, will also inhibit AA and reverse the appearance of suppressor cells (Kapusta et al., 1979).

However, the workers observed that Poly I:C (an interferon-inducer) has the ability to promote AA with MDP (Nagao and Tanaka, 1980) and the parainfluenza sendai virus which has

been reported to suppress AA by prior infection but does not influence AA if the virus was given at or after the time of adjuvant injection (Garlington and Van Housier, 1978).

Hence, it may be that interferon, a known suppressor of both humoral and cell-mediated responses (Baron et al., 1979) exerts its effect through actions on lymphocytes and macrophages rather than via an antiviral action. Before more convincing evidence is gathered the viral aetiology of AA must remain speculative.

1.5 Antigen-induced Arthritis in rabbits

A chronic synovitis, closely resembles human RA with regard to histopathology and chronicity, can be produced in rabbits by an initial systemic immunisation followed by injection of the same antigen into a knee joint (Cooke and Jasin, 1972). This method of induction of arthritis is based on the earlier observations of Dumonde and Glynn (1962) on fibrin-induced arthritis. The ensuing antigen-induced arthritis (AIA) affecting the knee required the presence of ~~CFA~~ antigen in the initial immunisation, the development of delayed hypersensitivity, and high titre of circulating antibodies to the inducing antigen (Cosden et al., 1971). Ovalbumin (OVA) (Consden et al., 1971) and bovine serum albumin (BSA) (Cooke et al., 1972) have been the most extensively used antigens in this model of chronic inflammation. Besides rabbits, the disease can be induced in

guinea-pigs (Dumonde et al., 1977), mice (Brackertz et al., 1977a), and chickens (Oates et al., 1973).

Blackham et al. (1974) have reported that there is a genetically-related, strain-dependent, susceptibility to AIA among rabbits with joint swelling and histological lesions appearing in decreasing severity in Old English, New Zealand White, and Dutch rabbits. The AIA is chronic as judged by the presence of villous hyperplasia of the synovial membrane, pannus, lymphocyte and plasma cell infiltration and bone and cartilage erosions (Hunneyball et al., 1978). This immune synovitis can be significantly changed by altering the schedules for intraarticular challenge (Goldlust et al., 1978).

The chronicity of the AIA might be explained by retention of antigen in the synovium. Glynn (1975) reported that some animals immunised and challenged with a single intraarticular injection still had active disease up to two years later. BSA or OVA used as the inducing antigen was specifically retained in the affected joints up to six times greater than that in control joints (Pearson, 1979). The injected antigen which subsequently binds to antibodies present in the synovial fluid produces immune complexes that become deposited on the articular tissues (predominantly articular cartilage, menisci, and ligaments) will result in the development of a type III hypersensitivity reaction to the antigen, after which T

lymphocytes enter the joint, producing a type IV (delayed) hypersensitivity reaction. The chronicity of the arthritic response is dependent on the cell-mediated immune response to the antigen. Animals having circulating antibodies but no cell-mediated responsiveness produce only a transient inflammatory response lasting 30 days (Glynn, 1968). The persistence of AIA following a single articular injection has yet to be explained fully. The elimination of antigen from the joints of immunised rabbits is relatively slow and retained antigen may be responsible for persistence on the arthritis for up to 6 months (Consden et al., 1971). Hollister and Mannik (1974) found retention half-lives of antigen of 17 days for ligaments, 31 days for articular cartilage, and 141 days for meniscus, which suggests that small amounts of antigen may persist for longer periods of time, but this would still not explain persistence beyond 12 months. The most logical explanation comes from Glynn (1972), who suggested that the AIA pursues a biphasic course, with the first phase representing a hypersensitivity reaction to the antigen and the second phase representing a true autoimmune response. The demonstration of spleen cell responsiveness to collagen and proteoglycans in rabbits with chronic arthritis induced by intraarticular injection of Mycobacterium butyricum in preimmunised animals (Champion and Poole, 1980) lends support to this hypothesis.

AIA in rabbits provides a model of arthritis that not only closely resembles RA at both macroscopic and histological level, its pattern of responsiveness to antirheumatic drugs is also very similar to that of RA. Hunneyball et al. (1979) have shown that prolonged daily oral treatment of rabbits with D-penicillamine (15-30 mg/Kg) beginning 16 weeks after the onset of chronic monoarticular arthritis, reduced the severity of chronic synovitis in a considerable proportion of animals. This was reflected in a reduction in joint circumference began 40 to 50 days after initiation of treatment and this parallels the delay observed in rheumatoid patients between the initiation of treatment and onset of effect. When D-penicillamine treatment was initiated prior to immunisation, a greater reduction in joint swelling, histopathology, and macroscopic score was obtained (Hunneyball et al., 1979). The reduction in synovitis may be attributable to a suppression of the cell-mediated immune response to the antigen (Goldberg et al., 1974), or possibly to a stimulation of the phagocytic activity of macrophages (Hunneyball et al., 1979).

The limitations of AIA in rabbit with regard to its use for pharmalogical screening are mainly due to the size of the animal and the duration of treatment required to demonstrate activity with disease-modifying compounds, particularly those of the slow-acting type such as D-penicillamine. The development of a comparable model of arthritis in the mouse should minimise these problems, provided that it can be

demonstrated that this model behaves in a similar manner to the clinical disease with regard to drug actions. However, both mouse and rabbit models suffer from the difficulty in continual assessment of the progress of the disease activity and, consequently, the actions of antirheumatic drugs (Hunneyball, 1984). The utility of these models for screening antiarthritic compounds will depend on the development of better methods of monitoring the disease process.

1.6 Collagen-induced Arthritis in rats

Immunological hypersensitivity to collagen may explain both the systemic nature and chronicity of inflammation in RA (Trentham et al., 1977). The demonstrations of antibodies to collagen in sera of patients with RA (Mestecky and Miller, 1975; Adriopoulos et al., 1976) support this premise. For this reason, an experimental model of autoimmunity to collagen, i.e. collagen-induced arthritis (CIA) was studied. This inflammatory polyarthritis can be induced in rats by intradermal immunisation with native type II collagen in Freund's Adjuvant (complete or incomplete). The onset of arthritis normally occurs at 14 - 16 days postimmunisation with a peak onset at 20 days (Trentham et al., 1977). These authors showed that the incidence and severity of arthritis increased as the amount of collagen injected was increased. The clinical features of CIA were similar whether heterologous or homologous type II collagen was the immunogen or whether

CFA or ICFA was used (Trentham et al., 1977).

The CIA in rats shares many similar features of human RA and AA in rats. Histopathologically, the primary lesion provoked by type II collagen is a chronic proliferative synovitis with intense mononuclear cell infiltrate of the synovial lining leading to erosion of the articular cartilage and subchondral bone. The invasion of mononuclear cells and their persistence in the synovium may suggest the importance of immune responses in the pathogenesis of this model (Trentham et al., 1977). Indeed, cellular and humoral immune responses to native type II collagen were found to be present in the rats after immunisation (Trentham et al., 1978; Stuart et al, 1979; Morgan et al., 1980). The arthritis can be transferred to native rats by cells (Trentham et al., 1978) or by antibody (gamma globulin fraction of serum taken from rats with CIA) (Stuart et al., 1982). Complement also appears to play a role in the initiation of this arthritis (Morgan et al., 1981).

The immune response towards type II collagen is under MHC (Ir-gene) control (Wolley et al., 1981) and only responder type animals appear to be susceptible to arthritis (Wolley et al., 1981; Griffiths et al., 1981).

The development of CIA is dependent on functional T lymphocytes as the arthritis cannot be induced in nude rats (Klareskog et al., 1983). Using antibodies specific for Ia

antigens and different T cell subsets in the rats, Klareskog et al. (1983) demonstrated immunohistochemically that T cells, predominantly of "helper" type and anti-Ia-reactive non-T cells were abundant in the arthritic synovial tissue.

Firth et al. (1984) demonstrated that arthritic rats have higher levels of IgG1, IgG2a and IgG2b subclass antibodies to native bovine type II collagen than do non-arthritic rats although no IgG2c subclass antibodies could be detected in immunised animals. All four subclasses will activate the classical complement pathway and IgG2b will also activate the alternate pathway (Medgyesi et al.,1981). As a role for complement in CIA has been suggested by Morgan et al. (1981), these subclass antibodies may contribute to the pathogenesis of CIA via complement fixation. Also, the IgG2a subclass of serum antibody is known to participate in macrophage binding (Medgyeshi et al., 1981) and to mediate the cytotoxic effect of eosinophils (Capron et al., 1978). Hence, IgG2a, like IgE, when bound to antigen, will cause mast cell degranulation. In fact, the greatest difference between arthritic and non-arthritic rats was observed in the IgG2a subclass. Therefore, these cellular responses mediated by IgG2a may also contribute to the production of CIA (Firth et al., 1984). In addition, William et al. (1983) found that all rats which developed CIA had serum antibodies to native type II collagen, but not all responded to tests for cell-mediated immunity. These findings suggest that antibodies to collagen are more associated with

the development of arthritis than is the cell-mediated immune response to collagen.

There are several reasons to believe that at least some of the pathogenic mechanisms that cause CIA are involved in human RA. This relationship has recently been reviewed (Stuart et al., 1982; Trentham, 1982).

The most important similarity between CIA and RA is their mutual association with collagen immunity. Several workers have identified both cellular and humoral reaction to collagen in RA (Adriopoulos et al., 1976; Trentham et al., 1978; Stuart et al., 1980). This reactivity is linked to the MHC locus, specifically HLA-DR4 (Sollinger et al., 1981). However, one must interpret this data with caution since mouse strains that respond to collagen immunisation with high levels of collagen immunity but do not develop arthritis have been identified (Wolley et al., 1981). Another similarity between CIA and RA is the proliferative synovitis, marginal lesions, and cartilage destruction that characterises both diseases (Caulfield et al., 1982). However, there are differences between the animal model and RA. In CIA, periostitis is a prominent lesion that is not usually seen in RA. In RA, there are vasculitis changes in the synovial tissue that are not seen in CIA. The relevance of CIA as a model for predicting the effects of drugs effective in RA was determined by Sloboda et al. (1981) and Phadke et al. (1982) also found some

correlations between the effects of nonsteroidal and steroidal anti-inflammatory drugs in CIA as compared to RA. D-penicillamine also has a beneficial effect that is reported to be similar to its effect in RA (Kerwar et al., 1981). Other antirheumatic drugs such as gold compounds (McCune et al., 1980) and levamisole (Stuart et al., 1981) have no or minimal effect in CIA.

The CIA is a versatile system with several advantages over other animal models of arthritis. The use of a purified and biochemically characterised antigen, as well as the ability to induce disease in a reproducible manner, appears to suggest that further studies of CIA in rats may be helpful and fruitful.

(C) Chemotherapy in Rheumatoid Arthritis

1.1 Anti-inflammatory drugs

The anti-inflammatory agents in current use may be divided into non-steroidal anti-inflammatory drugs (NSAID) and steroids, and these will be discussed separately.

(a) Non-steroidal anti-inflammatory drugs (NSAID)

The NSAID can relieve pain and stiffness, reduce swelling and improve joint function, but they do little to reverse the

underlying disease processes (Hart et al., 1982). NSAID such as aspirin, indomethacin, phenylalkanoid acids and others were first shown to inhibit prostaglandin (PG) synthesis by Vane (1971) and Smith and Willis (1971). Subsequent work from many laboratories has established that there is good correlation between the anti-inflammatory effects of nearly all classes of these drugs and their potency as PG synthesis inhibitors. The NSAID inhibit PG synthesis by a direct inhibition of the PG cyclooxygenase. All NSAID exhibit analgesic, anti-pyretic and anti-inflammatory actions as well as the characteristic gastrointestinal side effects. These toxic effects may be related to their common mechanisms of action. For example, PGs have been shown to inhibit gastric secretion in animals and man (Dilawari et al., 1973) and may have a protective effect on the gastric mucosa (Rainsford, 1982). Inhibition of these putative protective functions of PGs by the NSAID may explain some gastrointestinal toxicity. However, while clinical experience suggests, in general, that the NSAID are equally effective in patients, their potencies as cyclooxygenase inhibitors vary widely (Crook et al., 1976). Sodium salicylate, a less effective PG synthetase inhibitor, is clinically indistinguishable from aspirin. Also, cyclooxygenase activity in rheumatoid synovial tissue may be abolished in patients taking low doses of aspirin (600 mg./day) which do not exert detectable anti-inflammatory effects. Aspirin irreversibly inhibits PGE_2 synthesis of cultured human synovium, while other NSAID act reversibly

(Crook et al., 1976). Thus it seems that although NSAID undoubtedly inhibit PG synthesis, this is not their sole mechanism of action.

Various NSAID have also been found to inhibit lysosomal enzyme secretion (Robinson, 1978; Smith and Iden, 1980) and superoxide anion production (Simchowitz et al., 1979; Smolen and Weissmann, 1980; Edelson et al., 1982) by human polymorphonuclear cells and guinea-pig mø (Oyanagui, 1978). However, most of the above studies employed drug concentrations well in excess of physiological levels. More recently, NSAID have been shown to be sensitive inhibitors of chemiluminescence generated by H_2O_2-myeloperoxidase-Cl^- (Pekoe et al., 1982), the major cytotoxic system of neutrophils. Thus, it seems possible that part of the anti-inflammatory effect of NSAID is due to interference with the oxidative metabolism of neutrophils.

(b) Corticosteroids

Corticosteroids have numerous effects that probably contribute to their overall anti-inflammatory action. The drugs suppress the production and release of PGs (Lewis and Piper, 1975) by inhibiting phospholipase A_2 and thus preventing the formation of arachidonic acid from membrane phospholipid. Phospholipase A_2 inhibition is mediated by a protein, lipomodulin, the synthesis of which is induced by glucocorticoids (Hirata et

al., 1980). In addition to the inhibition of PG synthesis, lipomodulin production has a number of anti-inflammatory consequences. Suppression of phospholipase A_2 activity also prevents the formation of lipoxygenase products such as Leukotriene B_4 (LTB4) and interferes with the generation of Platelet Aggregating Factor (PAF). These effects on the production of the inflammatory mediators may help to explain the potency of steroids compared to NSAID. Corticosteroids also have effects on phagocytes especially monocytes. Steroid administration depresses monocyte bactericidal activity (Rinehart et al., 1975) and induces a profound monocytopaenia (Fauci et al., 1976).

The effects of glucocorticoids on the migration of various leucocyte populations may explain much of these drugs' anti-inflammatory and immunosuppressive actions (Parillo and Fauci, 1979). In addition to the monocytopaenia, bolus administration of hydrocortisone also causes a marked lymphocytopaenia with a selective loss of T cells (Fauci et al., 1976). This redistribution of mononuclear cells will prevent the accumulation of these cells in inflamed tissue and will thus exert a suppressive effect on the chronic inflammatory process.

1.2 Second-line antirheumatic agents

Most of the drugs in this category do not possess anti-

inflammatory activity. Current thoughts on their modes of action revolve around modulation of the immune response, but it is very likely that each compound in this group has a different mechanism where it exerts its antirheumatic effect.

(a) Gold compounds

The rationale for chrysotherapy has its roots deep in empiricism. The first recorded use was in China around 2500 B.C. (Walz, 1984). The basis of the early use of gold probably stems from folklore associated with precious metals. Forestier (1929) who reported positive results in patients with ankylosing spondylitis after treatment laid the cornerstone of modern chrysotherapy. Through the years, conflicting reports on the efficacy of gold compounds in the treatment of arthritis stimulated a number of controlled studies (Walz et al., 1974). The question of gold's efficacy was finally answered in 1956 when the Empire Rheumatism Council (ERC) (1961) conducted a well-controlled double-blind study with gold sodium thiomalate (GST) in a large patient population. The results from the study showed that, by practically all criteria, improvement was observed, the exception being improvement shown by X-ray examination. The beneficial effects of chrysotherapy persisted for more than a year even after therapy had been stopped. Sigler et al. (1974), in a double-blind study, confirmed the results of the ERC study and extended these findings. He described positive

radiological evidence that chrysotherapy significantly slowed the progression of the disease in the joints of RA patients.

Although 5 decades of experience with gold compounds in the treatment of RA have clearly established their clinical efficacy (ERC, 1961; Marshall, 1965), their mode of action is still uncertain.

A variety of enzyme systems are influenced by gold, particularly those requiring free SH groups (Zvaifler, 1979b). Gold inhibits several lysosomal enzymes in vitro (Persellin and Ziff, 1966; Ennis et al., 1968) and enzymes isolated from lysosomal fractions of mø's incubated with gold or hepatic lysosomes from gold-treated rats have diminished hydrolytic activity (Persellin and Ziff, 1966; Ennis et al., 1968). Addition of gold to tissue cultures of rheumatoid synovium and cartilage retards the rate of cartilage degradation (Hawkins et al., 1972), a process thought to be mediated by enzymes from synovial lysosomes.

In vitro the effects of gold compounds on the immune system have been investigated. Gerber (1974) has shown that the aggregation or denaturation of IgG is reduced in the presence of pharmacological concentrations of gold sodium thiomalate, presumably because of its ability to stabilise disulphide interchange. Gold has the ability to reversibly inactivate the CIs component of complement in vitro (Schultz et al.,

1974) and prevent the formation of C3 convertase by the alternative pathway (Burge et al., 1978).

In vitro responses of human peripheral blood lymphocytes to a variety of mitogens, tuberculin (PPD), and other lymphocytes in a mixed lymphocyte reaction were significantly inhibited by gold sodium thioglucose and thiomalate (Lies et al., 1977; Lipsky and Ziff, 1977). The latter authors have suggested that the inhibition of mitogen and antigen responses of normal human peripheral blood mononuclear cells is caused by the inhibition of monocyte accessory cell function and not by altering T cell function. Gold compounds have also been shown to suppress PWM-induced antibody formation by inhibiting the requisite accessory cell function of monocytes (Lipsky, 1982).

Administration of gold sodium thiomalate to rats was shown to depress the phagocytic activity of mø s in experimentally-induced inflammatory exudates and also to inhibit their capacity to migrate into sites of inflammation (Vernon-Roberts et al., 1973). Similarly, chrysotherapy diminished the augmented phagocytic capacity of blood monocytes from RA patients when tested in vivo by a modified skin-window technique although it had no effect on polymorphonuclear leucocyte phagocytosis (Jessop et al., 1973). In view of the critical role of mø s in both the induction and expression of immunologically-mediated inflammatory responses, interference with their function by gold compounds may well explain the

efficacy of chrysotherapy in reducing rheumatoid inflammation (Lipsky, 1982).

(b) D-penicillamine

D-penicillamine (D-pen) is a unique pharmacological agent in that it is used in treating many diverse clinical conditions. It is given for Wilson's disease due to its copper-chelating properties (Walshe, 1956). Since it forms a soluble mixed disulphide with cystine, it is used for treating cystinuria (Crawhall et al., 1963). D-pen was originally proposed for the treatment of RA because of its ability to dissociate macroglobulins (rheumatoid factors) by cleavage of their inter-subunit disulphide bonds (Deutch and Morton, 1957). However, Jaffe (1962) demonstrated that dissociation of rheumatoid factor (RF) only occurred following injection of penicillamine directly into the joints of RA patients and even then the effect was temporary. He also showed that prolonged administration of penicillamine could bring about a fall in RF (Jaffe, 1963) but later concluded (Jaffe, 1965) that this was unlikely to be due to simple splitting of the macroglobulins as it could not account for the time lapse between initiation of treatment and clinical effect.

The copper-chelating activity of D-pen exploited in the treatment of Wilson's disease may also play a role in the anti-rheumatic action of the drug. The resultant D-pen-copper

complex has been shown to afford protection from superoxide radicals by expressing superoxide-dismutase-like activity although at a relatively high concentration (Greenwald, 1981). Moreover, the reactivity of penicillamine with native thiol compounds is not necessarily restricted to mixed disulphide formation with extracellular cysteine or indeed, with circulating macroglobulins like IgM. For the drug may also react with free SH groups in other proteins such as serum albumin, resulting in the displacement of bound low molecular weight compounds or in SH-dependent enzymes (Stanworth, 1984). Indeed, Hall and Gillan (1979) have shown that D-pen enhances SH-SS exchange reactions and Gerber (1978) also reported that D-pen inhibits the SH-dependent heat aggregation of IgG *in vitro* and might therefore prevent the formation of autogenic IgG aggregates *in vivo*.

D-pen treatment also reduced the raised level of a disulphide bonded complex between IgA and alpha-$_1$-antitrypsin encountered in the serum and joint fluid of RA patients and this reduction is associated with clinical improvement (Stanworth, 1981). It has recently been shown that such complexes are capable of inhibiting phagocytosis by neutrophils, as well as promoting the release of acid hydrolases from mø's. These effects, if they occur *in vivo*, might contribute to the inflammation with the rheumatoid joints (Stanworth, 1984).

Based on the clinical observations that D-pen therapy frequently results in lowered antiglobulin titres (Jaffe, 1963; Multicentre Trial Group, 1973), decreased levels of circulating immune complexes (Epstein et al., 1979) and diminished levels of serum immunoglobulins (Bluestone and Goldberg, 1973; Epstein et al., 1979), Lipsky (1981) proposed that D-pen might exert an immunosuppressive action and inhibit the function of cells involved in humoral immune responses. D-pen, in the presence of copper, has been shown to inhibit the proliferative response of human peripheral blood T cells to nonspecific phytomitogens (Lipsky and Ziff, 1978). In these experiments, the T cell function appeared to be specifically inhibited while the monocyte accessory function was not affected. Later, these workers (1981) found that D-pen also inhibited the capacity of peripheral blood mononuclear cells to generate immunoglobulin-secreting cells in response to <u>in vitro</u> stimulation with T cell-dependent polyclonal B cell activators. Again, inhibition was observed only when cells were incubated with D-pen in the presence of copper ions. Other compounds, such as 2-mercaptoproprionyglycine and 5-thiopyridoxine also inhibited responsiveness in the presence of copper in this system (Lipsky and Ziff, 1981). The observation that pre-incubation with D-pen and copper ions did not alter the capacity of B cells to form immunoglobulin-secreting cells after appropriate stimulation, but rather diminished helper T cell activity, implies that the action of D-pen and other thiols results from

a selective inhibition of T cell function (Lipsky, 1981).

McKeown et al. (1984) have shown, using pokeweed mitogen stimulated IgG synthesis as the assay system, that monocytes from patients with active RA are defective and normal cell function could be restored either by treating cells in vitro with 2-ME or by treating the patients with D-penicillamine - an indication that monocyte accessory function may be dependent on cell surface SH groups.

Binderup et al. (1978) showed that very low percentage of rat peritoneal mø's, pretreated with D-pen, markedly enhanced in vitro rat lymphocyte proliferation. In addition, in vivo D-pen treatments of rats results in an increase of in vitro DNA synthesis of unstimulated and stimulated lymphocytes (Binderup et al., 1980). Both studies suggest a modulating effect of mø's on lymphocyte transformation, an effect itself modified by D-pen. Later, Arrigoni-Martelli and Binderup (1981) also reported that the suppressed response of lymph node lymphocytes in AA rats was caused by the inhibition by suppressive mø activity - an inhibition that could be abolished by D-pen treatment which also enhanced T helper cell function.

Although the mode(s) of action of D-pen remains unclear, the importance of the SH moiety of the drug for its immunomodulating effects, actions that may be shared by other

SH-reactive compounds (Jaffe, 1980) must be emphasised. The role of different SH groups in immunoregulation of autoimmune disease will be reviewed in the next section.

PART (III) THE ROLE OF SH GROUPS IN RHEUMATOID ARTHRITIS

1.1 Serum SH groups and reactivity in RA patients

Subnormal levels of serum sulphydryl (SH) groups have been observed in various connective tissue disorders including RA (Lorber et al., 1964). The depression of serum SH levels in RA correlates with active disease (Haataja, 1975). The erythrocyte membrane surface SH groups are also found to be deficient in RA and associate with the decrease in plasma protein SH levels (Lorber and Chang, 1968). The low levels of total serum SH in RA have been shown to increase towards normal with clinical improvement (Lorber et al., 1971) and with certain drug therapy, though early work suggested that they may alter with both antirheumatic drugs and some non-steroidal anti-inflammatory drugs (Evans, 1975).

It has been suggested that the low thiol levels in RA may be associated with a disturbance of the sulphydryl-disulphide (SH-SS) exchange reaction that could lead to the aggregation of serum proteins, particularly IgG, with the exposure of new antigenic sites leading to autoantibody production (i.e. rheumatoid factor). As a model for this hypothesis Gerber (1964) has shown that heat-aggregation of IgG is partly thiol dependent and may be inhibited by SH compounds such as sodium aurothiomalate and D-penicillamine (Gerber, 1974).

There are two distinct assay methods for thiols: the first measures the rate of SH-SS exchange reaction (reactivity), the second quantifies total serum sulphydryl. Gerber et al. (1967) have reported that a number of NSAIDs including large doses of aspirin, exhibit a high degree of specificity in accelerating the SH-SS interchange reaction between human sera and Ellman's reagent, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). Oronsky et al. (1969) have shown that sulphydryl binding agents are capable of suppressing the inflammatory process and that the anti-inflammatory activity of these agents and certain NSAIDs is, at least partly, related to their effects on SH groups. Serum SH-SS interchange reactions are severely impaired in AA and may be restored by administration of anti-inflammatory agents (Butler et al., 1969). However, Walz and Di Martino (1972) reported that the SH-reactive anti-arthritic drugs, sodium aurothiomalate and sodium aurothioglucose, produced a potent, dose-dependent inhibition of the SH-SS exchange reaction between rat serum and DTNB although the NSAIDs (phenylbutazone, indomethacin and aspirin) administered in vitro or in vivo, accelerated the interchange reaction in diluted rat serum.

In humans, three second-line antirheumatic drugs, namely aurothiomalate, D-penicillamine and levamisole, have been shown to stimulate serum protein SH reactions in vivo as measured by exchange with DTNB in vitro (Hall and Gillan, 1979). High concentrations of indomethacin were also shown to

stimulate the SH-SS exchange reaction between protein SH groups and DTNB in vitro (Hall and Gillan, 1979). These authors postulated that the mechanism whereby indomethacin stimulates protein SH reactivity towards DTNB differs from that of aurothiomalate, D-pencillamine and levamisole. The latter three compounds share the ability to interact directly with protein SH groups. Indomethacin and other NSAIDs are devoid of active SH groups and may stimulate thiol reactivity via an indirect or "allosteric" effect on protein conformation (Hall and Gillan, 1979). To lend support to this hypothesis, Hichens (1966) suggested that the increased rate of reaction associated with the NSAIDs may be indicative of free SH groups being uncovered by NSAIDs through an induced conformational change of the protein molecules. Human plasma has far more SS groups than SH groups. It contains little or no non-protein thiol and its protein SH concentration is about 0.4 - 0.6 mM (Lorber et al., 1970). Most of the SH groups reside in the serum albumin molecules. This protein has 17 cystine residues and a fractional SH of 0.65 - 0.68 per mole. The reason for the fractional SH titre of serum albumin is that a part of the protein has lost its free SH group in forming a mixed-disulphide with either a cysteine or to a lesser extent, glutathione (Jocelyn, 1972).

The phenomena of autooxidation and mixed-disulphide formation may account for the depressed serum SH level in RA. Hall et al. (1984) have shown that the lowering of serum SH levels in

RA may be due to oxidation caused by the release of hydrogen peroxide and oxidative intermediates from activated phagocytes (Badway and Karnovsky, 1980). Neutrophils generate relatively more hydrogen peroxide in vitro than do monocytes (Reiss and Roos, 1978). Thus, depressed serum SH content in early synovitis may be indicative of stimulated phagocytes, particularly neutrophils, liberating oxygen free radicals and other tissue damaging species (Hall et al., 1984). Since scavengers of superoxide anion and hydrogen peroxide are not easily detectable in rheumatoid serum or synovial fluid (Blake et al., 1983) these two reactive species may undergo an iron-catalysed reaction to produce hydroxyl radicals (Halliwell, 1978) which may cause degradation of lipids (Donato, 1981) and hyaluronic acid (McCord, 1974). These reactions may contribute to the inflammatory events seen in the synovial tissue and fluid. This mechanism may explain the association between low serum SH in early RA (active phagocytes generating hydrogen peroxide etc.) and more aggressive RA (production of hydroxyl radicals and subsequent lipid peroxidation and tissue damage).

In addition, as RA is an inflammatory polyarthritis, plasma levels of caeruloplasmin, an acute phase reactant, are increased (Conforti et al., 1982). This protein can rapidly oxidise certain low molecular weight thiols to formed disulphides (Albergoni and Cassini, 1978). Although it does not affect the thiol of albumin directly, presumably due to

the steric hindrance between the two proteins, the disulphides formed with the low molecular weight thiols may interact via SH-SS exchange reaction with the SH groups present in albumin (Foster, 1977) and this may also contribute to the lowering of serum protein SH levels in active RA.

1.2 Sulphydryl involvement in the immune system

The protein SH groups, unless masked in some proteins, are chemically the most reactive groups found in cells (Jocelyn, 1972) and participate in a wide range of biological processes (Jensen, 1959). More recently, many authors have reported that a variety of simple, low molecular weight thiol compounds augment various lymphocyte reactions in vitro (Click et al., 1972; Broome and Jeng, 1973) and this implicates a role for SH groups in immunoregulation.

2-mercaptoethanol (2-ME), one of the most effective thiols, has been used extensively as an additive to culture media in a variety of tissue-culture systems (Metcalf, 1976). At optimal doses, it has been found to (1) enhance the DNA-synthetic response of both human and rabbit lymphocytes to mitogens (Fanger et al., 1970) and to allogeneic lymphocytes in murine mixed lymphocyte cultures (MLC) (Heber-Katz and Click, 1972; Bevan et al., 1974), (2) enhance the murine primary humoral antibody response in vitro (Click et al., 1972), (3) propagate murine lymphoma cell line in vitro (Broome and Jeng, 1973),

(4) augment the generation of mouse cytotoxic thymus-dependent lymphocytes in the MLC (Cerottini et al., 1972; Engers et al., 1975), (5) allow the culture and propagation of clones of mouse B lymphocytes (Metcalf et al., 1975; Metcalf, 1976), (6) functionally replace mouse peritoneal macrophages in the primary humoral immune response (Chen and Hirsch, 1972) and (7) act as polyclonal activator of murine B cells (Goodman and Weigle, 1977) or cytotoxic T cells (Igarashi et al., 1977).

The stimulatory effect of 2-ME on the immune response in vitro was first described by Click et al. (1972). They showed that 2-ME restored the antibody-forming capacity of adherent cell depleted spleens to that of the unfractionated cell population. This phenomenon was not due to promotion of cell viability because daily inclusion of 2-ME into the culture was not necessary (Click et al., 1972). 2-ME is readily oxidised in tissue culture media and its half-life under these conditions is 5.9 hours (Broome and Jeng, 1973). Hence, the reducing agent may exert its effect on a very early event of lymphocyte activation (Click et al., 1972). Also, kinetic studies have shown that the development of plaque-forming cells in cultures with 2-ME progressed at an exponential rate earlier and for longer periods than in cultures lacking the reducing agent (Click et al., 1972).

2-ME could augment the primary antibody response in vitro not only to SRBC (a thymus-dependent antigen) but also to TNP-

Ficoll and TNP-LPS (thymus-independent antigens). It has been reported that mø̸s were necessary for antigen presentation to B cells to generate the immune response to TNP-Ficoll (Boswell et al., 1980a). However, the response to TNP-LPS requires neither mø̸s nor T cells (Boswell et al., 1980b). Ohmoshi and Yamamoto (1982) confirmed that depletion of either adherent cells or T cells from murine spleen cells did not affect the enhancement of the response to TNP-LPS by 2-ME. Therefore, B cells would be the target of 2-ME action, at least in this case. On the other hand, Chen and Hirsch (1972) and Lemke and Opitz (1976) have proposed that 2-ME can functionally substitute for mø̸s in the primary anti-SRBC response <u>in vitro</u>. They depleted the mø̸s from spleen cells by glass adherence (Chen and Hirsch, 1972) or by carbonyl iron treatment (Lemke and Opitz, 1976).

The above data have generated a question: Is the response of T and B cells to SRBC in the presence of 2-ME truly mø̸ independent? Hodes and Singer (1977) have shown that the primary response to TNP-protein, following depletion of accessory cells by Sephadex G-10 columns, was strictly dependent on accessory cells. 2-ME, however, has no effect in this system. Other workers have also demonstrated that the response to SRBC (Ly and Mishell, 1974) or TNP-Ficoll (Boswell et al., 1980a) was abrogated, even in the presence of 2-ME, when spleen cells were effectively depleted of adherent cells by passage through Sephadex G-10 columns. Furthermore,

Rosenstreich and Mizel (1978) reported that 2-ME could not replace the need for mø̸s in the lectin-induced response, although it would enhance the activity of a very small number of mø̸s added to the culture. These results indicate that 2-ME synergise with mø̸s to increase the response. Ohmori and Yamamoto (1982) have also confirmed that 2-ME could not substitute for the function of mø̸s in the Sephadex G-10 treated spleen cells. Hence, 2-ME could not totally replace the role of mø̸s, but rather a class of adherent cells which could not be removed by glass adherence or carbonyl iron treatment but could be depleted by Sephadex G-10 method might be involved in supporting the antibody response as accessory cells in the presence of 2-ME. However, it is also possible that in the presence of 2-ME, the antigen-presenting function may be performed by the B cell during its handling of Ag, particularly if these B cells are in high frequency, as in primed animals. This applies particularly to the SRBC response (Unanue, 1981).

The reducing activity of thiols in the culture medium was brief - a few hours (Broome and Jeng, 1973). Therefore, if the compounds acted directly on the lymphocyte, they should do it during the first few hours of culture. Chen and Hirsch (1972b), however, found that medium containing 10% foetal calf serum (FCS) treated with 2-ME retained its activity for promoting antibody formation for at least three days and suggested possible secondary effects. That the thiols might

have direct effects was suggested by experiments in which 2-ME was added to cells in the absence of protein, but the optimal growth potential required the addition of serum to the media (Lemke and Opitz, 1976). Similar observations were made by Goodman and Weigle (1977) who found that 2-ME showed a weak mitogenic activity in serum-free culture, which was however, enhanced by about ten-fold under serum-containing conditions. They suggested that 2-ME is able to activate lymphocytes directly. However, Opitz et al. (1978) stressed that 2-ME does not stimulate the DNA synthetic response by highly purified T lymphocytes and elicits negligible responses in B-lymphocytes in serum-free medium. This suggests that 2-ME has actually no direct lymphocyte-activating properties. The same authors showed that preincubation of FCS with 10^{-4} - 10^{-3}M 2-ME followed by removal of free 2-ME resulted in the conversion of a native serum factor (Pro-MaSF) in the FCS into the activated serum factor (MaSF) that could elicit the antibody response to SRBC (Lemke and Opitz, 1976) and anti-IgM-induced B cell proliferation (Sidman and Unanue, 1978) without further addition of 2-ME. Both Opitz et al. (1977) and Sidman and Unanue (1978) reported that 2-ME-activated serum factor(s) (i.e. MaSF) were eluted from Sephadex G-100 column near albumin. Indeed, Hewlett (personal communication, 1981) postulated that pro-MaSF was albumin and MaSF mercaptalbumin. The addition of 2-ME and, presumably, møs, reduced the albumin to mercaptalbumin, the active fraction for the induction of the immune response.

An alternative explanation for the effect of 2-ME on lymphocyte activity has been suggested by Ohmori and Yamamoto (1983b) who demonstrated that 2-ME-pulsed FCS can support the antibody response in the absence of additional 2-ME as effectively as native FCS in the presence of 2-ME. They postulated that the supporting activity of the antibody response in 2-ME-pulsed FCS was due to 2-ME molecules bound to FCS components via disulphide bonds. The thiol groups on the FCS components do not appear to exert the supporting effect of FCS but they would serve as a repository for 2-ME to maintain the appropriate 2-ME concentration in the culture medium to augment the antibody response (Ohmori and Yamamoto (1983b).

These authors also suggested that a major role of 2-ME in the augmentation of antibody response would be to stimulate the utilisation of cystine in both B cells and T cells through the formation of Cys-2-ME and that the 2-ME transformed into FCS-bound and oxidised forms could be used for the formation of Cys-2-ME via interaction with the lymphocytes.

However, other workers have shown that activation of lymphocyte reactions may be mediated via an alteration in cell surface SH groups. Chaplin and Wedner (1978) have reported that blockade of free SH groups on human peripheral blood lymphocyte cell surface by diamide and other sulphydryl reagents leads to an inhibition of lectin-induced lymphocyte activation, and that the inhibition is not due to decreased

intracellular levels of reduced glutathione or inhibition of binding of lectin to the lymphocyte. They suggested that the maintenance of free SH groups is important during the early induction of lymphocyte proliferation. Later, Wedner and Wilson (1983) demonstrated that when human peripheral blood T lymphocytes were incubated with ConA or PHA, washed and incubated with 5-iodacetamide salacylic acid (5-ISA), 5-maleimidyl salacylic acid (5-MSA) and assayed as cell suspensions or smeared on microscope slides, incubated with 3-maleimidyl-phenyl coumarin, and assayed using a microscope photometer, lymphocytes incubated with ConA or PHA showed a marked increase (peaked at 30-45 minutes) in free SH groups compared to control. The increase returned to baseline by 60 minutes. Lymphocytes depleted of glutathione had significantly more free SH, and peak increase seen with PHA or ConA was 15 minutes and decayed below control levels by 45 minutes. Since 5-MSA and 5-ISA are impenetrant this result suggests that lymphocyte activation is accompanied by rapid alterations in free SH groups located in or near the plasma membrane. The change in SH may involve proten-glutathione disulphide exchange reactions. These exchange reactions may be crucial stages in the early events leading to lymphocyte activation (Wedner and Wilson, 1983). In addition, Noelle and Lawrence (1981) have also demonstrated that oxidation of lymphocyte cell-surface thiol groups can ablate the ConA-induced response, and that this oxidative inhibition by O_2 or by the impermeant oxidant copper-phenanthroline could be

completely reversed by 2-ME.

In a study of the functional correlates of molecular structure of thiol compounds, Goodman and Weigle (1981) compared 2-ME with ethanol (lacking the SH group of 2-ME) and with ethanethiol (lacking the OH group of 2-ME) in culture when added either singly or conjointly. They showed that neither analogue was mitogenic and that only ethanethiol had a weak adjuvanticity. Thus, both the SH and hydroxyl groups had to be present on the same molecule to produce mitogenic and adjuvant activity. These results suggest that the SH group would play a major role in the augmenting effects of 2-ME on both the proliferative and the antibody responses while the hydroxyl group may contribute to make the compound nonvolatile and modify the interaction of the thiol group with the cells (Goodman and Weigle, 1981).

Low serum protein SH levels are generally a feature of active RA and have been shown to increase towards normal following clinical improvement (Lorber et al., 1971) and successful treatment with SH-containing antirheumatic drugs (Lorber, 1966; Hall et al., 1981). Since immune mechanisms are intimately linked to the pathogenesis of RA and can be affected by thiols, the blocked SH groups found in active RA may, therefore, contribute to the defective immune responses in this disease. As mentioned earlier, adjuvant arthritis (AA) in rats is an experimental model of chronic inflammation

that resembles RA both clinically and histopathologically (Pearson, 1979). Furthermore, defective immune mechanisms have also been implicated in the pathogenesis of AA. Using AA as a model, the aim of this project was (1) to determine the possible role and significance of SH groups in immunoregulation and their possible involvement in the immunopathogenesis of AA, and (2) to identify the immunomodulant effects of D-penicillamine in this model.

CHAPTER TWO: MATERIALS AND METHODS

MATERIALS

2.1 Materials	Suppliers
All chemicals, except where otherwise stated:	Sigma Chemical Co. Ltd., Poole.
All media reagents and tissue culture plastics, except where otherwise stated:	Gibco Bio-cult Ltd., Paisley, Scotland.
Concanavalin A (Lyophilised; from the jack bean Canavalia ensiforms):	Pharmacia, (G.B.) Ltd., Hounslow, Middx.
Ficoll-Hypaque (Density: 1.007 $\pm$ 0.001 g/ml); Sephadex G-10:	Pharmacia Fine Chemicals, (G.B.), Hounslow, Middx.
Heat-killed, mixed strains Mycobacteria; IL-2 (rat) standard:	Kindly donated by Lilly Research Centre, Windlesham.
IL-1 (Human) standard:	Genzyme, Koch-light Ltd., Suffolk.

Methyl [^{3}H]-Thymidine (Specific activity 5Ci/mol, 21 mCi/mg, Radioactive concentration ImCi/ml):	Radiochemical Centre Ltd., Amersham, Bucks.
Scintillation grade toluene:	British Drug Houses (BDH) Ltd., Poole.
Scintillation vials:	Richardsons of Leicester, Leicester.

2.2 Equipment	Suppliers
CE595 Double-beam digital U.V. Spectrophotometer:	Cecil Instruments, Cambridge.
Laborlux 12 microscope:	E. Ietz (Instruments) Ltd., Luton.
MSE Chilspin 2 Centrifuge:	MSE Laboratories Ltd., Irvine, Scotland.
Packard Tri-carb Liquid Scintillation Spectrometer:	United Technologies, Packard, Berkshire.
Skatron cell harvester:	Flow Laboratories Limited, Irvine, Scotland.

2.3 Animals

All animals used were bred in the Bath University Animal House except Sprague-Dawley rats and rabbits which were purchased from Interfauna, Huntingdon, Cambridgeshire.

2.4 Routine buffers and media

2.4.1 Calcium magnesium free salt solution (CMFSS) x 10 concentrate

	g/litre
Glucose	16.00
Sodium chloride	80.00
Potassium chloride	4.00
Disodium hydrogen phosphate	0.48
Potassium dihydrogen phosphate	0.60 pH 7.3

The above were dissolved in 1 litre of distilled water, filter sterilised, and stored at 4°C. Before use, a 10 fold dilution was performed with distilled water and the pH corrected to 7.3 with 1M NaOH.

2.4.2 Phosphate buffered saline (PBS) x 10

	g/litre
Sodium chloride	80.00
Potassium chloride	2.00
Disodium hydrogen phosphate	11.50
Potassium dihydrogen phosphate	2.00 pH 7.3

The above were dissolved in 1 litre distilled water, filter sterilised (Millipore filter 0.45 μm pore size) and stored at 4°C until required.

Before use, the solution was diluted to ten times its volume with distilled water. To this was added the equivalent of:

0.1 g/litre calcium chloride
0.1 g/litre magnesium chloride

The pH was adjusted to 7.3 with 1M sodium hydroxide solution.

2.4.3 Ammonium chloride solution

This solution was used for the lysis of extraneous red cells in the rat spleen cell suspension.

	g/litre
Ammonium chloride	8.290
Potassium hydrogen carbonate	1.000
Ethelenediaminetracetic acid	0.004

The above were dissolved in 1 litre distilled water and the pH corrected to 7.4 with 1M sodium hydroxide.

2.4.4 Lymphocyte culture medium RPMI 1640

	Volume (ml)
RPMI 1640 (x 10 strength)	10.00
Penicillin/streptomycin solution (5000 IU/ml of each)	2.00
200mM L-glutamine	2.00
7.5% sodium bicarbonate	2.70
Foetal calf serum	10.00 pH 7.4

The above mixture was diluted with 100 ml with sterile, distilled water. The pH was corrected to 7.4 with sterile 1M sodium hydroxide.

2.4.5 Scintillation fluid

To 2.5 litres of toluene was added:

12.50 g 2,5-Diphenyloxazole (PPO)

0.75 g 1,4-Di-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP)

METHODS

(A) Determination of the total serum SH levels and the rate of SH-SS exchange reaction (SH reactivity)

2.1 Preparation of sera

Peripheral blood was taken by venipuncture from healthy volunteers. The blood was allowed to clot at 37°C. The clotted blood was centrifuged at 1000 r.p.m. for 10 minutes. The serum extracted from the first centrifugation was spun again for 10 minutes to ensure complete removal of erythrocytes and white cells. Sera were similarly prepared from blood obtained from mice, rats and guinea pigs via cardiac puncture under ether anaesthesia and rabbits by puncturing the ear veins. Both fresh sera and frozen sera (stored at -20°C) were used for experiments.

2.2 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)

The reagent used for the determination of both total serum SH levels and SH reactivity was DTNB (Ellman, 1959). DTNB undergoes a SH-SS exchange reaction with the serum protein SH groups which may be represented by the reaction:

Protein-SH + DTNB -> Protein-S-S-TNB + TNB-SH (yellow)

The deep yellow product of this reaction, 5-thio-2-nitrobenzoic acid (TNB-SH,) may be determined spectrophotometrically at 440 nm (Butler et al., 1969). Only fresh DTNB solution was used. For the total serum SH assay the DTNB solution was added to a final concentration of 400 µM. For the SH reactivity measurements, DTNB solution was added to a final concentration of either 100 µM or 400 µM. The DTNB was protected from u.v. degradation with aluminium foil and always preincubated at 37°C during experiments.

2.3 The total serum SH assay

The experimental design was as follows:

'Blank' cuvette (1 ml)	'Test' cuvette (1 ml)
50 µl serum	50 µl serum
950 µl 0.1 M phosphate buffer, pH 7.4	750 µl 0.1 M phosphate buffer, pH 7.4
	200 µl DTNB solution (2mM)

The reaction mixtures were incubated for 5 minutes at 37°C and the absorbance in the test cuvette was measured against the blank cuvette at 440 nm.

Based on this method, a calibration curve for the total serum SH assay was determined, using different concentrations of reduced glutathione (GSH). The absorbance measurements were carried out in a CE 595 double-beam digital U.V. spectrophotometer (Cecil Instruments) which was linked to a CE 530 cell programmer coupled to a CE500/Control-Record Module/Series Two. The CE500/Control-Record Module was used to record the rate of SH-SS exchange reaction.

2.4 Determination of the rate of SH-SS exchange reaction (SH reactivity)

The experimental design was as follows:

'Blank' cuvette (1 ml)	'Test' cuvette (1 ml)
400 µl serum	400 µl serum
600 µl 0.1 M phosphate buffer, pH 7.4	400 µl 0.1 M phosphate buffer, pH 7.4
	200 µl DTNB solution

The method for measuring SH reactivity was almost identical to that of the total serum SH assay except that the amounts of serum and buffer used were altered.

The change in absorbance at 440 nm measured in the spectrophotometer (CE 595) was recorded via the CE500 Control-Recorder Module/Series Two which plotted the increase in absorbance against time (minutes) automatically. The gradient (absorbance/time) obtained from the graph plotted could be used to calculate the rate of SH-SS interchange reaction using the calibration curves shown in Figures 3.1.b and 3.1.c depending on the final concentration of DTNB added.

(B) Lymphocyte preparation

2.1 * Preparation of rat spleen and lymph node lymphocyte suspensions

Male Wistar or Sprague-Dawley rats (150 - 250 g) were killed under ether anaesthesia by cervical dislocation. The spleens and mesenteric lymph nodes were aseptically removed and kept in sterile Nunc universals containing ice-cold RPMI 1640 medium (pH 7.4) with Hepes buffer supplemented with 10% FCS, 2 mM L-Glutamine and Penicillin/Streptomycin (100 IU/ml each). The spleen or lymph node was pressed through a sterile tea strainer into a sterile petri dish containing 8 ml of ice-cold CMFSS. The cells were then passed through a sterile silk filter to give a single cell suspension.

* CFLP mouse splenic lymphocyte suspensions were obtained using the same method.

2.2 Lymphocyte separation

The single cell suspension obtained in B.2.1 was then layered onto 3 ml Ficoll-Paque (density 1.077 ± 0.01 g/ml) contained in a sterile Nunc centrifuge tube and centrifuged at 400 g for 30 minutes at 20°C. The mononuclear cells forming a discrete band at the interface were harvested and washed twice with CMFSS by gentle resuspension and pelleting at 400 g for 10 minutes. The final pellet was resuspended in culture medium: RPMI 1640 supplemented with 5% FCS, 2 mM L-Glutamine, 0.75% sodium bicarbonate solution and penicillin/streptomycin (100 IU/ml each).

2.3 Lymphocyte count and viabilities

Lymphocytes were enumerated using an improved Neubauer haemocytometer, and cell viabilities were assessed by trypan blue exclusion method.

2.4 Conditions of culture

Cells were diluted in culture medium. 200 µl aliquots of the diluted cell suspension were transferred to individual wells of a Nunc microtiter round-bottomed cell culture plate to which various compounds such as ConA, 2-ME etc. were added to stimulate lymphocyte proliferation. All cultures were performed in triplicate. The cell culture plates were

cultured in a humidified atmosphere of 5% CO_2 in air at 37°C.

2.5 Determination of lymphocyte proliferation

Cultures were harvested after 72 hours of incubation. For the final 4 hours before harvesting 0.5 μCi of methyl [^{3}H]-thymidine (specific activity 5 Ci/mmol) was added to each well. The cells were harvested with a Titertek cell harvester onto fibreglass filter papers. Cells were washed with distilled water, precipitated with 5% trichloroacetic acid (TCA) and dried with methanol. The filter papers were oven dried (37°C) overnight. Discs of filter paper were placed in mini-vials to which 2 ml of scintillation fluid were added. The resultant scintillation was determined with a Packard Tri-Carb liquid scintillation spectrometer and expressed as mean scintillation counts per minute (CPM).

(C) Interleukins

2.1 Interleukin-1 (IL-1) production

20 ml cold Phosphate buffered saline (PBS) supplemented with 2% FCS (PBS/2% FCS) were injected peritoneally into male Sprague-Dawley rat (200-250 g) and the abdomen of rat was massaged for 10 minutes. Peritoneal macrophages (møs) were collected and centrifuged at 400 g for 10 mins. The cells were washed three times with cold PBS/2% FCS by gentle

resuspension and pelleting at 400 g for 10 minutes. The final cell pellet was resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-Glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin and 0.75% sodium bicarbonate solution and counted in 0.2% trypan blue.

The cells were diluted in a concentration of 1×10^6 cells/ml in culture medium. The diluted cell suspension was transferred in 1 ml aliquots into 6 x 4 large Nunc tissue culture plates and pre-incubated for one hour in a humidified atmosphere of 5% CO_2 in air at 37°C with the following agents: 2-ME (2.5×10^{-5} M) and an irreversible non-penetrating SH blocking agent, p-hydroxymercuriphenylsulphonate (pHMPSA; 5×10^{-5} M) to allow the adherent cells (møs) to adhere to the plate.

At the end of the preincubation the plate was washed three times with cold PBS/2%/FCS to remove the non-adherent cells. 1 ml of culture medium (with or without 2.5×10^{-5} M 2-ME incorporated) containing 1 µg of Lipopolysaccharide (LPS) was added to the appropriate well which was initially preincubated with or without 2-ME. Finally, the plate was incubated in the same culture condition for 24 hours before the supernatant (which contained IL-1 released by the møs) was aspirated, centrifuged (at 500 g for 10 minutes) and filtered sterile through 0.22 µm filter prior to being aliquoted and stored at -20°C for later assay.

2.2 IL-1 assay

Thymuses aseptically removed from *C3H/HeJ mice (6-8 weeks old) were trimmed of adipose tissue and pressed through a sterile tea strainer into a petri dish containing 10 ml of cold PBS/2% FCS. The thymocyte suspension was prepared as described in B.2.1. However, the final cell pellet was resuspended in RPMI 1640 medium supplemented with 5% FCS instead of 10% FCS.

The adherent cells were separated by passage through a Sephadex G-10 column (10 ml) which had been pre-warmed to 37°C. The non-adherent cells were collected, washed with cold PBS/2% FCS and then adjusted to 10^7 cells/ml in culture medium (5% FCS) containing 2-ME (2.5×10^{-5} M).

The IL-1 dilution was performed using the ½ dilution design i.e. ½ -> 1/4096 in 12 wells. To the 100 µl/well diluted IL-1 in culture medium [containing 2-ME (2.5×10^{-5} M)], cells were added in 100 µl (1×10^6 cells) to give a total volume of 200 µl/well. Nunc microtiter cell culture plates with flat bottoms were used.

The experimental design was as follows:

(a) Culture medium (control) + cells

(b) Culture medium + IL-1 (Human) (commercial standard, Genzyme) + cells

(c) Culture medium + IL-1 (Human) + ConA (1µg/ml) + cells

(d) Culture medium + IL-1 (Rat) + cells

(e) Culture medium + IL-1 (Rat) + ConA (1 µg/ml) + cells

The cells were incubated for 72 hours in a humidified atmosphere of 5% CO_2 in air at 37°C.

For the final 4 hours of incubation 0.5 µCi of methyl [^{3}H]-thymidine (specific activity, 5Ci/mmol) was added to each well. The cells were harvested at the end of the 72-hour incubation and incorporation of radioisotope measured as previously described (see B.2.5 B.1.5). The capacity of each of the test supernatants to induce cell proliferation was calculated using probit-analysis and the following equation was used to convert the resulting titres (ED_{50}) into units.

$$\frac{ED_{50}\ (\text{test})}{ED_{50}\ (\text{standard})} \times 100\ \text{units/ml}$$

* C3H/Hej mice were used because they are LPS-resistant so that the thymocyte proliferation in the assay is not due to stimulation by LPS but through the IL-1 under test.

2.3 Interleukin-2 (IL-2) production

IL-2 was produced by a short term exposure of lymphocytes to ConA followed by the removal of ConA by extensive washing of the cells prior to subsequent incubation in culture medium free of ConA (Spiess and Rosenberg, 1981).

The spleen cell suspension was prepared as described in B.2.1 C.1.1.

The cell suspension was adjusted to 2 x 10^7 viable cells/ml in culture medium containing 20 µg/ml ConA. The cells were mixed thoroughly in Nunc centrifuge tubes and pre-incubated upright at 37°C in 5% CO_2 in air for 2 hours according to the following design:

Tube 1	Tube 2	Tube 3
No addition	+ 2-ME (2.5 x 10^{-5} M)	+ PHMPSA (5 x 10^{-5}M)

Following this incubation the tubes were centrifuged at 400 g for 10 minutes and the pellets washed three times with PBS/2% FCS. The final pellet was resuspended back to the original volume of ConA pre-incubation but in appropriate culture medium (with or without 2.5 x 10^{-5} M 2-ME) not containing ConA. The cells were not recounted. The cell suspension was then delivered in 1 ml aliquots to the wells of the large (6 x 4) Nunc tissue culture plates with flat bottoms. The plates were incubated for 24 hours in a humidified atmosphere of 5%

CO_2 in air at 37°C. The supernatant was then harvested, centrifuged at 500 g for 10 minutes and filtered through a 0.22 µm filter prior to being aliquoted and stored at - 20°C before assay.

2.4 IL-2 assay

Spleens obtained aseptically from C3H/HeJ mice 8-12 weeks old were pressed through a sterile tea strainer into a petri dish containing 10 ml of cold PBS/2% FCS and the spleen cell suspension was prepared as describd in B.2.1. The final cell pellet was resuspended in culture medium (RPMI 1640 supplemented with 10% FCS, 2mM L-Glutamine; Penicillin/ streptomycin (100 IU/ml each) 0.75% sodium bicarbonate solution) containing 2.5 x 10^{-5} M 2-ME and 3 µg/ml ConA.

The cells were distributed to the large (6 x 4) Nunc tissue culture plate at 2 ml/well and incubated for 72 hours at 37°C in a humidified condition with 5% CO_2 in air.

At the end of the 72-hour incubation the cells were harvested and spun at 400 g for 10 minutes. The cells were washed three times and the final cell pellet was resuspended in cultured medium containing RPMI 1640, 5% FCS, 2mM L-Glutamine, Penicillin/streptomycin (100 IU/ml each) 0.75% sodium bicarbonate solution, and 2.5 x 10^{-5} M 2-ME.

The cells were counted and diluted to 2 x 10^5/ml in culture medium containing 2.5 x 10^{-5} M 2-ME. These cells were used for IL-2 assay.

The IL-2 dilution was done using the same method as that of IL-1 assay, i.e. ½ dilution design; 1/2 -> 1/4096 in 12 wells. Round bottomed Nunc microtitre cell culture plates were used. To the 50 µl/well diluted IL-2 in culture medium containing 2.5 x 10^{-5} 2-ME, 50 µl of medium containing 2 x 10^5 cells/ml was added to give a total volume of 100 µl/well.

The experimental design was as follows:

(a) culture medium (control) + cells
(b) culture medium + IL-2 (PVG rat/Lilly)[&] + cells
(c) culture medium + IL-2 (male Sprague-Dawley rat) + cells

The cells were incubated for 24 hours and then pulsed with 0.5 µCi methyl [^{3}H]-thymidine (specific activity 5 Ci/mmol) for 4 hours before they were harvested and radioactivity determined as described in ~~B.1.5.~~ B.2.5. The capacity of each of the test supernatants to induce cell proliferation was calculated using probit analysis and the resulting titres (ED_{50}) were converted into units/ml. using the same equation shown in ~~C.1.2.~~ C.2.2.

[&] IL-2 (PVG rat/Lilly) was included as standard and control. It was kindly supplied by Lilly Research Centre, Windlesham.

(D) Short term treatment of adjuvant arthritis (AA) in rat with D-penicillamine

2.1 Experimental design

Male inbred Sprague-Dawley rats weighing 200-250 g were used throughout.

The animals were divided into 2 groups as follows:

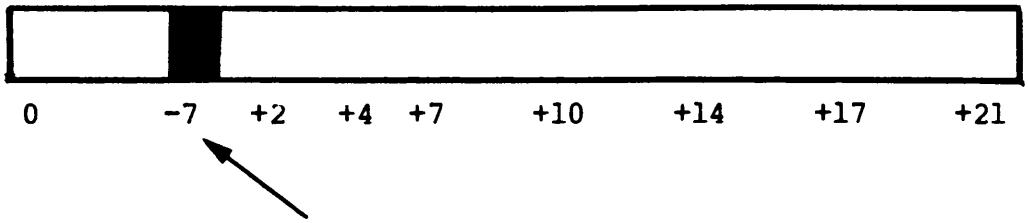
(a) Control (arthritic)

(b) Arthritic + D-penicillamine treatment

2.2 Induction of arthritis

Polyarthritis was induced by a single injection into the rat hind paws of 0.1 ml heat-killed, mixed strains of mycobacteria in mineral oil at a concentration of 5.5 mg/ml. The mycobacteria were ground in a pestle and mortar before adding to the mineral oil.

Certain groups of animals were dosed with D-penicillamine (50 mg/kg p.o. daily) according to the scheme outlined below.



Induction of arthritis

Duration of D-penicillamine treatment (50 mg/Kg/day, p.o.)

D-penicillamine was administered to the rats 7 days prior to induction of arthritis followed by daily treatment throughout the development of arthritis (up to day 21). Normal control animals received equivalent volumes of normal saline.

2.3 Evaluation of arthritis

Several parameters were used to assess the arthritis.

(1) Weight change (Winder et al., 1969). Changes in body weight and retardation in weight gain was used to monitor the course of the disease and the response to therapy with D-penicillamine. It was measured throughout the experiments.

(2) Size of hind paws: Circumferences (Aspinall and Cammarata, 1969) of the injected feet and the non-injected ones were measured using a modified caliper gauge with a Vernier scale.

(3) Severity of arthritic lesions (Currey and Ziff, 1968). This was based on the degree of joint involvement in the four

paws. Severity of arthritic lesions was graded using a scoring system of 1+ = detectable, 2+ = moderate, 3+ = severe for each inflamed paw. Max. score = 12+.

CHAPTER THREE: RESULT A

RESULT A: SERUM SULPHYDRYL LEVEL AND REACTIVITY

3.1 Serum sylphydryl levels and sulphydryl-disulphide (SH-SS) exchange reactions

There are two distinct assay methods for thiols: the first measures the rate of SH-SS exchange reaction (reactivity) and the second quantifies total serum SH. Total serum sulphydryls and their reactivity were initially studies in a number of healthy animal species including man. Later, the study was extended to include sera from adjuvant-induced arthritic (AA) rats and D-penicillamine treated AA rats.

3.1.1 Calibration curve for total serum SH assay

The calibration curve for total serum SH assay is illustrated in Fig. 3.1.a. It was determined using the reaction of known concentrations of reduced glutathione (GSH) solution with DTNB. The conversion of the absorbance of a given serum to total serum SH level (expressed as $\mu mol\ (SH)l^{-1}$) was achieved by reading directly from the calibration curve.

3.1.2 Total serum SH level in different species

The results presented in Table 1 show that healthy humans had significantly higher serum SH levels than any other of the animals studied ($P < 0.01$, student "t" test). The mean human

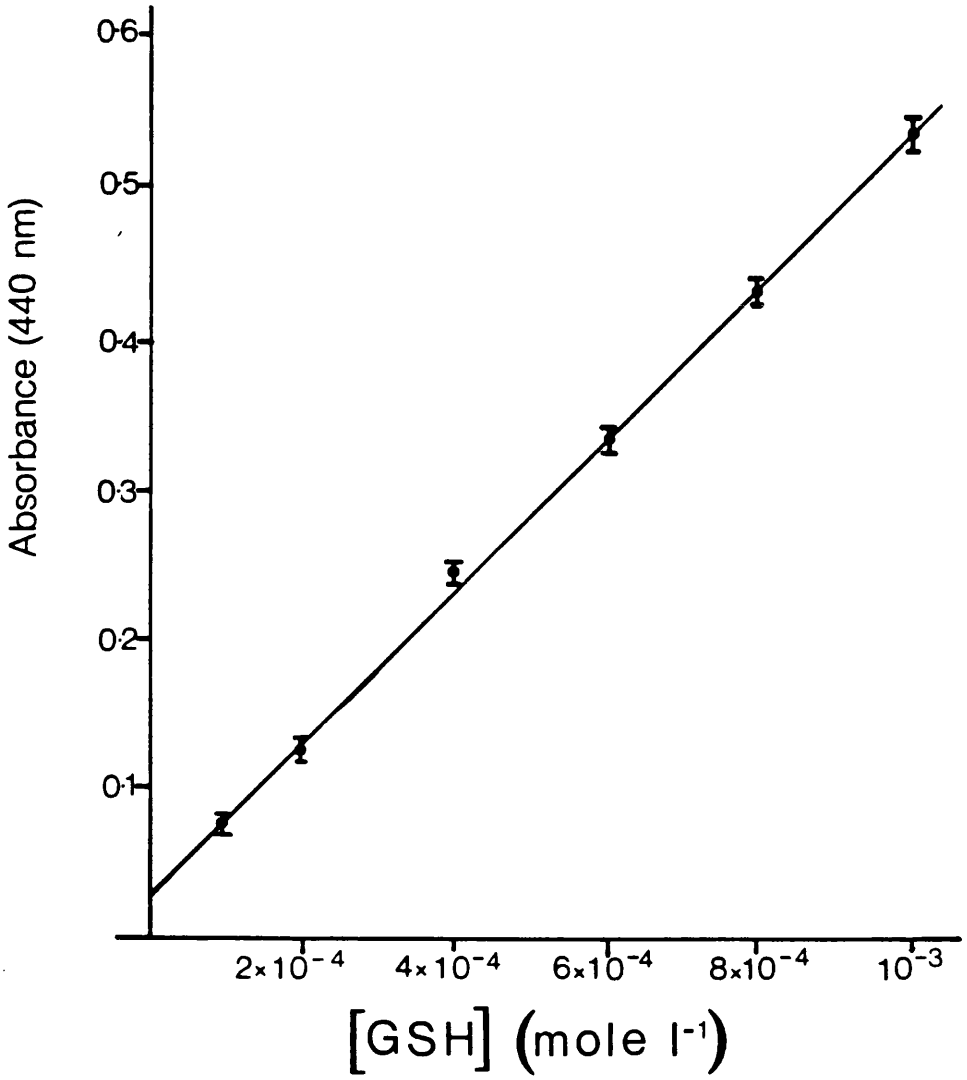


Fig. 3.1.a. The calibration curve for total serum SH assay. The results are expressed as mean ± 1 s.d. (n = 5, r = 0.99).

SPECIES NORMAL)	Serum SH LEVEL (μmol l^{-1})	
	x ± s.d	RANGE
HUMAN (n = 14)	538 ± 42	446—614
RABBIT (n = 15)	376 ± 45	308—468
GUINEA-PIG (n = 13)	270 ± 44	208—354
RAT (n = 19)	302 ± 33	230—358
MOUSE (n = 24)	388 ± 59	276—556

TABLE 1. The total serum protein SH level of different species. The results are expressed as mean ± 1 s.d.

serum SH level was 538 μmol (SH) l^{-1}. It is of interest to note that the mean total serum SH level of the mouse was significantly higher than that of the rat.

3.1.3 Determination of rate of serum SH reaction (reactivity)

Owing to the differences in serum SH reactivity of the animal species studied, two different DTNB concentrations had to be used to obtain comparative data. Preliminary experiments showed that the SH reactivity of the rat and mouse serum SH groups with a final DTNB concentration of 400μM was extremely fast and plateau was reached instantly. Since there is a non-linear increase in reaction rate with the increase of DTNB concentration, all reactions should be carried out at one DTNB concentration. However, this was not possible as the reaction rate of different serum samples varied considerably with a final DTNB concentration of 400μM. To enable a comparison, some species had their serum SH reactivity determined at DTNB concentration of 400μm. As the human and rabbit sera had reasonably fast reaction rates with the 400μM concentration, it was still possible to determine the serum SH reactivity of these species at the lower (100μM) DTNB concentration although the reaction rates were much slower. The serum SH reactivity of the rat and the mouse could only be measured at 100μM DTNB concentration. In contrast, the guinea-pig serum SH reactivity could only be determined at DTNB concentration of 400μM. The reaction rate was far too slow when 100μM DTNB

concentration was used instead.

For the purpose of comparison, the changes in absorbance during the first minute (between 0 and 1 minute) and the second minute (between 1 and 2 minutes) respectively, were used to calculate the reaction rates. The increase in absorbance between 1 and 2 minutes yielded more consistent data because the increase in absorbance was linear during this time interval. The change is absorbance during the first minute could vary considerably between individual samples (Hall and Gillan, 1979). The initial rapid reactivity may partly represent the interaction of non-protein low molecular thiols with DTNB (Jocelyn, 1962).

Table 2 shows the results of the serum SH reactivity of human, rabbit and guinea-pig measured at 400μM DTNB. The rabbit appeared to have the fastest serum SH reactivity at both first minute and second minute measurements. The statistical data in Table 3 show that the serum SH reactivity of the guinea-pig was significantly lower than those of both rabbit and human at both first and second minute measurements. The serum SH reactivity of rabbit was significantly faster than that of the man. Tables 4 and 5 show the results of the serum SH reactivity of human, rabbit, rat and mouse measured at 100μM DTNB. The rat has significantly faster serum SH reactivity than those of the rabbit and human at both first and second minute measurements. Although the serum SH reactivity of the

	SERUM PROTEIN SH REACTIVITY (μmol (SH)l $^{1}min^{-1}$)	
SPECIES (NORMAL)	1st Minute (0—1 minute)	2nd Minute (1—2 minute)
HUMAN (n = 14)	58 ± 6	43 ± 3
RABBIT (n = 18)	94 ± 16	52 ± 6
GUINEA-PIG (n = 12)	13 ± 3	11 ± 3

TABLE 2. The serum protein SH reactivity of different species measured at 400µM DTNB. The results are expressed as mean ± 1 s.d.

SPECIES (NORMAL)	1st Minute	2nd Minute
HUMAN vs RABBIT	P<0.01	P<0.05
HUMAN vs GUINEA-PIG	P<0.01	P<0.01
RABBIT vs GUINEA-PIG	P<0.01	P<0.01

TABLE 3. Statistical analysis of results of TABLE 2 using Student 't' test.

	SERUM PROTEIN SH REACTIVITY (μmol(SH)l^{-1}min^{-1})	
SPECIES (NORMAL)	1st Minute (0—1 minute)	2nd Minute (1—2 minute)
HUMAN (n = 14)	13 ± 3	8 ± 1
RABBIT (n = 18)	4 ± 1	4 ± 1
RAT (n = 18)	98 ± 14	35 ± 7
MOUSE (n = 11)	54 ± 19	29 ± 4

TABLE 4. The serum protein SH reactivity of different species measured at 100µM DTNB. The results are expressed as mean ± 1 sd.

SPECIES (NORMAL)	1st Minute	2nd Minute
HUMAN vs RABBIT	P<0.01	P<0.01
HUMAN vs RAT	P<0.01	P<0.01
HUMAN vs MOUSE	P<0.01	P<0.01
RABBIT vs RAT	P<0.001	P<0.001
RABBIT vs MOUSE	P<0.01	P<0.01
RAT vs MOUSE	P<0.01	N.S.

TABLE 5. Statistical analysis of results of TABLE 4 using Student 't' test.

rat is significantly faster than that of the mouse at the first minute measurement there is no difference in the second minute measurement. The rat serum SH reactivity is 7 times faster than that of the human in the first minute measurement and 4 times faster in the second measurement. It must be noted that both the rat and mouse sera oxidised rapidly when exposed to air. Rate measurements of their sera had to be made quickly (within an hour after their sera were extracted) to avoid oxidation of the free SH groups. By contrast, the serum SH groups of the human, rabbit and particularly guinea-pig remained relatively stable. The faster reaction rates of both the rat and mouse serum SH groups may be indicative of the intrinsically very reactive serum SH groups that these animals have. It is also interesting to see that at 400μM DTNB, the rabbit serum SH reactivity is significantly faster than that of the human while at 100μM DTNB the human serum SH reactivity is faster than that of the rabbit although not significantly.

Experiments were also carried out to find out if there is any difference in the total serum SH concentrations and reactivity between two different strains of rat i.e. male CFHB Wistar and male CFY Sprague-Dawley animals. The results in Tables 6 and 7 indicate that there was no significant difference in both the serum SH level and the reactivity between these two strains of rat. The lower values of the serum SH levels and reactivity were due to freezing (see below).

STRAIN OF RAT	Wistar (CFHB) (n = 6)	Sprague-Dawley (CFY) (n = 6)
SERUM SH LEVEL ($\mu mol.l^{-1}$)	222 ± 20	258 ± 11

TABLE 6. Comparing the total serum protein SH level of two different strains of rat i.e. male CFHB Wistar rat and male CFY sprague-Dawley rat. All rat sera were frozen at -20°C for 2 days prior being analysed. The results are expressed as mean ± s.d.

STRAIN OF RAT	Wistar (CFHB) (n = 6)		Sprague-Dawley rat (CFY) (n = 6)	
SERUM SH REACTIVITY ($\mu mol(SH)l^{-1}min^{-1}$)	1st Minute	2nd Minute	1st Minute	2nd Minute
	96 ± 12	34 ± 2	94 ± 6	32 ± 3

TABLE 7. Comparing the serum protein SH reactivity of male CFHB Wistar rat and male CFY Sprague-Dawley rat. All rat sera were frozen at -20°C for 2 days prior being analysed. The results are expressed as mean ± 1 s.d.

3.1.4 Effect of freezing on total serum SH levels and reactivity

As shown in the results of Tables 8 and 9, freezing process depressed the serum SH level and reactivity of both the rat and guinea-pig sera, respectively. The sharp fall in SH level and reactivity of both the rat and guinea-pig sera occurred during the first two weeks after storage at -20°C. Thereafter, the serum SH level and reactivity reached almost constant level but remained much lower than those of the fresh control sera. This observation suggests that the freezing process may induce certain conformational changes in the serum protein molecules resulting in less free SH groups available for reaction with DTNB and hence a reduction in serum SH level and reactivity.

3.1.5 Adjuvant arthritis (AA) in rat and its treatment with D-Pen

As will be seen later, a rather different pattern of serum SH level and reactivity was found in rats that developed arthritis after a single injection of complete Freund's adjuvant (CFA) and AA rats receiving daily oral D-Pen treatment (50 $mg.Kg.^{-1}$). Before looking at the results of these experiments it is appropriate to examine (1) the effect of induction of polyarthritis in male Sprague-Dawley rats and (2) whether D-Pen treatment affects the development of AA in

WEEKS AFTER STORAGE AT 20°C	SERUM SH LEVEL (μmol.l^{-1})	SERUM SH REACTIVITY (μmol(SH)l^{-1}min^{-1})	
		1st MINUTE	2nd MINUTE
0 (FRESH)	288 ± 6	92 ± 7	33 ± 6
2	94 ± 3	59 ± 8	25 ± 3
4	90 ± 5	53 ± 3	24 ± 3
6	102 ± 4	53 ± 5	22 ± 3

TABLE 8. Depressed rat serum protein SH level and reactivity over 6 weeks of storage at -20°C. The results shown above were based on the analysis of aliquots of pooled rat sera on a fortnightly basis. All determinations were performed in triplicate. The results are expressed as mean ± 1 s.d.

WEEKS AFTER STORAGE AT −20°C	SERUM SH LEVEL (μmol.l^{-1})	SERUM SH REACTIVITY (μmol(SH)l^{-1}min^{-1})	
		1st MINUTE	2nd MINUTE
0 (FRESH)	182 ± 6	27 ± 5	19 ± 4
2	120 ± 4	17 ± 2	17 ± 3
4	96 ± 4	15 ± 3	14 ± 3
6	108 ± 4	14 ± 2	13 ± 2
8	94 ± 5	14 ± 3	14 ± 3

TABLE 9. Depressed guinea-pig serum protein SH level and reactivity over 8 weeks of storage at -20°C. The results shown above were based on the analysis of aliquots of pooled rat sera on a fortnightly basis. All determinations were performed in triplicate. The results are expressed as mean ± 1 s.d.

these rats.

When 0.1 ml. of CFA containing heat-killed M. tuberculosis at a concentration of 5.5 $mg.ml^{-1}$ was injected into the right hind paws of male Sprague-Dawley rats, an intense but transient inflammatory response occurred. This initial phase of acute inflammatory response was local and produced no other obvious sign in the animals. In the next two weeks necrosis of the innoculation sites was observed but the animals appeared healthy. On about 14 days after CFA injection, a marked change occurred in the severely affected animals. There was extensive inflammation in areas remote from the injected sites, arthritis and periarthritis suddenly appeared in the joints of the extremities, especially in the distal small joints of the hind paws, which were characterised by redness and swelling or varying severity and painfulness. The forepaws and tails of the rats showed swelling and became slightly enlarged. The severely affected animals lost weight although a retardation in weight gain was recorded with almost all the animals that developed arthritis.

The majority of the animals studied responded well to the CFA and developed arthritis. However, it must be stressed that the arthritis induced in these Sprague-Dawley rats was not of a very severe nature. Non-articular lesions involving skin, eyes, nose and urogenital tract were not observed in some of the animals that developed arthritis although the tails and

forepaws of these animals were affected and showed signs of inflammation.

D-Pen (50 $mg.Kg.^{-1}$ day^{-1}) was administered orally to some rats 7 days prior to induction of arthritis followed by treatment throughout the development of arthritis (up to 21 days).

The effects of D-pen treatment on the various parameters used to assess the arthritis i.e. size of hind paws, arthritis score and weight change will now be examined.

As illustrated in Fig. 3.2.a a steady increase in size of injected paws occured from days 2 to 21. The increase was more marked after day 10 - the time of onset of AA. The non-injected paws showed no increase in size until day 7, thereafter a slow but steady increase in size was observed. The hind paw circumference of the normal control animals showed little increase. In vivo D-pen treatment did not induce any reduction in size of either injected or noninjected paws of AA rats. In fact, in vivo D-Pen treatment appeared to slightly exacerbate the swelling of the injected paws from days 14 to 21 and this was confirmed by the arthritis score shown in Fig. 3.2.b. In vivo D-Pen treatment did not improve the arthritis score from days 2 to 10. However, it tended to increase the arthritis score from days 14 - 21. Fig. 3.2.c shows the effect of in vivo D-Pen treatment on body weight change. Reduction in weight gain in AA rats started on day 2

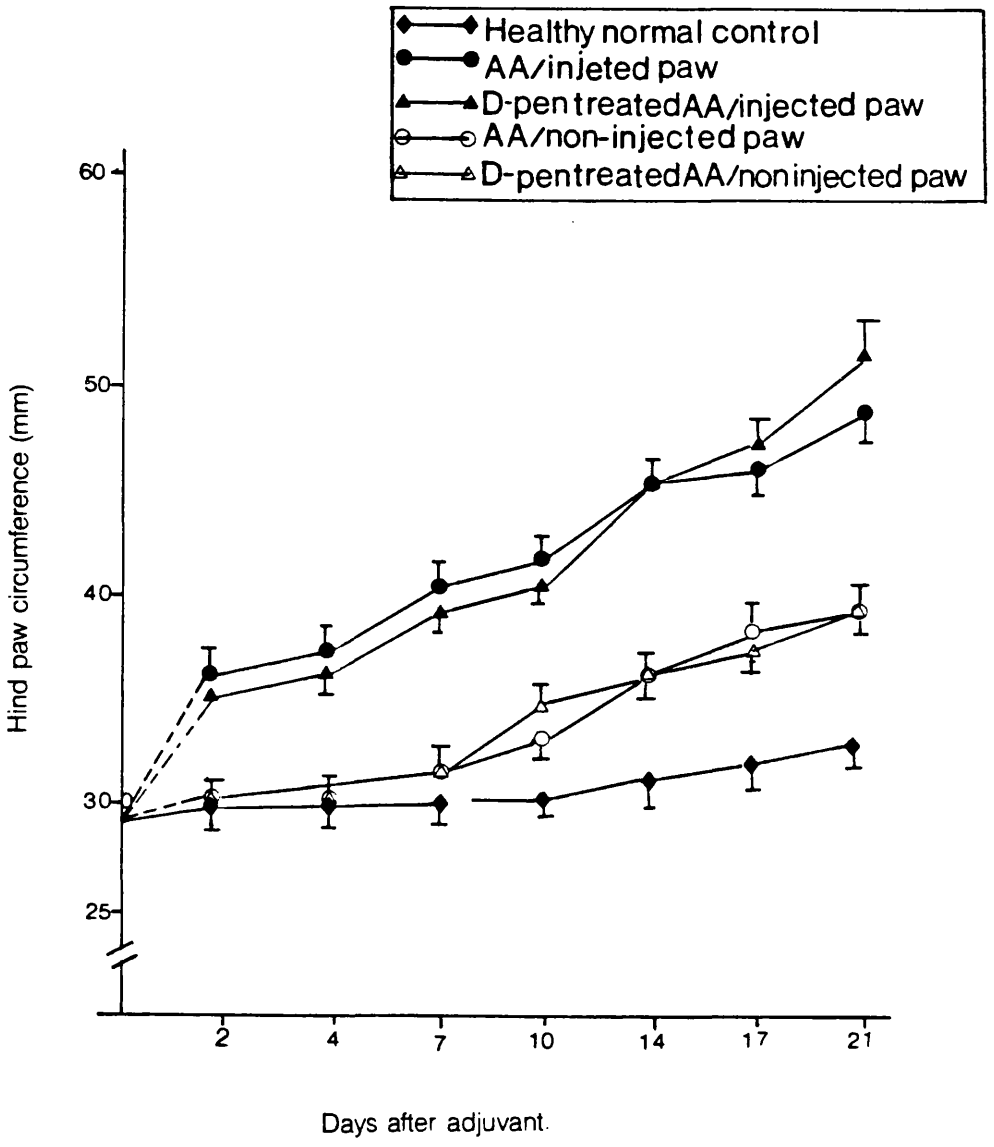


Fig. 3.2.a. The effect of D-penicillamine (D-Pen) treatment (50 mg.Kg.$^{-1}$day^{-1}, orally) on the hind paw circumference of Sprague-Dawley rats during development of AA (n = 6, at each time interval). The results are expressed as mean $\pm$ 1 s.d.

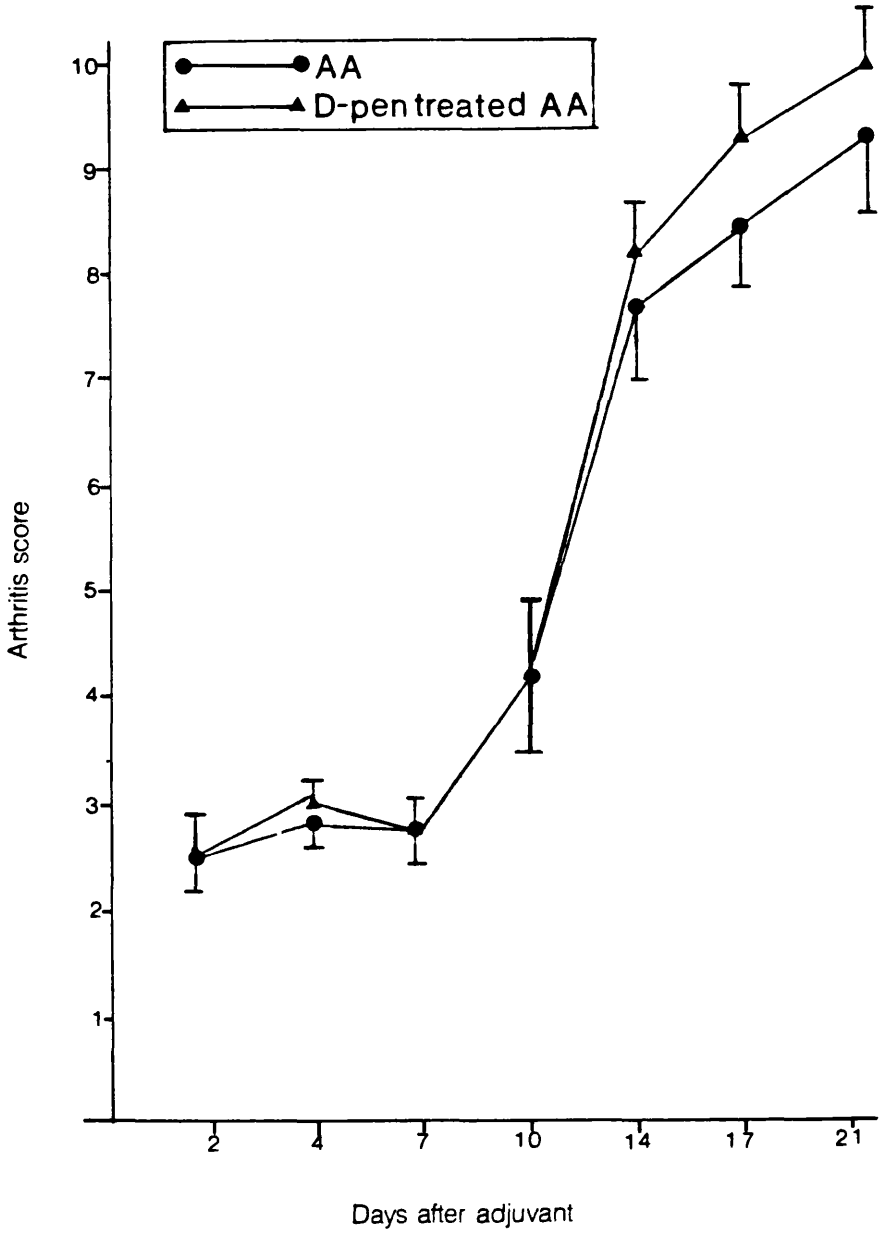


Fig. 3.2.b. The effect of D-Pen treatment (50 $mg.Kg.^{-1}day^{-1}$, orally) on arthritis scores during the development of AA in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean $\pm$ 1 s.d.

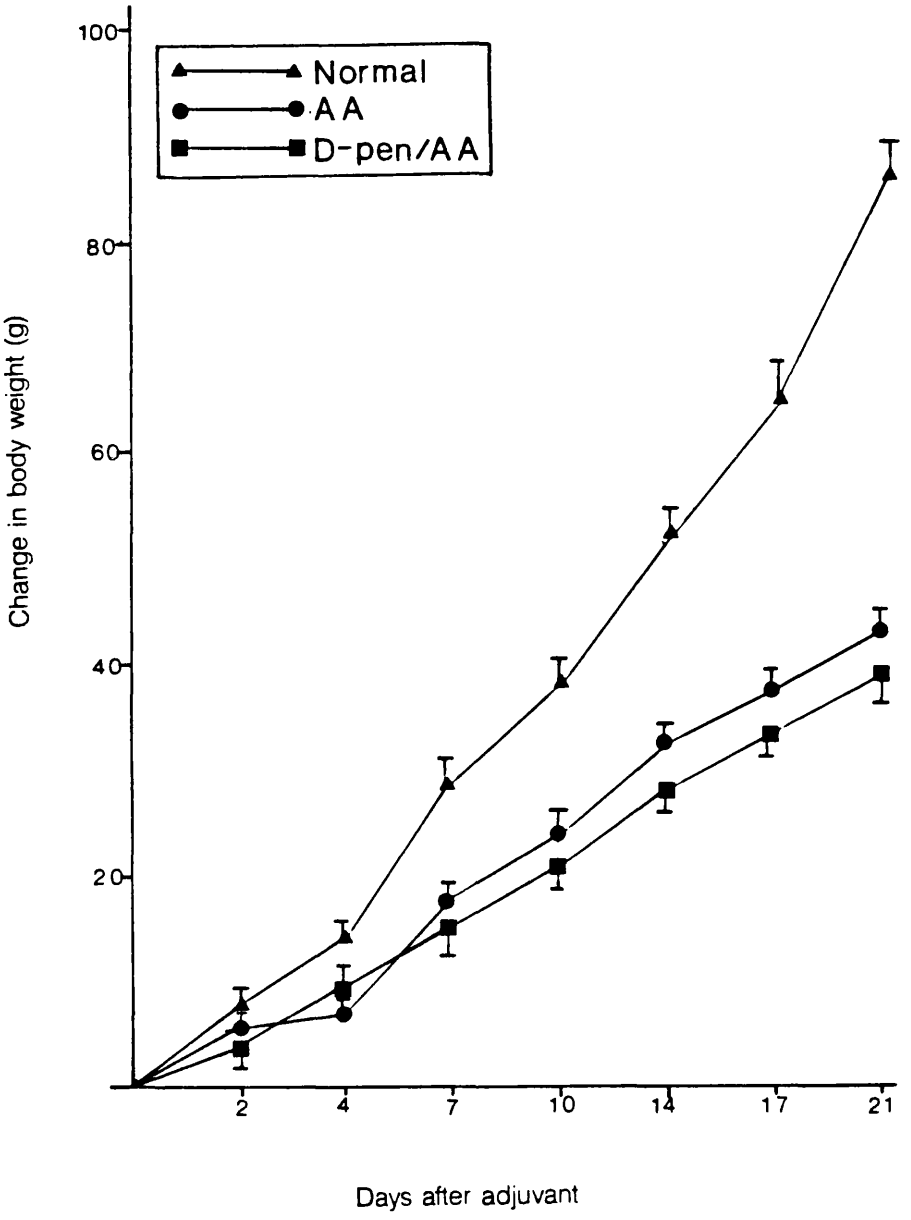


Fig. 3.2.c. The effect of D-Pen treatment (50 $mg.Kg.^{-1}day^{-1}$, orally) on body weight changes during the development of AA in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean $\pm$ 1 s.d.

when compared to normals. The AA rats gained only 40g. in weight compared to 86g. weight gain in normals. Again, *in vivo* D-Pen treatment did not improve the pattern of reduction in weight gain.

3.1.6 Total serum SH levels during development of AA

The serial changes which occurred in the total serum SH levels during the course of AA are shown in Fig. 3.3.a. A rapid fall in serum SH level occurred during the acute inflammatory phase, i.e. days 0 to 4 ($P < 0.01$ at day 2; $P < 0.01$ at day 4 Vs day 0). At about the time of onset of AA (between day 10 and day 17) a further decline in serum SH level was observed ($P < 0.001$ at day 14 Vs day 0). The serum SH level began to rise slightly from day 17 onwards although it still remained significantly lower than that of the control ($P < 0.01$ at day 21 Vs day 0).

As shown in Fig. 3.3.b similar serial changes in SH levels were observed in the D-Pen treated (50 $mg.Kg.^{-1}.day^{-1}$, orally) AA rat serum samples during the acute inflammatory period i.e. days 0 to 4 ($P < 0.05$ at day 2; $P < 0.01$ at day 4, Vs day 0). Thereafter, the serum SH level declined slowly until it reached its lowest level at day 10 ($P < 0.001$ Vs day 0) instead of day 14 with serum from AA rat not treated with D-Pen. *In vivo* D-Pen treatment increased the serum SH level of AA rats towards normality over days 14 - 21. D-Pen treatment

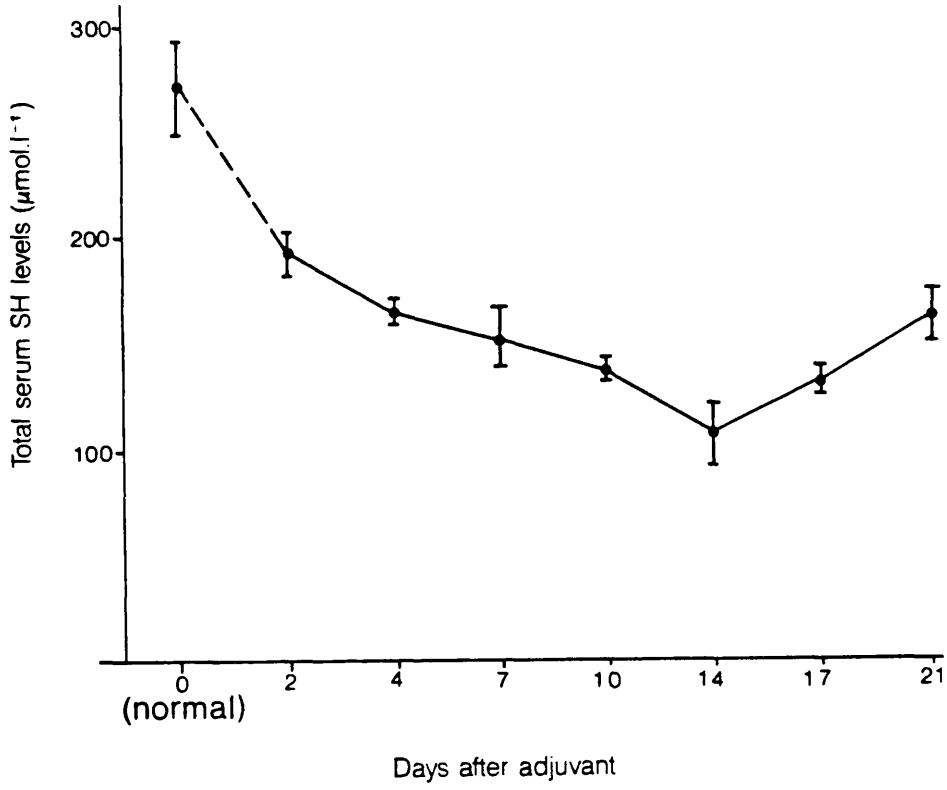


Fig. 3.3.a. The total serum protein SH levels during development of AA in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean ± 1 s.d. (Statistical analysis used: Student "t" test).

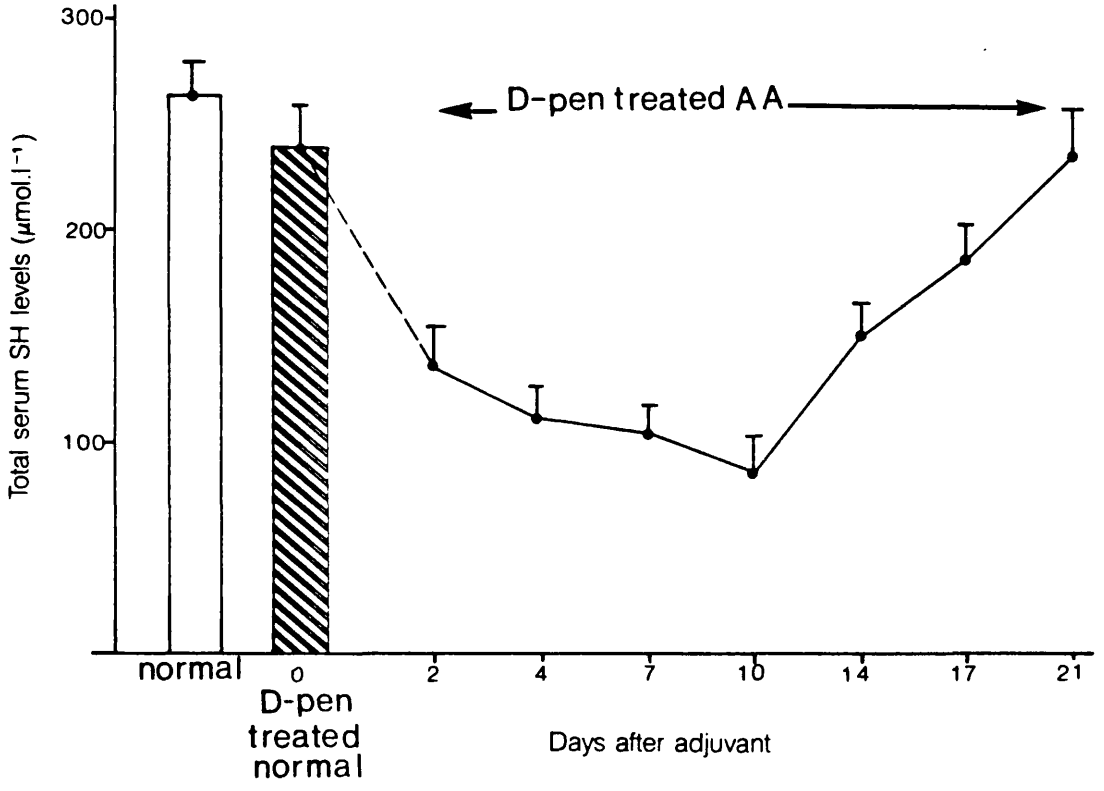


Fig. 3.3.b. The effect of D-Pen treatment on the total serum protein SH levels during development of AA in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean ± 1 s.d. (Statistical analysis used: Student "t" test).

had no significant effect on serum SH levels in normal rats.

3.1.7 Serum SH reactivity during development of AA

The serial changes in the serum SH reactivity during the course of AA are shown in Fig. 3.4.a. From the kinetic profile of the "first minute measurements", the changes in serum SH reactivity followed a similar pattern of the AA total serum SH level. A sharp fall in serum SH reactivity was observed in the acute inflammatory phase i.e. days 0 to 4 ($P < 0.01$ at day 2; $P < 0.001$ at day 4 Vs day 0). A further decline in serum SH reactivity was recorded about the time of onset of AA on days 10 to 14 ($P < 0.001$ on day 14 Vs day 0). However, the serum SH reactivity increased back to the level seen in the acute inflammation phase by day 17 ($P < 0.001$ Vs day 0) and was still rising at day 21 ($P < 0.05$ Vs day 0). The serial changes in serum SH reactivity measured via the "second minute method" presented a similar pattern as those measured via the "first minute method" although the depression of serum SH reactivity appeared to be less marked and more gradual. A sharp fall in serum SH reactivity was seen from days 0 to 2 ($P < 0.001$ Vs day 0). It then declined slowly from day 2 until it reached its lowest level at day 14 ($P < 0.001$ Vs day 0) i.e the time of onset of AA. Thereafter, the serum SH reactivity started to increase gradually towards normality although it was still significantly lower than that of the normal control level by day 21 ($P < 0.01$).

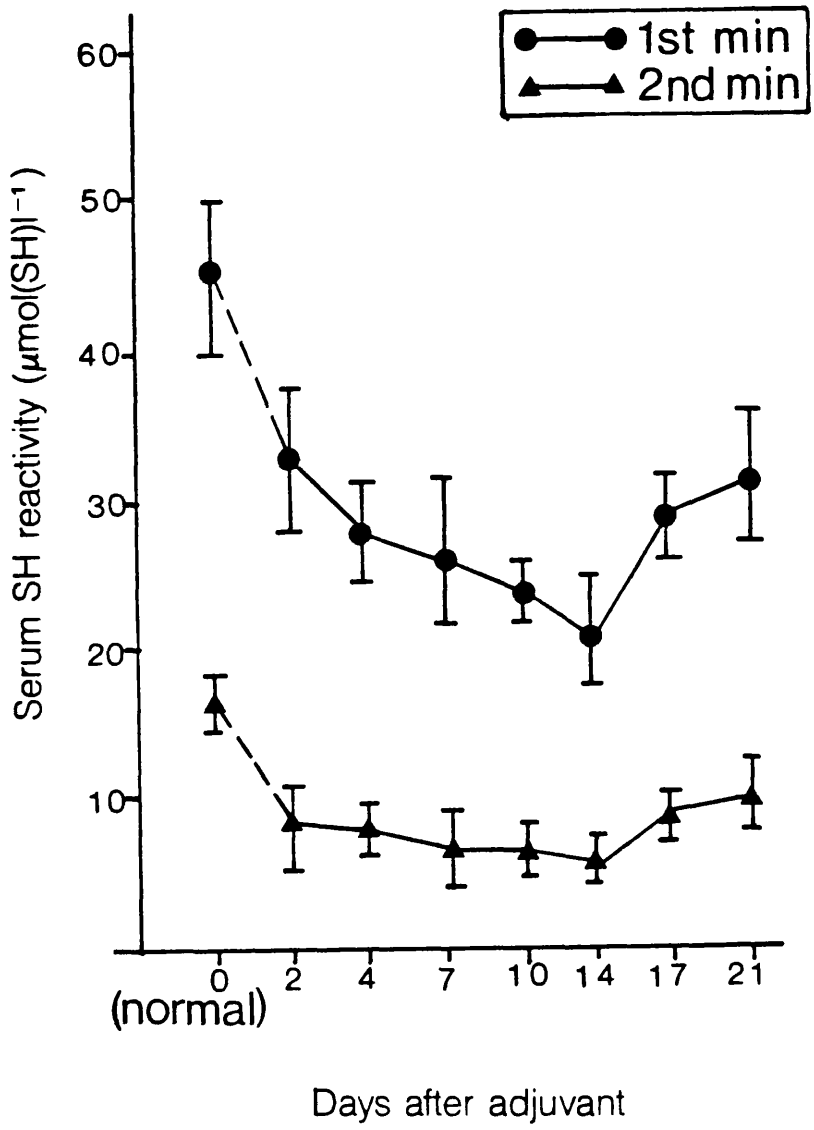


Fig. 3.4.a. Serum SH reactivity during development of AA in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean ± 1 s.d. (Statistical analysis used: Student "t" test).

From the kinetic profile of the "first minute curve" shown in Fig. 3.4.b the serial changes in the serum SH reactivity of the D-Pen treated AA rats were different from those seen in untreated AA. The serum SH reactivity fell sharply and rapidly to the lowest point at day 4 ($P < 0.01$ Vs day 0). Interestingly, the reactivity curve began to rise slowly towards normality by day 21. The kinetic profile of the "second minute curve" bore some resemblance to the "first minute curve". The serum SH reactivity curve fell steadily to its lowest level at day 4 ($P < 0.05$ Vs day 0). Then it started to rise slowly from day 4 to day 7 ($P < 0.05$ Vs day 0) and it reached normality at day 10 and was still rising at an even slower rate over days 17 - 21.

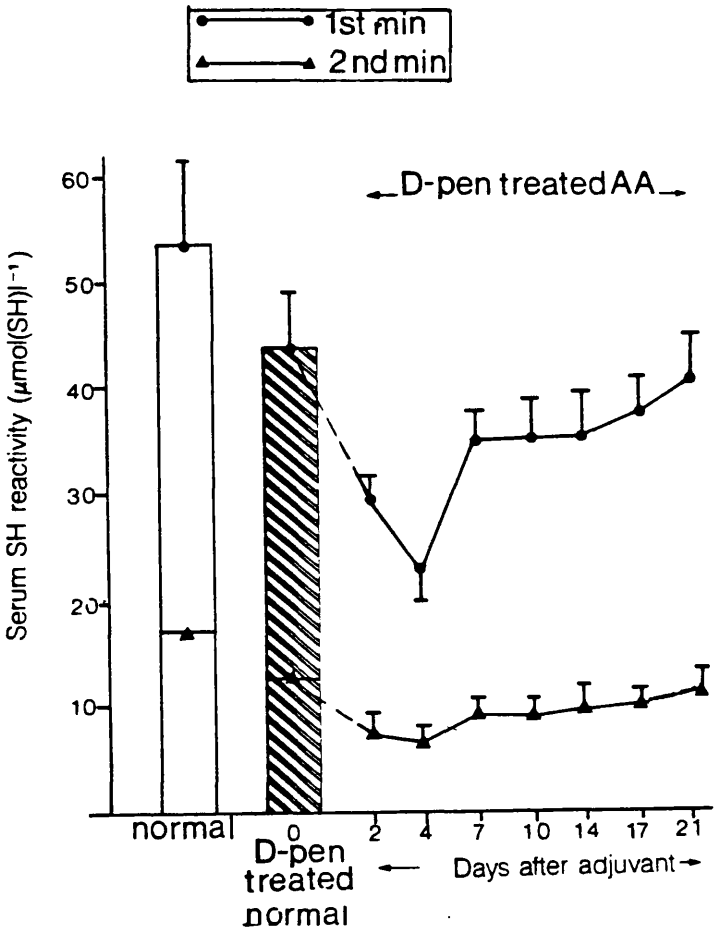


Fig. 3.4.b. The effect of D-Pen treatment on the serum SH reactivity during development of AA in male Sprague-Dawley rats. (n = 6, at each time interval). The results are expressed as mean ± 1 s.d. (Statistical analysis used: Student "t" test).

CHAPTER FOUR: RESULT B

RESULT B: EFFECTS OF 2-MERCAPTOETHANOL AND D-PENICILLAMINE ON THE IMMUNE FUNCTION OF RATS WITH ADJUVANT ARTHRITIS

4.1 Lymphocyte reactivity of rats with AA

The aim of this investigation was to determine (1) if the altered lymphocyte reactivity in RA also occurred during disease development of AA in rats, (2) whether the addition of 2-ME *in vitro* and D-Pen treatment *in vivo* might modulate immune function in AA rats and in mice. The investigation started off with determining the effect of 2-ME and a T-cell mitogen (ConA) on the proliferative responses of mouse spleen cells in culture and this was extended to study the effects of these agents and D-Pen treatment on the proliferative responses of AA rat spleen cells (SC) and lymph node cells (LNC) in culture.

4.1.1 Experiments with mouse spleen cell (SC) cultures

The ability of 2-ME to activate DNA synthesis was investigated in cultures of SC from male CFLP mouse. The results shown in Fig. 4.1.a show that 2-ME stimulated the incorporation of [^{3}H] thymidine into DNA in a dose-dependent manner. Peak [^{3}H] thymidine incorporation occurred at a 2-ME concentration of 5 x 10^{-5}M. Higher Lower 2-ME concentrations appeared to be inhibitory. The [^{3}H] thymidine incorporation stimulated by (5 x 10^{-5}M) 2-

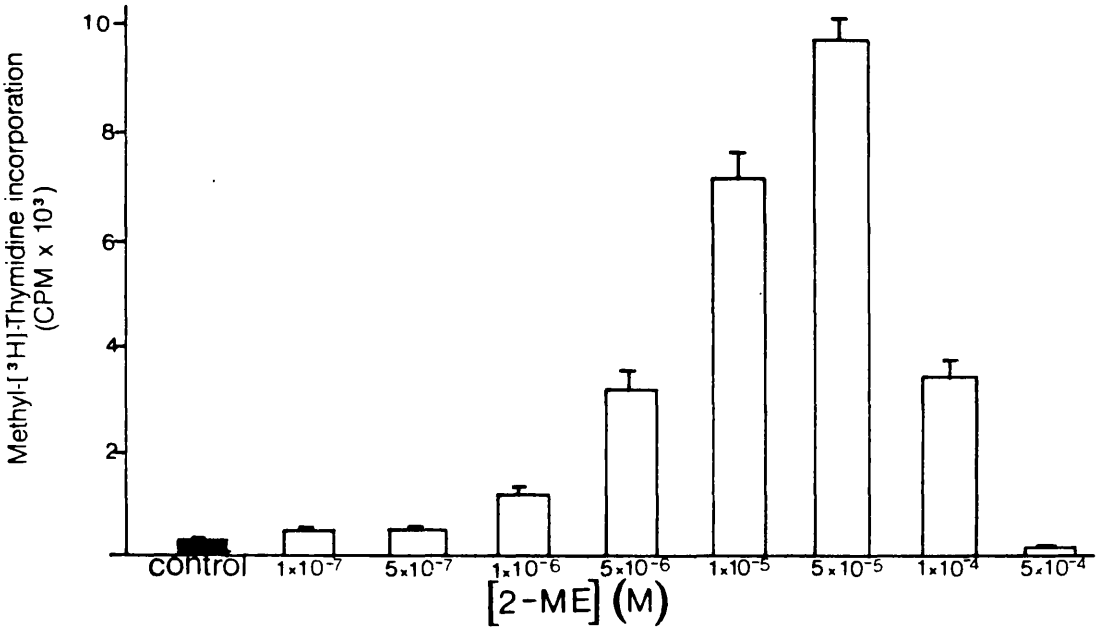


Fig. 4.1.a. The effect of 2-ME on the DNA synthesis of male CFLP mouse spleen cells in culture (n = 5). The optimal level of 2-ME required to stimulate spleen cell proliferation was 5×10^{-5}M. The results are expressed as mean $\pm$ 1 s.d.

ME was 33 times higher than the control level.

The results shown in Fig. 4.1.b indicate that ConA, like 2-ME, also promoted DNA synthesis in a dose-dependent fashion. Maximal [^{3}H] thymidine incorporation into DNA occurred at ConA concentration of 5 $\mu g.ml^{-1}$. A lower incorporation occurred at lower concentrations and inhibition was observed at very high concentration (50 to 100 $\mu g.ml^{-1}$). The peak ConA stimulated incorporation was 82 times higher than the control level.

The result in Fig. 4.1.c shows that the addition of 2-ME (5×10^{-5}M) to ConA stimulated mouse SC produced only an additive proliferative response at 1 to 10 $\mu g.ml^{-1}$ ConA. The peak proliferative response occurred at 5 $\mu g.ml^{-1}$ ConA in the presence of 2-ME (5×10^{-5}M).

4.1.2 Experiments with rat spleen and lymph node cell (LNC) cultures

The ability of 2-ME to stimulate DNA synthesis was also studied in male Wistar rat SC in culture. Fig. 4.2.a shows that 2-ME promoted the incorporation of [^{3}H] thymidine into DNA in the same way it did in the mouse system i.e. in a dose-related fashion. Again, peak proliferative response occurred at a 2-ME concentration of 5×10^{-5}M. Suboptimal doses resulted in suboptimal [^{3}H] thymidine incorporation and supraoptimal doses caused an inhibition of incorporation. The

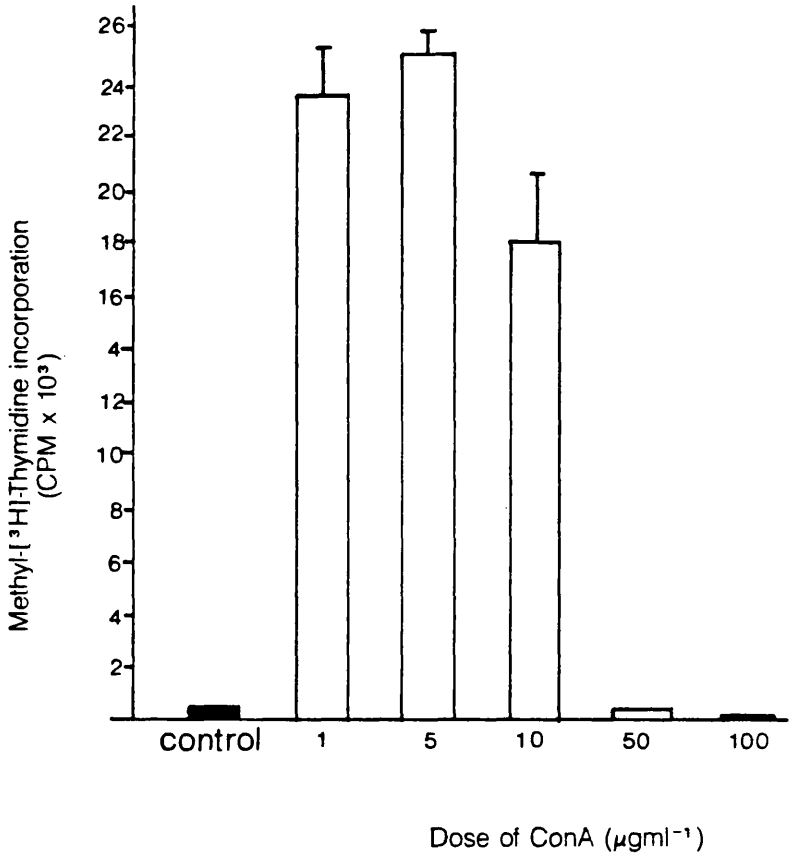


Fig. 4.1.b. The effect of ConA on the DNA synthesis of male CFLP mouse spleen cells in culture (n = 5). The optimal level of ConA needed to stimulate spleen cell proliferation was 5μg.ml.$^{-1}$. The results are expressed as mean $\pm$ 1 s.d.

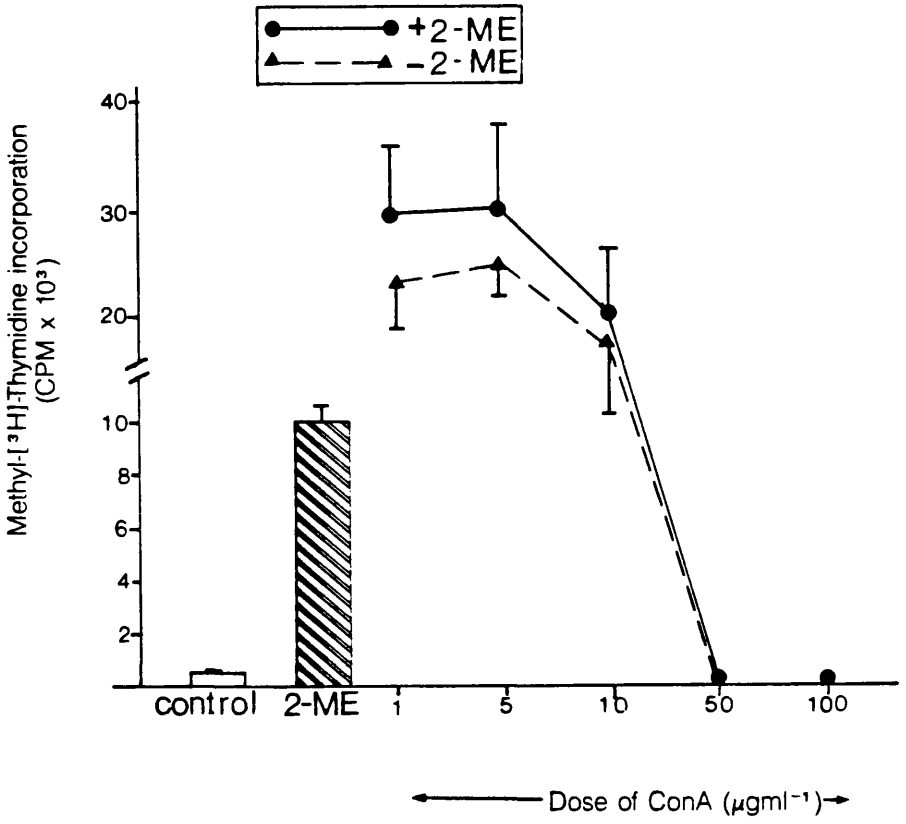


Fig. 4.1.c. The effect of various concentrations of ConA, in the presence of 5×10^{-5}M 2-ME on the proliferative response of male CFLP mouse spleen cells in culture (n = 5). The results are expressed as mean $\pm$ 1 s.d.

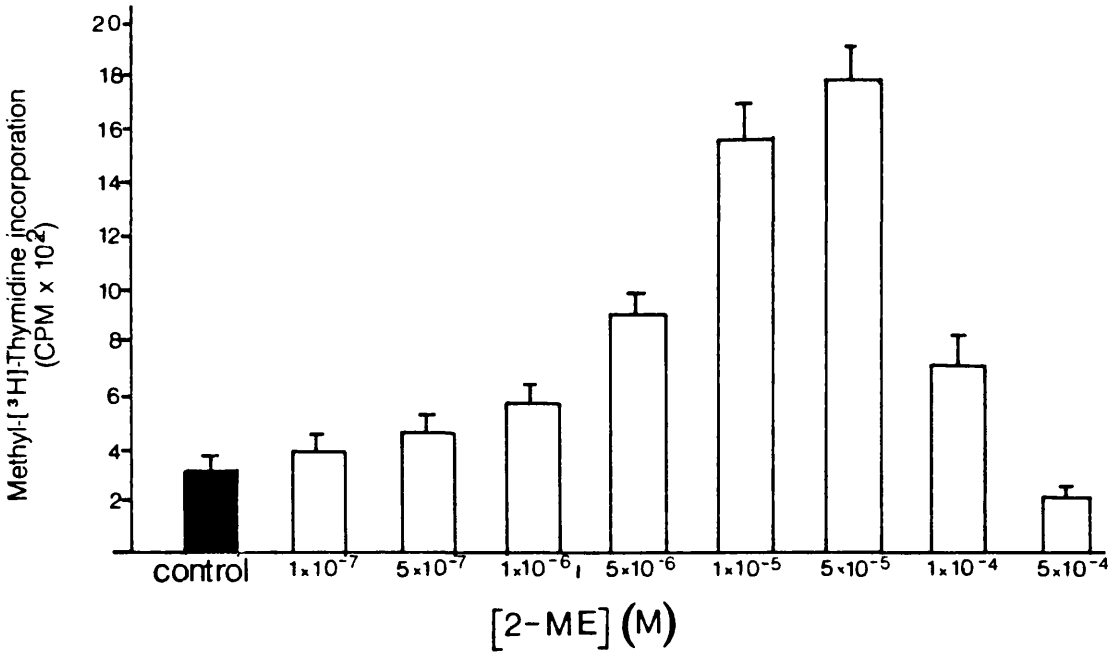


Fig. 4.2.a. The effect of 2-ME on the DNA synthesis of male Wistar rat spleen cells in culture (n = 5). The optimal dose of 2-ME required to stimulate spleen cell proliferation was 5×10^{-5}M. The results are expressed as mean $\pm$ 1 s.d.

peak 2-ME stimulated response was about 6 times the control value.

As shown in Fig. 4.2.b ConA stimulated DNA synthesis of male Wistar rat SC in culture in a dose-dependent manner. Maximal [^{3}H] thymidine incorporation occurred at 5 $\mu g.ml^{-1}$ ConA. High concentrations were inhibitory while lower concentrations induced suboptimal response. The peak ConA-induced response was about 6 times bigger than the control level.

The result in Fig. 4.2.c shows that the addition of 2-ME (5 x 10^{-5}M) to ConA stimulated rat SC produced a big enhancement of response at 1 to 10 $\mu g.ml^{-1}$ ConA. Maximal response occurred at 5 $\mu g.ml^{-1}$ ConA. 2-ME had no enhancing effect at high ConA concentrations (50 - 100 $\mu g.ml^{-1}$). It was probably because high doses of ConA poisoned the SC in culture. The peak 2-ME stimulated enhancement of ConA response (16830 $\pm$ 2899) was about 5 times bigger than the sum of individual responses of 2-ME (1660 $\pm$ 240) and ConA (1746 $\pm$ 462) respectively.

In order to establish the optimal time needed for SC in culture the kinetic profile of the proliferative responses of rat SC in culture was investigated. As the results in Fig. 4.3.a indicates that maximal proliferative response of control culture as well as those stimulated by 2-ME (5 x 10^{-5}M), ConA (5 $\mu g.ml.^{-1}$) and 2-ME + ConA occurred at day 3 (72 hours). Again, the addition of 2-ME greatly enhanced the ConA

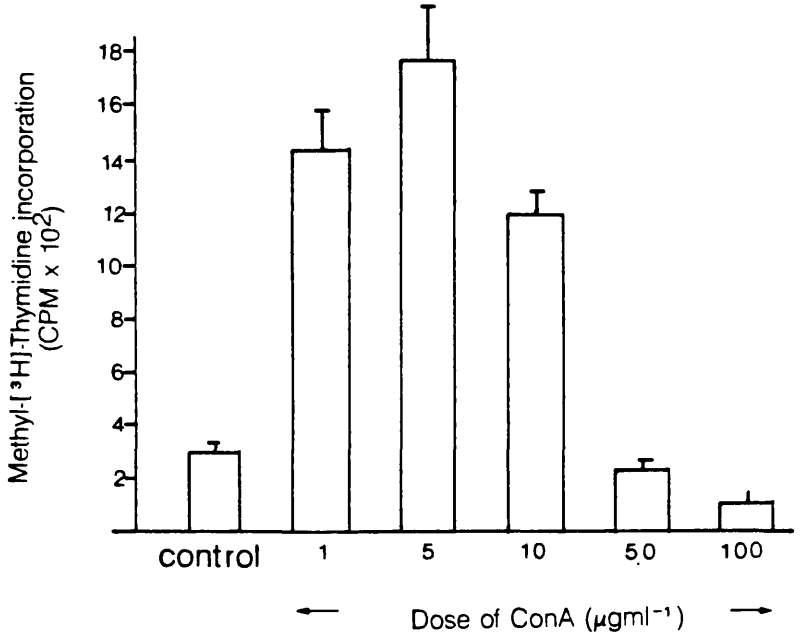


Fig. 4.2.b. The effect of ConA on the DNA synthesis of male Wistar rat spleen cells in culture (n = 5). The optimal dose of ConA needed to stimulate spleen cell proliferation was 5 $\mu g.ml.^{-1}$. The results are expressed as mean $\pm$ 1 s.d.

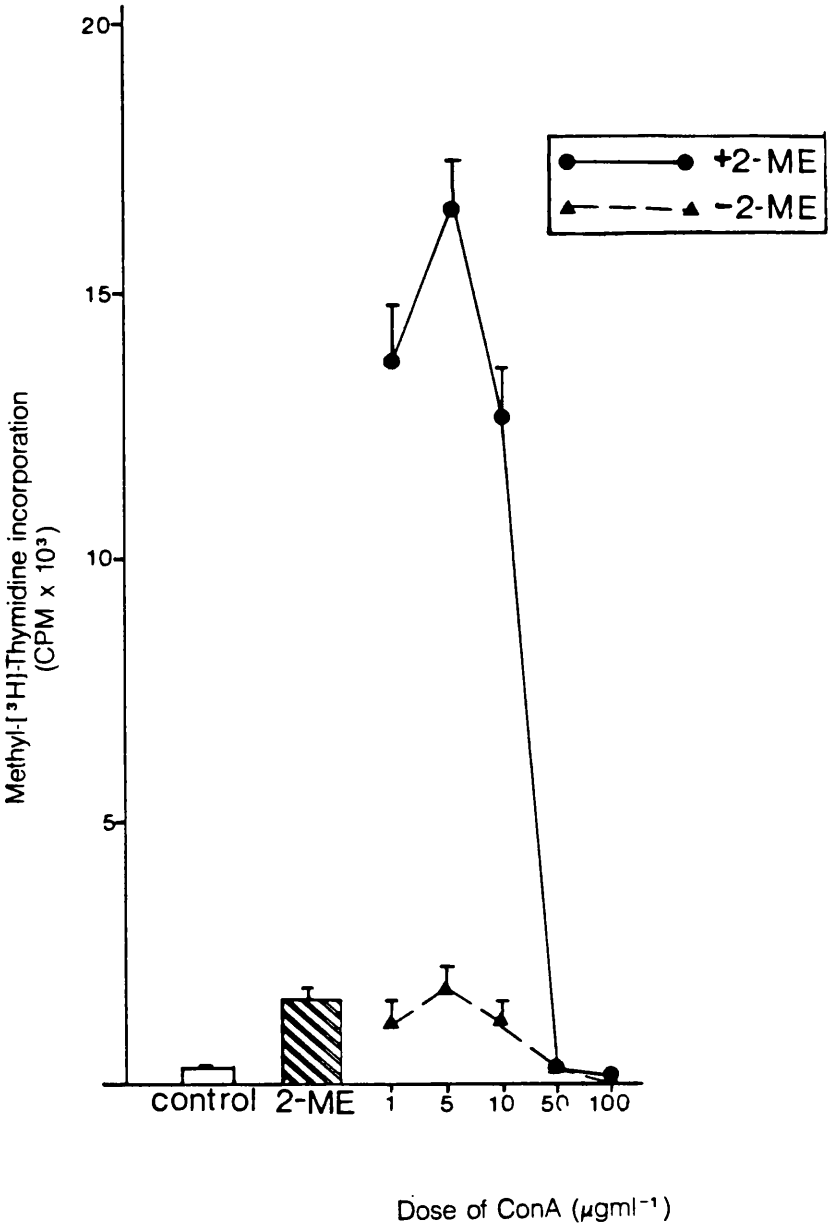


Fig. 4.2.c. The effect of various concentrations of ConA, in the presence of 5×10^{-5}M 2-ME on the proliferative response of male Wistar rat spleen cells in culture (n = 5). The results are expressed as mean $\pm$ 1 s.d.

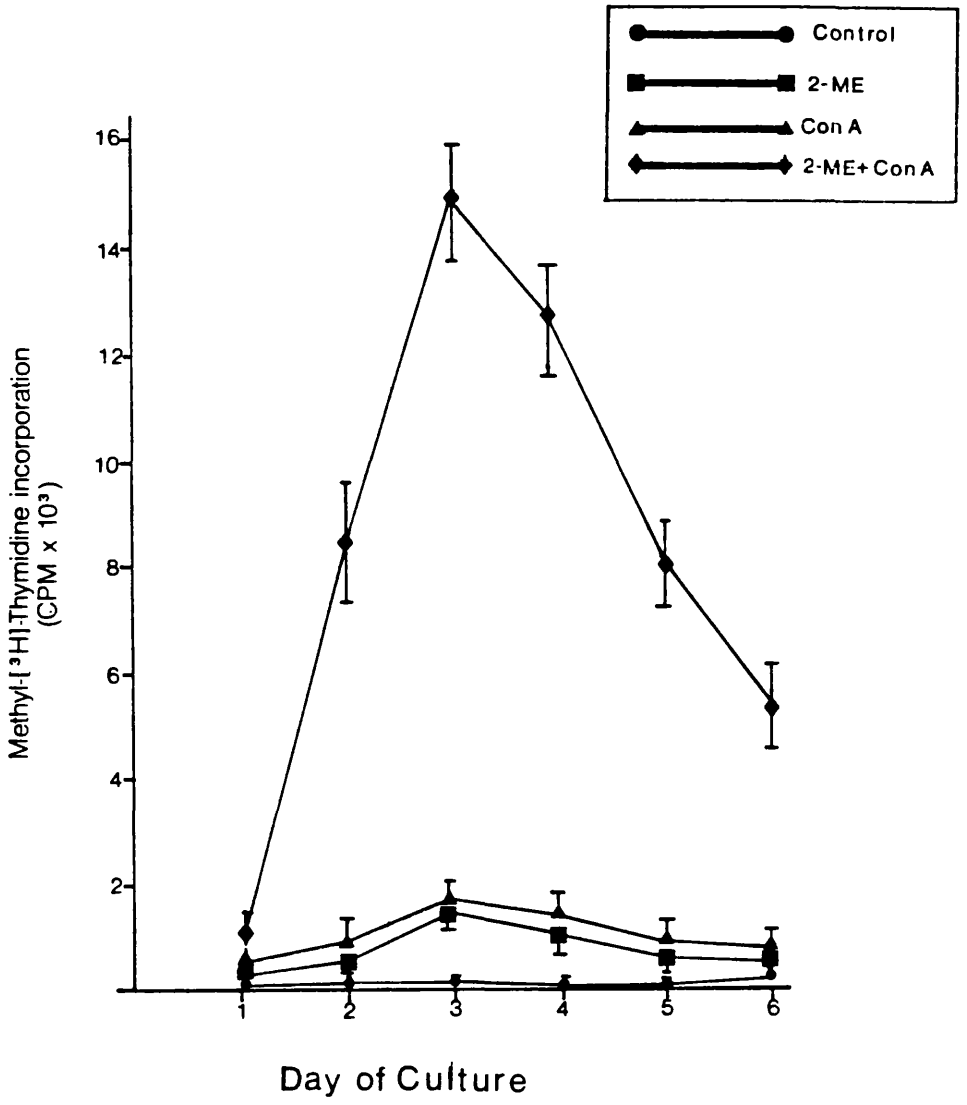


Fig. 4.3.a. Kinetic profile of the proliferative response of male Wistar rat spleen cells (SC) in culture (n = 3, at each time interval) to 2-ME (5×10^{-5}M), ConA ($5\mu g.ml.^{-1}$) and 2-ME + ConA. The results are expressed as mean $\pm$ 1 s.d.

stimulated responses starting from day 2, reaching the peak level at day 3, and declining thereafter.

As shown in Fig. 4.3.b the peak proliferative response of control Wistar rat LNC culture occurred at day 3. This was also true with the responses stimulated by 2-ME (5×10^{-5}M), ConA (5 $\mu g.ml.^{-1}$) and 2-ME + ConA. Again, enhancement of ConA stimulated responses by 2-ME was observed.

4.1.3 Effect of 2-ME, ConA and 2-ME + ConA on the proliferative responses of rat SC during development of AA

As illustrated in Fig. 4.4.a the proliferative response of AA rat SC stimulated with 2-ME (5×10^{-5}M) was markedly depressed 2 days after induction of disease ($P < 0.001$ Vs day 0). It increased modestly at day 7 and maintained at that level thereafter. The ConA (5 $\mu g.ml.^{-1}$) stimulated response increased sharply at day 2 of induction of disease. It then declined sharply until it became markedly depressed at day 14 ($P < 0.001$ Vs day 0) the time of onset of AA. Thereafter, it began to increase towards normal. The addition of 2-ME apparently enhanced the ConA stimulated response at all the time intervals studied. 2-ME not only restored the depressed ConA response at day 14, it also enhanced the ConA reponse to a level significantly higher ($P < 0.001$) than the control levels.

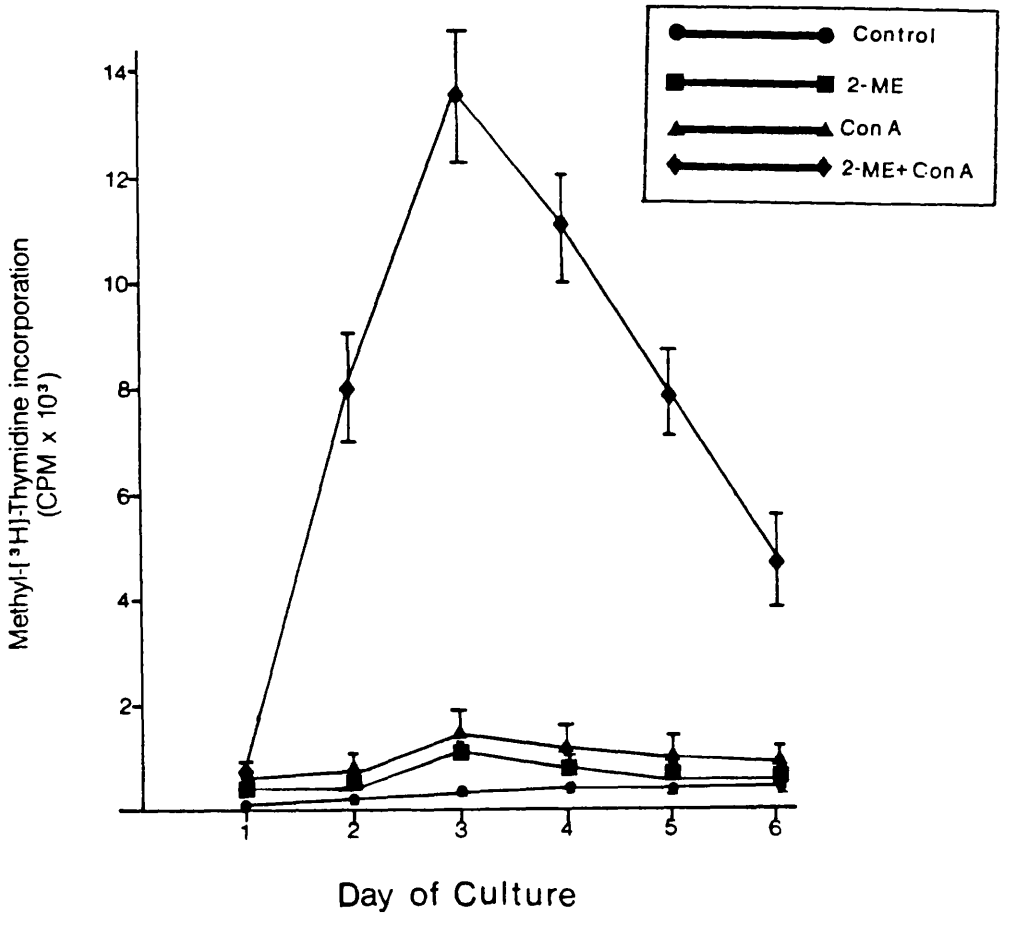


Fig. 4.3.b. Kinetic profile of the proliferative responses of male Wistar rat lymph node cells (LNC) in culture (n = 3, at each time interval) to 2-ME (5×10^{-5}M), ConA (5 $\mu g.ml.^{-1}$) and 2-ME + ConA. The results are expressed as mean $\pm$ 1 s.d.

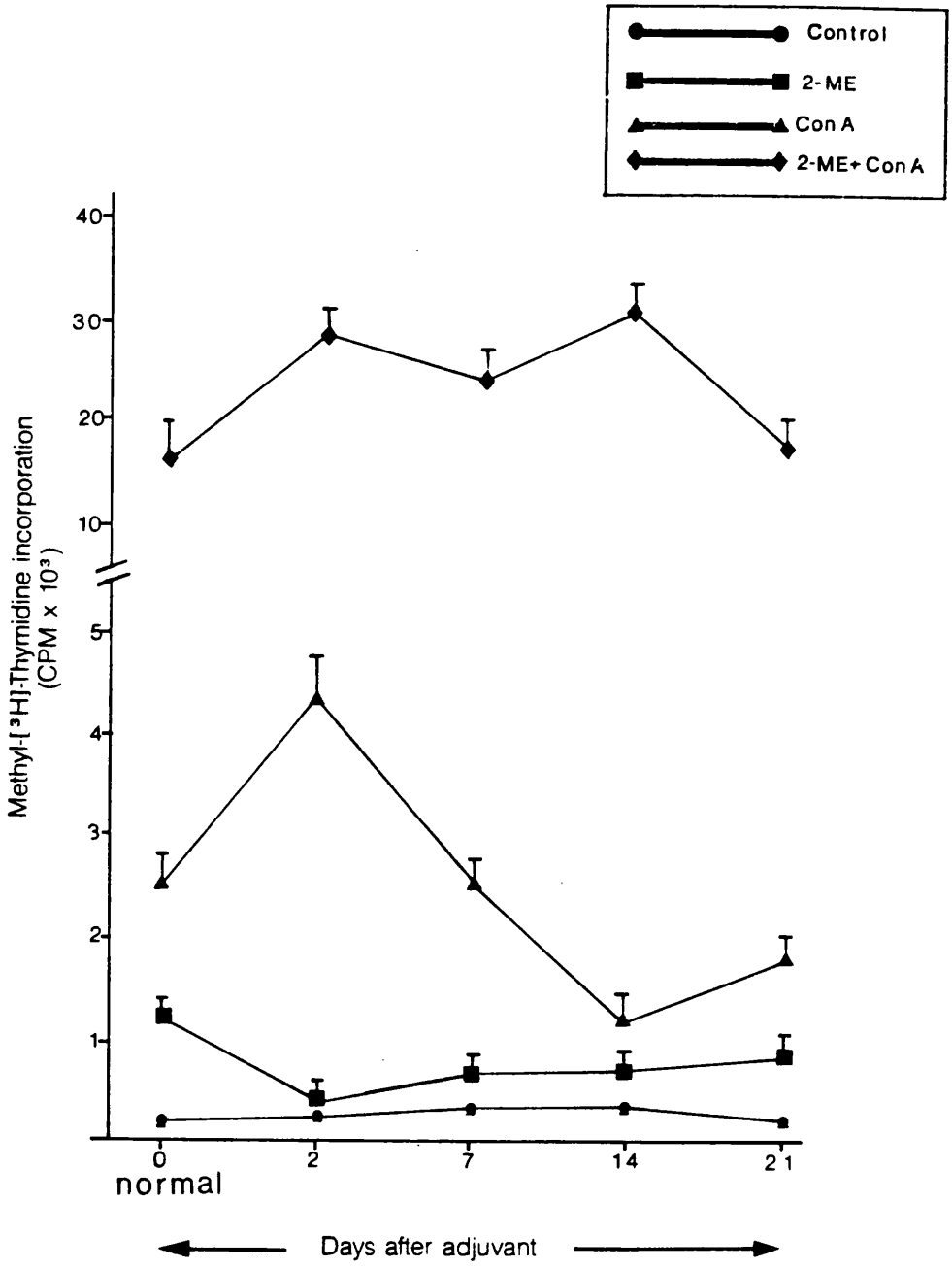


Fig. 4.4.a. The effects of 2-ME (5×10^{-5}M) and ConA (5 $\mu g.ml.^{-1}$) on the proliferative response of male Sprague-Dawley rat SC (n = 6, at each time interval) during the development of AA. The results are expressed as mean $\pm$ 1 s.d. (Statistical analysis: Mann-Whitney U test).

The tendency of the proliferative responses stimulated with 2-ME, ConA and 2-ME + ConA to normal levels as early as day 21 implies that the AA induced in these Sprague-Dawley rats was not of a very severe nature.

4.1.4 Effect of 2-ME, ConA and 2-ME + ConA on the proliferative response of D-Pen treated SC during development of AA

The result in Fig. 4.4.b indicates that there was a decrease in 2-ME (5×10^{-5}M) stimulated proliferative response of D-Pen treated AA rat SC at day 2 ($P < 0.001$ Vs day 0) and day 7 ($P < 0.01$ Vs day 0) of AA. Thereafter, it began to increase back to D-Pen treated normal level. The initial increase in Con A ($5\ \mu g.ml.^{-1}$) stimulated response at day 2 as seen in the AA rat SC was markedly decreased in D-Pen treated AA rat SC ($P < 0.001$). The markedly inhibited response to ConA on day 14 of AA rat SC was reverted to marked enhancement of response ($P < 0.001$). The ConA stimulated response fell slightly below the D-Pen treated normal level at day 21. The addition of 2-ME to the culture greatly enhanced the ConA stimulated response of D-Pen treated AA rat SC. Although the proliferative response induced by 2-ME + ConA initially decreased slightly below the D-Pen treated normal control level at day 2 it began to increase steadily thereafter and became significantly higher than the D-Pen treated normal control level at day 21 ($P < 0.001$).

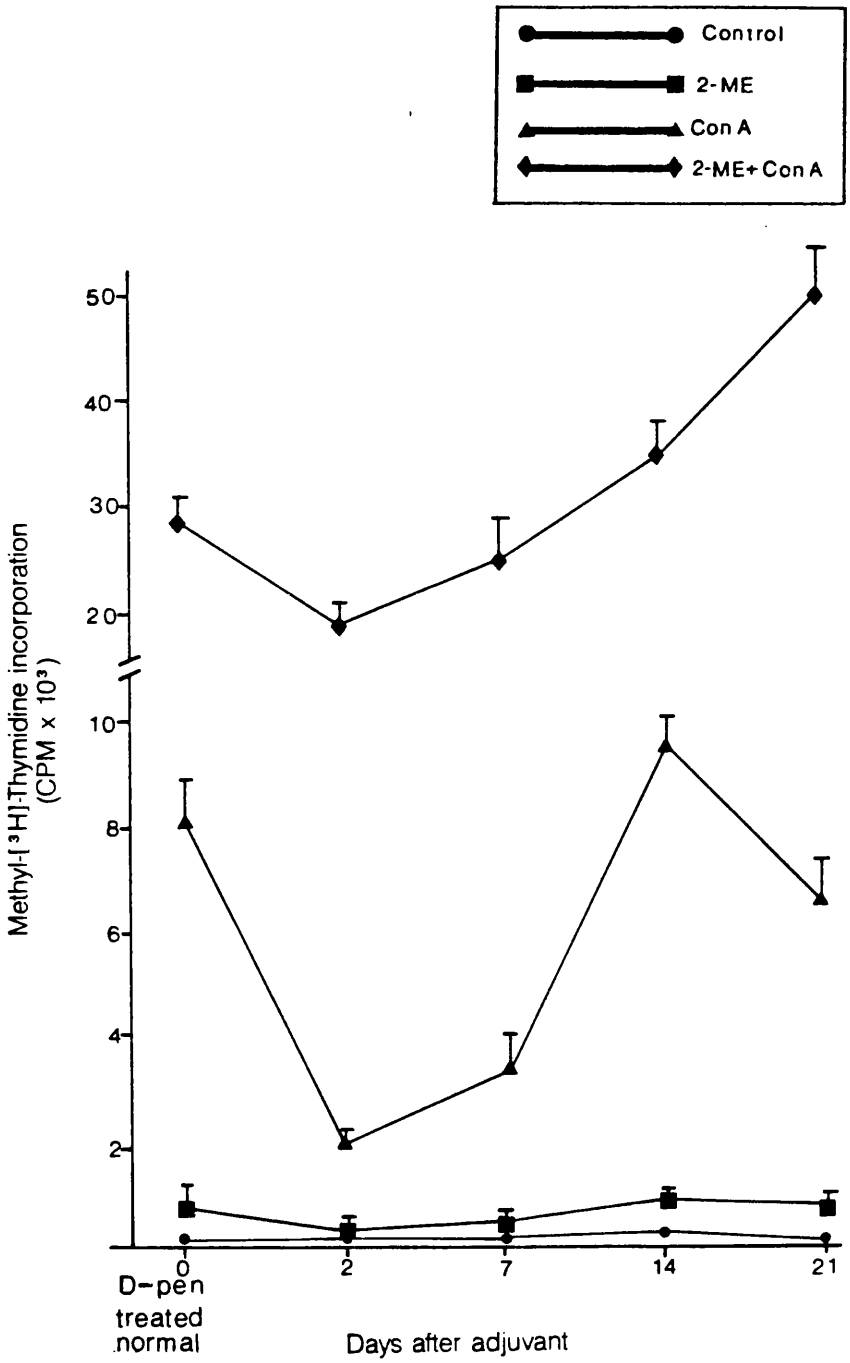


Fig. 4.4.b. The effects of 2-ME (5×10^{-5}M) and ConA (5 $\mu g.ml.^{-1}$) on the proliferative response of SC in D-Pen treated (50 $mg.Kg.^{-1}day^{-1}$, orally) AA induced in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean $\pm$ 1 s.d. (Statistical analysis: Mann-Whitney U test).

4.1.5 Proliferation response of normal and D-Pen treated normal rat SC

Short-term D-Pen treatment (50 mg.Kg.$^{-1}$.day^{-1} orally for 7 days) of normal Sprague-Dawley rats did not affect the background proliferative response of unstimulated SC as shown in Table 10. However, the 2-ME (5 x 10^{-5}M) stimulated response was significantly depressed in D-Pen treated normal rat SC ($P < 0.01$, Mann-Whitney U test). In vivo D-Pen treatment induced a marked increase in ConA stimulated response in normal rat SC ($P < 0.01$, Mann-Whitney U test). The enhancing effect of 2-ME on the ConA stimulated response was also significantly higher in the D-Pen treated normal rat SC than the untreated normal SC.

4.1.6 Effect of 2-ME and ConA on the proliferative response of rat LNC during development of AA

There was a significant increase in the ConA (5 μg.ml.$^{-1}$) stimulated proliferative response of AA rat LNC 2 days after the induction of disease ($P < 0.001$ Vs day 0) as illustrated in Fig. 4.4.c. However, by day 7 the ConA stimulated response dropped back to control level and became markedly depressed at day 14 ($P < 0.001$ Vs day 0) the time of onset of AA. However, it increased to slightly lower than normal control value at day 21. The addition of 2-ME (5 x 10^{-5}M) stimulated a variable pattern of response. A significant decrease in

ADDITION	NO TREATMENT	D-PEN TREATMENT (7 days)
0	217 ± 24	154 ± 71
2-ME	1213 ± 100	860 ± 159
ConA	2483 ± 358	8112 ± 2249
2-ME + ConA	15967 ± 2953	28911 ± 587

TABLE 10. The effects of 2-ME ($5x10^{-5}$M), Con A ($5\mu.ml^{-1}$) and 2-ME + Con A on the proliferative responses of normal and D-PEN treated (50 $mg.kg.^{-1}$ day^{-1}, orally for 7 days) normal Sprague-Dawley rat SC. The results are expressed as mean ± 1 s.d.

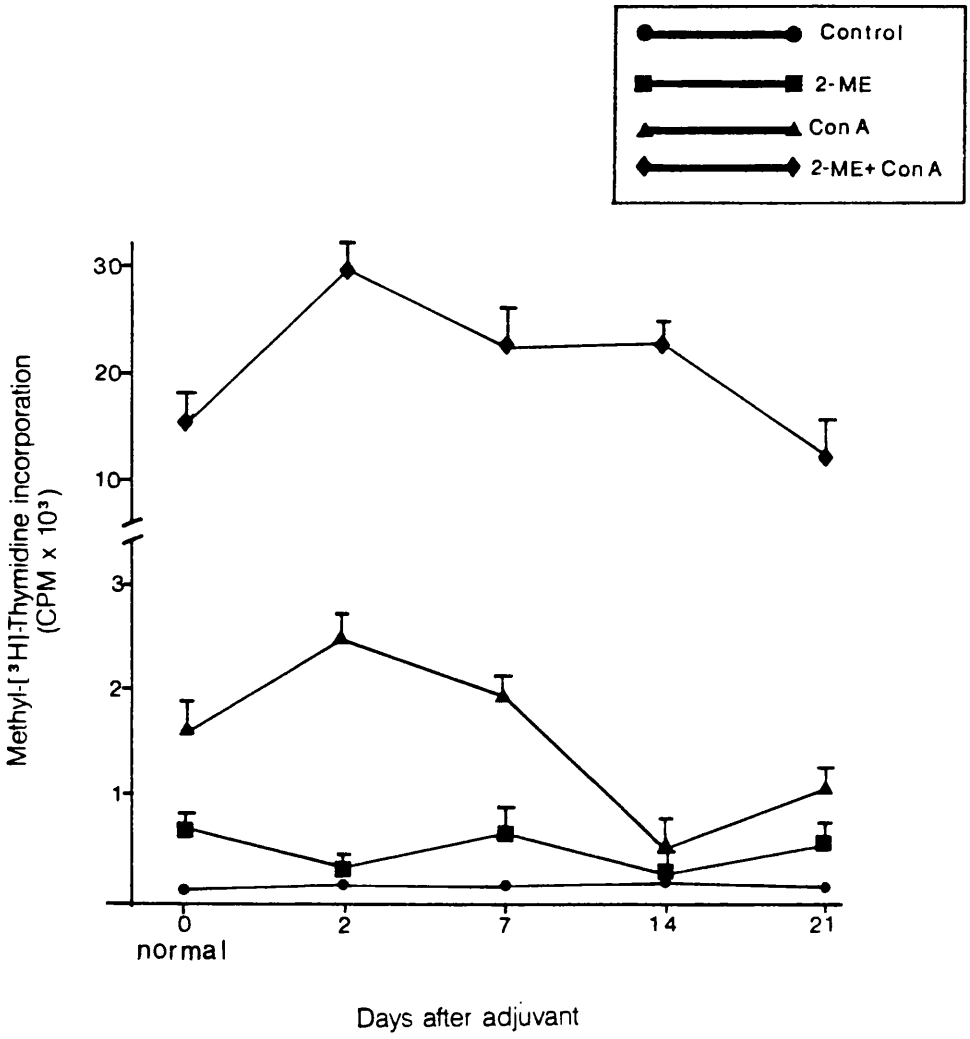


Fig. 4.4.c. The effects of 2-ME (5×10^{-5}M) and ConA (5 $\mu g.ml.^{-1}$) on the proliferative response of male Sprague-Dawley rat LNC (n = 6), at each time interval) during development of AA. The results are expressed as mean $\pm$ 1 s.d. (Statistical analysis: Mann-Whitney U test).

proliferative response occurred at day 2 of disease ($P < 0.001$) and it increased back to day 0 level at day 7. It showed a depressed response at day 14 and increased again to control level by day 21. 2-ME induced an enhancement of ConA stimulated response of AA rat LNC to above that of the normal control rat LNC at day 2 of disease ($P < 0.001$). The 2-ME + ConA induced response fell slightly at day 7 (but still significantly higher than the control value, $P < 0.001$) and remained at that level even at day 14 when the rat became arthritic. Thereafter it declined to control ConA stimulated level.

Again, the tendency of the proliferative response stimulated with 2-ME, ConA and 2-ME + ConA to normal levels at day 21 was observed, implying that the AA induced in these Sprague-Dawley rats was not of a very severe nature.

4.1.7 Effect of 2-ME and ConA on the proliferative response of rat LNC during D-Pen treatment of AA

Oral D-Pen treatment (50 $mg.Kg.^{-1}day^{-1}$) of rat with AA modified the pattern of proliferative responses of LNC as shown in Fig. 4.4.d. The initial increase in proliferative response on day 2 of disease was significantly reduced ($P < 0.01$), and the marked inhibition of the proliferative response to ConA (5 $\mu g.ml.^{-1}$) on day 14 was reverted to an enhanced response to ConA ($P < 0.001$). Thereafter, the ConA stimulated

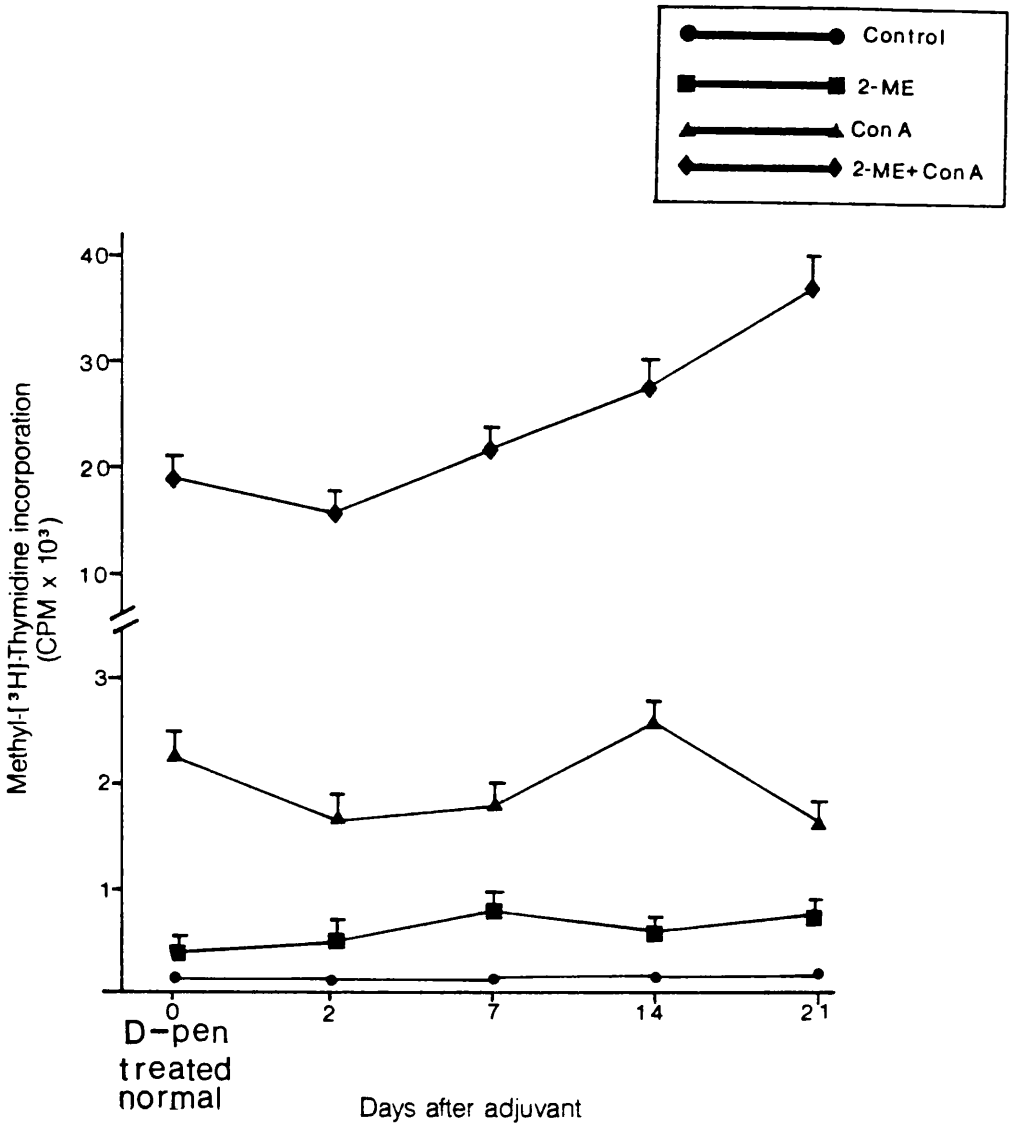


Fig. 4.4.d. The effects of 2-ME (5×10^{-5}M) and ConA (5 $\mu g.ml.^{-1}$) on the proliferative response of LNC in D-Pen treated (50 $mg.Kg.^{-1}day^{-1}$, orally) AA induced in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean $\pm$ 1 s.d. (Statistical analysis: Mann-Whitney U test).

response decayed below the D-Pen treated normal level. The addition of 2-ME (5 x 10^{-5}M) stimulated a significant increase in response at day 7 ($P < 0.01$) and day 21 ($P < 0.01$) when compared with that of D-Pen treated normal. 2-ME was found to enhance the ConA stimulated response of D-Pen treated AA rat LNC to above the D-Pen treated normal control level from days 7 to 21 ($P < 0.01$, at all times intervals) although a slight drop in response occurred at day 2 of disease.

4.1.8 Proliferative response of normal and D-Pen treated normal rat LNC

The results presented in Table 11 show that short-term D-Pen treatment (50 mg.Kg.$^{-1}$day^{-1}, orally for 7 days) of normal Sprague-Dawley rats produced no significant effect on the proliferative response of the unstimulated LNC. The addition of 2-ME (5 x 10^{-5}M) induced a significant decrease in the proliferative response of D-Pen treated AA rat LNC ($P < 0.05$, Mann-Whitney U test) although the ConA (5 μg.ml.$^{-1}$) stimulated response showed an increase. The combined effects of 2-ME + ConA stimulated an enhancment of proliferative response that was significantly greater in D-Pen treated normal rat LNC than the untreated normal rat LNC ($P < 0.05$, Mann-Whitney U test).

ADDITION	NO TREATMENT	D-PEN TREATMENT (7 days)
0	147 ± 13	136 ± 54
2-ME	755 ± 94	368 ± 169
ConA	1612 ± 244	2240 ± 693
2-ME + ConA	14962 ± 1880	18990 ± 3919

TABLE 11. The effects of 2-ME ($5x10^{5}$M), Con A (5μg ml^{-1}) and 2-ME + Con A on the proliferative responses of normal and D-PEN treated (50 $mg.Kg.^{-1}$ orally, for 7 days) normal Sprague-Dawley rat LNC. The results are expressed as mean ± 1 s.d.

4.1.9 The effect of indomethacin on the proliferative response of rat SC and LNC in culture

Prostaglandins (PGs) are established mediators of inflammatory responses and have been long suspected to play an important role in articular destruction (Zvaifler, 1973, Ziff, 1982). In addition they suppress certain *in vitro* immune functions, including mitogen proliferative responses and IL-2 synthesis (Goodwin and Webb, 1980; Rappaport and Dodge, 1982). By blocking PG synthesis with indomethacin suppression of the proliferative response may be removed. Hence, the blockade of PG synthesis with indomethacin was investigated in the male Sprague-Dawley rat SC and LNC culture system.

The solvent for indomethacin is ethanol. Before examining the effect of indomethacin, the effect of ethanol on the proliferative response of rat SC and LNC must be established. For ethanol, a structural analogue of 2-ME might be mitogenic.

As Figs. 4.5(a) and (b) show, the addition of ethanol (4.8×10^{-3}%) did not have any effect on the rat SC and LNC basal proliferative responses as well as those stimulated by 2-ME (5×10^{-5}M) ConA (5 $\mu g.ml.^{-1}$) and 2-ME + ConA.

The results of Figs. 4.5(a) and (b) also indicate that treatment of normal Sprague-Dawley rat SC and LNC *in vitro* with 10^{-6}M indomethacin did not produce any effect on the

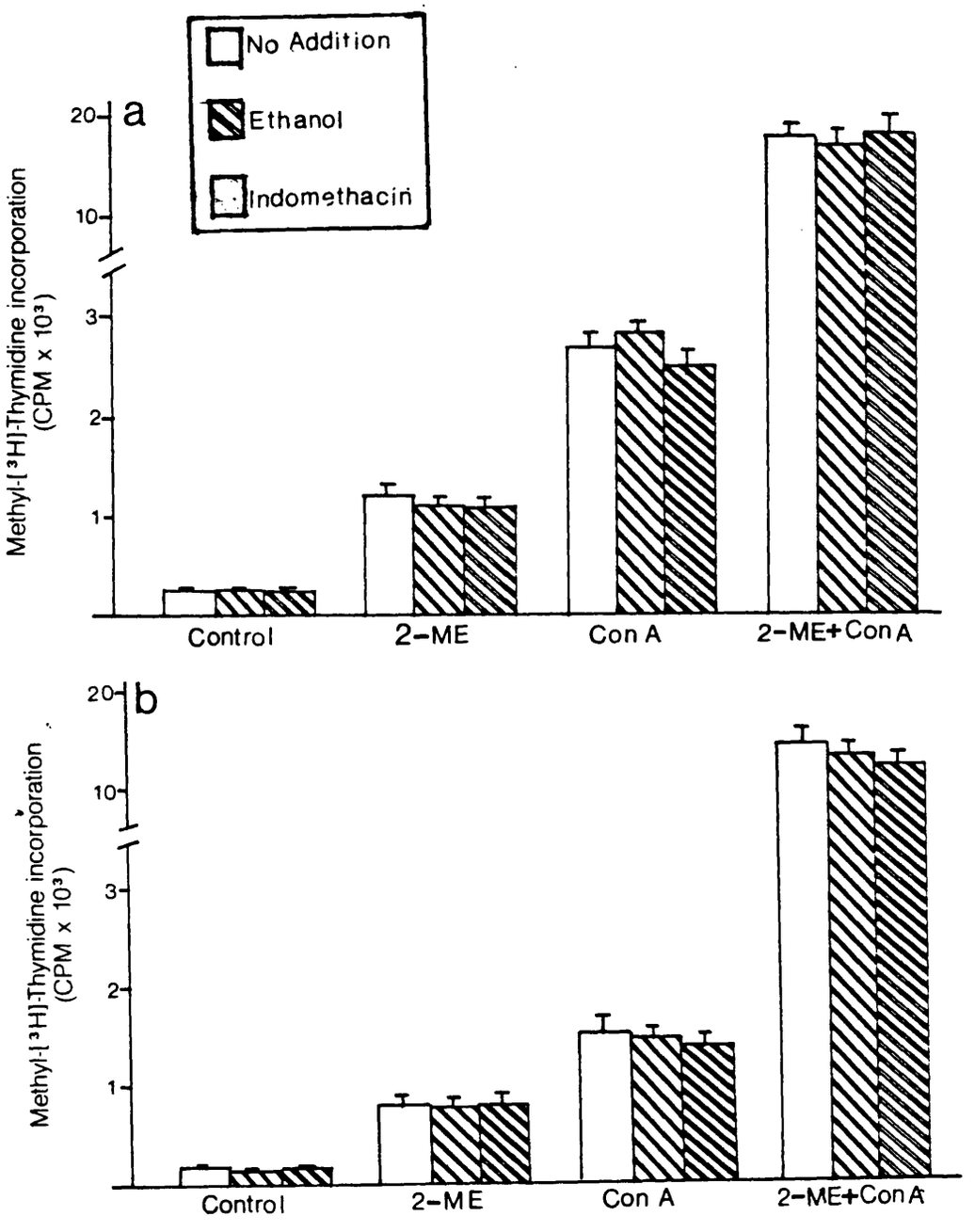


Fig. 4.5 (a), (b) The effects of Ethanol (4.8×10^{-3}%), Indomethacin (10^{-6}M), 2-ME (5×10^{-5}M) and ConA (5 μg.ml.$^{-1}$) on the proliferative response of normal male Sprague-Dawley rat (a) SC (n = 3) and (b) LNC (n = 3) in culture. The results are expressed as mean ± 1 s.d.

proliferative responses of these cells stimulated or not stimulated with 2-ME (5 x 10^{-5}M), ConA (5 μg.ml.$^{-1}$) and 2-ME + ConA. Hence PGs did not appear to play any significant role in the system.

4.2 The effect of PHMPSA on the proliferative response of rat SC and LNC in culture

It has been demonstrated that the addition of SH blocking agents to cell cultures results in the inhibition of lectin-induced proliferation (Chaplin and Wedner, 1978). Experiments were carried out to determine whether this inhibition was due to blockade of cell surface SH groups.

Figs. 4.5.1(a) and (b) show that blocking the cell surface SH groups of SC and LNC by incubation with the non-penetrating SH blocking agent, PHMPSA (5 x 10^{-5}M), resulted in the inhibition of the proliferative responses of these cells to 2-ME (5 x 10^{-5}M), ConA (5 μg.ml.$^{-1}$) and 2-ME + ConA.

4.2.1 Effect of indomethacin on the proliferative responses of AA rat (a) SC and (b) LNC in culture

Figs. 4.6(a) and (b) also show that indomethacin (10^{-6}M) did not restore the arthritic cells to normal indicating that PGs did not play a major part in contributing to the depressed mitogenic responses of 2-ME (5 x 10^{-5}M) and ConA (5 μg.ml.$^{-1}$).

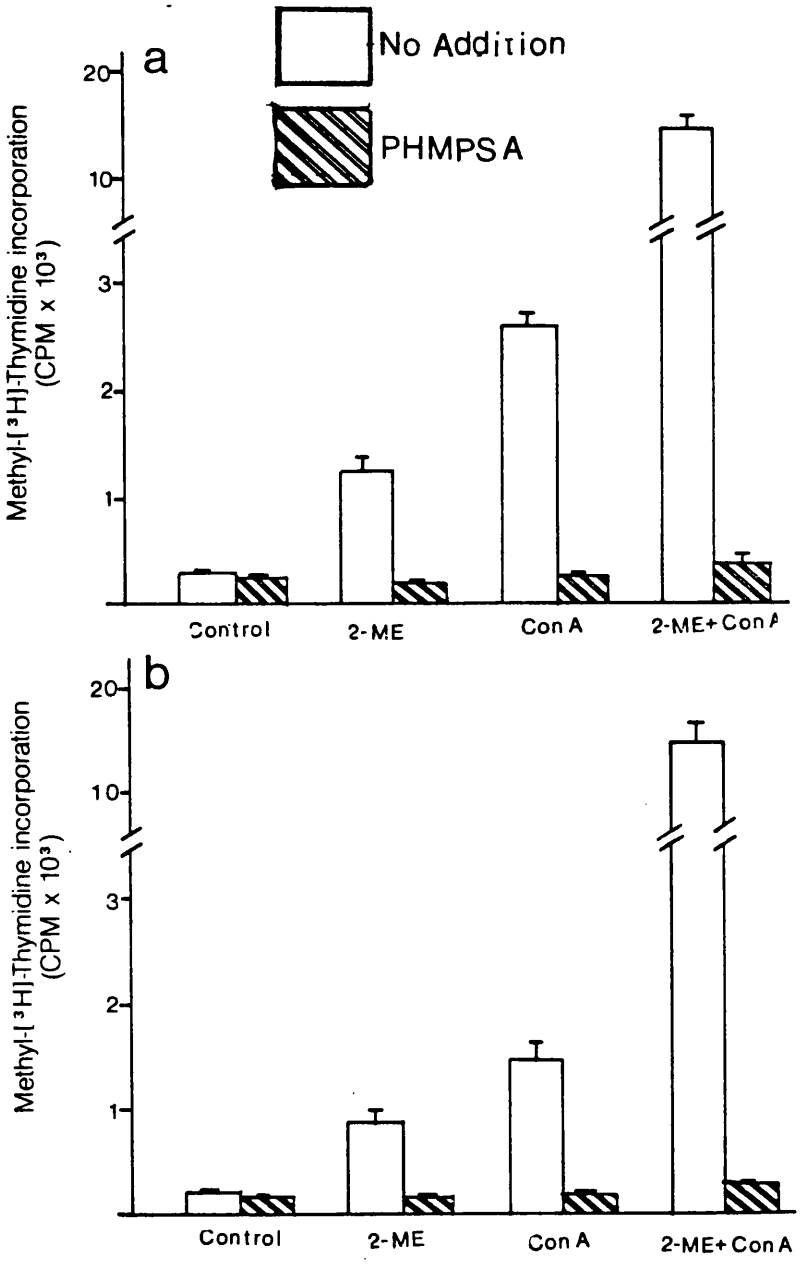


Fig. 4.5.1. (a), (b) The effects of PHMPSA (5×10^{-5}M), 2-ME (5×10^{-5}M) and ConA (5 $\mu g.ml.^{-1}$) on the proliferative response of normal male Sprague-Dawley rat (a) SC (n = 3) and (b) LNC (n = 3) in culture. The results are expressed as mean $\pm$ 1 s.d.

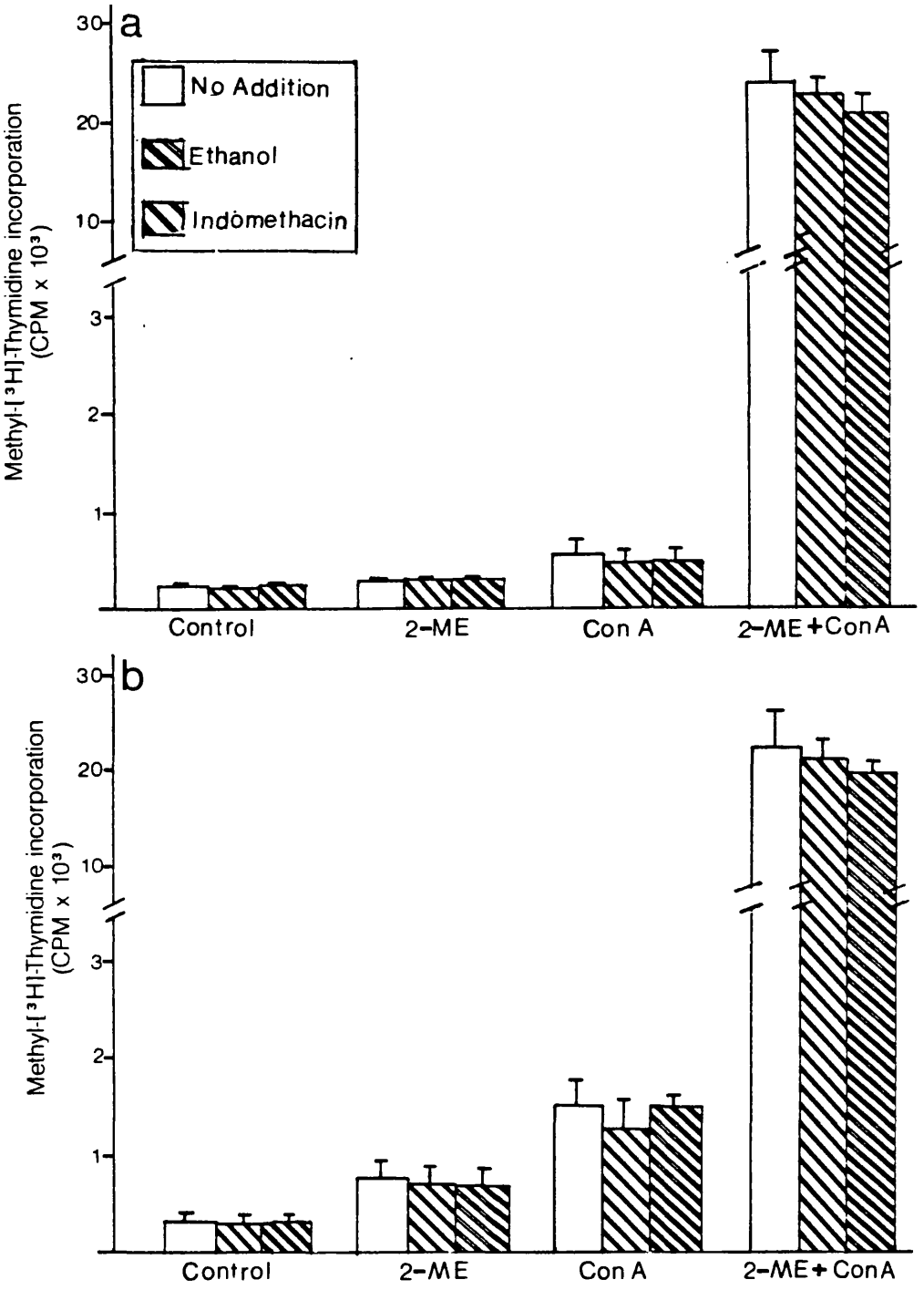


Fig. 4.6 (a), (b) The effects of Ethanol (4.8×10^{-3}%), Indomethacin (10^{-6}M), 2-ME (5×10^{-5}M) and ConA (5 μg.ml.$^{-1}$) on the proliferative response of AA (day 14) Sprague-Dawley rat (a) SC (n = 3) and (b) LNC (n = 3) in culture. The results are expressed as mean ± 1 s.d.

As Figs. 4.6(a) and (b) show, ethanol (48 x 10^{-3}%) did not have any mitogenic activity on the basal proliferative responses as well as those stimulated with 2-ME (5 x 10^{-5}M), ConA (5 μg.ml.$^{-1}$), and 2-ME + ConA. The 2-ME and ConA stimulated proliferative responses of both AA SC and LNC were depressed when compared with those seen in the normal rat (Figs. 4.5(a) and (b)). The mutual effects of 2-ME + ConA, however, appeared to enhance the proliferative responses of both the AA rat SC and LNC to above those seen in the normal rat. The background AA rat SC and LNC proliferative responses showed little difference from those of the normal rat.

4.2.2 Effect of PHMPSA on the proliferative responses of AA rat (a) SC and (b) LNC in culture

The results in Figs. 4.6.1(a) and (b) show that blocking the cell surface SH groups of AA rat SC and LNC with PHMPSA (5 x 10^{-5}M) inhibited all the proliferative responses stimulated with 2-ME (5 x 10^{-5}M), ConA (5 μg.ml.$^{-1}$) and 2-ME + ConA.

Again, ethanol (4.8 x 10^{-3}%) was found to exert no mitogenic effect on the proliferative responses of D-Pen treated AA rat SC and LNC stimulated or not stimulated with 2-ME (5 x 10^{-5}M), ConA (5 μg.ml.$^{-1}$) and 2-ME + ConA as shown in Figs. 4.7(a) and (b). The depressed ConA stimulated proliferative responses seen in the AA rat SC and LNC were restored by <u>in vivo</u> D-Pen treatment to beyond those seen in the normal rat. The 2-ME

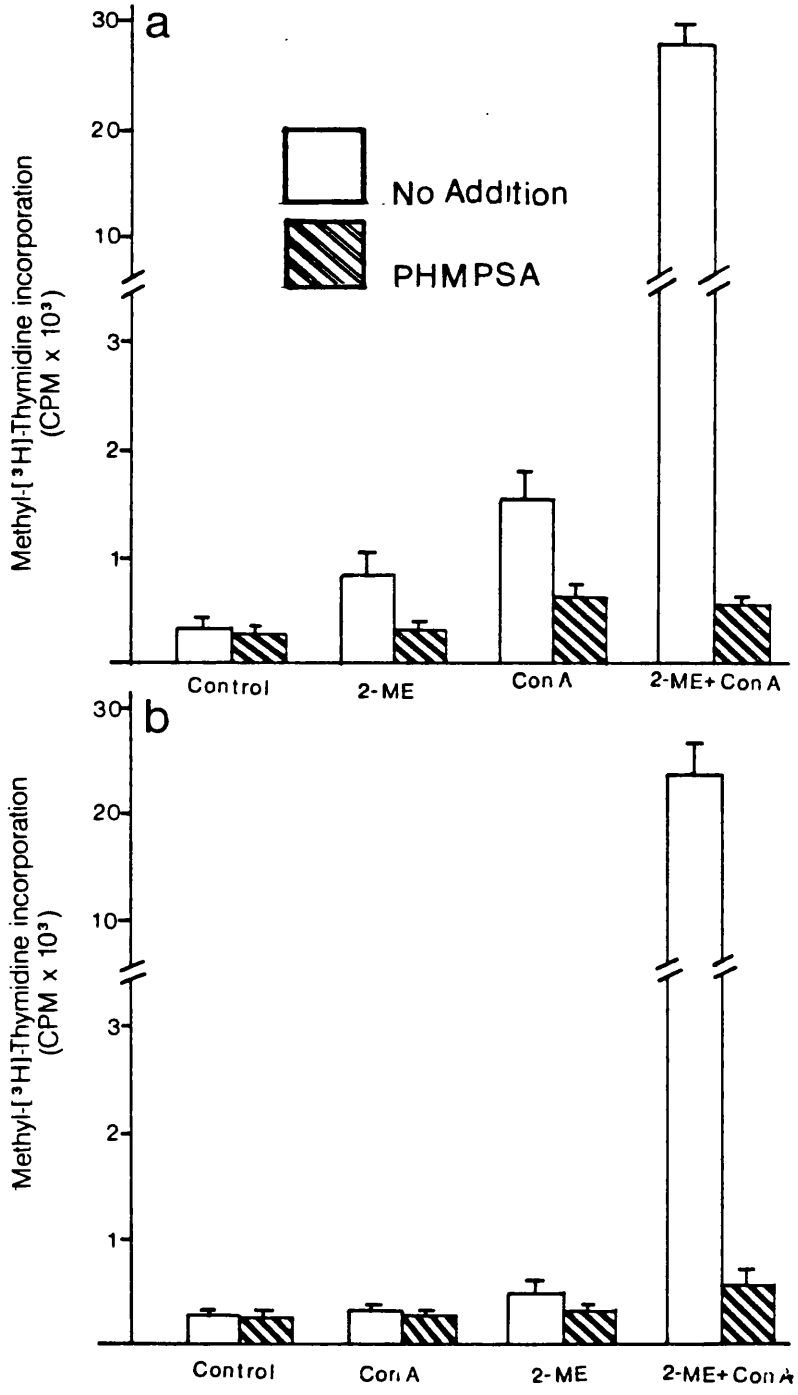


Fig. 4.6.1. (a), (b) The effect of PHMPSA (5×10^{-5}M), 2-ME and ConA on the proliferative response of AA (day 14) male Sprague-Dawley rats (a) SC (n = 3) and (b) LNC (n = 3) in culture. The results are expressed as mean $\pm$ 1 s.d.

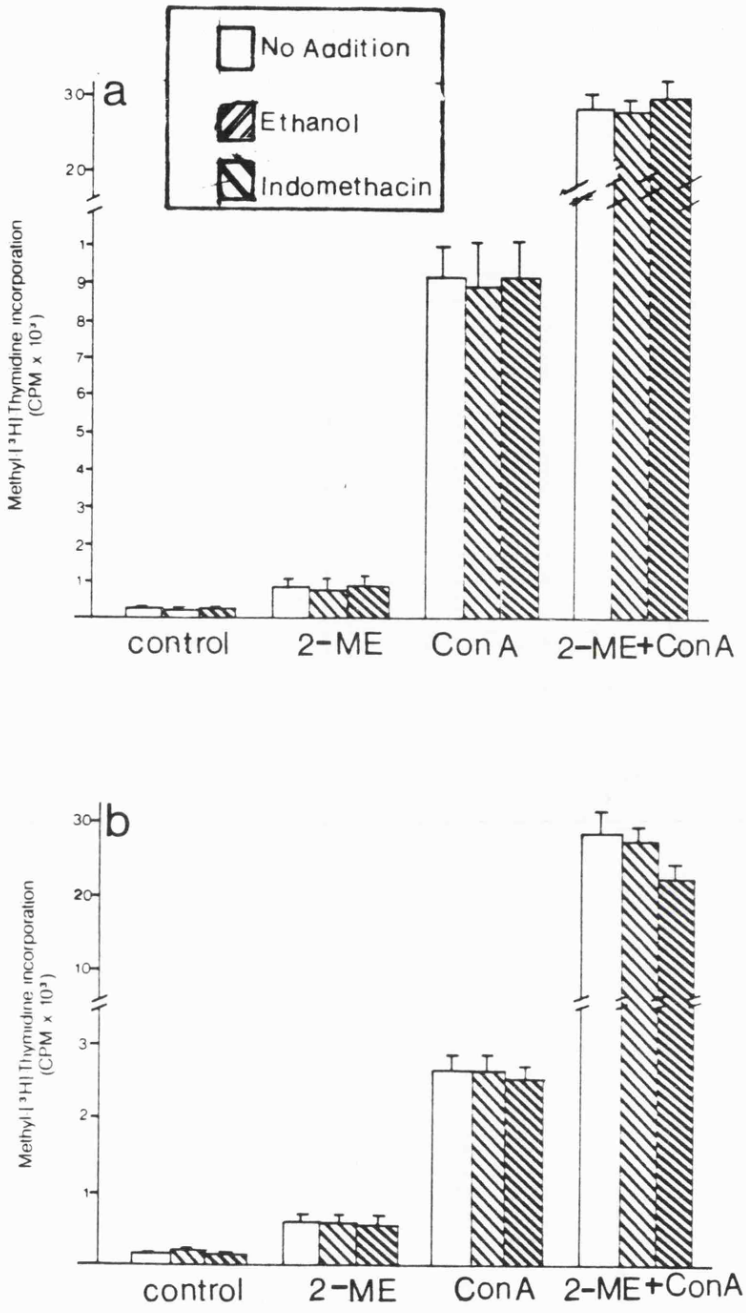


Fig. 4.7 (a), (b) The effects of Ethanol (4.8×10^{-3}%), Indomethacin (10^{-6}M), 2-ME (5×10^{-5}M) and ConA (5 μg.ml.$^{-1}$) on the proliferative response of D-Pen treated AA (day 14) Sprague-Dawley rat (a) SC (n = 3) and (b) LNC (n = 3) in culture. The results are expressed as mean ± 1 s.d.

stimulated response of D-Pen treated AA rat SC was lower than those of both the normal rat and AA rat. The 2-ME stimulated LNC response, however, showed a slightly depressed level compared with that of the normal rat LNC although it was slightly higher than that of the AA rat LNC. The 2-ME + ConA stimulated responses of D-Pen treated AA rat SC and LNC were greater than those seen in the normal SC and LNC although showed no difference when compared with those of the AA rat SC and LNC. No difference was found between the background proliferative responses of normal, AA and D-Pen treated AA rat SC and LNC.

Figs. 4.7.1(a) and (b) indicate that blockade of cell surface SH groups of D-Pen treated AA rat SC and LNC with PHMPSA (5×10^{-5}M) depressed all the proliferative responses stimulated with 2-ME (5×10^{-5}M), ConA (5 $\mu g.ml.^{-1}$) and 2-ME + ConA. The 2-ME + ConA stimulated responses of D-Pen treated AA rat SC and LNC were not inhibited to the extent seen in those of normal rat and AA rat.

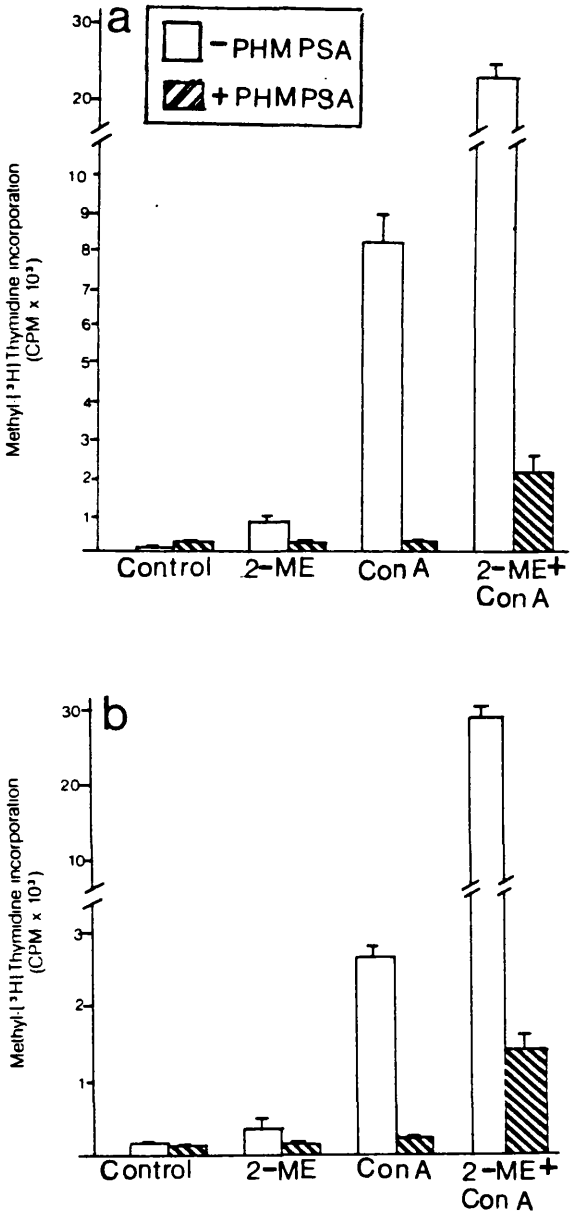


Fig. 4.7.1. The effect of PHMPSA (5×10^{-5}M), 2-ME (5 x 10^{-5}M) and ConA (5 $\mu g.ml.^{-1}$) on the proliferative response of D-Pen treated AA (day 14) male Sprague-Dawley rats (a) SC (n = 3) and (b) LNC (n = 3) in culture. The results are expressed as mean $\pm$ 1 s.d.

CHAPTER FIVE: RESULT C

RESULT C: EFFECTS OF 2-MERCAPTOETHANOL AND D-PENICILLAMINE ON INTERLEUKINS SYNTHESIS OF RATS WITH ADJUVANT ARTHRITIS

5.1 Interleukins

IL-1 is a mø derived mediator whose properties suggest that it can play a role in the pathology of arthritis. Activated møs release IL-1 which acts on T-lymphocytes to stimulate the production of IL-2 which in turn induces T-cell proliferation. Recently, the production of IL-2 was found to decrease in patients with RA (Alcocer-Varela et al., 1984; Combe et al., 1985).

The aim of the present investigation was to test (1) if IL-1 production by the LPS-activated peritoneal møs and IL-2 production by the spleen cells were altered during disease development of AA in male Sprague-Dawley rats and (2) whether in vivo D-Pen treatment of these AA rats could affect the production of IL-1 and IL-2 under these conditions.

5.1.1 IL-1 production during disease development of AA in Sprague-Dawley rats

Fig. 5.1.a shows the IL-1 synthesis by LPS-stimulated peritoneal møs obtained from male Sprague-Dawley rats during the course of AA. IL-1 secretion by AA rat peritoneal møs was

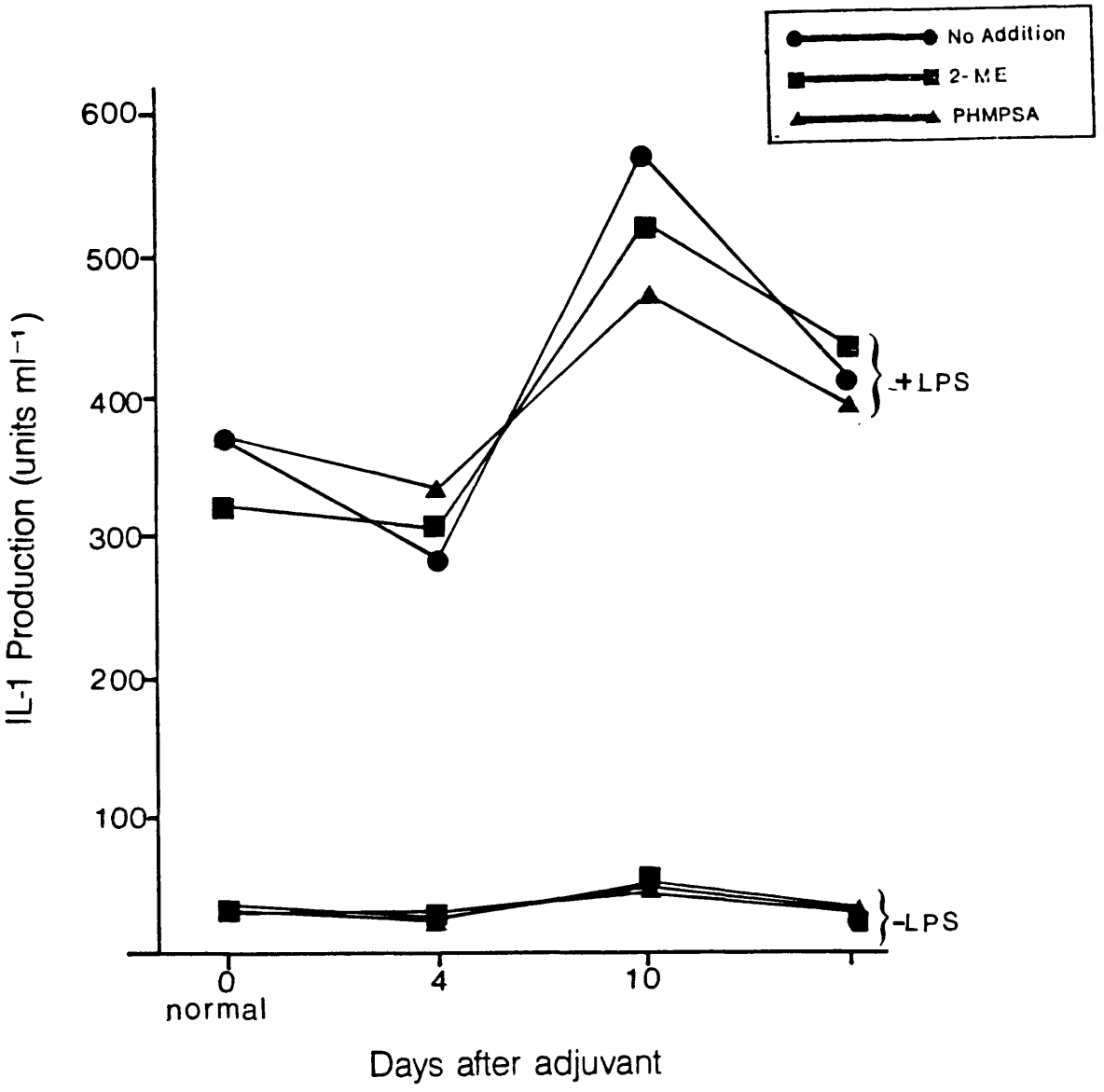


Fig. 5.1.a. IL-1 production during disease development of AA in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean ± 1 s.d. (The s.d. bars are omitted for clarity). (Statistical analysis: Mann-Whitney U test).

slightly reduced at day 4. However, at day 10 of disease the AA rat peritoneal møs released a significantly higher level of IL-1 than the normal control rat peritoneal møs ($P < 0.01$). The level of IL-1 production by the AA rat peritoneal møs was still higher (although not significantly) at day 17 of disease. Preincubation of these peritoneal møs with 2-ME (5×10^{-5}M) and the cell surface SH blocker, PHMPSA (5×10^{-5}M) did not alter the pattern of IL-1 synthesis by these cells. Both the normal and AA rat peritoneal møs not stimulated with LPS (1 $\mu g.ml.^{-1}$) produced little or minimal amount of IL-1. Again, preincubation of both the normal and AA rat peritoneal møs (not stimulated with LPS) with 2-ME (5×10^{-5}M) and PHMPSA (5×10^{-5}M) did not have any effect on the IL-1 production. This experiment shows that IL-1 production by both normal and AA rat peritoneal møs was not SH-dependent.

5.1.2 Effect of D-Pen treatment on IL-1 production during the course of AA in Sprague-Dawley rats

As Fig. 5.1.b shows a high level of IL-1 was produced by the <u>in vivo</u> D-Pen treated (50 $mg.Kg.^{-1}day.^{-1}$ for 7 days before induction of disease) normal rat peritoneal møs stimulated with LPS (1 $\mu g.ml.^{-1}$). IL-1 synthesis by D-Pen treated AA peritoneal møs began to decline from day 4 of disease and became significantly lower than that produced by D-Pen treated normal rat peritoneal møs at day 17 ($P < 0.05$). 2-ME (5×10^{-5}M) and PHMPSA (5×10^{-5}M) treatment did not alter the extent

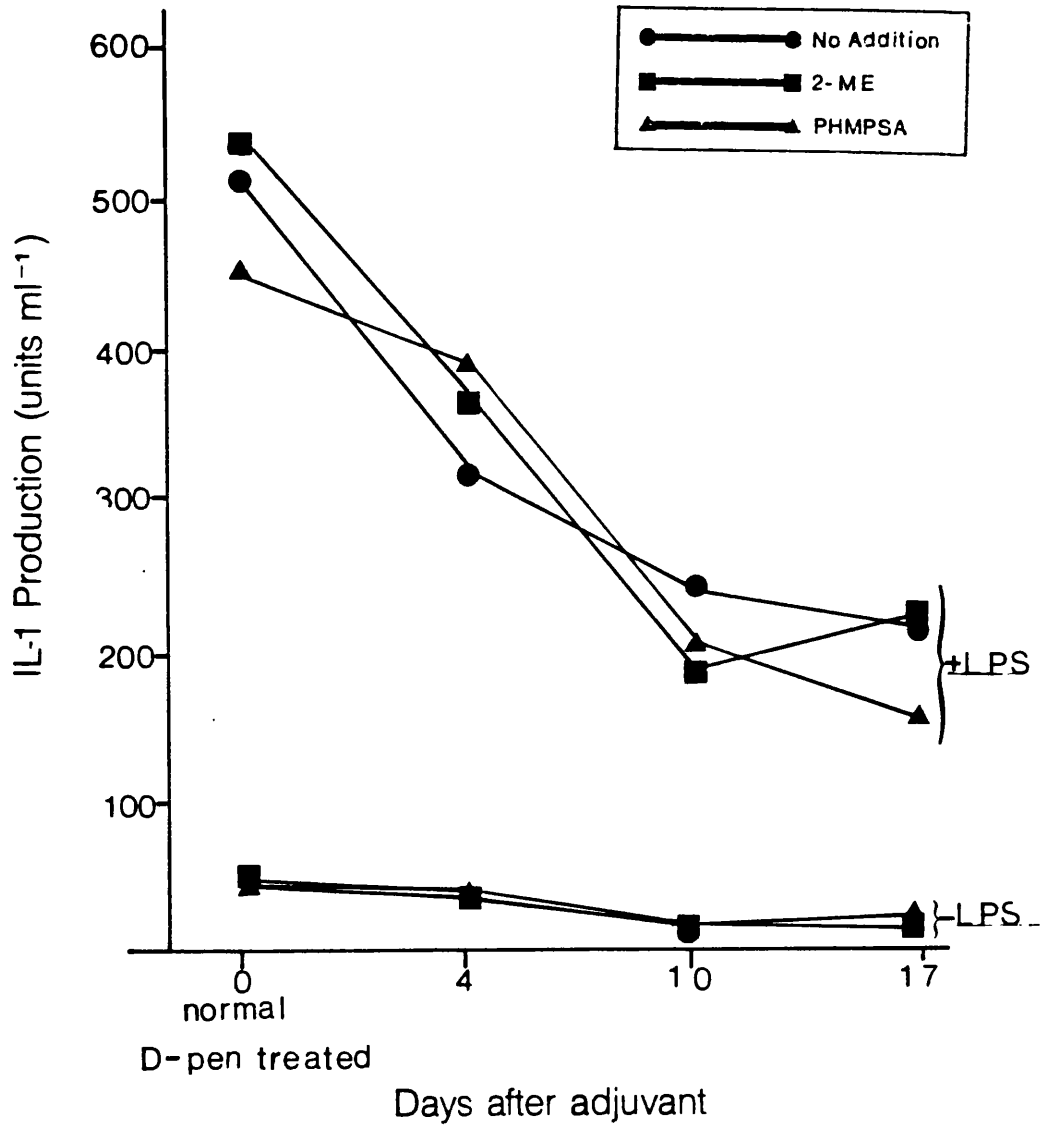


Fig. 5.1.b. Effect of D-Pen treatment (50 mg.Kg.$^{-1}$day^{-1}, orally) on IL-1 production during the course of AA in male Sprague-Dawley rats (n = 6, at each time interval). The results are expressed as mean $\pm$ 1 s.d. (The s.d. bars are omitted for clarity). (Statistical analysis: Mann-Whitney U test).

of IL-1 production in this system. Little or minimal level of IL-1 synthesis was again observed with both the D-Pen treated normal and D-Pen treated AA rat peritoneal møs not stimulated woth LPS (1 $\mu g.ml.^{-1}$). Preincubaion of these peritoneal møs not stimulated with LPS with 2-ME ($5 \times 10^{-5}M$) and PHMPSA ($5 \times 10^{-5}M$) did not affect the IL-1 production. Again, this demonstrates that the IL-1 production in the rat system was not SH-dependent.

5.1.3 IL-1 production by peritoneal møs from AA rat and D-Pen treated AA rat

Fig. 5.1.c shows that the <u>in vivo</u> D-Pen treatment (50 mg. $Kg.^{-1}.day^{-1}$ for 7 days) appeared to enhance the IL-1 synthesis by LPS-stimulated peritoneal møs in normal rats. IL-1 production was decreased in both the AA and D-Pen treated AA rats (more markedly in D-Pen treated AA rats) at day 4 of disease. However, D-Pen treatment depressed the high level of IL-1 synthesis on day 10 of AA ($P < 0.01$). IL-1 synthesis by AA peritoneal møs decreased to the normal control level at day 17. D-Pen treatment further depressed this reduced IL-1 secretion by AA rat peritoneal møs at day 17 of disease ($P < 0.01$).

5.1.4 Screening for IL-2 activity in the IL-1 supernatant

Activated peritoneal møs release IL-1 which acts on T-cells to

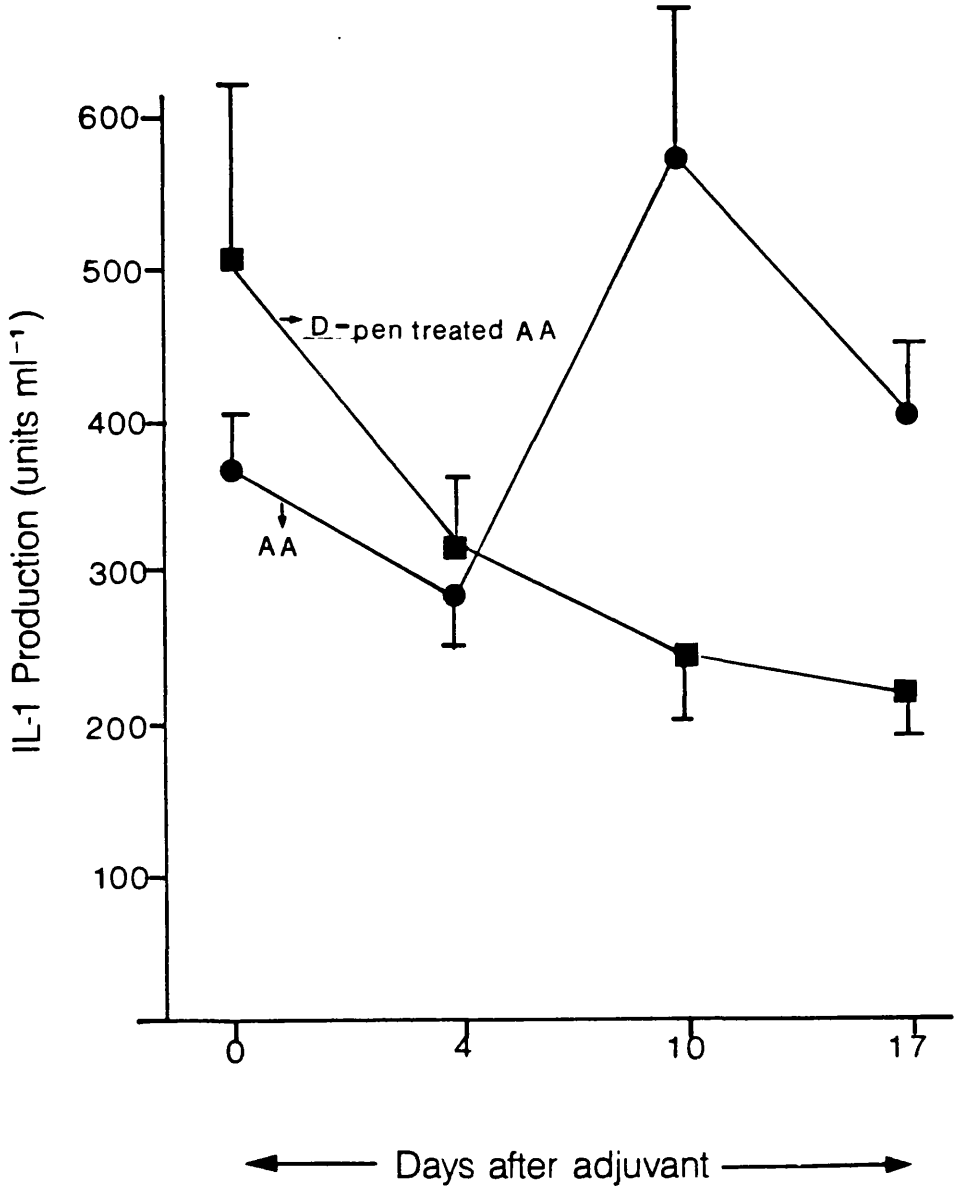


Fig. 5.1.c. IL-1 production by AA rats and D-Pen treated (50 mg.Kg.$^{-1}$day^{-1}, orally) AA rats (n = 6, at each time interval for both groups of rat). The results are expressed as mean $\pm$ 1 s.d. (Statistical analysis: Mann-Whitney U test).

promote the synthesis of IL-2 which in turn induces T-cell proliferation. As the IL-1 assay used in this study was not specific it would also detect IL-2 activity. Hence, IL-1 supernatants (obtained from normal, D-Pen treated normal, AA and D-Pen treated AA rat peritoneal mø's through activation with 1 $\mu g.ml.^{-1}$ LPS) were also screened for IL-2 activity. All these results proved negative (results not shown).

5.2 IL-2 production during disease development of AA in Sprague-Dawley rats

Fig. 5.2.a shows that a decreased level of IL-2 was produced by the ConA (20 $\mu g.ml.^{-1}$)-stimulated SC during the course of AA in male Sprague-Dawley rats. A significant decrease in IL-2 synthesis by the AA rat spleen cells occurred at day 4 of disease ($P < 0.01$). Although IL-2 production appeared to increase at day 10 of disease it began to decline again over the next 7 days. IL-2 production at day 10 and day 17 respectively, was lower than the normal control level (although not significantly). Preincubation of SC with 2-ME ($5 \times 10^{-5}M$) or PHMPSA ($5 \times 10^{-1}M$) did not affect the extent of IL-2 production. Thus, IL-2 production was not SH-dependent. Splenocytes of both the normal and AA rats not stimulated by ConA (20 $\mu g.ml.^{-1}$) produced minimal amount of IL-2.

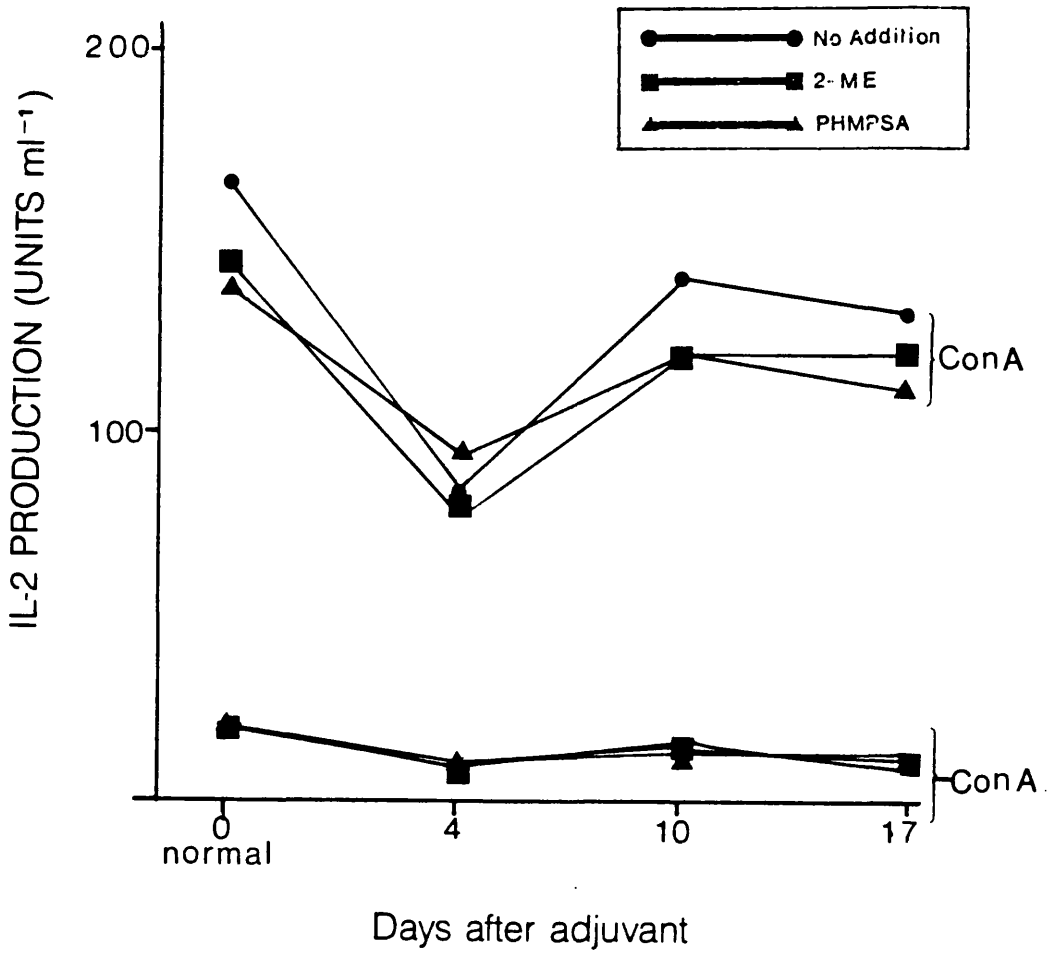


Fig. 5.2.a. IL-2 production during disease development of AA in male Sprague-Dawley rats (n = 6, per time point). The results are expressed as mean ± 1 s.d. (The s.d. bars are omitted for clarity). (Statistical analysis: Mann-Whitney U test).

5.2.1 Effect of D-Pen treatment on IL-2 production during the course of AA in Sprague-Dawley rats

The results of Fig. 5.2.b show that in vivo D-Pen treatment (50 mg.Kg.$^{-1}$.day^{-1}, orally) of AA did not affect the IL-2 synthesis by the ConA (20 μg.ml.$^{-1}$)-stimulated SC during the course of AA. In vivo D-Pen treatment also did not alter the IL-2 production by normal rat SC. Preincubation with 2-ME (5 x 10^{-5}M) or PHMPSA (5 x 10^{-5}M) did not alter the IL-2 synthesis by the SC obtained from both the D-Pen treated normal and D-Pen treated AA rats. This shows that IL-2 production in this system was not SH-dependent. SC of normal, AA and D-Pen treated AA rats not stimulated with ConA (20 μg.ml.$^{-1}$) release minimal amount of IL-2.

5.2.2 IL-2 production by SC from AA rats and D-Pen treated AA rats

The results in Fig. 5.2.c show that the in vivo D-Pen treatment (50 mg.Kg.$^{-1}$.day^{-1}, orally for 7 days) decreased the IL-2 synthesis by the normal rat SC. Although in vivo D-Pen treatment appeared to increase the low IL-2 synthesis at day 4 of disease to the D-Pen treated normal control level, it had no effect on the IL-2 synthesis by the AA rat SC at day 10 and day 17.

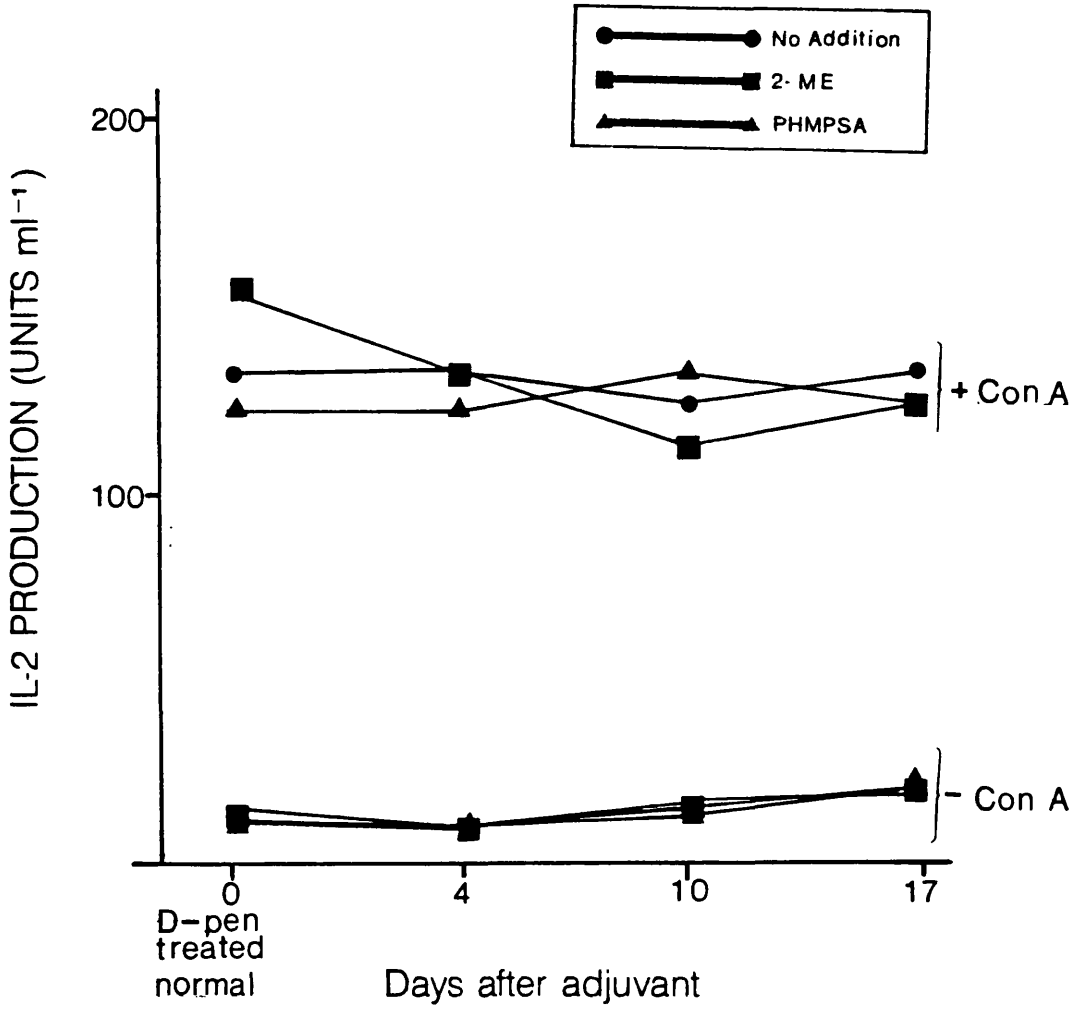


Fig. 5.2.b. The effect of D-Pen treatment (50 mg.Kg.$^{-1}$day^{-1}, orally) on IL-2 production during the course of AA in male Sprague-Dawley rats (n = 6, at each time point). The results are expressed as mean $\pm$ 1 s.d. (The s.d. bars are omitted for clarity). (Statistical analysis: Mann-Whitney U test).

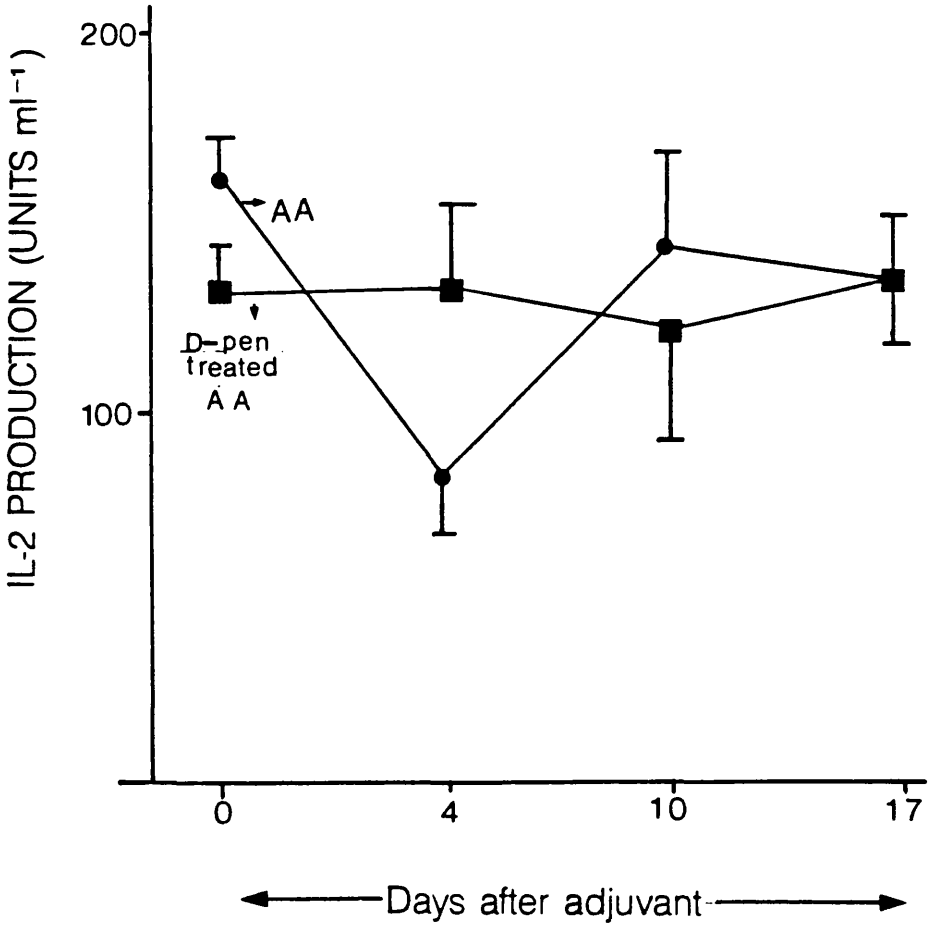


Fig. 5.2.c. IL-2 production by AA rats and D-Pen treated (50 mg.Kg.$^{-1}$day^{-1}, orally) AA rats (n = 6, at each time point for both groups). The results are expressed as mean $\pm$ 1 s.d. (Statistical analysis: Mann-Whitney U test).

CHAPTER SIX: DISCUSSION

Using DTNB as a reagent, the spectophotometric method for the determination of both the total serum protein SH level and reactivity is simple and convenient. Normal humans were found to have a mean total serum SH level of 538μmoles/l. This confirms the finding of Lorber et al. (1964) who reported that the SH content of normal human serum is stable and rarely fluctuates outside the 400-600 μmoles/l. range. The mean SH level of normal human serum is significantly higher than that of rabbit, guinea-pig, rat and mouse. The change in absorbance at 440 nm. during the first minute of SH-SS exchange reaction between DTNB and serum protein SH groups varied considerably between individual samples. This initial rapid reactivity represents partly the interaction of low molecular (non-protein) thiols with DTNB (Jocelyn, 1962). The increase in absorbance during the second minute (between 1 and 2 minutes) of the SH-SS exchange reaction appeared linear and hence more accurate and consistent data could be obtained. This finding is in line with the observation made by Butler et al. (1969) who found that the change in optical density between 1 and 2 minutes was strictly linear. It is interesting to find that although the mean total serum protein SH level of the rat is significantly lower than that of the mouse, its serum SH reactivity is significantly faster in the first minute of the SH-SS exchange reaction, although there is no difference in the second minute of the reaction. Indeed, the serum SH reactivity of the rat is significantly faster than that of any other animal species studied. The rat serum

SH reactivity is 7 times faster than that of the human in the first minute and 4 times faster in the second minute of reaction. This finding also agrees with the observation of Butler et al. (1969) that rat serum exhibits a much more rapid rate of reaction with DTNB than human serum. The fast reaction rate of the rat serum may indicate that serum from these animals contains more low molecular weight thiols which would catalyse the SH-SS exchange reaction. It may also be the case that the serum protein SH groups of rat are more reactive and sensitive to oxidation than those of the human. This would imply that the conformation of the albumin molecules in rat serum differs from that in man, the free SH group presumably being more accessible to oxidation and to reaction with DTNB. This suggestion would explain the low total SH levels and high SH reactivity observed with rat serum. Freezing was found to depress the serum protein SH level and reactivity of the various animal species studied. This supports the observations made by various investigators (Lorber et al., 1964; Butler et al., 1969; Hall and Gillan, 1979). The depressed serum protein SH level and reactivity observed with freezing may be due to the formation of disulphide dimers (via SH-SS exchange) between SH groups of the serum proteins present in the serum. The amounts of these dimers increase with storage leading to less free SH groups available for reaction with DTNB resulting in decreased SH level and reactivity.

In the present study, changes in total serum protein SH levels were studied during the course of AA in rats. A depression of serum protein SH levels and reactivity was found in rats that developed AA and this was associated with the persistent chronic inflammatory phase of the disease. The major depression of serum SH and reactivity occurred at the time of onset of AA (i.e. between day 10 and day 17) although the change was noticeable as early as 2 days after AA induction. These results confirm the findings of other workers (Butler et al., 1969; Lorber et al., 1975; Barbier et al., 1984). According to Lorber et al. (1975) the depressed serum SH level observed in rat serum was not due to the acute inflammatory phase since those rats in which AA was suppressed by the competing antigen did not show any depression. Neither was the depression due to decreased food consumption or loss of body weight (Lorber et al., 1975). This depression may be caused by oxidative stress due to the production of oxidative species, especially H_2O_2 by activated phagocytes (Hall et al., 1984) in the active inflammatory phase when gross tissue injury occurs. This may follow the same process as the finding of Thomas and Evans (1975) in rheumatoid patients. They showed that human serum SH levels are due almost entirely to cysteine side-chains within protein structures. The great majority of serum SH groups are present in albumin with the remainder distributed within the globulin fractions. The depression of serum SH in RA appears to be due to oxidation and subsequent mixed disulphide formation with a low molecular

weight thiol, cysteine, and with trace amounts of glutathione (Thomas and Evans, 1975).

Experiments with D-penicillamine treatment showed that although in vivo treatment with this SH-containing, second-line antirheumatic drug had no effect on the total serum protein SH level and reactivity of normal rats, it increased the serum SH level in AA rats towards normality over days 14-21. Furthermore, it also increased the serum SH reactivity towards normality by day 21 in the first minute reaction and by day 10 in the second minute measurement. This result suggests that D-penicillamine may act as a reducing agent, restoring the free SH groups that have been blocked in active AA via interaction with the mixed-disulphide formed from SH groups between albumin and other low molecular weight thiols due to oxidation caused by active inflammation. It must be stressed that although in vivo D-penicillamine treatment increased the AA serum SH reactivity to normal it did not stimulate the increase in SH reactivity significantly faster than that of normal serum as it does with RA serum SH reactivity as demonstrated by Hall and Gillan (1979). The difference may be due to (1) the pre-existence of more reactive low molecular weight thiols in AA rat serum than in RA serum and D-penicillamine treatment was no longer able to affect the fast reactivity of these reactive thiols, (2) the very much higher doses of D-penicillamine administered to AA rats than to RA patients. The increase in serum bound D-

penicillamine in AA rats may lead to a slower rate of SH-SS exchange reaction due to steric hindrance and formation of mixed disulphide with low molecular weight thiols. This is reminiscent of the dose-response profiles of 2-ME and ConA seen in mouse and rat spleen cell culture experiments where low doses lead to suboptimal proliferative responses and supraoptimal doses result in inhibition of responses.

The present study also showed that in vivo D-penicillamine treatment (50 mg./Kg./day, orally from day -7 to day +21) of AA had no effect on the various parameters used to evaluate disease severity (i.e. circumference of hind paws, weight change and arthritic score). These observations confirm the results of Liyanage and Currey (1972) who found that 200 mg./Kg. D-penicillamine had no effect on AA in rats. Similar results were also obtained by Dunn et al. (1984) who showed that oral pretreatment (1 to 3 months) of AA rats with D-penicillamine (100 mg./Kg. p.o.) followed by daily treatment after AA induction failed to modify the different parameters used to assess arthritic scores. In some studies, D-pencillamine has been shown to exacerbate the secondary lesions of AA (Binderup et al., 1976).

Although D-penicillamine does not affect the clinical course of AA it might modify the cell functions in the rats perhaps through the SH groups. Therefore, lymphocyte functions were investigated in rats with or without AA in order to identify

their SH dependence.

The addition of the simple low molecular weight thiol, 2-ME, to culture of both normal mouse and rat spleen cells resulted in a dramatic increase in uptake of [^{3}H] thymidine. The increase was more pronounced in the mouse system. This phenomenon occurred in a dose-related fashion with the peak [^{3}H] thymidine incorporation at 5 x 10^{-5}M 2-ME. The shape of the dose-response curve of the T cell mitogen, ConA, is similar to that of 2-ME, with maximal [^{3}H] thymidine uptake occurring at 5µg/ml. for both mouse and rat spleen cell cultures. In the mouse system, the addition of 2-ME to ConA stimulated spleen cell cultures produced only an additive response. However, in the rat system, the addition of 2-ME greatly enhanced the ConA stimulated proliferative response. The difference in the ConA stimulated spleen cell proliferative responses of these animals may be due to a difference in sensitivity of their surface SH groups to oxidation. The rat has a lower serum protein SH level than the mouse with a faster serum SH reactivity. This may reflect the more sensitive and reactive nature of the rat spleen cell surface SH groups to oxidation if it is assumed that the spleen cell surface thiols are equally susceptible to oxidation as serum SH groups. The ConA stimulated rat spleen cell surface SH groups oxidised quickly and became hyper-responsive to the presence of 2-ME in the culture medium thus giving rise to a much greater enhancement of response compared

with the mouse spleen cell response. The result also suggests that functionally free surface SH groups are needed for the rat spleen cells to proliferate and the addition of appropriate concentration of 2-ME to the cell culture helps maintain the cell surface SH groups in a reduced state.

Blockade of cell surface SH groups using the irreversible cell surface SH blocker, PHMPSA, abolished the ConA stimulated proliferative response. The addition of 2-ME to ConA stimulated cultures treated with PHMPSA could not reverse this inhibition. This result also indicates that cell surface thiols are necessary for proliferation of rat spleen and lymph node cells. This finding is in line with the observation of Chaplin and Wedner (1978) who demonstrated that blockade of free SH groups on human peripheral blood lymphocyte surface by diamide and other SH reagents led to an inhibition of lectin (PHA or ConA)-induced lymphocyte activation. The SH oxidising agent, diamide, was only effective when added within 30-60 minutes of lectin. This inhibition of lymphocyte activation was not caused by decreased intracellular levels of reduced glutathione or by inhibition of binding of lectin to the lymphocyte (Chaplin and Wedner, 1978). These results suggest that maintenance of free SH groups is important during the early induction of lymphocyte activation and indicate that an obligatory step or steps in the activation sequence may involve SH interactions.

Various mechanisms have been proposed to explain the dramatic effect of 2-ME on rat and mouse lymphocyte proliferation. Opitz et al. (1977) suggested that 2-ME does not act directly on lymphocytes, but it converts a native serum factor (Pro-MaSF) in the FCS into the activated serum factor (MaSF) that could elicit an antibody response to SRBC (Lemke and Opitz, 1976), anti-IgM-induced murine B cell proliferation (Sidman and Unanue, 1978) and murine T lymphocyte proliferation (Opitz, 1978) without further addition of 2-ME. Hewlett (personal communication, 1981) postulated that the Pro-MaSF was albumin and MaSF mercaptalbumin. The addition of 2-ME reduced the albumin to mercaptalbumin, the active fraction for the induction of the immune response. This is supported by the finding of Smith et al. (1978) who showed that in the culture medium, thiols such as 2-ME appear to function by altering albumin. Hoffeld and Oppenheim (1980), however, postulated that a major role of 2-ME in augmenting antibody responses <u>in vitro</u> is to convert oxidised glutathione (GSSG) in the serum into reduced glutathione (GSH), protecting it from further oxidation and preventing the accumulation of GSSG, an inhibitory product, by interchange with it. Ohmori and Yamamoto (1983b) proposed a third possible explanation for the effect of 2-ME on lymphocyte activity. They suggested that a major role of 2-ME in the augmentation of antibody responses would be to stimulate the utilisation of cystine in both B cells and T cells through the formation of Cys-2-ME. Both cysteine and Cys-2-ME would promote cystine uptake in the

initial phase of the culture. However, Cys-2-ME is more preferentially involved in the enhancement of cystine uptake throughout most of the culture period (4-5 days). Cys-2-ME itself could support the antibody response much more efficiently than cysteine. These workers also suggest that 2-ME is able to augment the antibody response because it has a durable stimulatory effect on cystine uptake. The superiority of 2-ME over other thiol compounds in its durable effect would be due to the fact that Cys-2-ME is also efficiently taken up by the lymphocytes. This would explain the observation that 2-ME added 3 days before the start of the culture could augment the antibody response as efficiently as freshly added 2-ME (Chen and Hirsch, 1972).

As discussed earlier, the addition of 2-ME to ConA stimulated rat spleen or lymph node cell cultures induced an enhancement of proliferative response and treatment of cells with PHMPSA abolished the 2-ME, ConA and 2-ME + ConA stimulated responses. These results suggest a fourth possibility, that the presence of cell surface SH groups is necessary for lymphocytes to proliferate, and that 2-ME maintains these SH groups in a reduced state. If this is the case, the role of MaSF (Opitz et al., 1977) present in the FCS-containing culture medium used for culturing rat spleen and lymph node cells in the present study might be as a reducing agent to restore the oxidised cell surface SH groups to that reduced state necessary for normal cell proliferative responses.

As FCS is known to contain both cysteine and GSH the role of 2-ME in promoting cell proliferation via a cystine uptake mechanism (Ohmori and Yamamato, 1983b) is a very plausible one. In the present study, 2-ME (5×10^{-5}M) was added to the rat spleen or lymph node cell at the beginning of cultures and incubated for 3 days without further daily addition. Under these conditions, the added 2-ME would be present as the disulphide form (2-hydroxyethyldisulphide or 2-ME_{ox}) and mixed disulphide with FCS components, cysteine and GSH (Ohmori and Yamamato, 1983b). It is likely that 2-ME_{ox} and FCS-bound 2-ME could stimulate the uptake of cystine into the lymphocytes through the formation of Cys-2-ME. Cys-2-ME would then be readily metabolised to GSH and cysteine after uptake. It was observed that free thiol was generated when 2-ME_{ox} was incubated with the lymphocytes (Ohmori and Yamamato, 1983b). Therefore, it appears that the rat spleen and lymph node cells would reduce FCS-bound 2-ME and 2-ME_{ox} to liberate free 2-ME that reacts with cysteine to produce equimolar amounts of Cys-2-ME. The liberation of 2-ME from 2-ME-pulsed FCS might be mediated by a small amount of thiol compounds released from the cells (Ohmori and Yamamato, 1983b). Hence, 2-ME promotes lymphocyte proliferation by stimulating the utilisation of cystine, an essential amino acid in both B cells and T cells through the formation of Cys-2-ME. 2-ME that was transformed into FCS-bound and oxidised forms could be used for the formation of Cys-2-ME via interaction with the lymphocyte, liberating free thiol and this reaction would help to maintain

the lymphocyte surface SH groups in a reduced state essentially for normal lymphocyte function.

The present study shows that induction of AA in Sprague-Dawley rats by the injection of heat-killed M. Tuberculosis in mineral oil led to an initial increase (at day 2) in the proliferative response of both the spleen and lymph node cells to ConA alone as measured by the incorporation of [^{3}H] thymidine into cellular DNA. This initial increase in response was followed by marked inhibition of [^{3}H] thymidine incorporation in ConA stimulated spleen and lymph node cells 14 days after the induction of disease. Thereafter, the response returned to control values. These results confirm the findings of others that spleen and lymph node cells from rats with active AA responded poorly in vitro to ConA (Arrigoni-Martelli and Binderup, 1981; Gilman et al., 1984). The return to normal of ConA stimulated spleen and lymph node cell proliferative response as early as day 21 indicates that the AA induced in the Sprague-Dawley rats was not of a very severe nature. The in vitro addition of 2-ME to the ConA stimulated AA rat spleen and lymph node cell cultures greatly enhanced the initial increase in [^{3}H] thymidine incorporation at day 2 to a level significantly higher than that of the normal control values. Furthermore, the presence of 2-ME was found to not only reverse but also enhance the markedly depressed ConA stimulated proliferative responses of AA rat spleen and lymph node cells (at day 14 of disease) to a level

significantly higher than the normal control values with 2-ME + ConA. The ConA stimulated spleen cell response was more markedly enhanced by 2-ME than the ConA stimulated lymph node cell response. The present experiment also shows that *in vivo* treatment with D-penicillamine during the course of AA modified the pattern of ConA stimulated proliferative response of AA rat spleen and lymph node cells. The results show that the initial increase in proliferative response of both ConA stimulated AA rat spleen and lymph node cells at day 2 of disease was significantly reduced and the marked inhibition of proliferative response to ConA at day 14 was reverted to an enhanced response to ConA compared with cultures from untreated arthritic rats and nonarthritic rats. The addition of 2-ME *in vitro* to the ConA stimulated cultures of the D-penicillamine treated AA rat spleen and lymph node cells further enhanced the already increased proliferative responses due to D-penicillamine treatment alone. The markedly enhanced ConA stimulated responses by 2-ME were still rising at day 21 of disease although in the respective AA rat, the 2-ME + ConA responses were back at normal control level at day 21. Again, the proliferative response of ConA stimulated D-penicillamine treated rat spleen cells was more markedly enhanced by 2-ME than that of the matched lymph node cells. This difference may be attributed to the existence of different subpopulations of lymphocytes in the lymphoid organs that express different responsiveness to 2-ME in their response to ConA. In the rat lymph nodes, about 30 to 40% are B cells, the remainder T

cells. In spleen, 60% B, 40% T (Gordon Van Arman, 1976). According to Goodman and Weigle (1977) mouse spleen B cells are much more 2-ME responsive than T cells. Assuming the rat B cells obtained from spleen or lymph node are equally 2-ME responsive as the mouse spleen B cells, it is quite obvious that the rat spleen cells containing 60% B cells should respond more markedly to 2-ME than the rat lymph node cells which contain only 30-40% B cells. Alternatively, the more marked spleen cell response may be related to the spleen cell sensitivity to suppressive mechanisms from macrophages or due to oxidative activity generated by activated phagocytes in active AA. The spleen cells may become hyper-reactive to 2-ME in vitro and D-penicillamine in vivo.

In vivo D-penicillamine treatment (50 mg./Kg./day, orally, for 7 days) of normal Sprague-Dawley rats did not increase the unstimulated incorporation of [^{3}H] thymidine into spleen and lymph node cells. This result is in contrast with that of Arrigoni-Martelli and Binderup (1981) who found that a short-term treatment with D-penicillamine (50 mg./Kg./day, orally, for 4 days) of normal Lewis rats increased the unstimulated incorporation of [^{3}H] thymidine into lymph node lymphocytes. However, the result presented shows that D-penicillamine treatment did increase the ConA stimulated proliferative response of both the AA rat spleen and lymph node cells compared with the respective controls. This confirms the result of Binderup et al. (1980) who also found that

administration of D-penicillamine (50 mg./Kg./day for 4 days) increased the uptake of [^{3}H] thymidine in ConA stimulated lymph node and spleen cells from Lewis rats. They suggested that D-penicillamine treatment in vivo enhances the responsiveness of lymphocytes dependent on the presence of functionally intact macrophages. The lymphocytes in D-penicillamine treated rats may have been primed in vivo by macrophages to an increased responsiveness such that the presence of adherent cells in vitro is no longer required for a response (Binderup et al., 1980; Arrigoni-Martelli and Binderup, 1981).

The altered cell functions of AA rats found in the present study may be due, at least in part, to the functional free cell surface SH groups of both the spleen and lymph node cells being oxidised (blocked). This is suggested by the serum protein SH level and reactivity of these AA rats also being markedly depressed at day 14 of disease and the addition of 2-ME in vitro and D-penicillamine treatment in vivo reversing and enhancing the markedly inhibited ConA stimulated responses. In addition, the reduced AA cell proliferative responses to ConA could be attributed to suppression from activated macrophages (Arrigoni-Martelli and Binderup, 1981) via a mechanism involving SH groups (see below). Removal of adherent cells or elimination of macrophages with the selective macrophage toxin, silica, abolished the inhibited proliferative responses at day 14 of disease (Arrigoni-

Martelli and Binderup, 1981). The production of prostaglandins by activated macrophages may also cause the altered cell functions since prostaglandins can suppress certain in vitro immune functions including mitogen stimulated proliferative responses (Goodwin and Webb, 1980). The macrophages from AA rats may thus exert their suppressive activity via release of inhibitory prostaglandins into the culture medium. By blocking prostaglandin synthesis the suppression of proliferative response may be removed. Hence, blockade of prostaglandin synthesis using indomethacin was investigated. The addition of indomethacin, however, did not abolish the observed inhibition of proliferative responses in AA rat spleen and lymph node cells. This result indicates that prostaglandin synthesis did not play a significant role in mediating the observed depression of proliferative responses of AA rat cells to ConA. The macrophages may exert their suppressive effects via other mechanisms. Binderup (1983 a, b) found that the function of macrophages was altered during the course of AA. Suppressor macrophages were found in the spleen and lymph node shortly after the induction of disease, these cells could inhibit lymphocyte responses to both B and T mitogens. Similar findings were reported by Gilman et al. (1985) who found that arthritic rat cells are not inherently hyporesponsive but the adherent suppressor cells were actively inhibiting T cell reactivity perhaps through the release of a soluble suppressor factor. Also, Aune and Pierce (1982) found that in the mouse system, ConA

stimulated murine T suppressor cells act via the mediator, soluble immune response suppressor (SIRS) which can nonspecifically suppress proliferative responses to alloantigen and mitogens in vitro. The target of SIRS is the macrophage. Incubation of macrophages with SIRS induces the release of macrophage derived suppressor factor (Mø-SF), which is directly responsible for the observed suppression of responses. Mø-SF can also be obtained by reacting SIRS with low concentrations of H_2O_2 in the absence of macrophages. Thus, macrophages may serve as a source of H_2O_2 and the mechanism of Mø-SF action appears to have an oxidative basis (Aune and Pierce, 1982). Mø-SF is lost following treatment with SH reagents such as 2-ME, dithiothreitol or cysteine. Furthermore, Mø-SF mediated inhibition can be reversed by high concentration of 2-ME or dithiothreitol under appropriate conditions (Aune and Pierce, 1982). It may well be that the induction of AA activates the macrophages to release H_2O_2 which reacts with SIRS to induce formation of Mø-SF which in turn suppresses the ConA stimulated proliferative response of AA rat spleen and lymph node cells at day 14 of disease. Alternatively, during active AA in rats the macrophages present in the spleen and lymph nodes may become defective (due to cell surface free SH groups being blocked or oxidised) in presenting the mitogen (ConA) to the lymphocytes for a normal immune response to occur. However, the work of McKeown (1984) (PhD thesis, data unpublished) with rat lymph node cells showed that removal of adherent cells by passage down a

Sephadex G-10 column abolished antigen stimulated lymphocyte proliferation. The proliferative response could only be recovered with added macrophages in the presence of 2-ME and not by preincubation of cells with 2-ME. There was no significant difference in lymph node cell proliferation whether macrophages were pulsed with antigen in the presence or absence of 2-ME (McKeown, 1984). These results show that the presence of free SH groups on macrophages are not obligatory for antigen presentation. Arrigoni-Martelli and Binderup (1981) showed that the altered cell function in AA may also be associated with a decrease in T helper cell activity at day 14 in cultures from untreated AA rats. This decrease was dependent on the presence of suppressor macrophages and was not observed in the corresponding cultures from D-penicillamine treated AA rats (Arrigoni-Martelli and Binderup, 1981). Hence, treatment with D-penicillamine in vivo may inhibit macrophage-mediated suppression, thereby permitting more effective T and B effector cell function. The increased T helper cell activity following D-penicillamine treatment may, through the production of lymphokines, activate macrophage clearance functions (Arrigoni-Martelli and Binderup, 1981) thus removing the macrophage-mediated suppression. The addition of 2-ME in vitro may inactivate the MØ-SF mediated inhibition. In vivo D-penicillamine treatment may prevent the generation of activated macrophages or alternatively it may inhibit the release of H_2O_2 by activated macrophages. Either one mechanism will lead to normalisation

of cell function because H_2O_2 is not secreted for reacting with SIRS to activate Mø-SF, resulting in depressed cell functions. D-penicillamine may also act by directly inactivating Mø-SF in the same way as 2-ME.

Because T cell proliferation in vitro is dependent on the production and utilisation of IL-1 and IL-2 (Smith, 1980; Dinarello, 1984) the possibility was examined that an imbalance in the regulation of this lymphokine cascade system could contribute to the depressed immune function in arthritic rats. In response to LPS the production of IL-1 by peritoneal macrophages from rats with AA was slightly reduced at day 4 followed by a significant increase at day 10. This may be due to the macrophages becoming highly activated in the process of developing an active inflammatory response (i.e. at day 10) before the rats became arthritic at day 14. The level of IL-1 synthesis was still higher than that of the normal control value at day 17 (the time when rats became arthritic). This observation is in line with the result of Gilman et al. (1984) who found that the synthesis of IL-1 by splenic or peritoneal macrophages from arthritic rats equalled or exceeded that of macrophages obtained from normal rats. Interestingly, rheumatoid monocytes, when stimulated with LPS, also produced enhanced amount of IL-1 compared with controls (McKeown et al., 1984). Thus, the proliferative defects of AA cells could not be accounted for by the insufficient availability of IL-1. However, the proliferative response of spleen cells from

arthritic rats to IL-1 was found to be markedly reduced (Gilman et al., 1985). This reduced AA rat spleen cell response may be caused by defective expression of IL-1 receptors by the arthritic rat spleen T lymphocytes which may be thiol dependent, and if the arthritic rat spleen cell surface SH groups are blocked, this may also result in defective expression of IL-2 receptors. The experiments of D-penicillamine treatment on IL-1 synthesis shows that D-penicillamine treatment *in vivo* decreased the production of IL-1 during the course of AA in Sprague-Dawley rats. This result suggests that D-penicillamine may act by partially preventing the activated macrophages from releasing large amounts of IL-1 thus preventing tissue damage mediated by this factor. Alternatively, D-penicillamine may act to prevent the generation of activated macrophages and thereby reduce the amount of IL-1 being produced. This would support the activated macrophage -> H_2O_2 -> SIRS -> Mø-SF hypothesis leading to altered cell function (see Fig. 6). D-penicillamine treatment of normal rats led to a high level of IL-1 being produced by LPS-stimulated peritoneal macrophages. The synthesis of IL-1 was not SH dependent as treatment with either 2-ME or PHMPSA did not affect IL-1 production by LPS-stimulated peritoneal macrophages from normal, D-penicillamine treated, normal untreated AA and D-pencillamine treated AA rats. Minimal amounts of IL-1 were produced by rat peritoneal macrophages not stimulated with LPS. The results of IL-2 production experiments show that a decreased amount of IL-2

was produced by ConA stimulated spleen cells during the course of AA in Sprague-Dawley rats. A significant decrease in IL-2 production occurred at day 4 of disease. Although the IL-2 production appeared to increase at day 10 it began to decline again at day 17. IL-2 production at day 10 and day 17, respectively, was lower than the normal control level (although not significantly). This result is less marked than the findings of Gilman et al. (1984) who reported that spleen cells from arthritic rats produced significantly reduced amounts of IL-2 following stimulation with ConA or PHA. Could this observation be the explanation for the reduced ConA stimulated AA rat spleen periferative response seen at day 14 of disease? According to Gilman et al. (1984), the reduced IL-2 production is not physiologically relevant, since bypassing the IL-2 synthetic defect by supplying an exogenous source of IL-2 did not restore their proliferative capacity. Furthermore, ConA stimulated spleen cells from AA rats failed to bind IL-2 or respond to this factor with increased [^{3}H] thymidine incorporation as did normal cells (Gilman et al., 1984). Thus, it could be that the principal defect in AA spleen cells is the failure to produce receptors for IL-2 and thereby receive the IL-2 mediated growth signal. Detailed analysis of IL-2 receptors using monoclonal antibody to the rat IL-2 receptor (for example, the human anti-Tac equivalent (Robb and Greene, 1983)) should throw light on this. The expression of IL-2 receptor may also be thiol dependent. The blocked surface SH groups on arthritic rat spleen cells may

lead to the failure of IL-2 receptor expression. D-penicillamine treatment did not affect IL-2 production by ConA stimulated spleen cells during the course of AA. In addition, D-penicillamine treatment did not alter the IL-2 synthesis by normal rat spleen cells. The production of IL-2 was not thiol dependent since treatment with either 2-ME or PHMPSA did not affect the IL-2 synthesis by ConA stimulated spleen cells obtained from the normal, D-penicillamine treated normal, untreated and D-penicillamine treated AA rats. Spleen cells obtained from normal, untreated AA and D-penicillamine treated AA rats not stimulated by ConA released only minimal amounts of IL-2.

The depressed spleen and lymph node cell responsiveness in AA rats is not simply a consequence of exposure of the animals to M. butyricum but is in fact related to the development of an arthritic response since spleen cell reactivity is normal in M. butyricum injected animal which failed to develop arthritis (Gilman et al., 1984).

From the data discussed so far, it appears that the altered cell functions in AA rats may represent the result of the interactions of a series of complex immunological events that occur during the development of an arthritic response initiated by the injection of CFA.

With these considerations in mind, a hypothetical scheme is

presented in Fig. 6 to account for the series of immunological events that lead to the altered cell functions in AA. According to this scheme, the mycobacterial material is considered to be playing a dual role in the induction of chronic arthritis in genetically predisposed strains of rats (e.g. Sprague-Dawley rats). First, the mycobacteria attract large numbers of leucocytes to the site of injection and to visceral loci, with subsequent release of proteolytic enzymes and an increase in hydrogen ion concentration at the extra cellular foci producing a granuloma with active inflammation. At the same time, mycobacterial cell wall materials enhance the immune response by activating macrophages. The activated macrophages may (1) release an increased amount of IL-1 and prostaglandins leading to further inflammatory events; (2) produce several active oxidative species including H_2O_2 causing further tissue damage. The H_2O_2 thus produced may also react with SIRS to form Mø-SF which may then interact with spleen cells or lymph node cells to cause inhibition of proliferative responses to ConA, (3) react directly with SIRS to suppress the proliferative responses to ConA; and (4) release other soluble suppressive factor(s) that can act on the AA rat lymphocytes to suppress the proliferative response to ConA. The failure of the spleen (or lymph node) cells to produce IL-1 and IL-2 receptors possibly due to blocked cell surface SH groups may also lead to the depressed ConA stimulated cell proliferative responses seen in AA.

The most likely conclusion of this work is that the addition of 2-ME *in vitro* and D-penicillamine treatment *in vivo* lead to the normalisation of cell functions of the AA rats due to the ability of these thiol compounds to react or interact with the various macrophage related inhibitory mechanisms discussed earlier.

Although *in vivo* D-penicillamine treatment can lead to normalisation of cell function it has no clinical effect on the disease development and severity of arthritic lesions of AA rats. The reason(s) are unclear. This may be due to D-penicillamine not being able to (1) inhibit leucocyte migration and hence subsequent proteolytic enzymes release, and (2) block synthesis of prostaglandins which are established mediators of inflammatory responses and have been long suspected to play an important role in articular destruction (Rainsford, 1982).

Alternatively, the development of AA may be due to viral infection since interferon (an anti-viral agent) inducing agents statalon (Kapusta and Mendenson, 1967), pyran copolymer (Kapusta and Mendenson, 1969), and tilorone (Chang and Hoffman, 1977) can inhibit AA. Virazole, an anti-viral agent which does not induce synthesis of interferon, will also inhibit AA. If this is the case, D-penicillamine cannot inactivate the virus or viruses that caused the AA and therefore has no effect on the clinical aspect of the disease.

Before more convincing evidence is gathered the viral aetiology of AA must remain speculative.

Although D-pencillamine modifies the mononuclear cell functions in AA rats it has no effect on the clinical course of AA. The significance of altered mononuclear cell functions in the pathogenesis of AA is therefore questionable and the value of AA as a model for screening novel antirheumatic drugs with immunomodulatory actions is limited.

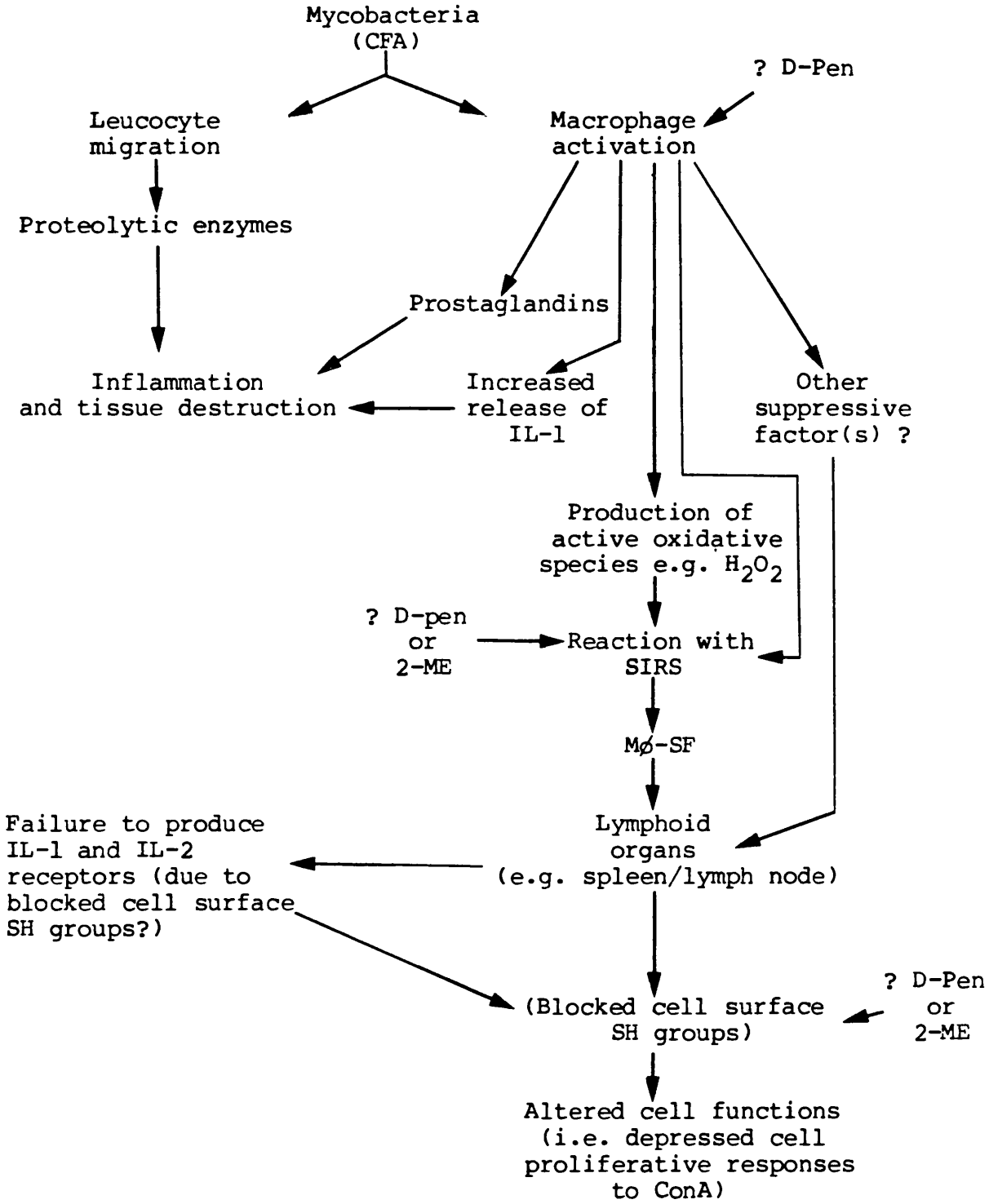


Fig. 6 Hypothetical scheme to depict the series of events leading to altered cell functions in AA.

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