THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

BY

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The data on drug toxicology which had been inadvertently left out of the original thesis on a few instances are now included. To facilitate searching for the data, a guide is provided below:

Drug	page/line in vitro	in vivo
Tetracyclines	74/8	80/1
Miconazole	91/8	93/13
Amphotericin B	117/1	107/5
Quinine	130/1	140/1
Primaquine	130/14	140/15
Mefloquine	134/16	140/15
Pyrimethamine	152/5	156/11

In general, the concentrations of these drugs chosen for in-vitro experiments were based on therapeutic blood levels that can be reached using conventional dosage schedules.

With regard to dosages in mice, their small size and much higher metabolic rate requires a much higher dosage on a per kg body weight basis compared to adult humans. As a general guidance, the dosages were those generally effective for treatment of experimental infections in mice.

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STATEMENT OF AUTHENTICITY

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

Y.H. Thong.

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ACKNOWLEDGEMENTS

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SUMMARY

Very little is known about the immunopharmacology of antimicrobial drugs. There are few reports in the scientific literature, and no mention of this subject can be found in recent editions of standard pharmacology textbooks. It is important to understand the immunopharmacology of antimicrobial drugs because immunomodulation by these drugs can affect the outcome of the infection being treated. Recovery from serious infections generally requires the participation of a reasonably intact immune system, even when appropriate and potent antimicrobial drugs have been prescribed. In such clinical situations, suppression of immunity can be detrimental to recovery while enhancement of immunity can contribute to recovery. This consideration is even more critical when the patients concerned are already immunocompromised by cancer or other debilitating diseases, or by anti-cancer or immunosuppressive therapy.

A systematic approach to the testing for the immunopharmacological properties of antimicrobial drugs was adopted in the present studies. The results of an in-vitro screening programme showed that the tetracyclines, miconazole, amphotericin B, the antimalarials and diaminopyrimidines have an effect on mitogen-induced human lymphocyte proliferative responses, whereas the penicillins, macrolides, aminoglycosides, cephalosporins and sulphonamides have little or no effect. In-vivo studies confirmed the immunosuppressive potential of tetracyclines, miconazole and amphotericin B, but not the antimalarials.

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Of particular interest was the discovery that pyrimethamine has immunopotentiating properties. There is current interest in such agents because of the potential for use in immunotherapy of cancer and other diseases where the immunological responses are inadequate, as well as their potential for use as probes to study immunological processes.

The results of the present studies suggest that caution should be exercised in selecting antimicrobial drugs for clinical use, especially in immunocompromised patients. The extensive and indiscriminate use of antimicrobial drugs by the medical community should be discouraged,not only because of toxic and allergic complications, the induction of resistant strains of micro-organisms, and the financial burden on the community and individual, but also because the immunosuppressive potential of some antimicrobial drugs can predispose to susceptibility to infection, birth defects, cancer, ageing and cardiovascular diseases, although the last three possibilities have not yet been realized, largely because epidemiological studies to date have not addressed themselves directly to the problem.

Further studies are required along a broad front in order to understand better the immunopharmacology of antimicrobial drugs, their sites and mechanisms of action, their interactions with each other and other classes of drugs, their clinical effects especially in complex clinical situations, and their long term complications.

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CHAPTER I

INTRODUCTION

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GENERAL INTRODUCTION

Infectious disease is one of the principal afflictions of mankind. Great epidemics of the past have wiped out whole populations. The era of modern medicine can be traced to the development of means for the control and treatment of infectious disease. Even before the germ theory of disease became generally accepted in the time (the 1880s) of Koch and Pasteur, ancient physicians had made the important observation that recovery from infectious disease conferred protection to subsequent infections. The Chinese physicians of the eleventh century were deliberately innoculating smallpox crusts into nostrils of patients to induce protective immunity. A systematic approach to the problem by Jenner and Pasteur led to the general adoption of immunization programmes. Antimicrobial chemotherapy became possible with the discovery of sulphonamides in the 1930's and antibiotics in the 1940's. Further contributions to control of infectious disease were provided by improvements in hygiene and public health. Except for occasional epidemics of influenza and cholera, and the special conditions produced by war, famine and tropical environments, the problem of infectious disease is now confined to susceptible individuals. They are the very young and very old, the ones with cancer or other debilitating illnesses, or those immunocompromised by intercurrent infection or immunosuppressive therapy.

These categories of patients represent a difficult therapeutic challenge for physicians involved in their care. Recovery from serious infections generally requires the participation of a reasonably

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intact immune system, even when appropriate and powerful antimicrobial drugs have been prescribed. The chance of success in critical situations may be increased by the use of antimicrobial agents which are without immunosuppressive properties. In contrast, antimicrobial drugs with immunopotentiating properties may enhance the possibility of the patient's recovery.

It is therefore important to determine the immunopharmalogical properties, if any, of the antimicrobial drugs available for clinical use. Yet, little is known about this important aspect of antimicrobial chemotherapy. Pharmaceutical companies do not appear to have systematic programmes for the testing of these drugs with regard to their immunopharmacological potential, either for drugs which have been previously released, or the ones recently introduced for clinical use.

The present studies were undertaken for such a purpose. The findings have implicated several very useful antimicrobial drugs as potent immunosuppressive agents. A less expected spin-off from these studies is the discovery that pyrimethamine has immunopotentiating effects. This drug may prove useful in the future for the manipulation of immune responses.

Background information will be provided in the next three sections dealing with (1) pharmacology of antimicrobial drugs, (2) physiology of the immune response, and (3) the mechanisms of action of drugs on the immune system. Individual antimicrobial drugs will be discussed in greater detail in the relevant chapters.

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PHARMACOLOGY OF ANTIMICROBIAL DRUGS

Antimicrobial drugs fulfil their function by possessing selective toxicity for microorganisms and relative sparing of host tissues. This in turn is dependent on differences between microbial and host cells in terms of structure and biochemical processes.   In general, antimicrobial drugs can be classified with regard to their mechanisms of action into four groups (Goodman and Gilman, 1975; Melmon and Morielli, 1978; Meyers et al, 1978).   At the cellular and molecular level, most of these drugs function by (1) inhibiting cell wall synthesis, (2) increasing cell membrane permeability, (3) interfering with protein synthesis, and (4) disruption of nuclei acid metabolism. A scheme of these various processes is illustrated in figure I-1 and table I-1, and discussed below.

Inhibition of cell wall synthesis

The drugs which accomplish their antimicrobial action by inhibition of cell wall synthesis include the penicillins, cephalosporins, bacitracin, cycloserine and vancomycin.   Bacteria maintain their structural integrity by means of a rigid cell wall.   This cell wall is made up of a complex polymer of polysaccharides and a highly cross-linked polypeptide.   This peptidoglycan layer is thicker in Gram positive bacteria than in Gram negative bacteria.   Penicillins and cephalosporins inhibit the activity of the transpeptidase enzymes responsible for the terminal cross-linking of the linear glycopeptides. Other drugs such as bacitracin, vancomycin and ristocetin affect early

steps in the biosynthesis of peptidoglycans.   In the presence of
these antibiotics, susceptible bacteria develop defective cell walls
without the rigidity to resist the high internal osmotic pressure
and results in the rupture of these bacteria.

Increase in cell membrane permeability

The drugs which have their major action on microorganisms by
interfering with cell membrane functions include amphotericin B,
nystatin, colistin and the polymyxins.   Amphotericin B and nystatin
are polyene antibiotics with an affinity for sterol groupings on
cell membranes.   Since sterol groupings are absent on bacterial
cell membranes, their activity is confined to fungal and protozoan
pathogens.   The polymyxins affect membranes containing
phosphatidyl ethanolamines by means of detergent action.   The cell
membrane encloses the cytoplasm of all living cells.   Injury to
the cell membrane disrupts biochemical processes essential to life
as well as cause leakage of cell components.

Inhibition of protein synthesis

The antimicrobial drugs with a predominant action on protein
synthesis include tetracyclines, aminoglycosides, chloramphenicol,
erythromycins and lincomycins.   The explanation for the selective
action of these drugs on protein synthesis in bacteria is based on
the difference in ribosomal structure between bacterial and

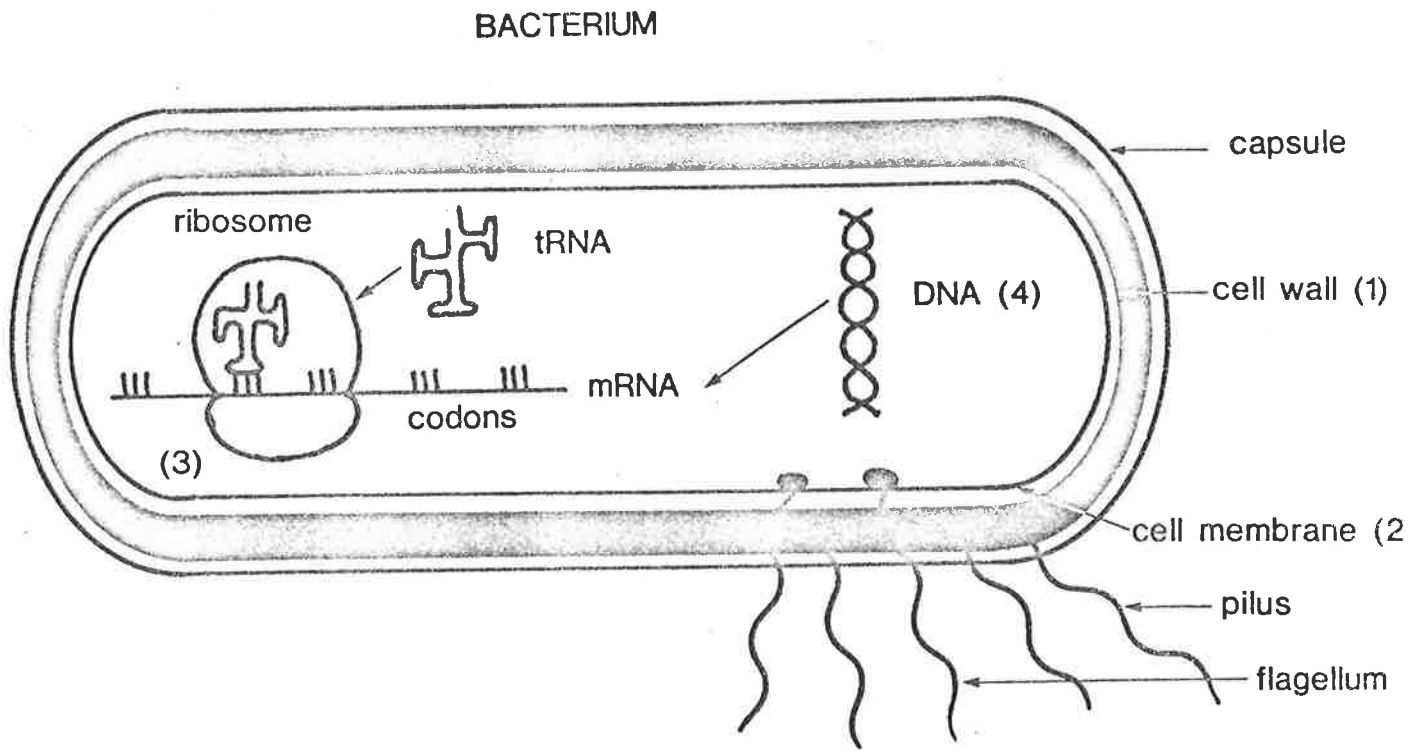THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

BACTERIUM

Figure I-1. Schematic diagram of bacterium showing the sites of action
of antimicrobial agents: (1) cell wall synthesis, (2) cell membrane, (3)
protein synthesis, and (4) RNA-DNA synthesis. The list of antimicrobial
agents with known actions at these sites are presented in table I-1.

Resistance of microorganisms to antimicrobial action are related to
(1) production of drug metabolising enzymes, (2) membrane alteration
resulting in decreased uptake, (3) production of drug antagonist, and
(4) alteration in drug receptor or susceptibility to binding by drugs to
microbial components.

Microorganisms can acquire drug resistance by means of mutation,
transduction, transformation and conjugation. Of all these mechanisms,
conjugation is the most important. This process consists of two factors:
the "R" factor which carries the information for resistance, and the
"RTF" factor which controls the transfer of the R factor during
conjugation. Coliform bacteria are the most active in terms of transfer
of multiple drug resistance. The antimicrobial resistances being
transferred include sulphonamides, tetracyclines, aminoglycosides,
chloramphenicol and penicillins.

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Table I-1. The sites and mechanisms of action of antimicrobial drugs on microorganisms.

Site of Action	Mechanism of Action
(1) Inhibition of cell wall synthesis	
Penicillins	inhibits transpeptidase
Cephalosporins	inhibits transpeptidase
Bacitracins	inhibits mucopeptide transfer
	changes cell membrane permeability
Vancomycin	inhibition of mucopeptide synthesis
Cycloserine	inhibition of alanine racemase
(2) Increase in cell membrane permeability	
Amphotericin B	affinity for sterol groupings
Nystatin	affinity for sterol groupings
Polymyxins	bind to phosphatidylethanolamines
(3) Inhibition of protein synthesis	
Tetracyclines	inhibits binding of tRNA to 30s ribosome
Aminoglycosides	inhibits function of 30s ribosome
Chloramphenicol	inhibits peptidyl transferase (50s ribosome)
Erythromycins	inhibits aminoacyl translocation
Lincomycins	inhibits initiation and aminoacyl translocation
(4) Inhibition of nucleic acid synthesis	
Sulphonamides	competitive inhibitors of para-amino benzoic acid
Trimethoprim	inhibits dihydrofolate reductase
Pyrimethamine	inhibits dihydrofolate reductase
Rifampin	inhibits DNA dependent RNA transcription
Novobiocin	inhibits DNA synthesis

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mammalian cells.   Tetracyclines and aminoglycosides affect the 30s subunit of microbial ribosomes, while chloramphenicol, erythromycins and lincomycins affect the 50s subunit.   These ribosomal subunits generally serve as receptors for the attachment of the drugs, thereby providing a foothold for the drugs to disrupt enzyme systems and interfere with the assembly of amino acids on to peptide chains.

Inhibition of nucleic acid synthesis

Several antimicrobial drugs interfere with microbial nucleic acid synthesis. These include sulphonamides, trimethoprim, rifampin and novobiocin.   Para-aminobenzoic acid is an essential requirement for the synthesis of folic acid in microbial but not mammalian cells. Folic acid in turn contributes to the biosynthesis of purines and pyrimidines, which are precursors of nucleic acids.   Sulphonamides act as competitive inhibitors of para-aminobenzoic acid because of a similarity in structure.   Trimethoprim and pyrimethamine act on the enzyme dihydrofolate reductase to inhibit folic acid synthesis. Rifampin on the other hand acts directly on DNA-dependent RNA transcription and blocks DNA replication.

PHYSIOLOGY OF THE IMMUNE RESPONSE

The multicellular style of living, because of its greater complexity, led to the development of specialized cells and cell products to cope with intrusions from the external environment.

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Later on, the immune system took on additional roles of homeostasis and surveillance in order to maintain the internal environment as well.   This evolutionary process is reflected in phylogeny, from the non-specific processes of inflammation and phagocytosis found in invertebrates, to a diffusely organized lymphoid system with production of archaic IgM molecules in primitive vertebrates, and its culmination in the sophisticated immune processes of higher vertebrates and man (Gell et al, 1975; Fudenberg et al, 1976; Bellanti, 1978).   The anatomical organization of this immune system, and the cells and cell products which make up the composite whole, will be discussed in the following two sections.   A further section will deal with the complex interplay of these various components of the immune system as they respond to invasion by microorganisms.

Anatomical organization

The development of an all pervasive circulatory system allows the deployment of immune cells and cell products to all areas of the body.   This logistically advantageous organization is complemented by high concentrations of immune cells at strategic locations, such as lymph nodes around the respiratory tract, lymph nodules lining the gastrointestinal tract, and the spleen within the circulation. In this regard, the thymus appears to be an exception.   It is situated in the mediastinum inaccessible from the external environment. It is even excluded from contact with the circulation by a perivascular epithelium.   This may have something to do with its

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function as an endocrine gland and staging post for maturation of
a subpopulation of lymphocytes.

Cells and cell products

Immune cells can be distinguished by morphology and function
into three broad groups.   These are (1) phagocytic cells,
(2) mediator cells, and (3) lymphoid cells.

The major phagocytic cells are neutrophils and macrophages
with eosinophils playing a minor role.   After release from the
marrow, neutrophils stay in the circulation for a few hours before
migrating into tissue spaces where they complete their life span
of a few days.   Eosinophils on the other hand stay in the
circulation for only thirty minutes.   Mononuclear phagocytes enter
the circulation in the form of monocytes, and enter the tissues
after one or two days where they mature into macrophages.
Phagocytic cells perform their function by ingesting, killing and
digesting microorganisms (figure I-2).   They reach the site of
infection by the process of chemotaxis.   Antibody and complement
receptors on their surfaces enhance their ability for phagocytosis.
They possess a variety of microbicidal substances and enzyme
systems (table I-2).

The mediator cells are so called because they produce a number
of chemical mediators with inflammatory and immunological effects.
These cells include the basophils and platelets of the blood, and
mast cells in the tissues.   They can be activated by physical,

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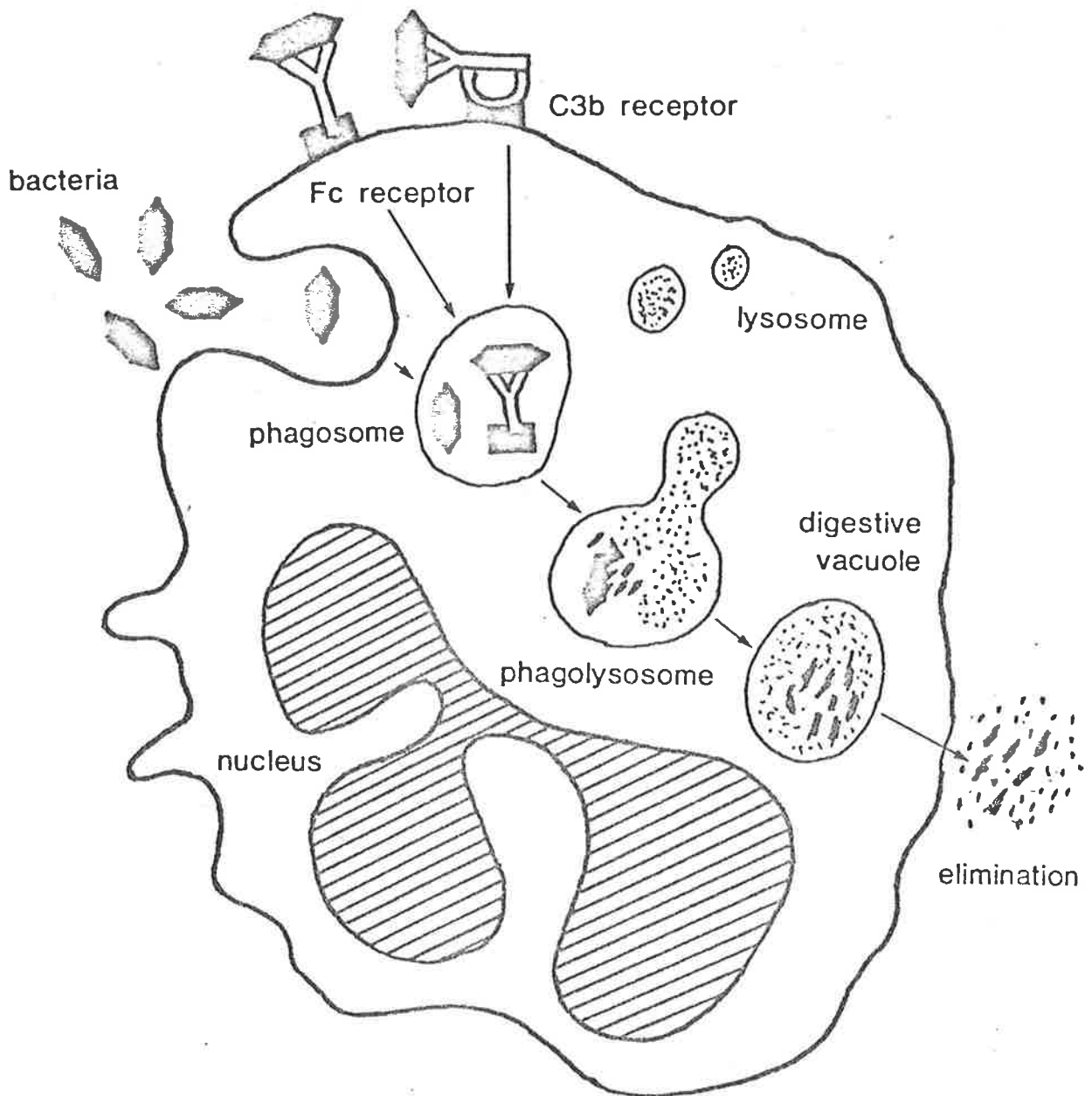
Figure I-2. A schematic diagram of opsonization, ingestion, killing and digestion of bacteria by phagocytic cell. In general, opsonization by antibody and complement enhances the rate of ingestion. A recent contention that complement does not enhance opsonization of Candida albicans (Yamamura and Valdimarson, 1977) has not been confirmed (Ferrante and Thong, 1979). Disorders of phagocytic function serve to illustrate the importance of these cells in host resistance to infection. These include disorders of chemotaxis, such as in the Shwachman syndrome (Thong, 1978; Aggett et al, 1979), failure of the oxidative respiratory burst, such as in Chronic Granulomatous Disease (Holmes et al, 1966), and failure of fusion between lysosome and phagosome, such as in the Chediak-Higashi Syndrome (Root et al, 1972).

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Table I-2.   The anti-microbial mechanisms of phagocytic cells.

Oxygen dependent	Oxygen independent
1. hydrogen peroxide	1. acid
2. superoxide anion	2. lysozyme
3. hydroxyl radical	3. lactoferrin
4. singlet oxygen	4. granular cationic proteins
5. ascorbate-peroxide-metalion	
6. amino acid oxidation	

A wide variety of substances are available as fail-safe systems for microbicidal activity.   Although oxygen dependent systems appear to be more potent, oxygen independent systems are important in anaerobic conditions, such as within abscesses. With regard to the myeloperoxidase-hydrogen peroxide-halide microbicidal system described by Klebanoff (1975), recent studies (Johnston, 1978) suggest that it may not be directly microbicidal. Studies from this laboratory also showed that patients with deficiency of myeloperoxidase have no increased susceptibility to infection (Robertson et al, 1979).

It is believed that the metabolic activity of neutrophils is kept to a minimum under normal conditions by factors present in serum (Thong, Rencis and Ness, 1978).   The unbridled metabolic activity of these cells can lead to oxidative damage of not only the neutrophils themselves (Ferrante, Beard and Thong, 1980), but also other body tissues (Weissman et al, 1978).

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chemical or immunological stimuli to release histamine and other vasocative amines (figure I-3 and table I-3).   In this regard, macrophages may also be considered as mediator cells because they produce some of the complement components.   The complement system consists of a number of serum proteins which interact with one another in a sequential manner (figure I-4).   Activation of the complete sequence results in the lysis of microorganisms.   Along the way, several cleavage products are formed;   C3a, C5a and $\overline{C567}$ are chemotactic for phagocytic cells, C3b has opsonic and immune adherent functions, C3a and C5a also increase vascular permeability.

Two subpopulations of lymphoid cells can be recognised in the circulation.   T-cells are the predominant lymphocytes of the blood, and can be found in the paracortical regions of lymph nodes as well as around the follicles and periarteriolar sheaths of the splenic white pulp.   They reach these sites after a complex maturing process within the substance of the thymus.   They respond to antigenic stimulation by clonal proliferation into several cell types with cytotoxic function, termed killer T-cells, regulatory functions termed T-helper and T-suppressor cells, and a memory function (figure I-5).   They also release a number of effector molecules called lymphokines, which have a controlling influence on the inflammatory process and other immune cells (table I-4).

The other subpopulation of lymphocytes, the B-cells, are found in the circulation, as well as in the germinal centres of lymph nodes and spleen.   They respond to antigenic stimulation by clonal

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PLATELET

STIMULI

immune complex ⟶

activated complement ⟶

antiplatelet Ab ⟶

collagen ⟶

enzymes ⟶
(trypsin, thrombin)

⟹ vasoactive amines

MAST CELL

IgE

antigen ⟶

anti-IgE ⟶

C3a, C5a ⟶

ATP ⟶

chemicals ⟶

enzymes ⟶

bee venom ⟶

heat, cold ⟶
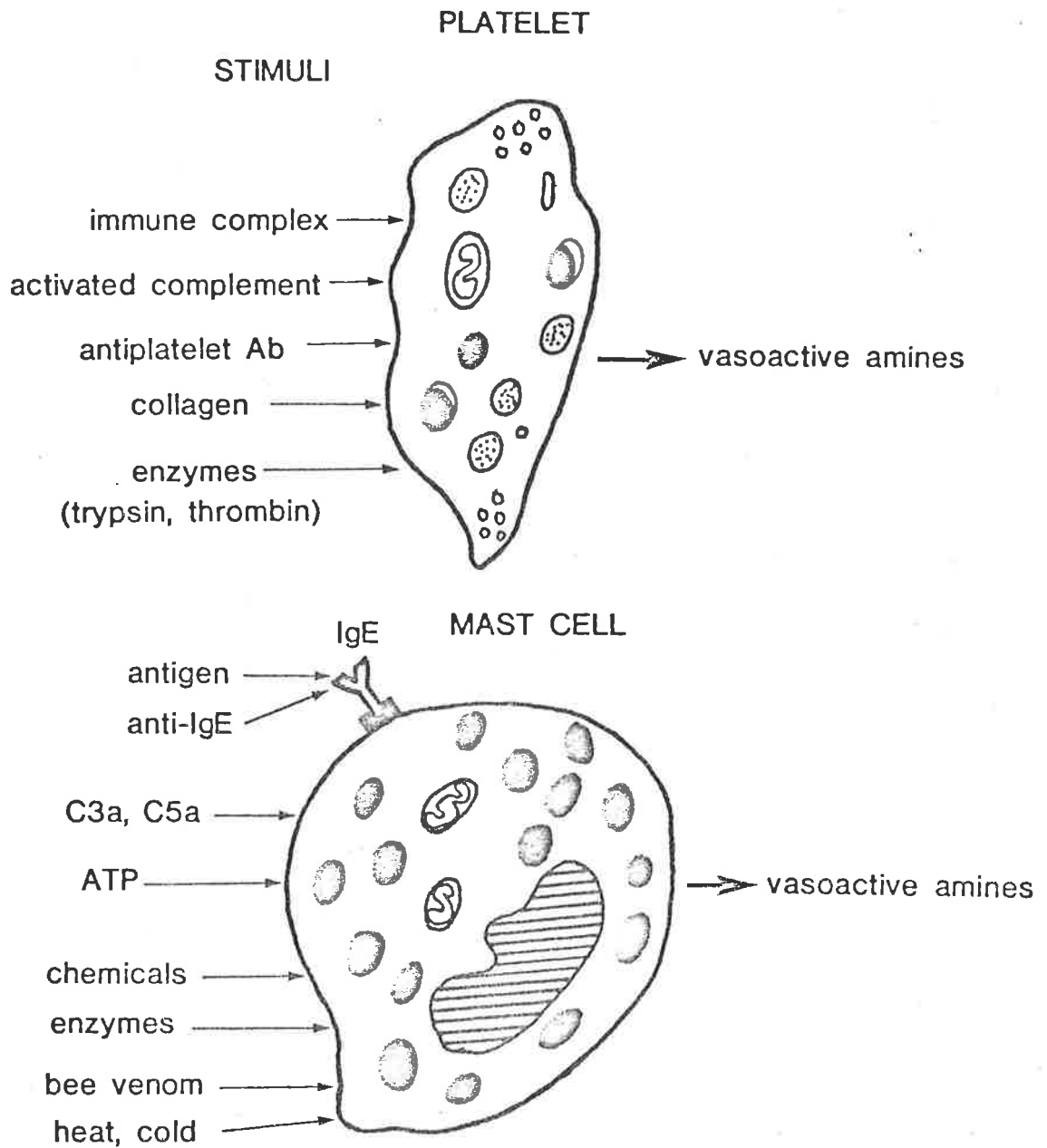
⟹ vasoactive amines

Figure I-3. The release of vasoactive amines from platelets and mast cells by a variety of stimuli. Platelets are generally more sensitive to stimuli arising from tissue damage, blood coagulation and immunological reactions. Mast cells are more active in immediate hypersensitivity reactions because of "arming" by IgE, which acts as a trigger on contact with allergen. Mast cells are also responsive to physical stimuli such as heat, cold and pressure. The basophil, another mediator cell, is not shown in this diagram.

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Table I-3.   Vasoactive amines as mediators of inflammation and
   immediate hypersensitivity.

Vasocative amines	Action
(1) Histamine	
H1 receptor	smooth muscle contraction, increase vascular permeability, mucous secretion
H2 receptor	increase gastric secretion, feedback control of vasoactive amine release
(2) Serotonin	increase vascular permeability, capillary dilation, smooth muscle contraction
(3) Kinins	
Bradykinin Lysylbradykinin	increase vascular permeability, lower blood pressure, smooth muscle contraction
(4) Slow reactive substance of anaphylaxis (SRS-A)	slow contraction of smooth muscle
(5) Eosinophil chemotactic factor of anaphylaxis (ECF-A)	attracts eosinophils
(6) Platelet·activating factor	platelet aggregation and secretion
(7) Protaglandins and precursors	platelet aggregation, smooth muscle contraction, leukocyte chemotaxis, vascular permeability, etc.

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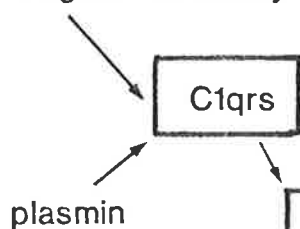
Figure 1-4. Schematic diagram of the complement system. The classical pathway is usually activated during the binding of antibody to antigen. In the absence of specific antibody, the complement system can be activated via the alterantive pathway. The alternative complement pathway may have a role in early defence against infection in individuals without prior exposure to the microorganism. Microorgansims capable of activating the alternative pathway include pneumococcus (Fine, 1975), Candida albicans (Ray and Wrepper, 1976; Thong and Ferrante, 1978), Cryptococcus neoformans (Diamond et al, 1974), and the amoeboflagellate Naegleria fowleri (Rowan-Kelly et al, 1980).

Inhibitors of complement activation acts as biological control mechanisms. Three such inhibitors are known, acting on C1, C3 and C6. Absence of C1 inhibitor occurs in Hereditary Angio-oedema Syndrome. Deficiency of main complement components predisposes to collagen-vascular and infectious diseases (Johnston, 1978; Thong, Simpson and Müller-Eberhard, 1980).

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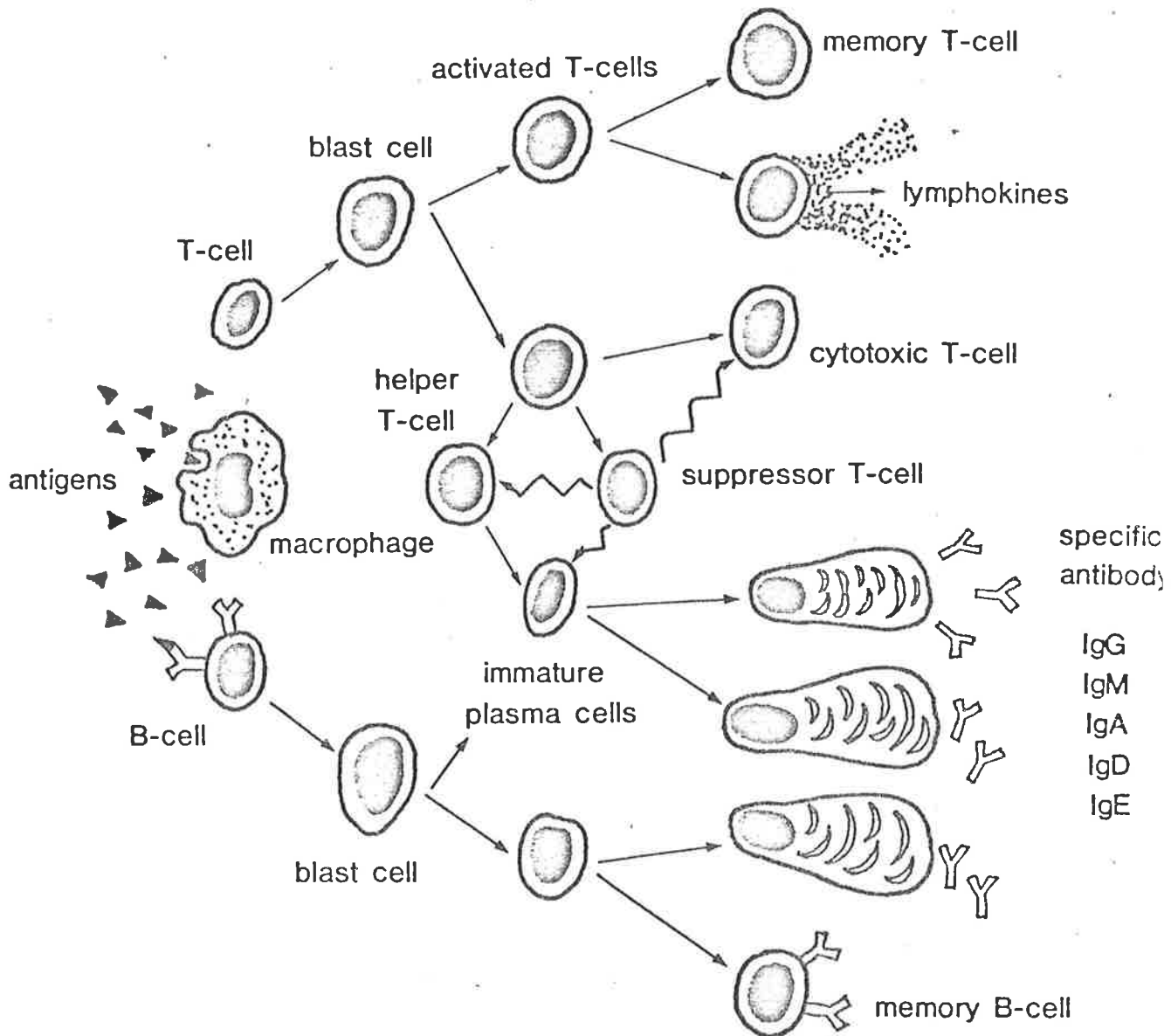
Figure I-5. A schematic diagram showing clonal proliferation of T and B-cells after exposure to antigen. Macrophages have a role in antigen-processing. Note the central role of T-cell subpopulations, which regulate the activities of other T-cells as well as the production of antibody (Waldmann, 1979). Self-regulation is a feature of most biological systems.

Deficiency of T-cell immunity, as in the DiGeorge Syndrome, predisposes to infections, especially fungi and viruses. This deficiency can be restored by transplantation of thymic epithelium (Thong et al, 1978a,) presumably by means of hormonal stimulation.

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Table I-4. Characteristics of some lymphokines produced by T and B-cells

Lymphokine	Source	Molecular Weight	Biological activity
1. Macrophage inhibitory factor (M.I.F.)	T&B	25-55,000	macrophage accumulation macrophage activation
2. Leukocyte inhibitory factor (L.I.F.)	T&B	68,000	neutrophil accumulation
3. Skin reactive factor	T	70,000	cutaneous reaction
4. Chemotactic factors	T&B	35-55,000	attracts macrophages and neutrophils
5. Mitogenic factors	T	25,000	lymphocyte transformation
6. Interferon	T&B	25,000	inhibits viral replication
7. Transfer factor	T	10,000	transfer of specific immune reactivity
8. Lymphotoxin	T	80-150,000	destroys target cells
9. Antibody	B	varies	varies (see table I-5)

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proliferation into antibody-producing plasma cells and memory cells.
Antibodies are made up of immunoglobulins of which five classes are
known (figure 1-6 and table 1-5).   IgA is found in secretions, and
is responsible for immunity at mucosal surfaces.   IgG is a small
molecule that penetrates into tissue spaces and crosses the placenta
easily.   IgM because of its larger size is confined to a great
extent within the circulation.   The function of IgD remains poorly
understood.   IgE binds to basophils and mast cells where they
trigger the release of vasocative amines on contact with antigen.

Immunophysiology

The complex interplay between various components of the immune
system can best be appreciated by contructing a series of hypothetical
situations which can occur after microbes have gained entry into the
body.   If the innoculum is a small one and the organisms have low
virulence, the acute inflammatory response followed by accumulation
of neutrophils at the site of infection is generally sufficient.
A large innoculum of virulent microbes, however, would require the
additional presence of macrophages and lymphocytes (figure 1-7).
Antibody is produced.   Killer T-cells enter the foray.   The
release of lymphokines recruit other immune cells and augment the
inflammatory reaction.   Phagocytic cells accumulate in even greater
quantities in response to chemotactic factors.   The microorganisms
are lysed by antibody and complement, or they are phagocytosed and
killed.   If the subject survives this infectious process, the next
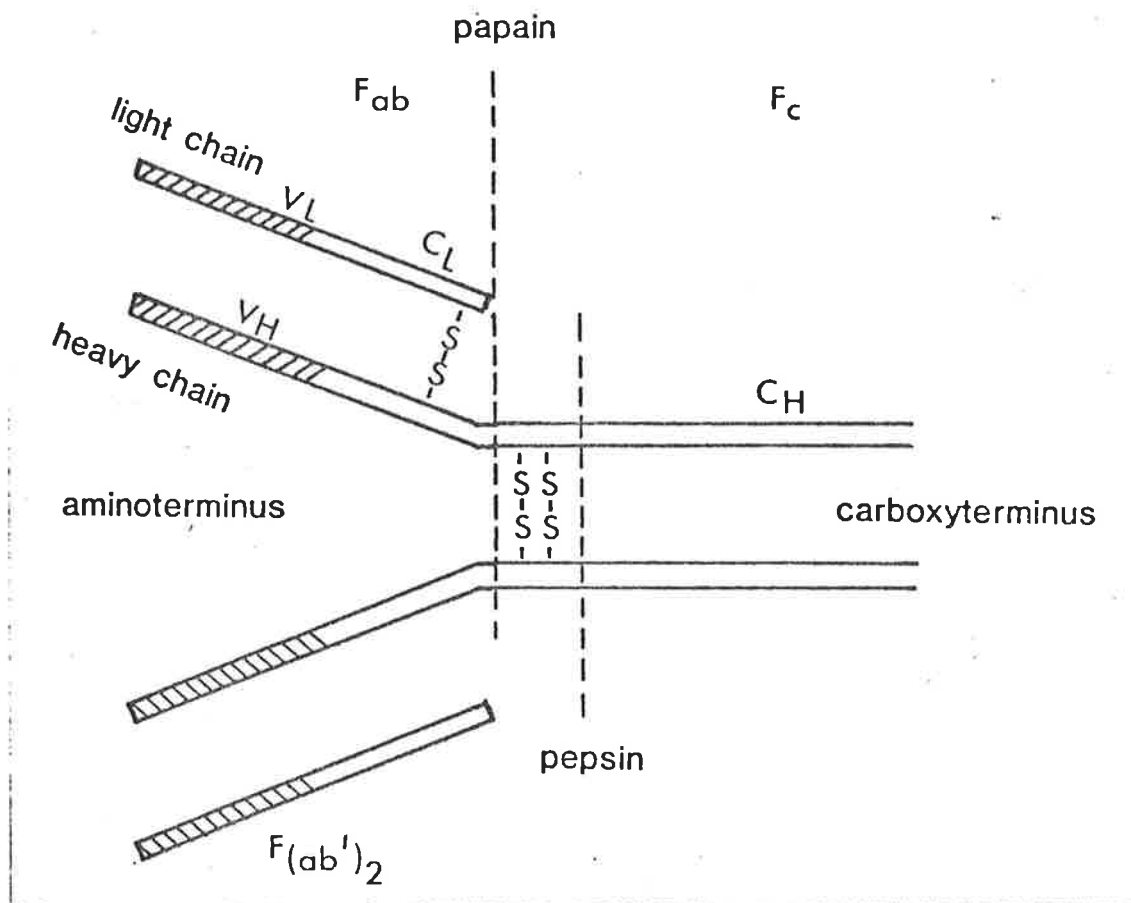
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papain

F_{ab}

F_c

light chain

V_L

C_L

heavy chain

V_H

S
S

C_H

aminoterminus

S S
S S

carboxyterminus

pepsin

$F_{(ab')_2}$

Figure I-6. A schematic representation of IgG_1 molecule showing 2 identical light and 2 identical heavy chains with intra-chain disulphide bonds. Each light chain has a molecular weight of about 22,000 and each heavy chain 55,000. Two types of light chains are found, termed kappa and lambda; and 5 types of heavy chains, one for each class of immunoglobulin, identified by the Greek-letter designation of their class names. The antibody-combining site is at the aminoterminus. Antibody specificity is determined by the variation in the 110 amino acids in the variable regions of light (VL) and heavy (VH) chains. Papain digestion divides the molecule into an antigen portion (Fc) and antigen-binding portion (Fab). Pepsin digestion splits the molecule into the F(ab')2 fragment which has 2 antigen-binding sites.

IgG, IgD and IgE exist in the basic four-chain unit depicted in the figure. IgM exists as a pentamer, held together by disulphide bonds and the J-chain, a small molecule with high sulphydryl content. IgA and IgG may also exist in polymeric form in serum. In secretions, IgA exists as a dimer, linked by the J-piece and secretory component. The secretory component protects the dimer from digestive enzymes in the gut.

By means of antigenic analysis, four IgG subclasses, and two subclasses each for IgA and IgD are known. They vary in terms of biological activity. For instance, IgG4 does not fix complement while the other 3 subclasses do.

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Table I-5. Characteristics of the Immunoglobulin classes in man.

Class	Mean serum Concentration (g/l)	Molecular Weight	Half Life (days)		Biological activity
IgG	12.4	150,000	23	1. 2.	fix complement cross placenta
IgA	2.8	170,000	6	1. 2.	secretory antibody alternative pathway
IgM	1.2	890,000	5	1. 2.	agglutination fix complement
IgD	0.03	150,000	3	1.	? surface receptor for lymphocytes
IgE	0.0003	196,000	1-2	1.	reaginic antibody

Immunoglobulin deficiency was first reported by Burton (1952) in a child
with repeated infections. Since then, many patterns of congenital
immunoglobulin deficiencies have been described. Selective deficiency
of IgA is the commonest of these disorders, with an overall incidence
of 1:500. Claims by Hobbs (1975) that selective IgM deficiency is the
second most common immunoglobulin disorder cannot be substantiated (Thong
and Maxwell, 1978).

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intrusion by an antigenically similar microoganism will be met with
an accelerated reaction because specific antibody may still be
present or memory cells remain operative.

Some microbes may escape from the area of infection into lymph
channells or the blood stream.   The lymph nodes and spleen come
into play as a second line of defense to prevent dissemination of
the infection.

Some microbes however have the capacity to resist the killing
mechanisms of phagocytic cells.   They may be either facultative
intracellular parasites such as Mycobacterium tuberculosis and
Toxoplasma gondii, or obligatory intracellular parasites such as
rickettsiae and viruses.   They are then carried into deeper tissues
within macrophages, where they multiply and infect other cells.
A chronic inflammatory process develops, leading to the destruction
of surrounding tissues, which are the innocent bystanders of combat
between microbe and immune system.

Under favourable circumstances, such as a previously healthy
subject in good nutritional status and with an intact immune system,
the result is elimination of invading microorganisms.   This process
is greatly facilitated by antibacterial chemotherapy.   In clinical
situations where the immune system has been compromised by debilitating
illnesses or immunosuppressive therapy, the use of appropriate and
potent antimicrobial agents can be the only means of recovery from
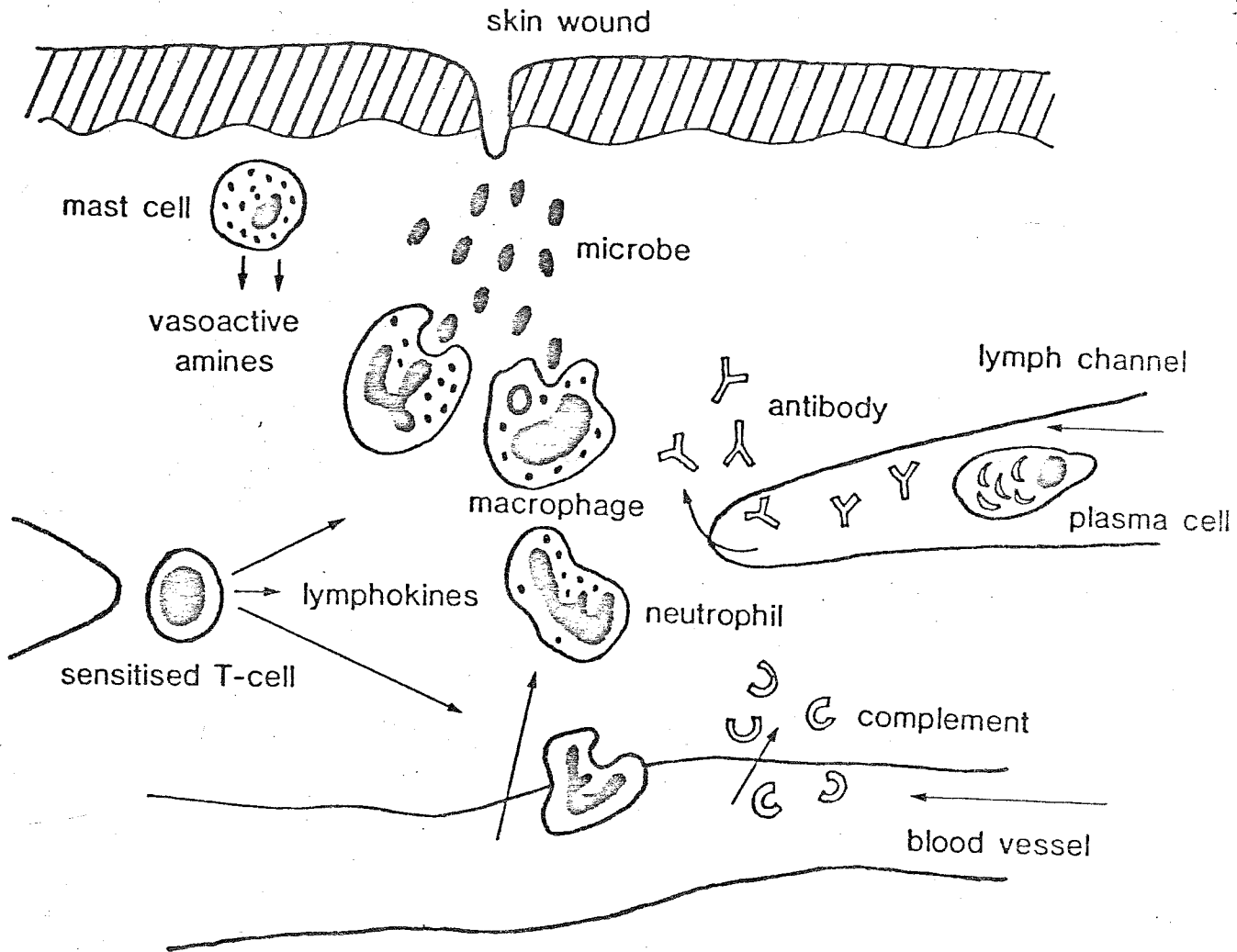infection.

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Figure I-7. A schematic diagram of the full repertoire of immunological responses to infection. Various elements of this immunological repertoire assume prominence under different circumstances. Phagocytic cells are most efficient for elimination of pyogenic bacteria. Toxin-producing bacteria such as tetanus or diphtheria organisms are best controlled by anti-toxic antibody. Tuberculosis and cryptococcus require the presence of activated macrophages, whereas viruses are best exposed from their intracellular location by T-cells and macrophages, so that antibody and complement can gain access to them.

The interaction between infectious organisms and host immunological responses may be more complicated than a simple parasitism-elimination model. It is now known that many infections can be accompanied by immunosuppression. The mechanism for this microbe-mediated immunosuppression is not completely understood, but in the case of viral infections, it is thought to be due to either a direct invasion of mononuclear cells, the induction of suppressor cells or factors, or direct antigenic competition (Notkins, 1975). In the case of malaria (McGregor 1972), circulating suppressor factors may play a role (Liew et al, 1979).

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IMMUNOPHARMACOLOGY

Much information has been gained about immunopharmacology in the past decade from clinical and experimental chemotherapy of cancer, autoimmune diseases and graft rejection. There is considerable overlap between anti-cancer and immunosuppressive drugs. These cytotoxic agents are more active on rapidly dividing cells whether they are cancer cells or immune cells. A cell cycle of five phases is now recognised (table 1-6). The phases of the cell cycle are usually well synchronised during the proliferation of immune cells because this usually happens as a response to antigenic stimulation. In contrast, tumour cells tend to be out of phase with regard to the cell cycle. Those tumour cells in the resting G_O phase are more resistant to selective toxicity by anti-cancer drugs, so that intermittent chemotherapy has a better chance of success by catching tumour cells as they emerge from the resting phase. A different strategy is employed for immunosuppressive therapy. This usually takes the form of continuous low dose regimens to control the proliferation of immune cells.

Anti-cancer drugs

The drugs employed for cancer chemotherapy generally fall into four broad categories (table 1-7). The alkylating agents affect a number of cellular functions, including glycolysis, respiration, nucleic acid and protein synthesis, and the integrity of biological membranes. The ionised forms of these drugs bind avidly to

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Table I-6. The kinetics of the cell cycle.

Phase	Duration	Biochemical events
G_1	18 hrs	preparation for DNA synthesis
S	20 hrs	DNA synthesis
G_2	3 hrs	RNA and protein synthesis
M	1 hr	mitosis
G_0	variable	resting period

Biosynthesis of enzymes in preparation for DNA synthesis occurs in G_1 phase. Actual DNA replication occurs in the S phase. In G_2, synthesis of RNA and protein is followed by assembly of the mitotic spindle. M is the phase of mitosis, after which the cell assumes a long resting G_0 phase.

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Table I-7. Some drugs useful for the chemotherapy of cancer.

Cytotoxic drug	Mechanism of action
1. Polyfunctional alkylating agents chlorambucil, mephalan, cyclophosphamide, nitrosoureas.	causes cytolysis and mutagenesis inhibits glycolysis and respiration inhibits protein and nucleic acid synthesis interferes with membrane function
2. Antimetabolites methotrexate, thioguanine, 6-mercaptopurine, allopurinol, cytosine arabinoside fluorouracil, azacytidrine.	substitutes for normal metabolite competes with normal metabolite for enzyme regulating site competes with normal metabolite for enzyme catalytic site binds and inactivates of enzyme catalytic site
3. Plant alkaloids and antibiotics vincristine, vinblastine	binds to microtubular protein of mitotic spindle
podophylotoxin	inhibits DNA synthesis, stops mitosis at metaphase
retinomycin D	inhibits DNA-dependent RNA synthesis
anthracyclines	binds to DNA, alkylating action
chromomycins	inhibits DNA-dependent RNA synthesis
mitomycin C	alkylating action
bleomycin	inhibits mitosis and DNA synthesis
asparaginase	competes with aminoacid L-asparaginase
4. Steroid hormones Androgens, oestrogens, anti-oestrogen, progestins, corticosteroids	steroid-receptor complex binds to DNA, disrupts nuclear membrane

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carboxyl, sulphydryl, amino and phosphate groupings. The most susceptible molecule for this alkylating process appears to be guanine, a purine constituent of DNA.

The antimetabolites are structural analogues of important substances required by cells for metabolism and growth. The major effect of these drugs is abberation of nucleic acid synthesis. Methotrexate inhibits the enzyme dihydrofolate reductase and prevents the formation of purine precursors of ATP, DNA and RNA.

The plant alkaloids, vincristine and vinblastine, act by inactivation of the mitotic spindle. Some antibiotics are also useful cytotoxic agents. Actinomycin D blocks the synthesis of ribosomal RNA. The anthracyclines bind strongly to DNA. Mitomycin C may have some of the attributes of an alkylating agent.

Steroid hormones appear to act by binding to specific receptors on the cell membrane. The steroid-receptor complex enters the cell to bind with DNA to disrupt transcription processes. It may also cause injury to the nuclear membrane. Cancer arising from the breast or prostate may be sensitive to estrogens and androgens. Corticosteroids are useful in the treatment of haematologic malignancies.

Anti-cancer drugs are also classified according to whether their cytotoxic capability is extended over all phases of the cell cycle (cell cycle non-specific) or restricted to the proliferative phases (cell cycle specific). The alkylating agents are cell non-specific while the antimetabolites tend to be cell cycle specific (table I-8).

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Table I-8.   Anti-cancer drugs in relation to kinetics of the cell cycle.

Cell cycle specific drugs (CCS)	Cell-cycle non-specific drugs (CCS)
Antimetabolites	**Alkylating agents**
methotrexate	mephalan
thioguanine	mechlorethamine
6-mecaptopurine	cyclophosphamide
cytosine arabinoside	busulphan
fluorouracil	thiotepa
azacytidine	carmustine
	lomustine
Plant alkaloids	
vincristine	**Antibiotics**
vinblastine	actinomycin D
	mitomycin C
Antibiotics	doxorubicin
bleomycin	daunorubicin
Miscellaneous	**Miscellaneous**
hydroxyurea	cis-platinum

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Immunosuppressive drugs

The complex interplay between various components of the immune system in response to infectious organisms has been discussed in a previous section. In circumstances where the antigen is persistent or the immune response inappropriate, tissue injury is likely to occur. Gell and Coombs have classified immunologically-mediated diseases into four types according to the dominant mechanism of tissue damage (table I-9). The source of antigen can be exogenous, such as in drug and pollen allergy, or allogeneic such as in organ transplantation and blood transfusion, orautologous such as in the auto-immune diseases.

A variety of anti-inflammatory and immunosuppressive drugs are available for amelioration of the pejorative effects of an aberrant immune response. The anti-inflammatory drugs inhibit or antagonise chemical mediators of inflammation (table I-10); some of them have inhibitory effects on phagocytic cells. The immunosuppressive agents have a direct action on antibody and cell-mediated immunological responses. Their nature and properties have been discussed in the previous section on anti-cancer chemotherapy. The clinically useful immunosuppressive drugs and a summary of their effects are presented in table I-11. The sites of action of these drugs on the immune response is illustrated in figure I-8. It can be seen that most immunosuppressive drugs have the ability to interfere with the proliferative phase of the immune response. It would appear from this that a good way to screen for immunosuppressive

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Table I-9.   Immunological mechanisms of tissue injury.

		Mechanism	Clinical manifestations (examples)
I	Anaphylactic	IgE and other Igs	Immediate hyper-sensitivity, hay fever, asthma
II	Cytotoxic	IgG, IgM, and phagocytes	Auto-immune haemolytic anaemia, Goodpasture syndrome
III	Arthus reaction	Antigen-antibody complexes	Serum sickness, systemic lupus erythematosus
IV	Delayed hypersensitivity	Sensitized T-cells	Contact dermatitis, caseating tuberculosis

This concept of the mechanisms of immunologically-mediated diseases introduced by Gell and Coombs has stood the test of time.   Because of the complex nature of the auto-immune diseases, more than one mechanism may be operative in the clinical situation.   In allergic bronchopulmonary aspergillosis, the combination of types I & III is responsible for tissue damage.   In extrinsic allergic alveolitis, types III & IV are responsible.

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Table I-10. Some mechanisms by which anti-inflammatory drugs produce
their effects.

Drug	Mechanism of action
Salicylates	I, II, III, IV, V, VI, VII, X
Phenylbutazone	I, II, III, IV, V, VI
Gold Salts	I, II, III, IV, XI
Indomethacin	I, II, VI, VII, IX
Colchicine	III, IV, V, VIII, IX
Chloroquine	I, IV, IX
Corticosteroids	IV, VII, VIII, IX, X, XI

I   inhibits prostaglandin synthesis
II   uncouples oxidative phosphorylation
III   inhibits cellular respiration
IV   stabilises lysosomal membranes
V   inhibits release of vasoactive amines
VI   inhibits synthesis of serotonin
VII   reduces capillary permeability
VIII   interferes with kinin formation
IX   inhibits neutrophil function
X   suppresses antibody formation
XI   suppresses cell-mediated immunity

This exhaustive list is by no means complete, nor does it
necessarily explain their anti-inflammatory effects, although recent
studies indicate that inhibition of prostaglandin synthesis may be the
principal mode of action of some of these drugs.

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Table I-11. The common immunosuppressive agents and their clinical uses.

Drug	Group	Clinical uses
Predñisone	Corticosteroid	Auto-immune diseases, organ transplantation
Cyclophosphamide	alkylating agent	auto-immune haemolytic anaemia, nephritis, organ transplantation
Chlorambucil	alkylating agent	auto-immune haemolytic anaemia
Azathioprine	antimetabolite	idiopathic thrombocytopaenic purpura, auto-immune haemolytic anaemia, renal and cardiac transplantation
Actinomycin D	antibiotic	renal and cardiac transplantation
Vincristine	plant alkaloid	idiopathic thrombocytopaenic purpura

The effects of these drugs on immunological responses sometimes depend on dosage and time of administration in relation to antigenic stimulation. For instance, a single dose of cyclophosphamide during induction with antigen, can preferentially affect suppressor T-cells, so that a paradoxical enhancement of delayed hypersensitivity is observed (Liew, 1977).
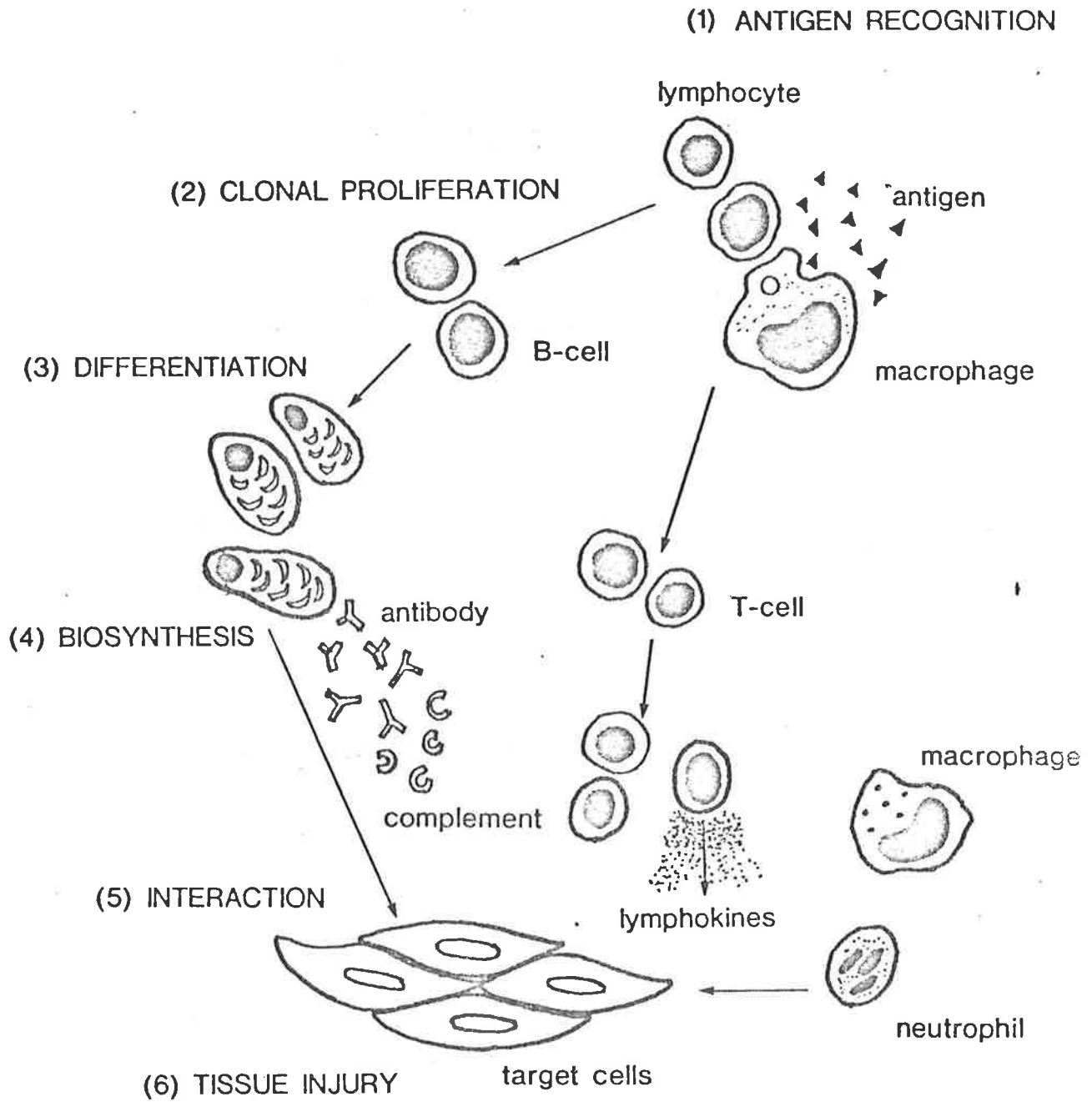
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Figure I-8. The sites of action of immunosuppressive drugs on the immune response. Most of the commonly used immunosuppressive drugs such as corticosteroids, azathioprine, methotrexate, cytosine arabinoside, and cyclophosphamide exert their major effect on the stage of clonal proliferation (2). Others, such as actinomycin D, also affect the stage of differentiation (3). Corticosteroids affect all 6 stages of the immune response (Vernon-Roberts, 1969; Thong et al, 1975; Fauci et al, 1976), as well as the inflammatory response, although the mechanism of action remains incompletely understood (Parillo and Fauci, 1979).

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potential of antimicrobial drugs would be to study the effect of these drugs on in-vitro lymphocyte proliferative responses.   These screening studies are described in chapter III.

TUMOUR IMMUNOLOGY

Two main strategies are followed in the management of patients with cancer.   The tumour mass is first reduced by a combination of surgery, radiotherapy and chemotherapy.   Next is to preserve, and if possible, augment immune function.   This would reduce the frequency of secondary infection.   It would also enable the immune system to control the remaining cancer cells.

The role of immune surveillance in the control of cancer is supported by clinical observations of a much higher incidence of malignancies in children with primary immunodeficiency disorders and patients on immunosuppressive therapy.   Tumour-associated antigens can be found in cancer cells.   In certain situations, oncofoetal antigens also appear on their cell membrane surfaces. Cancers can arise spontaneously, or as a result of interaction with certain chemicals and viruses (table I-12).   Tumour-associated antigens tend to be weak antigens, so that a strong immune reaction to them does not normally occur.   The immune surveillance theory has led to various devices for immunotherapy as a means of cancer control.   One way is to immunize patients with antigens derived from cancer tissue.   The other way is to stimulate the immune

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Table I-12. The antigenic characteristics of tumours.

Tumour induction	Specific agent	Tumour type	Antigenic specificity
1. Spontaneous	unknown	varied	specific for each tumour
2. Chemical agents	methylcholan-threne	carcinoma sarcoma leukaemia	specific for each tumour
3. Physical agents	sunlight	melanoma	specific for each tumour
4. Viruses RNA	avianleukosis virus	avian leukaemia	shared antigens, but also specific ones
DNA	adenovirus	sarcoma	shared antigens

In general, cells of identical genetic make-up transformed by the same chemical carcinogen gives rise to tumours, each with its own antigenic specificity, while transformation by oncogenic viruses results in tumours with cross-reacting tumour-specific antigens. Even cells of different morphology produce cross-reacting tumour-specific antigens if they are transformed by the same virus.

Also, oncofoetal or carcinoembryonic antigens can sometimes appear on the surface of transformed cells. These antigens usually found only in embryonic cells but not mature cells, are believed to reappear because of derepression of the genes concerned.

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response in a non-specific manner by means of certain biological and chemical substances (table I-13). Most of them are still under experimental evaluation. Clinical studies with BCG and levamisole have produced mixed results (Goodnight and Morton, 1978).

CONCLUSIONS

From this general review of current knowledge about the complex interaction between microorganisms and immune responses, the pharmacology of antimicrobial drugs, and the effects of immuno-suppressive and anti-cancer drugs on the immune system, it becomes apparent that antimicrobial drugs by themselves may have effects on the immune system. From the standpoint of general pharmacology, it would appear that some categories of antimicrobial agents are similar to anti-cancer and immunosuppressive agents with regard to their ability to interfere with protein and nucleic acid synthesis, while the polyene antibiotics, like the polyfunctional alkylating agents, disrupt cell membrane function.

Yet no discussion of the immunosuppressive potential of antimicrobial drugs can be found in the major text books of pharmacology, nor is this possibility mentioned in recent reviews of antimicrobial drugs (McHenry and Feiker, 1978; The New York Academy of Medicine, 1978). There are already some reports in the literature to indicate such a possibility. Chloroquine has been found to depress lymphocyte transformation (Hurvitz and

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Table I-13. Some substances that can potentiate the immune response.

Microbial origin	Chemicals
1. Mycobacteria (BCG)	1. Polynucleotides
2. Gram negative bacilli	2. Tilorone
3. Corynebacterium parvum	3. Glucan
4. Zymosan	4. Pyran Polymer
Mammalian origin	5. Levamisole
1. Transfer factor	Inorganic
2. Thymosin	1. alum
3. Immune RNA	2. silica

Clinical experience with BCG (Lamoureux, 1976) and levamisole (Willoughby and Wood, 1977) has instilled a certain degree of caution in the medical community about their value as immunostimulants. Some complications have been encountered with their use in patients. Also, levamisole has been found to have both immunosuppressive and immunostimulatory properties depending on dosage and time of administration. A better understanding of the immunopharmacology of immunopotentiating agents is needed before a rational approach to their use in patients can be devised.

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Hirschborn, 1968). Similar findings are reported for chloramphenicol (Pisciotta and Deprey, 1967; Becker et al, 1974; Ugazio et al, 1974). Rifampin has received the best documentation (Sanders, 1976), although the immunosuppression induced by this drug appears to have little effect on the ability of patients to recover from tuberculosis.

It thus became obvious that a systematic approach to the problem will be required in order to obtain useful information about the immunopharmacology of antimicrobial drugs. The knowledge gained from these studies would provide guidance for physicians in the choice of antimicrobial agents, especially in the treatment of serious infections in immunocompromised patients. It would also alert doctors to the possibility of unpleasant side-effects of immunosuppressive antimicrobial drugs which have been realized with the conventional immunosuppressive drugs, i.e. carcinogenicity and teratogenicity. The possibility also exists for the discovery of an antimicrobial drug with immunopotentiating properties, with the inherent benefits that can come from the judicious use of such drugs on immunocompromised patients. Finally, some of these immuno-active antimicrobial agents may become useful tools for the study of immunological processes.

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CHAPTER II

MATERIALS AND METHODS

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INTRODUCTION

A wide array of experimental methods are available for
investigation of immunopharmacological properties of antimicrobial
drugs.   The prime considerations for selection of suitable methods
are that they should be simple, well-tried and easily reproducible.
These are important considerations, because methods which have not
been studied in detail, and hence not well-understood, would
introduce another variable to confuse the issue.   These considerations
are also applied to the planning of experiments.   In general, a
simple approach is preferred to a complicated experimental design.
The in-vitro and in-vivo techniques employed in these studies are
described in the next few pages of this chapter.

ANTIMICROBIAL DRUGS

The antimicrobial drugs used in these studies were either
provided by Mr. Robert Fleetwood of the Adelaide Children's Hospital
Pharmacy Department, or obtained from Pharmaceutical companies with
his assistance.   In the latter situation, tetracyclines were
obtained from Pfizer Propriety Limited, New South Wales;   miconazole
was a gift of Dr. Leon Harris, Ethnor Private Limited, New South
Wales;   amphotericin B was purchased from two sources, either
E.R. Squibb & Sons, Inc., New York, or Flow Laboratories, New South
Wales;   the antimalarial drugs were purchased from either Wellcome

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Australia, New South Wales, or from Sigma, St. Louis, U.S.A.

Poorly soluble drugs were first dissolved in the appropriate solvent as a stock solution, and further dilutions made in RPMI 1640 for in-vitro studies. For in-vivo studies, poorly soluble drugs were either given orally as a suspension in distilled water, or injected as a suspension in saline.

EXPERIMENTAL ANIMALS

Two inbred strains of mice, BALB/c and C57B1/6J, were used in these studies at 6-10 weeks old. They were obtained from the breeding colonies at the Institute for Medical and Veterinary Science, Adelaide. For some experiments, the outbred LACA strain of mice were obtained from the breeding colonies at the Waite Institute, University of Adelaide. The mice were kept in plastic cages in an air-conditioned animal house, and allowed free acess to food and water.

REAGENTS AND CHEMICALS

The radio-isotope ^{3}H-thymidine was purchased from the Radiochemical Centre, Amersham, U.K., and RPMI 1640 culture medium and foetal calf serum from Commonwealth Serum Laboratories, Australia. Foetal calf serum was inactivated by heating to 56°C for 30 minutes prior to use.

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Antimicrobial drugs were dissolved in RPMI 1640 medium for in-vitro studies, and in sterile saline (0.5% w/v) for in-vivo studies.

Phytohaemagglutinin (PHA) was purchased from Wellcome, Australia; pokeweed mitogen (PWM) from GIBCO, U.S.A.: and Escherichia coli lipopolysaccharide (LPS) from DIFCO, U.S.A.

ISOLATION OF LYMPHOCYTES FROM HUMAN BLOOD

Blood was drawn by venepuncture from healthy adult donors and placed in heparized tubes, at a heparin concentration of approximately 25µg/ml.

The hypaque-ficoll gradient was made up of 20ml of 85% hypaque (28.33% sodium 3,5-diacetamido-2,4,6-triidobenzoate and 56.67% w/v of meglumine salt of 3,5-diacetamido-2,4,6-triidobenzoate) purchased from Winthrop laboratories, Australia, and 90ml of ficoll, a sucrose polymer of m.w. 400,000 dissolved in distilled water at a concentration of 9% w/v, purchased from Pharmacia, Sweden. The mixture has a density of 1.114g/ml.

About 5ml of blood was layered carefully on 3 ml of the hypaque-ficoll gradient in a 10 ml sterile plastic tube and centrifuged at 200G for 20-30 minutes at room temperature. Two bands formed at the interface (figure 11-1 and Table 11-1). The top band consisted of 98% mononuclear cells (Ferrante and Thong, 1978). The cells were washed twice and resuspended in RPMI 1640 medium.

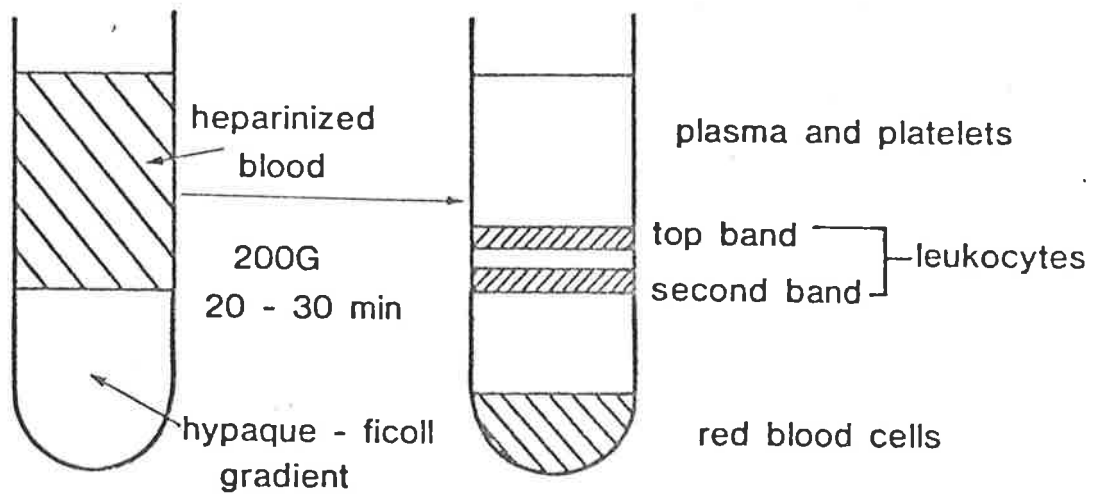THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

Figure II-1. Isolation of lymphocytes from human blood by gradient centrifugation.

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Table II-1. Cell types in mononuclear and polymorphonuclear cell fractions after hypaque-ficoll centrifugation.

Cell fractions	Differential count (%)*				
	Lymphocytes	Monocytes	Basophils	Neutrophils	Eosinophils
top band	83.9 ± 1.6	13.8 ± 2.3	0.5 ± 0.5	1.8 ± 0.8	-
second band	1.2 ± 0.4	-	-	96.4 ± 1.0	2.4 ± 1.0

* mean ±S.D. of 5 experiments using blood from 5 donors.

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ISOLATION OF LYMPHOCYTES FROM MOUSE SPLEEN

Mice were killed by cervical dislocation and the spleens removed with aseptic technique. They were pressed gently against a sieve to release the cells into ice-cold medium. Larger clumps were allowed to settle. The cell suspension was then removed and centrifuged at 45G for 30 seconds. The supernatant containing predominantly single cells was removed and centrifuged at 1000G for 5 minutes. The pellet was resuspended in 0.8% ammonium chloride and kept at $37^{o}C$ for 5 minutes to lyse red cells. The cells were washed 4 times and resuspended in RPMI 1640 medium. Viability of cells was determined by trypan blue dye exclusion: 0.1ml of cell suspension (2×10^{6}/ml) was mixed with 0.1 ml of 0.2% trypan blue in RPMI 1640 medium and kept for 5 minutes at $37^{o}C$, after which the number of stained cells was counted with a haemocytometer.

IN-VITRO LYMPHOCYTE RESPONSE TO MITOGENS

A microculture method was used for these experiments (Thong et al, 1973). For human lymphocyte studies, each well of a microtitre plate received 2×10^{5} cells in 0.1 ml medium containing 10% foetal calf serum and 0.5 ml of either phytohaemagglutinin (PHA) or pokeweed mitogen (PWM). To the test wells were added 0.05 ml of appropriate concentrations of antimicrobial agent, while control wells received 0.05 ml of medium or medium containing appropriate

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concentrations of diluent, as the case may be.   The final concentrations of PHA (1µg/ml) or PWM (50µg/ml) used in these experiments were previously determined to produce optimal stimulation. In some experiments, suboptimal concentrations of mitogens were also used.

For mouse lymphocyte cultures, a similar experimental design was used except that each well contained 1 x 10^6 spleen cells, and the final concentrations of PHA and LPS were 0.5µg/ml and 250µg/ml, respectively. These were previously determined to produce optimal stimulation.   In some experiments, suboptimal concentrations of mitogens were also used.

Human cell cultures were kept for 72 hours, and mouse cell cultures for 48 hours, at 37°C in a 5% CO_2-air atmosphere and high humidity.   The cultures were pulsed with 1µCi of ^{3}H-thymidine 6 hours prior to harvesting.   Harvesting was performed with the aid of a Skatron multiple sample harvester.   The cells were aspirated by the harvester on to glass fibre filter paper, automatically washed with saline and then dried for quantitation of radioactivity in a Packard Tricarb Liquid Scintillation Spectrometer.

IN-VIVO ANTIBODY RESPONSE TO SRBC

Sheep red blood cells (SRBC) kept in Elsevier's solution, obtained from Commonwealth Serum Laboratories, Australia, were

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washed 3 times in sterile saline prior to use.

The mice were immunized with 1 x 10^9 SRBC in 0.2 ml saline via the tail vein on day 0 and beld by intracardiac puncture at day 5. The blood was allowed to clot at room temperature and serum collected. The anti-SRBC haemagglutinating titre was determined by mixing 2-fold dilutions of sera in saline (0.025 ml) with the same volume of 1% (v/v) suspension of SRBC. The microtitre plates were incubated for 1 hour at $37^{\circ}C$ and observed for haemagglutination.

DELAYED-TYPE HYPERSENSITIVITY RESPONSE TO SRBC

For delayed-type hypersensitivity (DTH) responses, mice were immunized with 1 x 10^8 SRBC in 0.05 ml saline by subcutaneous (SC) injection behind the shoulder on day 0, challenged with 1 x 10^8 SRBC in 0.025 ml S.C. in the left hind footpad on day 5 and measured with a dial caliper (Mercer) on day 6. The DTH response was expressed as the percent increase in footpad thickness (Liew, 1977).

IN-VIVO MACROPHAGE CHEMOTAXIS

Mice were injected with 35µg of PHA intraperitoneally (i.p.), killed by cervical dislocation 48 hours later, and peritoneal lavage performed to determine the total number of macrophages present (Snyderman et al, 1976). The net macrophage influx into the peritoneal cavity is taken as an index of macrophage chemotaxis

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This is calculated by subtracting the number of macrophages in mice receiving PHA from those not receiving PHA.

PASSAGE OF B_{16} MELANOMA TUMOUR

The B_{16} melanoma used in these experiments was a gift from David Goh of the Departments of Surgery and Microbiology and Immunology, University of Adelaide.

The B_{16} melanoma cells were maintained by fortnightly i.p. injection into C57Bl/6J mice. For passage and experimental studies, the tumour was excised from the peritoneal cavity, passed through a sieve to obtain single cell suspensions in saline, checked for viability by trypan blue, and 5×10^5 cells in 0.2 ml injected i.p. into each mouse.

TRANSPLANTATION OF SKIN

Skin grafting was performed by the standard technique of Billingham and Medawar (1951). Donor C57Bl/6J mice were killed by cervical dislocation. Tail skin was obtained by circumferential incision at the base of the tail followed by a longitudinal incision to the tip of the tail. The skin was then stripped off, placed in a petri-dish containing sterile cold medium 199, and cut into 1 cm strips. Recipient BALB/c mice were anaesthetised with ether and an area of skin on the back similar in size to the donor skin was cut

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. off with a pair of sterile scissors, leaving the subdermis intact. Donor skin was then placed into this wound, a dry dressing placed on top, and the mouse bandaged around the chest with elastoplast. The dressing was removed on the eighth day for daily inspection of graft survival. Grafts with less than 10% surviving epithelium were considered to be rejected.

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CHAPTER III

IN-VITRO SCREENING FOR IMMUNOPHARMACOLOGICAL ACTIVITY

THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

INTRODUCTION

The proliferative response of lymphocyte cultures in the presence of mitogens is generally regarded as the in-vitro correlate of immune responsiveness (Ling & Kay, 1975). It is also evident from the review in Chapter I that most immunosuppressive drugs are active on the proliferative phase of the immune response. It was also discussed in the same chapter that antimicrobial agents that interfere with membrane function, protein synthesis and nucelic acid synthesis are much more likely to have immunosuppressive properties. By the same token, antimicrobial agents which interfere with bacterial cell wall synthesis are not likely to cause immunosuppression, because mammalian cells are not equipped with peptidoglycan cell walls. These deductions are well borne out by in-vitro experiments presented in this chapter.

Penicillin

Chain and Florey in 1940 followed up on the 1929 observation by Fleming that cultures of the mould Penicillium notatum have inhibitory effects on growth of streptococci, resulting in the ready availability of penicillin for clinical use. A number of semi-synthetic deviatives with advantages over penicillin G such as resistance to penicillinase, e.g. methicillin, and broad spectrum antimicrobial activity e.g. ampicillin, are also available. Conventional dosages produce therapeutic blood levels ranging from 0.6 to 6.0μg/ml. It can be seen from table III-1 that penicillin

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G, methicillin, ampicillin and carbenicillin, at concentrations of up to 10μg/ml did not affect mitogen-induced lymphocyte proliferative responses.

Cephalosporins

The fungus Cephalosporium acremonium produces a number of antibiotics with 7-aminocephalosporanic acid as a common nucleus. This group of antibiotics is resistant to penicillinase and has a broader spectrum of antibacterial activity. A large number of cephalosporins are now available. Therapeutic blood level is in the region of 1-10μg/ml. Studies with one of the analogues, cephalothin (table III-1) showed no inhibition of lymphocyte response to mitogens at these concentrations.

Tetracyclines

Chlortetracycline was the first of the tetracyclines to be isolated. A number of natural and semi-synthetic tetracycline analogues are now available for clinical use. They are among the most generally useful of antimicrobial agents. Their very broad spectrum of activity include Gram positive and Gram negative bacteria, anaerobes, rickettsiae, mycoplasmas, chlamydiae and protozoa. Therapeutic blood level is around 5-8μg/ml. Inhibition of mitogen-induced lymphocyte transformation was apparent at a concentration of 4μg/ml (table III-2).

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Table III-1. Effect of penicillin analogues and cephalosporins on mitogen-induced lymphocyte transformation.

Drug concentrations (μg/ml)	Cpm ^{3}H-thymidine uptake (mean ±S.D.)		
	No mitogens	PHA	PWM
Penicillin G			
0	488 ± 89	25,564 ± 2,835	11,009 ± 1,428
1	493 ± 125	26,641 ± 984	10,836 ± 280
4	330 ± 68	25,883 ± 1,238	11,861 ± 1,659
10	439 ± 99	25,775 ± 719	11,568 ± 865
Methicillin			
0	488 ± 89	25,564 ± 2,835	11,009 ± 1,428
1	492 ± 96	25,656 ± 1,705	13,521 ± 1,251
4	365 ± 70	25,624 ± 2,192	11,071 ± 1,260
10	542 ± 41	27,326 ± 7,063	10,662 ± 1,008
Ampicillin			
0	445 ± 74	29,352 ± 6,162	12,322 ± 1,578
1	380 ± 33	27,907 ± 761	11,264 ± 1,124
4	491 ± 42	26,236 ± 598	11,348 ± 527
10	503 ± 67	28,882 ± 1,616	11,878 ± 1,597
Carbenicillin			
0	445 ± 74	29,352 ± 6,162	12,322 ± 1,578
1	493 ± 140	22,189 ± 1,105	9,676 ± 1,481
4	384 ± 7	20,384 ± 2,077	10,911 ± 722
10	475 ± 42	28,001 ± 759	12,394 ± 932
Cephalothin			
0	361 ± 74	28,716 ± 3,131	10,576 ± 357
1	461 ± 114	26,153 ± 626	9,844 ± 683
4	339 ± 15	27,405 ± 1,301	11,068 ± 909
10	590 ± 166	23,904 ± 4,040	11,412 ± 1,574

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Chloramphenicol

Chloramphenicol is produced by Streptomyces venezuelae. Commercial production is by a completely synthetic process. It has a broad spectrum of antimicrobial activity including many bacteria and rickettsiae. It is clinically indicated for infections with Salmonella typhi, Haemophilus influenzae and Gram negative septicaemia. Therapeutic blood levels are in the region of 8-10 g/ml. At this concentration, little effect on proliferative responses of lymphocytes to mitogens could be detected (table III-2).

Erythromycin

Erythromycin is one member of a group of antibiotics known as macrolides, other examples of which are oleandomycin and spiramycin. It is most active against Gram positive organisms, but Neisseria, Haemophilus, Mycoplasma and Chlamydia may also be sensitive. Therapeutic blood level is usually around 2µg/ml. No effect on mitogen-induced lymphocyte transformation was seen at a concentration of up to 10µg/ml (table III-2).

Lincomycin

Lincomycin and clindamycin belong to the group of antibiotics known as lincosamines. Their spectrum of antimicrobial activity is similar to that of the macrolide antibiotics. Therapeutic blood levels vary between 2-5µg/ml. Even at the concentration of 10µg/ml, no inhibitory effect on mitogen-induced lymphocyte

Table III-2. Effect of tetracyclines, chloramphenicol, erythromycin and lincomycin on mitogen-induced lymphocyte transformation.

Drug concentrations (µg/ml)	Cpm ^{3}H-thymidine uptake (mean ±S.D.)		
	No mitogens	PHA	PWM
Tetracycline			
0	601 ± 81	18,644 ± 5,117	42,092 ± 7,553
1	560 ± 111	17,749 ± 6,478	35,990 ± 10,425
4	539 ± 117	13,888 ± 5,063	31,569 ± 8,644
10	414 ± 89	12,042 ± 5,349	32,030 ± 8,864
Chloramphenicol			
0	472 ± 154	56,661 ± 3,374	23,423 ± 2,545
1	558 ± 106	46,427 ± 1,550	20,334 ± 868
4	768 ± 220	43,129 ± 3,553	21,294 ± 1,452
10	778 ± 155	48,818 ± 1,520	21,884 ± 1,335
Erythromycin			
0	369 ± 57	28,496 ± 3,837	10,673 ± 939
1	441 ± 55	26,254 ± 1,936	9,932 ± 516
4	357 ± 46	27,522 ± 2,829	11,154 ± 488
10	331 ± 51	29,788 ± 1,859	10,837 ± 568
Lincomycin			
0	361 ± 74	28,716 ± 3,131	10,576 ± 357
1	430 ± 100	25,897 ± 1,969	12,178 ± 779
4	410 ± 98	24,970 ± 1,183	11,653 ± 787
10	409 ± 67	26,585 ± 5,015	13,270 ± 1,116

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transformation was observed (table III-2).

Aminoglycosides

The aminoglycoside antibiotics include streptomycin, kanamycin, gentamycin, tobramycin, amikacin and several others. The first of of the group, streptomycin was isolated by Waksman and colleagues in 1944 from streptomyces griseus. The antimicrobial spectrum of streptomycin includes Gram negative bacteria and mycobacterium tuberculosis. Gentamycin, which has additional activity against Gram positive organisms, can reach blood levels of 3-7µg/ml after conventional dosage schedules. At this concentration range, no effect was observed on mitogen-induced lymphocyte proliferative responses (table III-3).

Polyene Antibiotics

Nystatin and amphotericin B are polyene antibiotics with antifungal activity. Amphotericin B has clinical importance in the treatment of deep mycotic infections. Therapeutic blood levels range from 0.5-2µg/ml. Significant reduction of mitogen-induced lymphocyte proliferation was detectable at a concentration of 1µg/ml (table III-3).

Polymyxins

Polymyxins B and E are the only members of this group of basic polypeptide antibiotics with clinical application. They are

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Table III-3. Effect of gentamycin, amphotericin B, polymyxin E and sulphioxazole on mitogen-induced human lymphocyte transformation.

Drug Concentration (μg/ml)	Cpm ^{3}H-thymidine uptake (mean ±S.D.)		
	No mitogens	PHA	PWM
Gentamycin			
0	560 ± 98	81,526 ± 6,881	22,226 ± 647
1	721 ± 213	79,622 ± 15,324	20,362 ± 1,682
4	652 ± 167	86,202 ± 11,569	27,018 ± 5,358
10	589 ± 122	73,907 ± 14,211	24,235 ± 4,172
Amphotericin B			
0	1,168 ± 253	57,496 ± 2,012	24,916 ± 2,868
1	-	47,695 ± 4,384	19,959 ± 2,077
5	-	19,529 ± 3,942	2,841 ± 408
10	-	1,076 ± 187	965 ± 183
Polymyxin E			
0	606 ± 21	63,041 ± 7,209	25,091 ± 1,471
1	632 ± 38	63,490 ± 3,671	26,947 ± 2,023
4	378 ± 69	55,217 ± 5,734	22,083 ± 1,637
10	483 ± 74	30,370 ± 5,636	6,555 ± 283
Sulphioxazole			
0	1,397 ± 238	28,721 ± 8,785	7,539 ± 576
60	1,313 ± 414	19,295 ± 1,290	6,025 ± 834
120	345 ± 117	18,038 ± 936	4,751 ± 531

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particularly active against pseudomonas and coliform bacteria.
Therapeutic blood level is in the range of $1-4\mu g/ml$. Little
reduction in mitogen-induced lymphocyte transformation was observed
at the concentration of $4\mu g/ml$ (table III-3).

Sulphonamides and sulphones

Up to one hundred and fifty sulphonamide analogues have been
developed since its first introduction by Domagk in 1935. They
are active against Gram negative and Gram positive bacteria, nocardia,
chlamydia and protozoa. Therapeutic blood level is in the region
of $80-100\mu g/ml$. No effect on mitogen-induced lymphocyte
transformation was seen at a concentration of $60\mu g/ml$ (table III-3)
of sulphioxazole, and a slight affect at $120\mu g/ml$.

Diaminopyrimidines

The diaminopyrimidines were developed as a result of the search
for anti-malarial compounds during the second world war. Proguanil
was introduced in 1946 but had the disadvantage of inducing easy
development of resistant strains. At the present time, pyrimethamine
and trimethoprim are the most clinically useful of the
diaminopyrimidine compounds. Trimethoprim together with
sulphamethoxazole in a fixed-dose combination has broad spectrum
anti-bacterial activity. Pyrimethamine together with sulphadoxine
in a fixed-dose combination is useful in treatment of falciparum
malaria. Pyrimethamine by itself is useful in the treatment of

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toxoplasmosis. It can be seen that trimethoprim, at the concentration of 1 and 10μg/ml, has no effect on mitogen-induced lymphocyte transformation (table III-4). In contrast, pyrimethamine at concentrations of 1 to 10μg/ml, enhanced the lymphocyte response to mitogens (Chapter VII).

Quinine, chloroquine, primaquine and quinacrine

These are very useful antimalarial drugs. Quinine is an alkaloid derived from the bark of the South American native cinchona tree. Although used for the treatment of malaria as early as the seventeenth century, it was not specifically prescribed for the treatment of malaria until the nineteenth century when the diagnosis of malaria could be established by microscopic examination. It was superceded by synthetic anti-malarials in the early 1960's but the emergence of chloroquine-resistant strains has brought back quinine into frequent use. Chloroquine is a synthetic 4-aminoquinoline compound, very useful for the treatment of malaria. Primaquine is an 8-aminoquinoline with a different mechanism of action from quinine and chloroquine which bind to DNA; rather, it undergoes biotransformation into oxidants. It can be seen from table III-4 that at the concentrations of 1 to 10μg/ml which can be easily achieved with conventional dosage of these drugs, the proliferative response of lymphocytes to mitogens was significantly depressed.

Quinacrine is an acridine dye which binds to DNA. It has been used extensively in the chemoprophylaxis of malaria. It

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Table III-4. Effect of trimethoprim, quinine, chloroquine, primaquine and quinacrine on mitogen-induced lymphocyte transformation.

Drug concentration (µg/ml)	Cpm ^{3}H-thymidine uptake (mean ±S.D.)		
	No mitogens	PHA	PWM
Trimethoprim			
0	708 ± 101	28,254 ± 4,259	14,495 ± 1,429
1	-	21,045 ± 1,804	11,908 ± 429
10	-	26,229 ± 2,521	12,921 ± 684
Quinine			
0	522 ± 91	32,408 ± 13,549	20,180 ± 4,670
1	503 ± 70	25,033 ± 10,520	18,567 ± 3,442
4	472 ± 115	19,841 ± 11,043	15,900 ± 4,317
10	591 ± 98	16,416 ± 10,615	13,390 ± 4,097
Chloroquine			
0	422 ± 30	54,782 ± 4,909	25,072 ± 1,036
1	419 ± 11	47,960 ± 2,608	20,870 ± 2,430
4	326 ± 10	50,465 ± 2,942	23,702 ± 1,199
10	500 ± 158	39,732 ± 8,311	18,358 ± 2,182
Primaquine			
0	532 ± 201	35,449 ± 2,465	13,544 ± 5,389
1	784 ± 187	28,370 ± 4,658	10,519 ± 3,828
4	525 ± 251	18,207 ± 3,047	6,585 ± 2,865
10	428 ± 46	11,570 ± 5,498	3,495 ± 1,432
Quinacrine			
0	461 ± 110	58,352 ± 1,506	21,458 ± 2,238
1	249 ± 46	37,340 ± 2,163	16,220 ± 5,467
4	238 ± 32	125 ± 26	156 ± 28
10	271 ± 158	269 ± 42	186 ± 87

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use for treatment of tapeworm infestation has been superceded by less
toxic agents. Suppression of mitogen-induced lymphocyte trans-
formation can be demonstrated in-vitro (table III-4).

The imidazoles

Several of the imidazole derivatives have found useful clinical
applications. Metronidazole is a nitroimidazole first introduced
in 1959 for the treatment of trichomoniasis, and later proved
effective for the treatment of amoebiasis and anaerobic infections.
Tinidazole is another nitroimidazole with anti-amoebic properties.
Miconazole is an imidazole derivative recently introduced for the
treatment of systemic fungal infections.

Metronidazole has been shown to be carcinogenic in experimental
animals and mutagenic in bacteria. Tinidazole has been shown to
have immunosuppressive properties. It would appear that miconazole
may be similarly predisposed. The results from studies on
lymphocyte transformation (table III-5) indicate that it has a
potent inhibitory effect.

Nitrofurantoin

This is a furan derivative with a nitro group in the 5 position.
Furans have broad spectrum antibacterial activity, but this activity
is reduced markedly in the presence of blood and serum. Hence the
main use of nitrofurantoin is in the treatment of urinary tract
infections. No effect of this drug on mitogen-induced lymphocyte
transformation was observed (table III-5).

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Table III-5. Effect of miconazole, nitrofurantoin, pentamidine and
isoniazid on mitogen-induced lymphocyte transformation.

Drug concentration (μg/ml)	Cpm ^{3}H-thymidine uptake (mean ±S.D.)		
	No mitogen	PHA	PWM
Miconazole			
0	1,382 ± 989	30,873 ± 2,121	20,610 ± 6,365
1	−	26,105 ± 1,092	5,661 ± 354
10	−	238 ± 18	134 ± 15
Nitrofurantoin			
0	382 ± 49	90,759 ± 4,093	27,010 ± 3,289
1	451 ± 69	83,888 ± 3,245	23,474 ± 749
4	386 ± 83	86,219 ± 4,982	23,123 ± 2,054
10	561 ± 146	94,631 ± 4,743	18,354 ± 491
Pentamidine			
0	502 ± 54	44,548 ± 3,946	17,683 ± 1,339
1	343 ± 38	38,274 ± 1,251	15,311 ± 1,536
4	293 ± 80	24,630 ± 508	7,972 ± 475
10	289 ± 28	4,687 ± 656	1,041 ± 59
Isoniazid			
0	804 ± 67	35,210 ± 1,795	15,865 ± 995
1	−	33,342 ± 1,069	13,294 ± 1,310
5	−	34,866 ± 1,784	10,577 ± 909
10	−	35,393 ± 4,606	11,640 ± 982

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Pentamidine

Pentamidine is an aromatic diamidine synthesized for the treatment of trypanosomiasis. It has additional uses in the treatment of North American blastomycosis and Pneumocystis carinii pneumonia. A potent effect on mitogen-induced lymphocyte transformation was observed (table III-5).

Isoniazid

Isoniazid, a hydrazide of isonicotinic acid, is a valuable drug for the treatment of tuberculosis. The exact mechanism of action is unknown, but it appears to interfere with the biosynthesis of nucleic acid and mycolic acid. Therapeutic blood levels vary with genetic disposition. In slow inactivators, the drug can reach $1\mu g/ml$, whereas $0.2\mu g/ml$ is the usual level in rapid inactivators. The results show that even up to the concentration of $10\mu g/ml$, no effect of this drug on mitogen-induced lymphocyte transformation could be detected (table III-5).

Conclusion

The results of screening tests on 25 antimicrobial drugs show that at least five groups deserve further study. There are (1) the tetracyclines (2) the antifungal agents such as amphotericin B and miconazole, (3) the antimalarials such as quinine, chloroquine, primaquine, quinacrine (4) the diaminopyrimidines such as pyrimethamine and trimethoprim, (5) the aromatic diamidine, pentamidine.

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No screening programme is foolproof, nor is this programme designed to be exhaustive. Only commonly used antimicrobial drugs, or those considered to have clinical importance and personal interest have been included. Nevertheless, this screening study is sufficiently wide-ranging to have identified most of the antimicrobial drugs with immunomodulating potential.

A decision was made to study the first four groups mentioned above in greater detail for presentation in this thesis. These in-depth studies, both in-vitro and in-vivo, will be presented in the next five chapters.

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CHAPTER IV

IMMUNOPHARMACOLOGY OF TETRACYCLINES

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INTRODUCTION

The tetracyclines are extremely useful broad spectrum antimicrobial agents. The first of the tetracyclines to be isolated was chlortetracycline, obtained from streptomyces aureofaciens in 1948. The next, oxytetracycline, was obtained from S. rimosus in 1950. Catalytic dehalogenation of chlortetracycline yielded tetracycline in 1953. Since then, many more analogues have been made available by semi-synthetic manufacturing processes. Some of the newer analogues have pharmacological advantages over those introduced much earlier. Doxycycline for instance is better absorbed from the gastrointestinal tract, penetrates better in the tissues, has lower toxicity and does not accumulate in renal failure (Symposium on doxycycline, 1976).

All the tetracycline analogues have a basic four-ring structure illustrated in figure IV-1. They are crystalline amphoteric substances sparingly soluble in water. The hydrochloride salts have better solubility, and are more stable except for chlortetracycline. They bind strongly to divalent metal ions. Because of these properties, most tetracyclines are not well absorbed when given orally. Their continued presence in the gastrointestinal tract leads to changes in the intestinal flora, so that overgrowth of candida and other microbes can be a problem. At a conventional dosage schedule of two grams daily, blood levels of $5-8 \mu g/ml$ can be reached, but penetration into joint fluids and cerebrospinal fluid is much lower.
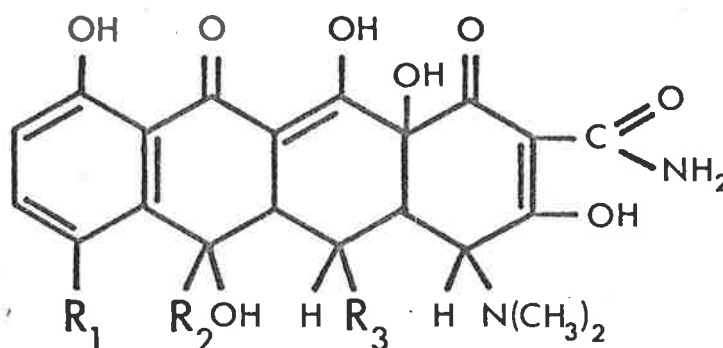
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Figure IV-1. Basic structure of the tetracyclines. They differ only with regard to the side chains at the R_1, R_2 and R_3 positions. For instance, chlortetracycline has the following side chains: R_1 = -cl, R_2 = -CH$_3$, R_3 = -OH. For oxytetracycline, it is: R_1 = -H, R_2 = -CH$_3$, R_3 = -OH. Whereas tetracycline has: R_1 = -H, R_2 = -CH$_3$, R_3 = -H. Doxycycline is slightly different: R_1 = -H, R_2 = -CH$_3$, R_3 = -OH; these substitutions are similar to oxytetracycline, except that doxycycline has no -OH at position 6.

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Higher concentrations of tetracycline in these fluids can be achieved by means of direct instillation of parenteral preparations.

Tetracyclines are indicated for the treatment of a wide variety of infectious diseases. They are the drugs of choice for cholera, mycoplasma and chlamydia infections. They are useful for the treatment of anaerobic infections in the chest, abdomen and genitourinary tract. They may be life-saving in a number of unusual infections including brucellosis, plague, tularaemia, ornithosis, Rocky Mountain Spotted Fever, scrub typhus and other typhus fevers, and falciparum malaria. The current position of tetracycline has been reappraised (The New York Academy of Medicine, 1978).

Treatment with tetracycline is associated with a number of side effects. Gastrointestinal complications are most common; these include nausea, vomiting and diarrhoea; candidiasis and staphlococcal enterocolitis are also encountered. They can cause hepatic injury, particularly in high dosages. Renal tubular acidosis has been reported with the use of outdated preparations. They also deposit in bones and teeth of young children. They cross the placenta easily; teratogenic effects have been demonstrated in experimental animals and observed in a few human infants. Allergic reactions to tetracyclines are not common and take the form of drug fever and skin rashes.

Little is known about the immunopharmacological properties of tetracyclines. A few reports have commented on the ability of

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tetracyclines to suppress neutrophil chemotaxis (Munoz and Geister,
1950; Forsgren et al, 1974), phagocytosis and metabolic activity
(Rubinstein and Pelet, 1973; Hill et al, 1974), as well as the
bactericidal effect of serum (Forsgren and Gnarpe, 1973).

Evidence for suppression by tetracycline analogues of both
in-vitro (Thong and Ferrante, 1979a) and in-vivo (Thong and Ferrante,
1980a) immunological responses are presented in the next two sections.

IN-VITRO STUDIES

Preliminary studies have established that the tetracycline
analogues, at concentrations used in these experiments, were not toxic
to lymphocytes for up to 4 days in culture, as assessed by trypan blue
dye exclusion (Thong and Ferrante, 1979a).

Effect of varying doses of tetracyclines on mitogen-stimulated lymphocytes

Among the three tetracycline analogues, doxycycline was found to
produce the greatest suppression of ^{3}H-thymidine uptake in both
PHA-stimulated and PWM-stimulated cultures (tables IV-1 and IV-2).
At a concentration of 10μg/ml, percentage inhibition in PHA-stimulated
cultures was 94.4 in the presence of doxycycline, compared to 24.0 in
tetracycline-treated and 23.0 in oxytetracycline-treated cultures.
The corresponding values for PWM-stimulated cultures were 92.2, 36.2
and 13.6 respectively.

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Table IV-1. Effect of tetracycline analogues on PHA-induced human lymphocyte proliferative responses

Drug concentration (μg/ml)	Tetracycline Ct/min ^{3}H-thymidine uptake	Doxycycline Ct/min ^{3}H-thymidine uptake	Oxytetracycline Ct/min ^{3}H-thymidine uptake
1	35,990 ± 10,425	34,592 ± 10,541	35,696 ± 6705
4	31,569 ± 8644*	26,849 ± 8299[†]	30,749 ± 8977*
10	32,030 ± 8864*	2282 ± 560[†]	31,945 ± 8395*

Results represent mean ±s.d. of nine experiments using cells from nine different donors. Uptake of ^{3}H-thymidine in control cultures was 42,288 ± 8127 ct/min (mean ±s.d.).

* p < 0.01.
† p < 0.001.

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Table IV-2. Effect of tetracycline analogues on PWM-induced human lymphocyte proliferative responses.

Drug concentration (µg/ml)	Tetracycline Ct/min ^{3}H-thymidine uptake	Doxycycline Ct/min ^{3}H-thymidine uptake	Oxytetracycline Ct/min ^{3}H-thymidine uptake
1	17,749 ± 6478	17,834 ± 7041	20,975 ± 9468
4	13,888 ± 5063*	12,378 ± 4193*	17,217 ± 7602
10	12,042 ± 5349	1423 ± 1071	16,295 ± 7537

Results represent mean ±s.d. of nine experiments using cells from nine different donors. Uptake of ^{3}H-thymidine in control cultures was 19,358 ± 9665 ct/min (mean ±s.d.).

* $p < 0.01$.
† $p < 0.001$.

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The inhibitory effect of doxycycline was dose-dependent (tables
IV-1 and IV-2).   This effect was found to be due to a direct
inhibition of blast transformation.   At 10µg/ml concentration, the
blastogenic index in PHA-stimulated cultures was 0.5 in the presence
of doxycycline compared to 26.1 in control;   in PWM-stimulated cultures,
it was 2.1 in the presence of doxycycline compared to 17.0 in controls.

Effect of delayed addition of doxycycline on mitogen-stimulated lymphocytes

Some characteristics of the inhibitory effect of doxycycline on
mitogen-induced lymphocyte proliferative responses were delineated
with further experiments.   It was found that marked suppression of
lymphocyte transformation occurred even when doxycycline was added
48 hours after the start of experiments (table IV-3).   This would
suggest that doxycycline has a maximum effect on blast cells, which
are present 48 hours after mitogen-stimulation, although its possible
effects on the early phases, such as mitogen-receptor binding,
glycolysis and RNA-synthesis cannot be excluded.

Effect of inhibition of mitogen-stimulated lymphocytes by doxycycline

Doxycycline may act by binding to cell receptors in irreversible
fashion.   This possibility was studied by incubating lymphocytes with
doxycycline for one hour prior to culture.   The inhibitory effect of
doxycycline could be reversed completely by washing (table IV-4),
suggestive of a lack of tight binding to receptors.

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Table IV-3. Effect of immediate and delayed addition of doxycycline on mitogen-induced human lymphocyte proliferative responses.

Time of addition of doxycycline (10μg/ml)	PHA ct/min ^{3}H-thymidine uptake	PWM ct/min ^{3}H-thymidine uptake
0	3102 ± 198	1100 ± 170
24 hrs	3808 ± 575	492 ± 38
48 hrs	5489 ± 683	1508 ± 419
None added	36,946 6601	16,063 3252

Results represent mean ±s.d. of three experiments using three different donors.

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Table IV-4. Reversibility of mitogen-induced human lymphocyte responses by doxycycline.

Treatment (doxycycline 10μg/ml)	PHA ^{3}H-thymidine uptake	PWM ^{3}H-thymidine uptake
Unwashed	2158 ± 631	1377 ± 660
Washed	22,618 ± 3519	11,042 ± 1619
Untreated control	26,922 ± 4236	13,633 ± 2754

Results represent mean ±s.d. of triplicate samples.

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IN-VIVO STUDIES

The highest daily dosage of 100 mg/kg used in these experiments was that needed for the successful treatment of experimental infections in mice and found to be well tolerated (Thong et al, 1978b).

Effect of doxycycline on DTH

Doxycycline was chosen for the first set of experiments to study dose and time-related activity on DTH responses to SRBC in mice. For these experiments, mice were divided into 4 treatment groups of 0, 30, 60 or 120 mg/kg doxycycline i.p. as a single dose. Each group was again divided into 3 lots. One lot received doxycycline 2 days prior to priming with SRBC, the second lot on the day of priming and the third on the day of challenge. The results showed thatthere was no effect on DTH when administered 2 days prior to priming (table IV-5). In contrast, there was significant reduction of DTH responses when doxycycline was given on the day of challenge. The increase in footpad thickness was 31.1, 25.6 and 11.7% in mice receiving 30, 60 and 120 mg/kg respectively, compared to 42.0% in controls. When given on the day of priming, suppression of DTH was only observed at high dose (120 mg/kg). It was concluded from this set of experiments that doxycycline was most effective in suppressing DTH at the manifestation phase of the immune response.

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Table IV-5. Effect of Doxycycline administered at various times on the DTH response to SRBC in mice.

Doxycycline dosage (mg/kg)	Day of Administration (% footpad increase ± S.E.)		
	-2	0	+5
0	43.8 ± 3.3	48.6 ± 3.8	42.0 ± 2.1
30	38.4 ± 3.1	44.5 ± 3.6	*31.1 ± 3.5
60	34.7 ± 2.2	38.0 ± 2.4	**25.6 ± 3.3
120	35.2 ± 2.8	*35.6 ± 1.8	†11.7 ± 2.1

Each experimental group consisted of 6 mice.
Doxycycline was administered at various dosages as a single i.p. injection on the day indicated. At day 0, the mice received 1×10^8 SRBC s.c. They were challenged with 1×10^8 SRBC in the hind footpad on day 5 and measured on day 6.

* p < 0.05.   ** p < 0.01. † p < 0.001.

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Effect of tetracycline analogues on manifestation phase of DTH

To discover whether other tetracycline analogues have similar capacity to suppress DTH, another set of experiments was performed with these drugs injected at the manifestation phase of DTH. The results showed that significant reduction (30-45%) of DTH occurred in groups treated with doxycycline, rolitetracycline and tetracycline (table IV-6). A similar degree of reduction occurred in the groups receiving oxytetracycline, but this did not approach statistical significance because of the wide variation in values.

Effect of doxycycline on macrophage chemotaxis

The manifestation phase of DTH not only depends on the integrity of sensitized lymphocytes, but also on the ability of macrophages to infiltrate subcutaneous tissue. Previous studies on mitogen-induced lymphocyte transformation suggested that sensitized lymphocytes are affected by tetracyclines. The present set of experiments were designed to evaluate the influence of these drugs on macrophage chemotaxis. For these experiments, mice were divided into 4 groups. Three groups received a single i.p. injection of either 25, 50 or 100 mg/kg doxycycline 24 hr prior to PHA-injection, while the fourth received i.p. saline and served as control. The results (table IV-7) showed that PHA-induced macrophage influx was significantly depressed in mice receiving 100 mg/kg doxycycline (3.4×10^6) compared to control (6.1×10^6).

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Table IV-6. Effect of tetracycline analogues on the manifestation phase of DTH response to SRBC in mice.

Tetracycline (100 mg/kg)	% increase footpad thickness (mean ± S.E.)
No drug	62.1 ± 7.4
Doxycycline	37.2 ± 3.6*
Rolitetracycline	33.6 ± 5.5*
Tetracycline	38.5 ± 6.9**
Oxytetracycline	39.2 ± 9.8

There were 6 mice per experimental group.
They received 1×10^8 SRBC s.c. at day 0, challenged with the same number SRBC in the footpad at day 5 and measured for DTH at day 6.
Tetracyclines were given as a single i.p. injection at day 5.

* $p < 0.01$. ** $p < 0.02$.

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Table IV-7. .Effect of doxycycline on PHA-induced macrophage accumulation in mouse peritoneal cavity.

Doxycycline dosage (mg/kg)	0	25	50	100
Macrophage influx (x 10^6)	6.2 ± 1.0	7.8 ± 1.0	4.4 ± 1.2	3.4 ± 0.8*

* $p < 0.05$

There were 11-12 mice per experimental group. The mice received i.p. doxycycline 24 hr prior to i.p. injection of PHA ($35\mu g$/mouse). Peritoneal washouts were performed 48 hours after PHA-injection. The values represent the mean (± S.E.) number of macrophages present in the peritoneal cavity in PHA-injected mice minus the number of macrophages (2.2×10^6) present in mice not given PHA.

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Effect of daily injections of doxycycline on antibody and DTH responses

In the clinical situation, tetracyclines are usually prescribed for several days. We therefore studied the effects of multiple doses of doxycycline on DTH and antibody responses to SRBC. The results confirmed previous findings that doxycycline has potent suppressive action on DTH (table IV-8). In contrast, there was no significant effect on antibody responses.

CONCLUSIONS

The results of the present studies indicate that tetracyclines, and in particular doxycycline, have potent suppressive effects on DTH in mice. The daily dose of 100 mg/kg used in these experiments is the usual regime for therapy of experimental infections in mice (Thong et al, 1978b), and is well tolerated. At the same dosage scehdule, antibody responses were more resistant to the influence of tetracyclines.

The most probable site of action of tetracycline appears to be on the effector cells of the DTH response. This interpretation is supported by three lines of evidence. Firstly, suppression of DTH was more pronounced when tetracyclines were administered during the manifestation phase of DTH, rather than at the induction phase. Secondly, a marked reduction in influx of macrophages into the peritoneal cavity occurred after tetracycline treatment. Thirdly, in-vitro studies showed that tetracyclines have direct action

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Table IV-8. Effect of multiple doses of doxycycline on antibody and DTH
responses to SRBC in mice.

Treatment	Anti-SRBC titer (log$_2$ mean ± S.E.)	% increase in footpad thickness (mean ± S.E.)
Nil	8.2 ± 0.3	38.9 ± 2.5
Doxycycline	7.7 ± 0.3	16.7 ± 2.8*

* p < 0.001.

There were 8-9 mice per experimental group. Mice were primed with
1 x 10^8 SRBC s.c. for DTH studies, and 1 x 10^9 i.v. for antibody studies
at day 0. Treated groups received 100 mg/kg doxycycline i.p. daily
until day 5, while controls received saline only. DTH groups were
challenged at day 5 and read at day 6. Antibody groups were bled at day
6. The treatment regime was well tolerated.

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on blast cells, since their suppressive effect on lymphocyte
transformation was just as potent when added 48 hours after the start
of cultures, a time when blast transformation had already occurred.

The mechanism by which tetracyclines adversely affect immune
function is not well understood.   This may be related to its metabolic
effects on microorganisms.   In this respect, its principal mode of
action appears to be inhibition of protein synthesis by binding to
the 30s ribosome to prevent the attachment of RNA (Gale et al, 1972).
At higher concentrations of 50-100μg/ml, tetracyclines also inhibit
DNA synthesis and alter membrane properties of Escherichia coli and
Bacillus subtilis (Pato, 1977);   the relevance of this observation
in human lymphocytes is not clear, since the effects on human
lymphocytes were seen with low concentrations of 1-10μg/ml.

CHAPTER V

IMMUNOPHARMACOLOGY OF MICONAZOLE

THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

INTRODUCTION

Miconazole is a recently introcuded broad spectrum antifungal agent with potential for the treatment of systemic mycotic infections (Van Cutsem and Thienpont, 1972; Hoeprich and Goldstein, 1974; Stevens, Levine and Deresinski, 1976). Blastomyces brasiliensis, Blastomyces dermatitidis, and Histoplasma capsulatum are sensitive in vitro to ≤ 0.1µg/ml. Madurella mycetomi, Coccidioides immitis, Paracoccidioides brasiliensis and Actinomyces israelii are sensitive to 0.1-1.0µg/ml. Cryptococcus neoformans, Nocardia asteroides, Sporothrix schenckii, Candida albicans, Aspergillus fumigatus and Gram-positive bacteria are sensitive to 1-10µg/ml. The mechanism of antimicrobial activity is not well understood. At low concentrations, it appears to act on the cell membrane to interfere with uptake of purines and glutamine. At higher concentrations it appears to affect the structure of microsomal membranes.

It is a synthetic imidazole deviative with a molecular weight of 479 daltons (figure V-1). It is practically insoluble in water and very stable. It is well absorbed from the gastrointestinal tract. An intravenous preparation is also available, consisting of 10 mg/ml of miconazole in 0.115 ml polyethoxylated castor oil, 1.0 mg lactic acid, 0.5 mg methylparaben, 0.05 mg propylparaben made up in water. This carrier solution has been reported to cause hyperlipidaemia, phlebitis and haematological abnormalities (Bagnarello et al, 1977; Sung and Grendahl, 1977). Miconazole
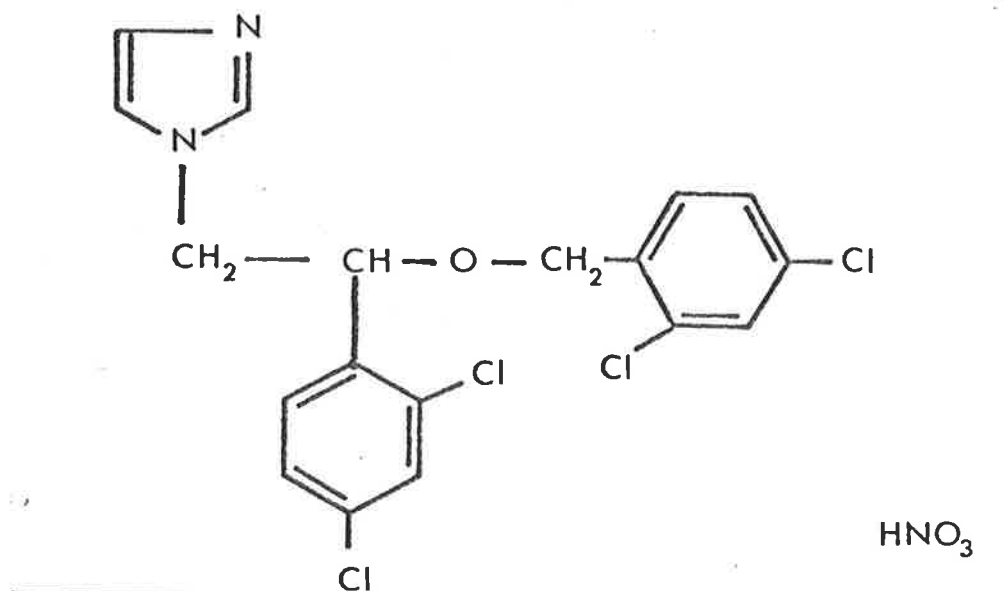
Figure V-1. Structural formula of miconazole nitrate. The chemical formula is 1-[2,4 -dichloro-β-(2,4-dichlorobenzyloxy) phenethyl]imidazole nitrate.

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itself is well tolerated.   It is metabolised in the liver and
excreted via the gastrointestinal tract.   Blood levels of 1-2µg/ml
by oral administration, and 1-10µg/ml by intravenous infusion, can
be reached at therapeutic doses.

IN-VITRO STUDIES

A stock solution of miconazole was prepared by disolving 5 mg
of the nitrate salt in 1 ml propylene glycol and stored at 4°C.
Further dilutions were made in RPMI-1640 medium and the pH maintained
at close to 7.4.   Preliminary studies showed that lymphocyte
Viability, as assessed by trypan blue dye exclusion was not affected
by miconazole in the concentrations used in these experiments for as
long as 4 days in culture (Thong and Rowan-Kelly, 1977).   The
concentrations of propylene glycol used in these experiments had
also been previously determined not to affect lymphocyte transformation.
In the next three sets of experiments reported here, appropriate
concentrations of this diluent were added to control cultures.

Effect of varying concentrations on lymphocyte transformation

A pronounced dose-dependent suppression of mitogen-induced
lymphocyte transformation was observed (table V-1).   At miconazole
concentrations of 1,5 and 10µg/ml, percentage inhibition was 12.6,
67.0 and 99.1 for PHA-stimulated cultures and 26.0, 76.2 and 97.8
for PWM-stimulated cultures, respectively.

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Table V-1. Effect of varying concentrations of miconazole on mitogen-induced lymphocyte proliferative responses.

MICONAZOLE (µg/ml)	PHA cpm ^{3}H-thymidine uptake	PWM cpm ^{3}H-thymidine uptake
0	43,388 ± 15,395	14,965 ± 6,902
1	36,668 ± 9,449*	9,808 ± 4,472**
5	15,959 ± 6,749[†]	3,268 ± 1,146[†]
10	387 ± 272[†]	236 ± 71[†]

The results represent mean ± S.D. of 7 experiments using 7 donors.

* $p < 0.05$. † $p < 0.001$. ** $p > 0.05$.

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Reversibility of inhibition by miconazole

Washing off miconazole after incubating with lymphocytes for 1 hour did not abrogate the inhibitory effect of this drug on lymphocyte transformation (table V-2). Percentage inhibition was 99.2 in washed cultures, and 99.6 in unwashed cultures.

Effect of delayed addition on lymphocyte transformation

There was a progressive reduction in percentage inhibition when miconazole was added at various time intervals after the initiation of cultures. Percentage inhibition was 91.8 at 4 hours, 57.5 at 24 hours and only 10.5 at 48 hours after the start of experiments (table V-3).

IN-VIVO STUDIES

In these experiments, miconazole nitrate was administered as a suspension in 0.3 ml of distilled water by oro-gastric tube (Thong and Ferrante, 1979b) or by i.p. injection (Thong, Ferrante and Secker, 1980a). The highest dosage used in these studies (300 mg/kg) was well below the LD50 dose of 578 mg/kg (Van Cutsem and Thienpont, 1972) and did not produce toxicity. Control mice received distilled water only.

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Table V-2. Non-reversible inhibition of mitogen-induced lymphocyte
proliferation by miconazole.

Miconazole Treatment (10μg/ml)	cpm ^{3}H-thymidine uptake PHA
Unwashed	173 ± 53
Washed	330 ± 175
Untreated control	41,577 ± 5,699

Results expressed as mean ±S.D. of triplicate samples.

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Table V-3. Effect of immediate and delayed addition of miconazole (10μg/ml) on mitogen-induced human lymphocyte proliferative responses.

Time of addition of miconazole (10μg/ml)	PHA cpm ^{3}H-thymidine uptake	PWM cpm ^{3}H-thymidine uptake
0 hrs	704 ± 213	232 ± 72
4 "	3,268 ± 2,269	2,436 ± 836
24 "	16,937 ± 1,319	6,230 ± 1,506
48 "	35,654 ± 1,996	20,028 ± 2,816
None added	39,818 ± 1,795	20,934 ± 2,209

Results represent mean ±S.D. of triplicate samples.

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Effect of miconazole on antibody responses in mice

For these experiments, 6-8 week old male BALB/c mice received either 100 mg/kg or 300 mg/kg daily in two divided doses, morning and evening, from 2 days before until five days after priming with SRBC. The mice were bled 6 days after priming for determination of haemagglutinating titre to SRBC. The results showed that no suppression of antibody responses occurred at the dosage of 100 mg/kg/day, and mild suppression occurred at 300 mg/kg/day (table V-4).

Effect of miconazole on skin graft survival

For these experiments, 6-8 week old BALB/c mice were also treated with a similar dosage schedule of miconazole, starting 2 days before skin grafting until complete graft rejecton had occurred. The results (figure V-2) showed that control mice rejected C57BL skin grafts by 13.6 ± 1.8 days (mean $\pm$ S.D.), compared to 14.9 ± 1.8 days for those receiving 100 mg/kg/day ($p<0.05$), and 18.6 ± 3.5 days for those receiving 300 mg/kg/day ($p<0.001$). These dosages of miconazole were well tolerated.

Effect of varying doses of miconazole on DTH

For these experiments, male BALB/c mice were divided into 4 groups. They were primed with SRBC on day 0. They received 100, 200 or 300 mg/kg miconazole i.p. daily from day 0 till day 5, while controls received saline only. The results (table V-5) show a dose-related suppression of DTH by miconazole. Significant

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Table V-4. Effect of miconazole on antibody responses in mice.

Miconazole dosage	Haemagglutinating antibody titre to SRBC*	
(mg/kg/day)	Experiment 1	Experiment 2
0	10.0 ± 0.6 (5)	9.9 ± 0.6 (9)
100	9.8 ± 0.4 (5)[†]	
300		9.0 ± 0.4 (11)[‡]

* Results expressed as $\log_2$ mean $\pm$S.D. titre. [†] $p > 0.05$. [‡] $p < 0.05$.
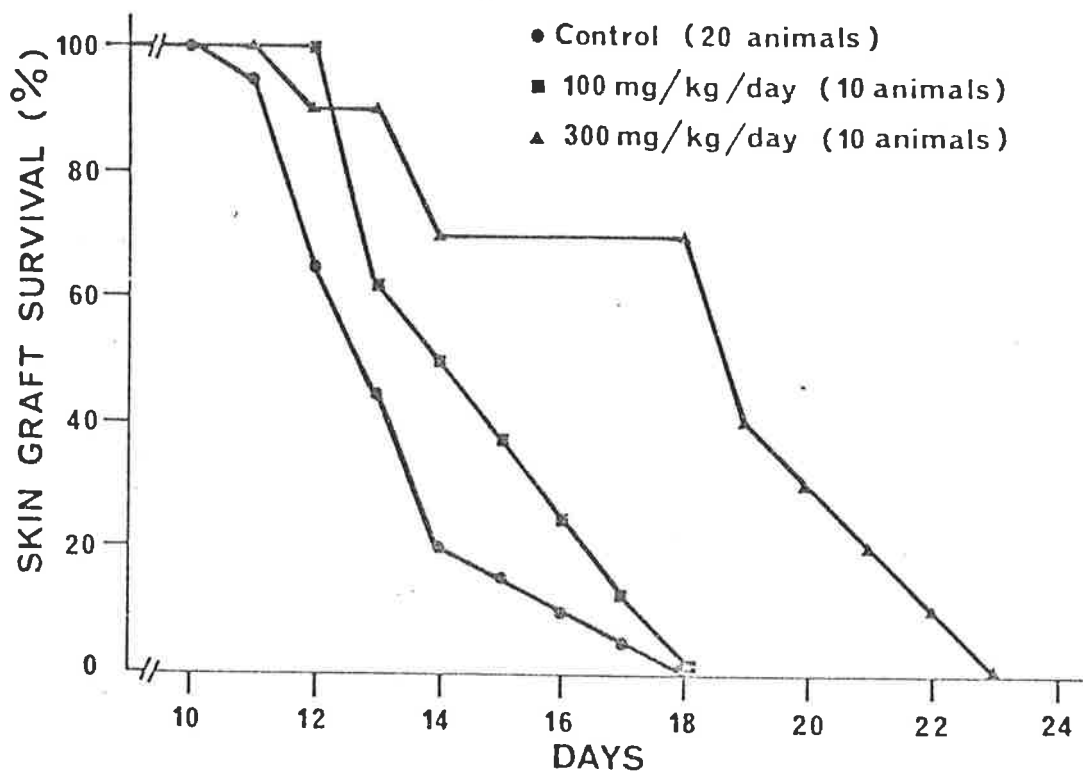
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Figure V-2. Effect of miconazole on skin graft survival in mice.
(●) Control, twenty animals; (■) 100mg/kg per day, ten animals and (▲) 300 mg/kg per day, ten animals.

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Table V-5. Effect of varying dosages of miconazole on DTH to
SRBC in BALB/c mice.

Treatment (mg/kg/day)	DTH (% increase in footpad thickness)	p value
0	50.3 ± 5.6	–
100	32.4 ± 2.1	< 0.02
200	14.6 ± 4.0	< 0.001
300	10.6 ± 2.8	< 0.001

There were 8 to 10 mice per experimental group - they were
primed with 1×10^8 on day 0, challenged with the same number
of SRBC on day 5, and measured for DTH on day 6.   They
received miconazole i.p. daily at the above dosages from
day 0 till day 5.

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suppression (p<0.02) of DTH occurred at the comparatively low dosage of 100 mg/kg which had no effect on skin graft rejection. Higher doses caused even greater suppression of DTH.

Effect of miconazole on induction or expression of DTH

The next set of experiments was designed to determine the phase of DTH on which miconazole produces immuno-suppression. For these experiments, a single i.p. dose of miconazole (300 mg/kg) was given to male BALB/c mice, either on the day of priming with SRBC (day 0) or on the day of challenge with SRBC (day 5). The results (table V-6) show that significant suppression occurred when miconazole was administered during the manifestation phase of DTH (p<0.001). Although some suppression of DTH occurred when the drug was given at day 0, this did not approach statistical significance (p>0.05).

Effect on DTH in tumour-bearing mice

The B_{16} melanoma is syngeneic in C57Bl/6J mice. For these studies, 6-8 week-old male mice were divided into 4 groups. Groups 3 and 4 received 1×10^6 B_{16} melanoma cells i.p. 7 days prior to priming with SRBC. At day 0, all mice were primed with 1×10^8 SRBC subcutaneously, and tested in the footpad at day 5, and read for DTH at day 6. Groups 2 and 4 received 200 mg/kg miconazole i.p. daily from day 0 till day 5, while controls (group 1) and tumour-bearing mice (group 3) received saline only.

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Table V-6.   Effect of miconazole on the induction and expression of DTH in BALB/c mice.

| Treatment (mg/kg) | DTH (% increase in footpad thickness) | |
	Exp. 1	Exp. 2
0	48.9 ± 6.2	60.0 ± 3.7
300	37.5 ± 7.6	28.6 ± 4.4
p value	>0.05	<0.001

There were 8 to 10 mice per group. They were primed with SRBC on day 0, and challenged with SRBC on day 5.

In exp. 1, the mice received a single 300 mg/kg dose of miconazole i.p. on day 0, while controls received saline only.

In exp. 2, the mice received a single 300 mg/kg dose of miconazole i.p. on day 5, while controls received saline only.

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The results (figure V-3) showed that DTH in tumour-bearing mice (group 3) were reduced to 36.1% compared to 66.8% in controls (group 1); this was statistically significant (p<0.001). Mice treated with miconazole (group 2) also showed depressed DTH of 19.7% (p<0.001). The most striking results were in group 4, where tumour-bearing mice treated with miconazole showed DTH of only 11.6%, much lower than either tumour-bearing alone or miconazole treatment alone (p<0.05).

CONCLUSIONS

In-vivo studies confirmed in-vitro findings that miconazole is a potent immunosuppressive agent, more so on DTH than on antibody production. This property may limit its value as a systemic antifungal agent because of a requirement for cell-mediated immunity to aid in elimination of fungal infections. One way to check on this is to include immune function monitoring into clinical trials now being conducted with miconazole. Since patients with cancer or other debilitating diseases are the usual ones to acquire deep mycotic infections, more information about immunosuppression by miconazole can be obtained by testing for DTH in tumour-bearing mice. The results indicate that DTH in tumour-bearing mice treated with miconazole are much lower than DTH in either category alone, i.e. the immunodepression associated with tumour-bearing is further compromised by miconaozle treatment.

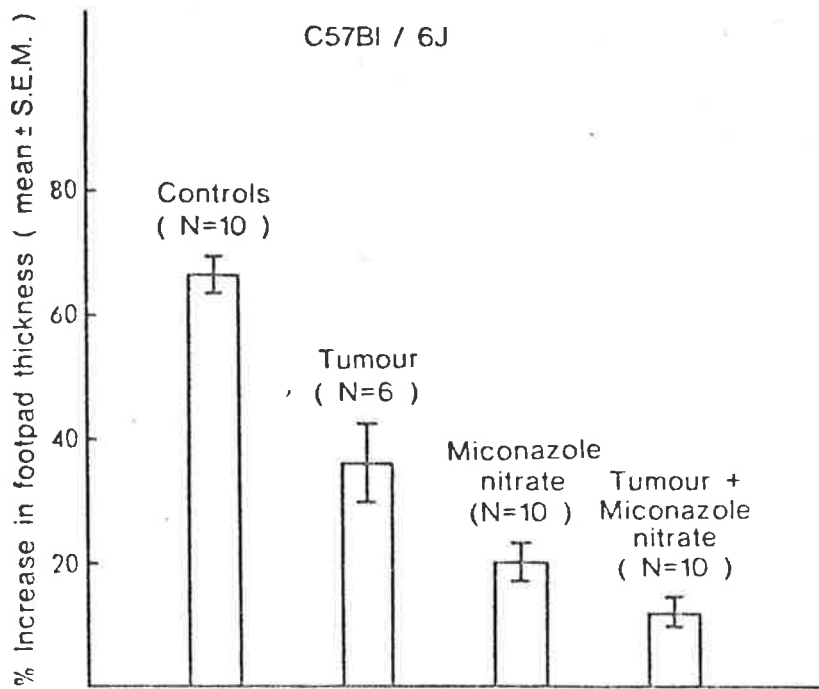THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

Figure V-3. Effect of miconazole on DTH in tumour-bearing
C57Bl/6J mice .   Dosage of miconazole was 200 mg/kg i.p.
daily from day 0 until day 5.   All mice were primed on
day 0 and challenged at day 5 with 1 x 10^8 S.R.B.C.   The
B$_{16}$ melanoma was given i.p. 7 days prior to priming with
S.R.B.C.

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On the other hand, the use of miconazole as an immunosuppressive agent in organ transplantation or autoimmune diseases should be further explored, because it appears to be very potent. The histocompatibility barrier between BALB/c and C57Bl mice is very strong, and to delay skin graft rejection in this situation is quite difficult. Yet miconazole, an imidazole derivative, and niridazole, a nitro-imidazole derivated (Mahmoud et al, 1975), can delay skin graft rejection for a number of days. If miconazole proves useful in clinical transplantation, it will have advantages over the conventional immunosuppressive drugs by possessing broad-spectrum antifungal and antibacterial activities (Van Cutsem and Theinpont, 1972), and even anti-Bacteroides activity (Thong and Ferrante, 1978a), so that opportunistic infections may be reduced in these patients.

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CHAPTER VI

IMMUNOPHARMACOLOGY OF AMPHOTERICIN B

THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

INTRODUCTION

Amphotericin A and B were isolated from Streptomyces nodosus in 1956. Amphotericin A proved too toxic for clinical use. Amphotericin B, although fairly toxic, has become very useful for the treatment of systemic fungal infections. It is an amphoteric polyene with an aminomethylpentose structure and the empirical formula $C_{46}H_{73}O_{20}N$. For intravenous and intrathecal administration, a colloidal preparation containing desoxycholate and phosphate buffer is used. Intramuscular injections cannot be given because of inflammation and tissue necrosis. Its spectrum of antifungal activity includes Candida albicans, Torulopsis glabrata, Cryptococcus neoformans, Coccidioides immitis, Blastomyces dermatitidis, Histoplasma capsulatum and Sporothrix schenckii. Renal, hepatic and marrow damage are serious complications of amphotericin B therapy.

The immunopharmacology of amphotericin B is not well understood. Enhancement of immunological responses occurred after a single injection of amphotericin B (Ishikawa, Narimatsu and Saito, 1975; Blanke et al, 1977), whereas its presence in culture suppressed mitogen-stimulated lymphocyte transformation (Tarnvik and Ansehn, 1974; Thong and Rowan-Kelly, 1978). It has also been shown to interfere with neutrophil chemotaxis (Bjorksten, Ray and Quie, 1976; Thong and Ness, 1977).

In the clinical situation, amphotericin B is administered once a day or every other day for many weeks, rather than the single dose employed in previous in-vivo studies. The immunopharmacological

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effects of daily injections of amphotericin B in mice are described in the next section; further in-vitro studies employing mouse lymphocytes are also described (Ferrante, Rowan-Kelly and Thong, 1979).

IN-VIVO STUDIES

The highest daily dosage of AmB (7.5 mg/kg/day) in these experiments had been used for the treatment of experimental infections in mice (Thong et al, 1978b). Toxicity was not evident at this dosage level.

The effect of AmB on the DTH response

Animals were divided into 6 groups of 6. Those in the first 3 groups received either 2.5, 5.0 or 7.5 mg/kg/body weight of AmB daily, while the other three groups were injected with the respective amounts of DOC. Preliminary studies had established that DOC, at the highest concentration used in these experiments (6 mg/kg) did not affect immunological responses. Treatment was started one day before immunization and terminated on the day of challenge (day 5). These dosage schedules were well tolerated.

The results presented in Figure VI-1 show that AmB markedly suppressed the DTH response. Significant suppression occurred in animals treated with 5.0 and 7.5 mg/kg body weight ($0.02 < p < 0.05$ and $p < 0.001$ respectively).
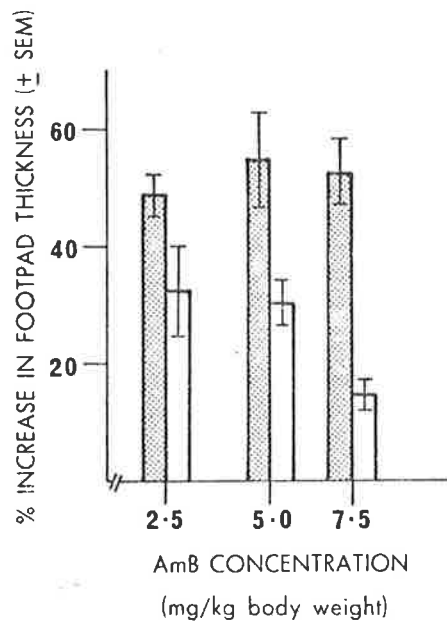
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Figure VI-1. The effect of daily AmB treatment on the DTH response. (▨) DOC treated; (☐) AmB treated. Suppression was significant at 5.0 and 7.5 mg/kg body weight ($0.02 < p < 0.05$ and $p < 0.001$ respectively).

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In order to determine if AmB affected the priming or the manifestation phase of the DTH response, further experiments were set up to find out if AmB was acting at one or both of these phases.

The effect of AmB on the priming and manifestation phase of the DTH response

In the following experiments the dose of AmB chosen to be injected into mice was 7.5 mg/kg body weight because this dose gave the best suppression in the experiments described above. Mice were divided into 10 groups of 5. The first 5 groups were treated with AmB as follows: Mice in groups 1, 2 and 3 were given daily treatment starting 1 day before, on the same day and one day after priming with SRBC respectively. Treatment was stopped on day 5. The remaining 2 groups were given single injections of AmB. Mice in group 4 received AmB on the same day as priming while those in group 5 were given AmB on the day of challenge (day 5).

The data presented in Figure VI-2 demonstrate that AmB given after the day of priming could still inhibit the response. In fact, a single dose administered on the same day as challenge markedly inhibited the DTH response. However, a single injection given on the day of priming had no significant effect. The results indicate that AmB affected the manifestation phase of the DTH response.

The effect of AmB on the anti-SRBC antibody response

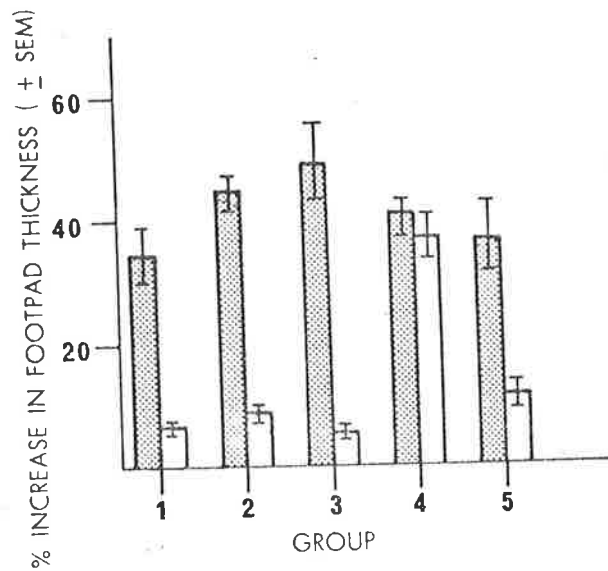The ability of AmB to inhibit the haemagglutination antibody

Figure VI-2. The effect of AmB on the priming and manifestation phase of the DTH response. (▨) DOC treated; (☐) AmB treated. Mice in groups 1, 2 and 3 were given daily treatment of either DOC or AmB starting 1 day before, on the same day and one day after priming respectively. Those in group 4 were given a single injection on the day of priming while those in group 5 received a single injection on the day of challenge for DTH response. Significant suppression occurred in groups 1, 2, 3 and 5 which had been treated with AmB (p<0.001).

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response to SRBC was tested in mice treated with 7.5 mg/kg AmB daily, beginning one day before priming with SRBC and terminating on day 5. Control mice were treated with the respective amounts of DOC. The results show that AmB had no effect on the antibody (Figure VI-3).

Effect of AmB on immunological responses in tumour-bearing mice

For DTH experiments the mice were divided into 4 groups. Groups 1 and 2 received saline injections (i.p.) at day 0, while groups 3 and 4 received 5×10^5 B_{16} melanoma cells. At day 7 all mice were primed, with 1×10^8 SRBC s.c. On the same day groups 2 and 4 received AmB (7.5 mg/kg) as a daily injection for 4 days. At day 13, all mice were challenged with 1×10^8 SRBC in the footpad. The DTH response was measured on day 14.

The results showed that tumour-bearing mice (group 3) had a reduced capacity to mount a DTH response (Figure VI-4); the percent footpad increase was 33.7 compared to 69.2 in controls (group 1). The results also confirm previous studies that multiple doses of AmB have a suppressive effect on DTH; the percent footpad increase was reduced to 35.7 (group 2). Of particular interest was the finding that the combination of tumour-bearing and AmB therapy resulted in a marked suppression of DTH, more than either tumour-bearing or AmB alone; the percent footpad increase was only 16.8 (group 4).

For antibody experiments, another lot of mice were divided into 4 groups according to the above experimental design, except
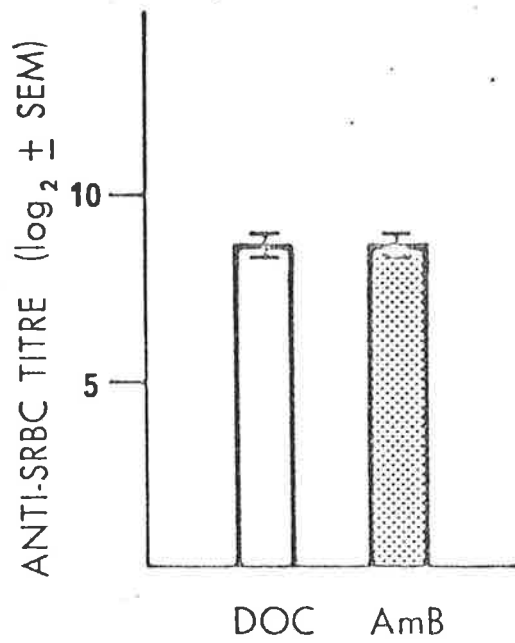
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Figure VI-3. The effect of AmB on the haemagglutinating anti-SRBC response in mice. Mice were treated with 7.5 mg/kg body weight of AmB daily, beginning one day before priming with SRBC and terminating on day 5. Controls received the appropriate concentration of the diluent, DOC.

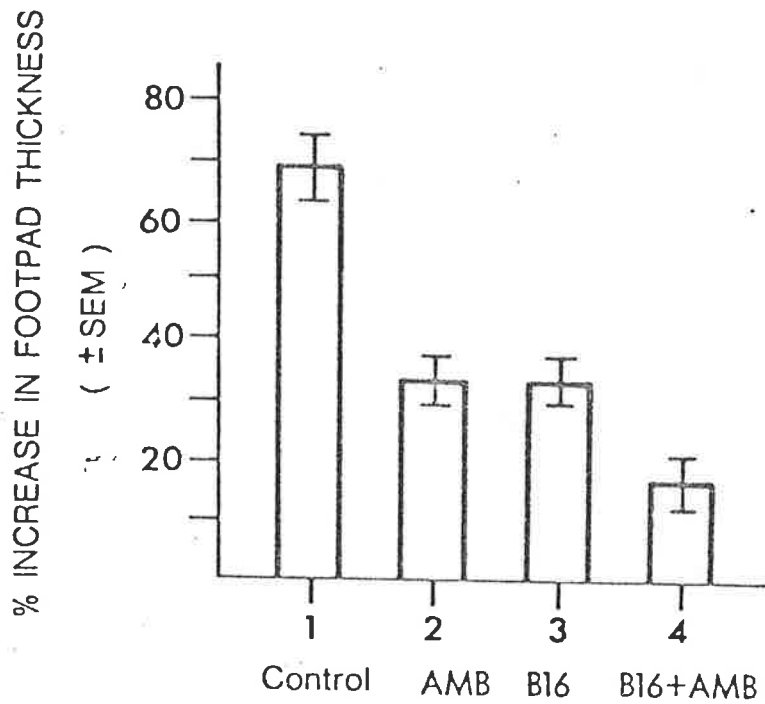THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

Figure VI-4. The effect of AmB treatment on the DTH response in normal mice and in mice bearing B_{16} melanoma. Mice injected with either AmB or B_{16} (groups 2 and 3 respectively) melanoma displayed a suppressed DTH response ($p<0.001$). Whilst mice bearing B_{16} melanoma and treated with AmB (group 4) showed a combined immunosuppression ($0.01<p<0.02$).

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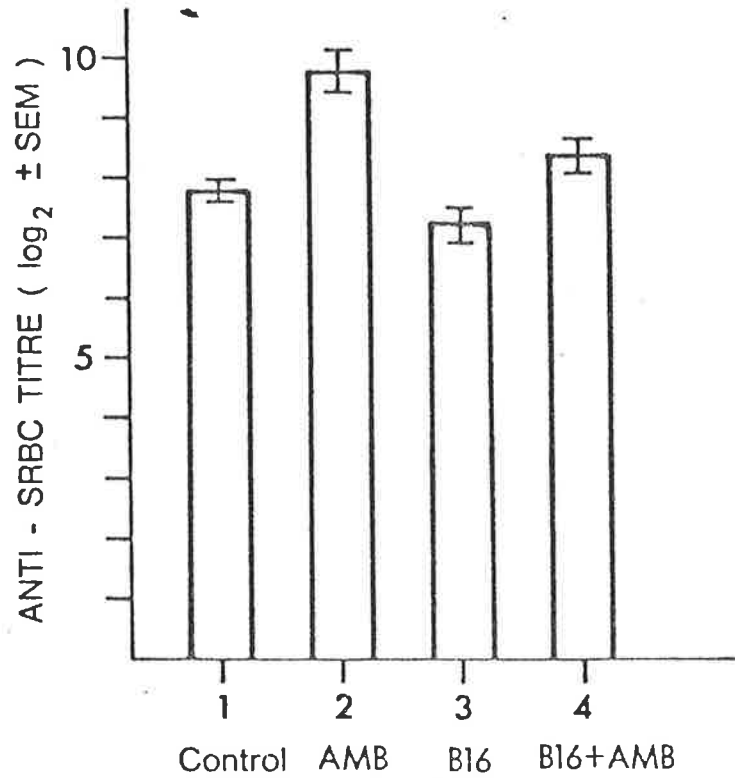
Figure VI-5. The effect of AmB treatment on the haemagglutinating anti-SRBC antibody response in normal mice and in mice bearing B_{16} melanoma. Neither AmB nor B_{16} melanoma (groups 2 and 3) caused a depression of circulating haemagglutinating antibody level. In fact, AmB treatment (group 2) caused enhancement of the antibody response ($p < 0.001$), although this was not observed if the mice were carrying B_{16} melanoma (group 4).

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that the mice were primed with 1×10^8 SRBC i.v. on day 7 and bled by intracardiac puncture on day 14. The results showed that tumour-bearing does not reduce the capacity to form antibody to SRBC (Figure VI-5); $\log_2$ mean titre was 7.3 (group 3) compared to 7.8 in controls (group 1). In mice receiving AmB (group 2), the antibody tire was significantly increased to 9.8. The tumour-bearing mice receiving AmB (group 4) also showed a raised antibody titre but this did not reach statistical significance.

IN-VITRO STUDIES

The effect of AmB on mitogen-induced lymphocyte proliferation

In order to define more clearly the effects of AmB on the immune response, the effect of this drug on the mitogen-induced lymphocyte response was studied.

Mouse splenic lymphocytes were incubated in the presence of either PHA or LPS and varying concentrations of AmB. Control cultures were incubated with the respective quantities of DOC as that present in the AmB solution. Preliminary studies in our laboratory showed that desoxycholate (DOC), at the highest concentration used in these experiments ($20\mu g/ml$) did not influence lymphocyte transformation. The results showed that AmB markedly inhibited the lymphocyte response to these mitogens (Table VI-1). Suppression was evident at $5\mu g/ml$ concentration of AmB. At this concentration it had a more profound effect on the PHA than the LPS response.

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Table VI-1. Effect of AmB on lymphocyte proliferation induced by mitogens.

AmB Concentration (μg/ml)	% Inhibition of ^{3}H-TdR incorporation	
	PHA	LPS
0	0	0
1.0	$42.4 \pm 10.9^*$	$21.5 \pm 12.1^+$
5.0	$78.8 \pm 5.5^\dagger$	48.8 ± 10.0^J
25.0	$99.3 \pm 0.2^\dagger$	$96.3 \pm 0.9^\dagger$

Results expressed as mean ± SEM of 7 experiments.

* $0.01<p<0.02$. + not significant. † $p<0.001$. ^{J} $0.001<p<0.01$.

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The effect of AmB on lymphocyte viability

Experiments were set up to determine if the immunosuppressive properties of AmB were related to its toxicity for lymphocytes. Splenic lymphocytes were incubated in microtitre plates in the presence ofmitogens, and AmB or DOC.   Ater 48 hr of incubation the cells were tested for viability using the trypan blue dye exclusion test (see materials and methods).   The results obtained demonstrated that AmB was not toxic for lymphocytes at 5µg/ml, although some reservation should be placed as to the accuracy of this test as an indicator of cell viability (Bhuyan et al, 1976).

The effect of AmB on lymphocyte proliferation in the presence of suboptimal concentrations of mitogens

It has been reported previously that some drugs may demonstrate dual effects (enhancement and suppression) depending upon the dose of mitogen used (Gery & Eidinger, 1977).

Lymphocyte cultures were set up with varying concentrations of PHA or LPS.   To one set of cultures was added AmB (5µg/ml), while the control set received DOC.   The results presented in Table VI-2 show that AmB also suppressed the lymphocyte response in the presence of suboptimal mitogen concentrations.

Reversibility of AmB inhibitory effect on lymphocytes

AmB once bound to sterol components of the membrane may cause irreversible changes to the membrane.   In the following experiments

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Table VI-2. Effect of AmB on lymphocyte proliferation in the presence of suboptimal mitogen concentration.

PHA Concentration (µg/ml)	cpm ^{3}H-TdR incorporated	
	DOC	AMB
0.5	49,701 ± 28,821	5,923 ± 448
0.05	3,488 ± 282	1,519 ± 39
0.005	4,248 ± 809	1,785 ± 255
0.0005	2,586 ± 111	1,675 ± 254
LPS Concentration (µg/ml)		
250.0	9,843 ± 756	4,825 ± 595
25.0	4,775 ± 272	2,216 ± 577
2.5	3,142 ± 74	1,604 ± 308
0.25	2,570 ± 266	1,747 ± 425

Results expressed as mean ± SEM of triplicate samples.

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Table VI-3. Reversibility of AmB induced inhibition.

Treatment	PHA		% inhibition
	cpm ^{3}H-TdR incorporated		
	DOC	AMB	
Non-washed	30,721 ± 2,263	5,457 ± 583	82.2*
Washed	28,395 ± 870	14,041 ± 1,120	50.6[+]

Treatment	LPS		% inhibition
	cpm ^{3}H-TdR incorporated		
	DOC	AMB	
Non-washed	9,874 ± 481	5,013 ± 818	49.2[†]
Washed	11,104 ± 721	12,191 ± 1,195	"0"

Results expressed as mean ± SEM of triplicate samples.
Similar results were obtained in one other experiment.

* $p<0.001$. + $0.001<p<0.01$. [†] $0.01<p<0.02$.

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lymphocytes were cultured in the presence of AmB (5μg/ml) in culture tubes for 1 hr at 37°C. These were washed thrice with medium and then added to wells in microtitre plates. To control tubes was added DOC in place of AmB. Control cell cultures in which the drug or diluent was not removed were also included. To the cultures was added either PHA or LPS.

The data in Table VI-3 shows that the inhibitory effect of AmB on the PHA-induced response could not be reversed by washing the treated cells. In contrast the inhibitory effect of the drug on LPS-induced response was totally removed by washing the treated lymphocytes.

The effect of delayed addition of AmB on lymphocyte responsiveness

The inhibitory effects of AmB could be related to its action at either the initiation phase or the proliferative phase of the mitogen-induced response. Experiments were set up to determine if AmB displayed inhibitory activity after the lymphocyte response had been initiated.

Lymphocyte cultures were set up in the presence of PHA or LPS. At different intervals of time, namely 0, 4, 24, and 42 hr, AmB (5μg/ml) was added to the respective wells. To control cultures was added diluent (DOC) only.

The data presented in Table VI-4 demonstrates that AmB had a pronounced effect on PHA and LPS induced response even when added 24 hr after initiation of cultures.

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Table VI-4. Effect of delayed addition of AmB on mitogen-induced responsiveness.

| Time (hr) | % Inhibition of ^{3}H-TdR incorporation | |
	PHA	LPS
0	80.3 ± 6.4*	36.0 ± 7.5[+]
4	67.3 ± 6.3*	35.4 ± 9.7[+]
24	59.4 ± 4.0*	23.4 ± 10.4[∫]
42	25.9 ± 22.1[∫]	11.4 ± 8.7[∫]

Results expressed as mean ± SEM of triplicate samples.
Similar results were obtained in one other experiment.

* $p<0.001$. + $0.01<p<0.02$. [+] $0.02<p<0.05$. [∫] Not significant.

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CONCLUSIONS

The first part of the study dealt with the effects of amphotericin B treatment on a daily basis for 6 days on immunological responses in mice. The results showed significant suppression of DTH at doses of 5 mg and 7.5 mg/kg/day; this dose has been used for treatment of experimental infections in mice and found to be non-toxic (Thong et al, 1978b). The probable site of action was identified as the manifestation phase rather than the elicitation phase of DTH. Antibody responses, however, were not depressed by AmB treatment. In-vivo studies also showed that the already depressed DTH of tumour-bearing mice was further depressed by AmB.

The in-vitro studies confirmed and extended these observations. Mitogen-induced lymphocyte transformation was markedly inhibited in the presence of $1\mu g/ml$ AmB, well under the concentration of $2\mu g/ml$ attainable in blood by conventional dosage schedules. The suppressive effect was still present after washing, which indicates that AmB binds irreversibly to receptors on the lymphocyte membrane. Its suppressive effect was still present 24 hours after the initiation of cultures, which suggests that it acts on the proliferative phase of the immune response.

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CHAPTER VII

IMMUNOPHARMACOLOGY OF ANTIMALARIAL DRUGS

THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

INTRODUCTION

Malaria continues to be a scourge in the tropics and subtropics with many deaths occurring among infants and young children. It is an important cause of chronic ill-health among the survivors.

There are four species of human malaria. Plasmodium falciparum is the most virulent of the four, and produces the clinical picture of malignant tertian malaria. Plasmodium vivax produces the clinical picture of benign tertian malaria; P. Ovale is a rare cause of benign tertian malaria. The term "tertian" refers to the spikes of fever every third day, which coincides with the release of merozoites from infected red cells. The fourth species of human malaria, P. malariae, gives rise to symptoms of quartan malaria.

Malaria is transmitted by anopheles mosquitoes. Sporozoites introduced by the bite first lodge in the liver where they multiply. They then invade the red blood cells, where they continue their cycle of multiplication. Every three or four days depending on species, the red cells burst to release merozoites, which go on to infect other red cells. Later on in the infection, male and female gametes are also produced. The bite of a suitable vector at this stage results in the transfer of some of these gaemtocytes into the gut of the mosquito. The parasite develops in the gut by means of sexual conjugation, and later migrates to the salivary glands, ready for infecting the next victim. This life cycle is illustrated in figure VII-1.

Anti-malarial drugs differ with regard to their selectivity for different parts of the life cycle. Quinine, chloroquine and
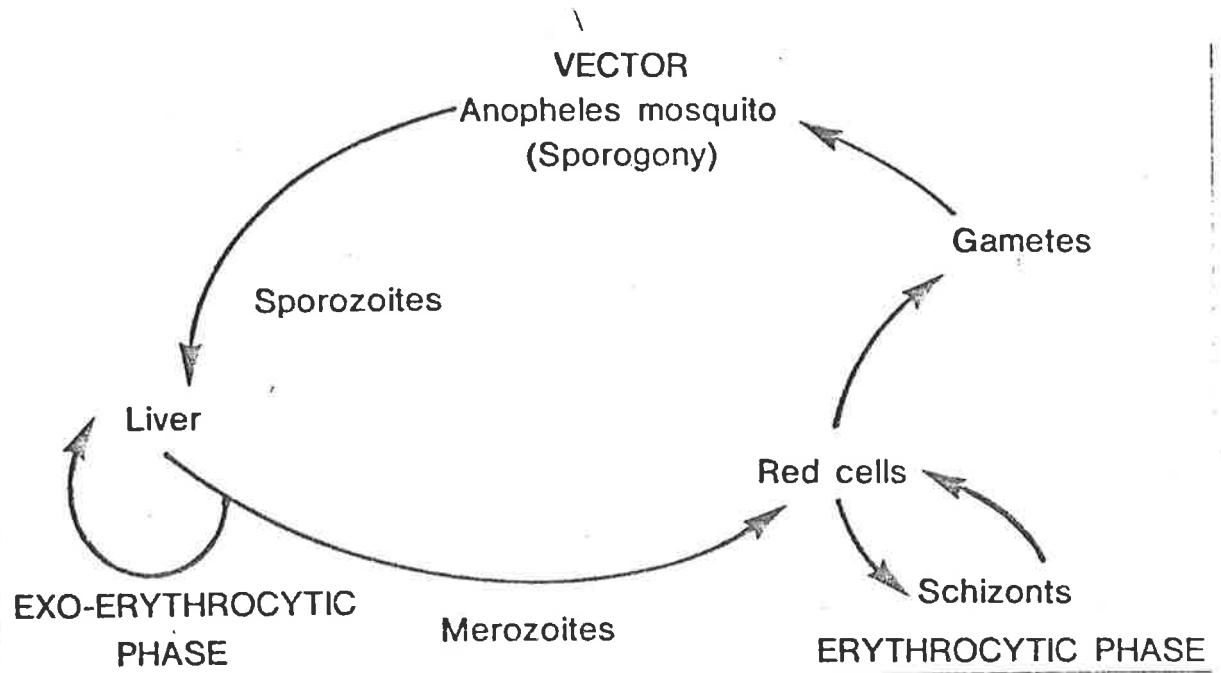
Figure VII-1. Life cycle of the malaria parasite. Only the female anopheles mosquito transmits malaria, because it requires a blood meal for its eggs to develop.

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mefloquine act most effectively on the schizont stage in the blood; they are therefore excellent for the suppression of clinical symptoms. Others such as primaquine, are gametocidal. Still others, e.g. pyrimethamine (Chapter VIII) prevent sporogony and multiplication within the mosquito. In the relapsing malarias, where a pool of parasites persists in the liver, tissue schizonticides such as primaquine, are required to eliminate the disease and prevent recrudescence.

Quinine

For three hundred years, from its discovery by Jesuits in the seventeenth century until the second world war, quinine was the only drug available for the chemotherapy of malaria. It was replaced by synthetic anti-malaria drugs until recent years, when the emergence of drug resistant strains required the re-introduction of quinine.

Quinine is a quinoline methanol, the chemical structure of which is illustrated in figure VII-2. It is the best absorbed of the four principal alkaloids of conchona bark. It can reach blood levels of 1-10μg/ml within 1-3 hours of ingestion. It appears to act by forming a hydrogen-bond complex with double standard DNA. This prevents DNA replication, RNA transcription and protein synthesis. Besides this, it also interferes with many enzyme systems. It is a blood schizonticide now used mainly in the treatment of chloroquine-resistant falciparum malaria. Side-effects

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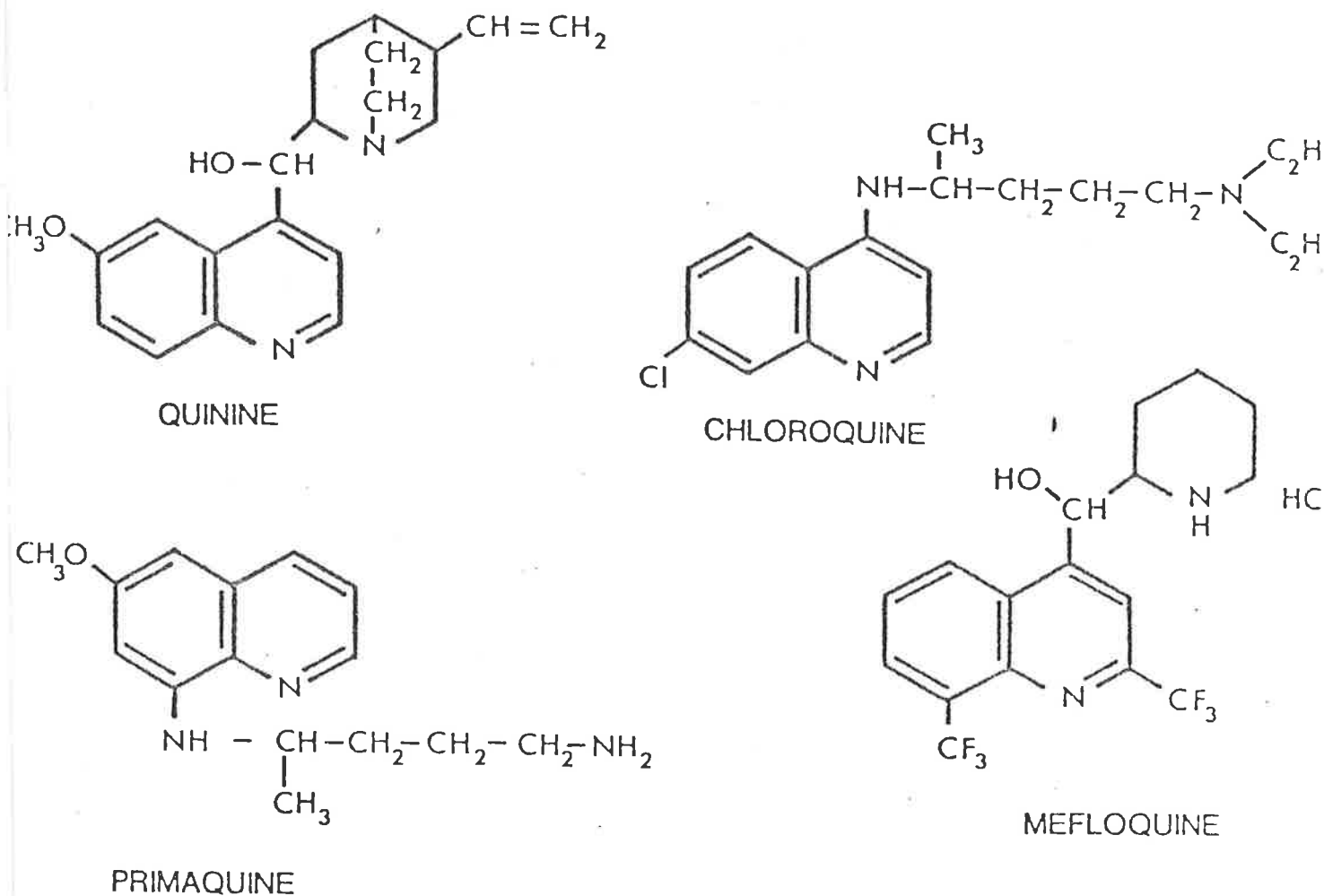
Figure VII-2. Structural formulae of the important anti-malaria drugs. Quinine contains a quinoline ring linked by a secondary alcohol at its fourth position to a quinuclidine ring. Chloroquine has a quinoline ring like that of quinine; the chlorine atom at the seventh position is critical for anti-malarial activity among the 4-aminoquinoline compounds. Primaquine is an 8-aminoquinoline. Mefloquine is a quinoline methanol like quinine.

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include cinchonism, which is a broadly descriptive term to cover symptoms of flushing, sweating, tinnitus, blurred vision, dizziness, nausea, vomiting and diarrhoea. More severe poisoning produces urticaria, deafness, blindness, coma, abdominal pain, and cardiac arrhythmias. Other side effects include haemolysis, leucopenia, thrombocytopenia and Blackwater fever.

Mefloquine

This is a newly synthesized methanol quinoline (Doberstyn et al, 1979). Early clinical trials indicate that it is effective as a suppressant of both falciparum and vivax infections. It is a good substitute for quinine or pyrimethamine-sulphadoxine in chloroquine-resistant malaria. It appears to be a safe drug, with gastrointestinal upset as the main side-effect. It is well absorbed orally, producing blood levels of 1-10μg/ml within 4 hours after oral administration. It has a prolonged metabolic half-life.

Chloroquine

This drug was first synthesized in Germany in 1934, and rediscovered in the United States in 1944. Its chemical structure, a 4-aminoquinoline, is illustrated in figure VII-2. The disulphate salt is a colourless, dimorphic crystalline powder with a bitter taste. It is very soluble in water especially at acid pH. It acts by forming a complex with DNA to block DNA and RNA synthesis. It is well absorbed, reaching blood levels of 1-10μg/ml within

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1-2 hours.   The half-life is around five days, with a high
concentration of the drug within the tissues.   Chloroquine is an
excellent drug for chemosuppression and chemoprophylaxis of
malaria.   It has occasional uses in amoebiasis, lupus erythematosus
and rheumatoid arthritis.   The side-effects include anorexia,
vomiting, diarrhoea, headache, vertigo and blurring of vision.
Alopecia, pruritus and exfoliative dermatitis are occasionally seen.
Central nervous system complications include psychosis and
peripheral neuropathy;   its passage through the placenta has
caused mental retardation and congenital deafness.   By far the
most serious complication of chloroquine usage is irreversible
retinopathy.

Primaquine

Primaquine is an 8-aminoquinoline synthesized for the treatment
of malaria after the loss of the cinchona crop in Java during the
second world war.   It is an orange-red crystalline powder.   It
is well absorbed from the intestine, with blood levels in the region
of 1-10µg/ml by 6 hours.   The active metabolites are quinoline-
quinone derivatives which are powerful oxidants.   Tissue schizonts
are very sensitive to oxidative damage, whereas erythrocytic stages
of malaria are quite resistant.   This is the most valuable property
of primaquine, in that no other drug can produce a radical cure in
vivax malaria.

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IN-VITRO STUDIES

Quinine

The viability of cultures remained at 95% or greater. Quinine, at the concentrations used in these experiments, was not toxic to lymphocytes. However, marked suppression of lymphocyte transformation by quinine was observed (Thong and Ferrante, 1978b). This inhibitory effect was found to be dose-dependent (table VII-1). There was a greater inhibitory effect on PHA-stimulated cultures than on PWM-stimulated cultures (p<0.01) at all concentrations of quinine used.

The inhibitiory effects of quinine were shown to be reversible by washing (table VII-2), suggesting that quinine does not bind irreversibly to receptors in lymphocytes. It was also found that the inhibitory effects of quinine remained quite substantial even when the drug was added at 18 or 42 hours after the start of experiments (figure VII-3).

Primaquine

Preliminary studies showed that lymphocyte viability, as assessed by trypan blue dye exclusion, was not affected by primaquine in the concentrations used in these experiments for as long as 4 days in culture (Thong, Ferrante and Rowan-Kelly, 1978). However, pronounced inhibition of lymphoproliferative responses by primaquine was observed. This suppressive effect was dose-dependent (table VII-3).

Some characteristics of this inhibitiory effect have been defined. The inhibitory effect of primaquine could be substantially

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Table VII-1.   Effect of varying concentrations of quinine on
mitogen-induced lymphocyte proliferative responses.

Quinine concentration (µg/ml)	% Inhibition* (mean ± S.D. of 6 experiments)	
	PHA[+]	PWM[‡]
0	0	0
1	24.0 ± 6.6	2.7 ± 6.0
5	41.8 ± 9.8	17.4 ± 11.5
10	51.4 ± 11.8	29.7 ± 13.1

* % inhibition $= \dfrac{\text{cpm untreated} - \text{cpm treated}}{\text{cpm untreated}} \times 100$

+ PHA, phytohemagglutinin

‡ PWM, pokeweed mitogen

Table VII-2. Reversibility of inhibition of mitogen-induced lymphocyte proliferation by quinine.

Treatment	Phytohemagglutinin	
	cpm ^{3}H-thymidine uptake*	% inhibition
Quinine (10µg/ml)		
Unwashed	10,631 ± 2,761	54.0[†]
Washed	19.046 ± 5,220	17.5[‡]
Untreated control	23,100 ± 5,026	Control

Treatment	Pokeweed mitogen	
	cpm ^{3}H-thymidine uptake*	% inhibition
Quinine (10µg/ml)		
Unwashed	4,433 ± 1,266	40.3[†]
Washed	8,544 ± 526	0[‡]
Untreated control	7,426 ± 1,714	Control

* Results expressed as mean ± S.D. of triplicate samples. Similar results were obtained in two other experiments using lymphocytes from different donors.
[†] Significant inhibition compared to control (p<0.001).
[‡] Statistically not significant compared to control (p>0.05).
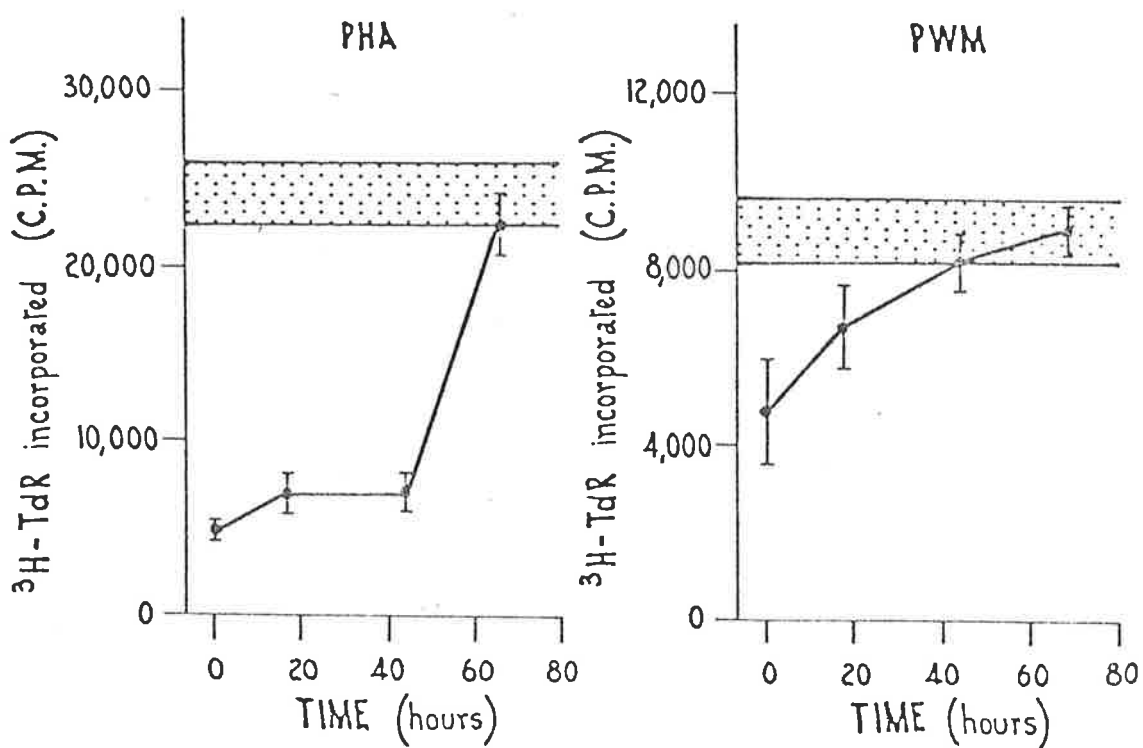
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Figure VII-3. Effect of immediate and delayed addition of quinine (10µg/ml) on mitogen-induced lymphocyte proliferative responses. Each point and bar represents mean ± S.D. of triplicate samples. The banded area represents 2 S.D. of control lymphocytes.

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reversed by washing.   For these experiments, lymphocytes were pre-incubated with 10μg/ml of primaquine for one hour, and then washed three times in medium before culture with mitogens.   There was 54.2 ± 9.8% (mean ± S.D. of triplicate cultures) inhibition of ^{3}H-thymidine incorporation in PHA-stimulated unwashed cultures compared to 5.3 ± 10.2% in washed cultures.   The corresponding values for PWM-stimulated cultures was 51.2 ± 5.3% and 17.2 ± 8.5%, respectively (table VII-4).

There was a substantial decline in the inhibitory capacity of primaquine when the drug was added at 4, 24 and 48 hours after the start of experiments (table VII-5).

Mefloquine

The results of in-vitro experiments are presented in table VII-6. At the mefloquine concentration of 1μg/ml, there was inhibition of ^{3}H-thymidine uptakes of 10.9%, 18.2% and 36.4% in cultures stimulated by PHA, PWM and E. coli lipopolysaccharide (LPS), respectively. The marked suppression of lymphocyte transformation in cultures containing 4μg/ml mefloquine was due to drug toxicity : cell viability was less than 15% as assessed by trypan blue exclusion.   In contrast, human lymphocytes were more resistant to mefloquine; cell viability remained at 50% at 4μg/ml (table VII-7) (Thong et al, 1979).

Table VII-3. Effect of varying concentrations of primaquine on lymphoproliferative responses.

Primaquine (μg/ml)	PHA ^{3}H-thymidine uptake (cpm)	PWM ^{3}H-thymidine uptake (cpm)
0	35,449 ± 2,465	13,544 ± 5,389
1	28,370 ± 4,658 (20.0)*	10,519 ± 3,828 (22.4)
4	18,207 ± 3,047 (48.7)**	6,585 ± 2,865 (51.4)*
10	11,570 ± 5,498 (67.4)**	3,495 ± 1,432 (74.2)†

The results represent mean ± S.D. of 5 experiments using 5 donors. Values in parenthesis denote percentage inhibition.
* p <0.05.   ** p <0.001.   † p<0.01.

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Table VII-4. Reversibility of inhibition of mitogen-induced lymphocyte proliferation by primaquine.

Primaquine treatment (10µg/ml)	% inhibition (mean ± S.D.) of ^{3}H-thymidine uptake	
	PHA	PWM
Washed cultures	5.3 ± 10.2	17.5 ± 8.2
Unwashed cultures	54.2 ± 9.8	51.2 ± 5.3

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Table VII-5. Effect of immediate and delayed addition of primaquine (10μg/ml) on mitogen-induced lymphocyte proliferative responses.

Time of addition of primaquine (hr)	PHA ^{3}H-thymidine uptake (cpm)	PWM ^{3}H-thymidine uptake (cpm)
0	15,571 ± 4,517 (60.8)	6,419 ± 1,042 (75.8)
4	21,974 ± 240 (44.7)	7,050 ± 1,069 (73.1)
24	33,568 ± 996 (15.4)	22,394 ± 2,348 (14.4)
48	39,646 ± 1,874 (0)	27,110 ± 3,575 (0)
Untreated control	39,671 ± 1,668	26,161 ± 1,166

The results represent mean ± S.D. of triplicate samples.
Values in parenthesis denote percentage inhibition.

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Table VII-6. Effect of mefloquine on mitogen-induced lymphocyte proliferative responses in mice.

Mefloquine (µg/ml)	cpm ^{3}H-thymidine uptake*			Cell Viability
	PHA	PWM	LPS	
0	56,428 ± 18,335	17,560 ± 7,590	9,672 ± 6,266	>95%
1	50,301 ± 17,002 (100.9)	14,367 ± 7,019 (18.2)	6,734 ± 4,912 (36.4)	>85%
4	5,826 ± 9,127 (89.7)	1,390 ± 1,835 (92.1)	656 ± 410 (93.2)	<15%

* Expressed as mean ± S.D. of 7 experiments.
Numerals in parenthesis indicate percent inhibition.

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Table VII-7. Effect of mefloquine on mitogen-induced human lymphocyte proliferative responses.

Mefloquine (μg/ml)	cpm ^{3}H-thymidine uptake*		Cell Viability
	PHA	PWM	
0	61,230 ± 25,453	25,717 ± 9,610	>95%
1	43,602 ± 13,950 (27.9)	20,957 ± 7,316 (18.5)	>95%
4	431 ± 235 (99.3)	230 ± 67 (99.1)	>50%

* Expressed as mean ± S.D. of 3 experiments using lymphocytes from 3 donors. Numerals in parenthesis indicate percent inhibition.

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IN-VIVO STUDIES

The dosages of antimalarial drugs used in these experiments were previously shown by Peters (1975) to be effective for successful treatment of immune malaria without toxic manifestations.

Mefloquine

LACA mice receiving 10 mg/kg x 6 days mefloquine showed diminished antibody titres to SRBC compared to controls (table VII-8). Mice receiving half this dose of mefloquine also showed lower levels of anti-SRBC antibody, but this difference was not statistically significant. The DTH responses to SRBC were not affected by mefloquine in either single or multiple dose regimens (table VII-9). Although slight inhibition of DTH responses was observed in the treated groups, this did not approach statistical significance. Higher doses could not be administered because of death from toxicity (Thong et al, 1979).

Comparison between mefloquine, chloroquine, primaquine and quinine

The effects of these major anti-malaria drugs on in-vivo immune responses were compared in syngeneic BALB/c mice. The dosage of 8.5 mg/kg/day is that required by mefloquine to cure 90% of mice infected with malaria (Peters, 1975). This dosage is in excess of that required for chloroquine (3.3 mg/kg/day), and primaquine (3.4 mg/kg/day), but well below that required for quinine (87 mg/kg/day). The results showed that at the dosage of 8.5 mg/kg/day, none

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Table VII-8. Antibody responses to sheep red blood cells in mice treated with mefloquine.

Mefloquine* Daily dose	Total dose	Anti-SRBC $\log_2$ titre[+]	p value
Nil	0	9.0 ± 0.6	--
5 mg/kg	30 mg/kg	7.4 ± 1.6	>0.05
10 mg/kg	60 mg/kg	7.6 ± 0.8	<0.02

* Mefloquine was administered by intraperitoneal injection one day before, until 4 days after immunization with 4×10^8 SRBC given via the tail vein.
+ Expressed as mean ± S.D. of 5-6 mice per experimental group.

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Table VII-9. Delayed-type hypersensitivity responses to sheep red blood
cells in mice treated with mefloquine.

Mefloquine Dosing Schedule *	Total dose (mg/kg)	Footpad thickness (% increase)+
Nil	0	25.4 ± 8.4
Day -1	10	24.8 ± 10.7
" 0	10	20.4 ± 8.3
" +1	10	21.5 ± 9.2
" -1 to +4	60	19.5 ± 11.9
" 0 to +4	50	21.9 ± 7.4
" +1 to +4	40	17.5 ± 10.0

* Mefloquine was administered as a daily intraperitoneal injection on the
  days indicated. At day 0, the mice were immunized with 1×10^8 SRBC
  subcutaneously. Footpad challenge was performed on day 5 and read at
  day 6.
+ Expressed as mean ± S.D. of 10 mice per experimental group.

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Table VII-10. Effect of antimalarial drugs on antibody and delayed-type hypersensitivity responses in mice.

Treatment	Antibody to SRBC ($\log_2$ mean ± S.E.)	DTH (% increase ± S.D.)
Control	10.1 ± 0.5	46.5 ± 11.3
Chloroquine	10.7 ± 0.5	42.9 ± 9.5
Primaquine	10.3 ± 0.5	51.2 ± 13.7
Mefloquine	9.9 ± 0.5	46.9 ± 10.8
Quinine	10.1 ± 0.5	44.9 ± 12.4

There were 6 mice in each group. Each mouse received 8.5 mg/kg/day by i.p. injection of either chloroquine, primaquine, mefloquine from day 0 to day 5 (6 doses), while controls received an equal volume of saline (0.2mg) only. Mice were either primed at day 0 with 1×10^9 SRBC i.v. and bled at day 6 for antibody studies, or primed with 1×10^8 SRBC s.c. at day 0, challenged with 1×10^8 SRBC in the footpad at day 5, and measured at day 6 for DTH studies.

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Table VII-11. Effect of therapeutic doses of quinine on antibody and delayed-type hypersensitivity responses in mice.

Treatment	Antibody to SRBC ($\log_2$ mean ± S.E.)	DTH (% increase ±S.E.)
Control	9.5 ± 0.2	52.7 ± 3.3
Quinine	*10.2 ± 0.2	*53.1 ± 3.1

*$p > 0.05$.

There were 10 mice per group. Each mouse received 87 mg/kg/day by i.p. injection of quinine from day 0 to day 3 (4 doses), while controls received an equal volume of saline (0.2ml) only. For antibody and DTH testing, see text and footnotes of table VII-10.

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of these drugs have an effect on antibody and DTH responses to SRBC in mice (table VII-10).

In the next set of experiments, the effect of therapeutic doses of quinine (87 mg/kg/day) was examined. The results showed that at this dosage, there was no significant suppression of DTH, nor was there suppression of antibody responses (table VII-11) (Thong, Ferrante and Secker, 1980b).

CONCLUSIONS

Now that the W.H.O. Malaria Eradication Programme, based on vector control, has failed, the antimalarials are once again the principal means of keeping the disease in check. This situation has become more precarious with the emergence of chloroquine resistant strains. Much more information is therefore required about these drugs and how they work, and more work needs to be done to develop better drugs for prevention and treatment of malaria.

It is therefore quite heartening to note that although the antimalarials havepotent in-vitro effects on lymphocyte transformation, they do not appear to suppress immunity in mice treated with the usual doses needed for adequate treatment of malaria (Peters, 1975). The clinical experience supports this conclusion to some extent, because in practice, antimalarials

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·are usually curative and do not appear to cause a prolongation of
infection.   However this applies to the usual short course of
treatment for clinical malaria, and no experimental or clinical
observations have been made about their long term use for
prophylaxis in malaria..

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CHAPTER VIII

IMMUNOPHARMACOLOGY OF THE DIAMINOPYRIMIDINES

THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

INTRODUCTION

In 1946, proguanil was developed by British scientists for the treatment of malaria. Two years later, pyrimethamine was introduced (Falco et al, 1951). Another 2,4-diaminopyrimidine, trimethoprim, was used as an antibacterial agent, until the emergence of drug-resistant strains of falciparum malaria stimulated the search for effective alternatives. This led to the discovery of synergism between 2,4-diaminopyrimidines and sulphonamides. The combination of pyrimethamine and sulphadoxine (25mg : 500mg), known as Fansidar, has proven to be useful in the chemoprophylaxis and chemosuppression of malaria. The combination of 25 mg pyrimethamine and 1 gm trisulphapyrimidine is useful for toxoplasmosis. The combination of trimethoprim and sulphamethoxazole (80mg : 400mg), known as Bactrim or Septrin, is a useful, broad spectrum antibacterial agent. The choice of the particular sulphonamide analogue in these combinations depends on the compatibility of their respective pharmacokinetic properties. The combination of 2,4-diaminopyrimidines and sulphonamides results in the double blockade of folic acid synthesis at two points along the metabolic pathway. The 2,4-diaminopyrimdines are slowly absorbed from the gastrointestinal tract, producing blood levels of up to $3\mu g/ml$, 3-7 hours after an oral dose. Significant quantities are still present in the tissues nine days after a single dose. Pyrimethamine is better tolerated than trimethoprim. Large doses over long periods can give rise to folic acid deficiency.
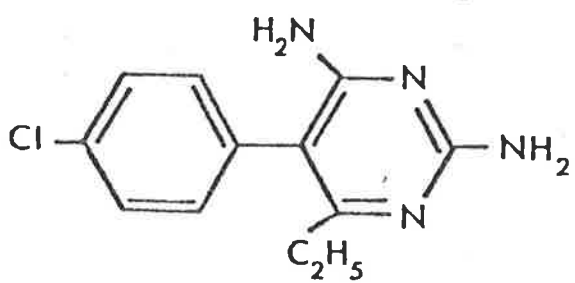
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Trimethoprim can produce marrow suppression. Their structural

formulae are shown in Fig. VIII-1.

IN-VITRO STUDIES

The inhibition of folic acid metabolism with these drugs results

in a decrease in production of DNA precursors such as thymine. Hence,

the addition of thymidine to lymphocyte cultures lacking in this

precursor would result in an increased rate of thymidine incorporation

into lymphocyte DNA. It has been shown for methotrexate, a potent

inhibitor of folic acid synthesis, that such cultures have a small

increase in thymidine uptake. Therefore, the results of in-vitro

experiments with pyrimethamine and trimethoprim must be interpreted

according to these considerations. It can be seen from table VIII-1

that trimethoprim did not have any effects on ^{3}H-thymidine incorporation

into lymphocyte cultures. In contrast, pyrimethamine caused a

striking increase of ^{3}H-thymidine incorporation in both PHA-stimulated

and PWM-stimulated cultures. Even cultures without mitogens showed a

significant increase above baseline. It is unlikely that such a

large increase of ^{3}H-thymidine uptake can be the result of precursor

deprivation, because methotrexate, a much more potent inhibitor of

folic acid synthesis produces only a slight increase in ^{3}H-thymidine

uptake. Pyrimethamine may in fact produce an actual increase in the

number of lymphoblasts; in parallel experiments, the blastogenic

index for PHA-stimulated cultures containing 1μg/ml of pyrimethamine

was 25.0 compared to 20.0 in PHA-stimulated cultures without

pyrimethamine. It is therefore concluded that pyrimethamine may

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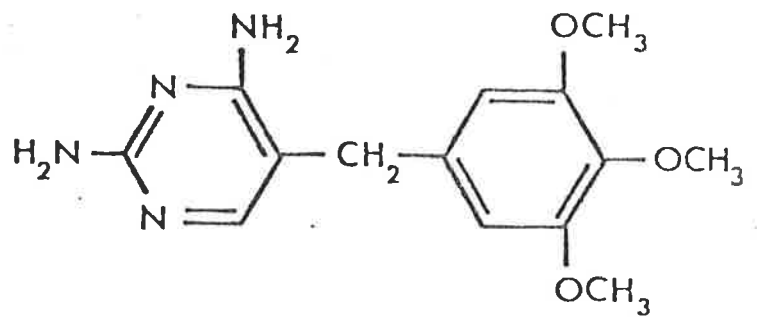
Figure VIII-1. Structural formulæ of pyrimethamine and trimethoprim.

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Table VIII-1. Comparison between the effects of trimethoprim and pyrimethamine on mitogen-induced lymphocyte transformation.

Drug concentration (μg/ml)	^{3}H-thymidine uptake		
	No mitogen	PHA	PWM
No drug	839 ± 161	25,967 ± 7,940	20,638 ± 2,862
Pyrimethamine			
1	1,896 ± 204*	64,801 ± 2,950**	48,457 ± 1,708*
4	2,053 ± 154*	56,498 ± 4,611**	47,441 ± 7,745*
10	1,852 ± 154*	52,493 ± 4,848*	39,577 ± 10,451*
Trimethoprim			
1	729 ± 112	20,005 ± 971	19,532 ± 1,951
4	769 ± 240	20,126 ± 1,093	18,744 ± 799
10	622 ± 177	26,601 ± 1,486	16,244 ± 510

Results represent mean ± S.D. of triplicate cultures.
* $p < 0.02$. ** $p < 0.001$.

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have a potentiating effect on mitogen-induced lymphocyte transformation.

Effect of varying concentrations of pyrimethamine.

A dose-response curve employing a wide range of pyrimethamine concentrations is presented in figure VIII-2. It can be seen that optimal concentrations of pyrimethamine are in the 1-10µg/ml range for PHA as well as PWM-stimulated cultures. Trypan blue exclusion tests showed that cell viability remained at 95% for concentrations of up to 25µg/ml, but decreased to 50% for 50µg/ml onward.

Effect of pyrimethamine on varying concentrations of mitogens.

In experiments where different dilutions of mitogen were employed (figure VIII-3), it can be seen that pyrimethamine at a fixed concentration of 10µg/ml potentiated lymphoproliferative responses at both sub-optimal and greater than optimal mitogen concentration.

Effect of pyrimethamine at various days in culture.

In experiments where optimal concentrations of mitogen were employed (figure VIII-4), it can be observed that pyrimethamine at a fixed concentration of 10µg/ml produced enhancement of lympho-proliferative responses from the third to the seventh day of culture in PHA cultures, and the third to the fourth day of culture of PWM cultures.
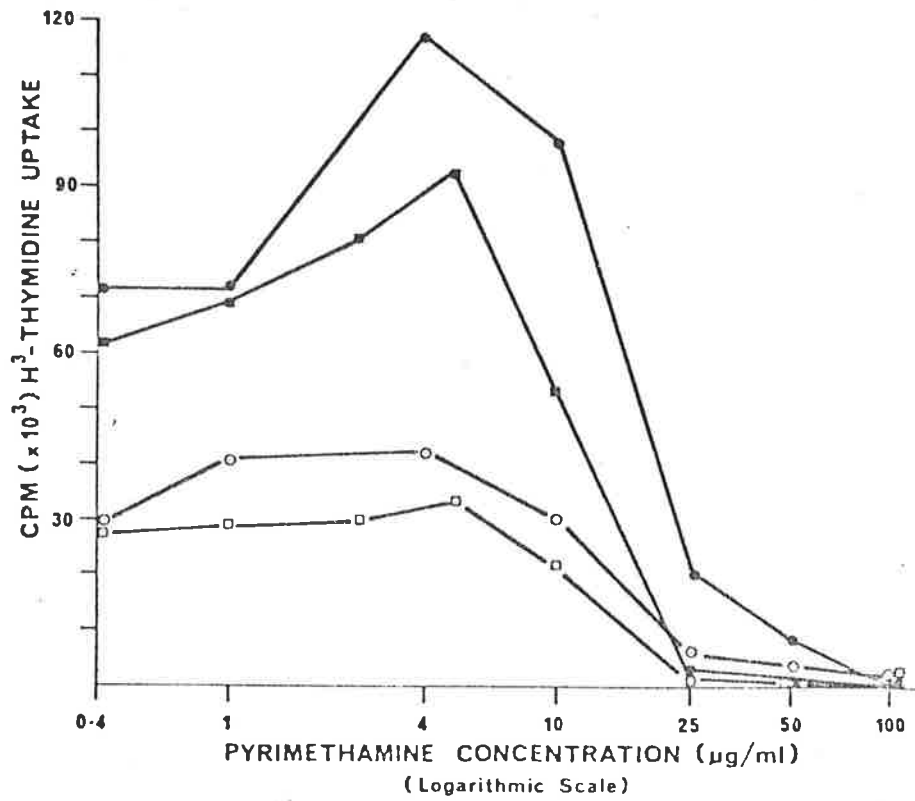
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Figure VIII-2. Effect of pyrimethamine on PHA-stimulated (square symbols) and PWM-stimulated cultures (circle symbols) after 3 days incubation. Cultures with pyrimethamine: closed symbols. Cultures without pyrimethamine: open symbols. Results are expressed as mean of triplicate samples. The S.E. did not exceed ±5%.

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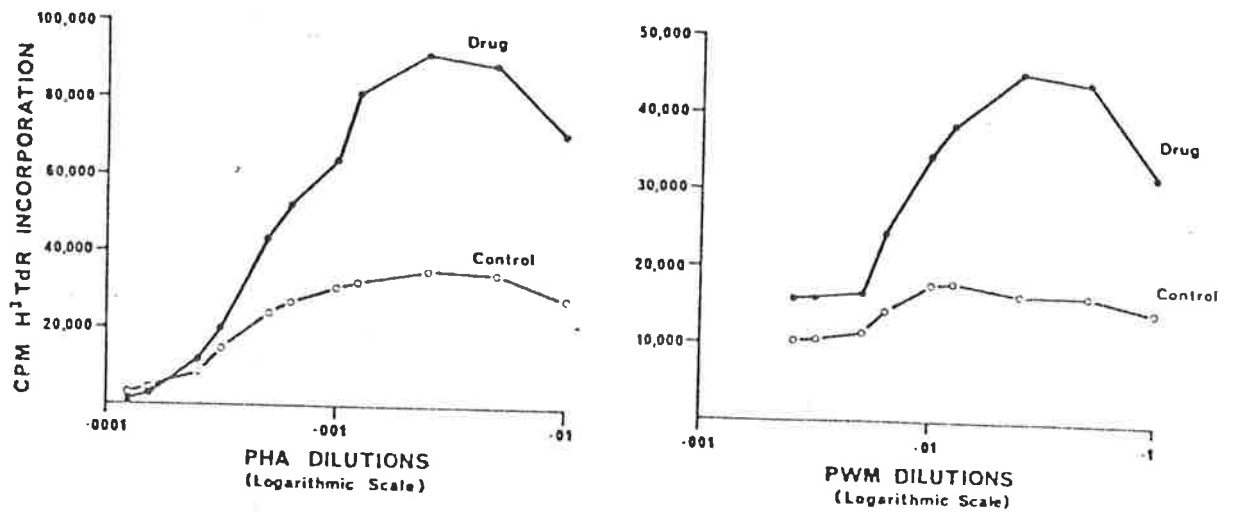
Figure VIII-3. Effect of pyrimethamine (10µg/ml) on lymphocyte proliferative responses to varying concentrations of mitogens. Results represent mean of triplicate samples harvested after 3 days in culture. The S.E. did not exceed ±5%.

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Figure VIII-4. Effect of pyrimethamine (10µg/ml) on mitogen-induced lymphocyte proliferative responses at various days in culture. The presence of ethanol (vehicle) at an appropriate concentration of 0.4% did not appreciably affect lymphocyte transformation. Results represent mean of triplicate samples. The S.E. did not exceed ±5%.
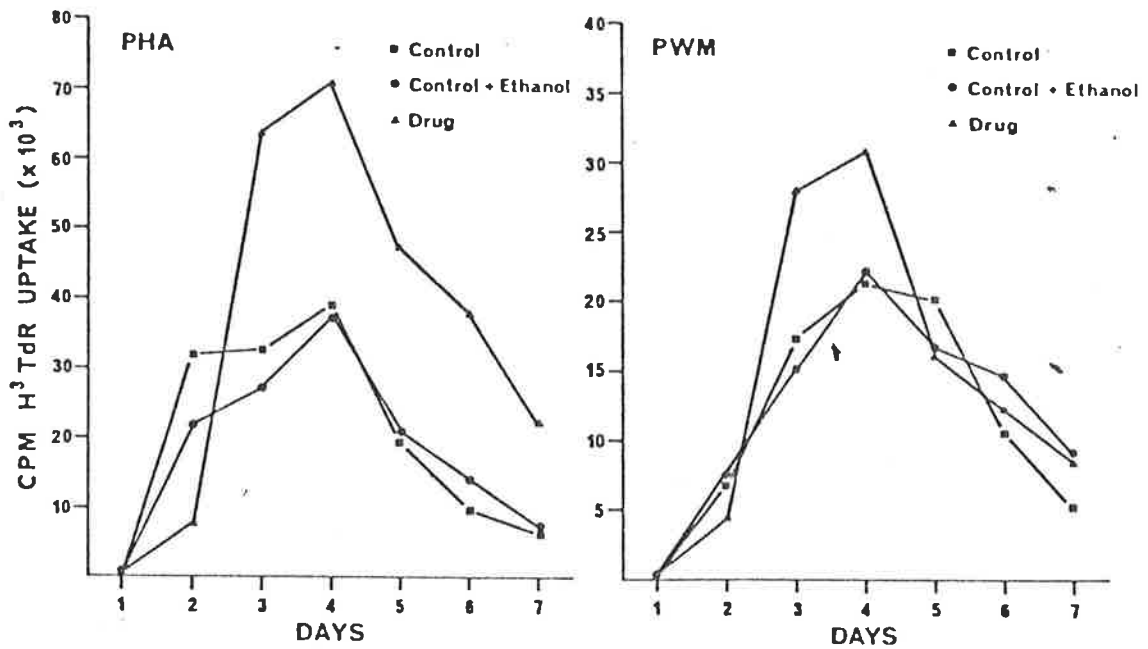
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Effect of immediate and delayed addition of pyrimethamine

Some of the characteristics of the potentiating effect of pyrimethamine on lymphoproliferative responses have been defined. Delayed addition of pyrimethamine, even up to 48 hours of culture, resulted in greater enhancement (table VIII-2).

Reversibility of pyrimethamine-induced enhancement of lymphocyte transformation.

The potentiating effect of pyrimethamine was also found to be compleltely reversible by washing (table VIII-3), which indicates that the drug does not bind tightly to cell receptors.

IN-VIVO STUDIES

The usual dosage of pyrimethamine for mice is 0.5 mg/kg (Peters, 1975) but it is a drug with very low toxicity (Goodman and Gilman, 1975) so that the higher doses chosen for these experiments were well-tolerated (Thong and Ferrante, 1980b).

Effect of different treatment schedules on DTH.

The results of single dose and multiple daily doses of pyrimethamine on the DTH response to SRBC in BALB/c mice are presented in table VIII-4. At the dose of 2.5 mg/kg/day, no effect on DTH was observed. In contrast, a single injection of 5 mg/kg on the day of priming with SRBC (day 0) resulted in marked enhancement

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Table VIII-2. Effect of immediate and delayed addition of pyrimethamine
on mitogen-induced human lymphocyte proliferative responses.

Time of addition of pyrimethamine (10 µg/ml)	PHA cpm ^{3}H-thymidine uptake	PWM cpm ^{3}H-thymidine uptake
0	42,011 ± 8,574	34,360 ± 2,975
4 hrs	*64,386 ± 9,259	32,853 ± 5,080
24 hrs	*68,980 ± 12,588	37,579 ± 2,674
48 hrs	**79,910 ± 2,154	**51,526 ± 2,744
None added	20,985 ± 1,227	12,337 ± 4,172

Results represent mean ± S.D. of triplicate samples.
Cultures harvested after 3 days incubation.
* $p < 0.05$.
**$p < 0.001$.

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Table VIII-3. Reversibility of pyrimethamine-induced enhancement of lymphocyte transformation.

Treatment (pyrimethamine 10μg/ml)	PHA ^{3}H-thymidine uptake	PWM ^{3}H-thymidine uptake
Unwashed	38,230 ± 2,401	27,748 ± 2,578
Washed	20,278 ± 1,924	8,136 ± 946
Untreated control	19,674 ± 1,938	8,400 ± 1,166

Results represent mean ± S.D. of triplicate samples.
In these experiments, lymphocytes were incubated in the presence of pyrimethamine for 1 hour and then washed three times prior to culture with mitogens for 3 days.

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Table VIII-4. Effect of different pyrimethamine treatment schedules on DTH responses to SRBC in BALB/c mice.

| Pyrimethamine treatment schedule | 5 mg/kg/day | | 2.5 mg/kg/day | |
	Total dosage (mg/kg)	Footpad thickness (% increase±S.E.)	Total dosage (mg/kg)	Footpad thickness (% increase±S.E.)
Nil	0	37.1 ± 3.0	0	40.7 ± 3.7
Day -1	5	43.2 ± 4.3	2.5	37.4 ± 2.4
" 0	5	66.1 ± 3.6*	2.5	41.1 ± 3.4
" +1	5	37.9 ± 4.4	2.5	36.6 ± 3.9
" -1 to +4	30	42.6 ± 3.4	15.0	39.6 ± 3.0
" 0 to +4	25	46.3 ± 2.1**	12.5	44.8 ± 3.3
" +1 to +4	20	57.1 ± 4.1†	10.0	40.6 ± 2.1
" 4	5	50.1 ± 2.7**	2.5	41.0 ± 2.7

There were 10 mice per experimental group. They received i.p. pyrimethamine at a dose of either 2.5 mg or 5.0 mg/kg/day on the days indicated, while controls received saline. On day 0, the mice were primed with 1×10^8 SRBC subcutaneously. Footpad challenge with 1×10^8 SRBC was performed on day 5 and measured on day 6.

* $p < 0.001$. ** $p < 0.05$. † $p < 0.01$.

of footpad response from 37.1% to 66.1% (p<0.001). A similar dose

given one day before or one day after priming did not enhance DTH.

Multiple daily doses also significantly increased DTH responses,

but not to the same extent as a single dose on the day of priming.

Effect of pyrimethamine on anti-SRBC antibody.

For these experiments, BALB/c mice were divided into four

groups. The first three groups received a single dose of either

2.5 mg/kg, 5 mg/kg or 15 mg/kg pyrimethamine i.p. while the fourth

group (controls) received sterile saline only. These mice were

immediately primed with 1×10^9 SRBC intravenously, and bled on day

6 for the determination of haemagglutinating antibody titre to SRBC.

The results (table VIII-5) showed that anti-SRBC antibody was

significantly enhanced by pyrimethamine. The log mean titre was

11.1, 12,2 and 13.0 in mice receiving 2.5 mg/kg, 5 mg/kg and 15 mg/kg

pyrimethamine respectively, compared to 10.7 in controls.

Effect of varying doses on DTH in C57B1/6J mice

C57B/6J mice were used in the next set of experiments in order to

determine whether the immunopotentiation of DTH by pyrimethamine

is reproducible in another strain of mice. The results (table VIII-6)

showed that enhancement of DTH also occurred after a single dose of

pyrimethamine given on the day of priming with SRBC. For this

strain of mice, enhancement of DTH was evident with the pyrimethamine

dosage of 2.5 mg/kg, which was not seen with BALB/c mice.

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Table VIII-5. Effect of pyrimethamine on anti-SRBC antibody titres in BALB/c mice.

Pyrimethamine dosage* (mg/kg)	Haemagglutinating titre ($\log_2$ mean±S.E.)**	p value
0	10.7 ± 0.2	–
2.5	11.1 ± 0.3	<0.01
5.0	12.2 ± 0.3	<0.001
15.0	13.0 ± 0.1	<0.001

* Given i.p. as a single injection on the day of priming with SRBC.
** There were 9-10 mice per experimental group.

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Table VIII-6. Effect of pyrimethamine on DTH response in C57B1/6J mice.

Pyrimethamine dosage* (mg/kg)	% increase in footpad tickness (mean±S.E.)**	p value
0	61.2 ± 2.3	-
2.5	81.9 ± 5.1	<0.01
5.0	76.2 ± 2.5	<0.01
15.0	75.6 ± 5.7	<0.05

* Given i.p. as a single dose on the day of priming with SRBC.
** There were 12-13 mice per experimental group.

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Effect of pyrimethamine on DTH in tumour-bearing mice.

For these experiments, C57Bl/6J mice were divided into four groups. Groups one and two received saline injections i.p. while groups three and four received 5×10^5 B16 melanoma cells. Seven days later, all four groups were primed with 1×10^8 SRBC subcutaneously; at the same time, groups two and four received pyrimethamine (5 mg/kg) i.p. while groups three and four received sterile saline. All four groups were challenged with 1×10^8 SRBC in the footpad 5 days after priming, and measured for DTH response the next day.

The results (table VIII-7) showed a significant impairment of DTH response in tumour-bearing mice (group 3) compared to control (group 1): increase in footpad thickness was 46.5% in tumour-bearing mice compared to 62.8% in controls ($p<0.01$). The results also confirmed previous studies that a single 5 mg/kg dose of pyrimethamine enhances DTH: pyrimethamine-treated mice (group 2) showed 88.8% increase in footpad thickness compared to 62.1% in controls ($p<0.001$). Of even greater interest is the observation that tumour-bearing mice treated with a single 5 mg/kg dose of pyrimethamine showed an increased of DTH to 77.4% (group 4) compared to only 46.5% in non-treated tumour-bearing mice (group 3) ($p<0.001$), and 62.1% in control mice (group 1) ($p<0.05$). These results suggest that pyrimethamine is an effective potentiator of DTH in tumour-bearing mice, even when DTH is already suppressed by the tumour.

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Table VIII-7. Effect of pyrimethamine on DTH response in C57B1/6J tumour-bearing mice.

Experimental groups*	% increase in footpad thickness (mean±S.E.)**	P- value (compared to control group)
1. Control	62.8 ± 4.0	-
2. Pyrimethamine	88.8 ± 4.5	<0.001
3. Tumour-bearing	46.5 ± 3.3	<0.01
4. Tumour and pyrimethamine	77.4 ± 4.3	<0.05

* Groups 2 and 4 were given a single dose of pyrimethamine (5 mg/kg) i.p. immediately after priming with 1 x 10^8 SRBC subcutaneously. Groups 3 and 4 were incoluated with 5 x 10^5 B16 melanoma cells 7 days prior to priming with SRBC.

** There were 12-13 mice per experimental group.

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CONCLUSIONS

Although much is known about purine and pyrimidine metabolism in mammalian cells, the regulation and interaction of these two pathways of nucleotide biosynthesis and degradation is not well understood (Ciba Foundation Symposium, 1977). An impetus to research in this area was provided by the discovery of immuno-deficiency syndromes associated with disorders of purine metabolism (Giblett et al, 1972; 1975). It appears that the functional integrity of purine metabolic pathways has a central role in maintenance of normal immunological responses. The precise role remains unclear, although toxic accumulation of metabolites such as adenosine and inosine can account for the depressed immunological responses of patients with adenine deaminase or purine nucleoside phosphorylase deficiency. Other mechanisms are possible, since these nucleotides are important precurosrs of not only nucleic acid synthesis, but also cyclic AMP and cyclic GMP synthesis, important regulators of cellular metabolism.

How pyrimethamine fits into all this is difficult to conjecture. The major mechanism for its antimicrobial activity is believed to be inhibition of folic acid synthesis (Goodman and Gilman, 1975). This in turn reduces the amount of purines and pyrimidines available to the cell for nucleic acid synthesis. Anti-folates such as methotrexate and trimethoprim are thought to produce immunosuppression in this manner, yet pyrimethamine, an analogue of trimethoprim, enhances rather than suppresses immunological responses.

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That pyrimethamine acts by inactivating suppressor cells and therefore indirectly enhances antibody and DTH responses is one possible explanation. Another hypothesis, which also seems plausible, is based on the structural formula of pyrimethamine. As a diaminopyrimidine, it might interfere with pyrimidines in the cell by substrate competition, which in turn upsets the balance of purine and pyrimidine biosynthesis and biodegradation, and ultimately influences cellular proliferation.

It is possible to construct other elaborate hypotheses, but further studies are necessary to establish the precise mechanism of immunopotentiation. Be that as it may, the results of the present studies suggest that pyrimethamine may be a valuable drug for immunopotentiation. In this regard, it appears to be superior to levamisole, which is only effective in situations where immune responses are depressed. In fact, the status of levamisole as an immunopotentiator remains unclear (Willoughby and Wood, 1977). However, caution must be exercised in the direct application of animal data to the clinical situation. Preferably, much more should be understood about the immunopharmacology of pyrimethamine before clinical trials are embarked upon.

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CHAPTER IX

DISCUSSION

THE IMMUNOPHARMACOLOGY OF ANTIMICROBIAL DRUGS

INTRODUCTION

In this chapter, an attempt will be made to assess the validity of the data presented in the previous chapters, to discuss the biological and clinical significance of the findings, and to indicate worthwhile areas for future research. In order to bring this subject matter into perspective, some information on the extent of antimicrobial drug usage, and the magnitude of the problems of inappropriate usage will be first examined.

USE AND ABUSE OF ANTIMICROBIAL DRUGS

Much more is known about prescriptive practice with antibiotics than with antimicrobial drugs as a whole. Although the information is mainly derived from American experience, it would be fair to assume that similar trends exist in other developed countries, and the urban areas of many of the developing nations.

It has been estimated that antibiotics account for up to 20% of prescriptions in general practice. It is in fact the most commonly prescribed class of drugs. Several factors suggest that most of these prescriptions are not really indicated. A culture is not usually taken. It is sometimes done by telephone. It is sometimes prescribed for a viral illness, and even for the common cold (Simmons and Stolley, 1974). In hospital practice, it has been found that up to 60% of patients treated with antibiotics have no

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evidence of infection.   In many instances, especially in surgical wards, antibiotics are prescribed for prophylactic purposes in routine fashion with dubious benefits.   In the United States alone, a total of 16.9 million pounds of antibiotics were produced in 1970; of this 9.6 million pounds were used on patients while 7.3 million pounds were used in animal feed.   Figures for 1971 showed that there were 31 million prescriptions for penicillins, 23 million for erythromycins, 21.5 million for ampicillins, 54 million for tetracyclines, and 33 million for other than the above.   Such large quantities of drugs can constitute a pollution problem by its presence in the environment, but the potential hazards can be greater than those of improper disposal of chemical wastes (Dunea, 1979) because these drugs are deliberately administered to millions of people around the world.   Evidence will be provided in the next section for the mutagenic and cancerogenic potential of antimicrobial drugs, in addition to the well-recognised problems of toxic and allergic side-effects, induction of resistant strains, and promotion of nosocomial infections, not to mention the financial burdens to the community and the individual.   Most of the discussion will be concerned with immunosuppressive side-effects, which is the theme of this thesis.

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ADVERSE EFFECTS OF ANTIMICROBIAL DRUGS

Toxic and allergic side-effects

In a 3-year prospective study, Caldwell and Cluff (1974) found an overall incidence of 4.5% of adverse reactions to antimicrobial drugs. The majority of patients who developed allergic or toxic side-effects required either further treatment for the iatrogenic illness or an extension of hospital stay. Loss of life was rare but nevertheless real.

Induction of antibiotic-resistant bacteria

It is believed that elimination of drug-sensitive bacteria by widespread use of antibiotics provides the opportunity for drug-resistant strains to persist and spread. The mechanisms of drug-resistance have been discussed in Chapter I, but suffice it to say that the recent emergence of penicillin-resistant Pneumococci (Editorial, 1977) and ampicillin-resistant Haemophilus influenzae (Jacobsen et al, 1976) exemplifies this aspect of the problem.

Nocosomial infections

Patients admitted to hospital for whatever reason are at risk to infectious agents within th hospital environment. The selective pressure of antibiotic usage within hospital generally means that these organisms are drug-resistant. It also means that Gram-negative intestinal bacilli, because of their propensity for transfer of

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resistance factors, are the predominant organisms, although penicillinase producing staphylococcus aureus seems to be holding its own.

Immunosuppressive side-effects

The immunosuppressive potential of antimicrobial drugs is of some concern in certain clinical situations. Since antibiotics are prescribed indiscriminately for viral infections, suppression of immunity in cases where a virulent virus is responsible for the infection can mean the difference between early and uneventful recovery, or serious complications and even death. In old people, pregnant women and infants, where there is physiological immuno-incompetence, even a not-so-virulent virus can gain the upper hand. Malnourished individuals, or patients debilitated by serious diseases, immunosuppressive or anti-cancer therapy, are frequently invaded by opportunistic microogansims. Antimicrobial drugs by themselves may not be effective under such circumstances because some contribution by host immune responses is generally required for the complete elimination of infectious organisms. Immune globulin injections and leukocyte transfusions can increase the chance of survival. Also, the correct choice of antimicrobial agent - one that the microorganisms is sensitive to, and is without immunosuppressive properties - can be critical for recovery.

Although these considerations are of obvious clinical importance, little is known about the immunopharmacological properties of

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antimicrobial drugs, and no mention has been made of this aspect of antimicrobial chemotherapy in textbooks of pharamcology.   There is now accumulating evidence to suggest that at least some of the antimicrobial drugs possess immunosuppressive properties.

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During the preparation of this thesis, several reports on this topic by other scientists have appeared in the literature.   These reports will be discussed in conjunction with data in this thesis in order to provide a more comprehensive and balanced account of current thinking on the subject.   Also, it became quite obvious earlier on in these studies that the results were important enough to warrant early publication, so as to create an awareness among other physicians and stimulate more research activity in this field.

Relevance of in-vitro screening on situation in-vivo

The blastogenic response of lymphocytes in culture on exposure to mitogens is generally regarded as an in-vitro correlate of immune processes (Ling and Kay, 1975).   Soon after contact with mitogen, a variety of biochemical events ensue which lead to biosynthesis of protein, RNA and DNA.   By the second day, lymphoblasts can be seen in the cultures.   Mitotic division results in clonal proliferation; in this case many clones are formed because plant mitogens are polyclonal stimulants.   The lymphocytes differentiate into

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immunoglobulin-producing cells, T-killer cells, T-helper cells, T-suppressor cells, memory cells, etc. This sequence of events is generally quantitated by ^{3}H-thymidine uptake, a direct measure of DNA synthesis, and quite indicative except for situations where drugs such as antifolates may produce thymine deprivation and cause spurious increase in uptake of ^{3}H-thymidine. It is always prudent to double check by visual counting of lymphoblasts in the cultures.

This assay enjoys widespread clinical and experimental usage because of sensitivity and reproducibility, and with the introduction of a semi-automated adaptation, there are added advantages of simplicity and convenience. It is obviously not a test of phagocytic function, and this aspect warrants further research because several antimicrobial drugs appear to interfere with function of phagocytes (Bjorksten et al, 1976; Thong and Ness, 1977). As a test of specific immunity, it appears to be fairly sensitive. On the basis of known mechanisms of action of antimicrobial drugs (Sanders and Sanders, 1979), this assay conforms to predictions that drugs acting on cell walls will have little or no immunopharmacological activity, while drugs acting on cell membrane or nucleic acid synthesis may have immunopharmacological activity. Confirmation of these results is provided by in-vivo tests, which show that most of the drugs identified in-vitro have in-vivo activity (Table IX-1).

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Table IX-1. Relevance of in-vitro tests to the in-vitro situation in relation to immunopharmacological properties of antimicrobial drugs.

Antimicrobial Drugs	Immunopharmacological Activity	
	in-vitro	in-vivo
Tetracyclines	Yes (1)(2)	Yes (1)
Miconazole	Yes (1)	Yes (1)
Amphotericin B	Yes (1)(3)	Yes (1)
Quinine	Yes (1)(4)(5)	No (1)
Chloroquine	Yes (1)(6)	No (1)
Primaquine	Yes (1)	No (1)
Quinacrine	Yes (1)	N.D.
Mefloquine	Yes (1)	Yes (1)
Pentamidine	Yes (1)	N.D.
Trimetoprim	No (1) Yes(7)	Yes (8)
Pyrimethamine	Yes (1)	Yes (1)
Rifampin	Yes (9)(10)	Yes (10)(11)(12)
Chloramphenicol	Yes (13)	N.D.
Clotrimazole	Yes (3)	N.D.
Niridazole	N.D.	Yes (14)
Metronidazole	N.D.	Yes (15)
Fusidic acid	Yes (2)	N.D.

(1) This thesis
(2) Forsgren and Banck, 1978.
(3) Tarnvik and Ansehn, 1974.
(4) Gold and Ben-Efraim, 1978.
(5) Gold et al, 1979.
(6) Hurvitz and Hirschhorn, 1968.
(7) Gaylarde and Sarkany, 1972.
(8) Ghilchik et al, 1970.

(9) Nilsson, 1971.
(10) Grassi and Pozzi, 1972.
(11) Dajani et al, 1973.
(12) Serrou et al, 1972.
(13) DaMert and Sohnle, 1979.
(14) Mahmoud et al, 1975.
(15) Grove et al, 1977.
N.D. = not done.

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The penicillins and cephalosporins

Penicillin is the first antibiotic discovered, and remains the most useful, especially now that broad-spectrum, acid-tolerant and penicillinase-resistant analogues have been developed. The penicillins, and cephalosporins to a lesser extent, are non-toxic to mammalian cells even in high concentrations, and generally safe except for allergic side-effects. They have no immunopharmacological properties in-vitro. Similar findings were reported by Forsgren and Banck (1978). In-vivo studies have not been done.

The macrolide antibiotics

These drugs inhibit protein synthesis in bacteria. They can inhibit mitogen-induced lymphocyte transformation at high concentrations of up to 50µg/ml (Forsgren and Banck, 1978), but this level cannot be achieved in serum by conventional dosage schedules. Data from this thesis shows no in-vitro effect at the realistic concentration of 10µg/ml. In-vitro studies have not been done, but may be worthwhile pursuing.

Chloramphenicol

This drug is also a protein synthesis inhibitor. Previous studies (Pisciotta et al, 1967; Becker et al, 1974; Ugazio et al, 1974) have shown in-vitro effects at high concentrations only. Clinically achievable concentrations did not suppress mitogen-induced lymphocyte transformation in this laboratory, but Damert

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and Sohnle (1979) recently showed that specific antigen-induced transformation was affected by low concentrations. In-vivo studies would seem worthwhile.

Tetracyclines

Results from this laboratory showed that doxycylcine suppressed mitogen-induced lymphocyte transformation at concentrations below 10µg/ml, whereas Forsgren and Banck (1978) showed suppression at concentrations greater than 10µg/ml. This disparity may be due to technical differences between laboratories, because in-vivo studies confirmed the in-vitro findings of immunosuppression.

The aminoglycosides

No effect on mitogen-induced lymphocyte transformation was observed; similar findings were reported by others (Forsgren and Banck, 1978). In-vivo studies have not been done.

Miscellaneous antimicrobials

The sulphonamides, polymyxins, and nitrofurantoin have no effect on mitogen-induced lymphocyte transformation (Chapter III).

Rifampin

This drug is the best documented as far as immunopharmacological properties are concerned. It has both in-vitro (Nilsson, 1971; Grassi and Pozzi, 1972) and in-vivo (Grassi and Pozzi, 1972; Dajani et al, 1973; Serron et al, 1972) immunopharmacological activities.

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The imidazoles

Miconazole and Clotrimazole are promising drugs for treatment of deep mycotic infections, miconazole being more promising. Results from this laboratory indicate that miconazole suppresses both in-vitro and in-vivo immunological responses. Clotrimazole has only been studied in-vitro (Tarnvik and Ansehn, 1974).

The nitroimidazoles

Mahmoud et al (1975) followed up the clinical observation that niridazole reduced granuloma formation during treatment for schistosomiasis, and found that it was a potent immunosuppressive agent in animals. In-vitro studies have not been done. A related drug, metronidazole - used for the treatment of trichomoniasis and amoebiasis - was also noted to be immunosuppressive in-vivo (Grove et al, 1977).

Amphotericin B

Earlier reports (Ishikawa et al, 1975; Blanke et al, 1977) suggested that this antifungal drug is an immunoadjuvant when mice are given a single injection. However, in the clinical situation, this drug is administered on a daily basis for days and sometimes weeks. In-vivo studies in this laboratory using a multiple dosage schedule confirm in-vitro findings that amphotericin B has potent immunosuppressive properties.

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The antimalarials

Chloroquine, primaquine, mefloquine and quinine were found to suppress mitogen-induced lymphocyte transformation. Similar in-vitro findings were reported by others for chloroquine (Hurvitz and Hirshhorn, 1968) and quinine (Gold and Ben-Efraim, 1978; Gold et al, 1979). However, in-vivo studies in this laboratory showed no effects on immunological responses. For these studies, mice were treated with a short course of the drug only. It is conceivable that long term treatment with these drugs for chemoprophylaxis of malaria may result in immunosuppression.

The diaminopyrimidines

A slight discrepancy exists for in-vitro effects of trimethoprim. No effect was found in this laboratory using lymphocytes from 2 donors, whereas Gaylarde and Sarkany (1972) found that some individuals are more susceptible than others. One other study (Ghilchik et al, 1970) showed a prolongation of skin-graft survival in rats. Pyrimethamine, on the other hand, was found to enhance ^{3}H-thymidine incorporation, and perhaps actual blastogenesis of lymphocytes. In any case, it appears to be a potent immunostimulant in-vivo.

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CONCLUSIONS

Several drugs with immunopharmacological properties have been identified from this general survey of commonly used or clinically important antimicrobial agents. This list is by no means complete, either because the in-vitro or in-vivo methods used have not been sesnitive enough to idenitify all of them, or because other aspects of immunity, such as phagocytic function, have not been incoprorated into the testing programme. Also, not all available antimicrobial drugs have been included in the study. Nevertheless, it is possible to conclude that some antimicrobial drugs may have immunopharmacological properties. This by itself is neither good nor bad. For instance, if miconazole proves useful in organ transplantation for its immunosuppressive effects, an additional boon will be its antifungal action because systemic fungal infections are common in this group of patients. The knowledge, as it becomes available, will provide a more rational basis for the choice of antimicrobial drugs.

Mutagenic and carcinogenic potential

It is the extensive and indiscriminate prescription of anti-microbial drugs by the general medical community that evokes the most concern. One other side-effect which has been mentioned in passing but not discussed in depth is the potential of some antimicrobial drugs as mutagenic and carcinogenic chemicals.

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This applies in particular to antimicrobial drugs which act by disruption of nucleic acid synthesis, either directly or indirectly. This mode of action is similar to that of conventional immunosuppressive drugs. A cell with its DNA damaged by chemicals can undergo mutation, and if it is a somatic cell may become cancerous, or if it is a germ cell, may be passed on as genetic defects in future generations (Ames, 1979). Other possible consequences of DNA damage are cataracts, aging and cardiovascular disease.

In this connection, a few of the antimicrobial drugs have been implicated as teratogens. This is best documented for tetracycline. Beverlander et al (1963) reported growth inhibition of human and rat newborns whose mothers were treated with tetracycline during pregnancy. Two other infants, one with limb abnormalities (Carter and Wilson, 1962), and the other with multiple skeletal and soft tissue abnormalities (Corcoran and Castles, 1977), were born to mothers on tetracycline treatment. Birth defects have also been noted following chloroquine treatment of pregnant women (Meyers et al, 1978). Metronidazole and its metabolites have been implicated as mutagens (Connor et al, 1977). More information is required about this aspect of antimicrobial drugs. Carcinogenecity has not been documented as a complication of antimicrobial drugs, perhaps because this can occur years after the event, so that the aetiological connection becomes blurred.

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Immunopotentiation by pyrimethamine

A most promising spin-off from this systematic approach to the study of the immunopharmacology of antimicrobial drugs is the discovery that pyrimethamine can potentiate immunological responses. This was not quite unexpected, although the possibility of finding such a drug must be considered as remote.   Many centres are actively engaged in the study of immunopotentiating agents, because of the potential for clinical application in patients with cancer, immunodeficiency disorders, collagen-vascular and infectious diseases, and also of their value as tools for the study of immunological phenomena.   In this regard, pharmacological agents have several advantages over other classes of immunopotentiating substances (e.g. BCG), since drugs can be administered and withdrawn at will, and their pharmacokinetics understood so that their effects can be regulated by suitable dosage schedules. Pyrimethamine may prove useful in these respects, but the extrapolation of animal studies to the clinical situation must be tempered with caution.

EPILOGUE

In 1909, Paul Ehrlich introduced Salvarsan for the treatment of syphilis; it was the result of a deliberate testing programme to develop drugs for chemotherapy of infectious diseases.   His ideas, however, did not come to complete fruition until the

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development of sulphonamides in 1935 and penicillin in 1941.   In
those intervening years, an opposing viewpoint,championed by
Metchnikoff and others, was that the stimulation of immune responses
offers the best hope of success for the treatment of infectious
diseases.   It was not without irony that the principal proponent
of this alternative viewpoint, Almroth Wright, came to witness
within his own laboratory the discovery of penicillin.

The story, however, has now turned full circle.   Medical
progress has created a large and important group of patients with
organ transplants, cancer and other debilitating diseases in whom
immunity has been severely compromised by drugs and irradiation.
There patients become unduly susceptible to infection by opportunistic
pathogens for which even appropriate andpotent antimicrobial drugs
may be ineffective, unless concurrent steps are also taken to
restore and enhance immunity.

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