FACTORS THAT MODIFY PENICILLAMINE-INDUCED LUPUS IN BROWN NORWAY RATS:

A Model of Idiosyncratic Drug Reactions

By

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ABSTRACT

Idiosyncratic drug reactions appear to be immune-mediated. Immune responses are driven by helper T cells (Th); Th1 responses promote cell-mediated immunity whereas Th2 responses drive antibody-mediated reactions. Th1 cytokines inhibit Th2 responses and Th2 cytokines inhibit Th1 responses; therefore, it may be possible to prevent idiosyncratic drug reactions by changing the Th1/Th2 cytokine balance.

We tested this hypothesis in an animal model in which penicillamine causes a lupus-like syndrome in Brown Norway rats. This syndrome has the hallmarks of a Th2-mediated response, and we tried to inhibit it with a polymer of inosine and cytosine (poly I:C), a Th1 cytokine-inducer. However, we found that a single dose of poly I:C, given at the onset of penicillamine treatment, significantly increased both the incidence (100% vs. 60%) and accelerated the onset (30 ± 4 vs. 39 ± 5 days) of penicillamine-induced lupus when compared with controls.

To rule out other effects of poly I:C that might overshadow the induction of Th1 cytokines, we directly tested the effects of the prototypic Th1 cytokine, interferon-γ. Although not as dramatic, interferon-γ-pretreatment also appeared to make the syndrome worse.

Conversely, when we used misoprostol, a prostaglandin-E analog that inhibits Th1 cytokines, it completely protected the animals. Just one dose of misoprostol prior to initiation of penicillamine treatment was sufficient to provide this protection.

Evidence suggests that nitric oxide (NO) promotes a Th1 response and would be expected to inhibit a Th2 response. The syndrome was also completely inhibited by aminoguanidine, an inhibitor of iNOS. Again, these results were the opposite of that expected from the Th1/Th2 paradigm.

In short, we have demonstrated that penicillamine-induced lupus in BN rats has the capacity to be influenced by the external factors. Poly-I:C, misoprostol and aminoguanidine were shown to effectively manipulate the incidence and severity of the syndrome in BN rats. However, our results suggest that the manipulatory effects of these agents on the development of the syndrome do not seem to correlate with the capacity of these agents to influence the Th1/Th2 cytokine balance in BN rats. Finally, our data implicate involvement of NO in the development of penicillamine-induced lupus in this animal model.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Name
ANA	antinuclear antibody
APC	antigen presenting cell
BN	brown norway
CD4+ cells	helper T cells
CD8+ cells	cytotoxic T lymphocytes
CNS	central nervous system
COX	cyclooxygenase
CYP450	cytochrome P450
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediamintetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fig.	Figure
GBM	glomerular basement membrane
HLA	human leukocyte antigen
ICOS	inducible co-stimulator
Ig	immunoglobulin
IL-	interleukin
INOS	inducible nitric oxide synthase
LEW	Lewis
LPS	lypopolysaccharide
MBP	myelin basic protein
MHC	major histocompatibility complex
mRNA	messenger RNA
NSAID	non-steroidal anti-inflammatory drug
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PCR	polymerase chain reaction
Poly-I:C	polymer of inosine and cytosine

PRP	pattern recognition receptors
PTU	propylthiouracil
RA	rheumatoid arthritis
rr	recombinant rat
SD	Sprague-Dawley
SLE	systemic lupus erythematosus
TBM	tubular basement membrane
TCE	trichloroethylene
TCR	T cell receptor
Th	helper T cell
Th1	helper T cell type 1
Th2	helper T cell type 2
TNF	tumor necrosis factor

LIST OF PUBLICATION AND ABSTRACTS

a) Publication

- Ebrahim Sayeh, Jack P. Uetrecht. Factors that modify penicillamine-induced autoimmunity in Brown Norway rats: failure of the Th1/Th2 paradigm. ***Toxicology 163 (2001) 195-211.***

b) Abstracts

- Sayeh E, Uetrecht JP (1998) Inhibition of penicillamine-induced lupus in Brown Norway rats by aminoguanidine, an inhibitor of nitric oxide synthase. ***Toxicological Sciences*** **42** (1-S):8

- Sayeh E, Uetrecht JP (1997) Poly I:C increases the incidence and severity of lupus in penicillamine-treated rats. ***The Toxicologist*** **36** (1 pt. 2):196

Chapter 1

General Introduction

1.1 An overview of research objectives and rationale

All drugs used to treat patients are associated with side effects. Idiosyncratic adverse reactions are often serious reactions that occur in a small number of patients treated with a specific drug. Although rare these reactions are often severe and sometimes fatal (Park B. K. et al 1998). Such reactions are not detected by pre-clinical toxicology testing in animals and, in general, the mechanisms involved are poorly understood. Due to their unpredictable nature, drug-induced idiosyncratic reactions have posed a difficult problem, both in medical practice and for present and future drug development. Thus, a better understanding of the mechanisms involved could lead to a significant improvement in drug therapy and simplify drug development.

Penicillamine, a drug used in the treatment of rheumatoid arthritis, is known to induce a range of idiosyncratic reactions of an autoimmune nature including drug-induced lupus (Howard-Lock H.E. et al 1986). Penicillamine also induces a lupus-like syndrome in Brown Norway (BN) rats (Donker A.J. et al 1984). The idiosyncratic nature and many of the features of the syndrome in BN rats are similar to penicillamine-induced lupus in humans. Therefore, penicillamine-induced lupus in BN rats represents a reasonable model for the study of the mechanism of drug-induced lupus in humans.

T helper (Th) cells play a central role in all immune-mediated reactions (Druet P. et al 1995). These (Th) cells can be directed towards developing into Th1 or Th2 cells. Th1 cells promote cell-mediated immunity, whereas Th2 cells drive antibody-mediated reactions (Mosmann T.R. et al 1986). Several lines of evidence have suggested that successful resolution of certain immune-mediated diseases depend on the activity of a specific Th subset (i.e., Th1), and is prevented by activation of the other Th subset cells (i.e., Th2). Thus, one important determinant of the type of adverse reaction induced by drugs may depend on the balance of Th1 and Th2 helper T cells. These observations suggest that knowledge of the type of T helper subset activated by drugs and the factors that differentially affect the activation of Th1 or Th2 cells might provide suitable means for modulation of several types of inappropriate immune responses including drug-induced lupus.

The objectives of this project were: a) to determine if altering the balance of Th1/Th2 responses influences the incidence of penicillamine-induced lupus in BN rats; b) to determine if a correlation exists between induction of specific patterns of Th1 or Th2 cytokine synthesis and the resulting clinical consequences; c) to investigate the possible therapeutic role of agents that have the capacity to influence Th1- or Th2-type responses for penicillamine-induced lupus in BN rats.

1.2 Hypothesis

The balance of Th1 vs. Th2 cytokines is a major determinant of the development of penicillamine-induced lupus in BN rats. Altering this cytokine balance provides a method of preventing drug-induced lupus and other idiosyncratic drug reactions.

1.3 penicillamine

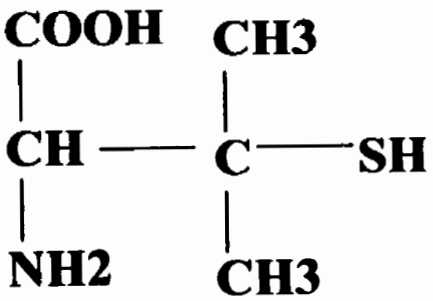
1.3.1 Structure

Penicillamine is a structural analog of the natural amino acid cysteine in which methyl groups replace the two hydrogen atoms in the beta carbon position (Fig.1-1). Penicillamine can exist as either the D or L stereoisomer due to its asymmetric carbon atom. The D form is the naturally occurring isomer and is the isomer used clinically. Thus, all further references to penicillamine in this thesis will specifically denote the D form. As shown in fig.1, penicillamine possesses three functional groups: alpha amino, carbonyl and sulfhydryl. The reactions of these three groups largely determine the biologically relevant chemistry of the compound.

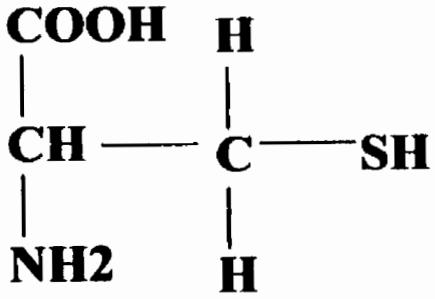
1.3.2 History

Penicillamine was first isolated from the acid hydrolysis products of penicillin by Abraham in 1943 and named penicillamine (Abraham E. P. et al 1943). Penicillamine is the most characteristic degradation product of the penicillin type of antibiotics and was first chemically synthesized in 1949. Walshe was the first to identify penicillamine in the urine of patients with advanced hepatic disease who were being treated with penicillin for intercurrent infections (Walshe J.M. 1956). He became interested in the compound because of its chemical structure and suggested that it should chelate divalent cations, particularly copper, and thereby promote its excretion in patients with Wilson's disease (Walshe J.M. 1956). This proved to be correct, and penicillamine is acknowledged to be the drug of choice for this inherited metabolic disorder that had previously led to severe morbidity and mortality. The drug may also mobilize other divalent cations, such as lead, and it is used in the treatment of intoxication with this and certain other heavy metals (Boulding J.E. & Baker R.A. 1957).

In 1954, Tabachnik demonstrated the *in vitro* formation of a mixed disulfide, penicillamine-cysteine (Tabachnick M. et al 1954). Ten years later, this observation was applied clinically in the management of yet another metabolic disorder, cystinuria (Crawhall J.C. et al 1963). Because of the enhanced solubility of the mixed disulfide resulting from the



penicillamine



cysteine

Fig. 1-1. Structures of penicillamine and cysteine.

penicillamine-cystine interaction *in vivo* (Fig.1-2), administration of the drug to patients with cystinuria results in a diminution of renal calculus formation, often with dissolution of pre-existing stones.

1.3.3 Clinical use

1.3.3.1 Wilson's disease

Wilson's disease is a rare, autosomal recessive, inherited disease characterized by both degenerative changes in the brain and cirrhosis of the liver due to deposition of copper. There is a decrease in the concentration of serum ceruloplasmin and total serum copper with an increase in non-ceruloplasmin copper.

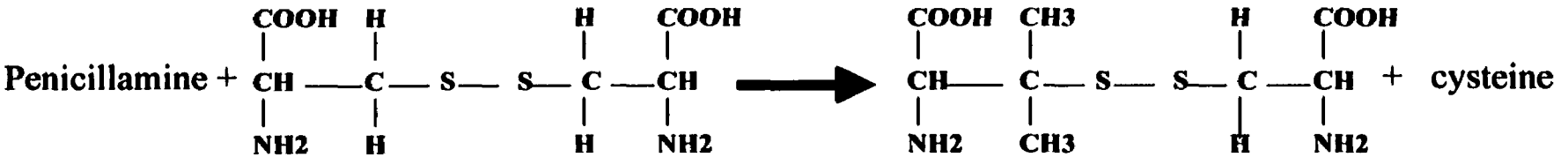
The treatment of Wilson's disease has two main objectives, to minimize the intake and absorption of copper and to promote the excretion of copper already deposited in the tissues. Penicillamine is prescribed for the latter purpose because it has the ability to chelate copper from the tissues and hence enhance its excretion in urine.

1.3.3.2 Cystinuria

Cystinuria is an inheritable disorder in which there is a defect in the transport of the amino acids, cystine, lysine, ornithine and arginine. Cystine has very low solubility at the usual pH of urine. Impaired renal tubular reabsorption of cystine leads to increased daily urinary excretion. Whereas in normal individual the daily urinary output of cystine is 40-80 mg, in cystinuria this may exceed 1 g a day. The formation of urinary cystine calculi is almost certain when the excretion exceeds 300 mg a day due to the poor aqueous solubility of cystine. However, this amino acid can be readily solublized by the formation of a mixed disulfide with penicillamine.

1.3.3.3 Progressive Systemic Sclerosis (Scleroderma)

Scleroderma is an autoimmune disease, which is characterized by excessive deposition of collagen in skin and by fibrosis involving viscera and vasculature (Kang B. et al 1982). Collagen is initially secreted by fibroblasts in a soluble form, which is a substrate for the enzyme lysyl oxidase. This enzyme oxidizes the ε-amino groups of lysine residues on soluble collagen to aldehyde groups. These groups from Schiff's bases with lysyl residues on other molecules of



cystine

penicillamine-L-cysteine

Fig. 1-2. Disulfide interchange between penicillamine and cystine. In this reaction penicillamine is oxidized and the disulfide is reduced.

soluble collagen, making a relatively weak crosslink. The crosslink then stabilizes. *In vitro* studies have shown that aldehyde groups of soluble collagen form thiazolidines with penicillamine, blocking formation of Schiff's bases and thereby preventing crosslinking (Fig.1-3) (Nimni M.E. 1977). Even after the Schiff's base crosslinks are formed; penicillamine is able to cleave them, again probably through thiazolidine formation. However, penicillamine is not capable of cleaving the mature crosslinks. Moreover, examination of collagen from systemic sclerotic patients largely supports the above explanation of penicillamine's effect *in vivo* (Light N. et al 1986).

1.3.4 Biochemical actions

Many of the biochemical modes of action of penicillamine depend on its ability to complex with copper ions and on its ability to disrupt disulfide bonds.

1.3.4.1 Heavy metal chelation

Due to the three functional groups, penicillamine can act as a ligand for a number of transitional metals under appropriate pH conditions (Chow S.T. et al 1973). Chelation of divalent cations, such as copper and lead, has been reported (Goldberg A. et al 1963; Weigert W.M. et al 1975). Chelation of cadmium, gold, zinc, iron, manganese, nickel and mercury by penicillamine has also been described (Fernandez L. et al 1977; Freeman H.C. et al 1976).

1.3.4.2 Disulfide interchange

Penicillamine will oxidize in the presence of a transitional metal and a mild oxidizing agent, such as oxygen, and forms a symmetrical disulfide (Faggiani R. et al 1984). It will also react with other thiols, such as cysteine or cystine, under similar circumstances to form mixed disulfides (Tabachnick M. et al 1954). The reactions are presented in figure 1-2.

Penicillamine is similarly shown to react *in vitro* with oxidized glutathione. In this reaction, the oxidized glutathion is reduced by penicillamine (Bir K. et al 1970; Drummer R. et al 1987). The symmetrical penicillamine disulfide, however, is relatively resistant to chemical reduction and thus does not participate in disulfide interchange reactions. This is possibly due to steric hindrance of the disulfide bond by the four surrounding methyl groups.

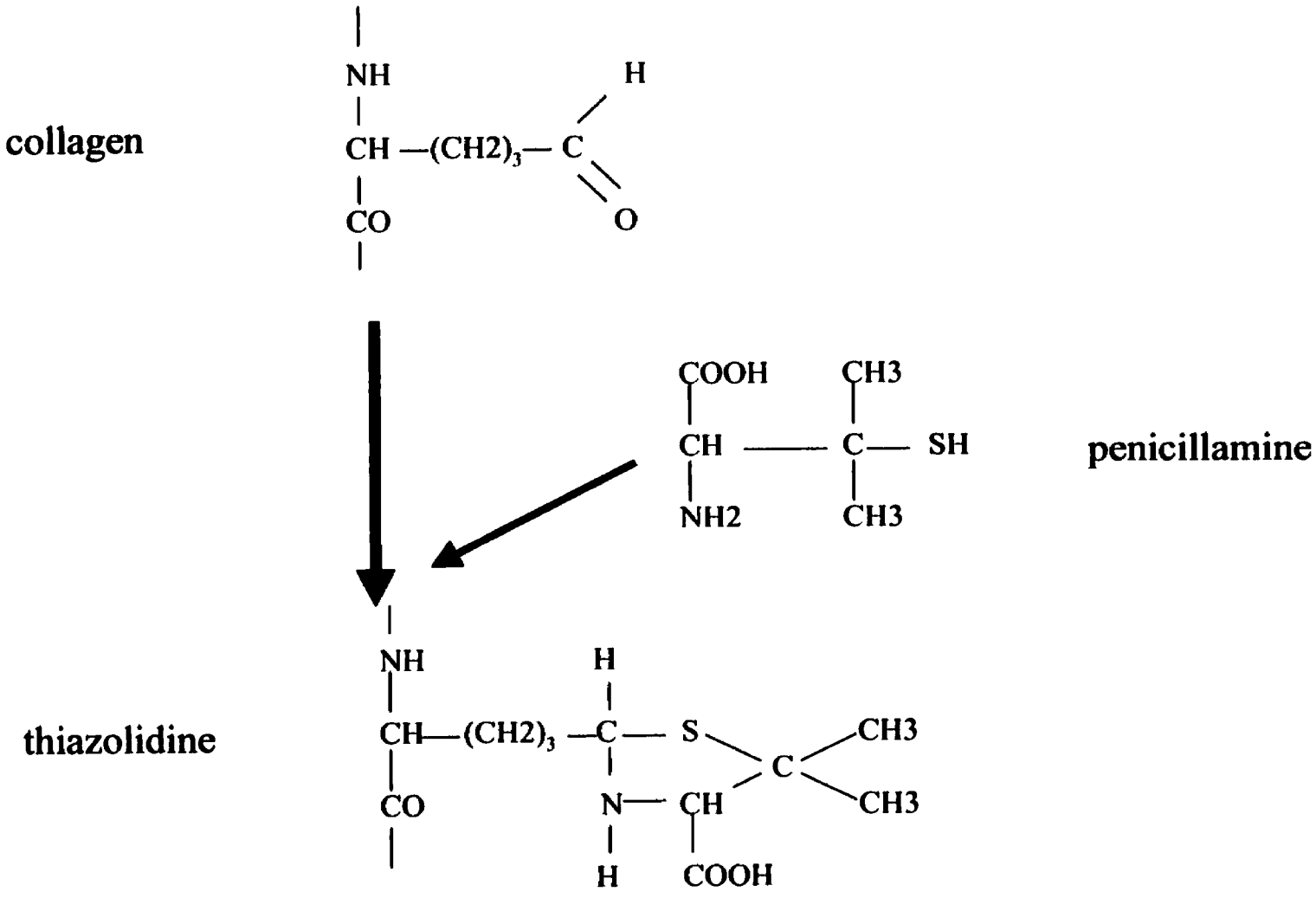


Fig. 1-3. Thiazolidine formation between penicillamine and aldehyde groups on soluble collagen.

1.3.4-3 Thiazolidine formation

Because of its particular chemical structure, penicillamine is able to form a thiazolidine ring by reacting with aldehyde or ketone groups (Howard-Lock H. E. et al 1985). Thiazolidines can also form with acetaldehyde (Nagasawa H.T. et al 1978) and pyridoxal (Heddle J.G. et al 1963) as well. Thiazolidine formation is relevant to the action of penicillamine in systemic sclerosis disease.

1.3.5 Pharmacokinetics of penicillamine

1.3.5.1 Absorption and bioavailability

Penicillamine is readily absorbed from the gut. Peak concentrations of free penicillamine occur between 1.5 and 4 hours after oral dosage in fasting patients (Kukovetz W.R. et al 1983). Bioavailability is reduced more than 50% by food and antacids and 35% reduced by ferrous sulfate (Osman M.A. et al 1983). Peak concentrations range from 5 μM after 150 mg to 27.5 μM after 800 mg after oral administration (Brooks P.M. et al 1984; Wiesner R.H. et al 1981).

1.3.5.2 Distribution

Despite the apparent high volume of distribution, penicillamine is not actively taken up by mammalian cells (Bergstrom R.F. et al 1981). Following oral administration of this drug in humans, free penicillamine concentrations in red blood cells are lower than serum concentrations (Russell A.S. et al 1979). Uptake by lymphocytes and macrophages is also limited (Dawkins R.L. et al 1981). The apparent high volume of distribution for penicillamine, despite limited entry to cells, may be due to binding to tissues, such as skin (Bergstrom R.F. et al 1981), aorta (Ruocco V. et al 1983) and plasma proteins (Joyce D.A. 1989), which occurs rapidly but is slowly reversible.

1.3.5.3 Excretion

Penicillamine has been shown to have a biphasic plasma clearance with approximately 20% being excreted rapidly in the urine, mostly as a disulfide (Bergstrom R.F. et al 1981; Kukovetz W.R. et al 1983). Symmetrical penicillamine disulfide (Perrett D. et al 1976) and disulfides between penicillamine and L-cysteine (Bergstrom R.F. et al 1981), homosysteine and plasma protein (Bergstrom R.F. et al 1981) are the major metabolic products of penicillamine.

The half-life of the rapid plasma clearance phase is about one hour, and the half-life for the slower clearance phase is about 8 days.

1.3.6 Penicillamine-associated adverse drug reactions

1.3.6.1 Hematologic toxicities

Hematological side effects are probably the most serious of the adverse reactions caused by penicillamine. Leucopenia, thrombocytopenia and aplastic anaemia have all been reported with thrombocytopenia being the most common. Hematological side effects lead to treatment cessation in 2 to 5% of patients (Capell H.A. et al 1990; Kean W.F. et al 1982; Situnayake R.D. et al 1987). Regular monitoring of blood counts will detect most of these side effects at an early stage.

1.3.6.2 Proteinuria

Proteinuria occurs in up to 13% of the penicillamine-treated RA patients but only in about 4% of patients with Wilson's disease (Capell H.A. et al 1990; Stein H.B. et al 1980). Renal biopsies of patients with proteinuria show typical immune-complex nephritis, with peak incidences usually occurring 6-9 months after the start of treatment. Should urinary protein excretion exceed 2 g per day, treatment should be withdrawn and urinary abnormalities may take up to 12 months to revert to normal (Howard-Lock H.E. et al 1986).

1.3.6.3 Skin reactions

Skin rashes are the most common of all the side effects (25 to 50%), and may occur after only two weeks treatment (Anderson J.A. 1992). The rashes are more prevalent in Wilson's disease than RA due to the higher initial starting dose. 'Late' skin rashes may occur after several months or even years of treatment and are usually unresponsive to antihistamines and require withdrawal of treatment (Howard-Lock H.E. et al 1986). About 1% of patients develop oral ulcers that require a reduction in the maintenance dose or, more usually, withdrawal of the drug.

1.3.6.4 Taste impairment

Impairment of taste is very common (up to 25%) and usually occurs within the first few weeks and persists for up to 3 months. This hypogeusia will normally clear up even though therapy is continued (Howard-Lock H.E. et al 1986). Nausea, anorexia and abdominal pain may

occur, usually in the first 6 months and more frequently with higher doses and rapid dose increments (Hill H.F.H. 1977). Gastrointestinal events cause approximately 5% of the treatment withdrawals (Capell H.A. et al 1990).

1.3.6.5 Induction of idiosyncratic autoimmune syndromes by penicillamine

What are autoimmune diseases? The word 'auto" is the Greek word for self. The immune system is a complicated network of cells and molecules that normally work to defend the body and eliminate infections caused by bacteria, viruses and other invading microbes. If a person has an autoimmune disease, the immune system mistakenly attacks self, targeting the cells, tissues and organs of a person's own body. There are many different autoimmune diseases and they can each affect the body in different ways. For example, the autoimmune reaction is directed against the brain in multiple sclerosis and the gut in Crohn's disease. In other autoimmune diseases, such as systemic lupus erythematosus, affected tissues and organs may vary among individuals with the same disease. One person with lupus may have affected skin and joints whereas another may have affected skin, kidney and lungs.

The genes a person inherits together with the way the person's immune system responds to certain triggers or environmental agents, such as chemicals or drugs, may influence the development of an autoimmune disease.

Penicillamine therapy is associated with a number of autoimmune diseases. Penicillamine has been reported to be involved in the development of a lupus-like syndrome (Harpey J.P. et al 1971; Oliver I. et al 1972; Walshe J.M. 1981), myasthenia gravis (Dawkins R.L. et al 1981), polymyositis (Ostensen M. et al 1980), dermatomyositis (Fernandez L. et al 1977) and pemphigus (Howard-Lock H.E. et al 1986).

Myasthenia gravis is a neuromuscular disease whose pathogenesis is largely mediated by immune mechanisms. Antibodies to the acetylcholine receptor can be demonstrated in most patients (Dawkins R.L. et al 1981). Although the precise role of this serum factor in the production of the physiologic abnormalities or their correlation with clinical severity is not quite clear, an association of acetylcholine receptor antibodies with the disease is not disputed. Penicillamine-associated myasthenic syndromes are common and quite similar to idiopathic myasthenia gravis (Dawkins R.L. et al 1981). Clinical and serological findings, e.g. the presence of anti-acetylcholine receptor antibodies in over 80% of patients, cannot help differentiate

between idiopathic and the drug-induced myasthenia. However, penicillamine-induced myasthenia may be associated with the HLA-DR1 phenotype, in contrast to idiopathic myasthenia, which is associated with the HLA-DR3 phenotype. The role of penicillamine is indicated by the chronological relationship between the onset of clinical/serological abnormalities and penicillamine therapy as well as their resolution after discontinuation of the offending drug. Most cases were noted in rheumatoid patients with only a few cases in patients with Wilson's disease.

Pemphigus is a disease in which epidermal cell-to-cell adhesion is abnormal (Stein H.B. et al 1980). This defective adhesion process is associated with an autoimmune antigen-antibody reaction occurring on surfaces of epidermal cells (differentiating keratinocytes). Pemphigus also noted in approximately 1% of penicillamine-treated patients (Howard-Lock H.E. et al 1986). Skin lesions may be more severe than in idiopathic pemphigus and can result in a fatal outcome. In contrast to idiopathic pemphigus, circulating pemphigus antibodies are often not detected in the sera of patients with penicillamine-induced pemphigus.

Polymyositis of varied severity (from mild muscle weakness to fatal evolution) has also been noted with penicillamine treatment (Ostensen M. et al 1980).

Penicillamine-induced lupus was first described in patients with RA by Harkcom et al. in 1978 (Harkcom T.M. et al 1978) and has been subsequently reported during penicillamine treatment of Wilson's disease (Walshe J.M. 1981) and cystinuria (Oliver I. et al 1972). The frequency of penicillamine-induced lupus in RA has been reported to be as high as 2% (Chalmers A. 1982), which is consistent with the frequency found in patients taking penicillamine for Wilson's disease (Walshe J.M. 1981).

Penicillamine-induced lupus may develop any time after 6 months and is usually associated with doses of 500 mg or more of penicillamine. Common presenting features include pleurisy, rash, cytopenias, proteinuria, or increasing muscle and joint pain after an initial response to therapy (Chalmers A. 1982).

Penicillamine should be withdrawn from patients who develop symptoms similar to any of the above syndromes. Most of these reactions resolve on stopping penicillamine but the drug should not be reintroduced. The mechanisms of the aforementioned penicillamine-associated autoimmune complications are unknown.

1.4 Adverse drug reactions

An adverse drug reaction is defined as any undesirable effect of a drug beyond its anticipated therapeutic effects occurring during clinical use (Pirmohamed M. et al 1998).

1.4.1 The impact of adverse drug-reactions

Adverse drug reactions are a major clinical problem. Approximately 5% of all patients treated with a course of medication have an adverse reaction related to therapy (Parker C.W. 1975), and approximately 5% of hospital admissions are due to adverse drug-reactions (Jick H. 1984). Adverse drug reactions are more common among hospitalized patients than among ambulatory patients, occurring in as many as 30% of hospitalized patients (Classen D.C. et al 1991), which considerably increases the length and cost of hospitalization. It is hard to estimate the economic burden of adverse drug reactions, but it has been estimated that the annual cost in the US alone for screening, diagnosis and therapy of adverse drug reactions is in the range of $ US 3 billion a year (Bates D.W. et al 1997).

Another cost associated with adverse drug reactions is their effect on drug development. Studies of the adverse effects of drugs and the legal costs associated with adverse drug reaction result in higher prices for new pharmaceutical agents.

In addition to the economic cost of adverse drug reactions, unfortunate patients who develop adverse drug reactions pay a price in terms of pain and suffering. Moreover, in the US, for example, morbidity and mortality related to adverse drug reactions may account for up to 140,000 deaths per year (Bates D.W. et al 1997).

1.4.2 Classification

Although many different classifications of adverse drug reactions have been described, a classification where adverse reactions are divided into two types, A and B, has been widely adopted in both experimental (Zbinden G. 1980) and clinical pharmacology (Breckenridge A.M. & Orme M.L.E. 1987). By separating adverse drug reactions into those that are normal, but

augmented, actions of a particular drug (type A reactions), and those that are totally abnormal, bizarre effects (type B reactions), it is possible to make a logical framework for considering the toxicity of drugs that has both theoretical and practical uses.

1.4.3 Type A adverse drug reactions

Type A adverse drug reactions are the result of an exaggerated, but otherwise normal, pharmacological action of a drug given in the usual therapeutic doses. Drowsiness with benzodiazepine anxiolytics and bradycardia with β-adrenoceptors antagonists are examples of type A reactions. Type A reactions are largely predictable on the basis of a drug's known pharmacology. They are usually dose-dependent and, although their incidence and morbidity in the community is often high, the risk of mortality is generally low.

1.4.4 Mechanisms of type A adverse drug reactions

When a group of individuals receive a drug, a spectrum of responses is observed. This variability manifests itself either as differing doses required to produce the same effect, or as differing responses to the administration of a defined dose, and this forms the basis of type A reactions. In some instances, type A reactions may be an excessive therapeutic effect, such as hypotension with antihypertensive drugs. In others, the reaction may occur as a result of a drug's primary pharmacological action at some other site, such as peptic ulceration and hemorrhage with nonsteroidal anti-inflammatory drugs. However, many type A reactions are not due to the pharmacological action of a drug that mediates its therapeutic effect but to some other property that it possesses. For instance, phenothiazines, some histamine H1-antagonists and tricyclic antidepressants have anticholinergic properties that result in atropine-like adverse reactions, including dryness of the mouth, difficulty with accommodation and, occasionally, retention of urine.

Variability in response to the administration of a defined drug dose is usually seen with anticoagulants, such as warfarin. The doses of warfarin that are required to achieve therapeutic anticoagulation may vary 20-fold between individuals. The reasons for this particular form of

interindividual variability are multifactorial. They are not solely due to differences in the manner in which the drug is distributed and metabolized; interindividual differences in the sensitivity at the site of action are also involved. There is thus considerable variation, between individuals, in the plasma warfarin concentrations required to produce similar degrees of anticoagulation (Routledge P.A. et al 1979). Type A reactions develop in individuals lying at the extremes of dose-response curves for pharmacological and toxicological effects.

1.4.5 Type B adverse drug reactions

Type B adverse drug reactions, which are sometimes referred to as "idiosyncratic, hypersensitivity or allergic reactions", involve aberrant effects that are not expected from the known pharmacological actions of a drug.

The term "idiosyncratic drug reaction" will be used in this thesis to describe a reaction that:

- does not occur in most patients who are treated with the drug, even at high doses
- is usually unpredictable
- is not observed during conventional pharmacological and toxicological screening tests.

1.4.6 Significance of idiosyncratic drug reactions

Idiosyncratic drug reactions are of major concern, both in medical practice and for drug development. Although these reactions are less common than predictable (type A) reactions, they are not rare, and they probably account for approximately 10% of all adverse drug reactions (Park B.K. 1986). In addition, there are a large number of drugs that are associated with serious and often life-threatening idiosyncratic reactions.

At present it is not possible to accurately predict the risk that a new drug will cause idiosyncratic reactions, and such adverse reactions are usually only detected at a relatively late stage in drug development. For this reason, many drugs, such as practolol, benoxaprophen, ticrynafen and zomepirac, were withdrawn shortly after they were released on the market. This is wasteful in terms of commercial investment and human effort.

1.5 Clinical characteristics of idiosyncratic adverse drug reactions

The clinical characteristics of idiosyncratic drug reactions can vary considerably between different drugs and also for the same drug in different individuals. The most common targets for idiosyncratic drug reactions are the skin, blood cells and the liver, but virtually any organ can be affected. Reactions are sometimes limited to specific organs such as the liver or bone marrow. However, in other instances, multiple organs may be affected simultaneously. In addition, the primary drug-induced lesion may be accompanied by general manifestations such as fever, skin rash, lymphadenopathy and eosinophilia suggestive of a hypersensitivity reaction.

1.5.1 Cutaneous reactions

Adverse cutaneous reactions to drugs are frequent and affect 2-3% of hospitalized patients (Park B. K. et al 1987). Fortunately, most skin reactions are mild and only require withdrawal of the drug for resolution. The majority of drug-induced skin reactions appear to be of an immunological nature. This has been attributed to the combination of metabolic activity and immunological competence of the skin. The skin contains small quantities of both phase I and phase II drug-metabolizing enzymes, including different forms of cytochrome P450. In addition, the skin is immunologically very active and contains specialized cells such as Langerhans cells and dendritic cells (Pirmohamed M. et al 1998).

The two most severe skin reactions are Stevens-Jonson syndrome, for which mortality rates approach 5%, and toxic epidermal necrolysis, which has a mortality rate of 30% (Pirmohamed M. et al 1998). Stevens-Jonson syndrome is a disease in which cutaneous lesions of erythema multiform bullosum are accompanied by severe mucous membrane involvement and systemic symptoms. This condition is occasionally fatal. In toxic epidermal necrolysis, the epidermis is infiltrated by activated T lymphocytes (the majority of which are CD8+ cells) and macrophages, which suggests a cell-mediated cytotoxic reaction against epidermal cells (Stiles D.P. et al 1990).

The drugs most frequently involved in life-threatening cutaneous reactions include antibacterials, such as long acting sulfonamides and penicillin, anticonvulsants and nonsteroidal anti-inflammatory drugs (NSAIDs) (Park B. K. et al 1998). Of the anticonvulsants, the aromatic compounds phenytoin, carbamazepine and phenobarbitone are particularly liable to cause skin eruptions (Uetrecht J.P. 1990).

1.5.2 Hepatotoxicity

Idiosyncratic drug reactions affecting the liver are common with more than 600 drugs having been reported to cause hepatic injury. Clinical features variably include liver enlargement, abnormal liver function test results, jaundice and fever (Pohl L.R. et al 1988). Offending drugs have included sulfonamides, phenylbutazone, allopurinol, halothane, phenytoin, quinidine and chlorpropamide (Park B. K. et al 1998). The pattern of liver injury is variable and includes hepatitis, cholangitis, steatosis, granuloma, cirrhosis and neoplasia. Idiosyncratic hepatotoxicity may be due to direct toxicity of a chemically reactive metabolite or secondary to an immune reaction. However, it is always difficult to determine whether the toxicity is chemical or immunological in nature because of the inaccessibility of the damaged tissue, especially at the time of the initial tissue injury. Fortunately, withdrawal of the offending drugs leads to clinical and histological resolution in the majority of cases. Although with some drugs, such as iproniazid, withdrawal has no effect and the hepatic injury progresses after discontinuation, suggesting an immunological etiology (Pirmohamed M. et al 1998). Overt hepatic disfunction due to drugs is uncommon, with drugs accounting for about 2% of all cases of jaundice (Descotes 1990), although asymptomatic liver enzyme elevation is more common, being as high as 20% for some drugs such as isoniazid (zimmerman H.G. 1978). Hepatic failure is the most severe result of liver injury of which idiosyncratic drug reactions account for approximately 20% of cases (zimmerman H.G. 1978).

1.5.3 Hematological reactions

Blood cells are a common target in idiosyncratic drug reactions. Drug-induced hematological toxicity can affect platelets, red cells, white cells or all the cellular elements of the bone marrow leading to thrombocytopenia, hemolytic anemia, agranulocytosis or aplastic anemia, respectively. The most serious of these reactions, because of its high mortality, is aplastic anemia. Although this is a rare reaction, it has been estimated that between 30% to 60% of all cases of this hematological abnormality are related to drug treatment (Uetrecht J.P. 1990). Aplastic anemia (failure of bone marrow to produce all of the blood cell lines) is presumably due to damage of the pluripotential stem cells (Uetrecht J.P. 1990). The mortality rate for aplastic anemia can be as high as 50% (Park B.K. et al 1987). In agranulocytosis only the granulocyte cell lines (mainly neutrophils) are depleted (granulocyte count of less than 500 cells/μL). This can occur either through depletion of granulocyte precursors in the bone marrow or peripheral destruction of neutrophils (Pirmohamed M. et al 1998). Agranulocytosis has been found to be the most likely cause of death among drug-induced blood dyscrasias. The incidence of agranulocytosis is dependent on the drug; clozapine 1 in 100 (Pirmohamed M. et al 1998), captopril 1 in 250 (Classen D.C. et al 1991) and phenothiazines 1 in 1300 (Pirmohamed M. et al 1998). Many drugs that cause agranulocytosis are also associated with aplastic anemia. The idiosyncratic nature of drug-induced agranulocytosis, and the time course of the reactions, especially on rechallenge with drug, may sometimes be strongly suggestive of the involvement of the immune system. However, direct evidence for such a mechanism is often limited. Hemolytic anemia and thrombocytopenia are other hematological reactions associated with the use of some drugs. These adverse reactions also usually appear to be immune-mediated.

1.5.4 Drug-induced lupus

1.5.4.1 Lupus

Lupus is a multisystem autoimmune disease affecting a variety of tissues and organs. The disease is characterized by a profound and widespread disturbance of immune mechanisms, leading to the formation of autoantibodies and immune complexes. Lupus characteristically

affects the vasculature (leading to renal, cerebrovascular, pulmonary and widespread organ involvement) as well as serosal surfaces (leading to pleurisy, pericarditis and peritonitis). While renal, central nervous system (CNS) and cardiac lesions are prognostically most important, the most frequent manifestations of lupus are of skin and of joint disease. The etiology of lupus is unclear, but hormonal factors, environmental toxins, infectious viruses, genetic predisposition and certain drugs have all been considered risk factors. Idiopathic lupus (when the cause of lupus is not known) is seen predominantly in young women, with a female: male ratio of approximately 10:1. Each patient is unique and may suffer from a variety of signs and symptoms. The disease is highly unpredictable, and most patients experience flare-ups or fluctuations. Drug-induced lupus is most notably different from idiopathic SLE in that:

- It resolves on discontinuation of the drug (possibly for this reason, it is a milder syndrome).
- There are no specifically predisposed age groups as affected patients merely reflect those more frequently treated with the drug.
- A greater percentage of Caucasian patients with less female predominance than reported in idiopathic lupus is evidenced in drug-induced lupus.
- Renal lesions may occur but less frequently than in idiopathic lupus. The central nervous system is rarely affected.
- High titers of antihistone antibodies are found in more than 95% of patients with drug-induced lupus. By contrast, native antiDNA and antiSM antibodies are generally considered as very specific for idiopathic lupus and hence extremely infrequent in drug-induced lupus syndrome (Descotes 1990).

1.5.4.2 Immunology of lupus

The immunological hallmark of lupus is the presence of circulating antinuclear antibodies (ANA). These antibodies are present in 96% of cases. The ANA may be directed against various components of the nucleus, the most characteristic being native or double-stranded DNA (dsDNA). Anti-dsDNA antibodies are seldom found in any condition other than lupus. Antibodies to single-stranded DNA are also found in lupus but are less specific. Antibodies to soluble proteins of the nucleus may also be found in lupus. By a strange convention these antigens are often named from the first two letters of the surname of the patient in whom they

were first discovered (e.g., Sm = Smith). Anti-Sm antibodies are highly specific for lupus (Chapel H. & Haeney M. 1984; Rose N. & Machay I. 1985).

In addition to ANA, autoantibodies may also be found in lupus to other cells and cellular components. For instance, many lupus patients have anti-phospholipid, -red blood cells, -platelets and -lymphocytes antibodies (Rose N. & Machay I. 1985). Lupus patients also have a marked polyclonal B cell activation and an associated hypergammaglobulinemia. Patients with active lupus usually have low complement levels, because in these individuals complement is consumed during the formation of the immune complexes (Rose N. & Machay I. 1985).

1.5.4.3 Causes of lupus

Why does the nuclear component in the lupus patient become antigenic? In some patients drugs are to blame. It is estimated that about 10% of lupus is drug-induced (Uetrecht J.P. 1990). Most commonly, hydralazine, isoniazid and procainamide are associated with the induction of lupus. Many of these compounds have an aromatic amine group, which is metabolized by acetylation. It is possible that a virus may also be responsible for the disease but none has yet been identified.

About 30% of SLE patients report an exacerbation of their symptoms by sunlight (Stiles D.P. et al 1990). It is also likely that ultraviolet radiation may denature DNA and render it antigenic.

Reactive oxygen species, which are released at sites of inflammation during the respiratory burst that accompanies phagocytosis, can also denature DNA (Stiles D.P. et al 1990).

1.5.4.4 Clinical manifestations of lupus

Skin and joints: The commonest features of lupus are inflammation of the skin (often in sun-exposed parts) and joints secondary to immune complex deposition. The arthritis of lupus is relatively mild and seldom damages the cartilage or causes deformities. The rash is often across the cheeks in a classical butterfly distribution. Occasionally the skin lesions are more severe and progress to ulcers or gangrene. Many patients also develop diffuse or patchy hair loss (Weatherall D.J. et al 1987). However, skin rash is less common in drug-induced lupus, and when it does occur, it is not the butterfly rash often seen in idiopathic lupus.

Kidney: The serious consequences of lupus arise mainly from involvement of the kidney, lung or brain. Circulating immune complexes, depending, in part, on their size, may cause

various patterns of kidney damage. Two clinical syndromes may result from renal damage. One is renal failure, which may happen very suddenly, and the other is the nephritic syndrome. In the latter situation the kidneys leak large amounts of albumin and the patient becomes edematous because there is no longer sufficient albumin in the circulation to maintain an adequate osmotic pressure.

Lung: Small blood vessel inflammation in the lungs may produce progressive breathlessness. The muscles of the chest wall and diaphragm may also become inflamed and weak (Rose N. & Machay I. 1985).

Brain: Inflammation in the brain may produce loss of consciousness or a variety of psychiatric syndromes. However, this is relatively uncommon (Rose N. & Machay I. 1985).

Thrombosis: As a result of the anti-cardiolipin antibody, which promotes coagulation, patients may develop recurrent thrombosis in the arteries or veins. These clots may cause strokes or myocardial infarctions (heart attacks). In pregnant women small clots in the placenta may cause miscarriages (Stiles D.P. et al 1990).

Blood related problems: Antibodies to red blood cells and platelets may be associated with anemia and bleeding respectively (Weatherall D.J. et al 1987).

As can be seen from the above description, the possible features of lupus are very diverse because many combinations of these features may occur together. In addition, patients may feel unwell with fever and they often experience a significant body weight loss.

There is a large degree of overlap between drug-induced and idiopathic lupus (when the cause of lupus is unknown) and it is impossible to differentiate them on the basis of clinical or laboratory manifestations (Uetrecht J.P. 1990). However, there are some specific diagnostic criteria for drug-induced lupus:

- There should have been no history suggestive of lupus before drug therapy was started.
- A positive antinuclear antibody should be detectable, with at least one clinical feature of SLE (fever, myalgias and arthralgias) during sustained treatment.
- There should be rapid improvement in the clinical symptoms and a gradual fall in the antinuclear antibodies and other serologic changes when the drug is withdrawn (Price E.J. & Venables P.J. 1995).

1.6 Possible mechanisms of drug-induced lupus

The mechanism of drug-induced lupus has been a matter of much speculation. Following are the mechanisms that have been proposed:

- One mechanism is that drugs could facilitate the expression of idiopathic lupus in predisposed patients (Alarcon-Segovia D. et al 1965). This hypothesis has not been substantially documented and there is no clear evidence that drugs inducing a lupus-like syndrome in humans can accelerate the development of lupus in animal models.
- Another mechanism possibly involved in the pathogenesis of drug-induced syndromes is the interaction of drugs with nuclear antigens. For instance, hydralazine has been reported to complex with nucleoproteins (Tan E.M. 1974). A possible mechanism is that hydralazine or procainamide could act as a hapten and react with histone proteins leading to the formation of antihistone antibodies. However, attempts to induce antinuclear antibodies by immunizing animals with procainamide bound to proteins were generally unsuccessful (Gold E.F. et al 1977).
- Another mechanism is the interference of drugs or their reactive metabolites with the immune system. Indeed, lupus-inducing drugs have been shown to impair some components of the normal immune response. As an example, α-methyldopa was found to impair phytohemagglutinin-induced proliferation of T-lymphocytes (Kirtland H.H. et al 1980) whereas procainamide, in addition to exerting the same effect (Bluestein H.G. et al 1979), may also specifically activate B-lymphocytes (Forrester J. et al 1988). Lymphocytotoxic antibodies were also found in patients with hydralazine or procainamide-induced lupus but their pathogenic significance is unclear (Bluestein H.G. et al 1979).
- An interference with the complement system has also been considered due to the established involvement of various complement defects in idiopathic lupus erythematosus. Even though several drugs that induce lupus syndromes, e.g. hydralazine, isoniazid or procainamide, have been shown to inhibit various complement components, in particular C3 and C4 (Sim E. et al 1985), the role of this interference in the induction of drug-induced lupus is unsettled.
- An interference with mononuclear phagocytes is another hypothesis (Uetrecht J.P. 1988). Drugs that have been associated with the induction of lupus syndromes are readily oxidized

to reactive metabolites within monocytes and macrophages. Interestingly, reactive metabolites produced within mononuclear phagocytes may bind to class II molecules of the major histocompatibility complex (MHC) at the surface of macrophages or other antigen-presenting cells and modify them in such a way that T cells recognize the modified epitopes as foreign. The molecule generated would be functionally equivalent to an allogeneic MHC molecule and would give rise to a strong stimulation of T-lymphocytes and the subsequent production of autoantibodies (Gleichman E. et al 1988) (interactions similar to those occurring in graft-*versus*-host disease).

- Inhibiting DNA methylation and altering expression of certain genes is another mechanism that has been postulated for the induction of lupus by some drugs, such as procainamide and hydralazine (Yung R.L. & Richardson B.C. 1994). These drugs have been shown to inhibit T cell DNA methylation and induce autoreactivity. In addition, T cells isolated from patients with active lupus were shown to have hypomethylated DNA and diminished DNA methyltransferase activity (Yung R.L. & Richardson B.C. 1994). Moreover, in an experimental model, it was shown that the adoptive transfer of murine T cells made autoreactive with DNA methylation inhibitors was sufficient to cause a lupus-like disease in otherwise healthy syngeneic recipients (Yung R.L. & Richardson B.C. 1994). These results and others propose a mechanism for drug-induced lupus, in which certain environmental agents modify T cells by inhibiting DNA methylation and altering expression of certain genes, thereby inducing autoreactivity. The autoreactive cells then interact with the host to produce a lupus-like disease (Richardson B. et al 1990).

1.7 Immune involvement in idiosyncratic drug reactions

1.7.1 The role and functions of the immune system

The immune response can be separated into two components, a cellular response and a humoral or antibody response (Fig. 1-4). These two systems act together to perform three major activities:

- Distinguishing "self" from "non-self" constituents,
- Attacking and eliminating "non-self" constituents,

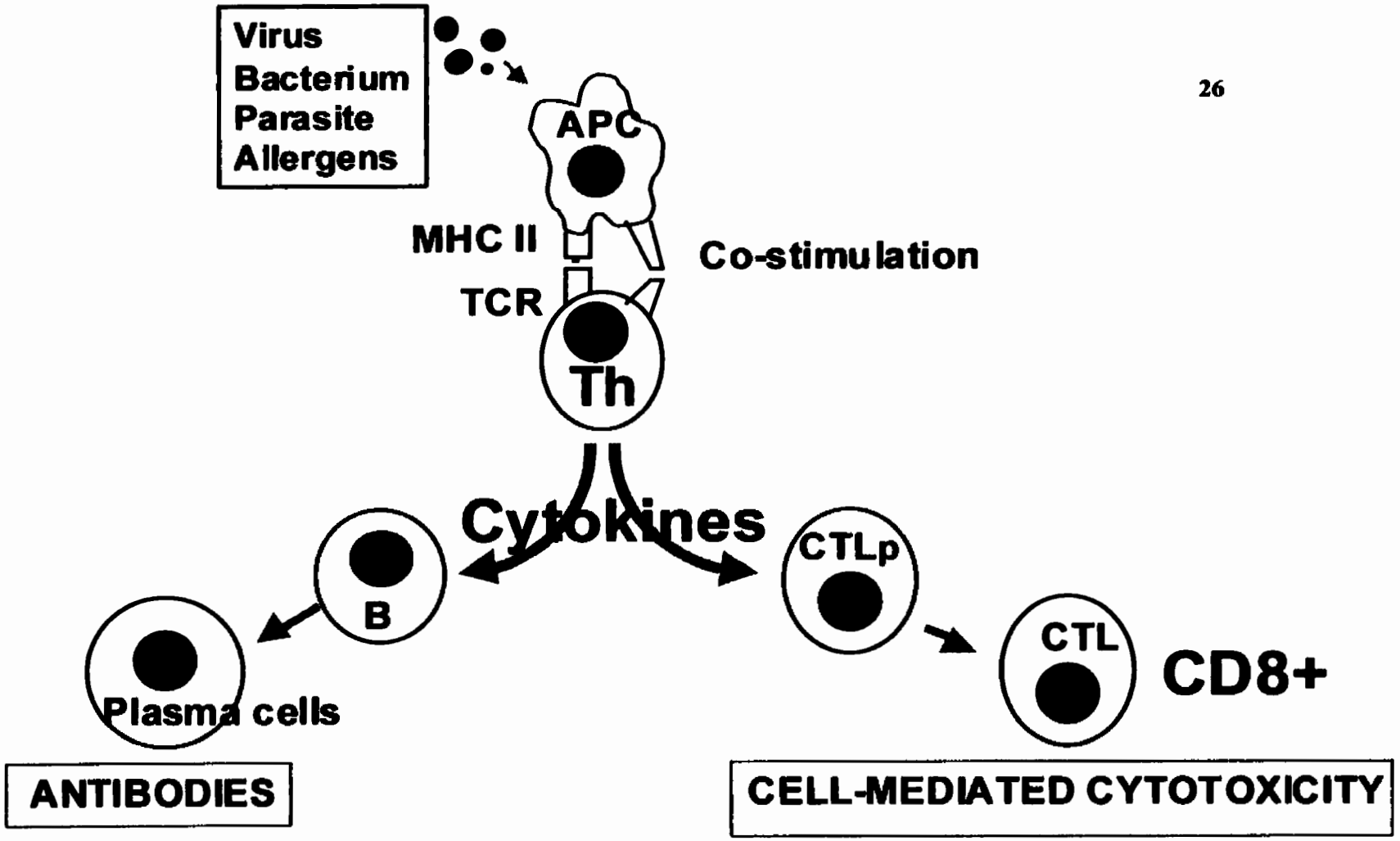


Fig. 1-4. The immune system can be separated into two components, a cellular response and a humoral or antibody response.

- Storing the determinants of these events in immune memory.

To be recognized by the immune system, antigens are internalized, partially degraded and displayed as antigenic determinants or epitopes on the surface of the cell in association with major histocompatibility complex (MHC) molecules. In the case of viral infections, this process leads to the marking of infected cells for destruction by the immune system. Cell-mediated cytotoxicity occurs through recognition of MHC-restricted antigen via the T-cell receptor complex, causing cellular activation, production of cytokines, clonal expansion and the recruitment of activated macrophages (Uetrecht 1997). In contrast, a humoral response is elicited by B cell production of antigen-specific antibody through the interaction of B cells with T cells that have been activated by antigen-presenting cells (APCs) (Uetrecht 1997).

As stated above, many idiosyncratic adverse reactions are thought to be mediated by the immune system on the basis of clinical criteria (Park B.K. 1986; Pirmohamed M. et al 1998; Pohl L.R. et al 1988). Adverse drug reactions that involve the immune system are referred to as hypersensitivity reactions (Uetrecht J.P. 1988).

The mechanism by which a drug leads to an immune-mediated adverse reaction is explained by the hapten hypothesis (Park B. K. et al 1987). In the following sections of this thesis the postulated mechanistic details of immune-mediated hypersensitivity reactions to drugs will be discussed (Fig.1-5).

1.7.2 Hapten hypothesis

The term hapten has been used to describe a substance that is not immunogenic *per se* but becomes immunogenic when conjugated to a macromolecular carrier.

An immunogen is a substance that is capable of eliciting a specific immune response manifested by the formation of specific antibodies and/or specifically committed lymphocytes.

The molecular mass of an organic molecule is one of the major determinants of its ability to act as an immunogen. It is generally believed that low molecular weight compounds (<1000 Da), such as drugs, are not immunogenic but may become so when they become covalently bound to a macromolecule, such as an endogenous protein, to form a hapten.

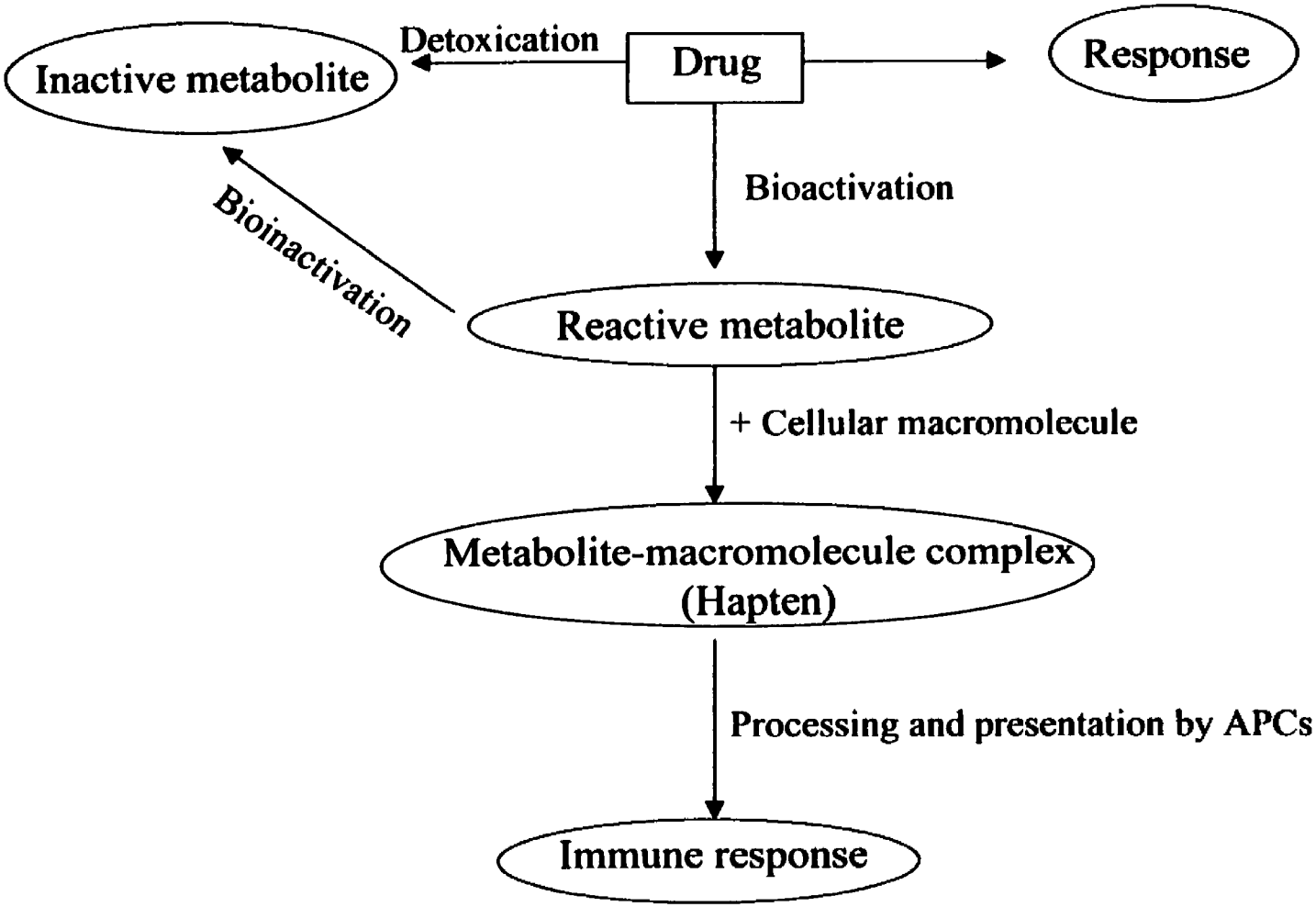


Fig. 1-5. Pathway for production of an immune response by reactive drug molecules associating with cellular macromolecules in the absence of adequate detoxification of reactive drug metabolites.

1.7.3 Mechanisms of hapten formation

1.7.3.1 Direct conjugation to proteins:

The classical model sensitizing agents, such as dinitrochlorobenzene and dinitrofluorobenzene, bind directly to proteins in a relatively nonselective manner and modify primarily lysine and cysteine residues (Park B. K. et al 1998). These compounds have been used in various animal models to investigate contact sensitivity, tolerance and antigen presentation.

Certain drugs, such as penicillin, have a reactive structure and have the capacity to bind covalently to proteins and carbohydrates by reaction with nucleophilic amino, hydroxyl, mercapto and histidine groups. A number of sites on the molecule are open to nucleophilic attack and, therefore, a number of antigenic determinants may be formed (Uetrecht J.P. 1990).

As explained at the beginning of this chapter, penicillamine is a trifunctional amino acid containing a free sulfhydryl group and a free amino group. It therefore has direct chemical reactivity toward a range of biological macromolecules. The drug can react with biochemical intermediates that contain a free carbonyl group, to form thiazolidine derivatives. Penicillamine can also participate in thiol-disulfide interchange reactions between the free sulfhydryl group in penicillamine and cystine groups in proteins, which governs its disposition in tissues. This is a spontaneous equilibrium process, which may result in the formation of disulfide-linked penicillamine-protein conjugates and the reduction of existing (cystine) disulfide bonds in proteins.

At present, all drugs or drug metabolites that can conjugate directly to proteins *in vivo*, by formation of a stable covalent bond, should be considered potential immunogens.

1.7.3.2 Drug-protein conjugation via bioactivation:

The majority of drugs do not bind directly to proteins *in vitro*, and it is therefore assumed that bioactivation to a chemically reactive metabolite, which is thought to function as the ultimate hapten, must occur *in vivo*. Bioactivation to chemically reactive species is usually dependent on oxidative phase I biotransformations, which are performed principally by cytochrome P450 enzymes. A number of drugs, such as halothane and tienilic acid, that are associated with hypersensitivity have been shown to undergo metabolism to chemically reactive metabolites in the presence of human and animal hepatic microsomes *in vitro* (Pohl L.R. et al

1988). Drug metabolism may also occur within leukocytes by non-P450 enzymes. Mature neutrophils have two enzymes, NADPH oxidase and myeloperoxidase, which together lead to the oxidation of drugs. NADPH oxidase converts oxygen to superoxide, which is reduced to hydrogen peroxide. Hydrogen peroxide oxidizes myeloperoxidase to form a compound that can oxidize drugs either directly, or indirectly by oxidation of chloride to hypochlorous acid; this can also bioactivate drugs to protein-reactive species (Uetrecht J.P. 1990).

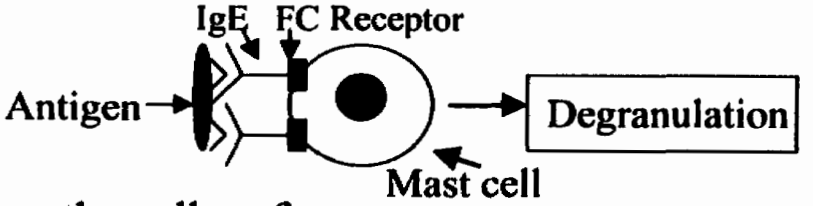
1.7.4 Classification of hypersensitivity reactions

In 1963, different types of hypersensitivity were classified by the Coombs and Gell (Coombs R.R.A. & Gell P.G.H. 1968) (Fig.1-6).

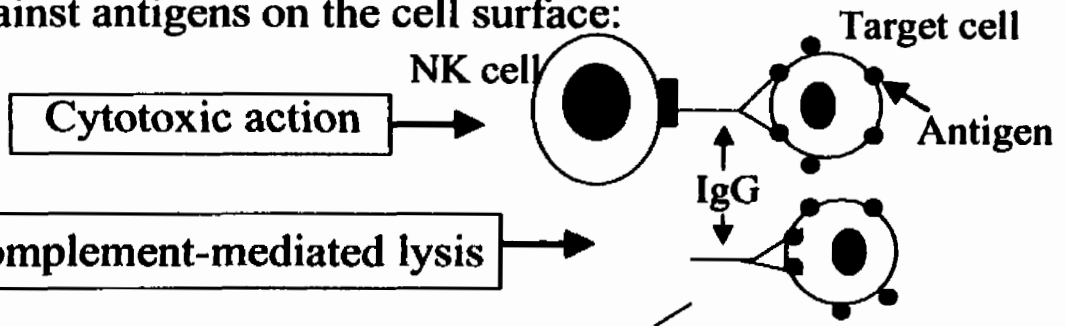
- Type I hypersensitivity refers to reactions initiated by antigen cross-linking between specific immunoglobulin E (IgE)-loaded mast cell Fc receptors. Cross-linking stimulates mast cell release of chemical mediators, such as histamine, serotonin prostaglandin, leukotrienes and heparin, which initiate inflammation, vasodilation and reduced platelet coagulation. Clinical symptoms of type I hypersensitivity may manifest as anaphylaxis, allergic rhinitis, urticaria and/or bronchospasm. The reactions can be local or systemic, and symptoms range from mild irritation to severe anaphylaxis (e.g. hives, swelling of mouth and throat, difficulty breathing, hypotension and shock).
- Type II hypersensitivity is mediated by processes in which antibodies (usually IgG) are directed against antigens present on cell surfaces. Antibody-antigen interaction leads to immune activation and cell damage via activated effector cells, such as NK cells, neutrophils and monocytes, or via activation of complement-induced lysis. Clinical manifestations of these reactions include disorders, such as hemolytic disease of the newborn, drug-induced hemolytic anemia and graft rejection in transplantation.
- Type III hypersensitivity describes processes mediated by immune complexes that are formed when antigen interacts with antibody that has not been cleared by the reticuloendothelial system. In the case of persistent infection, autoimmune disease (e.g., systemic lupus erythematosus) or repeated contact with antigens (e.g., occupational lung disease), chronic antigen-antibody interactions can lead to tissue deposition of immune complexes.

Fig. 1-6. Classification of hypersensitivity reactions as described by Gell and Coombs.

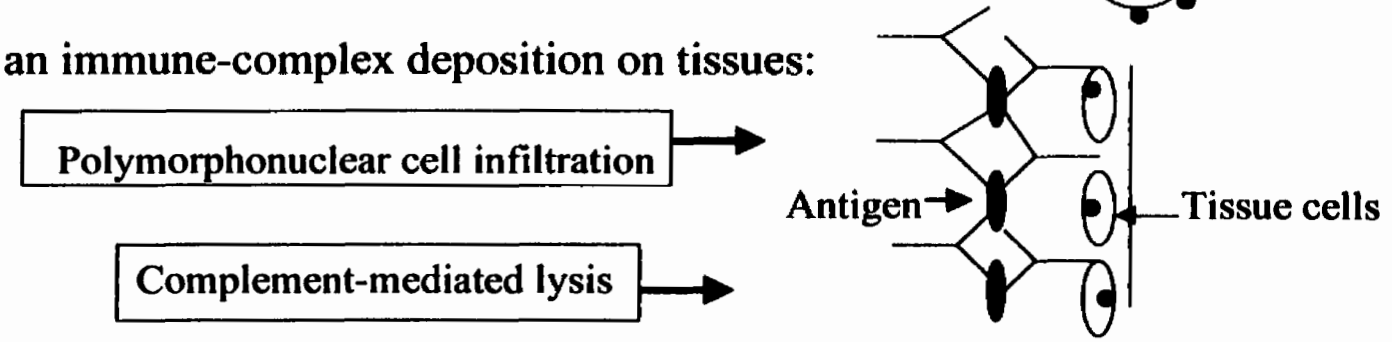
Type I is antigen-specific IgE directed against mast cell FC receptors:



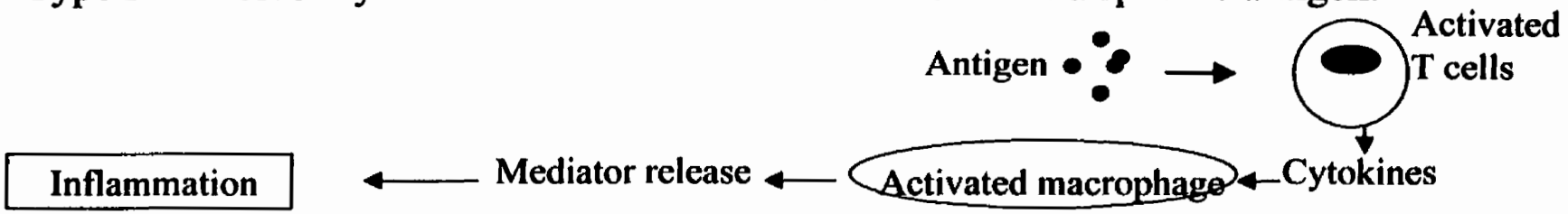
Type II is an antibody directed against antigens on the cell surface:



Type III involves an immune-complex deposition on tissues:



Type IV involves cytokine release from T cells sensitized to a specific antigen:



Complement activation and polymorphonuclear cell chemotaxis are the mediators of inflammation that damages the affected tissue.

- Type IV hypersensitivity (delayed-onset) describes reactions that take more than 12 hours to evolve; these reactions are mediated by T-lymphocyte responses to antigen. The activated T cells produce and secrete cytokines that, in turn, stimulate general inflammation and macrophage activation. Examples of delayed-onset hypersensitivity include contact dermatitis and tuberculin reactions, both of which occur within 72 hours of antigen challenge.

1.7.5 Non-hapten hypothesis

Besides hapten-induced unwanted immune responses, there are other ways in which a drug (or metabolite) could interact with the immune system that would result in the development of idiosyncratic reactions.

1.7.5.1 Drug-induced idiosyncratic reactions mediated by complement or alterations in the complement system

Complement: The complement system is a group of about twenty serum proteins whose overall function is the control of inflammation. Several of the components are acute phase proteins. The components interact with each other and with other elements of the immune system. For example, a number of microorganisms spontaneously activate the complement system via the so-called 'alternative pathway', which is an innate, non-specific reaction. This results in a coat of complement molecules on the microorganism, leading to its uptake by phagocytes. The complement system can also be activated by antibodies bound to the pathogen surface (the classical pathway) when it constitutes a specific, adaptive response.

Complement activation is a cascade reaction with each component sequentially acting on others in a similar way to the blood-clotting system. Activation of the complement system by either the classical or the alternative pathway generates peptides that have the following effects:

- Opsonization (coating) of microorganisms for uptake by phagocytes.
- Attraction of phagocytes to sites of infection (chemotaxis).
- Increased blood flow to the site of activation and increased permeability of capillaries to plasma molecules.

- Damage to plasma membranes on cells, gram-negative bacteria, enveloped viruses or other organisms that have induced the activation. This, in turn, can produce lysis of the cell.

A drug or its metabolite could directly modify activation of complement or clearance of immune complexes and result in the development of idiosyncratic reactions. An example of this type of mechanism is the idiosyncratic reaction induced by the radiographic contrast media. This chemical induces an anaphylactic-like syndrome in some people that does not appear to be mediated by immunoglobulin E (IgE). Although the pathogenesis of this adverse reaction is not completely known, radiocontrast material does predispose to activation of the alternate complement pathway (Arroyave C.M. et al 1976; Kolb W.P. et al 1978). Such activation could explain many of the features associated with the idiosyncratic reactions induced by radiographic contrast media, and this may play an important role in their pathogenesis.

A different type of interaction between drugs and the complement system that may lead to the development of idiosyncratic reactions has been reported (Sim E. et al 1985). Drugs that are good nucleophiles, such as hydralazines, have been shown to react with C4 or C3 components of the complement, and this leads to an increase in the concentration of circulating immune complexes (Sim E. et al 1988). Thus, it has been proposed that this type of enhancement in the levels of immune complexes may be the mechanism by which some drugs (e.g., procainamide, hydralazine and penicillamine) induce lupus (Sim E. & Law S.A. 1985).

Drugs such as penicillamine, which has several effects on cellular immunity including T-helper and B-cell functions, is likely to interfere with immunoregulation of humoral antibody production at various levels within the immune system. One should not therefore automatically assume that drugs that cause adverse reactions of an immunological nature do so by forming haptens.

1.8 Experimental models of drug or chemical-induced idiosyncratic reactions

1.8.1 Mercury-induced autoimmunity in BN rats

Mercuric chloride (1mg/kg, s.c.; 3 times a week) induces an autoimmune disease in BN rats (Sapin C. et al 1977). In the second week after the first $HgCl_2$ injection, an enlargement of spleen and lymph nodes is found due to proliferation of both CD4+ T-helper and B cells

(Pelletier L. et al 1988). Autoantibodies to anti-glomerular basement membrane (GBM), type II collagen and DNA are detected in these animals. Serum immunoglobulin concentrations, especially IgE, are increased. T cells from diseased BN rats can transfer the disease in naïve syngeneic recipients (Pelletier L. et al 1988). The susceptibility to mercury-induced autoimmunity is genetically controlled. Thus, Lewis rats are resistant in contrast to BN rats (Druet E. et al 1977).

1.8.2 Effect of mercuric chloride in other species

Mercuric chloride induces an idiosyncratic autoimmune disease in rabbits quite similar to the one observed in BN rats (Roman-Franco et al 1978). The susceptibility of mice to mercury-induced autoimmunity has been studied by several investigators (Hultman P. & Enestrom S. 1987; Hultman P. & Enestrom S. 1988; Pietsch P. et al 1989). As found in rats, susceptibility is genetically controlled. Lymphoproliferation, an increase in total serum IgG and IgE concentrations, circulating antinucleolar antibodies and an immune complex-type glomerulonephritis were described in A-SW, SJL and B10-S mice. In contrast, DBA/2 mice were completely resistant to mercury-induced autoimmune disease.

1.8.3 Penicillamine and gold salt-induced autoimmunity in rats

BN rats treated with penicillamine or gold salts display autoimmune disorders similar to the ones following mercuric chloride injections (Donker A.J. et al 1984; Tornade H. et al 1990). In mercuric chloride-injected BN rats, autoimmune disorders are more intense than in gold salt- and penicillamine-treated BN rats. Nephrotic syndrome or proteinuria is found in all the rats injected with mercuric chloride versus 50% in the gold salt- and none in the penicillamine-treated BN rats. Similarly, enhancement of serum IgE concentration is the highest in the first group when compared to those of other two groups (Prouvost-Danon A. et al 1981; Tornade H. et al 1990). Lewis rats are resistant to the induction of autoimmune diseases by all of these three drugs.

1.8.4 Penicillamine and gold salt-induced autoimmunity in mice

A-SW mice that are susceptible to mercuric chloride-induced autoimmunity also produce antinuclear antibodies when treated with either gold sodium thiomalate or D-penicillamine (Robinson C.J.G. et al 1986). An increase in total serum IgG and IgE concentrations was detected in A-SW mice when injected with gold salts (Pietsch P. et al 1989).

1.8.5 Gold salt-induced autoimmune nephritis in guinea pigs

Hartley guinea pigs injected with gold sodium aurothiomalate developed an autoimmune tubulointerstitial nephritis and an immune complex glomerulopathy (Ueda S. et al 1986). Anti-tubular basement membrane (TBM) antibodies were found linearly deposited along the TBM in those animals. Anti-brush border antibodies have been considered to be involved in the pathogenesis of the glomerular disease.

1.8.6 Cadmium-induced autoimmunity

Cadmium can induce antinuclear antibodies in ICR but not in BALB/c mice (Ohsawa M. et al 1988). Cadmium has been reported to induce an immune complex type glomerulonephritis in Sprague-Dawley rats (Johsi B.C. et al 1981).

1.8.7 Propylthiouracil-induced SLE in cats

Fifty to 70% of mongrel cats receiving propylthiouracil (PTU) develop an autoimmune disease marked by lymphadenopathy, autoimmune hemolytic anemia and autoantibodies such as anti-native DNA antibodies (Aucoin D.P. et al 1988). The presence of a free sulfhydryl group on PTU is required for autoimmunity to occur (Aucoin D.P. et al 1988).

1.8.8 L-canavanine-induced SLE

In monkeys, alfalfa sprouts can induce anemia, low serum complement, antibodies to DNA and deposition of immunoglobulins in skin and kidneys. This autoimmunity is attributed to the unusual amino acid constituent, L-canavanine (Malinow M.R. et al 1982).

1.8.9 Hydralazine and procainamide-induced autoimmunity

Hydralazine, given orally at the dose of 0.5-1 mg/day for 6-8 months increases the incidence of antinuclear antibodies in A/J and C57BL/6 mice (Cannat A. & Seligmann M. 1968). In one report, procainamide given orally at the dose of 20 mg/day for 8 months had the same effect in these two strains (Ten-Ven J.H. & Feltkamp T.E.W. 1972). However, clinical manifestations were absent.

1.8.10 Trichloroethylene-induced autoimmune response in MRL+/+ mice:

Trichloroethylene (TCE) is an organic solvent that is mainly used as a degreasing agent for metals. TCE has also been found as an indoor contaminant due to its use in adhesives, spot-removers, carpet-cleaning fluids and paint removers (Bruckner J. V. et al 1989). Human

exposure to has been associated with the development of autoimmune diseases such as systemic lupus erythematosus and scleroderma (Khan M.F. et al 1995).

The development of a spontaneous SLE-like disease in two MRL substrains of mice (MRL +/+ and MRL lpr/lpr) has been shown (Andrews B.S. et al 1978). Unlike the MRL lpr/lpr mice, which develop the SLE-like disease early in life, the MRL +/+ mice do not normally develop the clinical symptoms of autoimmunity until late in life (during their second year of life). However, MRL +/+ mice treated with TCE have been shown to exhibit an accelerated SLE-like autoimmune response early in life (Khan M.F. et al 1995). Using this mouse model, investigators have studied the capacity of TCE and its metabolites to induce autoimmunity (Griffin J.M. et al 2000). In addition, possible mechanisms by which TCE promotes an autoimmune response in this animal model have been studied (Gilbert K.M. et al 1999).

1.8.11 Penicillamine-induced lupus in BN rats:

Penicillamine has been shown to induce a lupus-like syndrome in Brown Norway (BN) rats (Donker A.J. et al 1984). Four to eight weeks after penicillamine administration in a dosage of 20 or 50 mg per day, 70% of BN rats became ill. The disease was characterized by weight loss, dermatitis and a high mortality rate. The plasma of these animals contained antinuclear antibodies and immune complexes. Thus, it appears that BN rats are particularly susceptible to developing a lupus-like disease after exposure to penicillamine. BN rats are also known to be the susceptible strain for the induction of lupus by mercuric chloride ($HgCl_2$) and gold salts (Druet E. et al 1977).

In general, susceptibility to autoimmune diseases is controlled by environmental and genetic factors, especially MHC genes (Uetrecht 1997). The association of MHC genotype with autoimmune disease is not surprising because autoimmune responses involve T cells, and the ability of T cells to respond to a particular antigen depends on MHC genotype. Thus the associations can be explained by a simple model in which susceptibility to an autoimmune disease is determined by differences in the ability of different allelic variants of MHC molecules to present autoantigenic peptides to autoreactive T cells. However, MHC genotype alone does not determine whether an individual develops disease. There are other less understood environmental factors that affect the immune system and the course of autoimmune diseases.

In rats susceptibility to penicillamine-induced autoimmunity is also genetically controlled. Unlike BN rats, Lewis (LEW) and Sprague-Dawley (SD) rats are resistant to developing autoimmunity with penicillamine (Donker A.J. et al 1984). The differences in susceptibility to penicillamine-induced lupus in these rat strains could be due to polymorphism of the MHC genes. A strain-dependent Th cell activation and IgE production has been shown in these animals with BN rats classified as high, but LEW and SD are low IgE responders (Goldman M. et al 1991). In addition, *in vitro* characterization of IFN-γ gene expression and secretion demonstrated differences between these rat strains. BN rats are known as low IFN-γ producers, while LEW and SD rats are classified as high IFN-γ responders (Druet P. & Pelletier L. 1996). These observations suggest a genetic predisposition to disease development in BN rats. However, BN rats initially receiving 5 mg of penicillamine per day (for four weeks) and subsequently 20 and 50 mg per day (for several weeks) do not develop the autoimmune disease. It appears that in these rats an initial, relatively low dosage of the drug induces tolerance to a dosage that otherwise would have been fatal (Donker A.J. et al 1984).

To compare penicillamine- and mercuric chloride-associated lupus, another group of investigators reproduced and evaluated the penicillamine-induced lupus animal model in more detail (Tornade H. et al 1990). Their findings in this experiment confirmed the previously reported results with regard to:

- Inducibility of the drug-related lupus in BN rats with a high incidence after treating animals for a relatively short period of time with 20 mg of penicillamine per day.
- Reproducibility of the drug-associated autoimmune disease in this experimental model.
- Lack of requirement for an immune stimulator (adjuvant) for the development of the drug-induced lupus in this animal model.

Penicillamine-induced lupus in BN rats can be termed idiosyncratic due to the following characteristics:

- It is not induced in all BN rats treated with penicillamine (the incidence of the development of lupus in BN rats is about 60% when the animals are treated with 20 mg/day of penicillamine for 8 weeks), and it is not possible to predict which BN rat will develop the lupus after treatment with penicillamine.

- It is not induced in other strains of rats, such as Sprague-Dawley or Lewis, with any dose of penicillamine.
- The mechanism of induction of lupus by penicillamine in BN rats is not known.

Thus, this experimental model is considered a representative model of drug-induced idiosyncratic reactions. In general, not many good animal models exist for drug-induced idiosyncratic responses. From a limited number of the available animal models of drug-induced idiosyncratic reactions, penicillamine-induced lupus in BN rats seems to be unique because of the following features:

- The drug (penicillamine) induces the idiosyncratic reaction (lupus) in susceptible animals (BN rats) with a high incidence (60%) and in a relatively short period of time (8 weeks).
- The model is reproducible both in the way it is induced and in the clinical features manifested.
- The animals exhibit clinical (rash, weight loss) and immunological features (ANA) analogous to the adverse reaction induced in some humans.

The usefulness of an animal model depends on how closely it resembles the disease to which it is compared. Rarely does a model show all the clinical, morphological, biochemical and functional features of the specific disease entity they resemble, yet they may still contribute significantly to a better understanding of the adverse reaction induced in humans. In respect to penicillamine-induced lupus in BN rats, it should be emphasized that although many of the features of this syndrome in BN rats are similar to the syndrome induced by penicillamine in humans, these two syndromes may not be exactly the same. In fact, not all human idiosyncratic reactions to a specific drug are the same and penicillamine-induced lupus in the BN rat is likely to involve a mechanism very similar to some human idiosyncratic drug reactions.

The existence of the above animal model, which closely resembles the adverse reaction that occurs in some humans treated with the same drug, offers an excellent opportunity to explore the mechanisms operative in drug-induced lupus idiosyncratic reaction. We have used this animal model to study the factors that may influence the incidence and severity of the penicillamine-induced lupus idiosyncratic reaction.

1.9 Therapeutic strategies and new approaches to inhibit drug-induced idiosyncratic reactions

The immune response, whether autoreactive or not, is initiated by an antigen presenting cell (APC) offering a peptide (either foreign or of self-origin) through its class II major histocompatibility complex molecules (MHC-II) to a T helper cell (Th) receptor. If the T cell receptor (TCR) has specificity for both self-MHC and the specific peptide presented, and also receives the necessary second signal, the T cell will proliferate and differentiate into effector cells and actively participate in the immune response. Theoretically, interference with any one of the above interactions should interrupt the immune response (Fig.1-7).

The pathogenesis of an autoimmune disease, such as lupus, is a complicated process that involves recognition of specific antigen(s), T cell activation, release of numerous cytokines, and the production of effector molecule(s), which, in turn, leads to target tissue damage, dysfunction and the development of clinical illness. Interruption of any step in such a process could serve as a target for clinical intervention.

1.9.1 Blocking of class II MHC

Removal or blocking of class II MHC on APCs should result in their inability to present peptide to the TCR. This can be achieved *in vitro* by using class II-specific monoclonal antibodies. However, if such antibodies could recognize all class II molecules, they would block all antigen presentation and have an undesirable non-specific immunosuppressive effect. As most individuals are heterozygous at each class II locus and as we all have multiple class II loci, a more logical approach would be to use only highly-specific monoclonal antibodies to a specific type of class II molecule, which is known to be associated with a particular disease.

Limited experimental work has been performed in animals in which class II monoclonal antibodies have been administered. Any beneficial effect of such treatment would be dependent on continuous administration and it seems unlikely that such treatment offers a viable option.

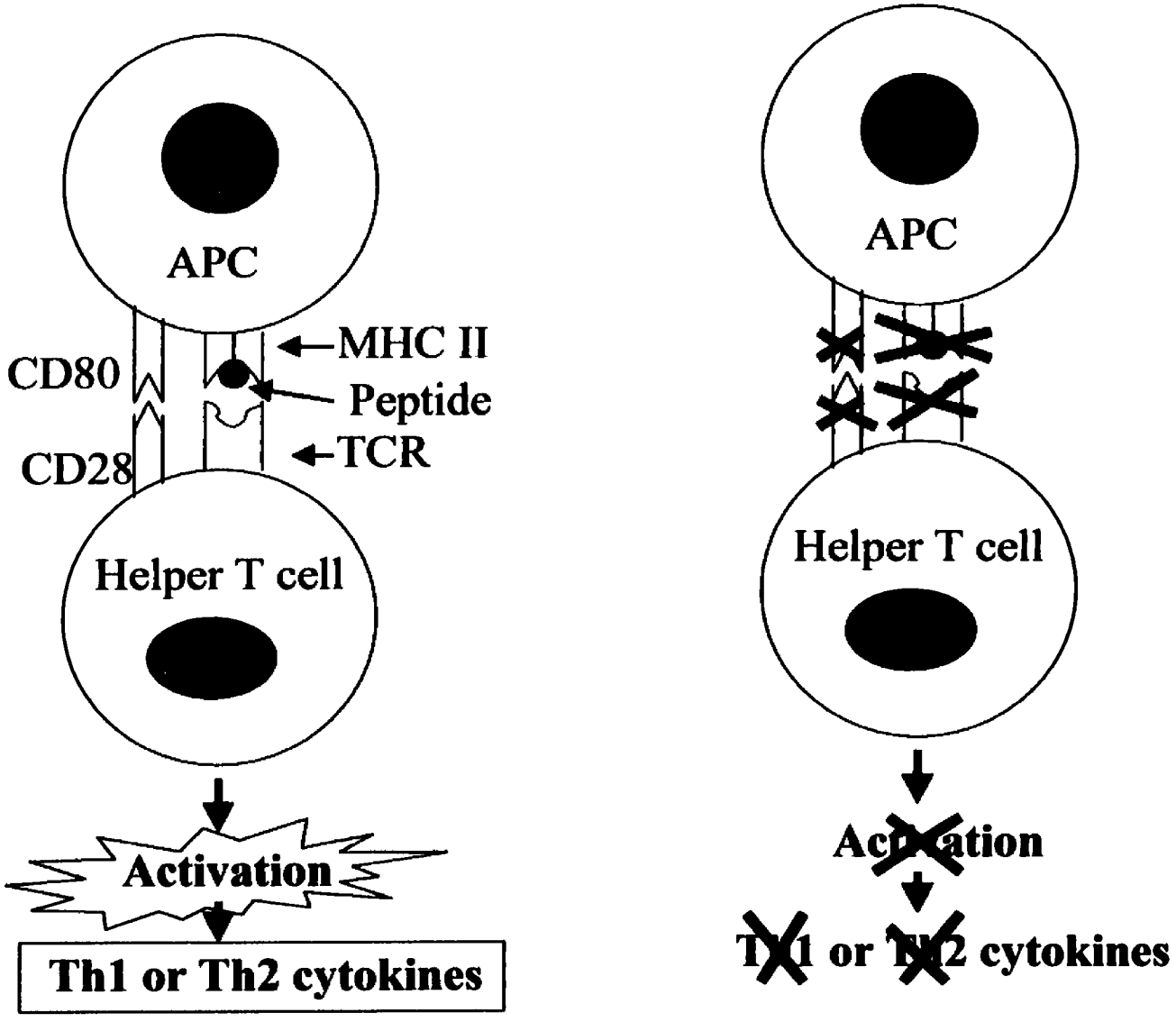


Fig. 1-7. Immune interactions and novel approaches to inhibit unwanted immune-mediated reactions.

1.9.2 Peptide therapy

A second possibility for therapeutic intervention is at the level of peptide binding to the class II molecule. If presentation of auto-antigen by a specific, HLA-associated molecule is an important component of the disease process, the administration of an alternative peptide with high affinity for the class II antigen presenting groove should displace and block the auto-antigen. There is considerable evidence that class II molecules can bind most peptides. However, some peptides have a high affinity for specific HLA molecules. If such a peptide could be found, which is also non-toxic, its use as a blocking agent could have great benefit (Adorini L. & Nagy Z.A. 1990). For example, in the EAE model, a peptide with high affinity for the MHC class II molecule I-A^u was shown to prevent induction of EAE. It is likely that this blocking peptide inhibited disease by competitive binding, as it was not immunogenic itself.

1.9.3 Manipulation of T cell recognition and tolerance

Manipulation of T cell recognition and tolerance may be induced specifically through procedures, such as T cell vaccination and induction of oral tolerance, or non-specifically by administration of monoclonal antibodies to T cell surface receptors.

1.9.3.1 T cell vaccination

T cell vaccination refers to the use of autoimmune T cells as vaccines to induce specific immunity to the T cells responsible for the disease. The effectiveness of T cell vaccination to confer resistance has been demonstrated in a number of animal models of autoimmune diseases including adjuvant arthritis, experimental autoimmune thyroiditis and type II collagen arthritis (Thompson H.S.G. & Staines N.A. 1991). The protection induced by T cell vaccination is specific and can only be induced by activated cells. Resting cells will neither passively transfer the disease nor confer resistance. This resistance is thought to be due to the generation of anti-idiotypic suppressor T cells.

Several clinical trials in humans are now under way. One such trial in RA uses T lymphocytes from synovial fluid exudates, expanded *in vitro* and then irradiated, to vaccinate patients (Lohse A.W. & Cohen I.R. 1990).

1.9.3.2 Orally induced tolerance to auto-antigens

The main interface between the immune response and external antigens is at the level of mucosal surfaces, such as gut. The gut encounters the largest challenge with ingestion of food and bacterial antigens. However, these antigens rarely immunize and this route of administration of antigens usually induces oral tolerance. This observation provided the basis for experiments where auto-antigens were given orally and the effects on disease outcome were measured.

Feeding either rats or mice with soluble type II collagen before giving the usual parenteral arthritogenic challenge of type II collagen had a significant effect (Thompson H.S.G. & Staines N.A. 1991). Fewer animals developed the arthritis and, in those animals that the disease was induced, the onset was delayed. Similarly in a rat model for EAE, oral administration of myelin basic protein (MBP) before giving a standard MBP encephalitogenic challenge reduces both the incidence and severity of EAE. This phenomenon is specific, as feeding animals with an irrelevant antigen confers no protection in these animal models.

Evidence from an animal model of multiple sclerosis, chronic relapsing experimental allergic encephalomyelitis, has provided some encouragement for considering oral tolerance induction as a treatment in humans. Guinea pigs already suffering from this condition benefit considerably from MBP given orally, with the frequency and severity of subsequent relapses being diminished. This approach is now being evaluated in clinical trials. MBP is being administered orally to multiple sclerosis patients. In addition, type II collagen is being given in capsule form as a treatment for RA.

1.9.4 T cell monoclonal antibodies

Another approach may come from using monoclonal antibodies to T cell surface markers and receptors. Such antibodies have been used in bone marrow and renal transplantation patients, and clinical trials of their efficacy in certain autoimmune diseases are under way. Antibodies to a number of T cell surface components may have therapeutic potential. These include CD4, CD5, CD7, the IL-2 receptor and the T cell receptor (α/β or γ/δ dimer) (Cooke A. & Wraith D.C. 1993).

Many of these antibodies fix complement and the reduction or removal of cells by cytotoxicity is likely. This strategy is also likely to lead to undesirable generalized immunosuppression.

1.9.5 Manipulation of T Helper cell subsets

The immune system employs a highly complex mechanism designed to generate responses to protect us against a variety of foreign pathogens while at the same time preventing responses against self antigens. In addition to deciding whether to respond, the immune system must also choose appropriate effector functions to deal with each pathogen. A cell critical in mediating and regulating these effector functions is the CD4+ T cell. Furthermore, it is the elaboration of specific cytokines from CD4+ T cells that appears to be the major mechanism by which they mediate their functions. Thus, characterizing the types of cytokines made by CD4+ T cells, as well as how their secretion is controlled is extremely important in understanding how the immune response is regulated.

Until 1986, although it was recognized that cytokines, such as interleukin-2 (IL-2), drove cellular immune responses and that (IL-4) influenced antibody production, the events controlling which cytokine was selected by the immune system for a particular immune response remained obscure. The breakthrough came when Mosmann et al. first described two polarized sets of mouse T helper cells (Th1 and Th2) with distinct cytokine secretion patterns (Fig.1-8) (Mosmann T.R. et al 1986). Subsequently described in humans and other species, Th1 cells secreted IL-2, interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β) and promoted cellular immune responses. Th2 cells secreted IL-4, IL-5, IL-6 and IL-10 and promoted B cell proliferation and antibody secretion. This new way of thinking was a great advance in understanding the control of immune responses.

It was also discovered that Th1 and Th2 cells cross-regulate each other's development, which provides a mechanism for regulation of immune responses (Reiner S.L. 2001). For example, IFN-γ, which is a product of Th1 cells, inhibits Th2 cell proliferation (Zhang Y. et al 2001). On the other hand, IL-4 and IL-10, which are products of Th2 cells, can downregulate Th1 cell responses (Boothby M. et al 2001). This interplay can result in a predominance of Th1

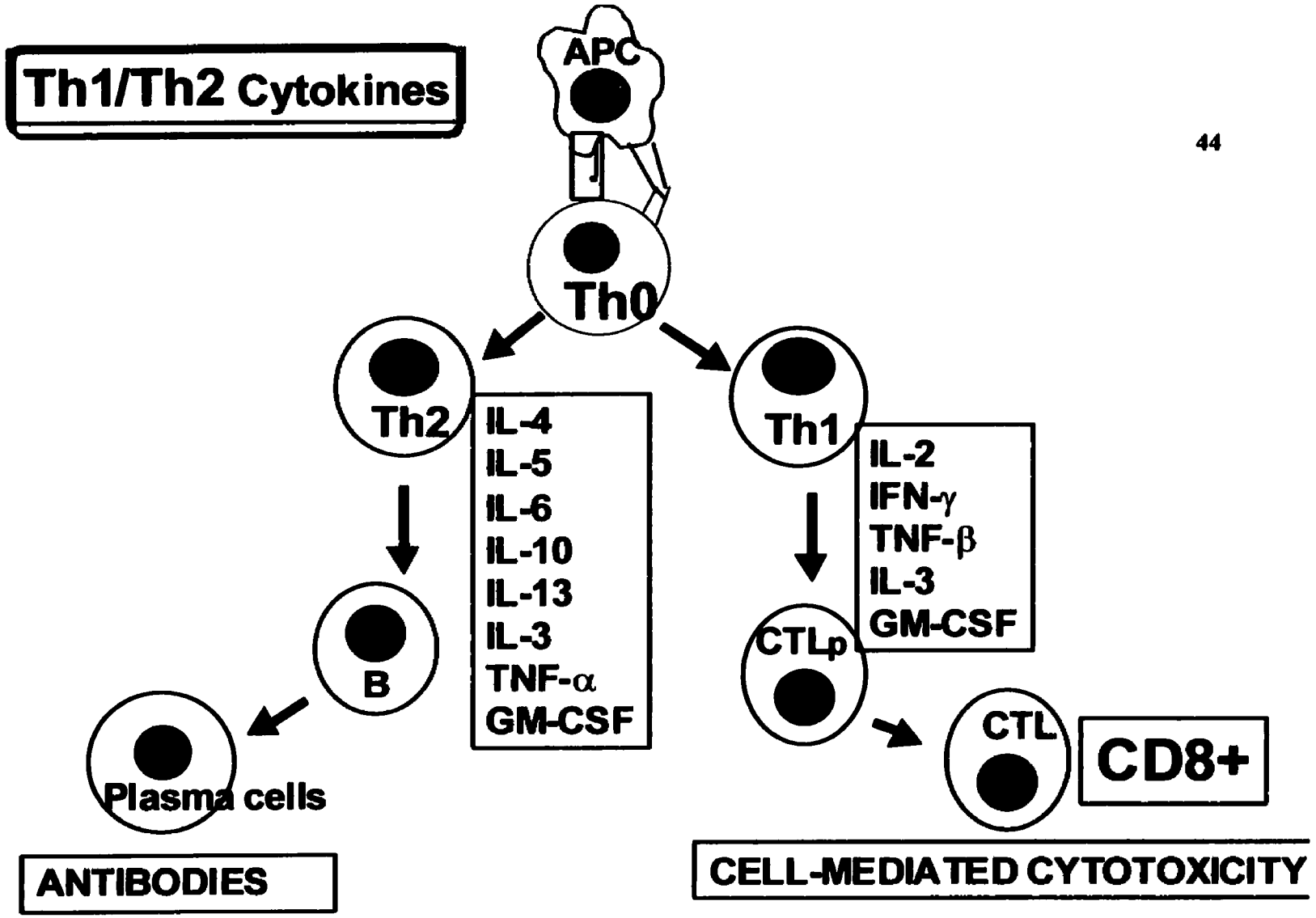


Fig. 1-8. Differentiation of naive CD4+ T cells into Th1 and Th2 subsets.

or Th2 cells following preferential stimulation of a particular Th subset. Strong cell-mediated immunity and often only a weak humoral response characterize the Th1 responses. In contrast, Th2 responses are usually associated with high titer antibody responses, but poor delayed-type hypersensitivity reactions.

During the course of an immune response, different immunoglobulin isotypes are also expressed. Initially, IgM is secreted and IgG levels subsequently increase. It has been shown that cytokines derived from T cells play a critical role in isotype switching. Thus, IL-4, a product of Th2 cells, allows B cells to switch from IgM to IgG1 secretion. Higher doses of IL-4 and more prolonged stimulation results in switching from IgG1to IgE. In contrast, IFN-γ, a product of Th1 cells, is associated with switching from IgM to IgG2a. Thus, stimulation of Th1 or Th2 cells may be expected to result in expression of different immunoglobulin classes and isotypes. The isotype pattern induced during an infection or following immunization is a clue as to which Th subset has become more prominent. For example, predominant IgG1 or IgE levels would suggest Th2 cell expansion, whereas predominance of IgG2a would imply more pronounced recruitment of Th1 cells.

The factors responsible for the Th cell differentiation into a predominantly Th1 or Th2 profile has been extensively investigated. Both environmental and genetic factors influence the Th1 or Th2 differentiation. The tendency to produce Th1 or Th2 responses to an antigen is determined, in part, by the genetic makeup of the host. However, scientists have been trying to determine what other factors may be involved in the induction of Th1 or Th2 responses. At least three different environmental factors seem to influence differentiation of Th cells. These factors are 1) availability of cytokines in the microenvironment of the responding Th cells (Fig.1-9), 2) antigen quantity and 3) the type of co-stimulatory signal that Th cells receive from antigen presenting cells.

1.9.5.1 Cytokines

The ability of a T cell to respond to both autocrine and exogenous cytokines depends on the ability to regulate expression of specific receptors. Precursors of Th cells (Th0) express receptors for IL-2, IL4 (Rocken M. et al 1992) and IFN-γ (Bach E.A. et al 1995), but do not express IL-12 receptors until activated. Thus Th0 cells can respond to the principal players in both Th1 and Th2 development. IFN-γ and IL-4 have direct effects on each other, down-

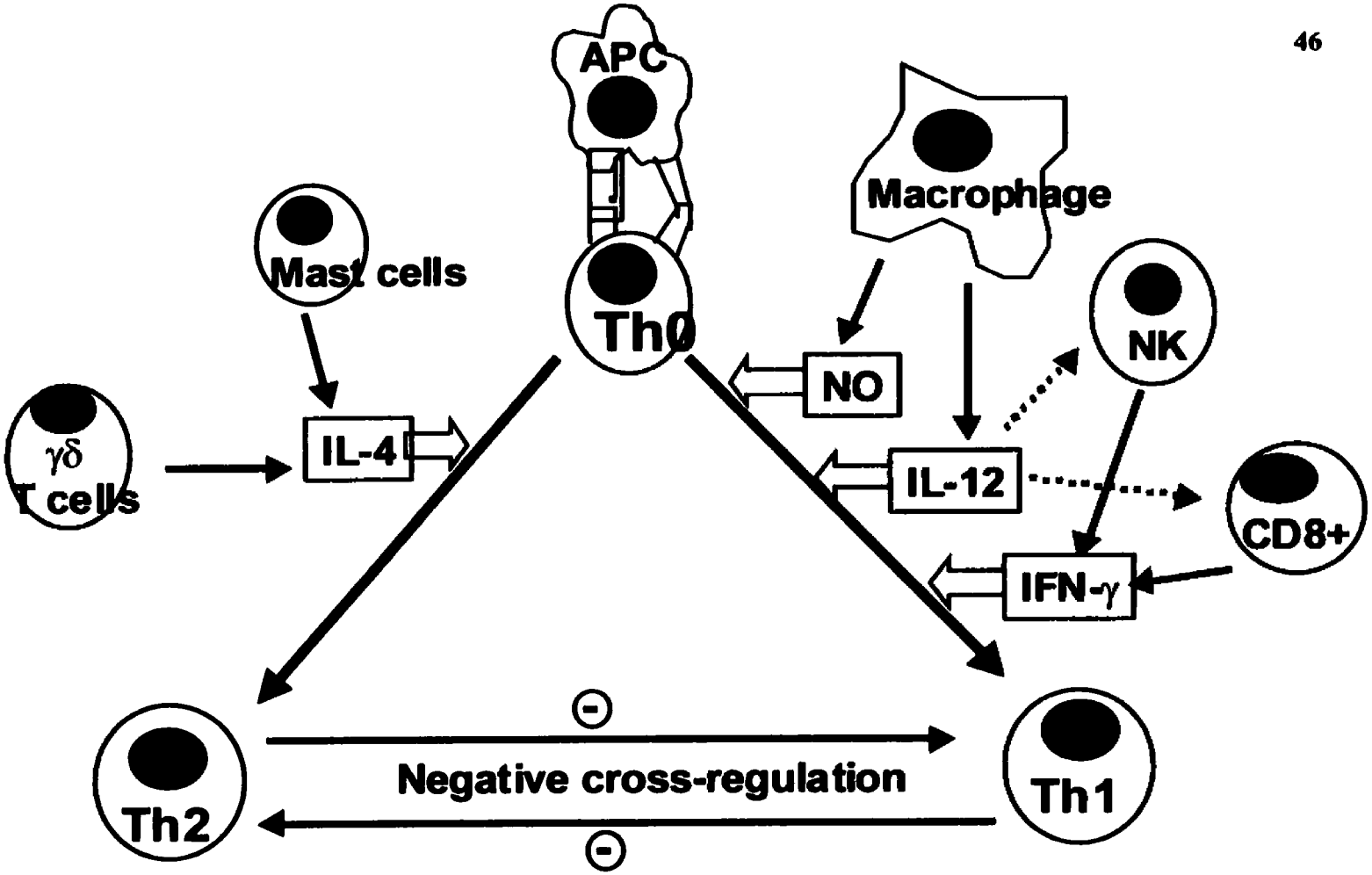


Fig. 1-9. Stimuli for the differentiation of Th0 cells into Th1 and Th2 subsets.

regulating each other's receptors and expression. However, while IL-4 directly down regulates IL-12 receptors, IL-12 has no direct effect on IL-4, working only via IFN-γ (Nakamura T. et al 1997). IL-12 is produced by APC and directly affects the T cells. Thus, if IL-12 is produced by the APC, a positive feedback loop of IFN-γ production results, down regulating IL-4. However, if IL-12 is not made by the APC, IL-4 produced by the T cell will rapidly down-regulate IL-12 receptors and the T cells will be free to continue to produce IL-4. This effect is amplified by IL-10 production by APC, via IL-10 strongly inhibiting IL-12 (Hsieh C. S. et al 1993).

1.9.5.2 Antigen quantity

The initial event in T cell activation is ligation of the T cell receptor (TCR) by antigen/MHC complexes on the surface of an APC. The strength of signal this delivers to the T cell depends on the affinity of the TCR for the antigen, and the amount of antigen available (Constant S.L. & Bottomly K. 1997). The results obtained from a study in which a range of peptide antigens was used to investigate the role of antigen in Th cell differentiation pattern demonstrate that the nature and abundance of an antigen does influence Th differentiation toward Th1 or Th2 (Constant S.L. & Bottomly K. 1997). In general, small quantities or low affinity of antigen for TCR/MHC, which results in less stimulation for T cells, has been thought to favor Th2 responses, while high affinity and abundance of antigen favors Th1 responses. Exactly why the degree of stimulation affects polarization between Th1 and Th2 responses is not clear yet. It has been hypothesized that low levels of antigen drive IL-4 production, while high levels may drive IFN-γ production (Rogers P.R. & Croft M. 1999).

1.9.5.3 Co-stimulatory and surface molecules

As discussed before, IL-12 and IL-10 produced by APC are crucial in driving Th1 and Th2 responses, respectively, but co-stimulation by surface molecules is also essential. Model systems studying individual co-stimulatory molecules have shown that ligation of the inducible co-stimulator (ICOS) molecule on T cells stimulates IL-10 production (Hutloff A. et al 1999). CD40 ligand will induce IL-12 production by dendritic cells (Shu U. et al 1995). Ligation of CD28 on T cells by CD80 (B7-1) can induce either a Th1 or Th2 response, whereas CD86 (B7-2) ligation of CD28 preferentially induces a Th2 response (Freeman G.J. et al 1995; Rulifson I.C. et al 1997). Also, LFA-1/ICAM family ligation on T cells tends to favor Th1 responses (Rogers P.R. & Croft M. 2000).

1.9.5.4 Application of Th1 and Th2 responses to prevent different immune-mediated disorders

In experimental models, a number of diseases can be prevented by switching immune responses from Th1 to Th2 or from Th2 to Th1 (Nicolson B.N. & Kuchroo V. 1996). For example, In experimental autoimmune encephalomyelitis (EAE) induced by the transfer of central nervous system (CNS) antigen-specific T cells, pathogenic T cells have universally been found to be of a Th1 phenotype (Luppi P. et al 1995). Th1 cells also appear to be involved in the early and late phases of diabetes development in the nonobese diabetic (NOD) mice, a spontaneous model for human type I diabetes (Luppi P. et al 1995). It has been shown that the mechanisms that activate Th2 cells and induce the secretion of a dominant Th2 cytokine pattern prevent the development of EAE and the progression of diabetes in diabetic animals (Luppi P. et al 1995).

Experimental autoimmune uveoretinitis (EAU) is an ocular autoimmune disease that can be induced in Lewis rats by a single immunization with the soluble retinal antigen (Saoudi A. et al 1993). EAU, which is a Th1-dependent autoimmune disease, can be prevented by *in vivo* treatment with monoclonal antibodies against IFN-γ or IL-2 receptor (Saoudi A. et al 1993).

Experimental *Leishmania major* infections in different mouse strains induce either a Th1 or a Th2 response. Mice that can generate Th1-type immunity in response to infection clear the organism (resistant), whereas mice that generate Th2 responses succumb to lethal Leishmania infections (susceptible). Many of the lesions in the mice that die are due to the inappropriate actions of the immune system itself rather than to direct effects of the infectious organism. Anti-IFN-γ antibody administered at the time of *Leishmania* infection altered the Th1 profile of resistant mice to a Th2 profile and made those mice susceptible to the infection. In contrast, Early treatment of susceptible mice with anti-IL-4 antibodies resulted in inhibition of the nonprotective Th2 response and establishment of a protective Th1 population (Coffman R.L. et al 1991).

Thus, manipulation of the Th1/Th2 balance may provide new approaches for the specific treatment of a wide range of immune-mediated disorders.

Chapter 2

Effect of Poly I:C, Misoprostol and Recombinant rat interferon-γ on the Clinical Outcome of Penicillamine-Induced Lupus in Brown Norway Rats

2.1 Abstract

Idiosyncratic drug reactions appear to be immune-mediated. Immune responses are driven by helper T cells (Th); Th1 responses promote cell-mediated immunity whereas Th2 responses drive antibody-mediated reactions. Th1 cytokines inhibit Th2 responses and Th2 cytokines inhibit Th1 responses; therefore, it may be possible to prevent idiosyncratic drug reactions by changing the TH1/Th2 cytokine balance. We tested this hypothesis in an animal model in which penicillamine causes a lupus-like syndrome in Brown Norway rats. This syndrome has the hallmarks of a Th2-mediated response, and we tried to inhibit it with a polymer of inosine and cytosine (poly I:C), a Th1 cytokine-inducer. However, we found that a single dose of poly I:C, given at the onset of penicillamine treatment, significantly increased both the incidence (100% vs 60%) and accelerated the onset (30 ± 4 vs 39 ± 5 days) of penicillamine-induced lupus when compared with controls. To rule out other effects of poly I:C that might overshadow the induction of Th1 cytokines, we directly tested the effects of the prototypic Th1 cytokine, interferon-γ. Although not as dramatic, interferon-γ-pretreatment also appeared to make the syndrome worse. Conversely, when we used misoprostol, a prostaglandin-E analog that inhibits Th1 cytokines, it completely protected the animals. Just one dose of misoprostol prior to initiation of penicillamine treatment was sufficient to provide this protection. These results, although dramatic, suggest that the effects of these agents were not mediated by their effects on Th1/Th2 balance but rather by some other mechanism, possibly by effects on macrophage activation.

2.2 Introduction

Most idiosyncratic drug reactions have characteristics, such as a delay between starting the drug and the onset of the reaction, that suggest they are mediated by the immune system (Park B. K. et al 1998). Some idiosyncratic reactions, such as drug-induced lupus, which is an autoimmune syndrome, are clearly immune-mediated. Immune reactions are believed to require T helper (Th) cells. In recent years it has been proposed that immune-mediated reactions involve a balance between T helper cell subtypes, namely Th1 and Th2, which are characterized by their pattern of cytokine secretion (Mosmann T.R. et al 1986). Th1 cells produce IL-2, lymphotoxin and interferon-γ (IFN-γ), with INF-γ being prototypic, and Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, with IL-4 being prototypic (Mosmann T.R. & Sad S. 1996). In general, Th2 cells drive a humoral (antibody-mediated) immune response, whereas Th1 cells are involved in the induction of cytotoxic T cell-mediated responses. The major stimulus for the induction of a Th1 response is IL-12 produced mainly by antigen presenting cells (Wenner C.A. et al 1996). The factors involved in the induction of a Th2 response are less clear because the major cytokine responsible is IL-4, but the major source of IL-4 is Th2 cells. It may be that the initial IL-4 to induce a Th2 response comes from mast cells and basophils or a $CD4^+$ $NK1^+$ T cell subset (Mosmann T.R. & Sad S. 1996). Alternatively, macrophage-derived IL-6 may be the stimulus for IL-4 production (Rincon M. & Flavell R.A. 1997). The cytokines produced by each subset are believed to act as their own autocrine growth factors, but they cross-regulate and inhibit the other subset's development and function (Morel P.A. & Oriss T.B. 1998; Nakamura T. et al 1997). At least in clones of T cells, the cells become irreversibly committed to either a Th1 or Th2 phenotype, and this commitment appears to be determined in the first 48 h after antigen stimulation (Morel P.A. & Oriss T.B. 1998). This model of cross-regulation has been successfully applied to explain a number of immune phenomena *in vivo* (Paul W.E. & Seder R.A. 1994; Romagnani S. 1994; Sher A. & Coffman R.L. 1992), although Th1 cells have been reported to proliferate in response to IL-4 under certain conditions (Oriss T.B. et al 1999). Thus it should be possible to influence an immune response by altering the balance of Th1/Th2 cytokines, especially early in the response, and such a strategy might be useful for the prevention and/or treatment of immune-mediated reactions.

The characteristics of idiopathic lupus in humans suggest that it is a Th2-driven reaction. A hallmark of lupus is autoantibodies, particularly antinuclear antibodies, which could be due to activation of autoreactive B cells by Th2 cells. In addition, it has been reported that patients with lupus have greater numbers of lymphocytes that produce IL-4 and IL-10 and decreased numbers that produce IL-2 and IFN-γ (Funauchi M. et al 1998). Furthermore, patients with recent onset lupus had decreased production of IL-12 and an increase in production of IL-10 (Horwitz D.A. et al 1998; Liu T.F. & Jones B.M. 1998). Drugs, such as procainamide, hydralazine and penicillamine, cause a lupus-like syndrome in humans (Enzenauer R.J. et al 1990; Harkcom T.M. et al 1978; Uetrecht 1997). In addition to lupus, penicillamine is known to induce a range of autoimmune idiosyncratic reactions, such as myasthenia gravis and pemphigus (Jaffe I. A. 1979; Jaffe I.A. 1981).

Animal models of drug reactions in which a drug has been observed to cause a similar idiosyncratic reaction in an animal species as it does in humans are rare, but penicillamine also causes a lupus-like syndrome in rats (Donker A.J. et al 1984). The reaction is idiosyncratic in that it appears to be specific for Brown Norway (BN) rats and does not occur in Lewis or Sprague Dawley rats. It is also idiosyncratic in that it only occurs in about 60% of the BN rats that are treated. The syndrome of penicillamine-induced lupus in BN rats is characterized by antinuclear antibodies, elevated serum IgE levels, proteinuria and immune complex glomerulonephritis, as well as clinical manifestations including weight loss and rash (Tornade H. et al 1990). The idiosyncratic nature and many of the features of this syndrome in BN rats are similar to penicillamine-induced lupus in humans. Therefore, penicillamine-induced lupus in BN rats may involve a similar mechanism and provide a reasonable model for the study of the mechanism of drug-induced lupus and possibly other idiosyncratic drug reactions in humans.

Penicillamine-induced lupus in BN rats is associated with elevated levels of IgE, and the major determinant of IgE production is believed to be IL-4, the prototypic Th2 cytokine (Ochel M. et al 1991; Romagnani S. 1990). In fact, IgE levels are often considered an indirect measure of IL-4 levels (Finkelman F.D. et al 1986). It is also known that penicillamine treatment up-regulates IL-4 mRNA expression in BN rats (Qasim F. J. et al 1997). The susceptibility of the BN rat appears to be due, at least in part, to defective IFN-γ synthesis in this strain (Druet P. & Pelletier L. 1996). Based on these facts, it has been assumed that penicillamine-induced lupus is

Th2 cell dependent (Goldman M. et al 1991). The counter-regulatory nature of Th cytokines predicts that Th1 cytokines would inhibit penicillamine-induced lupus. If the balance of Th1/Th2 cytokines is a major determinant of the nature of an immune response, altering this balance might provide a method of preventing drug-induced lupus and other idiosyncratic drug reactions. We set out to test this hypothesis with penicillamine-induced lupus in the BN model.

2.3 Materials and Methods

2.3.1 Animals

Studies were performed in male BN or Lewis rats weighing 170 to 200 g obtained from Harlan Sprague Dawley (Indianapolis, IN). After arrival, all animals were housed two to a cage in a 12 h light/dark cycle at 22°C. The rats were fed a standard rat chow (Agribrands, Purina Canada; Strathroy, ON) and allowed tap water for a week before starting the experiments.

2.3.2 Chemicals, Kits and Solutions

Penicillamine was a generous gift from Merck Frosst Canada Inc. (Montreal, QC). Poly-I:C was purchased from Sigma-Aldrich Canada (Oakville, ON). Misoprostol was a generous gift from Searle (Skokie, IL).

Recombinant rat IFN-γ was purchased from R&D Systems (Minneapolis, MN). The RNA extraction kit was obtained from Quiagen Inc. (Santa Clarita, CA). The cDNA synthesis kit was purchased from Promega (Madison, WI). The DNA purification kit was purchased from GIBCO BRL (Toronto, ON). Rumpun® (Xylasine) and Ketalin® (Ketamine) were purchased from Miles Canada Inc. (Etobicoke, ON) and MTC Pharmaceuticals (Cambridge, ON), respectively.

Poly-I:C was dissolved in cold normal saline over a period of 2 h with intermittent shaking, and misoprostol was diluted in normal saline immediately before injection.

2.3.3 Treatment of rats with penicillamine

Based on an average observed water intake of 56 ml/day/cage (28 ml/day/rat), penicillamine was dissolved in the drinking water of rats (based on 3 days water consumption) at concentrations of 120 mg/168 ml (20 mg/day/rat), 60 mg/168 ml(10 mg/day/rat) or 30 mg/168 ml (5 mg/day/rat).

2.3.4 Co-treatments to modify the incidence of penicillamine-induced lupus

Thirty BN rats were injected i.p. with poly-I:C at a dose of 10 mg/kg body weight (DeClercq E. 1981) on day zero of the experiment and, on the same day, treatment was initiated with penicillamine at a dose of 20, 10 or 5 mg/day in groups 1, 2 or 3, respectively (10 rats in each group). Another 30 BN rats from the same litters served as 3 groups of controls and were injected with 1 ml of normal saline and also started on penicillamine at a dose of 20, 10 or 5 mg/day, respectively (n=10 in each group). Eight age-matched BN rats served as untreated controls and received only a saline injection on day 0. All rats were killed a few days after the peak of the disease (showing the clinical scoring of 4 based on the Table 2-1) or 8 weeks after starting the experiments if an animal did not develop lupus.

To determine the effect of poly-I:C treatment on rats that are not genetically susceptible to penicillamine-induced lupus, 10 Lewis rats received a single i.p. dose of poly-I:C (10 mg/kg body weight) at the beginning of the experiment prior to penicillamine treatment (20 mg/day) as described above. Control groups of Lewis rats received either penicillamine (20 mg/day) and a single i.p. injection of saline (10 rats) or an i.p. injection of saline alone (4 rats). All rats were killed on week 8 of penicillamine treatment.

In another experiment, BN rats (n=10) were similarly treated with one dose of misoprostol (0.3 mg/kg body weight, s.c.) and then started on penicillamine at a dose of 20 mg/day in the drinking water. Groups of control animals, 10 in each group, were treated with penicillamine alone or misoprostol alone. All of the rats were killed at the end of 8 weeks of penicillamine treatment.

A group of BN rats (n=12) were injected s.c. once per day, with 750 units of recombinant rat (rr) IFN-γ on days 0, 1 and 2 of the experiment. After receiving the first injection of rrIFN-γ, the rats were started on penicillamine at a dose of 20 mg/day as described above for 8 weeks. Groups of control rats (n=12) were treated with penicillamine alone or rrIFN-γ alone (n=6).

2.3.5 Clinical Evaluation of drug-induced lupus in rats

Skin abnormalities are part of the syndrome of penicillamine-induced lupus in BN rats (Tornade H. et al 1990). The development of the skin lesions on BN rats after penicillamine treatment was quantified according to the arbitrary scale presented in Table 2-1. Rats that demonstrated an enhanced severity of the skin lesions after penicillamine treatment (score 4 based on the Table 2-1) were exsanguinated.

A significant drop in body weight usually followed the dermatological signs of penicillamine-induced lupus (Tornade H. et al 1990). All rats were weighed once a week from the beginning of the experiments to assess possible weight changes.

2.3.6 Blood, spleen and liver collection

At different intervals, rats from the test and control groups were selected and bled by retro-orbital puncture under light anesthesia (i.p. injection of 0.2 ml of a 1:1 mixture of xylasine (20 mg/ml) and ketamine (20 mg/ml) per rat). At the end of each experiment the rats were killed by cervical dislocation and their spleens were removed and stored at -80°C. The blood of each rat was allowed to clot at room temperature (10 min) and the serum was separated after centrifugation at 15,000 rpm for 5 min and stored at -20°C until tested for antibody content. At the beginning of the study, blood and spleens from untreated rats were also collected for use as control samples and stored at -80°C.

Livers were removed from animals from each treatment group when they were sacrificed, sliced and fixed with 10% buffered formalin. Embedded sections were stained with hematoxylin/eosin.

2.3.7 Analysis of serum IgE

The IgE immunoglobulin levels in the sera of rats were determined by a sandwich ELISA assay as described in detail elsewhere (Sapin C. et al 1984). Briefly, plastic microtiter plates

(Dynatec Laboratories, Alexandria, VA) were coated with 100 µl of mouse monoclonal anti-rat IgE (10 µg/ml; Immunocorp, Montreal, QC) in a coating solution (50 mmol/L of Tris (tris[hydroxymethyl]aminomethane) buffer, pH 7.8, containing 0.5 g/L of sodium azide) and incubated overnight at 4°C. Then the plates were washed three times with phosphate-buffered saline (PBS) containing 1% Tween 20 (Sigma-Aldrich Canada, Oakville, ON) followed by incubation with 50 µl of 1% bovine serum albumin (BSA; Sigma-Aldrich Canada, Oakville, ON) in PBS at room temperature for 1 h. The plates were then washed three times with PBS-Tween and 100 µl of serially-diluted IgE standard (Serotec Canada, Toronto, ON) or rat sera was added to the wells. Standard and individual test serum samples were diluted to various extents in the assay buffer (50 mmol of Tris solution, pH 7.8, containing 60 g of bovine serum albumin, 0.5 mol of KCl, 0.5 g of sodium azide, 50 ml of normal mouse serum and 5 g of Tween 20 per liter). The plates were incubated at room temperature for 1 h. Thereafter, they were washed 3 times with PBS-Tween and then 100 µl of goat anti-rat IgE mAb (Cedarlane, Hornby, ON) diluted in 6% BSA (1:5000) was added to each well. After a one h incubation at room temperature and washing 3 times with PBS-tween, 100 µl of an alkaline phosphatase-conjugated donkey anti-goat IgG antibody diluted in 6% BSA (1:5000) was added to each well. Then subsequent to a one h incubation at room temperature, 50 µl of 1 mg/ml p-nitrophenyl phosphate (Sigma, St. Louis, MO) in 100 mM diethanolamine-HCl pH 9.8 was added to each well. After a 10 min incubation at room temperature, the color reaction was quantified by an ELISA reader (Cyberfluor; Toronto, ON) at 405 nm. Rat myeloma IgE was used as a standard. Regression curves were constructed for each plate from duplicate samples of five dilutions of known IgE concentration between 1 and 500 ng/ml.

2.3.8 Analysis of IL-4 and IFN-γ mRNA

Reverse transcriptase-PCR (RT-PCR) was performed as follows:

Total RNA was extracted from rat spleen (20 mg) by guanidinium isothiocyanate method using a kit purchased from Qiagen. RNA levels in each sample were determined by UV absorbance at 260 nm. An absorbance of 1 unit at 260 nm (A_{260}) corresponded to 40 µg of RNA per ml.

DNase digestion of the RNA preparation with RNase-free DNase was performed by using a kit purchased from GIBCO BRL Canada Inc. After heat inactivation of the DNase, the purified RNA was used directly for the synthesis of cDNA.

Synthesis of the first-stranded cDNA for the above cytokines was performed using a kit purchased from Promega (Madison, WI). Two μg of total RNA was used for each cytokine.

A DNA thermal cycler (Perkin Elmer 2400; Foster City, CA) was used with 45 cycles of denaturing (94°C, 30 sec), annealing (57°C, 1 min) and extension (68°C, 2 min). After the final cycle, the reactions were incubated for 7 min at 68°C and stored at 4°C. Primers of the cytokines were prepared by the nucleic acid synthesis unit of University of Toronto (Toronto, ON).

Primer sequences were as follows:

IL-4 sense	5' -TGA TGG GTC TCA GCC CCC ACC TTG C 3'
IL-4 antisense	5' -CTT TCA GTG TTG TGA GCG TGG ACT C 3'
IFN-γ sense	5' -ATG AGT GCT ACA CGC CGC GTC TTG G 3'
IFN-γ antisense	5' -GAG TTC ATT GAC AGC TTT GTG CTG G 3'
β-actin sense	5' -ATG CCA TCC TGC GTC TGG ACC TGG C 3'
β-actin antisense	5' -AGC ATT TGC GGT GCA CGA TGG AGG G 3'

2.3.9 Analysis of PCR reaction products

Aliquots of the PCR products (5 μl from each sample) were analyzed by electrophoresis on a 2% agarose gel in Tris acetate EDTA buffer (10.8 g of Tris, 5.5 g boric acid, 4 ml 0.5 M ethylenediamine tetraacetic acid, pH 8.0 in 200 ml of distilled water). The gel was stained with ethidium bromide and a picture was taken under an UV light. Under the experimental conditions, the PCR product generated by IL-4 primers was between 300-400 base pairs while that generated by IFN-γ primers was between 400-500 base pairs and that generated by β-actin primers was 600 base pairs.

2.3.10 Statistical analysis

Data are presented as the mean ± SD. Comparisons between 2 groups of animals were done using the 2-tailed Student's t-test. Comparisons between multiple groups were done using an analysis of variance. *P* values less than 0.05 were considered significant.

2.4 Results

2.4.1 Induction of lupus in BN rats after penicillamine treatment

In a pilot study we reproduced and studied the penicillamine-induced lupus model. A group of BN rats (n=12) was treated with 20 mg of penicillamine per day for a period of 10 weeks. As it has been reported (Donker A.J. et al 1984), some of the rats in this group (n=8) developed early signs of skin lesions on the head and neck that became more severe with time until the animals died. In these rats a significant drop in body weight also followed the skin lesions. When the disease was allowed to run its course, the majority of the diseased rats (6 out of 8) died whereas 2 severely diseased rats started to recover despite continued treatment as evidenced by their normal weight gain and signs of improvement in the skin lesions. The rest of the rats (n=4) in this group did not develop any signs of skin abnormalities or weight loss despite continuation of penicillamine treatment until end of the experiment.

The serum of the penicillamine-treated rats contained IgG antinuclear antibodies (ANA; results not shown) and high levels of IgE antibodies as had been reported previously (Donker A.J. et al 1984; Tornade H. et al 1990).

2.4.2 Effect of poly-I:C administration on clinical manifestations of penicillamine-induced lupus in BN or Lewis rats

Time of onset, incidence and severity of the skin lesions were compared between different groups of rats. Significant differences in both the time of onset and the severity of the lupus were seen in poly-I:C + penicillamine versus penicillamine only-treated BN rats as illustrated in Fig. 2-1. The time to onset of clinical signs was decreased from 39 ± 5 days in penicillamine-treated rats to 30 ± 4 days in the poly-I:C + penicillamine-treated group. Likewise, as shown in Fig. 2-1-B, on day 42, five out of ten BN rats that received an injection of poly-I:C and were then treated with 20 mg penicillamine/day had already displayed an enhanced form of the skin

lupus score	clinical disease
0	No clinical sign of the disease
1	Redness of animal's ears, feet and tail
2	Appearance of rash on the ears and feet
3	Spread of rash over the whole body
4	loss of hair from rat's ears and legs and a swollen bloody snout

Table 2-1 Clinical scoring of penicillamine-induced lupus in Brown Norway rats.

Lupus was induced in Brown Norway rats by orally administration of 20 mg of penicillamine/day/rat. The appearance of skin abnormalities in penicillamine-treated rats were quantified according to the arbitrary scale presented in this Table.

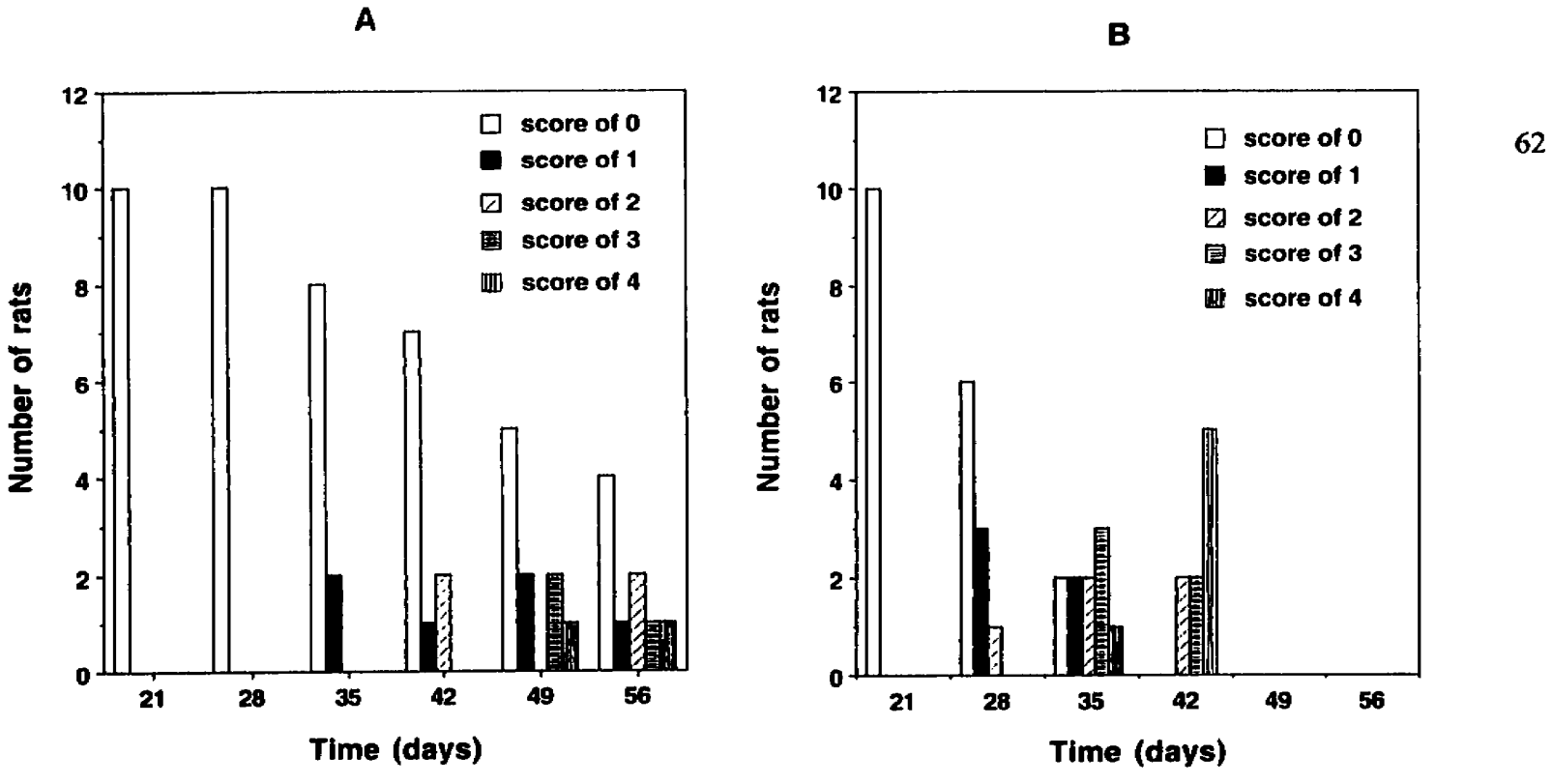


Figure 2-1. Effect of poly I:C on the severity of penicillamine-induced lupus in BN rats

Groups of rats (n=10 /group) were either treated with **A)** penicillamine alone (20 mg/day; p.o.) or **B)** a single dose of poly I:C (10 mg/kg; i.p.) followed by penicillamine (20 mg/day) for 56 days. The clinical scores were assigned using the scale presented in Table 2-1.

lesions (score 4 in Table 2-1) compared with zero out of 10 of penicillamine-treated controls (Fig. 2-1-A).

As illustrated in Fig. 2-2, by 42 days, 10 of 10 (100%) of poly-I:C + penicillamine-treated rats were diseased compared with 3 out of 10 (30%) of penicillamine-treated controls. In contrast, none of the poly-I:C + penicillamine-treated Lewis rats developed clinical signs of penicillamine-induced lupus (Appendix-Table 1). BN rats that were treated with polyI:C alone did not develop lupus (Appendix-Table 2). Nor did poly-I:C result in lupus in BN rats when co-administered with lower doses of penicillamine (5 or 10 mg/day/rat) for 56 days (Appendix-Table 2).

A general weight loss was seen after the development of the skin abnormalities in the rats following penicillamine treatment. As shown in Fig. 2-3, treatment with penicillamine at a dose of 20 mg/day did not significantly influence the body weight gain of BN rats until day 35 of post-treatment, but it did cause a decline in the body weight in some of the rats thereafter. Body weight loss, however, was seen in all BN rats that were injected with a single i.p. dose of poly-I:C prior to the 20 mg/day penicillamine treatment, and it started earlier (day 28 of post-treatment) and was more pronounced than that of penicillamine alone-treated rats (Fig. 2-3). Penicillamine treatment did not significantly influence body weight gain in any of the Lewis rats (results not shown).

2.4.3 Effect of misoprostol administration on clinical manifestations of penicillamine-induced lupus in BN rats

Administration of a single dose of misoprostol (0.3 mg/kg; s.c.) in BN rats prior to penicillamine treatment completely prevented skin lesions (Fig. 2-2) and weight loss (Fig. 3) when compared to rats who were treated with 20 mg of penicillamine/day alone.

2.4.4 Effect of recombinant rat IFN-γ on clinical manifestations of penicillamine-induced lupus in BN rats

As shown in Fig. 2-4, co-administration of rrIFN-γ during the first 3 days of penicillamine treatment appeared to decrease the mean time to the onset of clinical lupus from 37 ± 4 days in penicillamine only-treated group (n=12) to 28 ± 5 days (n=12); however the difference was not statistically significant. Administration of the rrIFN-γ did not significantly change the incidence of the lupus (7 out of 12 rats in the penicillamine alone-treated vs 8 out of 12 rats in the penicillamine + rrIFN-γ-treated group).

2.4.5 Serum IgE levels

Elevation of serum IgE levels is one of the characteristic features of penicillamine-induced lupus in BN rats, and it is also indicative of a Th2 response; therefore, it was one of the key parameters measured in this study. The effect of these treatments on total serum IgE levels in BN rats is shown in Fig. 2-5. There was no significant IgE increase in the serum of untreated control animals (mean serum level 0.9 ± 0.3 μg of IgE/ml), the poly-I:C-treated group (mean serum level 1.0 ± 0.4 μg of IgE/ml) or the misoprostol-treated group (mean serum level 0.8 ± 0.3 μg of IgE/ml). In contrast, the mean ± SD of the serum IgE levels increased in all BN rats treated with 20 mg of penicillamine per day. However, there was no significant difference in the serum IgE levels between BN rats that developed lupus and rats that did not develop the lupus after treatment with 20 mg of penicillamine per day (Appendix-Figure 4). The increase in IgE level was detectable at day 14 and reached a peak value during the forth week after the penicillamine treatment; IgE concentration then tended to decrease. There was no increase in serum IgE levels in BN rats treated with penicillamine at a dose of 5 mg/day (1.1 ± 0.3 μg of IgE/ml) compared to non-treated animals.

In BN rats treated with one dose of poly-I:C before penicillamine treatment, serum IgE levels showed a similar increase starting at week 2; however, the concentration of IgE was significantly higher at day 14 ($p=0.0001$) and 35 ($p=0.0001$) when compared with those treated

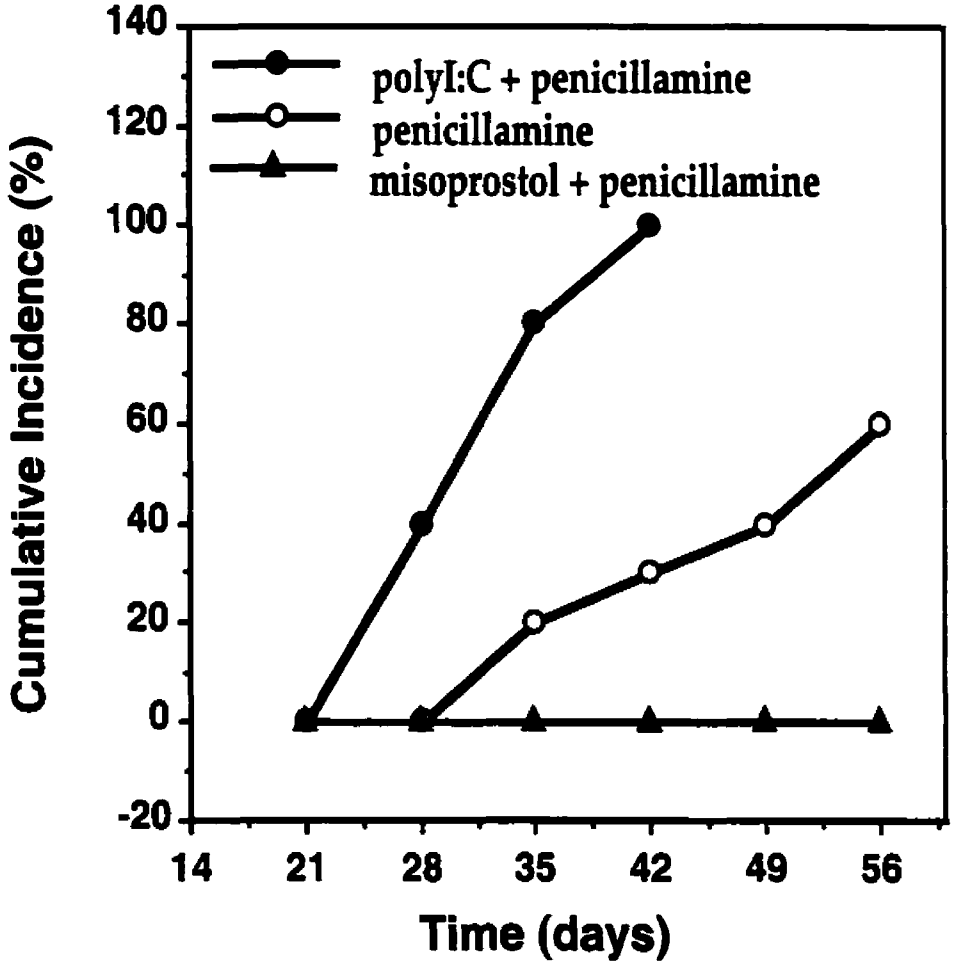


Figure 2-2. Effect of poly I:C or misoprostol on the incidence of penicillamine-induced lupus in BN rats.

Groups of rats were treated with penicillamine alone (20 mg/day; p.o.), a single dose of poly I:C (10 mg/kg; i.p.) followed by penicillamine (20 mg/day), or a single dose of misoprostol (0.3 mg/kg; s.c.) followed by penicillamine (20 mg/day) for 56 day (n=10/group). The total number of rats in each group that demonstrated the clinical signs of the lupus were enumerated weekly.

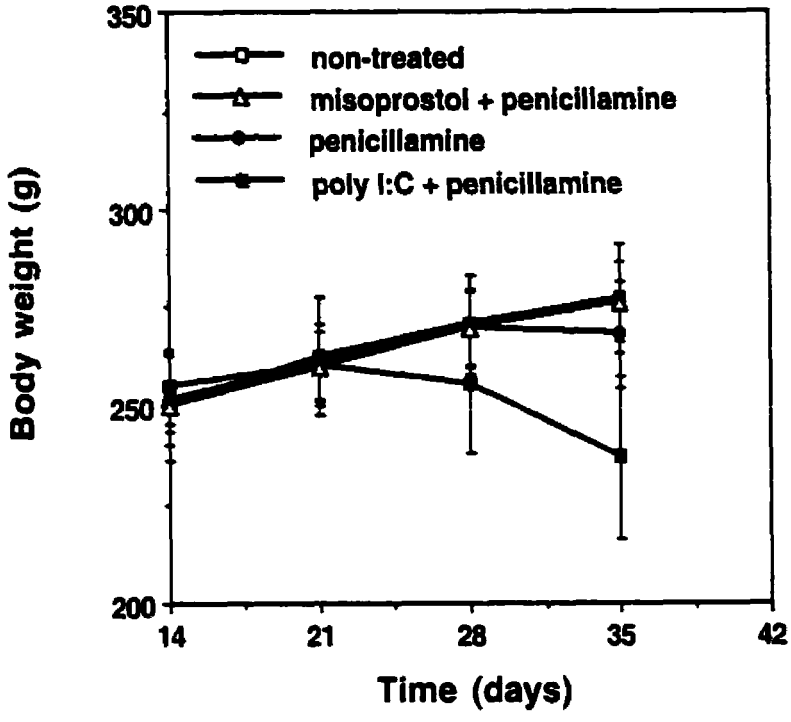


Figure 2-3. Effect of poly I:C or misoprostol on the body weight gains in BN rats.

Groups of rats were either treated with penicillamine alone (20 mg/day; n=10), a single dose of poly I:C (10 mg/kg; n=5), a single dose of misoprostol (300 μg/kg; n=5), a single dose of poly I:C followed by penicillamine (n=10), or a single dose of misoprostol followed by penicillamine (n=10). A group of untreated rats acted as the control (n=8). All rats were weight once a week from the beginning of the experiments and the mean ± SD of their body weight/group/week was compared.

with penicillamine alone and remained high until day 35 of the experiment as indicated in Fig. 2-5. The peak in average IgE levels in this group was observed at day 35 of post-treatment. In contrast, the levels of IgE in rats treated with one dose of misoprostol before initiation of penicillamine treatment initially increased at day 14, but then decreased to a level lower than those of rats treated with penicillamine alone at day 28 ($p=0.0003$) and 35 ($p=0.0001$) of post-treatment as illustrated in Fig. 2-5.

As expected, basal IgE levels were higher in BN rats (1 ug/ml) than in Lewis rats (0.3 ug/ml). Unlike BN rats, following 20 mg/day penicillamine treatment, there was no increase in mean ± SD of serum IgE levels in Lewis rats ($n=6$) compared to non-treated animals (Appendix-Figure 1).

As illustrated in Fig. 2-6, IgE levels in the serum of rats treated with both penicillamine and rrIFN-γ increased in parallel with the levels of IgE in the serum of penicillamine only-treated rats.

2.4.6 IL-4 and IFN-γ mRNA expression

The increased production of IgE associated with penicillamine-induced lupus in BN rats suggests that Th2 cells are dominant in this syndrome. For further evidence of Th2 dominance, we used the RT-PCR analysis to compare the expression of the Th1 cytokine (IFN-γ) and the Th2 cytokine (IL-4) mRNA in spleen cells from BN rats. These experiments demonstrated that mRNA for IFN-γ cytokine was expressed in the splenocytes of all treated and non-treated-BN and -Lewis rats (Appendix-Figures 2 & 3). In contrast, IL-4 mRNA was not detected in untreated control BN rats (Appendix-Figure 2) or penicillamine-treated BN rats that did not develop lupus (Appendix- Figure 5). However, it was detected in the BN rat spleen cells after development of lupus following treatment with penicillamine, penicillamine plus rrIFN-γ (Fig. 2-7) or penicillamine plus poly I:C (Appendix-Figure 2).

In Lewis rats, there was no detectable level of IL-4 mRNA expression in spleen cells after penicillamine treatment for 8 weeks (Appendix-Figure 3).

2.4.7 Liver histology

The liver from the polyI:C+penicillamine-treated rat demonstrated a number of large aggregates which were associated with necrosis of the contained hepatocytes. Furthermore, it showed diffuse portal and sinusoidal infiltration of plasma cells, lymphocytes, eosinophils, neutrophils and macrophages. In addition, there were numerous large foci of granulomatous lesions on the liver of this rat up to 2 mm in diameter. The histology of the liver from the penicillamine only-treated rat that had developed the clinical signs of the lupus showed similar but milder changes with rare large granulomatous lesions on the liver, when compared to that of polyI:C+penicillamine-treated animal (Appendix-Table 3).

In contrast, examination of the liver from the misoprostol+penicillamine-treated rat, the penicillamine-treated animal without clinical signs of the lupus and the non-treated control rat showed minimal focal inflammation with no large areas of necrosis (Appendix-Table 3).

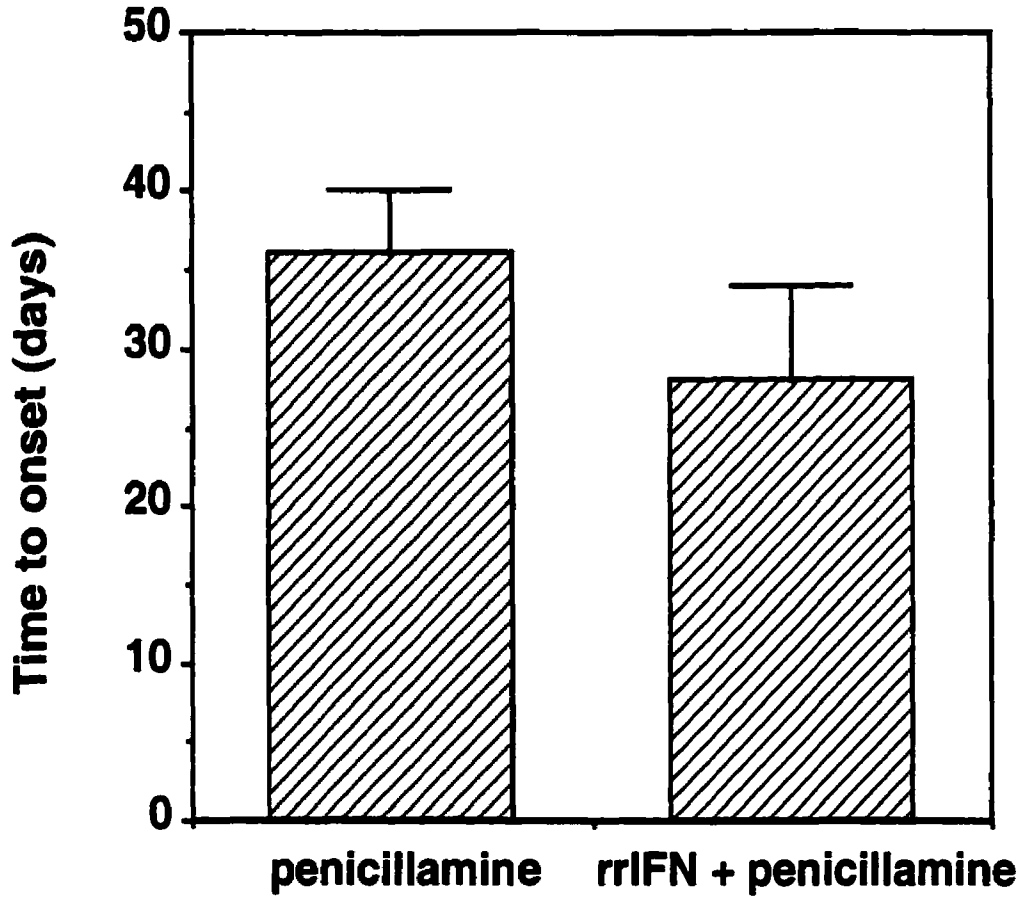


Figure 2-4. Effect of recombinant rat IFN-γ on the time to onset of penicillamine-induced lupus in BN rats.

Rats were either treated with penicillamine alone (20 mg/day; p.o.) or both recombinant rat interferon-gamma (rrIFN-γ; 750 units/day/rat for 3 consecutive days at the beginning of the experiment; s.c.) and penicillamine (20 mg/day; p.o.) for 8 weeks. The value for each group represents the mean ± SD of the time (days after starting the experiment) at which the animals first developed signs of lupus (score of 1 using the scale presented in Table 2-1; n=7/group).

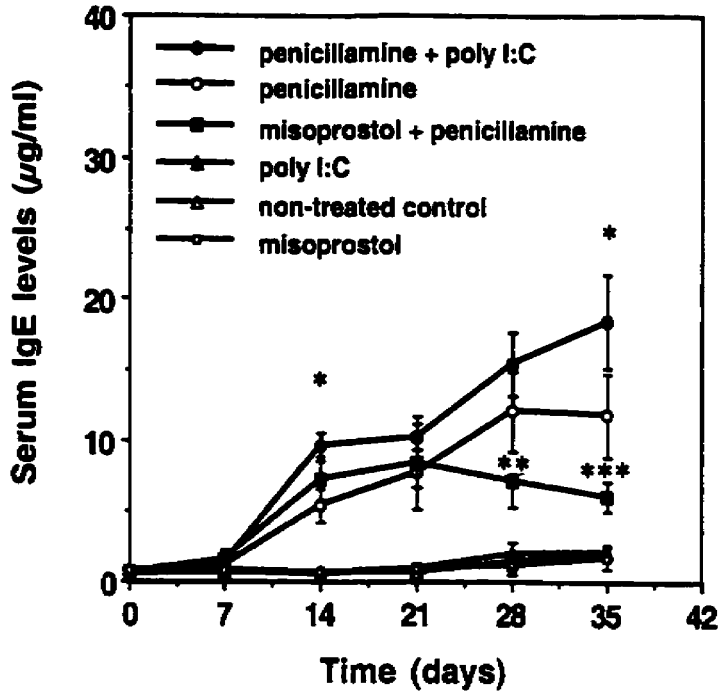


Figure 2-5. Effect of treatment with poly I:C or misoprostol on the serum IgE levels in penicillamine-treated BN rats.

Brown Norway rats were either treated with penicillamine alone (20 mg/day), or a single dose of poly I:C or misoprostol followed by penicillamine (n=10/group). Total serum IgE levels in BN rats were determined weekly by a sandwich ELISA assay as described in *Materials and Methods*. The data shown represent the mean ± SD of the serum IgE levels of rats in each group at each time point.

The serum IgE levels in poly I:C + penicillamine treated group were significantly higher (* p=0.0001) when compared with penicillamine-treated group.

The serum IgE levels in misoprostol + penicillamine-treated group were significantly lower (** p=0.0003, *** p=0.0001) compared to those treated with penicillamine alone.

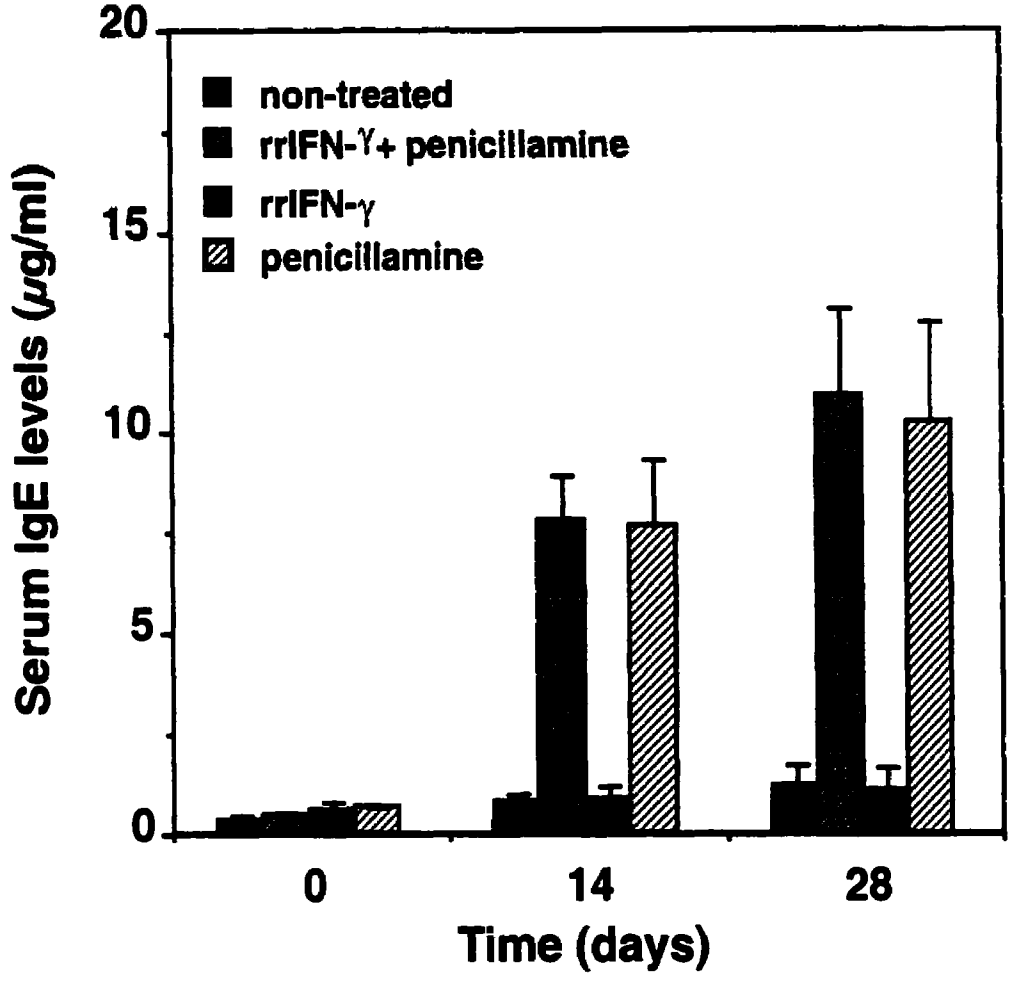


Figure 2-6. Effect of recombinant rat IFN-γ-treatment on serum IgE levels in penicillamine-treated BN rats.

Rats were either treated with penicillamine alone (20 mg/day) or both penicillamine and rrIFN-γ (n=12/group), as described in *Materials and Methods*, for 56 days. Total IgE levels in the sera of rats were determined at days 14 and 28 of post-treatments by a sandwich ELISA assay. The data shown are the mean ± SD of the serum IgE levels of rats in each group at each time point.

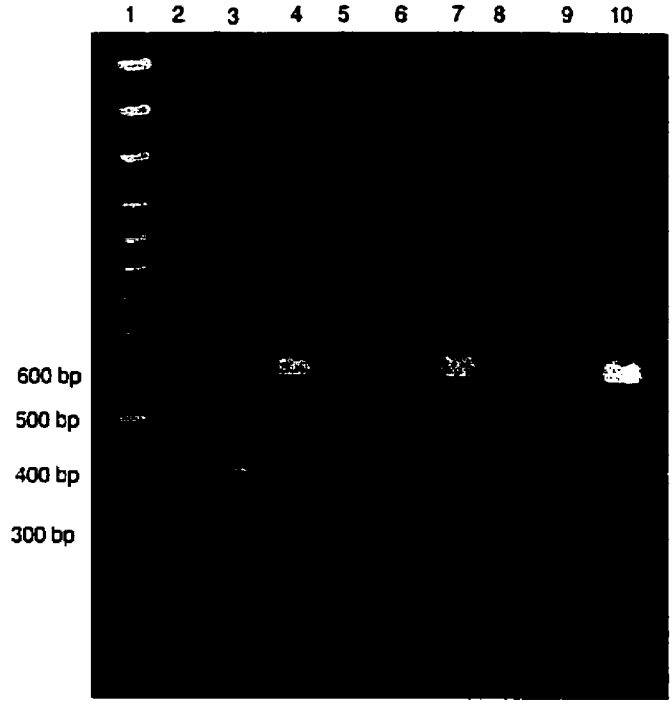


Figure 2-7. Effect of rrIFN-γ-treatment on the expression of mRNA for IL-4 and IFN-γ in penicillamine-treated BN rats.

Brown Noway rats treated with both penicillamine and rrIFN-γ (n=12). The data shown are for a representative 3 out of 8 lupus-rats.
Samples (2 ug) of total RNA extracted from frozen spleen of 3 randomly chosen lupus-rats were reverse transcribed into cDNA and amplified by PCR as described in *Materials and Methods*. The PCR products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining. Lanes 2, 5 and 8 represent IL-4 mRNA; lanes 3, 6 and 9 represent IFN-γ mRNA; and lanes 4, 7 and 10 represent β-actin mRNA expression. Lane 1 contains molecular mass markers.

2.5 Discussion

We first used poly-I:C, a synthetic double-stranded polyribonucleotide, to stimulate interferons (Field A.K. et al 1967) in an attempt to tip the balance toward a Th1 response. Presumably because of its structural resemblance to double-stranded viral RNA, poly-I:C elicits immune responses that mimic a viral infection and include stimulation of interferon-α (IFN-α) and IL-12 production by macrophages (Manetti R. et al 1995). As mentioned in the introduction, IL-12 is believed to be the major determinant of a Th1 response, and IL-12 knockout mice have severely impaired IFN-γ production and almost completely lack the ability to generate a Th1 response (Magram J. et al 1996). IFN-α is also known to stimulate a Th1 response and inhibit a Th2 response (Finkelman F.D. et al 1991; Rogge L. et al 1997; Wenner C.A. et al 1996). In view of the simple Th1/Th2 model with counter regulation of Th1 and Th2 cytokines, poly-I:C would be expected to inhibit a Th2-driven response and, therefore, inhibit penicillamine-induced lupus. In fact, we found that poly-I:C increased the incidence of penicillamine-induced lupus to 100% and it occurred earlier and was more severe than in those animals treated with penicillamine alone. However, the effects of Th1 cytokines on lupus in other studies have been contradictory and many studies have found that they exacerbate lupus. Specifically, IFN-γ appears to cause an exacerbation of lupus in humans (Machold K.P. & Smolen J.S. 1990) and IFN-α therapy leads to a lupus-like syndrome (Wandl U.B. et al 1992). In addition, IFN-γ appears to be required for lupus to occur in MRL-lpr mice (Balomenos D. et al 1998; Haas C. et al 1997). In animal models there is evidence that Th1 cytokines can accelerate lupus (Takahashi S. et al 1996), but results are not always consistent (Peng S. L. et al 1997). An added complication to the interpretation of these results is the fact that the effects of poly-I:C are complex. Although poly-I:C has been reported to induce IL-12 *in vitro*, the effects *in vivo* could be different, and IFN-α has been reported to inhibit the production of IL-12 (Biron C.A. 1998). Furthermore, the effect of poly-I:C is dose dependent and it can have opposite effects at different doses (Sobel D.O. et al 1998). Therefore, we tested the effect of the prototypic Th1 cytokine, IFN-γ, on the induction of lupus by penicillamine. Although there was a trend to an earlier onset of lupus in IFN-γ-treated animals, the effects were not significant and it is difficult to interpret the results because they might have been different with a different schedule of IFN-γ-treatment. On the other hand, IFN-γ

certainly did not decrease clinical manifestations of penicillamine-induced lupus as would be expected from the Th1/Th2 model. Furthermore, IFN-γ did not suppress the increase in IgE and the expression of IL-4 mRNA induced by penicillamine.

Misoprostol is a prostoglandin-E1 analog that binds to the prostaglandin EP4/EP2/EP3 receptors and increases T cell cAMP levels (Zeng L. et al 1998). It has effects on the immune system very similar to those of prostaglandins E1 and E2 (Haynes D.R. et al 1992) but has the advantage that it has significant bioavailability when administered orally. Prostaglandin E2 is a potent inhibitor of IL-12 (Van-der Pouw Kraan et al 1995) and IL-12 receptor expression (Wu C. Y. et al 1998). It inhibits the priming of T cells for the production of IFN-γ but not IL-4 (Abe N. et al 1997). Overall, it appears that the balance of IL-12 and prostaglandin E2 determine IFN-γ levels in activated Th cells (Hilkens C.M. et al 1996). *In vivo*, misoprostol has been shown to suppress both clinical and histological characteristics of experimental autoimmune encephalomyelitis, an animal model for human multiple sclerosis in Lewis rats (Reder A.T. et al 1994). Indeed, the beneficial effect of misoprostol in preventing this autoimmune disease, in which Th1 cells are implicated in the disease process, was shown to be achieved via suppressing the Th1-like pathway. Therefore, using the Th1/Th2 model, misoprostol would be expected to stimulate a Th2-driven response and increase the incidence of drug-induced lupus. Again the results in penicillamine-treated BN rats were the opposite those predicted by the Th1/Th2 model.

Although our results were not those expected from the Th1/Th2 model, they do fit with some other data on the effects of IFN-γ on lupus as stated above. This suggests that, although penicillamine-induced lupus in the BN rat has many characteristics of a Th2-driven reaction, the pathogenic mechanism is more complex and both Th1 and Th2 cytokines are required for the reaction. Other aspects of idiosyncratic reactions are difficult to explain on the basis of the Th1/Th2 model. For example, reactions that appear to be mediated by cytotoxic T cells, and therefore should be Th1 driven, are often associated with eosinophilia, and eosinophils are associated with an elevation of IL-5, which is a Th2 cytokine. The results are also consistent with other observations made in idiosyncratic drug reactions in humans. For example it appears that a viral infection, or even influenza vaccine, which are likely to have effects similar to poly-I:C can increase the risk of idiosyncratic drug reactions. Much of the work defining the Th1/Th2 model has been done *in vitro*, and it should probably not be surprising that *in vivo* the response may be

more complex. Although clones of T cells are irreversibly committed to either a Th1 or Th2 phenotype, an immune response can be initially dominated by a Th1 response and convert to a Th2 response, although the reverse does not seem to occur (Morel P.A. & Oriss T.B. 1998).

It is interesting to note that, although misoprostol completely prevented penicillamine-induced lupus in this model, it did not prevent the initial increase in IgE, but at 3 weeks it peaked and then decreased. This suggests that, although the cytokine profile is consistent with a Th2 response, it is not Th2 cytokines that initially drive the immune response. Alternatively, it could be the state of activation of the antigen presenting cells that determines whether there will be an active immune response or tolerance, and then it is the balance of cytokines that determines the nature of that response. It is known that IFN-γ increases the expression of MHC-II (Revel M. 1984). The innate immune system may also play a role in the induction of an immune response (Fearon D.T. & Locksley R.M. 1996; Medzhitov R. & Janeway C.A. Jr. 1997), and IL-12 increases NK cell cytotoxicity (Caspi R.R. 1998) and nitric oxide (NO) production (Huang F.P. et al 1996).

Genetics obviously plays an important role since lupus could not be induced in Lewis rats even in the presence of poly-IC. BN rats appear to be predisposed to a Th2 response, but it must also be acknowledged that Lewis rats did not develop an obvious Th1 type of response to penicillamine.

Although the results do not fit the Th1/Th2 model, they were dramatic. For example, misoprostol has a short biological half-life, and yet one relatively small dose administered just before starting the penicillamine treatment completely prevented penicillamine-induced lupus that would have otherwise occurred about 4 weeks later. Although it is impossible to extrapolate with confidence from these results in penicillamine-induced lupus in BN rats to humans with other idiosyncratic drug reactions, if these agents had similar effects in idiosyncratic drug reactions in humans, it might be possible to use an agent like misoprostol to prevent or treat some idiosyncratic drug reactions. In most cases the incidence of an idiosyncratic drug reaction is so low that it would not be practical to use an agent, even as safe as misoprostol, in everyone to prevent a rare idiosyncratic reaction to a drug. However, in some subsets of a population where the risk is higher, such as patients with a past history of a reaction to a particular drug or in patients with AIDS who have a very high incidence of idiosyncratic reactions to certain drugs, it

might be feasible. It will be difficult to test the ability of agents, such as misoprostol, to prevent idiosyncratic drug reactions in other animal models because of the lack of such models; therefore, it may be necessary to perform further tests in patients where the risk vs. benefit of a drug favors a trial of the drug even though the risk of an adverse reaction is high.

Chapter 3

Effect of Aminoguanidine on the Incidence and Severity of Penicillamine-Induced Lupus in Brown Norway Rats

3.1 Abstract

About 60% of Brown Norway rats (BN) develop an idiosyncratic lupus-like syndrome when treated with penicillamine for several weeks. Using this animal model we attempted to test the involvement of nitric oxide in the pathogenesis of penicillamine-induced lupus. Urine samples from individual rats were collected and assayed for the 24-hr nitrite excretion levels as an indirect measurement of *in vivo* nitric oxide production. In penicillamine-treated rats, the urinary nitrite levels increased substantially following the onset of the clinical syndrome and there was a good correlation between the increased urinary nitrite excretion and the severity of the clinical manifestations of lupus. Co-treatment of the rats with aminoguanidine, an inhibitor of inducible nitric oxide synthase (iNOS), completely protected the animals from penicillamine-induced lupus. Furthermore, addition of arginine, the cofactor in NO synthesis, abrogated the protective effect of aminoguanidine. These results suggest that nitric oxide plays an important role in the development of penicillamine-induced lupus in this model. Individuals with HIV infections also have elevated urinary nitrite levels and they also have a marked increase in the incidence of idiosyncratic drug reactions. This suggests that NO may also play a role in the pathogenesis of idiosyncratic drug reactions in humans, and inhibitors of iNOS might be of use in the treatment of such reactions.

3.2 Introduction

Idiosyncratic drug reactions are a major clinical problem (Park B.K. et al 1987). The term will be used here to indicate adverse reactions that do not occur in most patients at any dose and do not involve known pharmacological properties of the drug involved. As the name implies, little is known about the factors that influence whether a specific individual will have such a reaction to a specific drug. Such reactions have characteristics that suggest involvement of the immune system, but that has not been proven for most reactions. We utilized an animal model, penicillamine-induced lupus in the Brown Norway rat, to study factors that could influence the incidence of such reactions.

Penicillamine is a chelating agent that is also used to treat inflammatory diseases such as rheumatoid arthritis (Jaffe I. A. 1979). However, a major drawback of penicillamine therapy is the development of a wide range of autoimmune adverse reactions including a lupus-like syndrome (Taylor H.G. & Samanta A. 1992). Penicillamine-induced lupus in humans is idiosyncratic, and although the mechanism is unknown, lupus is an autoimmune syndrome and, therefore, it must be immune-mediated.

Like humans, Brown Norway (BN) rats develop a lupus-like disease when treated with penicillamine (Donker A.J. et al 1984). The syndrome is also idiosyncratic in rats in that it only occurs in BN rats, not Lewis or Sprague Dawley rats (Donker A.J. et al 1984), and even in BN rats only about 60% of treated animals develop the syndrome. The syndrome in rats is characterized by the production of antinuclear antibodies (ANA), elevated serum IgE levels, immune-complex glomerulonephritis and clinical manifestations, including weight loss and a rash (Tornade H. et al 1990). Many of the features of this experimental model are similar to penicillamine-induced lupus in humans. Thus, this animal model provides an opportunity to study the mechanism of drug-induced lupus and the results may be relevant to other idiosyncratic drug-reactions in humans.

In a previous experiment (reported in chapter 2 of this thesis) we attempted to manipulate the incidence of penicillamine-induced lupus in the BN rat by altering the balance of Th1/Th2 cytokines. Although the effects were the opposite of what we expected based on the apparent Th2 characteristics of this syndrome, they were none the less dramatic. One possible explanation

for the observed results is that, although the syndrome is Th2 driven, this is determined by the genetic predisposition of the BN rat to a Th2 response and can not be readily changed. If this is correct, the effects of the agents employed in the study (e.g. interferon-γ and misoprostol) must be mediated by other mechanisms, one of which could be due to their effects (stimulation and inhibition, respectively) on macrophages and other antigen presenting cells rather than on the Th1/Th2 balance. If this hypothesis is correct, other agents that have important modulatory roles with respect to macrophages may also be effective at controlling this idiosyncratic reaction. One agent that appears to play an important role in macrophage activation is nitric oxide (NO). Therefore, we investigated the role of NO production and inhibition of the inducible NO synthase (iNOS) on the development of penicillamine-induced lupus in the BN rat.

3.3 Materials and Methods

3.3.1 Animals

Studies were performed in male Brown Norway rats weighing 180 to 200 g obtained from Harlan Sprague Dawley (Indianapolis, IN). After arrival, all animals were housed two to a cage in a 12 h light/dark cycle at 22°C. The rats were fed a standard rat chow (Agribrands, Purina Canada; Strathroy, ON) and allowed tap water for a week before starting the experiments.

3.3.2 Chemicals and solutions

Penicillamine was provided as a generous gift by Merck Frosst Canada Inc. (Montreal, QC). N-(1-naphtylethylene-diamine), aminoguanidine, sulphanilic acid and L-arginine were purchased from Sigma-Aldrich Canada (Oakville, ON). Glacial acetic acid was purchased from Analar® (Toronto, ON).

3.3.3 Induction of lupus with penicillamine

The induction of lupus by penicillamine in Brown Norway rats at a dose of 20 mg/day has been previously reported (Donker A.J. et al 1984). Based on an average observed water intake of 28 ml/day/rat, penicillamine was dissolved in the drinking water of rats (based on 3 days water consumption) at a concentration of 120 mg/168 ml (20 mg/day/rat).

3.3.4 Animal monitoring

Rats from different groups were weighed once a week from the beginning of the experiments to assess possible weight changes. From week two of the experiments, the rats were evaluated daily for the onset of dermatitis and the severity of the skin lesions was determined according to an arbitrary scale of 0 to 4, once a week. The severity scale consisted of: 0= no sign of skin abnormalities; 1= redness of animal's ears, feet and tail; 2= appearance of rash on the ears

and feet; 3= spread of rash over the whole body; 4= loss of hair from the rat's ears and legs and a swollen bloody snout.

3.3.5 Co-treatment with aminoguanidine and/or L-arginine

Rats were injected i.p. with a single dose of 100 mg/kg of aminoguanidine (in normal saline) a day prior to commencement of penicillamine treatment. In addition, from day one of the experiment, the rats drinking water was dosed with aminoguanidine to a final concentration of 0.1% (wt/vol) to maintain a more constant level of the iNOS inhibitor. Dosage schedules similar to this have been used and found to be nontoxic and effective for inhibiting iNOS activity (Griffiths M.J.D. et al 1993).

In other experiments, both arginine and aminoguanidine, at a final concentration of 1% and 0.1% wt/vol, respectively, were added to the rat's drinking water from day one of the experiment.

3.3.6 Measurement of NO production

At certain time points during the experiments, 24-h urine samples were collected from individual animals in metabolic cages and frozen at -70°C until being assayed. Urine nitrite levels were measured by a colorimetric assay based upon the Greiss reaction as has been described (Vander-Meide et al 1995) with minor modification. First, 1 ml of 350 mM ammonium chloride buffer (pH 9.6) was added to 100 µl of urine. Then, after mixing, 2 ml of freshly prepared Griess reagent consisting of 5 mM-sulphanilic acid; 5 mM N-(1-naphtylethylene-diamine; glacial acetic acid (1:1:3 by volume) was added to the reaction mixture. The optical densities of the resulting solutions were measured at 555 nm after a 10-minute incubation in dim light at room temperature. The amount of nitrite in the samples was calculated using a standard curve constructed with a set of known dilutions of sodium nitrite ranging from 2 to 1000 µmol.

3.3.7 Spleen weight

At the peak of the severity of the clinical lupus or at the end of the experiments (day 56), rats were sacrificed and their spleens were removed and weighed. Spleen indices were calculated by dividing the spleen weight by the body weight as reported (Schorlemmer H.U. et al 1996). The value for the experimental animals was then calculated and used as the mean spleen index for the experimental group. For a better comparison between experiments, the ratio of the mean spleen index of the experimental group to the mean spleen index of the control group (relative spleen index) was calculated by the following formula:

$$\text{Relative spleen index} = \frac{\text{mean spleen index of experimental group}}{\text{mean spleen index of control group}}$$

3.3.8 Statistical analysis

Data are presented as the mean ± SD. Comparisons between 2 groups of animals were done using the 2-tailed Student's t-test. Comparisons between multiple groups were done using analysis of variance. *P* values less than 0.05 were considered significant.

3.4 Results

3.4.1 Effects of aminoguanidine on clinical manifestations of the lupus

The appearance of a typical rash on the head and face of BN rats after several weeks of penicillamine therapy is an early clinical sign of the development of lupus in these animals. As shown in Fig. 3-1, the administration of penicillamine (20 mg/day/rat) in a group of BN rats for 8 weeks induced the clinical manifestations of the lupus (clinical score of 1) in 6 out of 10 animals, and in those rats the first clinical signs of skin lesions was observed after an average of 4 weeks of therapy. However, co-administration of aminoguanidine with penicillamine completely prevented the skin abnormalities throughout the experiment.

Another measure of illness is a decrease in the normal weight gain of the animals. Fig. 3-2 summarizes values for the weight gain in the six groups of rats. Baseline body weights of rats were measured at the onset of the experiments and then weighed again at the end of the experiments before the animals were sacrificed (day 56 for rats that did not develop lupus and at the peak of the illness for rats with lupus). As shown in Fig. 3-2, animals that developed other signs of lupus also had a significant decrease in the normal weight gain. This occurred in 6 animals treated with penicillamine alone but in none of the animals treated with the combination of penicillamine and aminoguanidine.

3.4.2 Effect of aminoguanidine on urinary nitrite excretion

The values for 24-hrs urinary excretion of nitrite in different groups of rats studied are summarized in Table 3-1. After treatment of a group of BN rats with penicillamine for several weeks, mean urinary output of these rats was increased regardless of whether they developed penicillamine-induced lupus or not. However, as indicated in Table 3-1, rats that were treated with penicillamine and demonstrated early clinical signs of penicillamine-induced lupus (clinical scoring of 1 or greater) had a higher 24-hr urinary nitrite output (392 ± 26) compared to that of rats that did not develop lupus (clinical score of 0; 179 ± 13). Furthermore, the mean urinary

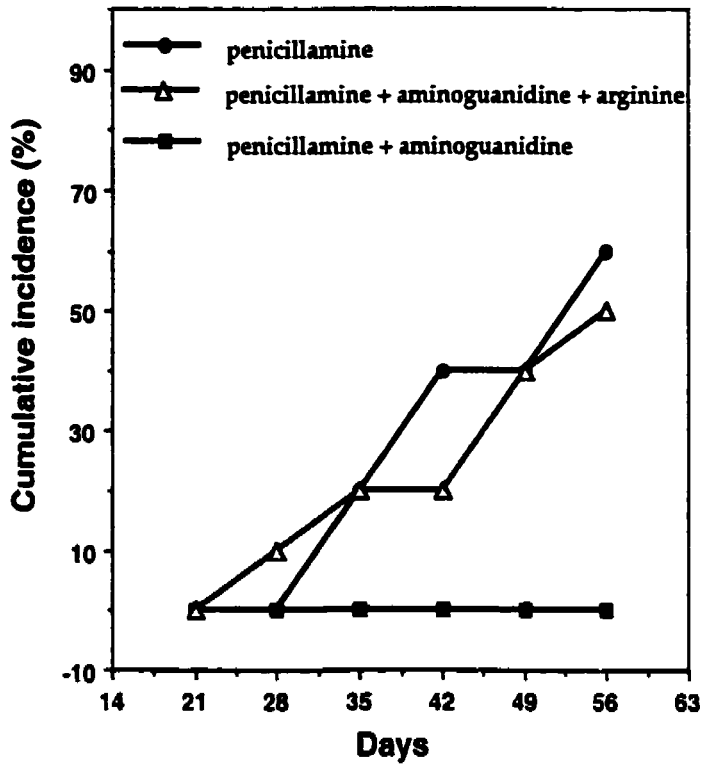


Figure 3-1. Effect of aminoguanidine and arginine on the incidence of penicillamine-induced lupus in Brown Norway rats

Brown Norway rats were treated with penicillamine alone (20 mg/day), penicillamine (20 mg/kg) plus aminoguanidine (a single i.p. injection of 100 mg/kg at the onset and addition of 0.1% wt/vol in the rat's drinking water throughout the experiment) plus L-arginine (1% wt/vol added to the rat's drinking water), or penicillamine (20 mg/day) plus aminoguanidine (same dose as above) for 56 days (n=10/group). The total number of rats in each group that developed clinical signs of the lupus was determined weekly.

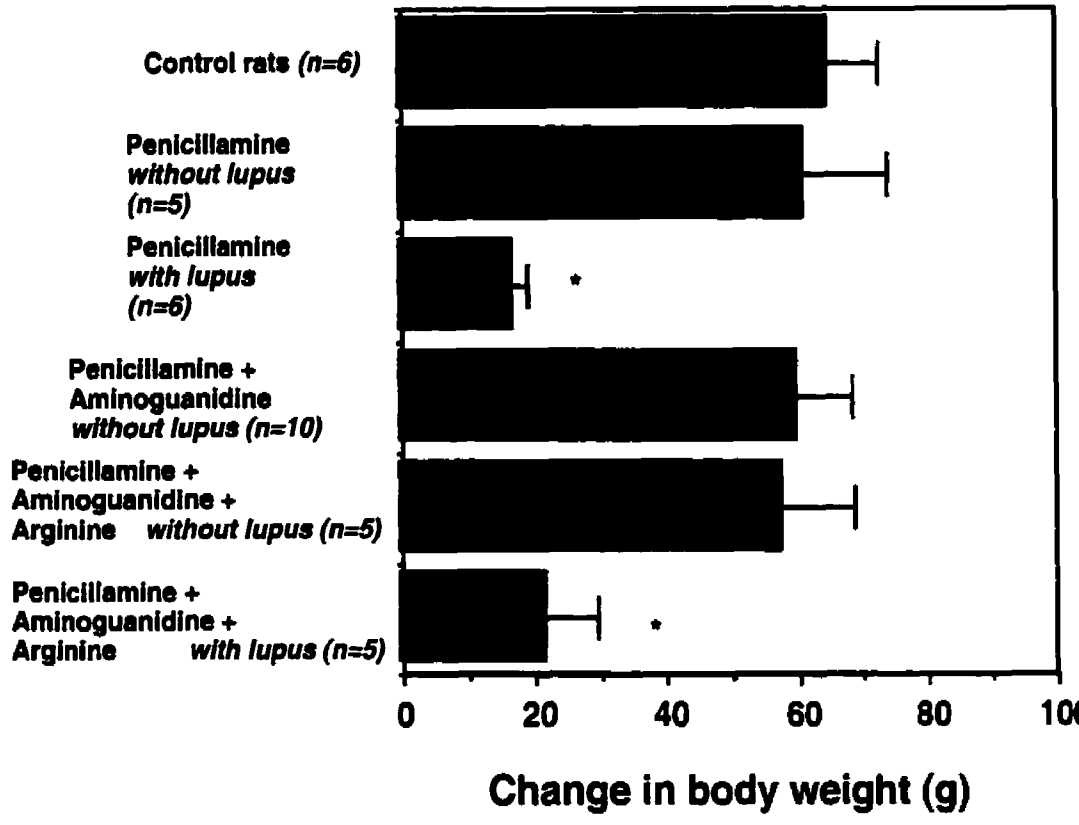


Figure 3-2. Effects of treatment and clinical lupus on weight gain

The change in body weight is the weight of rats on day 56 (for rats that did not develop lupus) or at the peak of the disease (for rats that developed lupus) minus that on day 0. Values are the mean ± SD of the indicated number of animals per group.

* $P < 0.003$ versus normal rats, by Student's *t*-test.

nitrite level was significantly higher (1008 ± 83, P<0.003) in rats with the most sever disease (clinical score of 4). As shown in Table 3-1, treatment of a group of BN rats with aminoguanidine in addition to penicillamine resulted in a mean urinary nitrite excretion level (228 ± 35) that was not significantly different from that of penicillamine-treated rats that did not develop lupus (179 ± 64).

3.4.3 Effect of aminoguanidine on spleen weight

Lymphocyte proliferation and spleen enlargement characterize penicillamine-induced lupus in BN rats (Donker A.J. et al 1984). Table 2 demonstrates the values for the mean spleen index of different groups of rats. The mean spleen index of BN rats that did not develop lupus after 56 days of penicillamine treatment was not significantly increased compared to that of non-treated controls (0.0020 vs 0.0019). However, the mean spleen index of rats that developed penicillamine-induced lupus was more than two-fold higher compared to that of non-treated controls (0.0040 vs 0.0019), or penicillamine-treated rats that did not develop lupus (0.0040 vs 0.0020). Treatment of rats with aminoguanidine plus penicillamine prevented splenomegaly, and as shown in Table 3-2, the mean spleen index of rats in this latter group after 56 days of treatment was not significantly different from that of control rats (0.0020 vs. 0.0019).

3.4.4 Effects of L-arginine on the reversal of the inhibitory effect of aminoguanidine

In a series of experiments, penicillamine-treated BN rats were also treated with L-arginine alone, aminoguanidine alone, or aminoguanidine plus L-arginine. As shown before, treatment with aminoguanidine suppressed clinical as well as other parameters associated with the development of penicillamine-induced lupus throughout the 8-week course of the experiment. However, co-treatment with L-arginine reversed the inhibitory effect of aminoguanidine on the development of penicillamine-induced lupus. As depicted in Fig. 3-1, in contrast to aminoguanidine-treated group, by 56 days, 5 out of 10 of aminoguanidine plus L-arginine-treated rats had developed penicillamine-induced lupus. Moreover, a decrease in weight gain was seen in all diseased rats in this group as shown in Fig. 3-2. Furthermore, L-arginine substantially

reversed the inhibition of nitrite excretion produced by aminoguanidine in a group of rats as shown in Table 3-1. In addition, the inhibitory effect of aminoguanidine on the enhancement of spleen weight by penicillamine was blocked as shown in Table 3-2.

Group (n)	Treatment	Clinical scoring	Nitrite excretion (nmol/24 hrs)
Normal rats (6)	-	0	148 ± 16
Rats without lupus (4)	Penicillamine	0	179 ± 13 *
Rats with lupus (4)	Penicillamine	1	392 ± 26 **
Rats with lupus (4)	Penicillamine	4	1008 ± 83 ***
Rats without lupus (4)	Penicillamine + Aminoguanidine	0	228 ± 35
Rats with lupus (4)	Penicillamine + Aminoguanidine + Arginine	4	903 ± 64 ***

Table 3-1. Effects of treatment and disease severity on urinary nitrite excretion

A score of 0 to 4 (with 0 = no evidence of disease to 4 = most severe disease) was assigned to rats based on the appearance and severity of skin lesions induced by penicillamine using an arbitrary scale as presented in *Materials and Methods*.

24-hour urinary nitrite excretion of rats was measured by the Greiss reaction assay.

Values are the mean ± SD.

* P <0.003 versus normal rats; ** P <0.003 versus penicillamine-treated rats that did not develop the lupus; *** P <0.0001 versus rats with the clinical score of 1, by Student's t-test.

Treatment Group (n)	Lupus syndrome	Spleen index	Relative spleen index
Normal rats (6)	no	0.0019 ± 0.0001	1
Penicillamine (5)	no	0.0020 ± 0.0004	1.053
Penicillamine (6)	yes	0.0040 ± 0.0004*	2.105
Penicillamine +Aminoguanidine (10)	no	0.0020 ± 0.0001	1.053
Penicillamine +Aminoguanidine +Arginine (5)	no	0.0019 ± 0.000	1
Penicillamine +Aminoguanidine +Arginine (5)	yes	0.0041 ± 0.0002*	2.158

Table 3-2. Effects of treatment and disease development on spleen size

Spleen indices were determined by weighing the animals and the spleens and calculating the spleen weight in relation to body weight. The ratio of the mean spleen index of the experimental group to the mean spleen index of the control group has been presented as "relative spleen index".

Data presented are the mean ± SD. * $P < 0.0001$ versus penicillamine-treated rats that did not develop lupus, Student's *t*-test.

3.5 Discussion

Our data implicate involvement of NO in the development of penicillamine-induced lupus in BN rats. First, not only did the urinary excretion of nitrite (NO oxidation product) increase prior to the onset of clinical symptoms, but there was also a good correlation between the increased urinary nitrite excretion and the severity of the clinical syndrome (Table 3-1). Second, aminoguanidine, a selective inhibitor of iNOS, completely protected the rats from the clinical changes that occur in penicillamine-induced lupus (Fig. 3-1). Third, the effects of L-arginine, the cofactor for NO production, indicated that the inhibitory effect of aminoguanidine on the development of penicillamine-induced lupus was due to its inhibition of iNOS (Figs. 3-1 and 3-2, Tables 3-1 and 3-2).

The data reported in Table 3-1 indicate that urinary nitrite excretion of rats was substantially enhanced upon penicillamine treatment. This suggests that penicillamine induces nitric oxide production, presumably by activation of macrophages in these animals.

Our observations in this study are in contrast to an earlier report that inhibition of NO synthase did not influence the incidence of autoimmunity induced by mercuric chloride in BN rats (Woolfson R.G. et al 1995). However, our results on the elevated NO production in the lupus rats are in accord with those obtained in human studies. Several human studies have examined the role of NO in autoimmune diseases. In those studies, patients with lupus had elevated serum NO levels when compared to healthy controls (Gilkeson G.S. et al 1996; Miesel R. & Zuber M. 1993). Furthermore, renal biopsy specimens from individuals with immune complex glomerulonephritis had enhanced immunostaining for iNOS compared to those from controls (Kashem A. et al 1996).

We used penicillamine-induced lupus in the BN rat as a model because this is one of the very few animal models of an idiosyncratic drug reaction, and it also shares many features with the similar idiosyncratic reaction that occurs in humans. However, the mechanisms of other idiosyncratic drug reactions could be very different from those responsible for penicillamine-induced lupus and our findings in this model may not extrapolate to other such reactions. One piece of clinical data that suggests that NO may be important in the induction of other

idiosyncratic drug reactions is that patients infected with HIV have a markedly elevated risk of idiosyncratic reactions to a wide range of drugs (Greenberger P.A. & Patterson R. 1987) and they also have elevated urinary and serum nitrite (Baldeweg T. et al 1996; Groeneveld P.H. et al 1996; Torre D. & Ferrario G. 1996) even though they are otherwise immunosuppressed. It would be reasonable to measure urinary levels of nitrite in non-HIV infected patients with severe idiosyncratic drug reactions to determine if increased NO synthesis is a general feature of idiosyncratic drug reactions. There are many different types of idiosyncratic drug reactions, and it may be that only specific types of reactions are associated with elevated NO levels. If elevation of urinary nitrite levels is found to be characteristic of specific types of idiosyncratic drug reactions, it would be reasonable to attempt to treat severe idiosyncratic drug reactions associated with increased NO synthesis with agents known to inhibit iNOS.

Chapter 4

Summary and discussion

4.1 Summary and discussion

The results obtained from the studies reported in this thesis can be summarized as follows.

- First, in this animal model, the idiosyncratic response that is observed in some rats after treatment with a higher dose of penicillamine (20 mg/day) has the capacity to be influenced by the external factors.
- Second, using this experimental model, it might be possible to predict the potential risk and beneficial factors for the development of penicillamine-induced lupus in BN rats.
- Third, poly I:C and misoprostol were shown to effectively manipulate the incidence and severity of penicillamine-induced idiosyncratic reaction in this animal model in a positive and negative manner, respectively. However, the manipulatory effects of poly I:C and misoprostol on the development of the syndrome in the rats do not seem to correlate with the capacity of these agents to influence the Th1/Th2 cytokine balance.

The serological characteristics of penicillamine-induced lupus in BN rats suggest that this idiosyncratic drug reaction is a Th2-driven immune response. Based on the counter regulatory nature of the Th1 and Th2 cytokines, it would be expected that poly I:C would inhibit penicillamine-induced autoimmunity by stimulating Th1 cytokines, which should inhibit Th2 cytokines. We observed a potentiation of both the syndrome and Th2 cytokines. Therefore, the hypothesis that changing the Th1/Th2 balance could be used to prevent the response was never really tested because Th2 cytokines were increased rather than decreased. Likewise, misoprostol, which was expected to inhibit Th1 cytokines and induce Th2 cytokines did not appear to induce Th2 cytokines. Therefore, either these agents did not have the effects reported in the literature, or the counter regulatory nature of the cytokines was overwhelmed by other effects.

The results obtained from the co-administration of misoprostol with penicillamine in this experimental model demonstrate an inhibitory role for prostaglandins on the development of penicillamine-induced lupus in BN rats. These results, on the other hand, suggest that prostaglandin inhibitors, such as non-steroidal anti-inflammatory drugs (NSAIDs), may potentiate the development and/or severity of this drug-induced idiosyncratic reaction in rats.

The therapeutical effects of NSAIDs are mediated primarily through inhibition of cyclooxygenase (COX) enzyme and prevention of subsequent formation of prostaglandins and related inflammatory mediators involved in pain and inflammation (Stiles D.P. et al 1990). There are two isoforms of the COX enzyme, COX-1 and COX-2 (Crawford L.J. 1997). COX-1 is a constitutive enzyme present in many tissues, including the stomach, kidneys, and platelets. This isoform is responsible for the production of prostaglandins involved in general "house-keeping" activities, such as the maintenance of gastric mucosal integrity, vascular hemostasis and regulation of renal blood flow. In contrast, COX-2 is predominantly an inducible enzyme at sites of inflammation. Expression of COX-2 is nearly undetectable in normal tissues, but it is upregulated in response to inflammatory stimuli, such as cytokines and bacterial lipopolysaccharides (Crawford L.J. 1997).

Conventional NSAIDs inhibit both COX-1 and COX-2 enzymes. Inhibition of inducible COX-2 is the principal anti-inflammatory mechanism of the NSAIDs. Nonspecific COX inhibition appears to be responsible for much of the adverse effects seen with NSAIDs. For example, reduced PGE-2 production in the gastrointestinal tract leads to gastritis and ulceration. The NSAIDs can also cause reversible renal failure, platelet dysfunction and bronchospasm (Schuna A.A. & Megeff C. 2000). New generations of NSAIDs have been developed that specifically inhibit the COX-2 enzyme and, therefore, are associated with fewer side effects in patients.

As a future study, it is suggested that the modulatory effects of a NSAID drug (i.e., a COX-2 inhibitor) on the course of the idiosyncratic drug reaction to penicillamine in BN rats be investigated. If the effects of the NSAID drug in the previous study were shown to be opposite to that have seen with misoprostol, as predicted, it would strongly suggest a regulatory role for the COX enzyme in the mechanism of this adverse drug reaction. Moreover, the importance of the COX-enzyme inhibition on the induction of this idiosyncratic drug reaction would offer a new therapeutical strategy for the future management of idiosyncratic drug reactions. Furthermore, the preventive role of misoprostol in the induction of this adverse drug reaction encourages a new study to investigate the potential therapeutic indications for the other members of the prostaglandin family in the prevention of idiosyncratic drug reactions.

- Fourth, the results presented in chapter three of this thesis implicate involvement of NO production in the development and severity of the syndrome in BN rats.

A small amount of nitric oxide is produced by vascular endothelial cells via a constitutive pathway that acts in a critical manner to relax smooth muscles in the cardiovascular, gastrointestinal and renal systems (Moncada S. & Higgs A.N. 1993). On the other hand, a large amount of nitric oxide is synthesized by macrophages via the inducible pathway which is believed to be responsible for the induction of a variety of pathologic effects (Kolb H. & Kolb B.V. 1992). Excessive production of NO appears to be crucial to the initiation and maintenance of penicillamine-induced lupus in the BN model because inhibition of NO synthesis by aminoguanidine abrogates the disease. NO is produced by macrophages. Macrophages perform many functions including synthesis of NO and prostaglandins in response to inflammatory stimuli (Bachmann S. & Mundel P. 1994; Tetsuka T. et al 1994). Several studies suggest that prostaglandins and NO production play a role in the development of lupus in both humans and experimental models of lupus. For example, alterations in prostaglandin production have been reported to occur in human and murine lupus nephritis (Kelley V.E. et al 1986; Patrono C. et al 1985; Spurney R.F. et al 1992). Prostaglandin E_2 and prostaglandin I_2, vasodilators that increase glomerular filtration rate, are decreased in lupus nephritis (Yoshida T. et al 1996). On the other hand, when prostaglandin E_2 was exogenously administered in murine lupus, it attenuated the disease progression primarily by systemic immune suppression (Strassmann G. et al 1994). Prostaglandin J_2, a recently described prostaglandin formed from dehydration of prostaglandin D_2, has been shown in vitro to inhibit activation of normal rat macrophages (Ricote M. et al 1998). Further studies demonstrated that this prostaglandin had antiproliferative effects on macrophages and proapoptotic effects in tumor cell lines (Jiang C. et al 1998; Kim H.S. et al 1996). Additional investigations indicated that prostaglandin J_2 inhibited NO production by activated peritoneal macrophages derived from normal rats (Ricote M. et al 1998). In another study it has been shown that prostaglandin J_2 and E_2 blocked the production of NO in LPS stimulated macrophages from lupus-prone MRL/lpr mice (Reilly C.M. et al 2000).

Viral infections have been shown to stimulate NO production by iNOS in several cell types including monocytes and macrophages (Heitmeier M.R. et al 1998). The effects of poly I:C on macrophage expression of iNOS and production of NO have been investigated. In these

studies it was shown that poly I:C, alone or in combination with IFN-γ, stimulated nitrite production and iNOS expression by mouse macrophages (Heitmeier M.R. et al 1998). Similarly, treatment of rat islets with poly I:C + IFN-γ stimulated the expression of iNOS and production of nitrite by these cells (Heitmeier M.R. et al 1999).

Based on the above observations, misoprostol may prevent the lupus in BN rats by inhibiting macrophage activation and NO production. On the other hand, poly:IC may activate macrophages leading to an increase in the expression of costimulatory molecules and an increase in NO production.

Macrophages are antigen-presenting cells and they also are one of the cell components of the innate immune system. The prominent role of macrophages in nitric oxide production and the involvement of nitric oxide synthesis in the development of penicillamine-induced lupus in BN rats suggest a role for the innate immune system in this idiosyncratic drug reaction.

In general, the immune system of higher vertebrates consists of two recognition systems: innate and adaptive. Immunologists have intensively studied the adaptive immune response for several decades. In this system, antigens are processed and presented in association with MHC-II molecule to helper T cells by antigen presenting cells (APCs). These cells (T cells), in turn, stimulate plasma cells to differentiate into antibody-producing B cells or stimulate cytotoxic T cells that recognize the same peptide antigen in the context of MHC-I molecule. This system, with the aid of gene rearrangements, is able to respond to an almost infinite number of antigens in a very specific manner. While adaptive immunity occurs only in vertebrates, all multicellular organisms have some forms of innate (natural) host defense system (Fearon D.T. & Locksley R.M. 1996). The cells most important for an innate immune response are granulocytes, macrophages, NK cells and γδ T cells (Fearon D.T. & Locksley R.M. 1996). Innate immune recognition is mediated by a set of germline encoded receptors which belong to several distinct protein families (Janeway C 1992). For example, mammalian macrophages have mannose, lipopolysaccharide and scavenger receptors for identifying components of yeast and bacterial cell walls. Plasma proteins, such as the complement cascade, C-reactive protein, and the mannose-binding protein of the collectin family, also recognize microbial carbohydrates (Janeway C 1992). These cellular and soluble proteins pre-exist, or are rapidly induced within hours of infection. They provide immediate defense against microorganisms by several mechanisms.

These receptors recognize conserved molecular patterns associated with microbial pathogens and are referred to as pattern recognition receptors (PRR). Because the pathogen-associated molecular patterns (PAMP) are produced only by microbes and not by host organism, their recognition by receptors on the innate immune cells can signal the presence of pathogens.

Triggering of the receptors on cells of the innate immune system by PAMPs can directly activate effector mechanisms of innate immunity, such as phagocytosis, induction of the synthesis of antimicrobial peptides and induction of nitric oxide synthase in macrophages. Additionally, PAMPs induce expression of a set of endogenous signals in the form of inflammatory cytokines. These signals control the recruitment of leukocytes to the site of infection, and regulate the activation of appropriate effector mechanisms, for example by controlling differentiation of T lymphocytes into effector cells of a particular type (Th1 or Th2). Finally, and most importantly, recognition of PAMPs by the receptors on cells of the innate immune system induces the expression of co-stimulatory molecules, such as B7.1 and B7.2, on APCs.

The current mode of helper T cell (Th) activation requires two signals. Based on this model, the presentation of antigen to a Th cell is necessary, but not sufficient, to induce proliferation. This is only an initiation event, termed signal one, which occurs when the T cell receptor and its co-receptors (CD4 or CD8) are ligated. Th cell proliferation will not occur until an additional costimulatory signal is received. This second signal is provided by surface ligands on antigen-presenting cells. The major costimulatory molecule expressed by Th cells is CD28, which binds to two related cell-surface molecules, B7-1 and B7-2, on antigen-presenting cells (Bluestone J.A. 1995). CD28-B7 interactions appear to be important in the development of an immune response to self as well as foreign antigens. The lack of costimulation after engagement of the Th cell receptor results in a lack of an immune response or tolerance. This places the CD28/B7 pathway at a key location for controlling the immune response.

For many years, innate immunity has been considered as a separate entity from the adaptive immune response and has been regarded to be of secondary importance in the hierarchy of immune functions. However, in recent years, evidence has been accumulating that the innate immune system may be the ultimate controller of adaptive responses. For example, Janeway argues that the activation of adaptive cells of the immune system is not fundamentally controlled

by recognition of self versus nonself antigens through the T cell- and B cell-receptors. Instead, he propose that innate receptors that recognize exogenous microbial molecules govern the activation of immunity through the activation of molecular and cellular innate pathways that turn on T-helper co-stimulation (Janeway C 1992; Janeway C.A. Jr. 1989).

The dependence of naive T cell activation on co-stimulatory signals is thought to be a mechanism that provides information for the T cell concerning the origin of the antigen (Janeway C 1992). Self-antigens, as well as environmental non-self antigens that are not of microbial origin, are not recognized by the innate immune system, and therefore do not induce the expression of co-stimulatory signals. These antigens, when presented by APCs, would not activate T lymphocytes. Recognition of MHC/peptide ligand by a T-cell receptor in the absence of co-stimulation results in clonal inactivation of lymphocytes (tolerance). In contrast, when the antigen is of microbial origin, it induces the expression of co-stimulatory molecules on the APC that presents the antigen. Recognition of these antigens by specific T cells results in their activation and clonal expansion (immune response).

In considering the emerging picture of the interplay between innate and adaptive responses, Matzinger has proposed that the activation of the immune system is based upon the discrimination between dangerous and nondangerous antigens (Matzinger P. 1994). The danger model that has been proposed by Matzinger suggests that the activation of an APC depends on the health of the cells in their neighborhood. She proposes that the default response of the immune system to antigens and haptens is tolerance, and it is "danger" rather than nonself that leads to an immune response. If a cell is injured or stressed, it sends activating signals to its local APCs, which then take up local antigens, travel to the draining lymph nodes and up-regulate the co-stimulatory molecules needed to activate T cells. Thus, according to this model, it is the innate immune system, which delivers the danger signal to the cells of adaptive immune system.

It has been proposed that the innate immune system might be involved in the mechanism of idiosyncratic drug reactions (Park B. K. et al 1998; Uetrecht 1997). There are several examples of idiosyncratic drug reactions with characteristics that suggest mediation by the innate immune system. For example, based upon published clinical studies (Safferman A.Z. et al 1992), if a patient with a recent history of clozapine-induced agranulocytosis is rechallenged with clozapine, it takes just as long, 6-weeks, for the onset of agranulocytosis as it did during first

exposure. The long lag time between reemergence of agranulocytosis upon reexposure to clozapine is very difficult to reconcile with an adaptive immune response, which would be expected to occur much faster. However, because there are no memory cells in the innate immune system (Fearon D.T. & Locksley R.M. 1996), it is possible that the cells of the innate immune system mediate clozapine-induced agranulocytosis. There are several other examples of drug-induced idiosyncratic reactions that have characteristics that suggest mediation by the innate immune system. Some of the examples include, amoxicillin- and ampicillin-induced skin rash in patients who have a viral infection. In these patients, there is usually a delay between starting the drug and the onset of rash and it often does not recur if the patient is rechallenged with the drug. Similar observations have been made in HIV positive patients in which the adverse reactions seen in these patients to sulfamethoxazole often do not recur on reexposure. These characteristics suggest that the above idiosyncratic reactions that only occur in a context of a danger signal, such as a virus, may be mediated by the innate immune system.

If this hypothesis (the innate immune system involvement in the mechanism of idiosyncratic drug reactions) is correct, a study of the factors involved in the induction of idiosyncratic drug reactions might make it possible to prevent, or at least decrease, the risk of these reactions.

There is an interesting observation with penicillamine-induced lupus experimental model that first was reported by Donker (Donker A.J. et al 1984). If a group of BN rats are treated with a small dose of penicillamine (5 mg/day) for about four weeks and then switched to a dose of 20 mg/day of penicillamine for the rest of the experiment, none of the animals develop penicillamine-induced lupus. This means, the lower dose (5 mg/day) subsequently protects animals from a dose that would normally induce lupus (20 mg/day). The mechanism of this drug-induced tolerance in BN rats has not been investigated, but it is likely to be important. There are other examples, such as nevirapine and lamotrigine, where starting a drug at a low dose and then increasing the dose decreases the incidence of an idiosyncratic reaction (Richens A 1994).

There are several mechanisms for induction of tolerance, but as discussed above, a major mechanism appears to involve antigen presentation to T cells in the absence of costimulation from APCs (second signal). Experimentally it has been shown that blocking CD28/B7

interactions with specific antibodies inhibits disease progression in a variety of experimental autoimmune disease models (Khoury S.J. et al 1995). For example, when lupus-prone NZB/W F1 mice were treated with antibodies to both B7-1 and B7-2 before the onset of the disease, the production of autoantibodies and the associated nephritis was completely prevented (Khoury S.J. et al 1995). Possible defects in the CD28/B7 system in SLE patients has also been investigated (Khoury S.J. et al 1995). These patients were found to have increased expression of the CD28 gene compared with normal individuals.

It has been shown that blocking CD28-B7 T cell costimulation by CTLA4- Ig in a rat renal allograft model induces tolerance to rat renal allografts. Immunohistological studies in these rats demonstrated that CTLA-4 Ig therapy significantly inhibited iNOS expression in the intragraft macrophages (Akalin E. et al 1996). In another study, the role of CD28-B7 costimulation blockade by CTLA-4 Ig was studied in a rat model of chronic cardiac rejection (Russell M.E. et al 1996). In rats that were treated with a single dose of CTLA-4 Ig 2 days after transplantation, allografts survived significantly longer than in untreated controls. In this study, reverse transcriptase-PCR and immunostaining analyses of tissue from CTLA-4 Ig-treated rats showed significant reduction in iNOS expression (Russell M.E. et al 1996). These results indicate that CD28-B7 blockade inhibits NO production.

As mentioned before, prostaglandins were shown to decrease, whereas poly I:C was shown to increase NO production in different systems. These observations suggest that the inhibitory effects of some of the agents used in our studies, such as misoprostol and aminoguanidine, on the development of penicillamine-induced lupus in BN rats might be mediated via inhibition of the co-stimulatory pathways. On the other hand, poly I:C might increase the expression of co-stimulatory molecules, which, in turn, could lead to T cell activation and increased production of NO. Further studies in this regard are needed to provide an answer to whether modifying the costimulatory signal (CD28/B7pathway) is the molecular mechanism by which these agents manipulate the incidence of penicillamine-associated lupus in BN rats. In addition, studies of the roles of CD28 and its B7 ligands in the pathogenesis of idiosyncratic reaction in BN rats by the 20 mg/day dose of penicillamine may also help us further understand the mechanism of tolerance induction by the smaller dose of penicillamine (5 mg/day) in this animal model.

Another way to study the mechanism of tolerance induction in this animal model is by preventing tolerance. A cytokine that appears to be very effective at preventing tolerance in different models is IL-1, and agents that stimulate IL-1 production, such as LPS and some NSAIDs (i.e., aspirin, indomethacin) inhibit tolerance (Weigle W.O. & Romball C.G. 1997). Thus, agents that stimulate IL-1 production are expected, at least in theory, to break the tolerance in BN rats when co-administered with a small dose of penicillamine. In a future study, the effect of IL-1 production on the induction of tolerance by the small dose of penicillamine in this animal model should be studied.

Alternatively, by applying the danger hypothesis to this idiosyncratic drug reaction, the 20 mg/day dose of penicillamine could lead to a small amount of cell death or modify a cytokine by formation of a disulfide bond to one of the high cystein-containing cytokines and provoke a "danger" signal. This, in turn, could lead to the activation of APCs and induction of the immune response. In contrast, the lower dose (5 mg/day) might lead to tolerance because it does not cause any harm to the animals. In this case, once tolerance is induced it is much more difficult for the antigen involved to invoke an immune response.

Similarly, poly I:C, which potentiated the immune response to penicillamine, could represent a danger signal because it is structurally similar to viral RNA, and as discussed before, there are many examples in which a viral disease increases the risk of an idiosyncratic drug reaction (Levy M. 1997), possibly by providing the "danger signal". In particular, AIDS is associated with a large increase in the incidence of idiosyncratic drug reactions.

On the other hand, misoprostol could inhibit cytokines or other factors involved in activation of antigen-presenting cells. Elimination of the second signal, could, in turn, lead to the induction of tolerance. Similarly, NO production (in large amounts) may represent a "danger signal" and aminoguanidine could induce tolerance in rats by preventing the cells from producing increased amounts of NO.

In summary, penicillamine-induced lupus in the BN rat represents a potentially useful model for the study of idiosyncratic drug reactions in humans. Prospective studies of these adverse reactions are virtually impossible because the incidence is usually low. Studies of patients after the adverse reaction has begun do not allow study of the early steps in the pathogenesis of the reaction. Studying animal models of idiosyncratic drug reactions, on the other

hand, permit prospective studies such as potential risk or beneficial factors as well as potential therapeutical measures.

In the current studies we used penicillamine-induced lupus in BN rats as an animal model of idiosyncratic drug reactions. There are few other animal models available for the study of idiosyncratic drug reactions. In order to see if our findings in the penicillamine-induced lupus model are applicable to the other animal models of idiosyncratic drug reactions, and by extensions to some idiosyncratic drug reactions in humans, further studies are needed. In this regard, the ability of agents, such as misoprostol and aminoguanidine, to modify the adverse reactions in other animal models of idiosyncratic drug reactions should be investigated.

Most probably a combination of environmental and genetic factors is involved in the induction of a drug-induced idiosyncratic reaction. Due to the involvement of these multiple factors, it may not be possible to prevent all idiosyncratic drug reactions, but a better understanding of the factors involved could markedly decrease the risk. In this respect, animal models are important for the study of these factors. With the understanding gained from such studies in animal models, it might be possible to proceed to high-risk situations, such as sulfonamides in AIDS patients, where the incidence of adverse reactions is high and where testing the hypothesis would be feasible. If such studies led to an understanding of the major risk factors involved in idiosyncratic drug reactions, and this understanding led to measures that substantially decreased the incidence of such reactions, it would be a major advance.

References

Abe N., Katamura K., Shintaku N., Fukui T., Kiyomasu T., Iio J., Ueno H., Tai G., Mayumi M., Furusho K. (1997) Prostaglandin E2 and IL-4 provide naive CD4+ T cells with distinct inhibitory signals for the priming of IFN-gamma production. Cell Immunol 181: 86-92

Abraham E. P., Chain E., W., B., R., a. R. (1943) penicillamine, a characteristic degradation product of penicillin. Nature 151: 107-110

Adorini L., Nagy Z.A. (1990) Peptide competition for antigen presentation. Immunol. Today 11: 21

Akalin E., Chandraker A., Russell M.E., Turka L.A., Hancock W.W., Sayegh M.H. (1996) CD28-B7 T cell costimulatory blockade by CTLA-4 Ig in the rat renal allograft model. Transplantation 62: 1942-1945

Alarcon-Segovia D., Worthington J.W., Ward L.E. (1965) Lupus diathesis and the hydralazine syndrome. N. Engl. J. Med. 272: 462

Anderson J.A. (1992) Allergic reactions to drugs and biological agents. J. A. M. A. 268: 2845-2857

Andrews B.S., Eisenberg R.A., Theofilopoulos A.N., Izui S., Wilson C.B., McConahey P.J., Murphy E.D., Roths J.B., Dixon F.J. (1978) Spontaneous murine lupus-like syndrome. Clinical and immunopathological manifestations in several strains. J. Exp. Med. 148: 1198-1215

Arroyave C.M., Bhat K.N., Crown R. (1976) Activation of the alternative pathway of the complement system by radiographic contrast media. J. Immunol. 117: 1866

Aucoin D.P., Rubin R.L., Peterson M.E., Reidenberg M.M., Drayer D., Hurvitz A.I., Lahita R.G. (1988) Dose-dependent induction of anti-native DNA antibodies in cats by propylthiouracil. Arthritis Rheum. 31: 688-693.

Bach E.A., Szabo S.J., Dighe A.S. (1995) Ligand-induced autoregulation of IFN-gamma receptor-beta chain expression in T-helper cell subsets. Science 270: 1215-1218

Bachmann S., Mundel P. (1994) Nitric oxide in the kidney: synthesis, localization and function. Am. J. Kidney Dis. 24: 112-117

Baldeweg T., Sooranna S., Das I., Catalan J., Gazzard B. (1996) Serum nitrite concentration suggests a role for nitric oxide in AIDS. AIDS 10: 451-452

Balomenos D., Rumold R., Theofilopoulos A.N. (1998) Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. J. Clin. Invest. 101: 364-371

Bates D.W., Spell N., Cullen D.J., Burdick E., Laird, N., Petersen M.D. (1997) The cost of adverse drug events in hospitalized patients. J. A. M. A. 277: 307-311

Bergstrom R.F., Kay D.R., Harkcom T.M. , Wagner J.G. (1981) Penicillamine kinetics in normal subjects. Clin. Pharmac. Ther. 30: 404-413

Bir K., Crawhall J.C., Mauldin D. (1970) Reduction of disulphides with sodium and potasium borohyrides and its application to urinary disulphides. Clinica. Chim. Acta. 30: 183-190

Biron C.A. (1998) Role of early cytokines, including alpha and beta interferons, in innate and adaptive immune responses to viral infections. Sem. Immunol. 10: 383-390

Bluestein H.G., Zvaifler N.J., Weissman M.H. (1979) Lymphocyte alteration by procainamide: relation to drug-induced LE syndrome. Lancet ii: 816

Bluestone J.A. (1995) New perspectives of CD28-B7 mediatedT cell costimulation. Immunology 2: 555-559

Boothby M., Mora A.L., Aronica M.A., Youn J., Sheller J.R., Goenka S., Stephenson L. (2001) IL-4 signaling, gene transcription regulation, and the control of effector T cells. Immunol. Res. 23: 179-191

Boulding J.E., Baker R.A. (1957) The treatment of metal poisoning with penicillamine. Lancet 2: 985

Breckenridge A.M., Orme M.L.E. (1987) Principles of pharmacology and therapeutics. In: Weatherall D. J., Ledingham G.G., Warrell D.A. (eds) Oxford textbook of medicine. 2nd edn. Oxford University Press, London, pp 7.1

Brooks P.M., Miners J.O., Smith K., S. M. D., Fearnley I., Birkett D.J. (1984) Dose, plasma concentration and response relationships of D-penicillamine in patients with rheumatoid arthritis. J. Rheumatol. 11: 772-775

Bruckner J. V., Davis B.D., Blancato J.N. (1989) Metabolism, toxicity and carcinogenicity of trichloroethylene. CRC Crit. Rev. Toxicol. 20: 31-50

Cannat A., Seligmann M. (1968) Induction by isoniazid and hydralazine of antinuclear factors in mice. Clin. Exp. Imunol. 3: 99-105.

Capell H.A., Marabani M., Madhok R., Torley H., Hunter J.A. (1990) Degree and extent of response to Sulphasalazine or penicillamine therapy for rheumatoid arthritis: results from a routine clinical environment over a two year period. Quarterly J. Medicine 75: 335-344

Caspi R.R. (1998) IL-12 in autoimmunity. Clin. Immunol. Immunopath. 88: 4-13

Chalmers A., T. D., Stein H.E., Reid G. and Patterson A.C. (1982) Systemic lupus erythematosus during penicillamine therapy for rheumatoid arthritis. Annals of Internal Medicine 97: 659-663

Chapel H., Haeney M. (1984) Essentials of clinical immunology. Blackwell Scientific Publications., Oxford

Chow S.T., McAullife C.A., Sayle B.J. (1973) Metal complexes of amino acids and derivatives-VIII: The reaction of DL-penicillamine with some transitional and non-transitional metals. J. Inorg. Nucl. Chem. 35: 4349-4354

Classen D.C., Pestotnik S.L., Evans R.S. (1991) Computerized surveillance of adverse drug-reaction is hospitalized patients in hospitalized patients. J.A.M.A. : 2847-2852

Coffman R.L., Chatelain R., Leal L.M.C., Varkila K. (1991) Leishmania major infection in mice: a model system for the study of CD4+ T-cell subset differentiation. Res. Immunol. 142: 36

Constant S.L., Bottomly K. (1997) Induction of Th1 and Th2 responses: The alternative approaches. Ann. Rev. Immunol. 15: 297-322

Cooke A., Wraith D.C. (1993) Immunotherapy of autoimmune disease. Curr. Opin. Immunol. 5: 925-933

Coombs R.R.A., Gell P.G.H. (1968) Classification of allergic reactions responsible for clinical hypersensitivity and disease. In: Coombs R.R.A., Gell P.G.H. (eds) Clinical aspects of immunology. 2nd edn. FA Davis, New York

Crawford L.J. (1997) COX-1 and COX-2 tissue expression: Implications and predictions. J. Rheumatol. 24: 15-19

Crawhall J.C., Scowen E.F., Watts R.W.E. (1963) Effects of penicillamine on cystinuria. Brit. Med. J. 1: 588

Dawkins R.L., Garlepp M., McDonald B., Williamson J. , Zilko P.J. (1981) Myasthenia gravis and D-penicillamine. J. Rheumatol. 8 (Suppl. 7): 169-172

DeClercq E. (1981) Interferon induction by polyncleotides, modified polynucleotides and polycarboxilates. Methods Enzymole 78: 227-235

Descotes, J. (1990) Drug-induced immune diseases. In: Dunkes, M. N. G. (ed.) Drug-induced disorders. Elsevier, New York, pp 14-15

Donker A.J., Rocco C.V., Vladutiu A.O., Brentjens J.R., Andres G.A. (1984) Effects of prolonged administration of D-penicillamine or captopril in various strains of rats. Clin. Immunol. Immunopathol. 30: 142-155

Druet E., Sapin C., Gnther E., Feingold N., Druet, P. (1977) Mercuric chloride-induced anti-glomerular basement membrane antibodies in the rats. Genetic control. Eur. J. Immunol. 7: 348-351.

Druet P., Pelletier L. (1996) Th2 and Th1 autoreactive anti-class II cell lines in the rat suppress or induce autoimmunity. J. Autoimmunity 9: 221-226

Druet P., Sheela R., Pelletier L. (1995) Th1 and Th2 cells in autoimmunity. Chem. Immunol. 63: 158

Drummer R., Routley L., Christophidis N. (1987) Reversibility of penicillamine disulfide formation in vitro. Clin. Exp. Physiol. Pharmac. Suppl. 10: 40-41

Enzenauer R.J., West S.G., Rubin R.L. (1990) D-penicillamine-induced lupus erythematosus. Arthritis Rheumatism 33: 1582-1585

Faggiani R., Howard-Lock H.E., Lock C.J.L., Martins M.L., P.S., S. (1984) Structural and spectroscopic studies of dipotassium 3,3,3',3'-tetramethylcystinate. Can. J. Chem. 62: 1127-1133

Fearon D.T., Locksley R.M. (1996) The instructive role of innate immunity in the acquired immune response. Science 272: 50-54

Fernandez L., Swinson D.R., Hamilton E.B.D. (1977) Dermatomyosis complicating penicillamine treatment. Annals of the Rheumatic Diseases 36: 94-95

Field A.K., Tytell A.A., Lampson G.P., Hilleman M.R. (1967) Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. Proc. Natl. Acad. Sci. 58: 1004-1010

Finkelman F.D., Katona I.M., Urban J.F., Snapper C.M., Ohara J., Paul W.E. (1986) Suppression of *in vivo* polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor-1. Proc. Natl. Acad. Sci. USA 83: 9675-9678

Finkelman F.D., Svetic A., Gresser I., Snapper C., Holmes J., Trotta P., Katona I.M., Gause W.C. (1991) Regulation by interferon-a of immunoglobulin isotype selection and lymphokine production in mice. J. Exp. Med. 174: 1179-1188

Forrester J., Golbus J., Brede D. (1988) B cell activation in patients with active procainamide-induced lupus. J. Rheumatol. 15: 1384

Freeman G.J., Boussiotis V.A., Anumanthan A. (1995) B7-1 and B7-2 do not deliver identical co-stimulatory signals, since B7-2 but not B7-1 preferentially co-stimulates the initial production of IL-4. Immunity 2: 523-532

Freeman H.C., Huo F., Stevens G.N. (1976) Metal binding by D-penicillamine: crystal structure of D-penicillaminatocadmium (II) hydrate. J. Chem. Soc. Chem. Commun. 1: 90-91

Funauchi M., Ikoma S., Enomoto H., Horiuchi A. (1998) Decreased Th1-like and increased Th2-like cells in systemic lupus erythematosus. Scand. J. Rheumatol. 27: 219-224

Gilbert K.M., Griffin J.M., Pumford N.R. (1999) Trichloroethylene activates CD4+ T cells: potential role in an autoimmune response. Drug Metab. Rev. 31: 901-916

Gilkeson G.S., Cannon C., Goldman D., Petri M. (1996) Correlation of a serum measure of nitric oxide production with lupus disease activity measures. Arthritis Rheum. 39: s251

Gleichman E., Vogeler S., Mirtschewa J. (1988) Possible pathogenic pathways of chemically induced autoimmunity: experimental studies in animals. In: Estabrook R.W., Lindenlaub E., Oesch F., Weck AL.de. (eds) Toxicological and immunological aspects of drug metabolism and environmental chemicals. Schattauer, Stuttgard, pp 419

Gold E.F., Ben-Fraim S., Faivesewitz A. (1977) Experimental studies on the mechanism of induction of anti-nuclear antibodies by procainamide. Clin. Immunol. Immunopathol. 7: 176

Goldberg A., Smith J.A., Lockhead A.C. (1963) Treatment of lead poisoning with oral penicillamine. Br. Med. J. 1: 1270-1275

Goldman M., Druet P., Gleichmann E. (1991) Th2 cells in systemic autoimmunity: insights from allogeneic diseases and chemically-induced autoimmunity. Immunol. Today 12: 223-227

Greenberger P.A., Patterson R. (1987) Management of drug allergy in patients with the acquired immunodeficiency syndrome. J. Allergy Clin. Immunol. 79: 484-488

Griffin J.M., Gilbert K.M., Pumford N.R. (2000) Inhibition of CYP2E1 reverses CD4+ T-cell alterations in trichloroethylene-treated MRL +/+ mice. Toxicol. Sci. 54: 384-389

Griffiths M.J.D., Messent M., MacAllister R.J., Evans T.W. (1993) Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br. J. Pharmacol. 110: 963

Groeneveld P.H., Kroon F.P., Nibbering P.H., Bruisten S.M., Van Swieten P., Van Furth R. (1996) Increased production of nitric oxide correlates with viral load and activation of mononuclear phagocytes in HIV-infected patients. Scand. J. Infect. Dis. 28: 341-345

Haas C., Ryffel B., Le-Hir M. (1997) IFN-gamma is essential for the development of autoimmune glumerulonephritis in MRL/lpr mice. J. Immunol. 158: 5484-5491

Harkcom T.M., Conn D.L., Holley K.E. (1978) D-Penicillamine and lupus erythematosus-like syndrome. Ann. Intern. Med. 89: 1012

Harpey J.P., Caille B., Molias R., Goust J.M. (1971) Lupus-like syndrome induced by penicillamine in Wilson's disease. Lancet 1: 292

Haynes D.R., Whitehouse M.W., Vernon-Roberts B. (1992) The prostaglandin E1 analog, misoprostol, regulates inflamatory cytokines and immune functions in vitro like the natural prostaglandins E1, E2 and E3. Immunol. 76: 251-257

Heddle J.G., McHenery E.W., Beaton G.H. (1963) penicillamine and vitamine B6 relationships in the rat. Can. J. Biochem. Physiol. 41: 1215-1222

Heitmeier M.R., Scarim A.L., Corbett J.A. (1998) Double-stranded RNA-induced inducible nitric oxide synthase expression and IL-1 release by murine macrophages requires NF-kB activation. J. Biol. Chem. 273: 15301-15307

Heitmeier M.R., Scarim A.L., Corbett J.A. (1999) Double-stranded RNA inhibits b-cell function and induces islet damage by stimulating b-cell production of nitric oxide. J. Biol. Chem. 274: 12531-12536

Hilkens C.M., Snijders A., Vermeulen H., Van-der Meide P. H., Wierenga E.A., Kapsenberg M.L. (1996) Accessory cell-derived IL-12 and prostaglandin E2 determine the IFN-gamma level of activated human CD4+ T cells. J. Immunol. 156: 1722-1727

Hill H.F.H. (1977) Treatment of rheumatoid arthritis with penicillamine. Semin. Arth. Rheum. 6: 361-388

Horwitz D.A., Gray J.D., Behrendsen S.C., Kubin M., Rengaraju M., Ohtsuka K., Trinchieri G. (1998) Decreased production of interleukin-12 and other Th1-type cytokines in patients with recent-onset systemic lupus erythematosus. Arthritis Rheum. 41: 838-844

Howard-Lock H. E., L., L. C. J., Smalley P. S. (1985) (S)-2,2,5,5-Tetramethylthiazolidine-4-carboxylic acid: a compound which exists in the amino acid rather than the zwitterionic form. Can. J. Chem. 63: 2411-2419

Howard-Lock H.E., Lock C.J., Mewa A., Kean W.F. (1986) D-penicillamine: chemistry and clinical use in rheumatoid disease. Seminar in Arthritis and Rheumatism 15: 261-281

Hsieh C. S., Macatonia S. E., Tripp C. S., Wolf S. F., O'Garra A., Murphy K. M. (1993) Development of TH1 CD4+ T cells through IL-12 produced by Listeria- induced macrophages. Science 260: 547-549

Huang F.P., Feng G.J., Lindop G., Stott D.I., Liew F.Y. (1996) The role of IL-12 and Nitric Oxide in the development of spontaneous autoimmune disease in MRL/MP-lpr/lpr Mice. J. Exp. Med. 183: 1447-1459

Hultman P., Enestrom S. (1987) The induction of immune complex deposits in mice by peroral and parenteral administration of mercuric chloride: Strain dependent susceptibility. Clin. Exp. Immunol. 67: 283-292.

Hultman P., Enestrom S. (1988) Mercury-induced antinuclear antibodies in mice: Characterization and correlation with renal immune complex deposits. Clin. Exp. Immunol. 71: 269-274.

Hutloff A., Dittrich A.M., Beier K.C. (1999) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 397: 263-266

Jaffe I. A. (1979) Penicillamine in rheumatoid arthritis: clinical pharmacology and biochemical properties. Scan. J. Rheumatol. Suppl. 28: 58-64

Jaffe I.A. (1981) Induction of auto-immune syndromes by penicillamine therapy in rheumatoid arthritis and other diseases. Springer Semin. Immunopathol. 4: 193-207

Janeway C, A. J. (1992) The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol. Today 13: 11-16

Janeway C.A. Jr. (1989) Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb. Symp. Quant. Biol. 54: 11-13

Jiang C., Ting A.T., Seed B. (1998) PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391: 82-84

Jick H. (1984) Adverse drug reactions: the magnitude of the problem. J. Allergy Clin. Immunol. 74: 555-557

Johsi B.C., Dwivedi C., Powell A., Holscher M. (1981) Immune complex nephritis in rats induced by long-term exposure to cadmium. J. Comp. Path. 91: 11-15.

Joyce D.A. (1989) D-penicillamine pharmacokinetics and pharmacodynamics in man. Pharmacology and Therapeutics 42: 405-427

Kang B., Veres-Thorner C., Heredia R., Cha E., Bose S., Schwartz M. (1982) Successful treatment of advanced progressive systemic sclerosis by D-penicillamine. J. Allergy Clin. Immun. 69: 297-305

Kashem A., Endoh M., Yano N., Yamauchi F., Nomoto Y., Sakai H. (1996) Expression of inducible-NOS in human glomerulonephritis: The possible source is infiltrating monocytes/macrophages. Kidney Int. 50: 392-399

Kean W.F., Anastassiades T.P., Dwosh I.L., Ford P.M. , Kelly W.G. (1982) Efficacy and toxicity of D-penicillamine for rheumatoid disease in the elderly. J. Am. Geriat. Soc. 30: 94-100

Kelley V.E., Sneve S., Musinski S. (1986) Increased renal thromboxane production in murine lupus nephritis. J. Clin. Invest. 77: 252-259

Khan M.F., Kaphalia B.S., Prabhakar B.S., Kanz M.F., Ansari G.A. (1995) Trichloroethene-induced autoimmune response in female MRL +/+ mice. Toxicol. Appl. Pharmacol. 134: 155-160

Khoury S.J., Akalin E., Chandraker A., Turka L.A., Linsley P.S., Sayegh M.H., Hancock W.W. (1995) CD28-B7 costimulatory blockade by CTLA-Ig prevents actively induced experimental autoimmune encephalomyelitis and inhibits Th1 but spares Th2 cytokines in the central nervous system. J. Immunol. 155: 4521-4524

Kim H.S., Lee J.H., Kim I.K. (1996) Intracellular glutathion level modulates the induction of apoptosis by PG-J2. Prostaglandins 51: 413-420

Kirtland H.H., Mohler D.N., Horowitz D.A. (1980) Methyldopa inhibition of suppressor-lymphocyte function. N. Engl. J. Med. 302: 825

Kolb H., Kolb B.V. (1992) Nitric oxide a pathogenetic factor in autoimmunity. Immuno. Today 13: 157-160

Kolb W.P., Lang J.H., Lasse E.C. (1978) Nonimmunologic complement activation in normal human serum induced by radiographic contrast media. J. Immunol. 121: 1232

Kukovetz W.R., Beubler E., Kreuzig F., Moritz A.J., Nirnberger G., Werner-Breitenecker L. (1983) Bioavailability and pharmacokinetics of D-penicillamine. J. Rheumatology 10: 90-94

Levy M. (1997) Role of viral infections in the induction of adverse drug reactions. Drug Safety 16: 1-8

Light N., Meyrick T. R.H., Stephens A., Kirby J.D.T., Fryer P.R. , Avery N.C. (1986) Collagen and elastin changes in D-penicillamine-induced pseudoxanthoma elasticum-like skin. Br. J. Derm. 114: 381-388

Liu T.F., Jones B.M. (1998) Impaired production of IL-12 in systemic lupus erythematosus. II. Production in vitro is correlated negatively with serum IL-10, positively with serum IFN-g and negatively with disease activity in SLE. Cytokine 10: 148-153

Lohse A.W., Cohen I.R. (1990) Mechanisms of resistance to autoimmune disease induced by T cell vaccination. In: Demaine, A. G., Banga, J. P., McGregor, A. M. (eds) The molecular biology of autoimmune disease. Springer-Verlag, Berlin, pp 333

Luppi P., Rossiello M.R., Faas S., Trucco M. (1995) Genetic background and environment contribute synergistically to the onset of autoimmune diseases. J. Mol. Med. 73: 381-393

Machold K.P., Smolen J.S. (1990) Interferon-gamma induced exacerbation of systemic lupus erythematosus. J. Rheumatol. 17: 831-832

Magram J., Connaughton S.E., Warrier R.R., Carvajal D.M., Wu C. Y., Ferrante J., Stewart C., Sarmiento U., Faherty D.A., Gately M.K. (1996) IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity 4: 471-81

Malinow M.R., Bardana E.J., Pirofsky B., Craig S. (1982) SLE-like syndrome in monkeys fed alfalfa sprouts: Role of a nonprotein amino acid. Science 216: 415-417.

Manetti R., Annunziato F., Tomasevic L., Gianno V., Parronchi P., Romagnani S., Maggi E. (1995) Poly I:C promotes T helper type 1-specific immune responses by stimulating macrophage production of interferon-a and interleukin-12. Eur. J. Immunol. 25: 2656-2660

Matzinger P. (1994) Tolerance, danger and the extended family. Annu. Rev. Immunol. 12: 991-1045

Medzhitov R., Janeway C.A. Jr. (1997) Innate immunity: impact on the adaptive immune response. Curr. Opin. Immunol. 9: 4-9

Miesel R., Zuber M. (1993) Reactive nitrogen intermediate, antinuclear antibodies and copper theonein in serum of patients with rheumatic diseases. Rheum. Int. 12: 95-102

Moncada S., Higgs A.N. (1993) The L-arginine-nitric oxide pathway. N. Engl. J. Med. 329: 2002-2012

Morel P.A., Oriss T.B. (1998) Crossregulation between Th1 and Th2 cells. Crit. Rev. Immunol. 18: 275-303

Mosmann T.R., Cherwinski H., Bond M.W. (1986) Two types of murine helper T cell clone: I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136: 2348

Mosmann T.R., Sad S. (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today 17: 138-146

Nagasawa H.T., Goon D.J., De Master E.G. (1978) 2,5,5,-Trimethylthiazolidine-carboxylic acid, a D-(-)-directed pseudometabolite of ethanol. Detoxication method of acetaldehyde. J. Med. Chem. 21: 1274-1279

Nakamura T., Lee R.K., Nam S.Y., Podack E.R., Bottomly K. (1997) Roles of IL-4 and IFN-gamma in stabilizing the T helper cell type 1 and 2 phenotype. J. Immunol. 158: 2648-2653

Nicolson B.N., Kuchroo V. (1996) Manipulation of the Th1/Th2 balance in autoimmune disease. Curr. Opin. Immunol. 8: 837-842

Nimni M.E. (1977) Mechanism of inhibition of collagen crosslinking by D-penicillamine. Proc. R. Soc. Med. 70: 65-72

Ochel M., Vohr H.W., Pfeiffer C., Gleichmann E. (1991) IL-4 required for the IgE and IgG1 increase and IgG1 autoantibody formation in mice treated with mercuric chloride. J. Immunol. 146: 3006-3011

Ohsawa M., Takahashi K., Otsuka F. (1988) Induction of antinuclear antibodies in mice orally exposed to cadmium at low concentrations. Clin. Exp. Immunol. 73

Oliver I., Liberman U.A., De Vries A. (1972) A lupus-like syndrome induced by penicillamine in cystinuria. Journal of the American Medical Association 220: 558

Oriss T.B., McCarthy S.A., Campana M.A., Morel P.A. (1999) Evidence of positive cross-regulation on Th1 by Th2 and antigen-presenting cells: effects on Th1 induced by IL-4 and IL-12. J. Immunol. 162: 1999-2007

Osman M.A., Patel R.B., Schuma A., Sundstrom W.R., Welling P.G. (1983) Reduction in oral penicillamine absorption by food, antacid and ferrous sulphate. Clinical Pharmacology and Therapeutics 33: 465-470

Ostensen M., Husby G., Aarli J. (1980) Polymyositis with acute myolysis in a patient with rheumatoid arthritis treated with penicillamine and ampicillin. Arthritis and Rheumatism 23: 375-377

Park B. K., Coleman J. W., Kitteringham N. R. (1987) Drug disposition and drug hypersensitivity. Biochem. Pharmacol. 36: 581-590

Park B. K., Pirmohamed M., Kitteringham N. R. (1998) Role of drug disposition in drug hypersensitivity: A chemical, molecular, and clinical perspective. Chem Res Toxicol 11: 969-88

Park B.K. (1986) Metabolic basis of adverse drug reactions. J. Roy. Coll. Phys. 20: 195-200

Park B.K., Coleman J.W., Kitteringham N.R. (1987) Drug disposition and drug hypersensitivity. Biochem. Pharmacol. 36: 581-590

Parker C.W. (1975) Drug allergy. N. Engl. J. Med. 292: 957

Patrono C., Ciabrattoni G., Remuzzi G., Gotti E., Bombardieri S., Munno O.D., Tartarelli G., Cinotti G.A., Simmonetti B.M., Pierucci A. (1985) Functional significance of renal prostacyclin and thromboxane A2 production in patients with systemic renal lupus erythematous. J. Clin. Invest. 76: 1011-1017

Paul W.E., Seder R.A. (1994) Lymphocyte responses and cytokines. Cell 76: 241-251

Pelletier L., Pasquier R., Guettier C., Vial M.C., Mandet C., Nochy D., Bazin H., Druet P. (1988) Mercuric chloride induces T and B cells to proliferate and differentiate in BN rats. Clin. Exp. Immunol. 71: 336-342

Peng S. L., Moslehi J., Craft J. (1997) Roles of interferon-gamma and interleukin-4 in murine lupus. J Clin Invest 99: 1936-46

Perrett D., Sneddon W., Stephens A.D. (1976) Studies of D-penicillamine metabolism in systinuria and rheumatoid arthritis: Isolation of S-methyl-D-penicillamine. Biochem. Pharmac. 25: 259-264

Pietsch P., Vohr H.W., Degits K., Gleichmann E. (1989) Immunopathological signs inducible by mercury compounds. II. Mercuric chloride and gold sodium thiomalate enhance serum IgE and IgG concentrations in susceptible mouse strains. Int. Arch. Alergy. Appl. Immunol. 90: 47-53.

Pirmohamed M., Breckenridge A.M., Kitteringham N.R., Kevin Park B. (1998) Adverse drug reactions. B. M.J. 316: 1295-1298

Pohl L.R., Satoh H., Christ D.D., Kenna J.G. (1988) Immunologic and metabolic basis of drug hypersensitivities. Ann. Rev. Pharmac. 28: 367-387

Price E.J., Venables P.J. (1995) Drug-induced lupus. Drug Saf. 12: 283-290

Prouvost-Danon A., Abadie A., Sapin C., Bazin H., Druet P. (1981) Induction of IgE synthesis and potentiation of anti-ovalbumin IgE response by mercuric chloride in the rat. J. Immunol. 126: 699-702.

Qasim F. J., Thiru S., Gillespie K. (1997) Gold and penicillamine induce vasculitis and up-regulate mRNA for IL-4 in the BN rats. Clin. Exp. Immunol. 108: 438-445

Reder A.T., Thapar M., Sapugay A.M., Jensen M.A. (1994) Prostaglandins and inhibitors of arachidonate metabolism suppress Experimental Allergic Encephalomyelitis. J. Neuroimmunol. 54: 117

Reilly C.M., Oates J.C., Cook J.A., Morrow J.D., Halushka P.V. Gilkeson G.S. (2000) Inhibition of mesangial cell nitric oxide in MRL/lpr mice by prostaglandin J2 and proliferator activation receptor-gamma agonists. J. Immun. 164: 1498-1504

Reiner S.L. (2001) Helper T cell differentiation, inside and out. Curr. Opin. Immunol. 13: 351-355

Revel M. (1984) Anti-viral drugs and interferon: The molecular basis of their activity. Martinus Nijhoff, New York

Richardson B., Scheinbart L., Strahler J., Gross L., Hanash S., Johnson M. (1990) Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis. Arthritis Rheum. 33: 1665-1673

Richens A. (1994) Safety of Lamotrigine. Epilepsia 35: S37-S40

Ricote M., Li A.C., Willson T.M., Kelly C.J., Glass C.K. (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 391: 79-82

Rincon M., Flavell R.A. (1997) T-cell subsets: transcriptional control in the Th1/Th2 decision. Curr. Biol. 7: R729-732

Robinson C.J.G., Balazs T., Egorov I.K. (1986) Mercuric chloride, gold sodium thiomalate and penicillamine-induced antinuclear antibodies in mice. Clin. Exp. Immunol. 58: 300-306.

Rocken M., Saurat J.H., Hauser C. (1992) A common precursor for CD4+ T cells producing IL-2 or IL-4. J. Immunol. 148: 1031-1036

Rogers P.R., Croft M. (1999) Peptide dose, affinity and time of differentiation can contribute to the Th1/Th2 balance. J. Immunol. 163: 1205-1213

Rogers P.R., Croft M. (2000) CD28, Ox-40, LFA-1 and CD4 modulation of Th1/Th2 differentiation is directly dependent on the dose of antigen. J. Immunol. 164: 2955-2963

Rogge L., Barberis-Maino L., Biffi M., Passini N., Presky D. H., Gubler U., Sinigaglia F. (1997) Selective expression of an interleukin-12 receptor component by human T helper 1 cells. J Exp Med 185: 825-831

Romagnani S. (1990) Regulation and deregulation of human IgE synthesis. Immunol. Today 11: 316-321

Romagnani S. (1994) Lymphokine production by human T cells in disease states. Annu. Rev. Immunol. 12: 227-257

Roman-Franco, A., Turiello, A., Albini, M., Ossi, E., Milgrom, F., Andres, G. A. (1978) Anti-basement membrane antibodies and antigen-antibody complexes in rabbits injected with mercuric chloride. Clin. Immunol. Immunopathol. 9: 464-481.

Rose N., Machay I. (1985) Autoimmune diseases. Academic Press, New York

Routledge P.A., Chapman P.H., Davies D.M., Rawlins M.D. (1979) Factors affecting warfarin requirements. Eur. J. Clin. Pharmacol. 15: 319

Rulifson I.C., Sperling A.I., Fields P.E., Fitch F.W., Bluestone J.A. (1997) CD28 co-stimulation promotes the production of Th2 cytokines. J. Immunol. 158: 658-665

Ruocco V., DE Angelis E., DE Luca M., Pisani M., Prota G. (1983) Specific incorporation of penicillamine into the epidermis of mice: an autoradiographic study. Br. J. Derm. 108: 441-444

Russell A.S., Saetre R., Davis P., Rabenstein D.L. (1979) A rapid sensitive technique to assay penicillamine in blood and urine. Journal of Rheumatology 6: 15-19

Russell M.E., Hancock W.W., Akalin E., Wallace A.F., Glysing-Jensen T., Willett T.A. (1996) Chronic cardiac ejection in the LEW to F344 rat model. Blockade of CD28-B7 costimulation by CTLA-4 Ig modulates T cell and macrophage activation and attenuates arteriosclerosis. J. Clin. Invest. 97: 833-838

Safferman A.Z., Liberman A.J., Alvir J.M., Howard A. (1992) Rechallenge in clozapine-induced agrnulocytosis. Lancet 339: 1296-1297

Saoudi A., Kuhn J., Huygen K., Kozak Y., Velu T., Goldman M., Druet P., Bellon B. (1993) Th2 activated cells prevent experimental autoimmune uveoretinitis, a Th1-dependent autoimmune disease. Eur. J. Immunol. 23: 3096-3103

Sapin C., Druet E., Druet P. (1977) Induction of anti-glomerular basement membrane antibodies in the Brown Norway rat by mercuric chloride. Clin. Exp. Immunol. 28: 173-178

Sapin C., Hirsch F., Delaporte J., Bazin H., Druet P. (1984) Polyclonal IgE increase after HgCl2 injections in BN and LEW rats: a genetic analysis. Immunogenetics 20: 227-236

Schorlemmer H.U., Brendel S., Bartlett R.R. (1996) Malononitrilamides prevent the development of Murine systemic lupus erythematosus-like disease in BDF1 hybrid mice and MRL/lpr autoimmune mice. Transplantation Proceedings 28: 3040-3042

Schuna A.A., Megeff C. (2000) New drugs for the treatment of rheumatoid arthritis. Am. J. Health Syst. Pharm. 57: 225-234

Sher A., Coffman R.L. (1992) Regulation of immunity to parasites and T-cell derived cytokines. Annu. Rev. Immunol. 10: 385-409

Shu U., Kiniwa M., Wu C. (1995) Activated T cells induce IL-12 production by monocytes via CD40-CD40 ligand interaction. Eur. J. Immunol. 25: 1125-1128

Sim E., Gill E.W., Sim R.B. (1985) Drugs that induce systemic lupus erythematosus inhibit complement component C4. Lancet ii: 422

Sim E., Law S.A. (1985) Hydralazine binds covalently to complement component C4: different reactivity of C4A and C4B gene products. FEBS. Lett. 184: 323

Sim E., Stanley L., Gill E.W., Jones A. (1988) Metabolites of procainamide and practolol inhibit complement C3 and C4. Biochem. J. 251: 323

Situnayake R.D., Grindulis K.A., B, M. (1987) Longterm treatment of rheumatoid arthritis with sulphasalazine, gold and penicillamine: a comparison using life table methods. Annals of the Rheumatic Diseases 46: 177-183

Sobel D.O., Goyal D., Ahvazi B., Yoon J.W., Chung Y.H., Bagg A., Harlan D.M. (1998) Low dose poly I:C prevents diabetes in the diabetes prone BB rat. J Autoimmun 11: 343-352

Spurney R.F., Fan P.Y., Ruiz P., Sanfilippo F., Pisetsky D.S., Coffman T.M. (1992) Thromboxane receptor blockade reduces renal injury in murine lupus nephritis. Kidney Int. 41: 973-978

Stein H.B., Patterson A.C., Offer R.C., Atkins C.J., Teufel A. (1980) Adverse effects of D-penicillamine in rheumatoid arthritis. Annals of Internal Medicine 92: 24-29

Stiles D.P., Stobo J.D., Fudenberg H.H., Wells J.V. (1990) Basic and clinical immunology. Appleton and Lange

Strassmann G., Patil-Koota V., Kinkelman F., Fong M., Kambayashi T. (1994) Evidence for the involvement of IL-10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. J. Exp. Med. 180: 2365-2372

Tabachnick M., Eisen H.N., Levine B. (1954) A new mixed disulphide: penicillamine-cysteine. Nature 174: 701-702

Takahashi S., Fossati L., Iwamoto M., Merino R., Motta R., Kobayakawa T., Izui S. (1996) Imbalance towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. J Clin Invest 97: 1597-1604

Tan E.M. (1974) Drug-induced autoimmune disease. Fed. Proc. 33: 1894

Taylor H.G., Samanta A. (1992) Penicillamine in Rheumatoid Arthritis. Drug Safety 7: 46-53

Ten-Ven J.H., Feltkamp T.E.W. (1972) Studies on drug-induced lupus erythematosus. I. Drug-induced antinuclear antibodies. Clin. Exp. Immunol. 11: 265-276.

Tetsuka T., Daphna-Iken D., Srivastava S.K., Baier L.D., DuMaine J., Morrison A.R. (1994) Cross-talk between cyclooxygenase and nitric oxide pathways: prostaglandin E2 negatively modulates induction of nitric oxide synthase by IL-1. Proc. Natl. Acad. Sci. 91: 12168-12175

Thompson H.S.G., Staines N.A. (1991) Could specific oral tolerance be a therapy for autoimmune disease? Immunol. Today 11: 396403

Tornade H., Pelletier L., Pasquier R., Vial M.C., Mandet C., Druet P. (1990) D-penicillamine-induced autoimmunity in Brown-Norway rats: Similarities with HgCl2-induced autoimmunity. J. Immunol. 144: 2985-2991

Torre D., Ferrario G. (1996) Immunological aspects of nitric oxide in HIV-1 infection. Med. Hypotheses 47: 405-407

Ueda S., Wakashin M., Wakashin Y., Yoshida H., Iesato K., Mori T., MoriY., Akikusa B., Okuda K. (1986) Experimental gold nephropathy in guinea pigs: Detection of autoantibodies to renal tubular antigens. Kidney Int. 29: 539-548.

Uetrecht J.P. (1988) Mechanism of drug-induced lupus. Chem. Res. Toxicol. 1: 133

Uetrecht J.P. (1990) Drug metabolism by leukocytes and its role in drug-induced lupus and other idiosyncratic drug reactions. Critic. Rev. Tox. 20: 213-235

Uetrecht, J. P. (1997) Current trends in drug-induced autoimmunity. Toxicology 119: 37-43

Van-der Pouw Kraan, Boeije L.C.M., Smeenk R.J.T., Wijdenes J., Aarden L.A. (1995) PGE2 is a potent inhibitor of human IL-12 production. J. Exp. Med. 181: 775-779

Vander-Meide, P. H., De Labie M.C., Botman C.A.D., Aten J., Weening J.J. (1995) Nitric oxide suppresses IFN-g production in the spleen of Mercuric chloride-exposed Brown Norway rats. Cell. Immunol. 161: 195-206

Walshe J.M. (1956) penicillamine: a new oral therapy for Wilson's disease. Amer. J. Med. 21: 487

Walshe J.M. (1981) penicillamine and the SLE syndrome. J. Rheumatol. 8 (Suppl. 7): 155-160

Wandl U.B., Nagel-Hiemke M., May D., Kreuzfelder E., Kloke O., Kranzhoff M., Seeber S., Niederle N. (1992) Lupus-like autoimmune disease induced by interferon therapy for myeloproliferative disorders. Clin. Immunol. Immunopathol. 65: 70-74

Weatherall D.J., Ledingham J.G., Warrell D.A. (1987) Oxford Textbook of Medicine. Oxford University Press, Oxford

Weigert W.M., Offermanns H., Scherberich P. (1975) D-penicillamine production and properties. Angew. Chem. Int. Edn. 14: 330-336

Weigle W.O., Romball C.G. (1997) CD4+ T cell subsets and cytokines involved in peripheral tolerance. Immunol. Today 18: 533-538

Wenner C.A., Guler M.L., Macatonia S.E., O'Garra A., Murphy K.M. (1996) Roles of IFN-g and IFN-a in IL-12-induced Th1 development. J. Immunol. 156: 1442-1447

Wiesner R.H., Dickson E.R., Carlson G.R., McPhaul L.W., Go V L.W. (1981) The pharmacokinetics of D-penicillamine in man. Journal of Rheumatology 8 (Suppl. 8): 51-55

Woolfson R.G., Qasim F.J., Thiru S., Oliveira D.B.G., Neild G.H., Mathieson P.W. (1995) Nitric oxide contributes to tissue injury in mercuric chloride-induced autoimmunity. Biochem. Biophy. Res. Communic. 217: 515-521.

Wu C. Y., Wang K., McDyer J. F., Seder R. A. (1998) Prostaglandin E2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. J Immunol 161: 2723-2730

Yoshida T., Kameda H., Ichikawa Y., Tojo T., Homma M. (1996) Improvement of renal function with a selective thromboxane A2 synthetase inhibitor, DP-1904, in lupus nephritis. J. Rheum. 23: 1719-1725

Yung R.L., Richardson B.C. (1994) Role of T cell DNA methylation in lupus syndromes. Lupus 3: 487-491

Zbinden G. (1980) Predictive value of pre-clinical drug evaluation. In: Turner, P. (ed.) Proceedings of the first world conference on clinical pharmacology and therapeutics. Macmillan, London, pp 9

Zeng L., An S., Goetzl E. J. (1998) EP4/EP2 receptor-specific prostaglandin E2 regulation of interleukin-6 generation by human HSB.2 early T cells. J Pharmacol Exp Ther 286: 1420-1426

Zhang Y., Apilado R., Coleman J., Ben-Sasson S., Tsang S., Hu-Li J., Paul W.E., Huang H. (2001) Interferon gamma stabilizes the T helper cell type 1 phenotype. J. Exp. Med. 194: 165-172

zimmerman H.G. (1978) The adverse effects of drugs and other chemicals on the liver. Appleton-Century Crofts, New York

Appendix

Rat strain	group number	penicillamine dose	poly I:C dose	number of rats / group	cumulative incidence (%) at week 10
Lewis	1	20 mg/day	10 mg/kg	10 males	0
	2	20 mg/kg	0	10 males	0
	3	0	10 mg/kg	4 males	0
	4	0	0	4 males	0

Appendix-Table 1.

Groups of Lewis rats were treated with penicillamine alone (group 2) or a single dose of poly I:C followed by penicillamine (group 1) for 10 weeks. The total number of rats in each group that demonstrated the clinical signs of the lupus were enumerated.

Rat strain	group number	penicillamine dose	poly I:C dose	number of rats / group	cumulative incidence (%) at week 8
BN	1	10 mg/day	10 mg/kg	10 males	0
	2	5 mg/kg	10 mg/kg	10 males	0
	3	0	10 mg/kg	4 males	0
	4	0	0	4 males	0
	5	10 mg/day	0	5 males	0
	6	5 mg/day	0	5 males	0

Appendix-Table 2.

Groups of BN rats were treated with penicillamine alone (groups 5 & 6) or a single dose of poly I:C followed by penicillamine (groups 1 & 2) for 8 weeks. The total number of rats in each group that demonstrated the clinical signs of the lupus were enumerated.

Summary of findings in liver histology studies

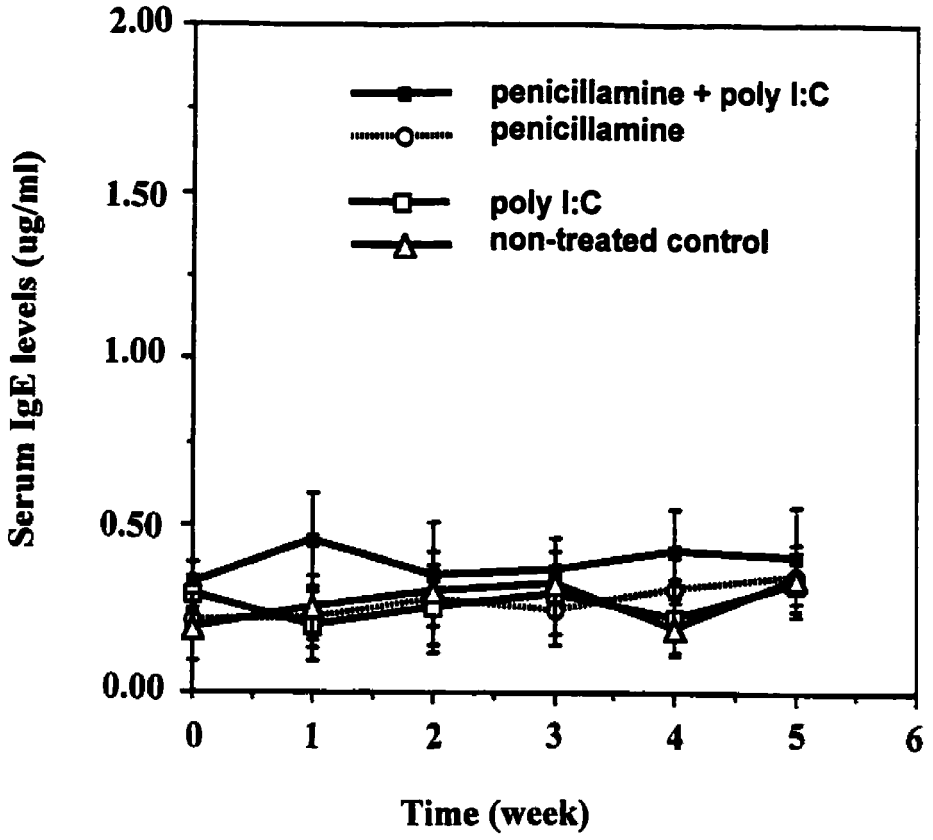
Treatment	Description of liver	Sinusoidal infiltrate	Portal eosinophils and neutrophils	Portal plasma cells	Granuloma (#/slice)
Control	Rare focal collection (0-1/slice)	0	0	0	0
Misoprostol + penicillamine	Occasional focal necrosis	0	0	0	0
Penicillamine without lupus	Occasional portal neutrophils and sinusoidal cells	1	1	0	0
Penicillamine with lupus	Mild mixed sinusoidal and portal infiltrate, no clusters of cells in sinusoids	2	1	1	0-1
Poly I:C + penicillamine	Heavy sinusoidal infiltrate (PC, L, PMN,Eos) forming numerous small clusters, focal granulomatous necrosis (0.5 – 2 mm)	4	3	3	4-11

Appendix-Table 3.

Livers were removed from animals from each treatment group when they were sacrificed, sliced and fixed with 10% buffered formalin.

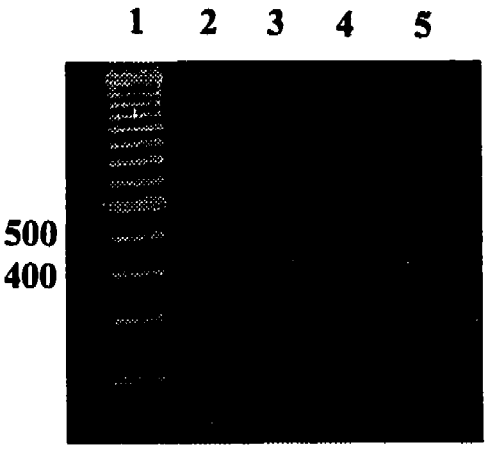
Embedded sections were stained with hematoxylin/eosin and studied under a light microscope.

Abbreviations used: PC, plasma cells; L, lymphocytes; PMN, polymorphonuclear cells; Eos, eosinophils.



Appendix-Figure 1.

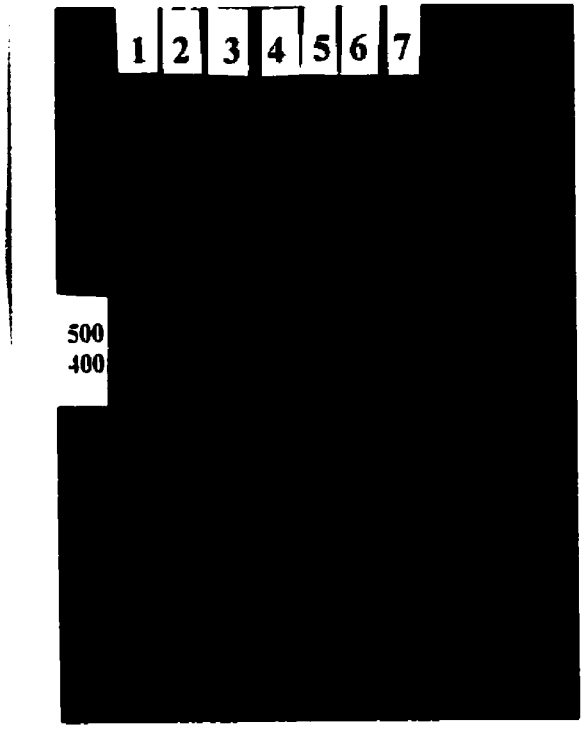
Effect of pre-treatment of 6 Lewis rats with 10 mg/kg of poly I:C on the serum IgE levels before treating rats with 20 mg/day of penicillamine for 5 weeks. Serum IgE level was analyzed by an immunoassay method (described in chapter 2 of this thesis). Results are presented as μg of IgE/ml based on the value obtained for the rat IgE standard.



Appendix- Figure 2.

Photograph of a typical gel demonstrating polymerase chain reaction products of IL-4 and IFN-γ reverse transcriptase (RT-PCR). Lanes 2 and 3 show cytokine mRNA expression from a spleen from an untreated control BN rat; lanes 4 and 5 show cytokine mRNA expression from a spleen from a poly- I:C + penicillamine-treated BN rat. Lane 1 size markers.

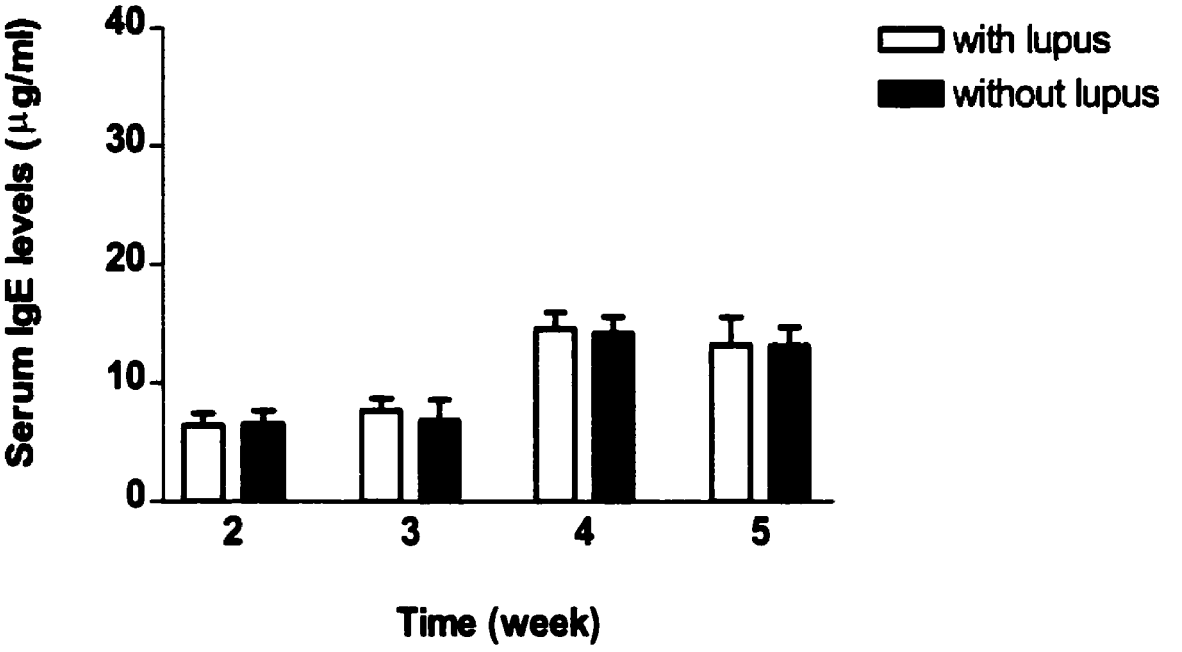
Samples of total cellular RNA extracted from spleen were reverse transcribed into cDNA and amplified by PCR as described in Materials and Methods (Chapter 2 of this thesis). The PCR products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining. Lanes 2 and 4 represent IL-4 mRNA expression, lanes 3 and 5 represent IFN-γ expression.



Appendix- Figure 3.

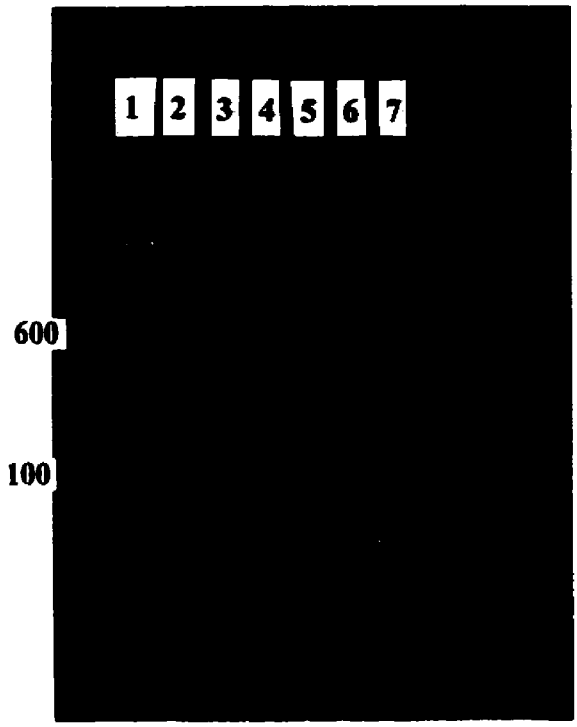
Photograph of a typical gel demonstrating polymerase chain reaction products of IL-4 and IFN-γ reverse transcriptase (RT-PCR). Lanes 2 to 7 show cytokine mRNA expression from 3 spleens from 3 penicillamine-treated Lewis rats. Lane 1 size markers.

Samples of total cellular RNA extracted from spleen were reverse transcribed into cDNA and amplified by PCR as described in Materials and Methods (Chapter 2 of this thesis). The PCR products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining. Lanes 2, 4 and 6 represent IL-4 mRNA expression, lanes 3, 5 and 7 represent IFN-γ expression.



Appendix- Figure 4.

Comparing serum IgE levels between a group of BN rats that developed the clinical manifestations of the lupus (n=6), and rats that did not develop the lupus (n=6) after being treated with 20 mg/day of penicillamine for 8 weeks. Total serum IgE levels were determined weekly by an ELISA method. The data shown represent the mean +/- SD of the serum IgE levels of rats in each group at each time point.



Appendix- Figure 5.

Photograph of a gel demonstrating polymerase chain reaction products of IL-4 and IFN-γ reverse transcriptase (RT-PCR). Lane 1 contains size markers. Lanes 2 and 3 show cytokine mRNA expression from a spleen from a penicillamine-treated BN rat that did not develop lupus.

Samples of total cellular RNA extracted from spleen were reverse transcribed into cDNA and amplified by PCR as described in chapter 2 of this thesis. The PCR products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide staining. Lane 2 represents IL-4 mRNA expression. lane 3 represents IFN-γ expression. Lane 7 represents β-actin mRNA expression. Lanes 4-6 empty.