

**A MONOKINE WHICH BINDS TO CLASS II
HISTOCOMPATIBILITY PROTEINS.**

by

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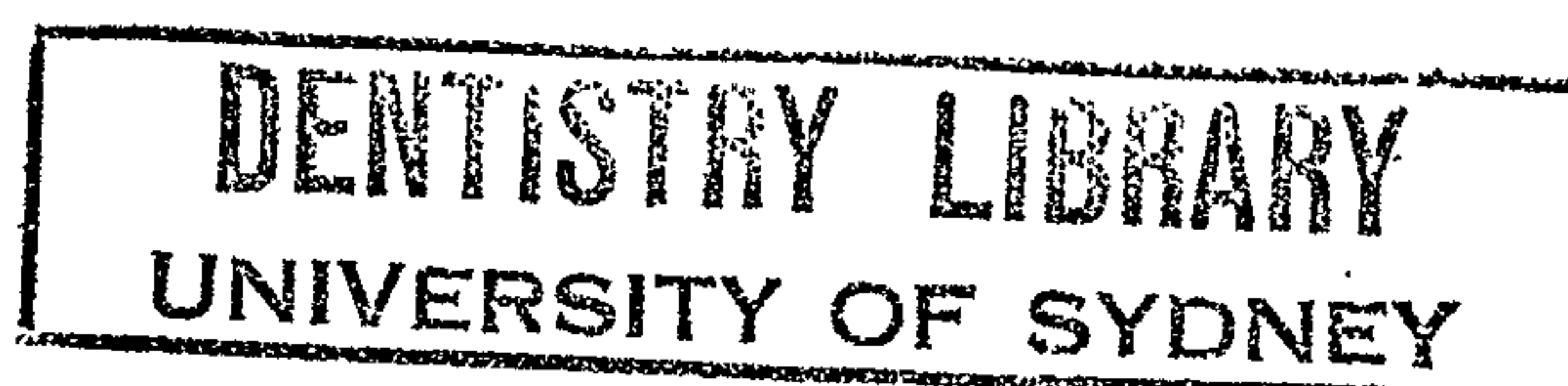
A thesis submitted in fulfilment of
the requirements for the degree of
Doctor of Philosophy (Dentistry)

in the

Department of Preventive Dentistry

The University of Sydney

June 1988



DECLARATION

The experiments described in this thesis were carried out in the Institute of Dental Research at the United Dental Hospital of Sydney, between Lent Term 1985 and Michaelmas Term 1987. The experiments are the original work of the author and have not been submitted, in whole or in part, for any other degree.

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ACKNOWLEDGEMENTS

During this project I was the recipient of a Postgraduate Dental Research Scholarship from the National Health and Medical Research Council of Australia. I am grateful to this organisation for their generous financial assistance. In addition I would like to thank the following people, without whom this project would not have been possible.

Dr. Neil Hunter, Principal Scientific Officer at the Institute of Dental Research, who supervised this project and provided advice and encouragement,

Professor Noel Martin, for allowing me to do this degree in the Department of Preventive Dentistry,

Professor Ken Knox, for making all of the facilities of the Institute of Dental Research so readily available,

Dr. D.W. Mason, for his generous gift of the monoclonal antibody, MRC-OX 52,

Robert Van Luyn and Peter Holloman, for their assistance with photography,

The research staff at the Institute for their assistance and encouragement,

Paul Kirwan, at the Royal Prince Alfred Department of Electron Microscopy, for his assistance with the preparation of specimens,

Professor Alan Ng, for his assistance with the preliminary histopathological studies,

Janet Jeffreys, Joan Thwaite and Christine Crocker for their assistance in researching the topic,

my parents for their practical assistance and support

and finally, my husband Andrew for his co-operation, support and encouragement throughout the time that this project has taken.

FOREWORD

This thesis is presented in two sections, each of which is prefaced by a review of the relevant literature. The first section deals with a clinical problem, oral lichen planus and the difficulties inherent in diagnosing the disease. Two studies have been carried out in an attempt to distinguish this disease from other oral mucosal problems.

In the course of these preliminary experiments, the need for an animal model, to study the interaction of T lymphocytes; macrophages and epithelial cells, became apparent. The system which evolved for this purpose, provides the basis for the experiments in the second part of the thesis. In this section, a monokine is described. The production of the monokine, its binding site and its effect on thymocyte proliferation are examined and potential roles for the monokine, in various situations, are discussed.

SUMMARY

This thesis describes a series of experiments which were designed to study the interaction of macrophages, epithelial cells and thymocytes in a rat model system. The need for such a system arose from two preliminary studies of an oral mucosal disease, lichen planus, in which the aetiology and pathogenesis, are the subject of considerable controversy.

I. CLINICAL STUDIES

In the first study, an attempt was made to define more precisely, the histopathological parameters that would be diagnostic of oral lichen planus despite the variability of its clinical presentation. In addition, by studying the association of these parameters, it was hoped that more information about the pathogenic mechanisms involved, would be obtained.

In the second of the preliminary studies, the expression of the class II histocompatibility protein, HLA-DR by keratinocytes in oral lichen planus was studied and compared with other oral mucosal lesions. The finding of an association between expression of HLA-DR and a particular disease process would have been of diagnostic value. However, it appeared to be an indicator of cell-mediated immune reactions, in general, rather than of a specific disease.

II. STUDIES IN A RAT MODEL SYSTEM

There is a marked decrease in the viability, in vitro, of dense, immature, rat thymocytes over a four hour incubation period. The addition of an Mr 36,000 monokine derived from cultured rat peritoneal macrophages which had been previously stimulated with lipopolysaccharide prevented these cells from dying. The early release of this factor, together with preliminary results of its physical and functional properties suggest that it is distinct from the well characterized monokines. The macrophage factor was found to bind in competition with a monoclonal antibody directed against a common determinant on Ia but to bind non-competitively with a monoclonal antibody to a rat strain specific epitope on Ia. Based on the results of experiments using a fractionated thymic cell model, it would appear that this monokine binds to the Ia protein complex on the cell surface of thymic epithelial cells causing them in turn to release an activity which is ultimately responsible for the survival of thymocytes.

The addition of picogram quantities of bacterial products to rat thymic cells in culture produced a doubling of the proliferative response of these cells to sub-optimal levels of concanavalin A. This effect could be prevented by the depletion of adherent cells which comprised less than 0.1% of the total population. The response was restored by the addition of supernatants from peritoneal macrophages which had been stimulated two hours previously with lipopolysaccharide. Treatment of these

supernatants with phenylglyoxal, an inhibitor of interleukin 1, did not prevent the stimulatory effect. Augmentation of the thymocyte proliferative response could also be achieved by the addition of either the partially purified, Mr 36,000 monokine or a monoclonal antibody to a common determinant on Ia.

Fractionation of the thymic cells on a density gradient yielded a buoyant population which accounted for the majority of the proliferative activity and a dense cell fraction which was poorly responsive to the mitogen. The addition of the monokine to this latter fraction produced a significant increase in proliferation in response to concanavalin A. This suggested that the factor had promoted the survival of these cells but, in addition, had also caused the maturation of the thymocytes to a point at which they were capable of responding to the mitogen.

Experiments were designed to provide more definitive data on the production of this Mr 36,000 monokine. The results of these studies indicated that production occurred in response to either lipopolysaccharide or muramyl dipeptide but that the activity was greatest in response to 20 µg/ml of lipopolysaccharide when the supernatant was collected two hours after the cells were challenged. Increasing the culture time beyond two hours resulted in a decrease in activity at three hours and a complete loss of activity from four hours on to forty-eight hours. The effect of three protein synthesis inhibitors, on production, were tested and found to have no effect on the initial release of the factor but to effectively inhibit production after fifteen minutes. Lysis of unstimulated macrophages, produced protective activity suggesting that it was constitutively present in all cells.

Furthermore, attempts to remove the activity from stimulated macrophages by treatment with trypsin, prior to lysis, failed and it was assumed, on this basis, that the activity was not present on the surface of these cells.

The results of preliminary experiments designed to characterise the thymic epithelial cell factor are also presented.

In addition, potential roles for the monokine in cell-mediated immune reactions, macrophage biology and thymic biology are proposed.

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ABBREVIATIONS

PBS - phosphate buffered saline

kD - kilodalton

cAMP - cyclic adenosine monophosphate

MHC - major histocompatibility

Mr - molecular mass

ATPase - adenosine triphosphatase

DNA - deoxyribonucleic acid

LPS - lipopolysaccharide

TNF tumour necrosis factor

Il-1 - interleukin 1

cDNA - copy DNA

pI - iso-electric point

EDTA - ethylene diamine tetra acetic acid

PAGE - polyacrylamide gel electrophoresis

F'ab - fragment of proteolysis which retains antibody activity.

Those fragments derived by papain digestion are univalent.

F'c - cleavage product of the immunoglobulin molecule which contains no antibody activity.

IgG - immunoglobulin G

IgM - immunoglobulin M

FCS - foetal calf serum

DCF - dense cell fraction

DTEC - dense thymic epithelial cells

MF - macrophage factor

Ia-ve DCF - dense cell fraction with Ia-+ve cells removed

Con A - concanavalin A

MRCOX6 - antibody to a common determinant on Ia

MRCOX3 - antibody to Ia that is rat strain specific in its
recognition

MRCOX52 - antibody to rat T cells

W3/25 - antibody to CD4

CD4 - T helper cell marker in humans, equivalent to T4.

MF-TECF - macrophage factor induced thymic epithelial cell
factor

MRCOX6-TECF - Thymic epithelial cell factor induced with an
antibody to a common determinant on Ia.

PUBLICATIONS

The work described in this thesis has been published in the following papers:

Hedberg, N., Ng, A. & Hunter N. (1986) A semi-quantitative assessment of the histopathology of oral lichen planus. *Journal of Oral Pathology*. 15, 268-272.

Hedberg, N.M. & Hunter, N. (1987) The expression of HLA-DR on keratinocytes in oral lichen planus. *Journal of Oral Pathology*. 16, 31-35.

Hedberg, N. & Hunter, N. (1988) A monokine which binds to class II histocompatibility proteins. *Immunology and Cell Biology*. (In Press).

Hedberg, N. & Hunter, N. (1988) The augmentation of mitogen-induced thymocyte proliferation by bacterial products is mediated by an Mr 36,000 monokine. *Immunology and Cell Biology*. (In Press).

Hedberg, N. & Hunter, N. (1988) Characteristics of the production of an Mr 36,000 monokine. (In preparation).

PREFACE TO PART ONE

The first part of this thesis contains a brief literature review of the histopathology of oral lichen planus with emphasis on the difficulties inherent in diagnosing the disease. This is followed by two studies of oral lichen planus, in which the histological features (Chapter Two) and the expression of HLA-DR (Chapter Three) were examined.

CHAPTER ONE

LITERATURE REVIEW - THE HISTOPATHOLOGY OF LICHEN PLANUS

1.1 Introduction

Oral lichen planus is a relatively common disease which appears to represent the manifestation of a mucosal reaction to a variety of aetiological factors.

The appearance of lichen planus on the oral mucosa is variable and there have been numerous attempts to classify the disease on the basis of its clinical appearance. Essentially, the disease varies from the more debilitating, erosive forms characterized by widespread areas of ulceration which can persist for years (Plate 1) to a non-erosive presentation, consisting of leukoplakic white striae (Plate 2).

The incidence, site distribution, age and geographical distribution, prognosis and treatment of the disease have been extensively reviewed by Scully and El-Kom (1985). The present discussion will be confined to the histology of lichen planus and its possible implications in the pathogenesis of the disease.



Plate 1. The clinical presentation of oral lichen planus in a case where oral ulceration predominated.

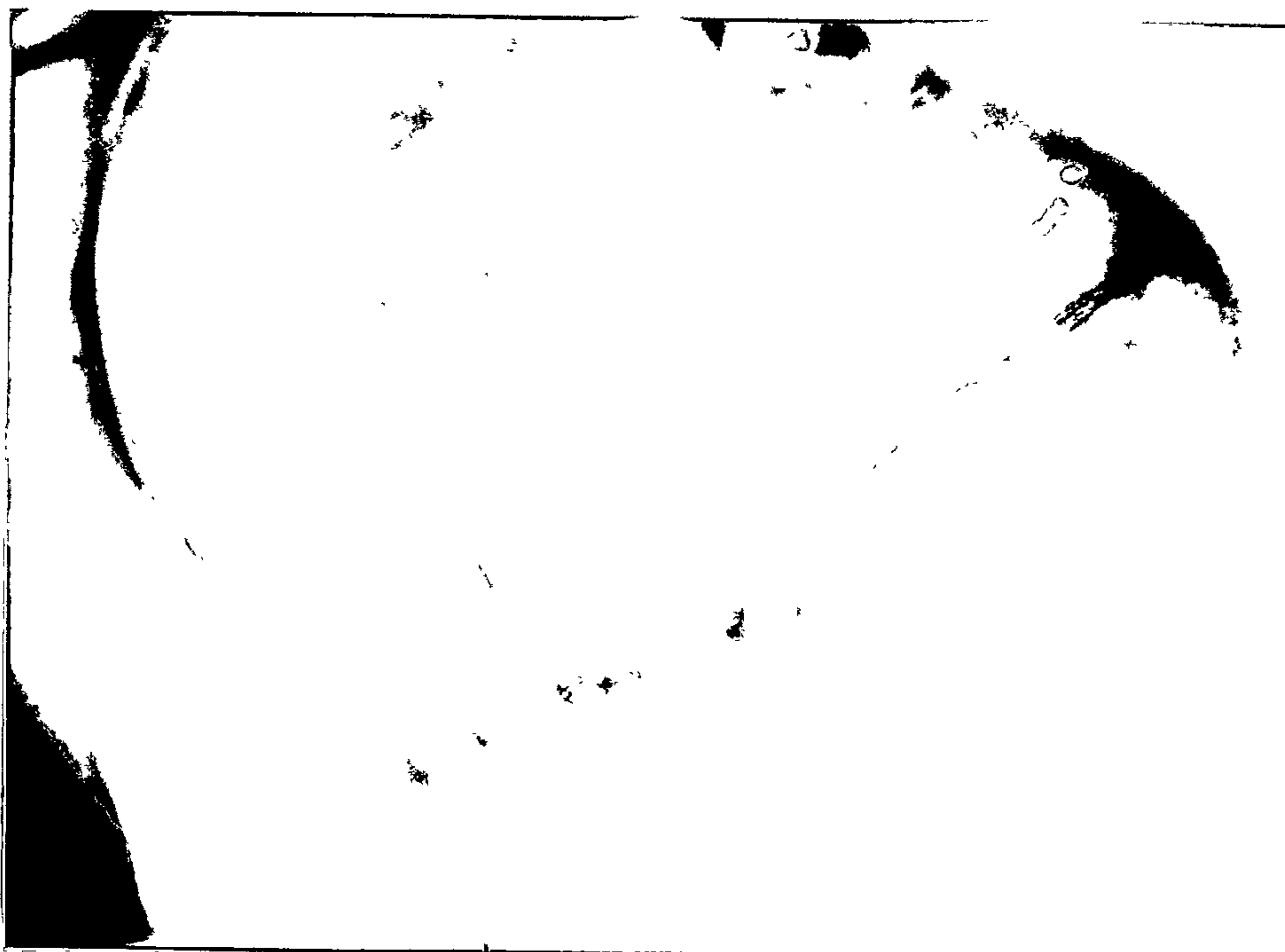


Plate 2. The clinical presentation of oral lichen planus in a case where lichenoid striae predominated.

1.2 Histopathology

(1). Light microscopic findings

While the histological findings in dermal lichen planus are considered to be characteristic of the disease on the skin and as such can be used to make a diagnosis (Thyresson & Moberger, 1957 and Ellis, 1967), there is still considerable controversy regarding the microscopic findings in oral lichen planus. For the purpose of making a microscopic diagnosis of oral lichen planus, Shklar and Meyer (1961) described a triad consisting of (i) parakeratosis (ii) hydropic degeneration of the basal layer, and (iii) a bandlike chronic inflammatory infiltrate adjacent to and beneath the epithelium. The importance of a supportive clinical diagnosis was emphasised as often the microscopic picture was merely one of non-specific inflammation. McClatchey et al., (1975), reported histological and clinical agreement in diagnosing a large number of various forms of lichen planus. They found hyperkeratosis and a variable infiltrate in all cases but observed basal degeneration in only 59% of the biopsy specimens studied. Similarly, Kramer et al., (1970) examined the frequency of tissue changes in lichen planus and other oral keratoses and found mononuclear infiltration and basal layer degeneration to be the most common findings in lichen planus. Andreassen (1968), reported similar findings to those of Shklar and Meyer, (1961) with the addition of a marked stratum granulosum, however, the only consistent finding in his study was the chronic subepithelial inflammation.

(2). Electron microscopic findings

Ultrastructural studies have been carried out on the epithelium of affected tissues and the structural abnormalities observed are consistent with light microscopic findings (Johnson & Fry, 1967, Pullon, 1969, Whitten, 1970, Tyldesley & Appleton, 1973, El-Labban & Kramer, 1975, Medenica & Lorincz, 1977). It was generally felt that most of the observed changes were not specific to lichen planus but rather, were indicative of epithelial disruption. The most striking changes were in the keratinocytes of the basal layer which showed loss of nuclear membrane, disappearance of nucleoli, homogenization of nuclear material and aggregation of tonofilaments around the nucleus. The lamina densa of the basement membrane was intact and of variable thickness in places but in other areas there were defects of variable sizes. The smaller defects were occupied by epithelial projections into the connective tissue while the wider defects were filled with phagocytic cells and remnants of the lamina densa could be seen (El-Labban & Kramer, 1975). A small fraction of the cells in the dermal infiltrate showed changes such as membrane discontinuities, disintegration of cytoplasm and breaks in the nuclear membrane (Medenica & Lorincz, 1977).

(3). Nature of the infiltrate

Various techniques have been used to identify the cells in the subepithelial infiltrate, in lichen planus, as being predominantly T cell in nature (Tan et al., 1975, Walker, 1976, Alario et al., 1978, Bjerke & Krogh, 1978, Regezi et al., 1978, Dockrell & Greenspan, 1979 and Braathen et al., 1979). The presence of

variable numbers of macrophages and a few B lymphocytes was also reported. Accumulations of macrophages close to the basement membrane and associated with damaged cells have been demonstrated and the proportion of macrophages is thought to be higher in early lesions than in older ones (Matthews et al., 1985).

More recent studies have focussed on characterizing the T cell subsets present in the infiltrate in an attempt to learn more about the pathogenesis of the disease. McMillan et al., (1981) used monoclonal antibodies in conjunction with an immunoperoxidase technique to demonstrate that most of the cells in the infiltrate were T helper cells. Immunofluorescent staining showed that the majority of T cells reacted with monoclonal antibodies specific for helper/inducer cells while a minority of the cells appeared to be of the cytotoxic/suppressor type (Bhan et al., 1981 and Bjerke, 1982). In contrast, Becker et al., (1983) found that suppressor/cytotoxic cells predominated in the stromal infiltrate, particularly at the epithelial/connective tissue interface. This may merely reflect different stages in the disease process because it has been demonstrated that the ratio of helper/inducer cells to suppressor/ cytotoxic cells is higher in early lesions than in later lesions (De Panfilis et al., 1983). It has also been demonstrated that the T lymphocytes are positive for HLA-DR and therefore represent an antigen-stimulated population (De Panfilis et al., 1983).

(4). Apoptosis/ Civatte bodies

Thyresson and Moberger (1957) produced evidence that the degeneration of basal epidermal cells was one of the primary events in lichen planus. It was later recognized that a morphologically distinct type of cell death, apoptosis (Kerr *et al.*, 1972), was involved (Weedon, 1974, El-Labban & Kramer, 1974). More recent studies have confirmed the role of apoptosis in the formation of Civatte bodies (Hashimoto, 1976, and Medenica & Lorincz, 1977) and the later extrusion of filamentous rich apoptotic fragments into the connective tissue to form colloid bodies. Other authors use the terms colloid and Civatte interchangeably (Ebner & Gebhart, 1977). Civatte bodies are well defined round or ovoid structures which reach a maximum size of approximately 20 μm and occur singly, or in clusters, in the epithelium or in the connective tissue papillae. Ultrastructurally, they consist of a dense fibrillar network and are occasionally connected to adjacent keratinocytes by desmosomes. They are eosinophilic, Periodic acid Schiff positive and negative for elastin or Congo red. Immunofluorescent staining for IgM, IgG and complement C₃ are frequently positive while IgA is rarely positive (Ebner & Gebhart, 1977).

The structural changes necessary for their formation occur in two phases. In the first stage there is nuclear and cytoplasmic condensation and the breaking up of the cell into a number of membrane-bound structures. In the second stage these apoptotic bodies are shed from the epithelium or are taken up by other cells and are rapidly degraded by lysosomal enzymes. Apoptosis

is involved in cell turnover in many healthy adult tissues and is responsible for the focal elimination of cells during normal embryonic development. It appears to occur spontaneously in untreated malignant neoplasms and is thought to participate in some cases of therapeutically induced tumour regression. Apoptosis is also thought to play a role in the physiological involution and atrophy of tissues (Kerr et al., 1972). These authors also speculate that hyperplasia might sometimes result from a decreased apoptosis rather than an increased mitosis. The areas of focal hyperplasia seen in lichen planus could certainly be explained in this manner.

(5). Expression of HLA-DR

HLA-DR, the human analogue of Ia, is a class II histocompatibility antigen which has been found to occur in increased amounts in dermal (Tjernlund, 1980, De Panfilis et al., 1983 and Lampert, 1984) and oral (Sloberg et al., 1984 and Regezi et al., 1985) lichen planus. These antigens are thought to play a role in the induction of immune responses. Whereas B lymphocytes may recognize an antigen as such, directly or with concomitant T cell activity, T lymphocytes will only recognize antigens when they are presented with either class I or class II major histocompatibility proteins. T helper/inducer lymphocytes require the presence of class II molecules in the membrane of antigen presenting cells before they can recognize antigen while cytotoxic T cells co-recognize the antigen in combination with a class I histocompatibility antigen. Thus, in cell-mediated immune responses, such as delayed type hypersensitivity, the activation

of T lymphocytes is restricted by class II histocompatibility antigens.

It has been suggested that whenever there is damage to the epidermis induced by cellular immunity, a lymphokine may induce Ia expression in the keratinocytes (Suiitters & Lampert, 1982). In their study, designed to assess the stimuli for the expression of Ia by keratinocytes, they found contact sensitivity to be a cause whereas chemical and physical irritation were not.

Ia antigens have also been detected on keratinocytes in graft-versus-host disease in rats (Mason et al., 1981 and Lampert et al., 1982), the skin manifestations of which are regarded as being a cell-mediated immune reaction and is histologically similar if not identical to lichen planus (Saurat & Gluckman, 1977). In normal epidermis the expression of this antigen is confined to the bone marrow-derived Langerhans cells (Klareskog et al., 1977). The expression of Ia by the skin during graft-versus-host disease may result in the recognition of alloantigen by the injected T lymphocytes used to induce the disease. It was suggested that the T cells would not recognize naturally occurring antigens unless they were presented with Ia and, as such, the epithelial cells of the skin take on an antigen presenting function in graft-versus-host disease.

Tjernlund (1980) reported HLA-DR as a consistent finding in dermal lichen planus, being found on virtually all mononuclear cells in the dermal infiltrate and on cell surfaces or in an intercellular pattern in the epidermis. Another study (De Panfilis et al., 1983), designed to demonstrate activated T cells in the

dermal infiltrate, failed to confirm these findings. In contrast, Lampert (1984) demonstrated the presence of HLA-DR in dermal lichen planus in the basal keratinocytes with one case showing a weaker staining throughout the epidermis.

Ia antigens have been demonstrated in the epithelium of patients with oral lichen planus (Sloberg et al., 1984 and Regezi et al., 1985). Both authors examined the relative numbers and staining intensity of Langerhans cells in the diseased tissue. Sloberg et al used an anti-T6 antibody and found an increased expression of the antigen compared to normal while the number of Langerhans cells remained the same. Regezi et al., however, used anti- S-100 antibody to positively identify the Langerhans cells and concluded that in active areas of oral lichen planus, these cells expressed HLA-DR more intensely and showed a real increase in number.

These disparate findings may be explained by the fact that different monoclonal antibodies will recognize different parts of the molecule, which could be variably preserved in tissue processing. In addition, Taylor (1981) has reported the potential for artefactual relocation of antigens, by diffusion in frozen sections.

1.3 Pathogenesis of the Disease

It has never really been established in the pathogenesis of lichen planus whether the disease has its origin in the connective tissue, with the infiltrate actually invading the dermis or whether the pathological process primarily affects the basal

epithelial cells, with the infiltration being a secondary phenomenon (Black, 1972). In either case the antigenic stimulus is unknown although preliminary studies have revealed a lichen planus specific epidermal antigen (Olsen et al., 1984).

Black (1977) has postulated that lichen planus is a cell-mediated auto-immune response in which the target cells are the basal cell layer of the epidermis. His conclusions were based on the predominance of T cells in the infiltrate, together with the similarity of this disease to the chronic manifestations of graft-versus-host disease. Dvorak et al., (1976) studied the morphological characteristics of delayed hypersensitivity reactions and described necrosis, hyperplasia and intercellular oedema as being characteristic of the epithelial changes which occur in contact sensitivity in man. The histological findings in lichen planus, although variable, would tend to support a delayed hypersensitivity reaction on this basis. The epithelial cells have been shown to be HLA-DR positive and could therefore act as antigen presenting cells (Tjernlund, 1980, Lampert, 1984 and Regezi et al., 1985). The demonstration that the majority of the infiltrating lymphocytes are also HLA-DR positive suggests that they are in an activated state and are therefore reacting to an antigen. This is further supported by the finding of lymphocytes close to all epidermal cells undergoing apoptosis. Lymphocyte attachment to epithelial cells, which would be necessary for lymphocyte-mediated cell killing, has not been detected but the transient nature of this process might account for its not having been observed (Weedon, 1980). The expression of HLA-DR by epithelial cells does not appear to be a specific

marker for lichen planus (Walsh et al., 1985). If HLA-DR expression is considered to be an inducible function (Walker et al., 1984 and Collins et al., 1984), then epithelial reactivity would be expected to occur in other inflammatory conditions, perhaps in response to gamma interferon secreted by activated T cells

Macrophages also act as antigen-presenting cells and therefore might be important in initiating the lesion or, in their phagocytic role, might provide a scavenger function in long-established lesions. In order to act as antigen presenting cells, the macrophages would have to be positive for HLA-DR which is in contrast to the findings of Becker et al., (1983) but consistent with the findings of Tjernlund (1980). More recently, it has been suggested that they may assume a phagocytic role at the epithelial/ connective tissue interface where epithelial cells are either degenerating or are being destroyed by cytotoxic T cells (Regezi et al., 1985).

A humoral immunodeficiency in the aetiology of lichen planus has also been suggested but was dismissed on the basis that no significant differences in serum immunoglobulins were detected in patients with the disease, compared to controls (Scully, 1982). In contrast, Sklavounou et al., (1983) showed significant increases in serum IgG and reductions in serum IgA while IgM and C₃ levels remained unaltered. In the same study it was argued that the predominance of T lymphocytes in the cellular infiltrate of lichen planus would suggest that humoral immunity played little part in the pathogenesis of the disease.

Autoimmunity has been considered in the aetiology of lichen planus, however the evidence is largely against it. In their review, Scully and El-Kom (1985) state that while autoantibodies are uncommon, antinuclear antibodies have been detected in low titre in a minority of patients and anti-DNA antibodies have not been found. Several studies for lesional immune deposits have failed to reveal specifically diagnostic patterns and it was suggested that the immunoglobulins found in Civatte bodies as well as the fibrin in the basement membrane region might represent non-specific extravasation of serum proteins, particularly as there was no correlation between the deposits and the clinical pattern of the disease.

The presence of lichenoid lesions in graft-versus-host reactions may provide some clues as to the pathogenesis of lichen planus. Graft-versus-host reactions follow the grafting of allogeneic marrow cells into immunosuppressed patients and are thought to be caused by the reaction between immunocompetent donor cells and host tissues (Saurat & Gluckman, 1977). The skin is one of the main targets but there is controversy as to whether this represents a direct attack on the epithelium by lymphocytes or whether the host or donor lymphocytes liberate lymphokines which induce a secondary epithelial lesion. The skin is not affected until the early phase of the graft-versus-host reaction has subsided. For this reason, it is postulated that there is primary epidermal cell damage in the basal layer and that this evolves via a cell-mediated immune response into the histological picture that is characteristically seen in lichenoid lesions (Saurat & Gluckman, 1977). In support of this, Sarkany & Gaylarde (1971)

have observed liquefaction degeneration of the basal layer in the absence of an inflammatory infiltrate in lichen planus.

From the evidence provided, lichen planus would appear to represent a cell-mediated immune response. Dvorak et al., (1986) have emphasized both the complexity and heterogeneity of the histological findings in cell-mediated immunity. The common denominator in all of these reactions, however, is the presence of a subset or subsets of sensitized lymphocytes which, on exposure to antigen lead to recruitment and collaboration with other circulating bone marrow derived cells. Although the reactions generally resemble chronic inflammation by virtue of the infiltrating lymphocytes and macrophages, cell-mediated immunity represents a common immunological response, by the host, to a broad spectrum of exogenous and endogenous antigens and therefore has a variable presentation.

CHAPTER TWO

SEMI-QUANTITATIVE ASSESSMENT OF THE HISTOPATHOLOGY OF ORAL LICHEN PLANUS.

2.1 Introduction

The purpose of the present investigation was to attempt to describe more precisely the parameters which are useful in making a histopathological diagnosis of oral lichen planus. No attempt has been made to subdivide the lesions clinically as there is often great variation in the clinical manifestations of the disease in the one patient, with time. Rather, it was hoped that a set of parameters could be defined which would be diagnostic of the disease despite the variability of its presentation. In addition, by studying the associations of these parameters it was hoped that information about the pathogenic mechanisms involved might be obtained.

The histological findings in dermal lichen planus are considered to be characteristic of the disease on the skin and the disease can be diagnosed on this basis (Thyresson & Moberger, 1957 and Ellis, 1967). There is, however, still considerable controversy regarding the microscopic findings in oral lichen planus. Shklar & Meyer (1961) described three histologic features which were useful in diagnosing oral lichen planus. They were; (i) parakeratosis or hyperkeratosis, (ii) hydropic degeneration of the basal layer, and (iii) a bandlike chronic inflammatory infiltrate adjacent to and beneath the epithelium. It was also

recommended that a histological diagnosis be supported by a clinical one, as often the oral lesions represent a microscopic picture of non-specific inflammation. A more recent study (McClatchey et al., 1975) reported clinical and histological agreement in diagnosing a large number of various forms of lichen planus. The same authors found hyperkeratosis and a variable inflammatory infiltrate in all the cases they studied but observed basal degeneration in only 59% of the biopsy specimens.

Kramer et al., (1970) examined the frequency of various histological changes in lichen planus and in other oral keratoses. In agreement with other authors, they found the mononuclear infiltration and basal layer degeneration to be the most frequent findings in lichen planus. As a further diagnostic aid, discriminant analysis of these findings was used to distinguish lichen planus from other oral keratoses.

Andreasen (1968) studied 97 cases of oral lichen planus and found that only the reticular and papular lesions exhibited the histological changes described as being characteristic of dermal lesions. The most constant findings were in agreement with those reported earlier by Shklar & Meyer (1961), with the addition of the presence of a marked stratum granulosum. The only consistent finding in all the various clinical forms of oral lichen planus was the chronic subepithelial inflammation which is common in other oral mucosal diseases.

2.2 Materials and Methods

A retrospective study was carried out on 112 biopsy specimens from patients at the United Dental Hospital of Sydney. The cases selected for study met 3 criteria: 1) the provisional clinical diagnosis was oral lichen planus; 2) a medical history eliminated systemic conditions with oral manifestations which could mimic lichen planus; and 3) sections showed at least three of the histological parameters listed in Table 1, in addition to a mononuclear infiltrate which closely apposed the epithelium. The group comprised 84 females and 28 males of mean age 57.3 ± 12.6 years with ages ranging from 19 to 82 years. Approximately 10 % of these patients also had dermal lesions.

The biopsies were taken from various sites including buccal mucosa (71%), tongue (19%), alveolar mucosa (8%) and palate (2%). The normal baseline for tissue variability at these different sites was taken into account in the quantitation of the parameters studied.

Of the cases selected for study, 41% were described clinically as being erosive, 10% as non-erosive and in the remaining 49% the presentation was not specified. In cases where the lesion was erosive, these areas were avoided in the quantitation of parameters because of their acute inflammatory nature. The areas adjacent to the ulceration showing the histological criteria stated previously were taken to be representative. Similarly, areas of normal tissue were excluded.

Parameter	% of cases showing parameter	Method of assessment of parameter
Keratin	10	Assessed as being present or absent and graded + → ++++ according to width
Parakeratin	93	Cell layers counted
Granular layer	83	Cell layers counted
Acanthosis	79	Cell layers counted
Intercellular edema	57	Assessed as being present or absent and graded as + → ++++ according to severity (see Fig. 3)
Intracellular edema	62	Assessed as being present or absent and graded as + → ++++ according to severity (see Fig. 3)
Basal layer degeneration	84	Assessed as being present or absent and graded as + → ++++ according to severity (see Fig. 3)
Mononuclear infiltrate	100	Assessed as being present or absent and graded according to the density of the infiltrate (see Fig. 3)
Bandlike distribution of infiltrate	46	Assessed as being present or absent
Civatte bodies	35	Counted
Focal separation of epithelium and connective tissue	29	Assessed as being present or absent
"Saw-toothed" rete ridges	9	Assessed as being present or absent
Widening of the basement membrane zone	61	Assessed as being present or absent
Lymphocytic predominance in infiltrate	99	Assessed as being present or absent
Dilated vessels in connective tissue	48	Assessed as being present or absent
Atrophy	14	Assessed as being present or absent
Hyperplasia	35	Assessed as being present or absent
Areas of atrophy and hyperplasia	51	Assessed as being present or absent

Table 1. Incidence of parameters studied and the method of their assessment.

All tissues studied were fixed in neutral buffered formalin (10% v/v) embedded in paraffin and 6µm sections were cut. They were stained with haematoxylin and eosin.

The parameters listed in Table 1 were scored according to the method of assessment outlined. Scoring was carried out by only one observer with frequent referral to a reference slide in order to optimize the reproducibility of the assessments made. All sections were scored along their length. The sections were coded and read blind and all data were gathered prior to analysis to aid in the objectivity of the study.

2.3 Results

The results of this study show that mononuclear infiltration beneath and adjacent to the epithelium and degeneration of the basal layer were consistent findings in oral lichen planus. Parakeratinization, acanthosis and a prominent granular layer were also frequent findings (Table 1).

The density of the mononuclear infiltration and the severity of the basal degeneration were studied more closely (Figures 1 & 2).

Plate 3 shows a representative grading for mononuclear infiltration and basal degeneration. Plate 4 represents a grading of 1 for mononuclear infiltration. The relationship of basal degeneration and mononuclear infiltration was studied and linear regression analyses revealed a positive correlation between these two parameters that was significant at the 1% level (Figure 3).

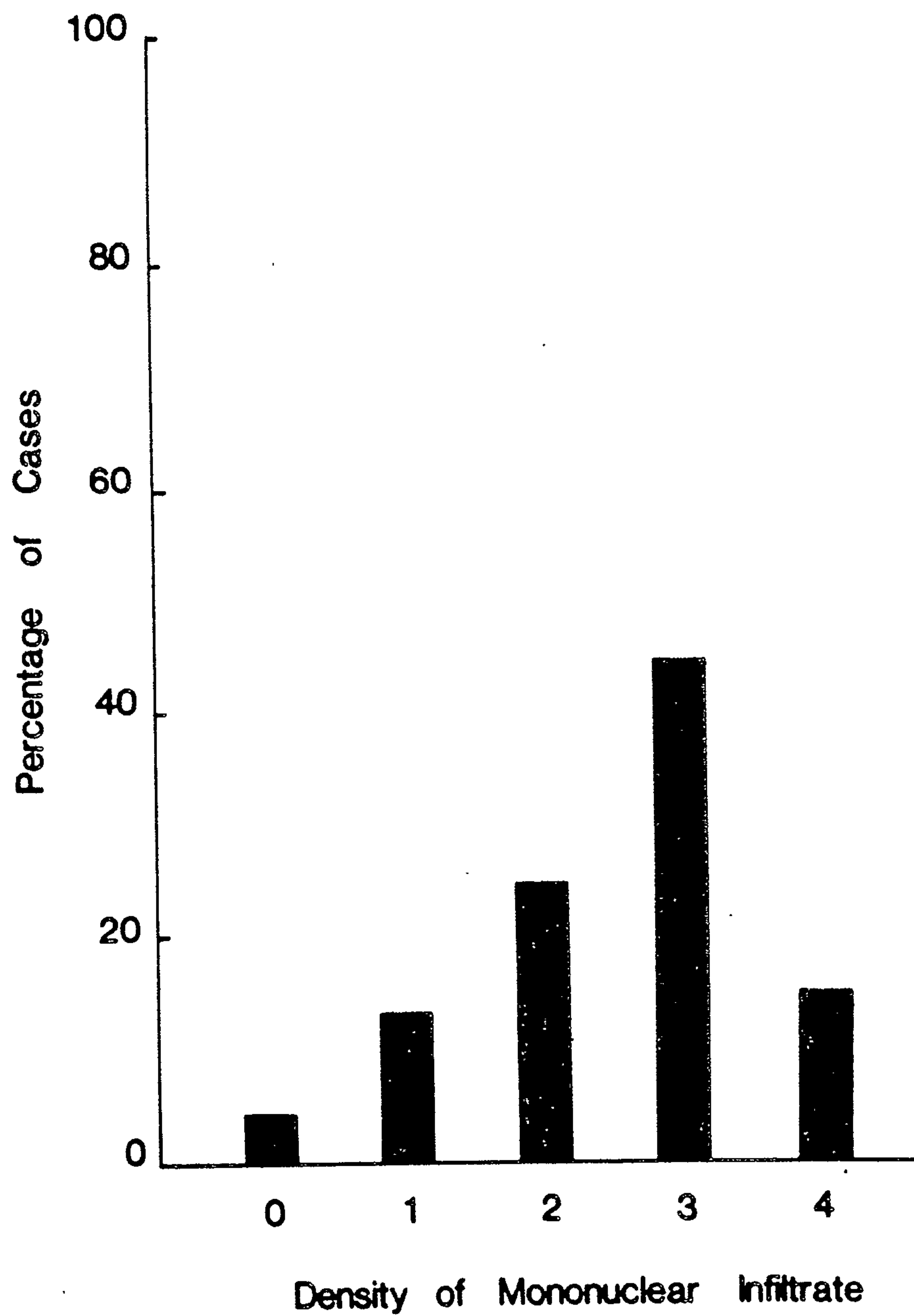


Figure 1. Quantitative assessment of density of mononuclear infiltrate versus percentage of cases.

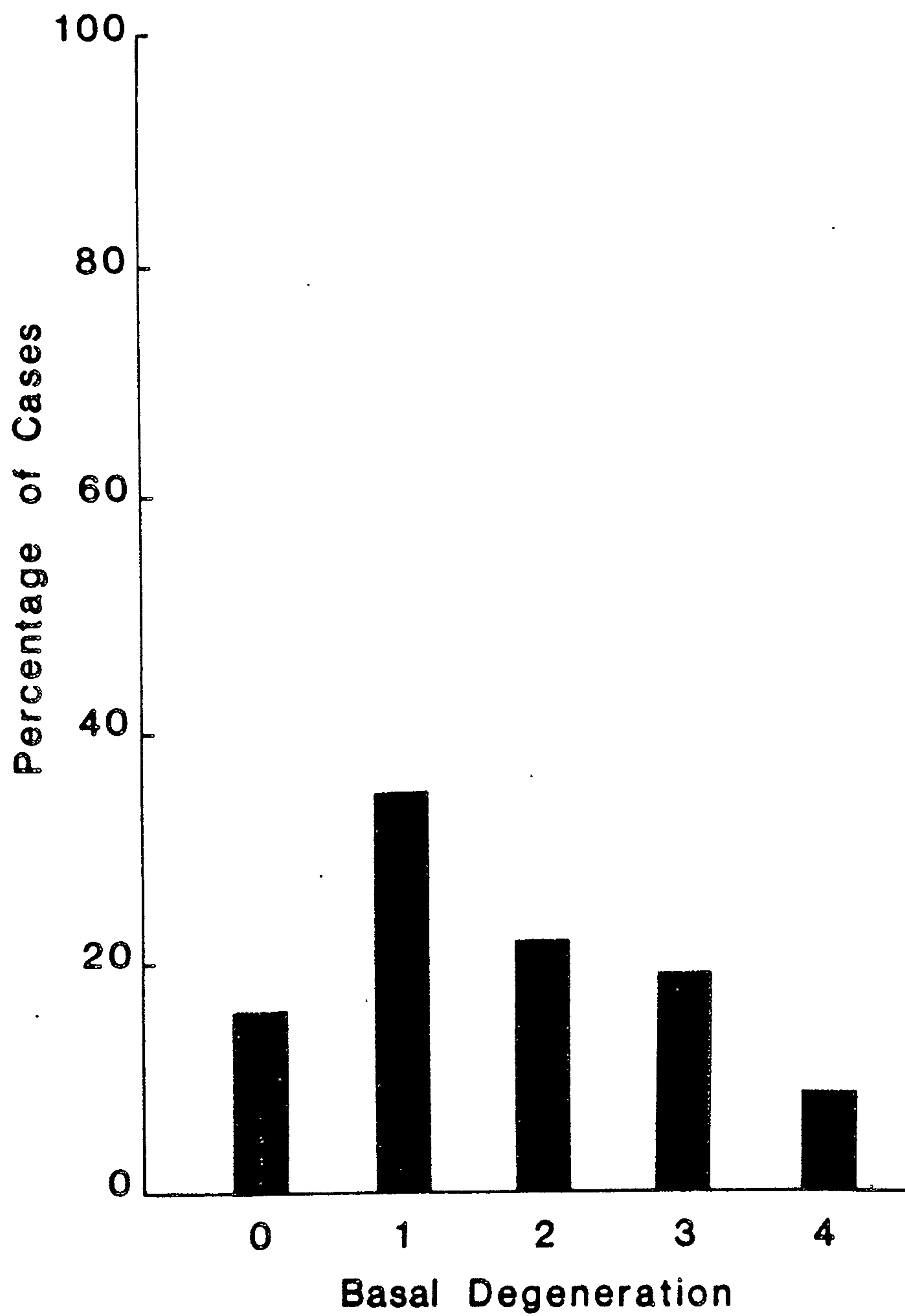


Figure 2. Quantitative assessment of severity of basal degeneration versus percentage of cases.



Plate 3. Photomicrograph of lichen planus showing (i) +++ (dense) infiltration and (ii) +++ basal layer degeneration (severe).

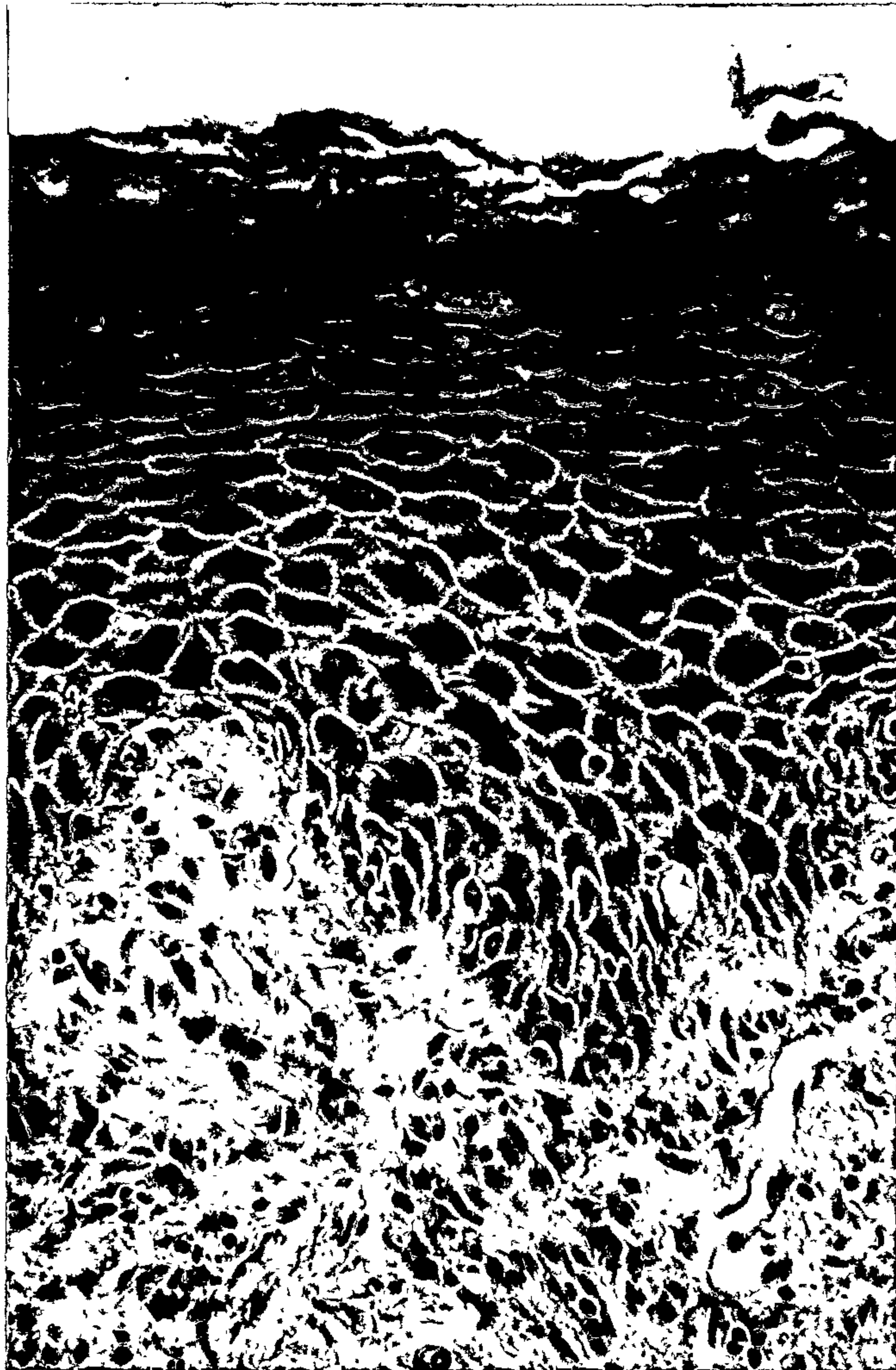


Plate 4. Photomicrograph of lichen planus showing a sparse or + (1) grading for mononuclear infiltration.

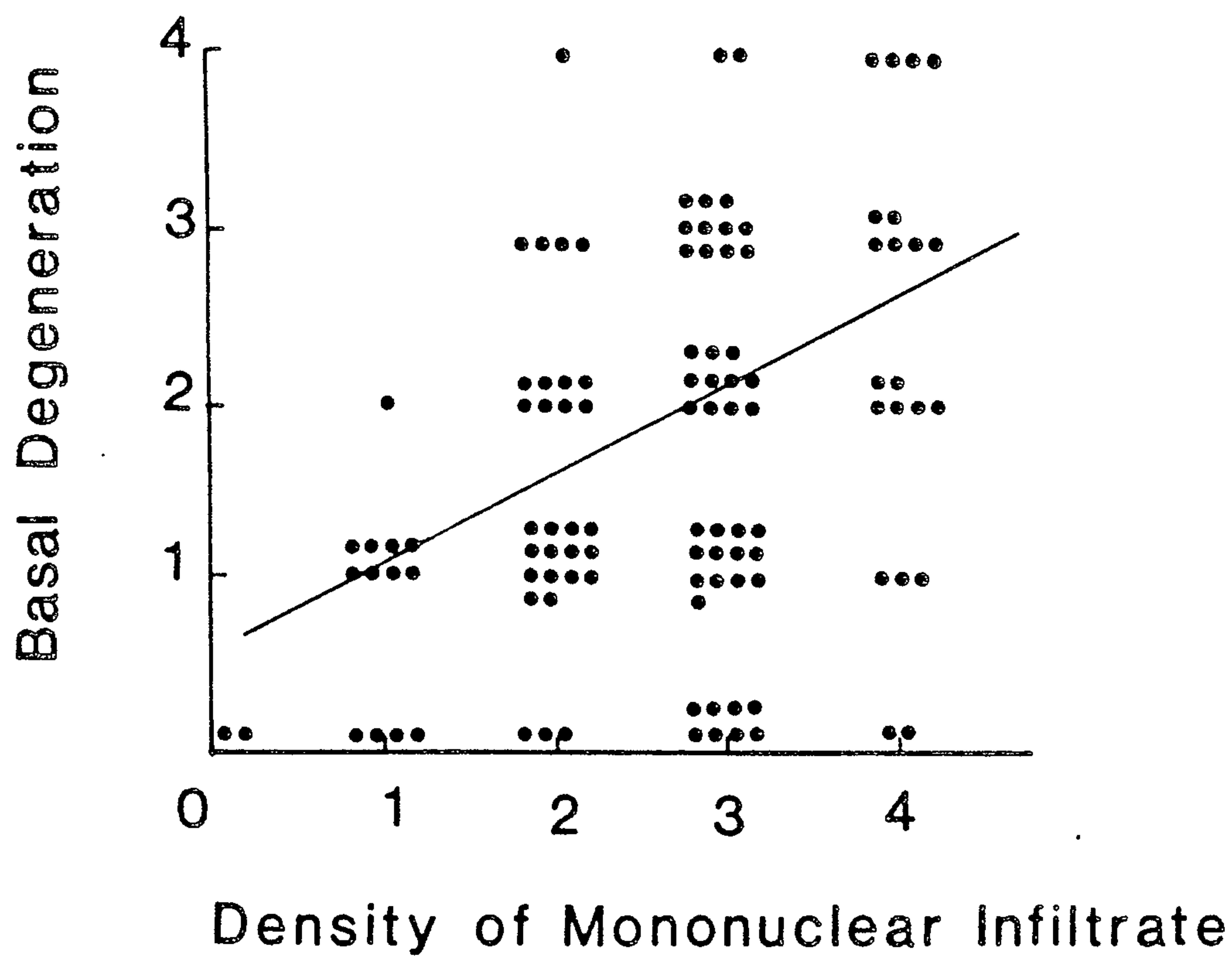


Figure 3. Scattergram indicating a positive linear correlation between the density of mononuclear infiltration and the severity of basal layer degeneration.

As parakeratosis was such a frequent finding, its relationship to both mononuclear infiltration and basal layer degeneration was studied. Linear regression analysis of mononuclear infiltration versus the number of layers of parakeratin revealed an inverse correlation that was significant at the 5% level (Figure 4). There was no linear correlation between parakeratinization and basal layer degeneration. Linear regression analyses of the amount of intracellular oedema versus the density of the mononuclear infiltrate, intercellular oedema versus the density of the infiltrate and the number of Civatte bodies versus the infiltrate showed no linear relationships.

2.4 Discussion

This study of an Australian population confirms the findings of previous authors (Thyresson & Moberger, 1957 and Ellis, 1967) that basal layer degeneration and mononuclear infiltration are frequent histopathological findings in oral lichen planus. It was found, however, that parakeratosis was a more consistent finding than basal degeneration, being present in 93% of the cases studied. This was even higher than the incidence of 86% previously reported by Andreassen (1968). Acanthosis and the presence of a prominent granular layer were also found more often than other parameters in this study.

Although it is generally accepted that the pathology of lichen planus represents tissue damage as the result of some form of immunological response, there does not appear to have been any

previous attempt to evaluate the association of these parameters in relation to possible pathogenic mechanisms for the disease.

It has been proposed that the tissue response represents a cell-mediated immune attack against the basal layer of the epithelium (Sloberg et al., 1984). The data presented shows a significant and positive correlation between the severity of the basal layer degeneration and the density of the mononuclear infiltrate which is compatible with a cell-mediated type of response. The association of several other parameters studied, however, failed to show any correlation. Dvorak et al., (1976) listed necrosis and hyperplasia of the epithelium and the presence of intercellular oedema as being forms of epithelial change found in contact hypersensitivity reactions in man. These parameters were examined, but did not correlate with the density of the mononuclear infiltrate in this study.

Other support for a cell-mediated response is provided by immunoperoxidase studies using monoclonal antibodies which demonstrated a predominance of T helper cells within the infiltrate (McMillan et al., 1981 and Bhan et al., 1981). A subsequent study (Becker et al., 1983) suggested, however, that suppressor/cytotoxic type lymphocytes were more common at the epithelial/connective tissue interface.

The epidermal changes in the early chronic phase of graft-versus-host disease are both clinically and histologically very similar to lichen planus. Basal keratinocytes are seen to be damaged and the infiltrate is confined to the papillary dermis (Janin-Mercier et al., 1981). Saurat et al., (1977) considered the

graft-versus-host reaction to be a cell-mediated or delayed-type hypersensitivity reaction in which basal epidermal cells are the target of an attack by lymphocytes. Skin biopsies suggest, however, that basal layer damage is the primary event and that the mononuclear infiltration occurs secondarily. In relation to this, Sarkany & Gaylarde (1971) observed liquefaction degeneration of basal cells even in the absence of an inflammatory response in lichen planus. This may represent an early stage in the disease process.

In the so-called "drug-induced" (Hay & Reade, 1978 and Chau et al., 1984) or "amalgam-induced" (Finne et al., 1982 and Lind et al., 1984) oral lichenoid lesions, it has been argued that the specific drug or substance increases the antigenic stimulus for this attack to occur.

A humoral response has also been postulated (Sklavounou et al., 1983) for the pathogenesis of lichen planus. Direct immunofluorescent studies, although positive (Schiødt et al., 1981, Goldstein & Katz, 1979, Laskaris et al., 1982 and Baarte de la Faille-Kuyper & Baarte de la Faille, 1973) are generally not interpreted as being conclusive evidence for a humoral response, but rather are seen to occur as a secondary event, after the basal layer has been damaged. Furthermore, the T cell nature of the mononuclear infiltrate does not support a humoral response.

Recently, the discovery of HLA-DR expression on epithelial cells in lichen planus (Sloberg et al., 1984 and Tjernlund, 1980) has made the argument for a contact hypersensitivity type of reaction more tenable. In normal epidermis, expression of class II

alloantigens is restricted to the bone marrow derived Langerhans cells. The expression of this Group D histocompatibility marker on epithelial cells, in lichen planus, means that the requirements for Class II histocompatibility restriction will be met (Thorsby, 1984) and that the epithelium can then function in antigen presentation. As such, the cells could also be the target of a subsequent immune attack, initiated by T cells recognizing antigen and producing lymphotoxins which directly attack the epithelial cells. Alternatively, the sensitized T cells could stimulate macrophages which are then responsible for destroying the epithelial cells.

The stimulus for the expression of HLA-DR on epithelial cells in lichen planus is unknown. One possible mechanism could be that T cells in the infiltrate produce gamma or immune interferon. This lymphokine is known to induce HLA-DR expression on previously negative macrophages and umbilical vein endothelial cells (Walker et al., 1984). This would lend further support to an important role for cell-mediated immunity in the pathogenesis of lichen planus.

CHAPTER THREE

THE EXPRESSION OF HLA-DR IN ORAL LICHEN PLANUS

3.1 Introduction

HLA-DR, the human analogue of murine Ia, is a class II histocompatibility antigen which has been found to occur in increased amounts in both dermal (Tjernlund, 1980, DePanfilis et al., 1983 and Lampert, 1984) and oral (Sloberg et al., 1984 and Regezi et al., 1985) lichen planus. The expression of this determinant was initially thought to be limited to B lymphocytes and some macrophages and monocytes but has since been detected on a variety of cells (Engleman et al., 1980, Foulis, 1986 and Carr et al., 1986).

These proteins are thought to play a major role in the induction of immune responses. B lymphocytes may recognize an antigen as such, directly or with concomitant T cell activity. T lymphocytes, however, can only recognize antigens when they are presented with either class I or class II major histocompatibility antigens. T helper cells require class II molecules in the membrane of antigen presenting cells before they can recognize antigen whereas cytotoxic T cells co-recognize the antigen in combination with a class I histocompatibility protein (Thorsby, 1984). Therefore a cell-mediated immune response such as delayed-type hypersensitivity is restricted by these class II antigens.

Suitters & Lampert (1982) suggested that whenever there was damage to the epidermis induced by cellular immunity, a lymphokine could induce Ia expression in the keratinocytes. In their study, designed to assess the stimuli for the expression of Ia in keratinocytes, they showed contact hypersensitivity to be a cause whereas chemical and physical irritation were not. This further supported the hypothesis that Ia plays a major role in cell-mediated immune reactions. Ia antigens have also been detected on keratinocytes in graft-versus-host disease induced in rats (Mason et al., 1981 and Lampert et al., 1982). This process is widely regarded as being a manifestation of cell-mediated immunity and also as being similar to lichen planus histologically (Saurat & Gluckman, 1977). The reported staining in graft-versus-host disease was mostly peripheral with some cytoplasmic staining seen in the basal 2 or 3 layers of keratinocytes and hair follicles. In some specimens the entire length of the epithelium was involved, while in others the staining occurred in a focal distribution. The expression of Ia in the skin during graft-versus-host disease may result in the recognition of alloantigen by injected T cells used to induce the disease. It was suggested that T cells would not recognize naturally occurring antigens unless they were presented with Ia and, as such, the epithelial cells of the skin take on an antigen-presenting function in graft-versus-host disease.

Tjernlund (1980) reported Ia as a consistent finding in dermal lichen planus, being found on virtually all the mononuclear cells in the dermal infiltrate and on cell surfaces or in an intercellular pattern in the epidermis. He found no positive

staining in the various other dermatoses studied except some slight staining in one case of discoid lupus erythematosus. In contrast, in a study designed to demonstrate activated T cells in the infiltrate of dermal lichen planus, DePanfilis et al. (1983) reported that they were unable to confirm the findings of Tjernlund. Lampert (1984), however, demonstrated the presence of HLA-DR in keratinocytes in dermal lichen planus, discoid lupus erythematosus and eczematous dermatitis. In all of the three cases of lichen planus studied, the author reported positive staining of the basal keratinocytes. In one of the three cases, the basal staining was associated with a weaker staining of keratinocytes throughout the epidermis. Sloberg et al., (1984) used anti-Ia-like antibodies to demonstrate the presence of HLA-DR in oral lichen planus and in healthy mucosa. In lichen planus, the Ia-like antigens were distributed in a suprabasal position within the epithelium. In a similar study, Regezi et al., (1985) used a monoclonal antibody to HLA-DR on formalin-fixed and paraffin-embedded sections of oral lichen planus and showed that keratinocytes expressed membrane-associated HLA-DR activity. Both authors examined the relative numbers and staining intensity of Langerhans cells in the diseased tissue. Sloberg et al., (1984) used an anti-T6 antibody and found an increased number of HLA-DR antigens on Langerhans cells in oral lichen planus compared to normal mucosa. Regezi et al., (1985) used an anti-S100 antibody to identify Langerhans cells and concluded that in active areas of lichen planus these cells expressed HLA-DR more intensely and showed a real increase in number. They further postulated that the expression of HLA-DR

by keratinocytes in oral lichen planus suggested a role for these cells in the recognition of antigen.

These disparate findings may be explained by the fact that different monoclonal antibodies will recognize different determinants on the class II molecule, which are variably preserved. In addition, Taylor has reported the potential for artefactual relocation of antigens, by diffusion, in frozen tissue sections.

The purpose of the present investigation was to compare the expression of HLA-DR in oral lichen planus and other oral mucosal lesions. Paraffin sections were used firstly because of their convenience, and, secondly to compare them to cryostat sections for use in immunofluorescent tests. It would be of diagnostic value to find an association between a particular disease and the expression of the HLA-DR antigen. It appears, however, that it may be an indicator of cell-mediated immune responses in general rather than of a specific disease.

3.2 Materials and Methods

Staining for HLA-DR antigen

The amounts of the various reagents used for the indirect immunofluorescence tests were optimized so that non-reactive cells were negative. There was trace reactivity of skeletal muscle with the monoclonal antibody but this did not interfere with the interpretation of positive or negative staining. The primary antibody, rat immunoglobulin against HLA-DR and mouse H-A, I-A sub-region, from hybridoma supernatant culture

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fluid (Sera Lab Clone YE2-136 HLK), was used at a concentration of 20 µg/ml. The second antibody, sheep anti-rat immunoglobulin conjugated to fluorescein-isothiocyanate (Wellcome), was diluted to 160 µg/ml and added to equal parts of calf serum. Deparaffinized sections were pre-incubated with calf serum for 10 minutes then rinsed in phosphate buffered saline (PBS) (pH 7.2). Pre-digestion with trypsin was tried but discarded as it appeared to eliminate staining. The sections were air dried then incubated with the primary antibody for 60 minutes. All incubations were carried out in a moist chamber at room temperature. After 3 x 10 minute washes in PBS, the sections were incubated for a further 60 minutes with the second antibody. Finally the sections were washed for 30 minutes with three changes of PBS. Sections were mounted in Kaiser's glycerol jelly and examined the same day with a Leitz Orthoplan microscope equipped for incident fluorescence.

The brightness of the staining was assessed semi-quantitatively as being + where the staining was slight but nonetheless positive to ++++ where it was very bright. The distribution of the staining was mapped using pencil drawings with reference to haematoxylin and eosin stained sections from the same region of the block.

Photomicrographs were taken using an Orthomat camera and Ilford HP5, black and white film, ASA 400.

In order to test that the antigenic determinant recognized by this antibody survives formaldehyde fixation when in position at the cell surface, human buffy coat leukocytes were stained both

without fixation and also with fixation in 10 % neutral-buffered formalin at 37'C for 60 minutes. The fixed cells were embedded in paraffin wax and sections were cut and stained according to the method described.

Selection of specimens for study.

Sixteen cases were selected from the previous retrospective study of 112 cases (Chapter 2) in which the clinical and histopathological diagnosis was oral lichen planus. The cases chosen were those exhibiting the more frequent histological findings in oral lichen planus, including mononuclear infiltration, basal layer degeneration, parakeratosis and either hyperplasia or atrophy of the epithelium.

Additional cases of lesions involving the oral mucosa and with varying amounts of inflammatory infiltrate were selected for comparative study. This group included squamous cell carcinoma, verrucous carcinoma, fibroma, hyperplasia, amalgam tattoo, Sjogrens syndrome, fibro-epithelial polyp, aphthous ulcer and periodontitis. Further controls were carried out as follows: The monoclonal antibody was replaced with PBS and also with a commercial preparation of rat immunoglobulin (Wellcome). Normal tissue was examined. Frozen sections of recent cases of oral lichen planus which fulfilled the same criteria as the retrospective cases were compared to paraffin sections from the same specimen.

3.3 Results

Specificity and reactivity of fixed cells for anti-HLA-DR

Forty-seven per cent of unfixed human buffy coat leukocytes stained with the antibody when used at 20 µg/ml. The pattern of staining varied in intensity and in distribution from a uniform fluorescent halo to discrete fluorescent spots at the periphery. Formalin fixation and paraffin embedding did not alter the percentage of reactive cells or the distribution of cell surface staining but did reduce the brightness of the fluorescence. Under the staining conditions used for this study, normal tissues were negative for the primary and secondary antibody except for trace staining of skeletal muscle and patchy intercellular staining in the epithelium. The controls where the monoclonal antibody was omitted or substituted for were all negative. It was essential to include a calf serum step to eliminate non-specific binding. As further proof of specificity and reactivity many of the cells of the mononuclear infiltrate showed a fluorescent rim of staining (Plate 5). Trypsin digestion eliminated staining indicating that a proteinase susceptible determinant was necessary for antibody binding. As a further control, unfixed cryostat sections gave a similar but less well-defined pattern of staining compared to formalin-fixed, paraffin-embedded tissue from the same biopsy specimen.

The localization and intensity of epithelial staining

The epithelium in each of the 16 cases chosen for this indirect immunofluorescence study showed some reactivity for the

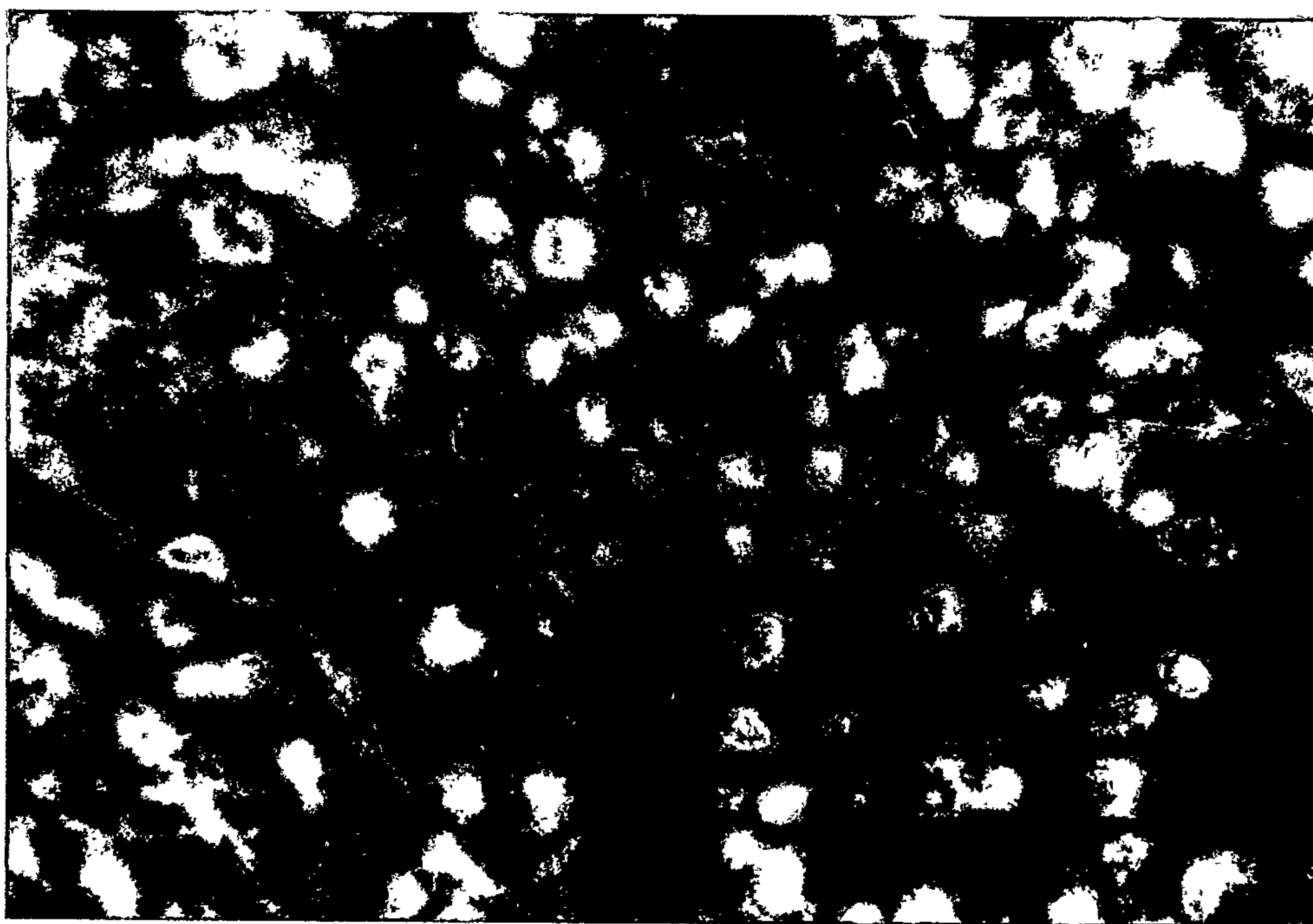


Plate 5. Photomicrograph of mononuclear infiltrate showing a fluorescent rim of staining of some cells.

monoclonal antibody to HLA-DR (Plate 6). In all cases, the basal layer of the epithelium was unreactive. In contrast, all of the spinous cell layers stained for HLA-DR, although there was considerable variation in the intensity of the staining as outlined in Table 2. There did not appear to be any association between the amount of fluorescence of the spinous cells and the intensity of the mononuclear infiltrate.

Normal tissue sections showed no cytoplasmic staining within the epithelium. There was, however, patchy extracellular or intercellular staining which probably represented the dendritic processes of Langerhans cells.

The sections of other mucosal conditions also showed some reactivity with the primary antibody, as outlined in Table 3. The staining was less intense, although still suprabasal in these lesions but the distribution of the staining was different.

3.4 Discussion

The monoclonal antibody used in this study selectively stained buffy coat leukocytes in a pattern similar to that reported by the group who produced this reagent (Brickell et al., 1981). The high percentage of positive cells in the present study is probably due to some lysis of the granulocytes which were not positive, during preparation of the buffy coat and subsequent staining.

The results of this study indicate that this antibody can be used to label HLA-DR in formalin-fixed, paraffin-embedded tissues. Wilson et al., (1984) and Regezi et al., (1985) have similarly

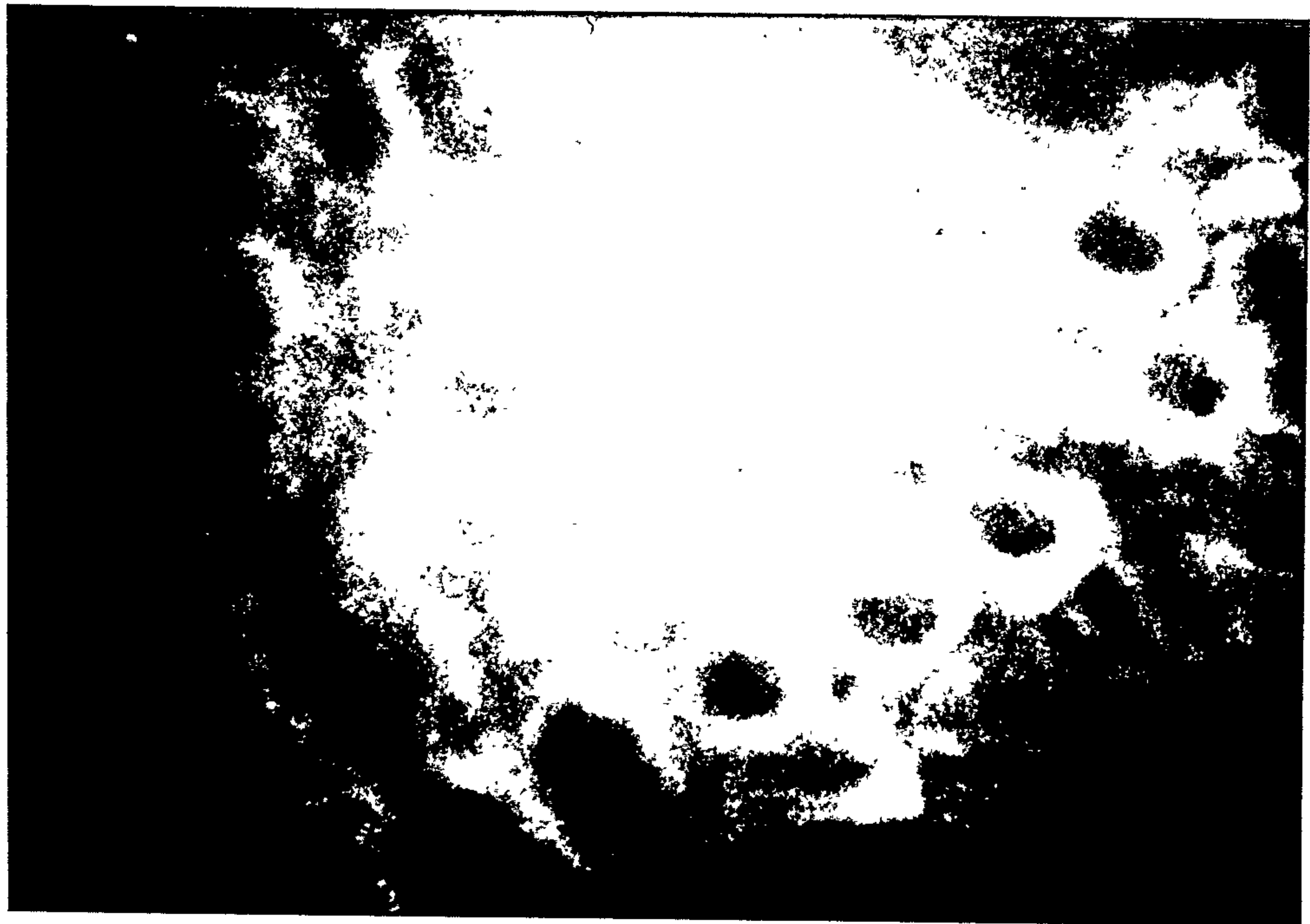


Plate 6. Photomicrograph showing epithelial specificity for monoclonal antibody to HLA-DR.

Case	Cytoplasmic staining in the spinous layer	Zones of brighter cytoplasmic staining	Extracellular cell surface staining
1	++++	-	-
2	++	Present	-
3	++++	-	-
4	++	Present	-
5	+++	Present	-
6	+	Present	-
7	+++	Present	-
8	+++	Present	Present
9	+++	Present	-
10	+++	Present	-
11	+	Present	-
12	++	Present	-
13	++	Present	-
14	+++	-	-
15	+++	Present	-
16	++	-	Present

Table 2. Incidence and distribution of staining for HLA-DR in lichen planus.

Lesion	No. of cases	Type and intensity of staining	Spinous cells showing staining
Squamous cell carcinoma	3	+ - ++ Cytoplasmic	Upper layers only
Verrucous carcinoma	3	+ Cytoplasmic	80% total
Fibroma	3	± + + Intercellular/ cell surface	Lower layers only
Amalgam tattoo	2	+ + ++ Cytoplasmic	30% total
Sjögrens syndrome	1	+ Cytoplasmic	Upper layers only
Fibroepithelial polyp	3	++ Cytoplasmic	50% total
Aphthous ulcer	3	+ - ++ Intercellular/ cell surface	Upper layers only
Periodontitis	2	++ - +++ Intercellular/ cell surface	50% total
Hyperplasia	4	+ Cytoplasmic	20 - 40% total

Table 3. Incidence of staining for HLA-DR in a variety of oral mucosal diseases.

reported the use of a monoclonal antibody to stain fixed, embedded tissues for HLA-DR. The clone was, however, not maintained. The monoclonal antibody used in the present study binds to an ancestral part of the HLA-DR molecule and it also recognizes a common determinant in mouse Ia (Brickell et al., 1981). In keeping with the results using buffy coat leukocytes, the intensity of fluorescence was reduced in fixed embedded tissue compared with unfixed cryostat sections. The less well-defined pattern of staining in the cryostat sections of lichen planus may have been due to relocation of the antigen, by diffusion (Taylor, 1981).

The epithelium in oral lichen planus lesions was consistently positive for HLA-DR. This is general agreement with other authors (Tjernlund, 1980 and Lampert, 1984) and two previous studies (Sloberg et al., 1984 and Regezi et al., 1985) on oral lichen planus. In addition, the fluorescence involved the whole of the spinous zone. In contrast to other reports using different monoclonal antibodies and cryostat sections, the fluorescent staining in this study was clearly cytoplasmic. A peripheral pattern of fluorescence indicating cell surface or extracellular antigen was noted in a few cells of only two of the lesions of lichen planus.

The peripheral pattern of staining in other studies (Tjernlund, 1980 and Sloberg et al., 1984) leaves unanswered the question of the localization and origin of this antigen. It could be that this staining represents the dendritic processes of Langerhans cells which are known to be HLA-DR positive, or cell surface antigen on keratinocytes or that it represents antigen released from the

underlying inflammatory process. Volc-Platzer et al., (1984) presented evidence for the synthesis of HLA-DR by cells within the epithelium using a monoclonal antibody which recognized a determinant on a transport chain which was not part of the surface determinant. The cytoplasmic fluorescence of the whole spinous layer, as found in the present study, strongly supports the synthesis of HLA-DR by keratinocytes.

One of the aims of this study was to provide further information about the nature of the pathologic process in oral lichen planus. In particular, it was considered that the expression of HLA-DR by keratinocytes would favour a delayed-type hypersensitivity response. While the data of this study and others support this concept in general terms, the complete lack of basal layer reactivity detracts from this idea because it is the presumed target tissue. In addition, the lack of association between the intensity of the fluorescence of the spinous cells and the severity of the inflammatory infiltrate is a negative finding.

The expression of HLA-DR by keratinocytes does not appear to be a specific marker for lichen planus. This was demonstrated by the reactivity of the epithelium in a variety of lesions of the oral mucosa. The sections of periodontitis in this study showed a similar expression of HLA-DR to that found on gingival keratinocytes in chronic gingivitis (Walsh et al., 1985). If HLA-DR expression is considered to be an inducible function, perhaps in response to gamma interferon secreted by activated lymphocytes (Walker et al., 1984 and Collins et al., 1984), then keratinocyte reactivity would not be expected to occur only in lichen planus.

The intensity and the pattern of epithelial staining did, however, allow us to distinguish lichen planus from the other mucosal lesions studied. In this sense, the use of this reagent on fixed, embedded tissues could provide a useful adjunct to differential diagnosis.

PREFACE TO PART TWO

In the second part of this thesis, an animal model was used for the experimental studies. The need for this model arose from questions that were posed in the studies on the histopathology of lichen planus. The experiments in this model system are preceded by literature reviews of three areas which are relevant to the findings that are presented. Following on from the studies of lichen planus, where HLA-DR expression on keratinocytes was demonstrated, literature reviews of the following areas are included;

- (1) Ia and HLA-DR with emphasis on the potential function of class II histocompatibility antigens as signal transducer molecules,
- (2) the thymus and in particular, the intrathymic development of thymocytes and the signals and interactions that induce either maturation or apoptosis of precursor cells and
- (3) the production of monokines

These literature reviews are followed by a series of experiments, the results of which are discussed in the light of their possible implications in thymic biology, macrophage function and mucosal disease.

CHAPTER 4

LITERATURE REVIEW - IA & HLA-DR

4.1 Introduction

There are two classes of major histocompatibility (MHC) proteins which are primarily involved in the recognition of self. Class I molecules are expressed on the surface of most cell types whereas class II molecules have a more limited distribution and were initially thought to be found only on B cells and some macrophages, but which have since been detected on a variety of other cells.

The structures of both class I and II proteins in man and in mouse show considerable homology between the two species. The class I antigens consist of a 45 kD polymorphic chain complexed to β_2 -microglobulin. Type II antigens, also called immune-response-associated antigens (Ia), contain two polymorphic chains of 33kD and 28 kD and an invariant chain which is involved with the transport of the complex to the cell surface, but which is not part of the surface complex. In both classes of antigen, the polypeptide chains, but not the invariant class II chain, are constructed by domains similar to those of immunoglobulins, suggesting a common ancestry for these molecules. Histocompatibility antigens are unique in their polymorphism which is so extensive that, in a randomly bred population, all individuals are expected to differ in at least some of the antigens of this complex.

The present discussion will be confined to class II proteins and in particular to the products of the R region genes (HLA-DR) and the analogous Ia antigens of rats and mice.

4.2 Distribution of Class II Histocompatibility Proteins

The study of the distribution of HLA-DR and Ia antigens has been facilitated by the availability of numerous monoclonal antibodies. Since their discovery on B lymphocytes and monocytes, Ia antigens have been detected on bone marrow precursor cells, Langerhans cells of the epidermis, vascular endothelial cells, a variety of tumours and more recently on activated T cells (Engleman et al., 1980). They have also been found on interdigitating reticular cells, Kupffer cells and some epithelial cells including thymic epithelial cells, lactating mammary tissue, small intestine, lingual and tracheal epithelium and bronchial glands (Foulis, 1986), and on normal keratinocytes (Carr et al., 1986).

There have also been attempts to relate the increased expression of these antigens to certain disease states although the findings have been inconclusive (Ritzmann, 1976). As previously described, HLA-DR antigens are thought to occur in increased amounts in both oral and dermal lichen planus (Sloberg et al., 1984, Regezi et al., 1985, Tjernlund, 1980, Lampert, 1984 and De Panfilis et al., 1983).

The expression of class II antigens has been shown to be an inducible function on some cell types. Collins et al. (1984)

demonstrated that recombinant human gamma interferon induced the de novo appearance of transcripts of multiple class II antigen genes, including the α and β genes of HLA-DR as well as of the invariant chain, with subsequent surface expression of these antigens. It was also demonstrated that after the removal of gamma-interferon, the class II specific mRNA's disappear relatively rapidly while the expression of the proteins remained on the cell surface at relatively constant levels for several days.

Walker et al., (1984) demonstrated that either recombinant murine gamma-interferon or conditioned medium from concanavalin A-activated rat spleen cell cultures could stimulate a macrophage tumour line to produce a second activity which, in turn, was capable of inducing cell surface Ia expression on a monocyte line. The Ia-inducing factor is neither shed plasma membrane Ia glycoprotein molecules nor any known form of murine interferon. It was stressed that the release of this second factor, after gamma-interferon activation, represented a mechanism whereby macrophages and perhaps other cells of the lymphoid system could amplify the direct effect of gamma-interferon stimulation.

This finding was supported by a later study which looked at supernatants from human mixed leukocyte cultures with respect to their MHC class II antigen-inducing capacity (Groenewegen et al., 1986). These authors found a mediator which was different from gamma-interferon, but which was potentiated by gamma-interferon, and had a molecular weight of 32 kD. This mediator was also capable of inducing Ia expression

in the absence of gamma-interferon suggesting that the new mediator was directly responsible.

Similarly, Tabibzadeh et al., (1986), showed that endometrial epithelial cells, which are HLA-DR negative under normal culture conditions, can be induced to actively synthesize these molecules in vitro under the influence, at least in part, of gamma-interferon.

The expression of Ia has been induced on kidney epithelial cells as the result of primary immunization with alum-precipitated protein antigen, or after secondary challenge with the soluble antigen used for the priming (Andrew & Parkhouse, 1986). Although the mechanism was unclear, the authors postulated that either prostaglandins or gamma-interferon might be produced after the initial stimulation.

Recently, Tripp et al., (1986) evaluated the role of exogenous and endogenous arachidonic acid metabolites on the expression of Ia molecules by macrophages and found that prostaglandins E₂ and I₂ had a suppressive effect. This effect was thought to be mediated via cyclic AMP (cAMP) production since both prostaglandins stimulate its production, and also because dibutyryl cAMP suppressed Ia expression while cyclooxygenase inhibitors potentiated its expression. It was further proposed that the removal of prostaglandins together with the stimulatory effect of Ia were necessary for an immune response to develop normally and altered levels of these two factors could either down-regulate or alternatively increase the level of the immune response.

Polla et al., (1986) confirmed earlier findings that gamma-interferon could regulate the expression of Ia and also demonstrated that the T cell derived lymphokine, B-cell stimulatory factor could induce the expression of Ia genes as well.

More recently, it has been demonstrated that class II antigens could be induced on a murine macrophage cell line (WEHI 3) by tumour necrosis factor- α or by murine gamma-interferon (Chang & Lee, 1986). Furthermore, stimulation with a combination of the two cytokines resulted in a more than additive effect relative to the individual potential of each agent.

In contrast to the findings with most other cell types, Pujol-Borrell et al., (1987), found that human pancreatic islet cells could not be induced to express class II molecules by gamma-interferon alone. They demonstrated, however, that the combination of either tumour necrosis factor or a lymphotoxin with gamma-interferon induced class II expression on islet cells. It was therefore proposed that multiple signals with a degree of tissue and cell specificity were necessary for the most efficient induction of class II expression and that this synergy of mediators might explain the marked heterogeneity of expression in various cell types.

Rhodes et al., (1986) confirmed the role of gamma-interferon in enhancing the expression of class II molecules and clarified the potential effect of alpha-interferon in this context. They showed that interferon- α 2 did not increase Ia expression and conversely that interferon- α 1 did promote it. In addition, while

corticosteroids have been shown to diminish the expression of class II antigens in murine systems, it was shown that hydrocortisone could increase their expression on human monocytes.

4.3 Structure of Class II Histocompatibility Proteins

The structure of MHC class II molecules has been reviewed by Shackelford *et al.*, (1982) and Giles & Capra (1985). Initial studies involved the isolation of whole molecules in the molecular weight range of 28,000-34,000 from cell lines. The development of monoclonal antibodies in this area allowed not only the identification of these molecules but also their isolation, for biochemical analysis. A large number of allelic forms of the individual class II genes have been cloned and sequenced (Germain & Malissen, 1986). The allelic polymorphism is concentrated in the amino terminal domains of both the alpha and the beta chains and within these domains there are three or four hypervariable regions.

The alpha chain has a molecular weight of 34,000 and consists of 229 amino acids and 191 of those are exposed on the outer surface of the plasma membrane. The portion embedded in the plasma membrane is thought to consist of 23 hydrophobic amino acids and the remaining 15 amino acids form a hydrophilic tail which is localized in the cytoplasm. The extracellular part, which appears to have two domains, has two asparagine-linked carbohydrate moieties in positions 78 and 118. The second, membrane proximal domain contains one disulphide bond and

shows strong homology in its amino acid sequence to the constant region of immunoglobulin as well as to the second domain of the beta chain of class II proteins.

The beta chain sequences (molecular weight, 28,000), consist of 237 amino acids and have two immunoglobulin-like disulphide loops and a 22 amino acid segment integrated in the plasma membrane. In the cytoplasm there is a 16 amino acid portion. The beta chain has a single carbohydrate moiety linked to an asparagine residue in position 19. The terminal amino acid sequence contains 91 residues which are homologous with the corresponding region of class I heavy chains. There is also homology between β 2-microglobulin and the heavy chains of the immunoglobulin-like domains of HLA-A, B- and C- and the beta chain residues from 91-192.

Despite the apparent understanding of the biochemical structure of the HLA-DR molecule, there is still an enormous amount to be learned concerning the biosynthesis, processing, regulation, glycosylation and/or the phosphorylation of these molecules.

4.4 Function of HLA-DR and Ia

Ia molecules are known to have a role in the development of mature T cells and of normal immunocompetence.

(1) It has been demonstrated that T lymphocytes will only react to a foreign antigen on an antigen-presenting cell, if that antigen is presented in conjunction with an autologous Ia molecule (Rosenthal & Shevach, 1972). On this basis it was possible to

specifically block the interaction between antigen-presenting cells and antigen-specific T lymphocytes with an anti-Ia antibody (Shevach et al., 1972).

(2) At the same time it was observed that the interactions between helper T cells and the B cells that they regulate were also restricted by these class II molecules (Kindred & Schreffler, 1972). The helper T cell is restricted to interact only with a B cell bearing an autologous Ia molecule, identical with the one on the accessory cell which originally presented the T cell with antigen. Therefore T helper cells are specific for both autologous Ia antigens and foreign antigens presented simultaneously. More recently, it has been demonstrated that T cells specific for HLA-DR antigens bear T4 glycoprotein in their membrane (Meuer et al., 1982).

(3) Ia antigens are also important in controlling the primary stimulatory determinants in the mixed lymphocyte reaction, however the exact mechanism is unknown (Shackelford et al., 1982). Palacios (1982) demonstrated that it was the alpha chain and not the beta chain which was responsible for enabling resting T cells to respond to interleukin 2 and induce the production of interleukin 2 in autologous mixed lymphocyte reactions.

(4) The selection of clones of thymocytes reactive with autologous MHC antigens is thought to occur as a consequence of specific interaction with Ia-bearing autologous accessory cells, in the thymus (Benacerraf, 1985).

The majority of functional studies on the role of Ia molecules have utilized either anti-sera or monoclonal antibodies, with restricted antigenic specificities and concentrated on their effects on various immunological reactions. It is now well recognized that antisera that interact with determinants encoded by the Ia region or its human counterpart, the HLA-DR locus, are capable of interfering with the ability of monocytes and lymphocytes to respond to an antigenic stimulation.

- 8 Shevach et al., (1972) showed that the addition of anti-Ia sera to cultures of T lymphocytes responding mitogenically to antigen, in the presence of macrophages, effectively inhibited the proliferative response.

The effects of anti-Ia serum on the mitogenic response of spleen cells to lipopolysaccharide stimulation have been studied (Niederhuber et al., 1975). Pretreatment of spleen cells with anti-Ia and complement reduced the response by more than 90%, indicating that the B cells, necessary for an LPS response, must have Ia antigens on their surface. It was also observed that pretreatment of these cells with the anti-Ia antibody in the absence of complement also inhibited the response to LPS.

Marrack & Kappler (1977) conducted experiments to study the B cell responses to antigens in cultures in which the only targets of added anti-Ia sera were B cells themselves, or, in other experiments, the cytokines used to stimulate the B cells. When B cells were the only targets, there was an inhibition of the response despite the absence of macrophages. By contrast, anti-Ia sera had no effect on the activity of a T-cell derived, non-

specific, B-cell helper mediator. It was concluded that the binding of anti-Ia sera to B-cell surfaces inhibited B-cell responses to antigen, either by competing directly with the binding of signal molecules or alternatively by delivering an inhibitory signal to the B cell such that it became refractory to any further stimulation.

Accolla et al., (1981) examined the effect of two monoclonal antibodies, with specificity for two distinct epitopes on human HLA-DR molecules, on the mixed lymphocyte response. It had previously been established that the binding of one of the antibodies to Ia-positive cells was not influenced by the presence of a saturating dose of the other antibody. The addition of either antibody, up to Day 3 in culture resulted in an inhibition of the proliferative response normally seen in mixed lymphocyte reactions. While it had been generally assumed that the inhibition of proliferative responses was due to the masking of relevant Ia determinants on stimulating cells, the observed inhibition with xenoantisera directed against nonpolymorphic epitopes did not support this theory. The possibility that stimulating cells might have been eliminated in an antibody-dependent cell-mediated cytotoxic reaction was excluded by showing that the F'ab portion of one of the monoclonal antibodies also caused inhibition of the proliferative response. There was also evidence that pretreatment of the responder cells with monoclonal antibody to Ia and complement, without carryover of these agents into the mixed lymphocyte culture, reduced the proliferative response. Similarly treated, purified T cells were also incapable of responding in mixed lymphocyte cultures but

had no effect on the proliferative response of non-treated T cells suggesting that the effect of the monoclonal antibody and complement was directed at the T cell.

T cells stimulated in mixed leukocyte cultures expressed both Ia and an Mr 120,000 protein on their surfaces and neither of these could be detected on resting T cells. On this basis, Moretta et al., (1982) analyzed whether antibodies to either of these markers had any effect on interleukin-2-dependent T cell proliferation. Strong inhibition of cell growth was observed in cultures supplemented with an antibody to a common determinant on Ia but not in cultures supplemented with an antibody to the differentiation marker. Inhibition of cell growth was therefore assumed to be the result of a direct interaction of monoclonal antibody to Ia with activated T cells.

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ε Muchmore et al., (1982), used an in vitro assay that measured antigen-specific proliferation of human lymphocytes and found that both a heteroantiserum from rabbits and an alloantiserum which recognized determinants encoded for by the HLA-DR locus, dramatically blocked any proliferative response. Monocytes pulsed and then washed free of any excess antisera failed to promote proliferation in the presence of untreated T cells and soluble antigen whereas untreated monocytes and identically pulsed T cells responded normally, suggesting that the antisera acted at the level of the monocyte. On the basis that the addition of unpulsed monocytes failed to restore the antigen-specific reactivity, the authors proposed that the observed effect was not merely due to simple steric hindrance but rather that the

combination of anti-DR antisera with DR antigens caused a suppressive signal.

Vazquez et al., (1982) attempted to explain the obvious disparity between results by looking at the effect of three different monoclonal antibodies to HLA-DR on the in vitro antibody response of B cells to trinitrophenyl polyacrylamide beads. The three monoclonal antibodies used were directed against non-polymorphic determinants of the HLA-DR molecules but precipitated an identical HLA-DR molecule and were cytotoxic at comparable dilutions for the same proportion of peripheral blood mononuclear cells. Only one of the three antibodies inhibited the response. A cytotoxic effect was excluded on the basis that the bovine serum in the cultures was heat-inactivated and more importantly, the F'ab fragment was also inhibitory, showing that the inhibition was due to the specific recognition of an HLA-DR epitope on the responding cells. Neither of the other antibodies was inhibitory or even suppressive. The different functional effects of the antibodies could not be attributed to differences in their biological properties, as they all recognized the same molecule so a more likely explanation was thought to be that the antibodies recognized different epitopes on the same HLA-DR molecule. It was also postulated that HLA-DR molecules are implicated in several aspects of cellular co-operation and this would partially account for the apparent differences in effect of monoclonal antibodies to class II proteins that had been observed.

8 It is recognized that the induction of proliferative and antibody-specific responses of lymphocytes, to antigen or mitogen, requires accessory cells. However, the importance of Ia

molecules in both these responses has been questioned (Gerrard et al., 1983a & Gerrard et al., 1983b). It was demonstrated that while Ia-bearing accessory cells were necessary for antigen-induced responses, an Ia negative myeloid tumour line (K562) could replace monocytes in the proliferation of a monocyte-depleted population of lymphocytes in response to mitogen. This was supported by the finding that an anti-DR monoclonal antibody inhibited antigen-induced T cell response and T cell-dependent B cell responses but not mitogen-induced proliferation. In contrast to the findings of Muchmore et al., (1982), Gerrard et al., (1983b) found that anti-DR treatment of monocytes inhibited antigen-induced responses but that the response could be reconstituted by the addition of untreated monocytes. They also found that the antibody did not directly inhibit interleukin 1 secretion since the addition to cultures, of interleukin 1, did not restore the response. Furthermore, DR negative monocytes, selected using an antibody/complement lysis technique, were capable of secreting interleukin 1 but not presenting antigen. They were also able to support a mitogen-induced T cell proliferative response where interleukin 1 was an essential mediator. These authors supported the concept of steric blocking of the class II molecule which had previously been dismissed by Muchmore et al., (1982).

In direct contrast, Gilman et al., (1983) demonstrated that a monoclonal antibody, with specificity for a public rat Ia antigen, inhibited the production of interleukin 1 by peritoneal exudate cells activated with lipopolysaccharide and also inhibited the production of interleukin 2 by spleen cells stimulated with

Concanavalin A. The addition of an exogenous source of interleukin 1 to spleen cell cultures restored the proliferative response, suggesting that the effect of the antibody was to bind to Ia and block interleukin 1 production, which in turn limited the production of interleukin 2 and ultimately, T cell proliferation. It was also noted that the inhibition of interleukin 1 synthesis required that the antibody be present, in excess, throughout the entire culture period.

Palacios et al., (1983) proposed an acceptor/transducer function for HLA-DR molecules in T helper cell dependent B cell responses. They demonstrated that two monoclonal antibodies, (147 & 164), directed against monomorphic determinants on the HLA-DR molecule could replace T helper cells in the induction of proliferation and immunoglobulin secretion of B cells stimulated with pokeweed mitogen. Two other antibodies, one an anti-DR antibody (231) and the other an anti-human T cell antibody (UCHT1), and both of the same isotype as the previous two anti-DR antibodies, failed to exhibit similar helper activity on B cells. It was proposed that antibody 231 did not react with a functionally relevant epitope on B cells and might therefore interfere, by steric hindrance, with the interaction of T helper cell derived signals with corresponding DR receptors on B cells. In support of this, antibody 231 competitively inhibited the binding of antibody 164.

A panel of 22 monoclonal antibodies, against different epitopes on Ia were probed over a wide range of concentrations for their ability to inhibit the various stages of lipopolysaccharide-induced proliferation and maturation of B cells (Forsgren et al., 1984). A

similar study and results were reported by Clement et al., (1986) who used 6 monoclonal antibodies to HLA-DR on purified human B cells. The finding that all the antibodies were inhibitory to some degree meant that no correlation could be found between the effect and epitope specificity of these antibodies. They argued that the Ia receptor and the lipopolysaccharide receptor might be in close association on the cell surface membrane and that the binding of anti-Ia antibodies could either result in the steric hindrance and/or co-capping of the lipopolysaccharide receptor such that the binding of lipopolysaccharide, with its receptor, was prevented. This phenomenon of co-capping has been described previously between Ia and Fc receptors (Dickler & Sachs, 1974). Whatever the mechanism of the response, these results indicated some form of functional interaction among membrane molecules where the binding of a specific ligand could influence signal generation at another receptor when exposed to the appropriate activating ligand.

In contrast to the earlier findings of Gilman et al., (1983), Palacios (1985) found that monoclonal antibodies to HLA-DR stimulated monocytes to produce interleukin 1. Interleukin 1 activity, induced by monoclonal antibodies to HLA-DR, could be detected in monocyte cultures 24 hours later with peak levels being attained at 3-4 days. The antibodies were effective at concentrations of 1µg/ml or higher and only on monocytes which were positive for class II antigens, whereas both positive and negative monocytes will produce interleukin 1 after stimulation with lipopolysaccharide. This further supported the concept that Ia could act as a transducing molecule by acting as a signalling

channel between lymphocytes and Ia bearing cells. On this basis, it is possible that anti-Ia antibodies which recognize functionally relevant epitopes could equally well inhibit interleukin 1 secretion by monocytes which is driven by antigen-stimulated T cells and, in addition, these antibodies could inhibit the maturation of B cells. These concepts are difficult to test because of the difficulty of separating the restrictive function of Ia molecules in presenting foreign antigens from this putative function of signal transduction.

Corley et al., (1985) evaluated the role of class II molecules as transducing elements in T helper cell-dependent activation of B lymphocytes. The results suggested that while both the I-A and I-E molecules on a B cell lymphoma line were active in antigen presentation, the I-E molecule was necessary for the activation and subsequent production of antibody by these cells. This confirmed previous findings of a bifunctional nature of class II molecules so that while Ia molecules are ligands for the T cell receptor in the activation of T lymphocytes, the T cell receptor is itself a ligand for the Ia molecule in B cell excitation. In view of the widespread expression of Ia on a variety of cell types it is unlikely that this transducer function for Ia molecules is limited to B lymphocytes as demonstrated here and to monocytes, as reported by Palacios (1985).

In support of this, Kim et al., (1986) also found that Ia molecules were involved in two different ways in the T cell recognition of foreign antigen. This followed from findings that antigen-stimulated antigen presenting cells that were glutaraldehyde fixed and treated with monoclonal antibodies to Ia were unable

to stimulate T cells to proliferate. The ability to proliferate, as determined by interleukin 2 production, could be restored by the addition of glutaraldehyde fixed, non antigen-treated accessory cells to cultures in the presence of anti-Ia antibodies. This second function was attributed to Ia molecules because it could be replaced by the addition of cell membranes of antigen presenting cells or alternatively by soluble membrane extracts. Further experiments showed that T cells incubated with monoclonal antibodies to Ia and the calcium ionophore, ionomycin would proliferate. This was not the case with the tumour promoter, phorbol myristate acetate. Glutaraldehyde fixed antigen presenting cells added together with either ionomycin or phorbol myristate acetate to T cells did not result in proliferation. On this basis, Ia molecules were thought to contribute in two ways, the first being the presentation of foreign antigen and the second, a non antigen-specific interaction between Ia and T cells which is essential to provide additional intracellular activation signals.

Chen et al., (1987) provided the first molecular evidence that Ia proteins could act as signal transducing molecules. Specifically, it was found that ligand binding to either the I-A or I-E molecule caused a rapid (within 2 minutes) and transient translocation of protein kinase C from the cytosol to the nucleus. The process appeared to require the cross-linking of Ia molecules since it was induced by divalent F'(ab)₂ but not monovalent F'ab fragments of anti-Ia antibodies. Immunofluorescent studies indicated that after the cells were stimulated, the protein kinase C became associated with the nuclear envelope or some molecular complex associated with the nuclear envelope and this association could

not be disrupted with non-ionic detergents suggesting that the enzyme was not lipid bound. The mechanism whereby the cross-linking of Ia leads to translocation of protein kinase C to the nucleus is unknown, although there is evidence that it is mediated via cyclic AMP and the activation of a cyclic AMP-regulated kinase which in turn modulates the localization of protein kinase C. It has been proposed that the nucleus associated enzyme may in fact be in an inactive state and may have to be activated, possibly via a nuclear calcium ion store, phosphatidylserine and constitutive diacylglycerol. Chen et al., (1987) proposed that the observations of Palacios (1985), that is that monoclonal antibodies against Ia could stimulate the production of interleukin 1 by monocytes, would suggest that the translocated protein kinase C plays an active role.

Chen et al., (1987) also proposed that the lack of consistency of findings in previous studies on the role of Ia, could simply reflect the use of inappropriate assays and moreover suggested that it was unrealistic to expect soluble antibodies to mimic totally, the sum of T cell signalling required for B cell growth and/or antibody production.

The mechanisms by which extracellular signals are received and transduced across cell membranes have been reviewed by Berridge (1985) and Hamilton & Adams (1987). Essentially, the messages are transmitted across a membrane via a series of proteins, each of which induces a conformational change in the protein next in line. At some point in this process the information is assigned to a small molecule or to ions within the cytoplasm and these are the "second messengers" which allow a

signal to be propagated rapidly throughout a cell. Despite the fact that the number of different second messengers is very small, they are still able to direct a vast array of biochemical and physiological processes. Two major pathways of signal transduction have now been described: One of these employs cyclic AMP as a second messenger and the other uses a combination of calcium ions and inositol triphosphate and diacylglycerol. In both pathways, the receptor molecule at the surface of the cell transmits information via a series of G proteins (membrane proteins which are activated by binding guanosine triphosphate) and these in turn, activate an "amplifier" enzyme on the inner surface of the membrane which converts the precursor molecules, which are generally highly phosphorylated, into second messengers. These second messengers function similarly in both pathways by acting in one of two ways. It can bind directly to the regulatory component of the protein and trigger a conformational change (as in skeletal muscle where calcium binds to troponin C and triggers the conformational change which leads to the muscle contraction). The alternative mechanism is more common and indirect and involves the activation of a protein kinase which then phosphorylates a protein and this induces the conformational change in the protein.

Hamilton & Adams (1987) discussed various implications of signal transduction using mononuclear phagocytes as a model. Their findings on the effect of lipopolysaccharide on signal transduction in macrophages is of relevance to the present investigations, although the precise sequence of events is not yet fully

established. Lipopolysaccharide induces two types of functional responses in macrophages: (1) there are the rapid responses such as the secretion of metabolites of arachidonic acid, neutral proteases and lysosomal hydrolases which occur in a matter of hours and (2) the induction of cytotoxicity towards both microbes and tumour cells occurs over a period of days. At a molecular level, concentrations of lipopolysaccharide which are effective triggering signals will initiate hydrolysis of phosphatidylinositol-4,5-biphosphate and the subsequent generation of the 1,4,5 isomer of inositol triphosphate. This isomer has been associated with increases in intracellular calcium in other cells and the lipid moiety of lipopolysaccharide has been shown to effect a rapid but smaller rise in intracellular calcium. There is also a characteristic pattern of protein phosphorylation which occurs and is thought to be mediated by protein kinase C which would in turn have been stimulated by diacylglycerol, generated during the hydrolysis of phosphatidylinositol-4,5-biphosphate. Changes in early gene expression, in response to lipopolysaccharide, are also thought to contribute to macrophages reaching a level of competence and it has been observed that it can cause a marked increase in the peak expression of some genes as early as thirty minutes. There appears to be a second pathway by which lipopolysaccharide can initiate a cascade of events in responsive macrophages and this involves the independent expression of genes and it is manifested by the rapid and transient synthesis of a set of short lived proteins.

These preliminary reports on transmembrane signalling are clearly only the beginning of a more complex story which will ultimately unfold.

In this context, the CD4 molecule which is expressed on T helper cells, is thought to bind to a monomorphic region of class II molecules on target cells. There is evidence that CD4 does more than simply promote adhesion between the cells. Gay et al., (1987) have proposed that an additional function of CD4 might be to transduce signals across the T-cell membrane. An alternative is, that the recognition of class II molecules by CD4 and the T-cell antigen receptor might facilitate the formation of a T-cell antigen receptor/antigen/MHC tertiary complex. In either case, an interaction between the CD4 molecule and a ligand would be essential and Gay et al., (1987) have proposed that the class II molecule could function as such a ligand.

CD4 is a glycoprotein (Mr 55,000) of the immunoglobulin supergene family which is expressed on the surface of T helper cells and which is essential in the activation of T lymphocytes. The species equivalents of CD4 are T4/Leu 3 in humans, L3T4 in mice and W3/25 in rats (Littman, 1987 and Clark et al., 1987). While there has been interest in the role of the CD4 molecule as a marker of T-cell differentiation, few experiments have addressed the potential role of CD4 in the development of thymocytes. Of relevance to the results presented in this thesis, however, is the observation that the administration of antibodies against class II molecules to mice, in the neonatal period, blocks the development of class II-restricted CD4-positive cells intrathymically (Kruisbeek et al., 1985).

In summary, while the function of class II histocompatibility antigens in antigen presentation is well recognized, there are increasing reports that these molecules might also function in signal transduction. In relation to this it has been proposed that class II histocompatibility proteins might play some role in the production of cytokines by providing intracellular activation signals (Palacios, 1985). It was also proposed that the different functional effects of various monoclonal antibodies to Ia could be attributed to their binding at different epitopes on the same molecule (Vazquez et al., 1982). These findings have implications for the experimental model to be presented in this thesis.

CHAPTER FIVE

LITERATURE REVIEW - THYMUS

5.1 Introduction

The mammalian thymus is an encapsulated, bilobed organ situated in the thorax, overlying the heart and major blood vessels. The lobes are divided into lobules containing lymphoid cells and are arranged into an inner medulla and an outer cortex. Thymocytes differentiate and mature in the thymus. Haemopoietic precursors colonize the organ, mature into functional T cells and are finally exported to the peripheral lymphoid organs. Several thymocyte subsets can be defined on the basis of their cell surface phenotypes which are thought to reflect discrete stages in maturation (Yeh et al., 1986). It is in the thymus that thymocytes are selected to seed peripheral organs and those thymocytes with reactivity against 'self' antigens are eliminated (Yoshikai et al., 1986). The thymus gland also imparts to T cells the property of H-2-restricted recognition of antigen, such that T cells will only react with foreign antigens which are presented in association with self H-2-gene products (Lo & Sprent, 1986). Although the precise sequence of events is unknown, most available evidence suggests that the receptor genes first undergo rearrangement and subsequently become expressed, within the thymus (Snodgrass et al., 1985 , Born et al., 1987 and Bluestone et al., 1987). It has been shown that the course of T cell differentiation within the thymus is not

simply a progression towards maturity but includes different lineages with different fates. It has been estimated that about 90% of cells generated within the thymus will die there and thus never leave the thymus to participate in peripheral immune responses (Kinnon et al., 1986 and Adkins et al., 1987).

5.2 Embryology of thymus

In mice, the thymic rudiment arises in the region of the third and possibly the fourth pharyngeal pouches and clefts. Histological evidence suggests that the epithelial component of the thymus arises from the endoderm of the third pharyngeal pouch with a substantial contribution coming from the ectoderm of the third pharyngeal cleft (Jenkinson, 1981 and Nicolas, 1985). Jenkinson reviewed the sequence of events involved in the embryological development of the mouse thymus as follows; initially there are two lobes, each containing a central cleft which represents the original pouch-cleft cavity. The cleft disappears and by Day 13, the lobes which are now separate from the pharynx, begin their caudal migration to lie adjacent to one another in the thorax. It is during this phase that the thymic rudiment is colonized by large, basophilic lymphoblasts. At this time, also, each lobe acquires a thin, mesenchymally-derived outer capsule which later dips down into the epithelium of the lobe and initiates its division into smaller segments. At this stage the epithelial cells still form a relatively compact mass but by Day 14 they begin to show the desmosomes and tonofilaments characteristic of the reticulo-epithelium of the

adult thymus. At the same time, the lymphoid cells start to increase exponentially in number and simultaneously decrease in size. Finally by Day 18, the adult architecture of cortical and medullary regions with a lymphoid population of predominantly small cells, has emerged.

£ In this context, it is interesting to note the existence of a nude mutation in mice (Pantelouris, 1968) and a similar mutation in rats (Festing et al., 1978 and Douglas-Jones et al., 1985), and which contain a small and highly abnormal thymus rudiment. It is thought that these rudiments differ in that there is a persistence of the central cleft (Owen et al., 1975). Other studies have suggested that the abnormal development results from a failure in the ectodermal contribution, leaving only the endoderm-derived epithelial portion (Cordia & Heremans, 1975). Despite cross-breeding of nude mice with other strains of mice and genetic rearrangement of nude mice, it has never been possible to separate the genes responsible for the absence of the thymus from those responsible for the absence of mature body hair (Edelson & Fink, 1985). These animals, because of their lack of thymus, show a depressed or absent T cell function and have therefore been exploited by immunologists as an experimental model for studying the influence of the thymus gland on T cell differentiation and maturation.

5.3 Anatomy of the Thymus

Studies of thymus glands in rats (Hwang et al., 1974), mice (Weiss, 1963) and humans (Rouse & Weissman, 1981) have shown

that the thymus gland is organized into an outer cortex which is densely packed with cells and an inner, more sparsely populated medullary area. The two compartments differ in their organization of lymphoid cells as well as their non-lymphoid, stromal components. In the cortex, the majority of stromal cells are epithelial in nature and have long thin processes which reticulate among the cortical thymocytes. In addition, the cortex contains a small number of macrophages which reside in the region adjacent to the medulla. The medulla contains fewer thymocytes and the epithelial cells have shorter, more truncated processes resulting in a denser network of cells. Macrophages as well as interdigitating dendritic cells can be found in the medulla (Adkins et al., 1987).

It has been observed that involution of the thymus gland is almost totally confined to the lymphoid cell elements and that the epithelial cell network remains largely intact (Henry & Anderson, 1987). When involution is complete, sheets of thymic epithelial cells can be demonstrated in the remaining remnant.

The evidence provided for the existence of a blood-thymus barrier (Weiss, 1963 and Raviola & Karnovsky, 1972) is of functional significance in studying the anatomy of the thymus. Raviola & Karnovsky assessed the permeability of the thymic vessels in young adult mice using ultrastructural tracers of different molecular weights. These findings suggested that lymphoid cells in the cortex, in contrast to those in the medulla, are protected from circulating macromolecules by a twofold mechanism: impermeability of the endothelial junctions and the strategic location of the few cortical macrophages along the

vessels. They proposed that the absence of a barrier in the medulla might allow circulating substances to act as a trigger for thymocytes to complete their maturation and leave the thymus.

It is generally believed that the thymus gland parenchyma contains few or no lymphatics. Hwang et al., (1974), however, observed a few lymphatics in the medulla of rat thymus glands which extended to the cortico-medullary junction but were never seen in the cortex.

5.4 Cellular Components of the Thymus

The present discussion of the cellular components found in the thymus will be confined to the post-natal thymus. Basically, the cells can be divided into three broad categories:

- (i) epithelial
- (ii) lymphoid
- (iii) other

Epithelial Cells

The 'reticulo-epithelium' of the thymus gland which makes up the supporting framework consists of a large number of interdigitating epithelial cells accompanied by a well developed basement membrane. Studies in the rat (Hwang et al., 1974) have shown that these epithelial cells differ in the cortex and the medulla. In the outer cortex, they are somewhat stellate in shape with long cytoplasmic processes which insert into the fibrous capsule. The nuclei are oval, often indented, and show

evenly dispersed chromatin and prominent nucleoli. Tonofilaments and small, radiolucent membrane-bound vesicles are present in the cytoplasm. The Golgi apparatus and endoplasmic reticulum are not prominent. Desmosomes can occasionally be seen between adjacent cell processes. The epithelial cells are anchored to the capillaries by a fusion of their basement membrane with the basement membrane of the capillaries.

The epithelial cells of the inner cortex are similar but there is more anastomosing between the cell processes. In addition, many of the epithelial cells are anchored to the walls of post-capillary venules at the cortico-medullary junction.

In the medulla, the epithelial meshwork is denser than in the cortex and the resulting inter-reticular spaces are smaller. The epithelial cells in the medulla of the rat show cellular polymorphism, a finding which has been confirmed in studies in mice (Nicolas et al., 1985 and Nabarra & Andrianarison, 1987). In both species, the cells described as existing in the cortex are also found in the medulla. In addition, Hwang et al., (1974) describe epithelial cells which are more globular in shape and possess polymorphic membrane-bound inclusions or small uniform membrane-bound inclusions which correspond with the Type II and Type III epithelial cells described in mice. Type II cells are those exhibiting a highly developed alveolar network within their cytoplasm, possibly due to a modification of the Golgi apparatus. Type III cells contain one or two cytoplasmic cavities which are pseudo-cystic and have been linked to secretory functions. Nicolas et al., (1985) were also able to define a distinct

subset of medullary thymic epithelial cells on the basis of their reactivity with three different monoclonal anti-keratin antibodies.

The epithelial cells in both the cortex and the medulla express class I and class II major histocompatibility complex antigens (Van Ewijk et al., 1980 and Jenkinson, 1981) and are thought to be important in the selection of thymocytes which show class II antigen recognition (Zinkernagel et al., 1978 and Lo & Sprent, 1986). Thymic epithelial cells have also been shown to be the source of three thymic hormones, namely thymopoietin, thymosin α 1 and thymulin, which are able to induce the appearance of T-cell markers and functions (Berrih et al., 1985). More recently, thymic epithelial cells have been shown to be a source of interleukin 1, in humans (Le et al., 1986), presumably leading to thymocyte proliferation within the thymus.

The functional significance of the morphological differences and the distribution of the thymic epithelial cells has yet to be fully elucidated. However, it has been postulated that different types of T cells mature in different regions of the thymus and that each type of epithelial cell has a unique function in this role (Nabarra & Andrianarison, 1987).

As part of a discussion of thymic epithelial cells, the existence of thymic nurse cells, previously called lymphoepithelial complexes, should be included. Thymic nurse cells are epithelial cells, which are associated with up to two-hundred intact, actively dividing thymocytes which are surrounded by vacuolar membranes (Wekerle et al., 1980, Ritter et al., 1981, Kyewski & Kaplan, 1982

and De Waal Malefijt et al., 1986). It is not yet clear whether the associated thymocytes are in intimate association with or are actually enclosed within the epithelial cells (Adkins et al., 1987). They are derived mainly from the thymic cortex. Associated with each nurse cell there is a heterogeneous population of thymocytes, the majority are immature and cortisone-sensitive although there is a minor population of cortisone-resistant cells expressing the phenotype of mature precursors of the T-helper lineage. Nurse cells are presumed to play a role in both the early and the late stages of thymocyte maturation (De Waal Malefijt et al., 1986).

Lymphoid Cells

The majority of studies of the lymphoid cell populations in the thymus gland have been undertaken in mice and in humans because of the availability of a large and diverse range of monoclonal antibodies for phenotyping, in those species. The intrathymic differentiation of T-cell precursors to mature T lymphocytes is dependent, in part, on the expression of various antigens on their cell surfaces, including

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- (1) the T-cell receptor for antigen
- (2) growth factor receptors
- (3) the associative recognition antigens which are thought to participate in interactions with class I and class II histocompatibility structures (Lyt-2 and L3T4, respectively, in the mouse)

(4) homing receptors involved in the homing of thymus emigrants to peripheral lymphoid organs and

(5) an abundance of molecules (e.g. Thy-1 and Ly-1) which have been characterized but, as yet, have no assigned functions (Adkins et al., 1987).

In mice, the major markers used to separate thymocytes into phenotypically distinct subsets are Lyt-2 (equivalent to the glycoproteins on most human cytotoxic T cells which are recognized by T8 monoclonal antibodies in humans), L3T4 (equivalent to the molecules on T-helper cells which are recognized by T4 monoclonal antibodies in man), Thy-1 (equivalent to the simultaneous expression of T1, T11 and T3 which appear on the majority of T cells in humans), Ly-1 (found on the majority of T-helper cells and equivalent to T1 in man), H-2K (part of the MHC gene complex), PNA (binding site for the plant lectin peanut agglutinin) and MEL-14 (identifies a homing receptor for thymocytes emigrating from the thymus to peripheral tissues). Using these markers it has been possible to demonstrate that the majority of the cells in the cortex are either immature cells or the nonmature cells which die without further proliferation or differentiation. By comparison, the medullary cells are more mature with some cells bearing the same phenotype as peripheral T cells. The expression of these markers on thymocytes does not occur in an all-or-none manner but rather in degrees of staining intensity above background, making the distinguishing of these subpopulations a difficult and somewhat arbitrary task.

In addition to these markers, thymocytes have been divided into groups on the basis of their sensitivity or resistance to cortisone (Oosterom & Kater, 1979). Cortisone causes lysis of cortical thymocytes but it has no effect on the medullary thymocytes (Weissman, 1986).

A further method for categorizing lymphocytes as mature or immature is to look for the appearance of a nuclear enzyme, terminal deoxynucleotidyl transferase. This enzyme catalyses the formation of DNA sequences without template direction and its distribution in foetal thymus varies from that found in the post-natal thymus where it is mainly restricted to the cortical area (Janossy et al., 1981).

Despite the availability of a wide range of markers in mice and humans and despite the importance of understanding T-cell maturation and function, there is still a limited understanding of T-cell development, within the thymus. In the rat, there are fewer available monoclonal antibodies with which to work and therefore, even less is known about thymocyte maturation.

Other Cells

In addition to thymocytes and epithelial cells, macrophages and dendritic cells have been described in the thymus (Hwang et al., 1974, Jason & Janeway, 1984, Lo & Sprent, 1986, Kaneshima et al., 1987 and Nabarra & Andrianarison, 1987).

The thymic dendritic cells have been characterized in cultures from normal human thymus or in fixed tissues (Pelletier et al.,

1986 and Barthélémy et al., 1986). In culture, the dendritic cells were distinguished from epithelial cells and macrophages by their long fine processes, their irregular nucleus, by dense membrane-bound granules and that they never displayed phagolysosomes, tonofilaments or desmosomes. The dendritic cells also stained positively for Ia, ATPase, S-100 protein and vimentin and negatively for esterase, lysozyme and keratin. By contrast, the macrophages had numerous lysosomes and phagolysosomes and were positive for esterase, lysozyme and vimentin staining and negative for S-100 protein and keratin. In situ, dendritic cells and macrophages differed in shape and distribution. The dendritic cells were more irregular in shape and were located in the medulla and at the cortico-medullary junction while the macrophages were rounder and were randomly distributed throughout the cortex, medulla and connective tissue septa.

The function of these additional cell types is at present unknown, although there has been some evidence to suggest that they may have a role in shaping T-cell specificity (Longo & Schwartz, 1980 and Ready et al., 1984). These authors showed that the induction of H-2 restricted specificity failed to occur in thymus glands which had previously been treated with deoxyguanosine, a procedure which selectively removes macrophages and dendritic cells and early T cells but spares epithelial cells. Lo and Sprent (1986), were unable to confirm these findings but suggested that they may play a part in the induction of tolerance, as opposed to H-2 restriction, by removing those T-cells expressing a high affinity for self-H-2 determinants.

Small numbers of B lymphocytes, fibroblasts, plasma cells, granulocytes and mast cells are reported to occur within the thymus but almost always in the fibrous capsule and septa, or in the perivascular regions (Rouse & Weissman, 1981).

5.5 Differentiation and Maturation of Thymocytes within the Thymus

(1) Colonization of the thymus

(2) Intrathymic differentiation

(i) intrathymic hormones

(ii) direct contact of precursors with stromal cells

(iii) T-cell growth factors

(iv) MHC products

Colonization of the thymus

T lymphocytes are derived initially from haemopoietic stem cells which transform into lymphoid stem cells which are found in foetal liver and adult bone marrow. It has not yet been established whether these progenitor cells differentiate into pre-T cells before they enter the thymus, however, this would appear to be the case (Adkins *et al.*, 1987).

In animals, there is a massive migration of these progenitor cells into the thymus only in foetal life. The bone marrow does not develop as a significant site for haemopoiesis until shortly before

birth so the migrating cells are presumably derived from the foetal liver. In mice, this colonization of the thymus occurs at about eleven days of gestation while in birds, seeding of the thymus occurs in three successive waves in response to surges of unidentified chemotactic peptides (Jenkinson, 1981). In contrast, experiments have suggested that the adult thymus relies on an intrinsic intrathymic pool of self-renewing precursor cells to maintain its lymphoid population (Scolley et al., 1986) as well as precursors derived from bone marrow (Fowlkes et al., 1985). This is further supported by the fact that the adult thymus contains a small population of cells which bear a striking resemblance to the thymocytes which colonize the foetal thymus (Adkins et al., 1987).

Thymic colonization has been studied under experimental conditions using various systems including whole body irradiation followed by intravenous transfer of syngeneic, congenic or allogeneic bone marrow cells. However, the cellular influx into the thymus has been shown to be dependant on the dose of radiation (Goldschneider et al., 1986). Another method for studying colonization of the thymus has been to use organ cultured thymus lobes and exploit the selective toxicity of deoxyguanosine for early T cells, to produce alymphoid lobes which could be recolonized (Kingston et al., 1985 and Williams et al., 1986). Using this method, it was shown that a single micromanipulated stem cell could recolonize an embryonic thymus lobe and produce several phenotypically, distinct T-cell populations. In this context, Ezine et al., (1987) have proposed that different microenvironments within the thymus might be

responsible for the predominant maturation of the various mature T cell subsets. Kyewski (1987) also favoured thymic compartmentalization for the maturation of thymocytes but emphasized that the interpretation of his results was based on the assumption that isolated lymphostromal complexes represented a direct correlate in vitro of specific cell-cell interactions in vivo and that this was not necessarily the case.

Intrathymic Differentiation

The differentiation and maturation of thymocytes appears to be mediated by thymic humoral factors and via direct contact of thymic precursors with epithelial cells of the thymic reticulum. In addition, the action of lymphokines, produced by T cells, as well as the exposure of precursors to histocompatibility antigens on thymic epithelial cells has been shown to play a role.

Intrathymic hormones

The role of thymic humoral factors in T-cell differentiation is well established. They have been shown experimentally to be capable of restoring, in part, the immunocompetence of thymectomized mice in vivo and in vitro (Sztein et al., 1986). It has also been demonstrated that thymic hormones will augment the mitogen-induced, proliferative response of human, murine and rat lymphocytes (Hensen et al., 1978, Nieburgs et al., 1985 and Kruisbeek et al., 1977). More recently, thymic hormones have been shown to modulate the expression of the interleukin-2

receptor on activated thymocytes (Sztein, 1986). The early work on thymic humoral factors was carried out using supernatants collected from cultured explants of thymic stromal tissue which complicated the interpretation of the exact origin of the factors. The development of cloned lines of thymic epithelium has allowed more detailed studies of the roles and origins of thymic hormones (Beardsley et al., 1983 and Pfeifer et al., 1986).

Several thymic polypeptides have been isolated, characterized and sequenced, including;

(1) thymosins - a set of low-molecular weight peptides which have immunomodulatory effects including increasing the production of interleukin-2 (Sztein et al., 1986)

(2) thymopoietin - molecular weight 5500, shown to induce thymocyte maturation in vitro and in vivo (Bach & Papiernik, 1981)

(3) thymic humoral factor - increases cyclic AMP production in lymphocytes of neonatally thymectomized mice and augments graft-versus-host reactions (Bach & Papiernik, 1981).

(4) serum thymic factor - molecular weight 847, also called thymulin (Berrih et al., 1985), originally isolated from porcine serum by its ability to induce T-cell markers on thymocytes. Involution of the thymus gland is accompanied by a parallel decline in the serum level of this protein (Bach & Papiernik, 1981).

(5) interleukin-1 - initially thought to be produced by phagocytic cells of the thymic reticulum, in culture (Papiernik &

Homo-Delarche, 1983). More recently, the production of interleukin-1 by human thymic epithelial cells has been described (Le et al., 1987).

(6) prostaglandin E_2 - is also thought to be a product of thymic phagocytic cells (Papiernik & Homo-Delarche, 1983).

These hormones have been shown to be important in T-cell differentiation, particularly in its later stages. In the early stages, the direct contact of thymocytes with the thymic epithelium is essential (Bach & Papiernik, 1981).

Direct Contact of Thymic Precursors with Thymic Stromal Cells

The role of direct contact of thymocyte precursors and thymic epithelium, in the differentiation of T-cells is suggested, firstly, by their migration into the thymus, which if thymic hormones alone, were effective, would be unnecessary. Secondly, the migration of foetal liver cells to the thymus occurs at a time when the influence of thymic hormones is unable to achieve the maturation of T-cells (Stutman, 1978).

Singer et al., (1986), developed methods for the long term culture of thymic epithelial cells and rosette-forming assays for thymic epithelial cell-thymocyte binding and were able to demonstrate that, in humans, thymocytes bound to thymic epithelial cells but not to epidermal keratinocytes or thymic fibroblasts. These authors also demonstrated that the type of T-cells which bound were non-specific and that the binding could not be inhibited by antibodies to class I or class II histocompatibility antigens. They

postulated that the thymocyte CD-2 antigen bound to a receptor, found specifically on thymic epithelial cells and resulted in the activation of the thymocytes. A later study showed that thymocyte-thymic epithelial cell binding was necessary, but not sufficient, for the activation of thymocytes (Denning et al., 1987). These authors suggested that an intrathymic source of cytokines, such as interleukin-1 (Le et al., 1987), was necessary to induce interleukin-2 receptor expression followed by the secretion of interleukin-2 and the subsequent activation of T-cells. In this context they proposed that thymic epithelial cells could be a source of activation signals for the differentiation of thymocytes as well as providing accessory cell signals for intrathymic T-cell response to antigens. Wolf Vollger et al., (1987) proposed a receptor-ligand interaction between the CD-2 molecule on thymocytes and the LFA-3 molecule on thymic epithelial cells by demonstrating that antibodies to both these molecules inhibited the binding of the two cell types.

In this context, the concept of the thymic nurse cell could be important. The nurse cell complex has been proposed as a specialized site for the division and perhaps education of immature thymocytes such that they reach functional maturity prior to phenotypic maturity (Adkins et al., 1987). It is still uncertain whether the thymocytes are actually enclosed within the nurse cell or are merely in close association with it.

Kyewski et al., (1982), described a thymocyte rosette formed by the association of about ten to fifteen thymocytes with a single, central macrophage or dendritic cell, of which, half expressed Ia. These cell rosettes appear to play a role in the processing and

presentation of antigen (Kyewski et al., 1984 and Kyewski et al., 1986), although the exact mechanism is unclear.

T Cell Growth Factors

Thymic medullary lymphocytes, have long been considered to be the cells emigrating from the thymus to peripheral tissues. However, Elliott (1977) demonstrated that the majority of these cells were permanently resident in the thymus. It was later shown that these cells, in the presence of antigen or mitogen, secreted interleukin-2 (Gillis et al., 1978) and could thus represent an effective amplifier in T-cell differentiation. More recently, it has been demonstrated that both interleukin-1 and 12-O-tetradecanoyl-phorbol -13-acetate can induce maturation of human thymocytes to a point at which subpopulations of these cells are capable of producing interleukin-2 and responding to phytohaemagglutinins (de Vries et al., 1983).

Functional studies have shown that immature thymocytes, of the phenotype found in the thymic cortex, express receptors for interleukin-2, but do not proliferate significantly in response to interleukin-2. The basis for the expression of an interleukin-2 receptor by immature thymocytes is at present unknown (Ceredig, 1986, Lowenthal et al., 1986 and Shimonkewitz et al., 1987).

Histocompatibility Antigen Products

Ε T lymphocytes will only recognize a foreign antigen when it is presented with histocompatibility products identical to those on its own surface (Zinkernagel, 1976) and the capacity to recognize these proteins is acquired in the thymus (Zinkernagel et al., 1980). The thymic stromal cells are thought to be responsible for both class I and class II restriction of proliferative responses to antigen. However, the precise role of the various cell types has yet to be clarified.

The induction of tolerance has also been studied. Deoxyguanosine-treated embryonic thymus lobes could be transplanted across major histocompatibility barriers, without being rejected, in spite of their continued expression of foreign class I and class II antigens (Ready et al., 1984). There was, however, proliferation of the lymphocytes within the graft and also within the mature T-cell population in response to the foreign antigens, indicating that tolerance had not developed. These results were interpreted as demonstrating that the cells responsible for the induction of tolerance existed in the non-epithelial portion of the thymus gland.

It has been proposed that self antigens not only shape the repertoire of maturing T cells but also have a role in the development of responses for non-self antigens (Adkins et al., 1987).

5.6 Apoptosis - a Form of Programmed Cell Death

It has become increasingly well recognized that the majority of thymocyte precursors will die within the thymus (Kinnon et al., 1986 and Adkins et al., 1987). This phenomenon is known as apoptosis. The term apoptosis was first proposed by Kerr et al., (1972) to describe a mechanism of cell depletion which appears to be complementary to mitosis in the regulation of cell populations. The morphology of apoptosis suggests that it is an active, inherently programmed phenomenon and it has been shown that a variety of pathological and physiological stimuli can inhibit or initiate it. Structurally the apoptotic changes occur in two phases. In the first, there is nuclear and cytoplasmic condensation and breaking up of the cell into a number of membrane bound, ultra-structurally, well-defined fragments. In the second stage, these cell remnants, or apoptotic bodies, are shed from epithelial lined surfaces or are taken up by other cells, where they undergo autolysis within phagosomes followed by rapid degradation by lysosomal enzymes from the ingesting cells.

Apoptosis appears to be an important mode of controlled cell death which contributes to the homeostasis of cell populations. Histological sections of normal healthy tissues exhibit apoptotic bodies although little is known about their frequency or the factors that determine which cells will be affected.

Focal apoptosis also plays a role in embryogenesis, in the formation of luminal structures, tubular structures, the formation of limbs and interdigital clefts as well as the

involution of phylogenetic vestiges. It is obviously precisely controlled and this is thought to be the function of diffusable substances.

Malignant neoplasia is another area where the continuous death of cells, despite proliferation, is an inherent property. Studies on human basal and squamous carcinomata and 7,12-dimethylbenz(a)anthracene induced rat mammary tumours have been demonstrated to contain apoptotic bodies which are identical to those in non-neoplastic tissues. Mitotic figures and apoptotic bodies are often numerous in malignant tissues and it is the balance between the two processes which determines the rate of enlargement of the tumour.

Similarly, in atrophic and involuting tissues and organs, there is a decrease in cell numbers as well as in cell size. This has been demonstrated in rats where a reduction in the serum concentration of adrenocorticotrophic hormone results in the appearance of numerous apoptotic bodies in the cortex of the adrenal gland, an occurrence which can be prevented by the administration of exogenous hormone.

Little is known about the substances which induce apoptosis or the cellular mechanisms which are activated before the characteristic morphological changes. The withdrawal or administration of steroid hormones have, as previously described, been shown to affect the process. In areas of coagulative necrosis, where there are dense clusters of apoptotic bodies, ischaemia is thought to play a role but the mode of action of ischaemia in this process is unknown.

In this context, Russell et al. (1980) attempted to distinguish between target cell death mediated by cytotoxic T lymphocytes and cell death mediated by antibody and complement. In both cases, cell death has been thought to be the result of an increasingly large lesion in the target cell membrane, sustained by the influx of extracellular fluid in an attempt to balance the intracellular osmotic pressure from cytoplasmic proteins. They demonstrated that in fact the physiological consequences to the target cell were different in T-cell mediated cytotoxicity from those induced by antibody plus complement and that the difference was reflected in the state of the nucleus. They presented two hypotheses to explain the nuclear destruction by cytotoxic T cells. The first was that the T-cell could inject degradative enzymes as part of a lytic event and the second was that the target cell could autolyze in response to lytic signals received from the cytotoxic T cell. Subsequently, Russell & Dobos (1980) showed that the difference in the fate of the nucleus in antibody/complement lysis and cell-mediated cytotoxicity is evident within minutes of the induction of lysis. They proposed that while nuclear disintegration is not a prerequisite for cell death, the nucleus can be viewed as a marker to distinguish between different physiological processes. On this basis, they suggested that in cell-mediated lysis, nuclear destruction was an early event and that in response to this change, the plasma membrane is ruptured.

More recently, it has been demonstrated that near physiological concentrations of glucocorticoid hormones cause the death of several types of normal and neoplastic lymphoid cells (Wyllie,

1980). The morphological changes seen were characteristic of those in apoptosis, but in addition, it was shown that there was multiple double-stranded cleavage of nucleosome chains from nuclear chromatin, apparently through activation of an intracellular, non-lysosomal, endonuclease.

Duke et al., (1983) confirmed that within minutes of exposure of target cells to cytotoxic T lymphocytes there is fragmentation of nuclear DNA. In addition, it was shown that a similar pattern of DNA fragmentation occurred during glucocorticoid-induced killing of mouse thymocytes, an event which involves a specific endonuclease which is activated by calcium and magnesium ions but inhibited by zinc ions. This endonuclease yields multiples of 180-base-pair subunits of DNA. Zinc was also shown to inhibit DNA fragmentation and ^{51}Cr release induced in target cells by cytotoxic T cells, suggesting a common biochemical pathway for both types of cell death. The enzyme could also be activated in the isolated nuclei of thymocytes, spleen cells and lymphocytes by incubation with calcium and magnesium ions. In contrast to glucocorticoid-mediated killing of thymocytes where protein synthesis was shown to be necessary for the activation of the endonuclease, protein synthesis inhibitors had no effect on DNA fragmentation observed in target cells incubated with cytotoxic T cells. Further studies are required to localize the source of the endonuclease to either the target cell or the effector cell.

The calcium and magnesium dependant endonuclease is found constitutively in the nucleus of all thymocytes, however mature thymocytes lack the glucocorticoid-inducing mechanism for activating it. If fresh immature thymocyte nuclei are incubated

with dexamethasone or corticosterone and the appropriate ions, as much as 77% of their DNA is cleaved within ninety minutes (Cohen & Duke, 1984). These authors proposed that the protein synthesis involved in steroid-induced thymocyte death was not for the endonuclease itself but was in some way involved in its activation, possibly as part of a system for transporting calcium into the nucleus.

Further studies on cytotoxic T lymphocyte-mediated lysis of target cells have shown that 5,8,11,14-eicosatetraenoic acid and other inhibitors of the lipoxygenase pathway, at concentrations which inhibited arachidonic acid metabolism in mixed lymphocyte culture, will prevent lysis from occurring (Taylor et al., 1985). Although their study was designed to investigate the possibility for a direct role of arachidonate metabolites in the transduction of signals which would result in the lysis of cells, they were unable to exclude the possibility that exogenously produced metabolites might play a role in cell-mediated lysis.

More recently, Ucker (1987) suggested that unlike complement-mediated lysis, the processes of glucocorticoid-mediated and cell-mediated cytotoxicity seemed to require that target cells be active in their own programmed death. This was demonstrated for the glucocorticoid-mediated death of thymocytes by showing that the hormone-induced death of transformed thymoma cells occurred at a limiting dilution in the absence of other cell types and had to be cell autonomous. The implications of these findings to target cell lysis by cytotoxic T cells are unknown. However, it was proposed that if colloid osmotic lysis of the target cell were to

occur then the cytotoxic lymphocytes must be inducing an autolytic process in their targets.

CHAPTER SIX

LITERATURE REVIEW - MACROPHAGES AND THEIR PRODUCTS

6.1 Introduction

Macrophages are present in most tissues and as such have the potential to exert a regulatory role in tissue homeostasis and in local immunological and inflammatory responses. They have been shown to have multiple functions as follows:

1. They interact with extracellular molecules such as proteins and polysaccharides which may be free in solution or exist as part of the structure of microbes. The macrophage is capable of internalizing these particles and degrading them.

2. Macrophages secrete some one hundred products whose molecular masses range from 32 (superoxide anion) to 440,000 (fibronectin) and which have diverse activities. They include proteinases and some of their inhibitors, complement components, polypeptide hormones, coagulation factors, extracellular matrix or cell adhesion proteins, binding proteins for metals, lipids and biotin, arachidonate derivatives, reactive oxygen and nitrogen intermediates, some purine and pyrimidine products and $1\alpha,25$ -dihydroxyvitamin D₃ (Nathan, 1987).

3. Macrophages interact with both T and B lymphocytes directly and via the release of monokines and lymphokines.

4. Macrophages are strategically distributed in the tissues being situated close to the microvasculature and close to surrounding epithelial cells and mesenchymal cells. This phenomenon has been described in the thymus where macrophages and epithelial cells are present in large numbers adjacent to post-capillary venules at the corticomedullary junction (Raviola & Karnovsky, 1972).

5. Macrophages possess surface receptors for lymphokines allowing them to respond to and become activated by these products.

While it is recognized that the phagocytic role of macrophages is extremely important, the present discussion will be confined to the activation, the transduction of signals by and the secretory products of macrophages. The diverse role that macrophages play is emphasised by their early involvement in the events that lead to the stimulation of lymphocytes and the induction of an immune response and their continued involvement in the later reactions that characterise cell-mediated immunity.

6.2 Activation of Macrophages

Cells of the mononuclear system leave the bone marrow in a rather immature state and enter the circulation briefly. They then randomly leave the blood stream or attach to the walls of

sinusoids where they remain as relatively quiescent tissue macrophages until challenged with one or more stimulatory signals (Cohn, 1975 and Adams & Hamilton, 1987). The morphology of macrophages is heterogeneous owing to the varied endocytic histories and degrees of maturation of the cells (Adams, 1976).

The term "activated macrophage" has been used to describe an enhanced microbicidal activity of macrophages from animals with an acquired immunity to infection by facultative intracellular parasites (North, 1978 and Adams, 1982). Attempts have been made to distinguish between (1) "mature" macrophages which show increased endocytic and degradative abilities, in vitro, compared with monocytes (Blanden, 1968), (2) "activated" macrophages, as previously described and (3) "stimulated" macrophages which have an increased capacity to destroy neoplastic cells (Cerottini, 1974). It has not been established whether they represent different stages of the same state of altered function or whether they are in fact separate physiologic states (Adams, 1976).

The concept of activation of macrophages has been reviewed by North (1978). It developed from studies of tuberculosis which provided evidence for a cell-mediated immune mechanism based on the ability of macrophages to accumulate at the site of infection, engulf the micro-organism, destroy the tubercle bacilli more quickly if a second infection occurred and all of this happened without the participation of humoral immunity. Direct evidence came from studies which showed that macrophages harvested from vaccinated animals and infected in vitro with tubercle bacilli, in contrast to control macrophages, displayed an

enhanced ability to inhibit the growth of tubercle bacilli (Lurie, 1942). A similar role for macrophages in acquired immunity to infection was demonstrated with other micro-organisms including Salmonella, Listeria and Brucella. It was demonstrated that macrophages from immune animals destroyed the micro-organisms and in addition, that macrophages from normal animals were destroyed by intracellular bacterial multiplication. Evidence was also presented that the presence of "immune" serum in the normal macrophage cultures made little difference to their capacity to destroy the bacteria.

It was also observed that macrophages harvested from animals that had been immunized as a result of infection with one type of bacteria were phagocytic for other bacteria, that is, the immunity that developed was non-specific. Mackaness (1969) demonstrated that the activation of macrophages, although non-specific, depended on the generation of a more specific response, a delayed-type hypersensitivity response. Furthermore, he demonstrated that the non-specific resistance to infection was relatively short-lived, compared to the specific resistance and delayed sensitivity that developed (Mackaness, 1964). This was followed by the finding that macrophages could be activated in vivo by the transfer of sensitized lymphocytes to non-immune animals (Blanden & Langman, 1972 and North, 1973). These findings were reproduced in vitro by incubating normal macrophages with both Listeria and sensitized lymphocytes and demonstrating that the macrophages became activated against the microorganisms (Simon & Sheagren, 1972). It was at this point that the concept which is currently accepted, arose, that

is, the activation of macrophages is mediated by soluble factors, collectively known as "lymphokines" and they are produced by specifically sensitized T lymphocytes.

More recently, the concept of the activated macrophage has taken on a broader role and has been shown to include the intrinsic adaptive changes first described by Mackaness (1962). Amongst these were the pronounced ruffling of the plasma membrane of activated cells, an increased capacity to adhere to and spread on glass and plastic surfaces and an increase in phagocytic activity, the number of phagolysosomes and endocytic vesicles. Cohn (1978) has attempted to explain some of the biochemical changes which are seen to occur in the activation process. The increase in size of macrophages is attributed to an increased protein content and the observed spreading is thought to be due to a cleavage product of the alternate complement pathway, a polypeptide (Factor Bb) with a molecular weight of 65,000. On the cell surface, exteriorly disposed plasma membrane polypeptides show restricted labelling when macrophages are activated compared to resident cells. The enzyme, 5'-nucleotidase undergoes a marked decrease with progressive activation until in the activated cell, the synthesis is unable to compete with its degradation. In contrast, alkaline phospho-diesterase increases to more than double its amount in resident cells when macrophages are activated. Levels of ATP are increased markedly above those in control cell populations and the production of superoxide anions also occurs. As a result of enhanced phagocytic and endocytic activity, there is an increased intracellular accumulation of lysosomal hydrolases. In addition to these

changes it has been observed that activated macrophages secrete a vast array of products with a diverse range of activities (Adams, 1982 and Nathan, 1987). Of relevance to the present studies are two monokines in particular, interleukin 1 and tumour necrosis factor and they will be discussed in some detail. The expression of class II histocompatibility proteins by activated macrophages has also been the subject of considerable controversy and will be discussed later.

6.3 Macrophage Response to Lipopolysaccharide (LPS)

The cell wall of Gram-negative bacteria contains proteins, lipid and lipopolysaccharide (LPS), the latter sometimes being responsible for the type specific antigenicity of the micro-organism and of relevance to the present discussion. Chemically, LPS is composed of three principal regions (Skidmore et al., 1975) (i) the O-polysaccharide which is covalently linked to, (ii) the core polysaccharide, which in turn, is linked to, (iii) a lipoidal acylated glucosamine- disaccharide termed, Lipid A, via 2-keto-3-deoxyoctanoic acid. Functionally, the O-polysaccharide has been characterised as the major antigen and the Lipid A portion is responsible for the adjuvant and mitogenic activities. Lipid A has been shown to be a specific mitogen for bone marrow-derived B lymphocytes in mice (Chiller et al., 1973) and also to act as an adjuvant for the specific antibody response to the O-polysaccharide antigens in LPS, leading to an increased response over and above the response that would be achieved with polysaccharide antigen alone (Von Eschen et al., 1974). In this

context, the C3H/HeJ mouse is of interest because it is refractory to the effects of LPS while other B cell mitogens will stimulate mitogenic responses in this strain (Watson & Riblet, 1975). The C3H/HeJ mouse will support immune responses to the O-polysaccharide antigens but the defect lies in the augmentation by the Lipid A moiety which is not apparent.

More recently, Koga et al., (1985) compared the chemical, biological and immunochemical properties of LPS preparations from Bacteroides gingivalis and Escherichia coli and showed that LPS-nonresponsive C3H/HeJ spleen cells showed good mitogenic responses to both butanol-water-extracted and phenol-water-extracted LPS from B. gingivalis and also to butanol-water-extracted LPS from E.coli. These authors noted that the chemical compositions of LPS preparations differed considerably with different extraction procedures. They made no attempt, however, to explain the observed mitogenic effects of these LPS preparations on spleen cells which were previously considered to be refractory to the effects of LPS.

In relation to this, it has been demonstrated that N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), the core unit of peptidoglycan, injected into either normal mice or C3H/HeJ mice produces a rapid elevation in the level of monocyte-macrophage derived colony stimulating activity (CSA) (Galleli & Chedid, 1986). LPS, however, induced CSA in the serum of normal mice but was completely inactive in the C3H/HeJ hyporesponsive mice.

The mechanism of the interaction of LPS with monocytes and macrophages has been extensively studied. Warner et al., (1985)

examined the interaction between radioiodinated LPS from E. coli and human peripheral blood monocytes at 37° C and showed that it was dependent on binding to a carbohydrate in the cell membrane with subsequent internalization of the molecule. The reaction was not saturable with time up to two hours nor with concentration of LPS over a range of 0.1-10 µg/ml. The association of LPS with monocytes could be inhibited by homologous and heterologous LPS and O-polysaccharide but not by Lipid A or polymixin B, suggesting that the O-polysaccharide is important in the process. In support of this, Liang-Takasaki et al., (1983) used a murine macrophage cell line to demonstrate that the rate of phagocytosis of Salmonella organisms is dependent on this moiety.

Recent studies have focussed on the molecular mechanisms of signal transduction, in macrophages, in response to various stimuli (Hamilton & Adams, 1987 and Adams & Hamilton, 1987). These authors reported that LPS and Lipid A, at concentrations of as low as 10ng/ml initiated the rapid hydrolysis of phosphatidylinositol-4,5- biphosphate (PIP₂) resulting in inositol biphosphates and ultimately inositol. In addition it was found that the breakdown of PIP₂ was proportional to the dose of LPS or Lipid A applied to the macrophages and that Lipid A caused rapid and large rises in the intracellular level of calcium while LPS caused rapid, small but consistent rises, thought to be enough to trigger other transductional events and functional changes. Both stimulants caused increases in the phosphorylation of some proteins and the effects could be detected as early as fifteen minutes after exposure to LPS or Lipid A. There was a

strong suggestion in their studies that phosphorylation induced by LPS was mediated via protein kinase C.

LPS and Lipid A also appear to initiate the synthesis of new polypeptides (in the 35-85 kD range) as early as thirty minutes after stimulation and their synthesis stops within two to six hours, even in the continued presence of the stimulant. It was proposed that the early appearance, brief synthesis and short half-lives of these proteins favoured their having a regulatory rather than an effector function.

Stimulation of human monocytes with LPS induces the secretion of various proteins. The efficiency of LPS, from different species, as activators of human interleukin 1 synthesis and secretion has been studied by Newton (1986) who demonstrated that under low endotoxin conditions, monocytes synthesized little intracellular interleukin 1 and did not secrete detectable interleukin 1 into the extracellular fluid and that previous reports of the spontaneous secretion of interleukin 1, by tissue macrophages, failed to control for the presence of endotoxin in their systems. In Newton's study, it was demonstrated that as little as 10 pg/ml of LPS from S. minnesota could induce the secretion of measurable amounts of interleukin 1 activity. In contrast, LPS from E. coli required ten-fold higher levels to induce the same amount of interleukin 1 activity and LPS from V. cholerae or P. aeruginosa required more than 10,000 times more material. It was also observed that the timing and the level of interleukin 1 activity appeared to be directly related to the degree of exposure to LPS. Exposure at threshold levels of Salmonella minnesota (< 100 fg/ml) lead to intracellular interleukin 1 activity while higher

levels (> 1 pg/ml) were required for the secretion of interleukin 1. It should be noted that in Newton's study, the thymocyte proliferation assay, using thymocytes from C3H/HeJ mice, was used to measure interleukin 1 activity. In view of the observed mitogenic response of C3H/HeJ spleen cells to various forms of LPS (Koga et al.,1985), this assay may not be an appropriate method for measuring interleukin 1.

6.4 Specific monokine production

As mentioned earlier, macrophages secrete a large array of substances and are therefore capable of influencing several aspects of immune and inflammatory responses. Two factors, interleukin 1 and tumour necrosis factor have been well characterized and will be discussed in some detail. They are both LPS-induced macrophage products and therefore of relevance to the findings in this thesis. The multiple and often overlapping activities of these monokines have been reviewed by Le & Vilcek (1987). One obvious problem in studying the secretory products of macrophages is the propensity of the various products to affect one another (Libby et al.,1986).

Tumour Necrosis factor (TNF)

The term tumour necrosis factor was first used by Carswell et al. (1975) to describe a factor found in the serum of mice, with endotoxic shock which had been induced by repeated intravenous injections of endotoxin and mycobacteria. The serum containing this factor was cytotoxic or cytostatic for a number of cultured

cell lines (Campbell et al., 1980) and caused necrosis of some transplanted mouse tumours in vivo.

Beutler et al., (1985) isolated cachectin, now thought to be identical to tumour necrosis factor (Vilcek et al., 1986), produced by a macrophage cell line stimulated 22 hours previously, with LPS from E. coli. The isolated protein was shown to suppress lipoprotein lipase activity in the adipocyte line, 3T3-L1, to have a subunit molecular weight of 17kD and to bind to adipocytes and some other cell types via specific high affinity receptors.

Tumour necrosis factor has also been isolated from cultures of human peripheral blood monocytes which had been stimulated with phytohaemagglutinin and the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate. It was detected using antisera to TNF. Gel filtration demonstrated that the molecular weight of the native form was approximately 40,000 while SDS-PAGE revealed a sharp single peak of 16,500 + 500 (Chroboczek Kelker et al., 1985).

In a similar study, Kornbluth & Edgington (1985) demonstrated that the production of TNF by human monocytes, required as little as 1 pg/ml of bacterial lipopolysaccharide as a stimulus but it was not produced spontaneously; the cells had to be previously primed.

Le and Vilcek (1987) have summarized the molecular properties of purified TNF as being a non-glycosylated protein with a molecular weight (M_r) of 17 kD and an iso-electric point (pI) of 5.3. It has two cysteine residues which make up a single intramolecular disulphide bond. It arises from a precursor

molecule which contains 76 additional amino acids which are attached to the N-terminal sequence of the mature protein and they are shed prior to the release of TNF from the cell. Unlike other secretory proteins, TNF lacks a hydrophobic peptide sequence and the amino acid sequences of TNF are largely conserved among different animal species (about 80% between murine and human TNF). It should be noted that tumour necrosis factor and lymphotoxin (also called lymphocyte-derived TNF) have been shown to be structurally related, bind to the same cell surface receptor (Aggarwal et al., 1985) and have some indistinguishable biological activities and have therefore been designated as TNF α and TNF β respectively (Shalaby et al., 1985). The present discussion will be confined to macrophage derived TNF, that is, TNF α .

In addition to its cytostatic and cytotoxic effects, tumour necrosis factor- α has been shown to have diverse activities including

- (a) the induction of interleukin 1 gene expression by human endothelial cells in culture (Libby et al., 1986),
- (b) a limited role in the induction of the acute phase response (Darlington et al., 1986),
- (c) inhibition of lipoprotein lipase activity as described earlier,
- (d) stimulation of human umbilical vein endothelium to produce granulocyte/macrophage colony stimulating activity (Broudy et al., 1986),
- (e) the induction of Class I MHC antigens (Collins et al., 1986),
- (f) the stimulation of Ia antigen expression by a murine macrophage cell line, an effect which could be enhanced by the

addition of gamma interferon (Chang & Lee, 1986). In contrast to this, it has more recently been reported that TNF alone did not cause Ia expression on thyroid cells but produced a synergistic effect with interferon-gamma (Pujol-Borrell et al., 1987 and Weetman & Rees, 1988),

(g) an antiviral activity and it has been demonstrated whereby TNF synergizes with interferons in the induction of resistance to both RNA and DNA viral replication (Wong & Goeddel, 1986 and Mestan et al., 1986),

(h) the induction of osteoblast stimulated osteoclastic bone resorption with a degree of potency comparable to that of parathyroid hormone (Thomson et al., 1987). Recombinant interleukin 1 is similarly reported as being a potent and powerful stimulator of bone resorption in vitro (Gowen & Mundy, 1986),

(i) stimulation of synovial cells and fibroblasts to produce both prostaglandin E2 and collagenase (Dayer et al., 1985),

(j) a possible role in the pathogenesis of malaria infection on the basis of its accelerating the appearance of the terminal features of the disease in animals with low parasitaemias of Plasmodium vinckei (Clark et al., 1987),.

(k) Highly purified recombinant human TNF has been shown to cause stimulated cell growth of 4 different lines of human fibroblasts at concentrations of as little as 10 pg/ml, that is, lower than those required for its cytotoxic activity (Vilcek et al., 1986). Maximal stimulation occurred around 10 ng/ml or higher and was more sustained in the presence of 10% or more foetal calf serum than in medium with less than 5% or in the absence of foetal calf serum. Similar growth stimulatory effects on

fibroblasts have been observed with interleukin 1 (Schmidt et al., 1982). In this context, it is interesting to note that Nitta et al., (1986) use a fibroblast proliferation assay as a measure of interleukin 1. The possibility of TNF contamination of their culture supernatants was not considered.

Radioiodination of recombinant murine and human tumour necrosis factor revealed a single class of specific high affinity receptors for the factor on TNF-sensitive murine L cells and human HeLa S2 cells (Smith et al., 1986). Species specificity was also observed in cytotoxicity assays. Gel filtration chromatography showed that both the murine and human TNF-receptor complexes had an apparent Mr of 350,000. Non cross-linked recombinant tumour necrosis factor has an Mr of 17,200 on SDS PAGE gel and TNF has a propensity for aggregation, so it could not be determined whether a monomer or dimer was linked to one or more receptor subunits to make up the Mr of 350,000.

It has also been shown that steroids have a modulating effect on the production of TNF by lipopolysaccharide-stimulated human monocytes (Waage & Bakke, 1988). Concentrations of cortisol in the range 10^{-7} to 10^{-6} M, and dexamethasone ranging from 10^{-8} to 10^{-6} M suppressed the production of TNF in a dose-dependent manner. Using dexamethasone, 48 hours was required to reduce TNF production to 21% of the level found in control cultures and its effectiveness was decreased with shorter incubation times. There have been similar reports of hydrocortisone-induced suppression of interleukin 1 production by LPS-stimulated

murine macrophages using similar levels of steroid (Snyder & Unanue, 1982 and Besedovsky et al., 1986).

Interleukin 1 (Il-1)

Gery et al., (1972) first described lymphocyte activating factor as being a murine thymocyte mitogenic factor which was produced by stimulated human and murine adherent cells. It had the ability to augment thymocyte proliferation in the presence of sub-optimal concentrations of T cell mitogens. This finding provides the basis of an accepted biologic assay for interleukin-1, the thymocyte proliferation assay.

In 1979, the term interleukin 1 was proposed to refer to the monokine previously called lymphocyte activating factor and also to include B cell activating factor, endogenous pyrogen, leukocyte endogenous mediator, thymocyte proliferation factor and helper-peak-1, all of which had been described and had been subsequently shown, in functional assays to be identical to or to belong to a closely related family of molecules (Dinarello, 1984).

Although monocytes and macrophages are recognized as the main cellular sources of the cytokine, Il-1 activity has been shown to be produced by a variety of different cell types including thymic epithelial cells (Le et al., 1987) which is of relevance to the studies in this thesis.

The LPS-induced production of Il-1, by monocytes, can be seen in cell lysates as early as 1 hour and at 2 hours in the supernatant of stimulated cells with appreciable accumulation seen over a 6 hour period after which the level remains constant until 24

hours (Newton, 1986). Production can be enhanced by the addition of interferons (Arenzana-Seisdedos & Virelizier, 1983). There is some controversy as to whether Il-1 is secreted constitutively as it has been detected in the supernatant of unstimulated monocytes (Treves et al., 1983) and in unstimulated, homogenized epidermis (Hauser et al., 1986). However, as discussed earlier there were no controls in these experiments to eliminate the possibility of endotoxin contamination. There are numerous inducers of Il-1 production which have been reviewed by Dinarello (1984) but LPS is the most effective (McKernan & Lergen, 1983) and is the one of relevance to the present investigations.

Molecular studies have confirmed the presence of at least two species of human interleukin 1, termed interleukin-1 α (Il-1 α) and interleukin-1 β (Il-1 β), which although quite different in structure, exhibit similar biological activities (Gubler et al., 1986). March et al. (1985) demonstrated that the primary translation products for the two forms of Il-1 cDNA are 271 and 269 amino acids long and that the carboxy-terminal 159 and 153 amino acids are responsible for the biological activity of the molecules. The same authors reported 26 % homology between the amino acid sequences of Il-1 α and Il-1 β . Lomedico et al., (1984) showed that murine Il-1 cDNA coded for a polypeptide precursor of 270 amino acids (molecular weight 31,026) and similarly that the active portion was in the carboxy-terminal 156 amino acids. These findings were confirmed by DeChiara et al., (1986) and these authors postulated that the Il-1 precursor processing occurred extracellularly or on the cell membrane.

A membrane-associated Il-1, which is antigenically similar to the soluble form, has been described in macrophages (Kurt-Jones et al., 1986). It can be induced in one of two ways, firstly via direct cell to cell contact between the T cell receptor and the Ia/antigen complex on the macrophage and secondly via the release of a lymphokine, as yet unidentified (Weaver & Unanue, 1986). Membrane-associated Il-1 has also been induced on human endothelial cells and dermal fibroblasts stimulated with either recombinant tumour necrosis factor or lymphotoxin (Kurt-Jones et al., 1987) and in a human macrophage tumour cell line pretreated with phorbol myristic acid (Merluzzi et al., 1987). It has also been demonstrated that the activation of T cells in the anti-CD3 antibody-induced T cell response requires the presence of membrane-associated interleukin 1 (Hurme, 1987) and furthermore, that it could not be replaced by soluble Il-1.

There are numerous reports on the biochemical properties of Il-1 in various species. The Il-1 secreted by the murine macrophage line P388D₁ has a molecular weight between 12kD and 19kD and an iso-electric point between 4.6 and 5.2 (Mizel & Mizel, 1981). The production of an interleukin 1 precursor, with a molecular weight of 33kD, by the same cell line, was described by Giri et al., (1985). It was not feasible to determine whether this intracellular protein was biologically active as it was extremely labile and rapidly degraded to the bioactive form (17-19kD) during extraction and prolonged incubation in the thymocyte proliferation assay.

Using a different murine cell line RAW 264.7, and shorter culture times (48 hours as opposed to 6 days in Mizel's study),

McKernan & Largen (1983) found thymocyte mitogenic and comitogenic activity in three peaks on gel filtration: > 70kD, 30-40kD and 12-18kD. The low molecular weight form eluted from the column at 3 separate pH's: unbound material at > pH 7.4 and bound material at pH 5.2 and pH 4.8. It was observed, in addition, that the activity of the low and intermediate molecular weight forms, in the thymocyte comitogenic assay, depended on the number of thymocytes present in the cultures. Lower thymocyte concentrations (2×10^6 cells/ml) gave almost identical dose response curves with both preparations while higher thymocyte concentrations (1×10^7 cells/ml) altered the curve significantly. The activity of the intermediate molecular weight form was decreased at this dilution and this was proposed as an explanation for the failure of other investigators to detect activity in this fraction. It was also suggested that the length of time that the monocytes were initially cultured was important and that the higher MW proteins might be degraded in cultures of longer than 2 days. In this context, a 35-40 kD macrophage-derived factor has been described by Beller & Unanue.(1977) in a murine model and has been shown to promote the differentiation of thymocytes and have some comitogenic and mitogenic activity on thymocytes.

Studies on the molecular nature of human Il-1 are numerous. In a recent study, Auron et al., (1987) described three, distinct species of human Il-1, each with a molecular weight of 18kD and with iso-electric points of 7, 6, and 5. Evidence was also presented for the existence of a high (32kD) molecular weight precursor molecule which is the predominant intracellular form

of Il-1 and that it was processed either during or after transport to the extracellular environment. Previous reports of low molecular weight forms of intracellular Il-1 (Matsushima et al., 1986 and Lepe-Zuniga et al. 1985) were thought to be due to artefactual degradation of the precursor during extraction procedures.

Wood et al., (1985) had previously described four biochemically distinct species; two were 15kD in size and two were 35kD and within each pair there were two species, one with an iso-electric point of 5.5, the other with an iso-electric point of 7. All four fractions showed similar activity as mitogens for thymocytes. Identical species were identified by Kimball et al., (1986) who further proposed that each of the 17kD forms was derived directly from the 35kD form with the corresponding iso-electric point. This was demonstrated using 2 heterologous antisera raised against purified Il-1. The neutral one neutralized both pI 7 forms of Il-1 and conversely, the acidic form neutralized the pI 5 forms.

Schmitt et al., (1986) described two forms of Il-1 derived from normal human epidermis. A 17kD form was identified as having a pI of 5.7 and a 30kD form which was thought, as in other studies, to represent a precursor.

In a study designed to provide evidence for common antigenic determinants between Il-1 in different species, it was found that an antibody to human Il-1 with a pI of 6.9 neutralized that form of human Il-1 but not a pI 5.2 form. This antibody also had activity against a rabbit Il-1 with a pI of 7.4 but not against a

4.6 form from the same species and it failed to neutralize rat spleen cell-derived Il-1 or Il-1 from the murine cell line P388D₁ (Simon & Lee, 1986). These findings lent support to the concept that different pI types of Il-1 from the same species are both antigenically and biochemically distinct molecules and that Il-1 with similar pI's from different species may share antigenic determinants.

There are limited reports on Il-1 in the rat, however, Lovett et al., (1983) described a factor from LPS-activated rat peritoneal macrophages, the activity of which extensively co-purified with Il-1. The factor was shown to be heat-labile (80°C) and was inactivated by phenylglyoxal, the arginine-specific reagent, which has been shown to inhibit the activity of Il-1 (Klamfeldt, 1985 and Krakauer, 1985).

The release of Il-1-like activity by rat peritoneal macrophages, cultured for 48 hours in the presence of 10µg/ml of LPS has been described (Bird et al., 1985). Thymocyte comitogenic activity was observed in the expected molecular weight range of 12-16 kD but in addition, there was activity in the ultrafiltrates which had molecular weights below 10kD and 5kD. It was proposed that these fractions might represent degradation products of Il-1 which had retained their activity.

Giulian et al., (1986) described Il-1 produced by rat microglial cells as being an 18kD peptide, the activity of which could be neutralized by an antiserum specific for murine Il-1.

The biological activities of Il-1 are numerous and will not be discussed in this review. Two functions, which are of relevance

to the following investigations, are the comitogenic effect of Il-1 in augmenting thymocyte proliferation and the reported increase in fibroblast proliferation which occurs in the presence of Il-1.

The most characteristic function associated with Il-1 is the activation of T cells which occurs in response to antigen or mitogen and requires Il-1 (Gery et al., 1972). Il-1 is involved in the induction of Il-2 synthesis by T cells during mitogenic stimulation (Larsson et al., 1980). This is the basis of the thymocyte proliferation assay which is a widely accepted biological assay for measuring Il-1; thymocytes from LPS resistant, C3H/HeJ mice are cultured in the presence of sub-optimal doses of mitogen and test samples containing Il-1. Thymocyte proliferation is measured by the uptake of tritiated thymidine, 12 hours after its addition to cultures and 72 hours after cultures were established (Mizel et al., 1978). Other assays, based on the Il-1 dependent conversion of a cell line, LBRM-33, from an Il-2 nonproducer to a producer phenotype have been proposed (Conlon, 1983 and Larrick et al., 1985). The use of an immunoassay to measure Il-1 is unfeasible because of the multiple forms and therefore differing reactivities of Il-1.

The second biological activity of relevance to the present studies is the mitogenic effect of Il-1 for human fibroblasts (Schmidt et al., 1982). This observation forms the basis of another biological assay, used by Nitta et al., (1986), for measuring Il-1. This assay has been used in preference to the thymocyte proliferation assay for measuring Il-1, in this thesis (Chapter 7).

CHAPTER SEVEN

A MONOKINE WHICH BINDS TO CLASS II HISTOCOMPATIBILITY PROTEINS

7.1 Introduction

It is well recognized that macrophages play an essential role in immune and inflammatory processes, both at a cellular level and via the release of mediators such as interleukin 1 (Dinarello, 1984), tumour necrosis factor (Le & Vilcek, 1987) and colony stimulating factor (Burgess & Metcalf, 1980).

The aim of this chapter is to report the finding of a new monokine which is biochemically and functionally distinct from the previously well characterized monokines. Evidence is presented that this protein binds at the Ia antigen complex on the surface of thymic epithelial cells thereby stimulating them to release a protective factor which is directly responsible for preventing the death of thymocytes.

In relation to this, it has been estimated that approximately 90% of the cells which are generated within the thymus die in situ (Kinnon et al., 1986). This population of thymocytes is derived from the thymic cortex and the cells are characterized by their high density and susceptibility to cortisone-mediated apoptotic death (Weissman, 1986). The essential role of thymic epithelial cells in thymocyte maturation is well documented by studies in nude mice and rats in which the ectodermally derived epithelial

cells have failed to develop (Douglas-Jones et al., 1981). In the cortex of the normal thymus, the majority of the stromal cells are epithelial in nature and these have long processes which reticulate among the cortical thymocytes. In addition, the cortex contains a small number of macrophages which are primarily located in the region adjacent to the medulla (Adkins et al., 1987).

The epithelial cells in both the cortex and the medulla express both class I and class II histocompatibility antigens (Van Ewijk et al., 1980) and are thought to induce or select for self class II antigen recognition by thymocytes (Lo & Sprent, 1986). Thymic epithelial cells have also been shown to be the source of thymic hormones which induce the appearance of maturation markers on thymocytes (Berrih et al., 1985). More recently, the epithelial cells have also been shown to be the source of interleukin 1 (Le et al., 1987). A further function for thymic epithelial cells, that is, the rescue of thymocytes from apoptotic death is discussed. The relationship of this monokine, which induces this function in the epithelial cells, to thymic biology is discussed.

7.2 Materials and Methods

Materials

Animals. Inbred Wistar-Furth rats were obtained from the Blackburn Animal House, University of Sydney. They were bred to produce the four to eight week old animals which were used for this study. New Zealand white male rabbits were obtained from the Tillside rabbit stud, Yanderra, NSW. Both rabbits and

rats were housed in cages which had wire mesh bottoms. The animals were allowed water and pellets ad libitum.

Antibodies. The following monoclonal IgG antibodies from hybridoma culture supernatants were used: MRCOX52, a rat pan T cell and thymocyte marker (Robinson et al, 1986), a gift from Dr. D.W. Mason, Oxford University; MRCOX6, directed against a common determinant on rat Ia (Williams et al, 1977), obtained from Sera Labs.; MRCOX3, directed against a rat strain specific epitope on Ia (includes Wistar-Furth) (McMaster & Williams, 1979), also from Sera Labs. For indirect immunofluorescence studies Wellcome fluorescein-labelled sheep anti-rabbit immunoglobulins and Sigma fluorescein-labelled goat anti-mouse immunoglobulins were used.

Reagents. Purified rat lysozyme was a gift from Dr. E Osserman, Columbia University, New York. Tissue culture media including foetal calf serum and RPMI 1640 were obtained from Flow Laboratories. Dulbecco phosphate buffered saline tablets (PBS) were from Oxoid and pyrogen-free water was obtained from Travenol Laboratories. Iodo-beads were obtained from Pierce Chemical Company and ^{125}I , as NaI, from New England Nuclear. Freund's complete and Freund's incomplete adjuvant as well as guinea pig complement were from Difco. Lipopolysaccharide from Salmonella enteriditis, papain (type III), trypsin (type III), pepsin (twice crystallized, 2940 units/mg protein), thermolysin (protease type x), papain immobilized on beaded agarose, naphthol AS-B1 phosphate and fast red violet LB salt were obtained from Sigma while pronase (from Streptomyces griseus) was supplied by Calbiochem.

Separation techniques. Protein A-Sepharose, Percoll, Sephacryl S200 and Sephadex G10 were obtained from Pharmacia. LKB ampholine gels (pH range 3 to 9) were obtained from Linbrook International and ultrafiltration membranes were supplied by Amicon Ltd. Glutaraldehyde-activated silica matrix was supplied by Boehringer-Mannheim Ltd.

Plasticware. Lux 5350 25 sq. cm. tissue culture flasks and Linbro 76-232-05 flat bottom 96 well plates were from Flow Laboratories.

Cells. 3T3 Swiss fibroblasts were obtained from Flow Laboratories.

Methods

Antisera. Rabbits were immunized to produce specific antisera to rat lysozyme and to rat keratins. Keratin was purified from rat hair and from foot pad callous (Shimizu et al., 1974) to yield a preparation which contained twelve polypeptides by iso-electric focusing on LKB ampholine gel. For both proteins, rabbits were immunized with 50 µg protein emulsified in Freund's complete adjuvant, by injection in the back. Three weeks later they were given subcutaneous booster injections of 50 µg protein in Freund's incomplete adjuvant and bled from the marginal ear vein, one week later. Boosting was continued at monthly intervals until precipitating antibodies were produced.

An antiserum which produced precipitating antibody titres against a soluble keratin preparation was chosen for further

processing. The antiserum was dialysed against 0.1M sodium phosphate buffer and applied to a Protein A-Sepharose column equilibrated with the same buffer. The column was then washed with the running buffer containing 1M NaCl to remove non-specifically bound proteins and then with the running buffer containing 3M KSCN to recover the bound IgG fraction. This fraction was dialysed against 0.1M sodium phosphate buffer, pH 7.0, which also contained 0.9% NaCl and was applied to an affinity column. The affinity matrix was prepared by mixing 10 mg of purified soluble keratin with 200 mg of glutaraldehyde-activated silica in 2 ml of the above buffer at 4° C for twenty hours. The matrix was placed in a column and washed exhaustively with 0.1M sodium phosphate buffer, pH 7.0, containing 1.5% NaCl to remove unbound keratin. The remaining reactive groups on the absorbant were blocked with 0.3M glycine, pH 8.0, for one hour and the column was then washed extensively with 0.1M sodium phosphate buffer, pH 8.0, containing 0.9% NaCl. The IgG fraction in this buffer was added to the column, allowed to bind for one hour and then proteins were eluted at 6ml/hour. Non-specifically bound protein was removed by washing with the running buffer containing an additional 1M NaCl. The anti-keratin antibodies were then eluted with 3M KSCN and dialysed against PBS.

Preparation of F'ab. Four hundred micrograms of the mouse IgG monoclonal antibody MRCOX6 in a 2 ml volume was dialysed against 20 mM Na H₂PO₄ + 20 mM cysteine.HCl + 10 mM EDTA, pH 6.2 and incubated with ten units of immobilized papain for four hours at 37° C on a rotating drum. The supernatant was

dialysed against PBS, pH 7.4, and applied to a column containing 5ml Protein A-Sepharose equilibrated with the same buffer to separate the F'ab from the F'c fragments and undigested IgG. The sample was run at 6ml/hour and the eluate containing the F'ab was concentrated and a sample analysed by electrophoresis under non-reducing conditions on a 10% PAGE gel followed by Coomassie blue stain. There was no detectable intact IgG and only one band, consistent with F'ab, was observed.

Cell preparation. Macrophage cultures were prepared from the peritoneal washings of Wistar-Furth rats, six to eight weeks old. The cells in RPMI 1640 with 10% FCS were seeded into tissue culture flasks at a density of 1×10^6 cells/ml (5×10^6 /flask) and allowed to adhere for one hour at 37° C. The monolayers were then washed five times with PBS, 5 ml serum free medium was added/ flask and the cells were stimulated with 20 µg/ml lipopolysaccharide. The supernatant was harvested at two hours, filtered through an XM300 filter (Mr 300,000 cut off) to remove high molecular weight components and dialysed against PBS and concentrated over a PM10 membrane filter (Mr 10,000 cut off).

Thymus glands were removed from four to eight week old male and female rats taking care to dissect this organ free from surrounding tissues including lymph nodes. Thymuses were washed in RPMI 1640 with 10% FCS, sliced and pressed through a 0.5 mm pore size stainless steel wire mesh. The resulting thymic suspension was fractionated on a gradient of Percoll according to the method of Salisbury et al., (1979). The dense fraction was then washed three times in RPMI 1640 with 10% FCS.

Purified dense thymic epithelial cells and dense thymocytes were prepared by treating whole thymus suspensions with antibody and complement prior to fractionation on Percoll. Thymus cell suspensions containing 1×10^8 cells in 5 ml RPMI 1640 were treated with monoclonal antibody (MRCOX52 for thymocytes and MRCOX6 for Ia positive cells) at 2.5 $\mu\text{g/ml}$ final concentration together with complement at 2% final concentration. The preparations were incubated for one hour at 37° C and then layered onto Percoll.

The isolated populations were washed and characterized by indirect immunofluorescence and by enzyme histochemistry. For the demonstration of cytoplasmic or surface markers, cells were fixed for one minute in cold methanol, washed in PBS and then incubated with monoclonal antibodies at 2.5 $\mu\text{g/ml}$ or with 1/10 anti-rat lysozyme antiserum or with 100 $\mu\text{g/ml}$ affinity purified anti-rat keratin. Second stage antibodies were also used as 10% solutions of the original concentrations. Preparations were examined with a Leitz Orthoplan microscope equipped with incident fluorescence. Acid phosphatase activity was determined in cells fixed for one minute in cold formol-acetone and incubated with naphthol AS-B1 phosphate and Fast red violet LB salt in 0.1M acetate buffer pH 5.2.

The red blood cells used for the absorption of the macrophage factor (MF) were obtained by bleeding the rat from the heart and preparing and removing the buffy coat. The spleen cells, for similar experiments, were obtained by teasing a spleen apart and lysing the red blood cells in sterile water. Spleen cells depleted of Ia positive cells were obtained by complement

mediated lysis of spleen cells prepared, as outlined, and incubated with the MRCOX6 antibody.

Thymocyte viability assay. The dense cell fraction was divided into aliquots of 1×10^7 cells in 1 ml RPMI 1640 and incubated for four hours at 37° C. Duplicate samples were taken at hourly intervals and viability was assessed by Trypan blue exclusion (0.4% solution of Trypan blue in PBS). In those cases where the variation in counts was greater than 10%, fresh samples were taken.

Radio-iodination. Proteins were labelled by the method of Markwell (1982) using N-chloro-benzenesulphonamide derivatized polystyrene beads. Proteins were separated from free iodine by Sephadex G10 chromatography.

Fibroblast stimulation. Macrophage culture supernatant preparations were tested for their capacity to stimulate 3T3 fibroblasts to proliferate both in the presence and absence of FCS according to the method of Nitta et al., (1986) with the modification that 1×10^5 cells were used per well.

Electron microscopy. Purified dense thymic epithelial cells were pelleted and fixed in Karnovsky's fixative followed by osmium and embedded in Spurr's low viscosity resin. Sections were stained with uranyl acetate and lead citrate and examined in a Phillips 200 electron microscope.

Absorption procedures. Aliquots containing 1×10^7 cells were used to absorb 100µl of macrophage supernatant. The procedure was carried out on ice with occasional mixing for one hour. The

cells were spun out and the supernatant was absorbed twice more on new aliquots of cells, prior to testing in the thymocyte viability assay.

7.3 Results

The effect of macrophage products on the viability of thymic cells.

Aliquots of dense thymic cell preparations were counted and the number of viable cells was expressed as a percentage of the starting population. The results shown in Figure 5 indicate a rapid loss in viability. This was typically in the range of 40% to 70% of the starting population after four hours incubation. The addition of 50 μ l of supernatant from macrophage cultures which had been stimulated with 20 μ g LPS/ml two hours previously, protected the dense cell fraction from loss of viability (Figure 5). Neither lipopolysaccharide (from 1 to 20 μ g/ml) alone or supernatant from unstimulated macrophages protected the dense cell fraction. The supernatant from stimulated macrophages was titrated and was found to be protective at an end point dilution of 1/500. It was determined that approximately half of the peritoneal cells which were seeded into culture flasks became adherent macrophages. This gave 2.5×10^6 cells/ flask in 5 ml medium. Therefore 50 μ l of supernatant represents the release from 2.5×10^4 macrophages. This equates to this number of macrophages releasing enough activity to protect 500×10^7 dense thymic cells or 1 macrophage protecting 2×10^5 dense thymic cells. The protective effect was found in whole supernatant and

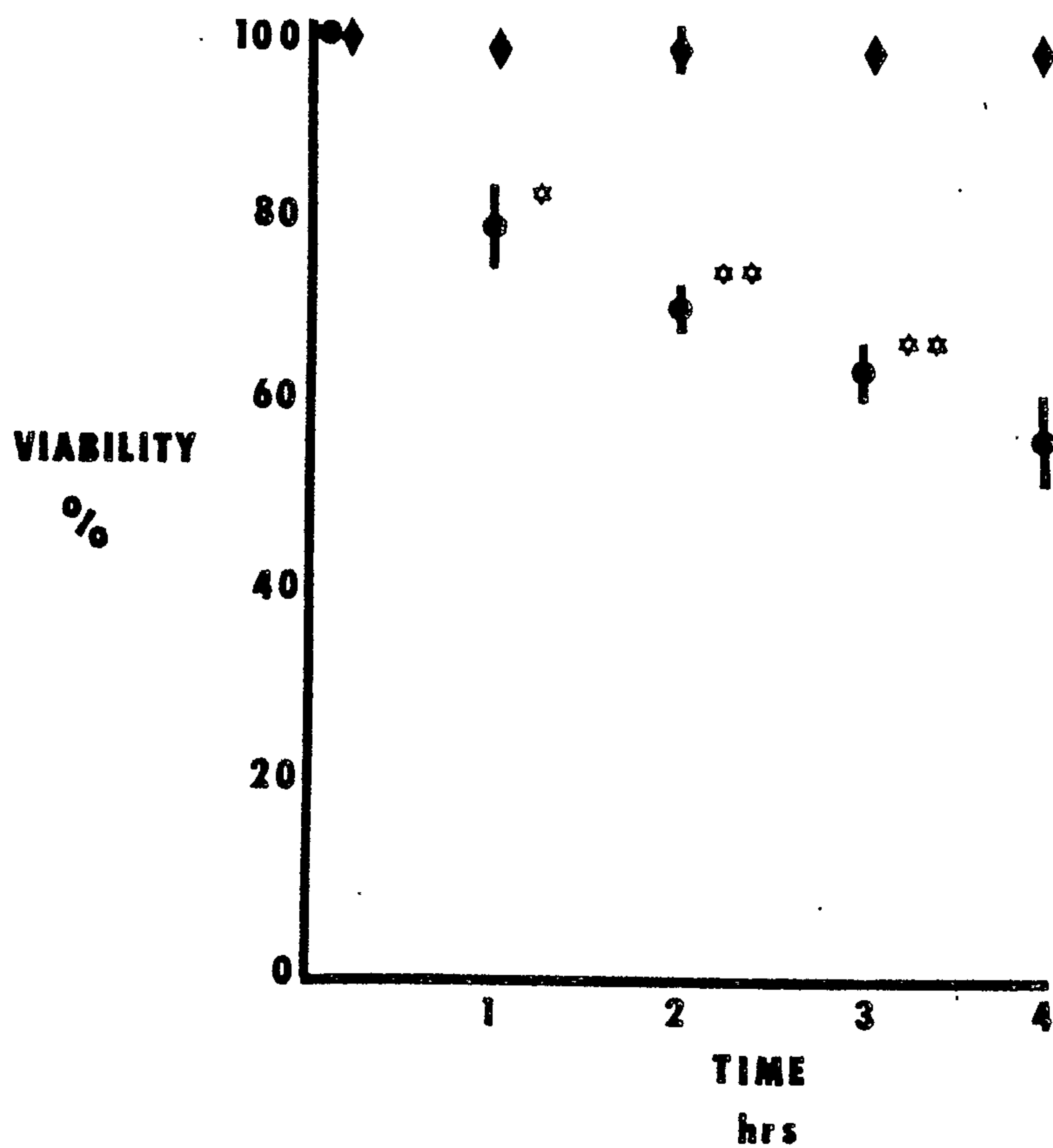


Figure 5. Viability of the dense thymic cells. Percentage cell viability as a function of time. ● Dense cell fraction (DCF) alone; ◆ DCF with the addition of stimulated macrophage supernatant. Results are the mean $\pm$ SEM for 25 experiments. By student 't' test, ☆ $p < 0.05$ and ☆ ☆ $p < 0.01$.

in the fraction retained above the PM10 membrane. Although these membranes have a nominal cut off of Mr 10,000 it was found that, under the conditions used, soybean trypsin inhibitor (Mr 23,000) passed through the filter while bovine serum albumin (Mr 67,000) was retained. Repeated assessment of macrophage cultures by examining at least 200 cells per culture showed that $99.6\% \pm 0.6\%$ of the adherent cells were positive for lysozyme by indirect immunofluorescence staining. The specificity of the anti-rat lysozyme serum was demonstrated by a single arc against a rat neutrophil lysate in immunoelectrophoresis and against the purified protein used for immunization. The control of second stage antibody alone, was negative. The cells in the cultures were 100% positive for acid phosphatase, the reaction being characterized by diffuse cytoplasmic staining of variable intensity. For further studies a batch of fractionated macrophage supernatant was prepared from the peritoneal washings of forty Wistar-Furth rats. This preparation (MF) was used at a concentration equivalent to a 1/250 dilution of whole supernatant in subsequent protection studies.

Mediation of the protective effect via thymic epithelial cells.

The dense cell fraction (DCF) was depleted of Ia positive cells by treatment with MRCOX6 and complement as described in Methods. Similarly, dense thymic epithelial cells (DTEC) were prepared by lysis of thymocytes using MRCOX52 and complement. Cell populations were characterized as described in Table 4 which displays the results for ten thymic preparations. To assess possible contamination with extraneous tissue,

<u>Population</u>	<u>Preparation</u>	<u>Identification</u>
1. Dense cell fraction of thymus (immature thymocytes & dense epithelial cells)	Fractionation of whole thymus on Percoll colloidal silica density gradient	(1) $12.8 \pm 1.9\%$ keratin +ve (2) $12.6 \pm 2.4\%$ remain after complement mediated lysis of thymocytes (3) Non responsive to lectins (4) All cells -ve for lysozyme (5) All cells -ve for acid phosphatase
2. Dense cell fraction depleted of Ia +ve cells	MRC OX6/complement mediated lysis of DCF	(1) Remaining cells lysed with MRC OX52 and complement
3. Dense thymic epithelial cells	MRC OX52/complement mediated lysis of DCF	(1) $96.9 \pm 3.7\%$ +ve for keratin (2) $98.9 \pm 1.6\%$ +ve for MRC OX6 (3) $99.0 \pm 1.5\%$ +ve for MRC OX3 (4) All cells -ve for lysozyme (5) All cells -ve for acid phosphatase (6) Homogeneous population - ultrastructure
4. RBC	Rat bled from heart	Morphology only
5. Rat spleen cells	Spleen teased apart, RBC lysed in H_2O	
6. Rat spleen cells depleted of Ia +ve cells	MRC OX6/complement mediated lysis of the above spleen preparation	

Table 4. Identification and preparation of cell populations.

randomly chosen thymuses were fixed in formalin and embedded in paraffin. Haematoxylin and eosin stained sections showed no evidence of contamination with lymph node tissue. The dense thymic epithelial cells stained weakly to moderately with affinity purified anti-keratin antibodies. The specificity of the anti-keratin antibodies was determined by the positive staining of epithelial cells in sections of rat skin with connective tissue components being negative. At least 200 cells were counted for each parameter under study. There was good agreement between the number of cells positive for keratin and those remaining after lysis of thymocytes, in the dense thymic fraction. The presence of macrophages and non-epithelial dendritic cells in this fraction was excluded on the basis of the buoyant nature of these cells and that all of the dense cells were accounted for by either anti-keratin staining of epithelial cells (12.8%) or MRCOX52 staining of thymocytes (87.2%). In addition, none of the cells in this fraction gave a positive reaction for lysozyme or for acid phosphatase. Furthermore, the purified epithelial cells appeared to be a uniform population by electron microscopic study.

The dense thymic epithelial cells maintained viability over twenty-four hours in culture while the dense Ia negative thymocytes showed 100% mortality over this time period. Fifty microlitre aliquots of MF failed to protect 1×10^7 thymocytes depleted of Ia positive cells (Figure 6). The incubation of 50 μ l MF with 1.2×10^6 dense thymic epithelial cells (in proportion to the percentage in the dense cell fraction) did, however, result in the release of protective activity by these cells (Figure 6).

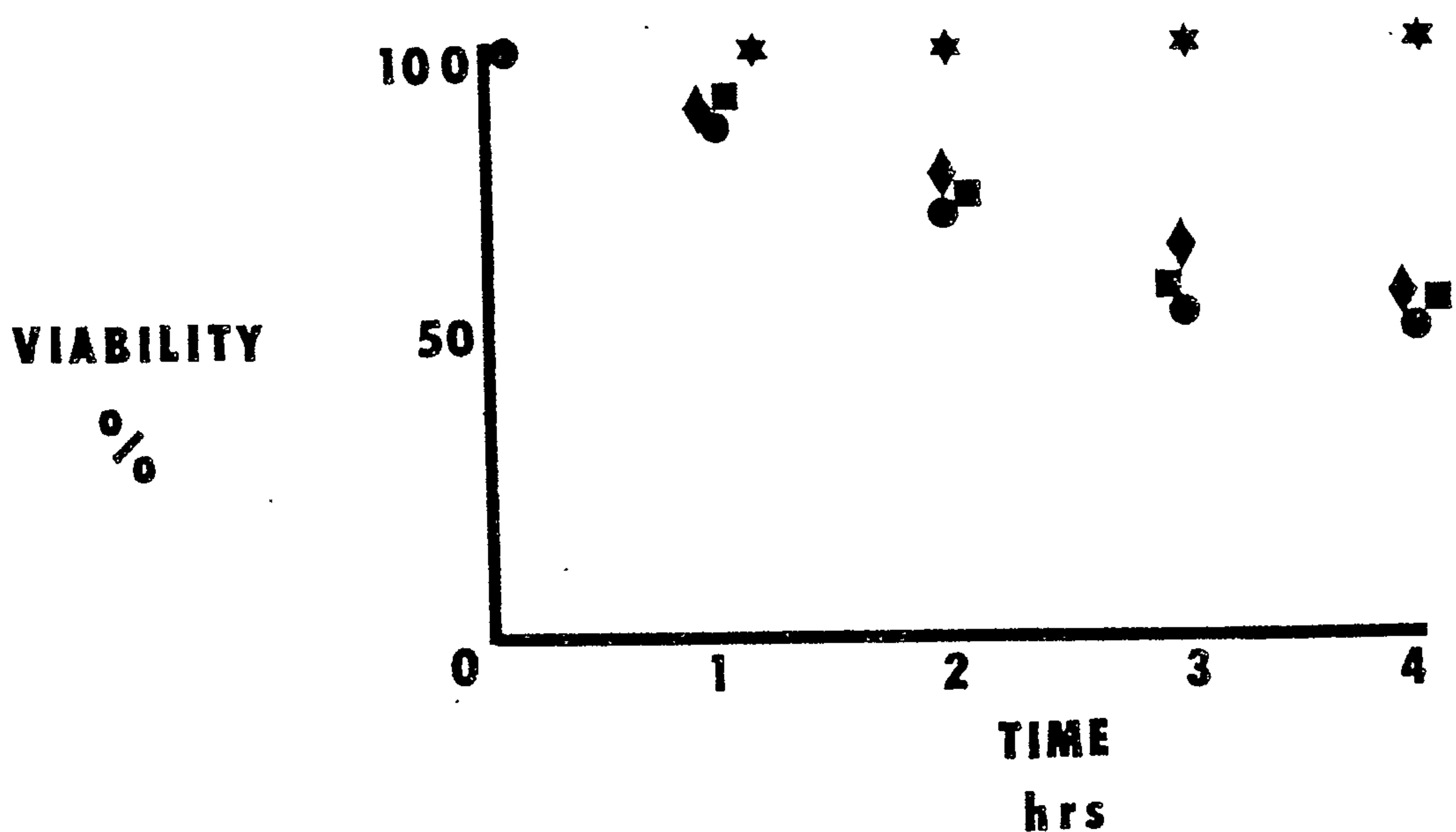


Figure 6. The role of thymic epithelial cells in thymocyte viability. Percentage cell viability as a function of time - representative experiment. Note that in every experiment the unprotected populations were less than 70% of the control population by four hours incubation. ■ Control, dense cell fraction depleted of Ia +ve cells (using MRCOX6 and complement in a lysis technique), ["Ia -ve DCF" = dense cell fraction after the depletion of Ia +ve cells], ♦ Ia -ve DCF with the addition of MF (reproduced over four experiments), ● Ia -ve DCF with 1 hour supernatant from unstimulated thymic epithelial cells (reproduced in 2 experiments), ★ Ia -ve DCF with supernatant from thymic epithelial cells incubated for 1 hour at 37' C with MF (reproduced in 2 experiments)

The role of the Ia molecule as a receptor for the monokine.

During the course of optimizing procedures for the depletion of Ia positive cells it was observed that MRCOX6, in the absence of complement, promoted thymocyte survival (Figure 7A) thus mimicking the effect of MF. As expected this antibody had no effect on the survival of the Ia negative DCF. When incubated with isolated thymic epithelial cells at $1.2 \times 10^6/\text{ml}$, it promoted the release of thymocyte survival activity. In contrast, MRCOX3, which recognizes a rat strain specific epitope on Ia, failed to protect.

The possibility that MF also bound Ia was initially investigated by attempting to deplete MF activity by absorption with different types of cells as outlined in Methods. As shown in Figure 7B, the DTEC effectively removed the MF activity. Spleen cells also removed the activity whereas spleen cells depleted of Ia positive cells, did not. Red blood cells were also ineffective. Similarly, MF was absorbed onto spleen cells which had previously been incubated, at 0°C , with either MRCOX6 or MRCOX3 and the supernatant was tested for protective activity. In order to negate the possibility of carryover of MRCOX6 into the viability assay, the antibody was labelled with ^{125}I as described in the Methods. The specific activity of the dialysed product was 2.09×10^5 dpm/ μg protein. No carryover of radioactivity into the viability assay was detected. The results shown in Figure 7C indicate that prior binding of MRCOX3 did not prevent absorption of MF whereas prior binding of MRCOX6 did so. This provided evidence that MF bound to surface Ia at a site which was the same as or nearby the site recognized by

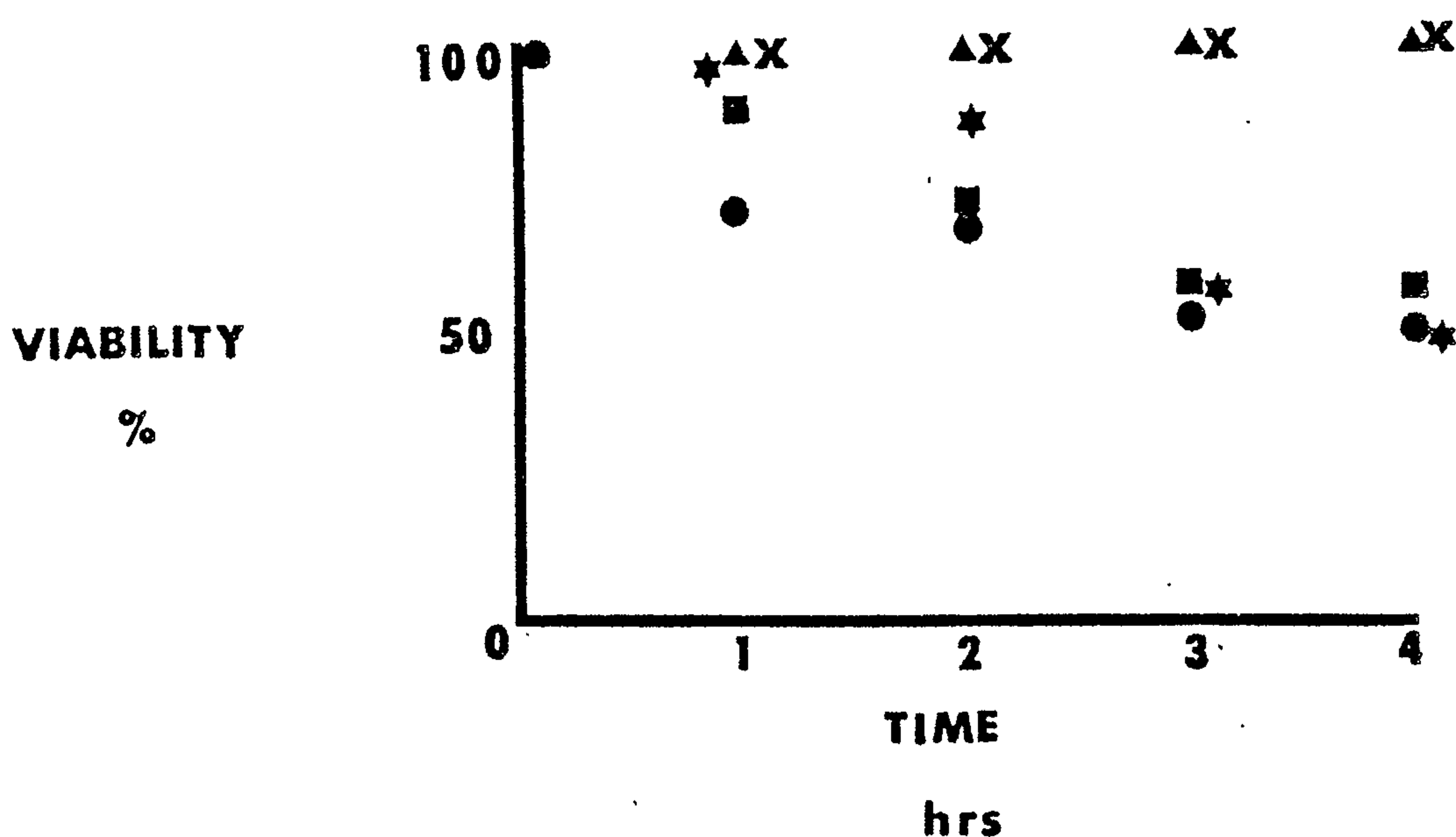
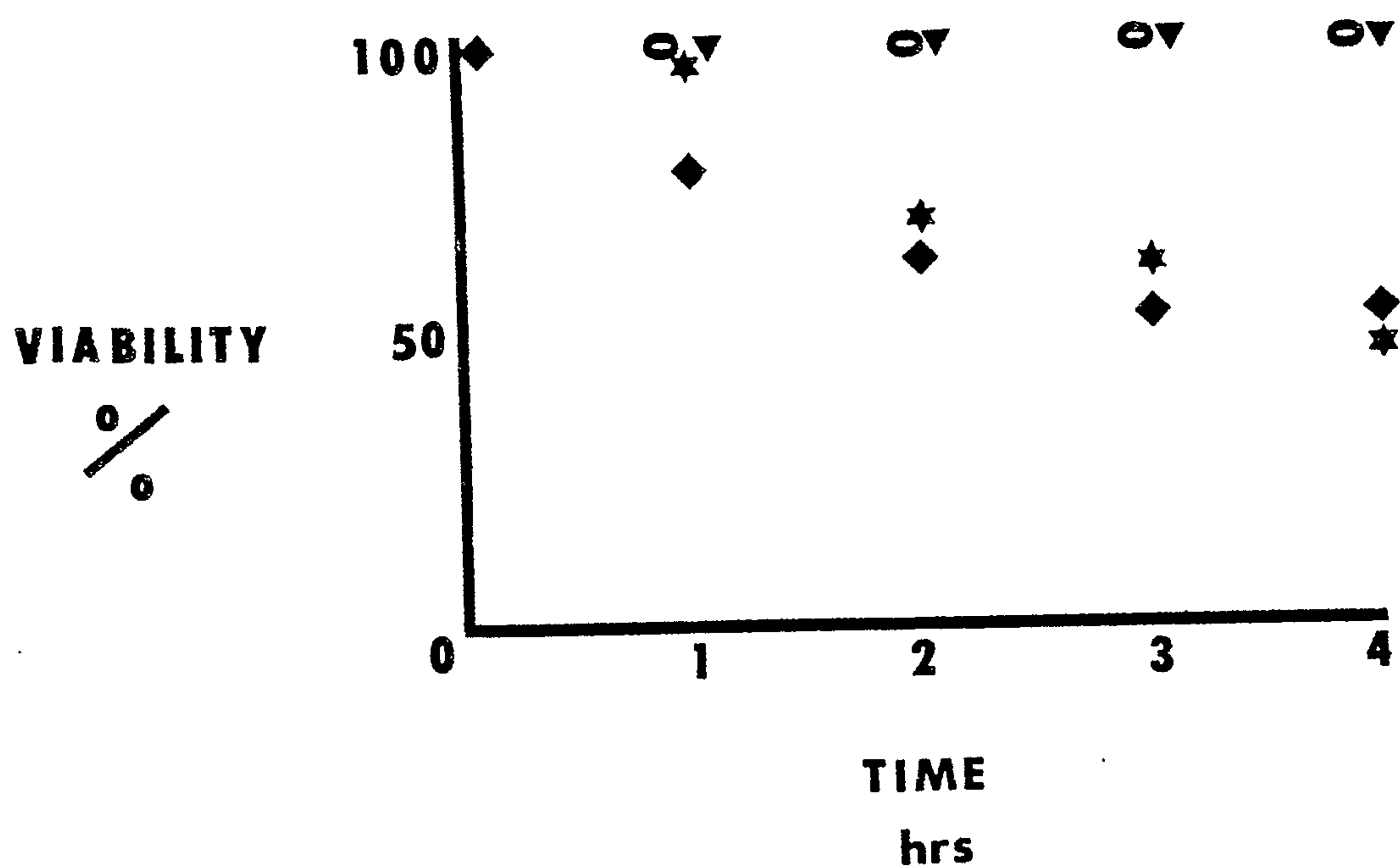


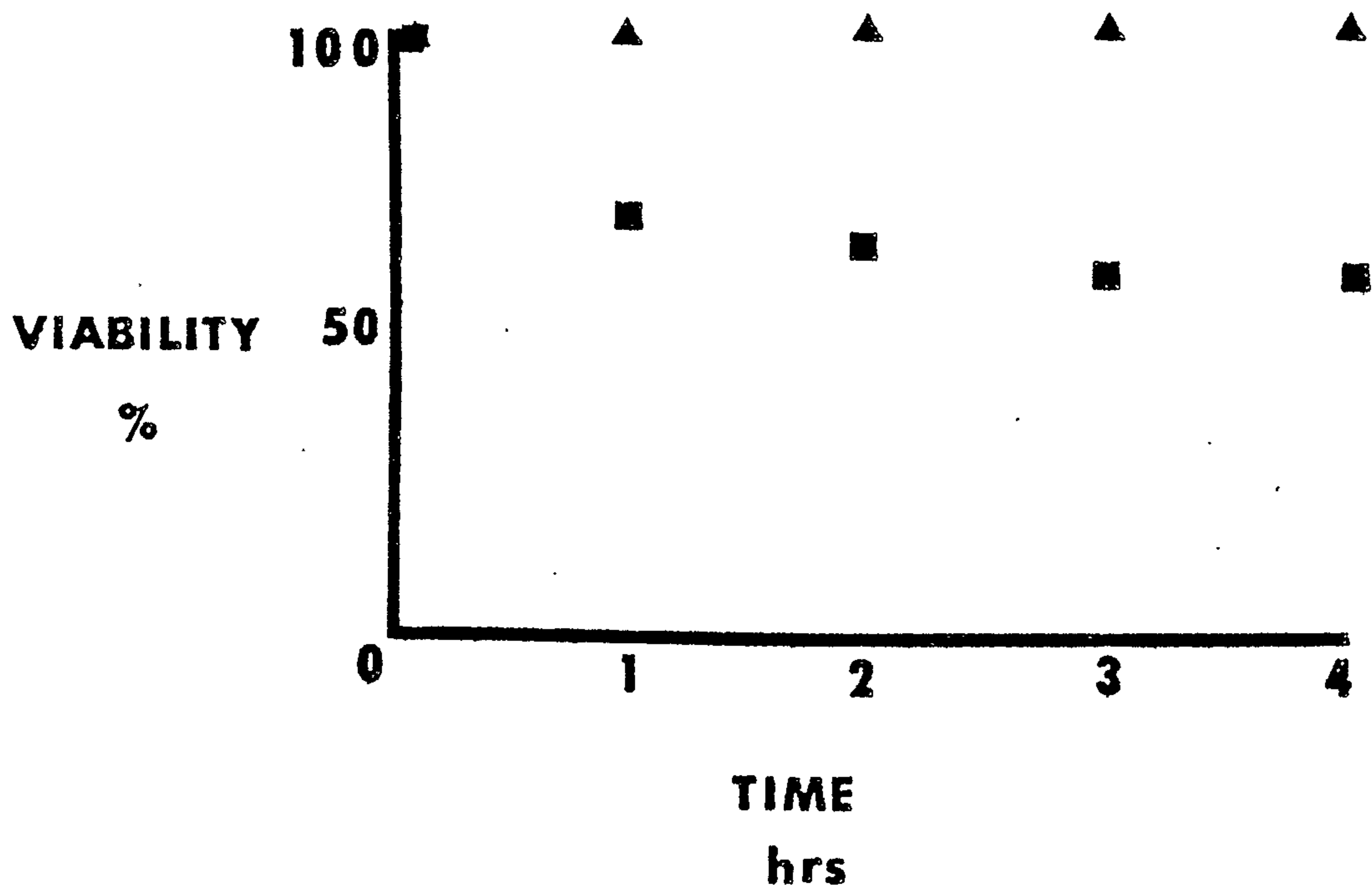
Figure 7. The role of the Ia molecule as a receptor for the monokine. Representative experiments are shown. In every experiment, the unprotected populations were less than 70% of the control population by four hours incubation.

7(A) Stimulated macrophage supernatant can be mimicked by a monoclonal antibody to Ia (MRCOX6). This effect is also mediated via an epithelial cell.

● Control DCF, ▲ DCF with 2.5 μ g/ml MRCOX6 added (reproduced in 4 experiments), ★ Ia -ve DCF with 2.5 μ g/ml MRCOX6 added (reproduced in 3 experiments), × Ia -ve DCF with supernatant from thymic epithelial cells stimulated with 2.5 μ g/ml MRCOX6, ■ DCF + 2.5 μ g/ml MRCOX3, the monoclonal antibody to rat Ia which is strain specific in its recognition (reproduced in 3 experiments)



7(B) Removal of MF activity by its absorption onto Ia +ve cells. ◆ MF absorbed onto thymic epithelial cells prior to addition to DCF. (reproduced in 4 experiments). ★ MF absorbed onto rat spleen cells. (reproduced in 3 experiments). ▼ MF absorbed onto rat spleen cells depleted of Ia +ve cells (using MRCOX6/complement lysis). (reproduced in 3 experiments). ● MF absorbed onto rat red blood cells. (reproduced in 2 experiments).



7(C) The binding site of the MF on spleen cells is blocked by an antibody to Ia (MRCOX6).

▲ MF absorbed onto spleen cells (as in 7B) but spleen cells previously incubated with 2.5 $\mu\text{g/ml}$ of MRCOX6. The possibility of carryover of MRCOX6 into the assay system was eliminated by labelling the antibody with ^{125}I . (reproduced in 3 experiments). ■ MF absorbed onto spleen cells previously incubated with rat strain specific antibody to Ia (MRCOX3). (reproduced in 3 experiments)

MRCOX6. The finding that MRCOX3 did not block absorption of MF mitigated against an intermolecular steric blocking of MF by MRCOX6. The binding site of the MF was further examined by studying the competitive effect of univalent F'ab MRCOX6 antibodies prepared as described. F'ab OX6 at 2.5 $\mu\text{g/ml}$ failed to protect the dense thymic fraction in the thymocyte viability assay. Pre-incubation of the dense cell fraction with this antibody preparation for fifteen minutes at 37° C did, however, effectively block the protective action of MF, indicating a close relationship between the binding site of the antibody and of the macrophage product (Figure 8).

Characterization of the monokine.

Physical properties

The molecular weight of the MF was determined by gel filtration fractionation on Sephacryl S200. The results are shown in Figure 9A. MF eluted at Mr 36,000.

The iso-electric point of the MF was determined by iso-electric focusing. Standard proteins and 100 μl of MF were loaded onto a broad range (pH 3-9) LKB ampholine gel and run to equilibrium. A standard curve was prepared from the reference proteins as shown in Figure 9B and in relation to this, strips of the gel were cut at known intervals. The strips were homogenized, dialysed against PBS and tested. A second run was used to further narrow the range. The activity was localized between pH 6.3 and 6.4.

The effect of heating was assessed by treating aliquots of MF for 30 minutes at 40°C, 56°C, 60°C, 70°C and 80°C. Activity was

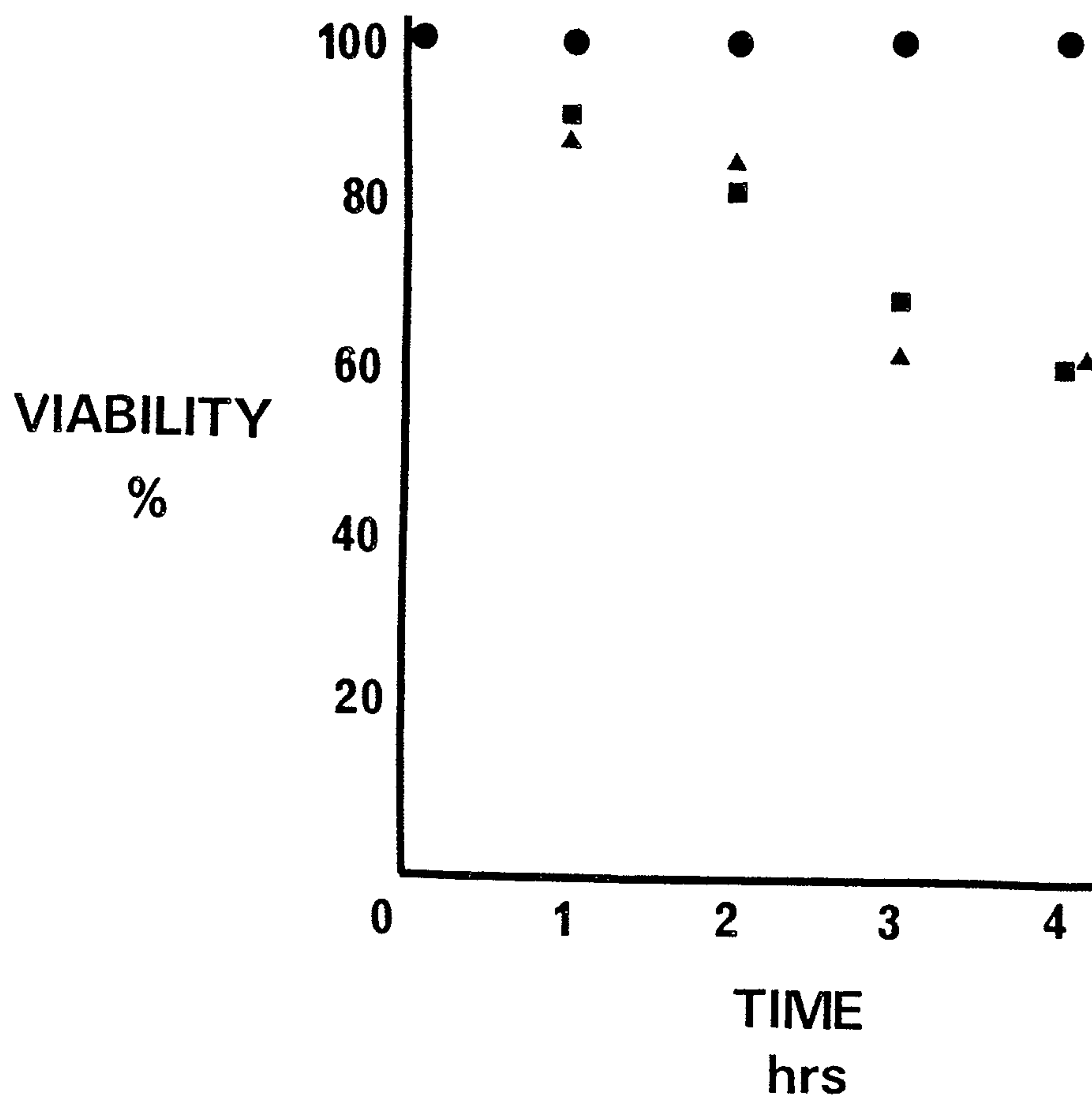


Figure 8. The role of the Fab portion of MRCOX6 in stimulating protective activity and in blocking the binding of MF.

● DCF + MRCOX6, ■ DCF + Fab MRCOX6, ▲ DCF previously incubated with Fab MRCOX6 + MF.

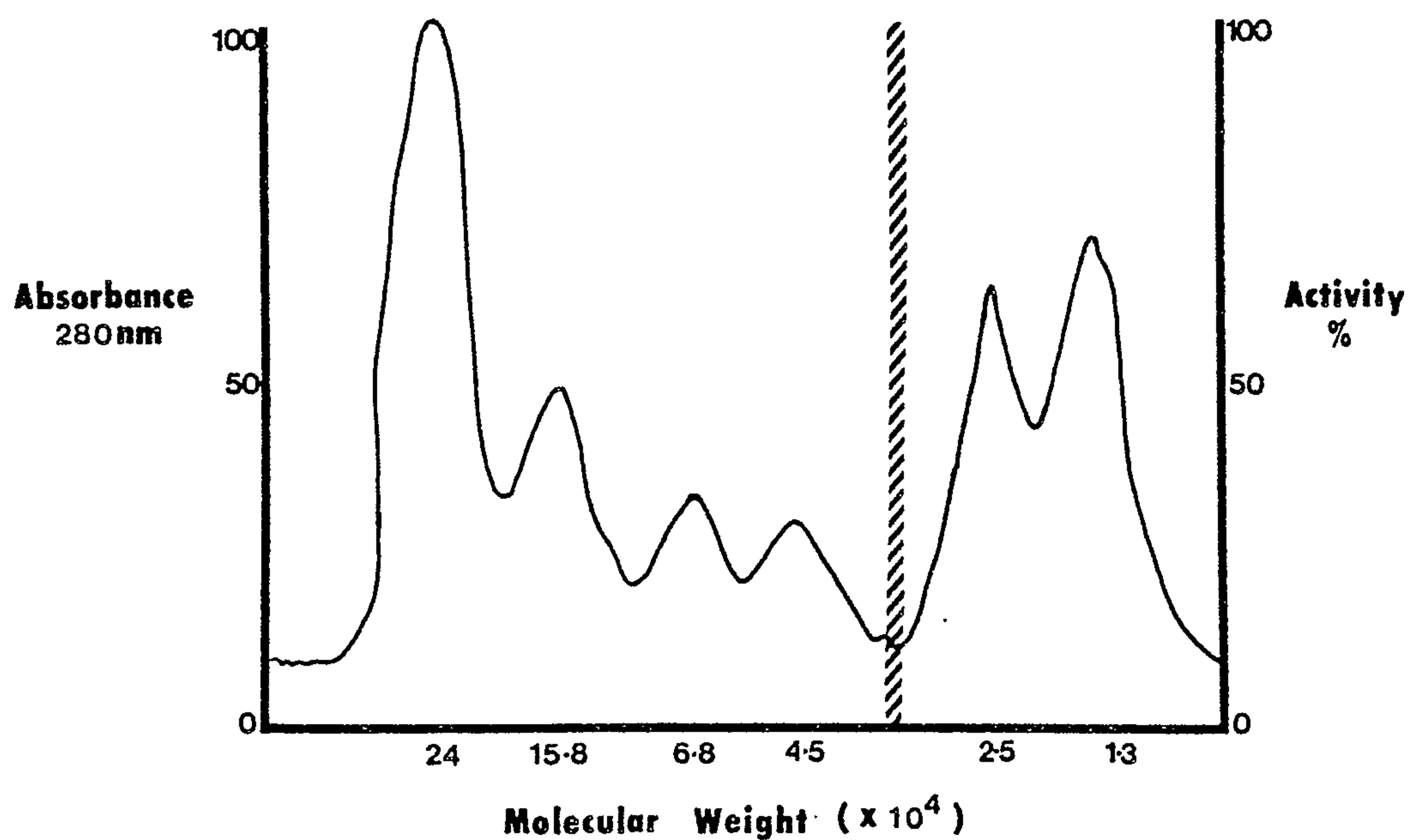


Figure 9A. Molecular weight determination of the macrophage factor. Sephacryl S200 column run. A column (100cm x 1.6cm) calibrated with Combithek II proteins in PBS with 0.1 M NaCl was run with a 5ml sample under identical total conditions. Flow rate 10.8 ml/hr. Sensitivity x 0.1. Chart speed 2cm/hr. Run time 17 hr. All of the fractions were tested and MF activity was detected in only one (0.8ml) fraction. Hatched area indicates activity.

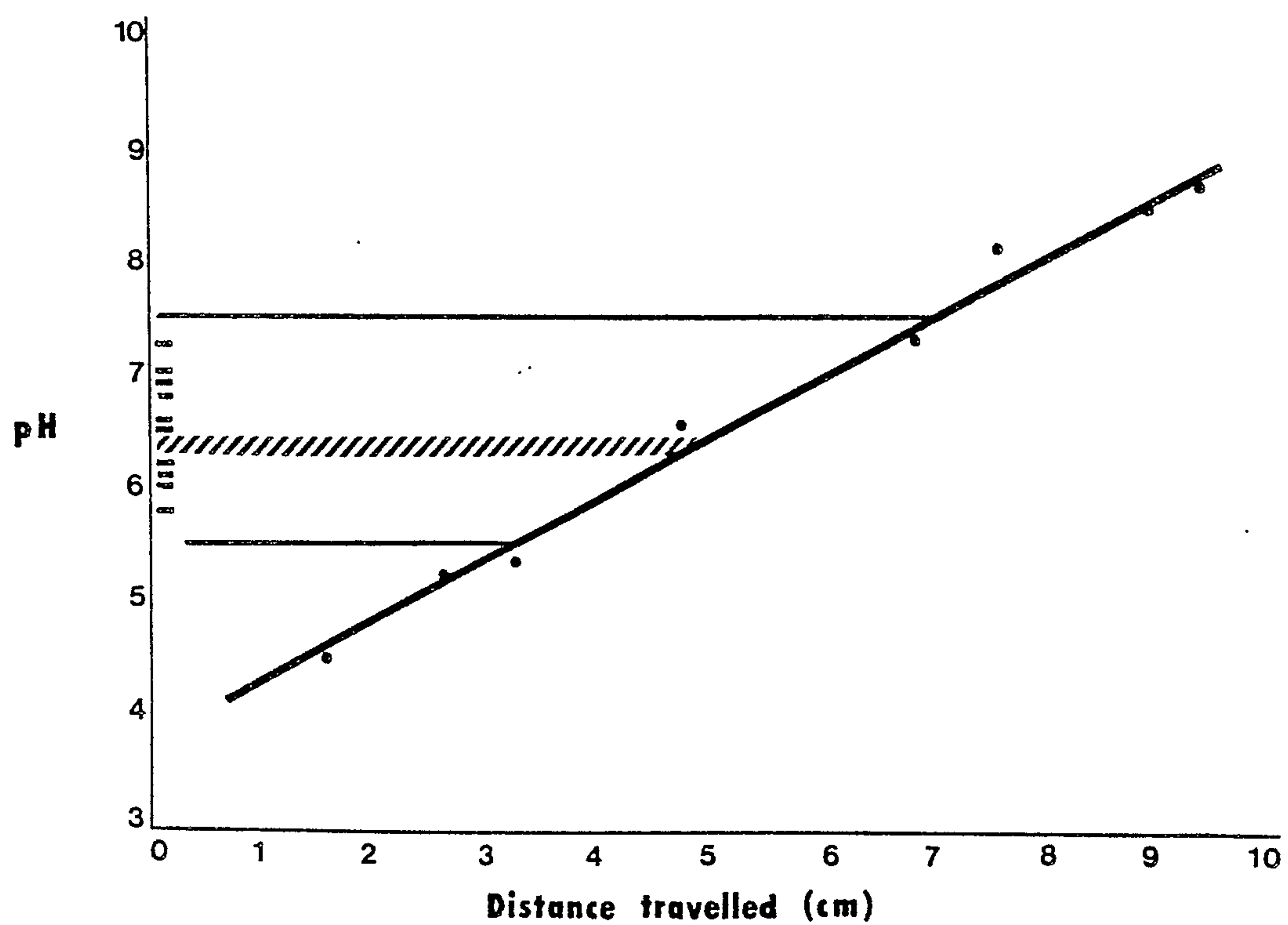


Figure 9B. Iso-electric point.

LKB Broad range ampholine gel (pH 3-9). Initial run, activity localized between 6 and 7 with reference to a standard curve. Second run, sample applied as a 10cm band with standard proteins at either end. Standard curves were identical and 2mm strips of gel were taken between pH 6 and 7 and the activity was localized to one strip: pH 6.3 - 6.4. Hatched area indicates activity.

destroyed at 80°C but was stable at lower temperatures. pH stability was tested by adding MF to appropriate 0.1M buffers at pH 2, 4, 6, 8 and 10 for 60 minutes at 37°C. Samples were then dialysed against PBS and tested for activity. MF was stable over this pH range. MF activity was lost following reduction with 5% 2 mercapto-ethanol.

The effect of proteinases on MF activity was assessed by adding MF to 10µg/ml solutions of enzyme for 60 minutes at 37°C. The proteinases were papain, pronase, thermolysin and trypsin which were used in RPMI 1640, pH 7.4 and pepsin in RPMI 1640 adjusted to pH 4.0 with 1 N HCl. The enzyme-MF mixtures or enzyme controls were then added to DCF in RPMI 1640 containing 10% FCS as a source of proteinase inhibitors. Proteinases alone had no effect on thymocyte survival whereas all five proteinases destroyed MF activity. The results of these studies are summarized in Table 5.

Functional properties

MF was tested for its ability to stimulate 3T3 fibroblasts to proliferate both in the presence and the absence of FCS. Proliferation of fibroblasts was expressed as the mean of quadruplicates of counts per minute of ³H thymidine uptake by the cells. As controls, fibroblasts alone and fibroblasts with whole macrophage supernatant were tested. The results shown in Table 6 indicate that while the whole supernatant has stimulating activity both in serum-containing and in serum-free conditions, MF has none under either condition.

Parameter	Monokine
Molecular Weight	Mr 36,000
Isoelectric Point	6.3 - 6.4
Receptor	Common part determinant on Ia
Heat Stability	Stable at 70°C Activity lost at 80°C
pH Stability	Stable between pH 2 and pH 10
Proteinases	Activity destroyed by papain, pepsin, pronase, thermo- lysin and trypsin
5% 2-Mercaptoethanol	Destroys activity
8M Urea	Destroys activity

Table 5. Physical characteristics of the monokine.

<u>Sample</u>	<u>Serum Free</u>	<u>10% FCS</u>
1. Control (fibroblasts alone)	1010 ± 99 cpm	1880 ± 315 cpm
2. Whole macrophage supernatant	5075 ± 434 cpm	4305 ± 1300 cpm
3. Partially purified macrophage supernatant (MF)	903 ± 125 cpm	1586 ± 588 cpm

Table 6. The effect of macrophage culture supernatants on fibroblast proliferation.

7.4 Discussion

The results of the present investigation describe an activity and a possible binding site for an Mr 36,000 monokine which is released by cultured peritoneal macrophages, two hours after stimulation with lipopolysaccharide. The factor has been shown to protect immature thymocytes from inevitable death in vitro. The loss of viability in vitro was confined to the dense, immature population and did not affect the buoyant fraction or the thymic epithelial cells alone. By titration it was shown that the described MF activity was increased at least five hundred times over that in control, unstimulated macrophage cultures. The cultured peritoneal cells were identified as macrophages by the criterion of adherent cells with 99.6% showing a positive reaction for lysozyme. This does not, however, exclude the possibility that trace contamination by other cell types could either account for the production of the factor, or modify its production by macrophages.

Removal of the Ia positive cells in the DCF left a population which displayed the described loss of viability over 4 hours. The addition of macrophage supernatant failed to prevent cell death as did the addition of supernatant from unstimulated thymic epithelial cells. The addition of supernatant from thymic epithelial cells which had previously been incubated with MF was protective, suggesting that a cascade was occurring in which macrophage products were acting on thymic epithelial cells resulting in the release of a factor responsible for the survival of thymocytes.

The monoclonal antibody to a common determinant on rat Ia (MRCOX6) was able to mimic the effect of the MF on thymocyte survival suggesting that Ia was the receptor for this monokine. The failure of MRCOX3 to induce protection demonstrated the site specificity of the binding of MF to the Ia molecule. The ability of Ia positive cells to effectively deplete the activity of the monokine provided more direct evidence for its binding at the Ia molecule on the cell surface and this was further substantiated by showing that MRCOX6 could completely block the absorption of MF onto spleen cells. Further confirmation of the binding site was provided by the finding that Fab OX6 blocked the protective effect of MF. From the data presented, it would appear that the Ia complex was the receptor for this regulatory cytokine and that the binding site was the same as or nearby to the site recognized by MRCOX6.

The results of the preliminary characterization of this macrophage factor are shown in Table 2 and 3. The protein nature of this product was established by its susceptibility to heating and to proteolytic attack. Some caution is needed in extrapolating a separate identity for this monokine because there have been few detailed studies on the characteristics of important monokines such as interleukin 1 and tumour necrosis factor in the rat (Lovett et al., 1983 and Schmitt et al., 1986). The failure to stimulate fibroblast proliferation in either the absence or presence of serum (Vilcek et al., 1986 and Rupp et al., 1986) does, however, suggest that the factor is neither of these monokines. This is supported by its apparent homogeneity with respect to both molecular weight and iso-electric point and

contrasts with both interleukin 1 and tumour necrosis factor for which a number of molecular forms have been described (Smith et al., 1986 and Wood et al., 1985). In addition, our data clearly point to a common region on Ia being the receptor for this monokine.

An Mr 35,000-40,000 macrophage-derived thymocyte maturation factor has been described in a murine model (Beller & Unanue, 1977). It expressed weak mitogenic activity but was not further characterized so that its relationship to the work presented here is unknown. De Luca and Mizel (De Luca & Mizel, 1986) have shown that interleukin 1 is able to replace, in part, the need for thymic epithelial cells in the development of T cells within the thymus. In the system suggested here, it is possible that the thymic epithelial cells may be producing interleukin 1 as the second part of the cascade mechanism. It is unlikely, however, on the basis of its physical and functional properties, that the first signal, that is the macrophage factor, is interleukin 1.

There has been considerable interest in the possible functions of Ia molecules in addition to their role in antigen presentation (Unanue & Allen, 1986 and Mason & Barclay, 1984). Palacios (1985) has postulated a transducer function for Ia based on the effect of anti-Ia monoclonal antibodies in mediating interleukin 1 secretion. The results presented here, point to a receptor function for Ia which, in addition, has been matched with a cytokine released from stimulated macrophages. It is well known that Ia glycoproteins are distributed throughout multiple cell types in foetal and mature animals. These include multiple lineages of the haemopoietic system, some histiocytic

macrophages, epithelial cells of the thymus and some areas of the gut, dendritic cells, B lymphocytes and activated T lymphocytes (Natali et al., 1981 and Fitchen et al., 1982). This distribution of Ia throughout the body would suggest that in addition to its role in immune responses there may be a broader role for this protein complex as a recognition system.

The functional significance of these findings for thymocyte maturation within the thymus is unknown. The absence of a blood-thymus barrier at the corticomedullary junction has been described together with the presence of macrophages and Ia positive thymic epithelial cells lying along the post-capillary venules (Raviola & Karnovsky, 1972). The macrophages are therefore in a position to be stimulated by incoming microbial antigens, to produce a factor which could then bind at the Ia molecule on epithelial cells, leading to the survival and possible maturation of thymocytes.

In mice, the foetal thymus is colonized by cells derived from foetal liver at about eleven days of gestation (Jenkinson, 1981) whereas the adolescent thymus relies on an intrathymic pool of self-renewing precursor cells to maintain its population and in order to achieve this, an intrathymic source of cytokines would appear to be necessary (Scollay et al., 1986). Epithelial cells have been proposed as an intrathymic source of activation signals for the differentiation of thymocytes as well as providing accessory cell signals for intrathymic T-cell responses to antigens (Denning et al., 1987)

Functional studies have demonstrated that immature cortical thymocytes are not capable of proliferating in response to interleukin 2 (Shimonkewitz et al., 1987). On this basis, the described release of factors by non-lymphoid thymic cells might represent an early stage in a mechanism whereby immature, non-proliferating lymphocytes are able to survive and differentiate to a point at which they are capable of responding.

CHAPTER EIGHT

THE AUGMENTATION OF MITOGEN-INDUCED THYMOCYTE PROLIFERATION BY BACTERIAL PRODUCTS IS MEDIATED BY AN Mr 36,000 MONOKINE

8.1 Introduction

An Mr 36,000 monokine which was released by rat peritoneal macrophages two hours after stimulation with lipopolysaccharide and which protects dense immature thymocytes from inevitable loss of viability in vitro was described, in Chapter 7. The cytokine was shown to exert its protective effect by binding at Ia on thymic epithelial cells and thereby stimulating these cells to release a protective factor for the thymocytes. The protective effect of the macrophage factor could be mimicked by a monoclonal antibody to a common determinant on Ia, but not by a monoclonal antibody to a strain specific epitope on Ia. In relation to this effect, Ia positive thymic epithelial cells are well known to play an essential role in thymocyte maturation via the release of a number of hormones (Bach & Papiernik, 1981). Activation of T lymphocytes and thymocytes, on the other hand, requires two signals, one being the monokine interleukin 1 (Unanue & Allen, 1987) and the other being a mitogen or an antigen presented in association with a histocompatibility protein. The murine thymocyte proliferation assay, first described by Gery et al., (1972) is accepted as a biological assay for measuring interleukin 1. In this system,

activity is usually measured by an enhancement in the proliferation of mitogen-stimulated cultures.

In the present investigation the thymocyte proliferation assay was used as a model for further study of the interactions of accessory cells and their products with thymocytes in the rat. The division of the thymus suspension into two fractions has revealed a mechanism whereby immature thymocytes in the presence of Ia-positive thymic epithelial cells and the products of stimulated macrophages can be induced to proliferate in response to a mitogen. A role for the Mr 36,000 monokine in this system is proposed.

8.2 Materials and Methods

Materials

Animals. Inbred Wistar-Furth rats were obtained from the Blackburn Animal House, University of Sydney. They were bred to produce the four to eight week old animals which were used for this study. New Zealand white male rabbits were obtained from Tillside rabbit stud, Yanderra, NSW. Both rats and rabbits were housed in cages which had wire mesh bottoms. The animals were allowed water and pellets ad libitum. The rats were killed using ether anaesthesia.

Antibodies. The IgG monoclonal antibodies, MRCOX6, directed against a common determinant on rat Ia (Williams et al., 1977), and MRCOX3, directed against a rat strain specific determinant on rat Ia (McMaster & Williams, 1979), were obtained from Sera

Labs. For indirect immunofluorescence studies, Wellcome fluorescein-labelled sheep anti-rabbit immunoglobulin was used.

Reagents. Purified rat lysozyme was a gift from Dr. E. Osserman, Columbia University, New York. Tissue culture media including foetal calf serum, penicillin, streptomycin and RPMI 1640 were obtained from Flow Laboratories. Dulbecco phosphate buffered saline (PBS) tablets were from Oxoid and pyrogen-free water was obtained from Travenol Laboratories. Freund's complete and incomplete adjuvant were from Difco. Naphthol AS-B1 phosphate and Fast red violet LB salt were purchased from Sigma. ^3H thymidine and Aquasol-II (Du Pont) were purchased from New England Nuclear. Mash II glass fibre filter pads (Whittaker M.A., Bio-Products) were from Commonwealth Serum Laboratories. Lipopolysaccharide (LPS) from Salmonella enteriditis and muramyl dipeptide were obtained from Sigma while cell wall fragments were obtained from group A variant streptococci and were prepared as described previously (Hunter et al., 1979). Concanavalin A (Jackbean haemagglutinin, lyophilized in NaCl) was purchased from Hoechst-Behring Diagnostics.

Separation techniques. Percoll and Sephacryl S200 were obtained from Pharmacia. Ultrafiltration membranes were from Amicon Ltd.

Plasticware. Lux 5350, 25 sq. cm. tissue culture flasks and Linbro 76-232-05 sterile flat bottom, 96 well plates were from Flow Laboratories. Scintillation vials were from Packard.

Methods

Cell preparation. RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum and 100U/ml penicillin and 100µg/ml streptomycin was used for experimental procedures.

Thymus cells. Thymus glands from 4-8 week old rats were sliced and pressed through a 0.5 mm pore size stainless steel wire mesh and the resulting suspension was used either whole or fractionated, in the proliferation assay. The thymus was split into a dense fraction containing the epithelial cells and immature thymocytes and a buoyant fraction with mature mitogen-responsive thymocytes, macrophages and dendritic cells (Gery & Waksman, 1972), on a Percoll colloidal silica density gradient according to the method of Salisbury et al., (1979). The various cell types were identified as described in the previous chapter and showed similar purity. Cells from whole thymus or from the dense or buoyant fractions were washed three times and then used in the thymocyte proliferation assay at a density of 1×10^7 cells/ml (1×10^6 cells/microwell).

Macrophages. The macrophages from which the culture supernatants were collected were prepared from the peritoneal washings of 6-8 week old Wistar-Furth rats. Cells were seeded into tissue culture flasks at 1×10^6 cells/ml (5×10^6 cells/flask) and allowed to adhere for one hour at 37° C in 5% CO₂ in air. Monolayers were washed five times, serum-free medium was added and the cells were stimulated with 20µg/ml of lipopolysaccharide. The resulting culture supernatant was

harvested at two hours and aliquots were treated in one of three ways, as outlined below.

Macrophage culture supernatants. In preliminary experiments and subsequently as a control, the peritoneal macrophage supernatant was used directly from the culture. Secondly, the supernatant was partially purified to leave an Mr 36,000 fraction, selected on the basis of its ability to maintain the viability of dense immature thymocytes as described in the previous chapter and which did not possess interleukin 1 activity as determined by a fibroblast proliferation assay. In brief, the supernatant was filtered through an XM300 membrane filter to remove high molecular weight components, then dialysed against PBS prior to concentrating over a PM10 membrane filter. The resulting supernatant was further fractionated over a Sephacryl S200 column calibrated with standard proteins (Combithek, Boehringer Mannheim). The Mr 36,000 fraction retained after this procedure was used in the thymocyte proliferation assay. The third treatment employed was to incubate the macrophage supernatant with 10mM phenylglyoxal on the basis of its inhibiting the activity of interleukin 1 (Klamfeldt, 1985). The incubated supernatant was dialysed against PBS prior to its use in the thymocyte proliferation assay.

Thymocyte proliferation assay. Thymocyte suspensions were cultured in sterile 96 well flat bottomed microtitre plates in a total volume of 200 μ l. Cultures were incubated at 37° C in 5% CO₂ for forty eight hours after which they were pulsed with 0.5 μ Ci ³H thymidine per well. The cells were harvested twelve

hours after the addition of radioactive label, using a Mash II cell harvester, onto Mash II glass fibre filter pads. The radioactivity was measured in a liquid scintillation counter (Beckman) with a toluene-based scintillant and was expressed as counts per minute of thymidine uptake. In all thymocyte proliferation studies, test samples and controls were assayed in quadruplicate. In assays using a thymic cell population depleted of macrophages, 5 ml aliquots of thymus suspension were placed into tissue culture flasks at 37°C for one hour, after which the non-adherent cells were recovered for use in the thymocyte proliferation assay.

Preparation of antisera. Rabbits were immunized with rat lysozyme, and the antisera were characterized as described previously.

Indirect immunofluorescence tests. For demonstration of lysozyme in macrophages, cells were fixed for one minute in cold methanol, washed in PBS and then incubated with 1/10 anti-rat lysozyme anti-serum followed by a 1/10 dilution of the second stage antibody.

Acid phosphatase activity was determined by fixing the cells for one minute in cold formol-acetone prior to incubating with naphthol AS-B1 phosphate and Fast red violet LB salt in 0.1 M acetate buffer pH 5.2.

8.3 Results

Augmentation of thymocyte proliferation by bacterial products.

Whole thymus suspensions were used to test the effects of bacterial products on thymocyte proliferation in the rat. The results shown in Figure 10 indicate that 0.5 μg of LPS added to 1×10^6 cells increased thymidine uptake over a range of concentrations of Con A but did not change the shape of the profile. This effect was reproduced over ten experiments so that for 0.5 μg Con A, the ratios of the means was 2.3 ± 0.87 ($p < 0.01$). Similar increases were obtained by the addition of 0.5 $\mu\text{g}/10^6$ thymus cells of streptococcal cell wall fragments (two experiments) or 1 $\mu\text{g}/10^6$ cells of muramyl dipeptide (one experiment).

The effect of varying concentrations of microbial products on the response to 0.25 $\mu\text{g}/\text{ml}$ Con A was examined. A representative experiment for LPS is shown in Figure 11. One picogram/well of LPS, the smallest concentration tested, produced a sharp increase in thymidine uptake over control with gradual further increases up to 1mg LPS/well. This effect was reproduced over three experiments. Similar increases were produced by muramyl dipeptide (two experiments) and particulate streptococcal cell wall (two experiments) where again picogram levels were effective stimulants.

The effect of depletion of adherent cells.

The depletion of adherent cells from whole thymus suspensions, as described in Methods, resulted in a decrease in thymocyte proliferation in response to varying levels of Con A and 0.5 μg of LPS compared to a control population. This was confirmed over 6 experiments such that the mean of the ratios of the control

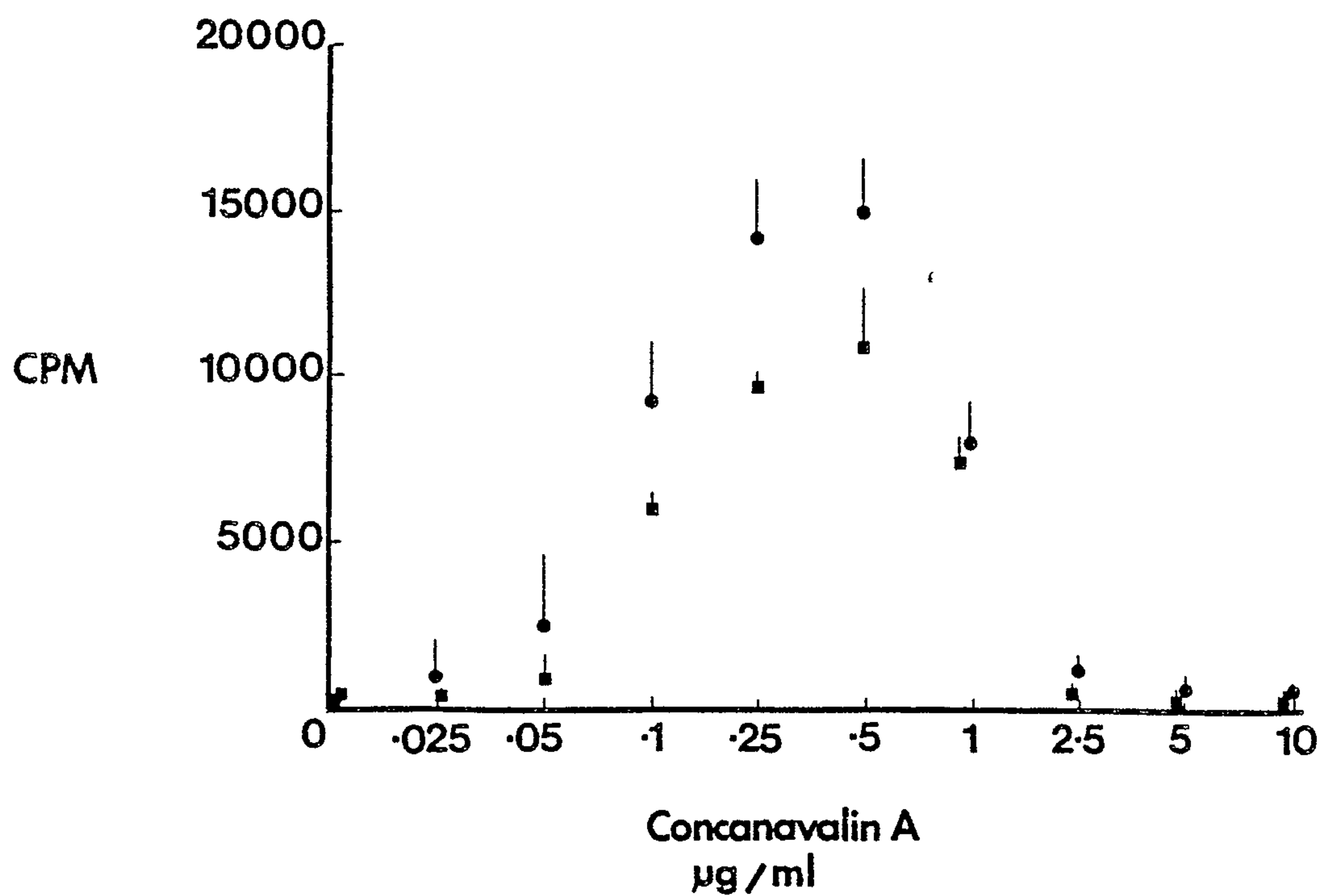


Figure 10. The synergistic effect of lipopolysaccharide and concanavalin A on rat thymocyte proliferation.

Cells were cultured at 1×10^6 cells/ 200µl/ well in the presence of 0.5 µg of LPS and increasing concentrations of Con A. Representative experiment of 10 runs showing the mean value $\pm$ the standard deviations for quadruplicate cultures. Results expressed as cpm of ^3H thymidine uptake. ■ Con A alone, ● Con A + LPS

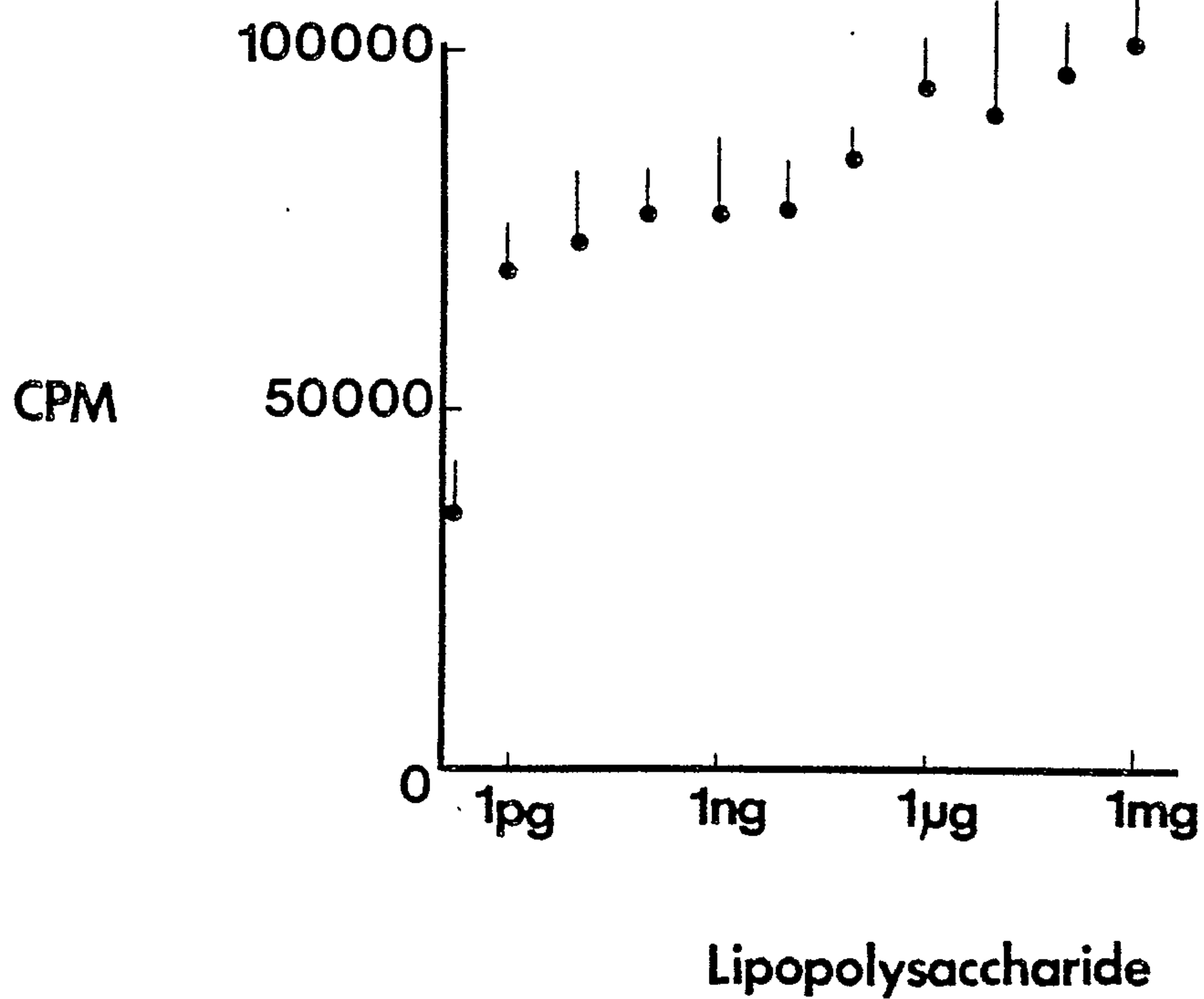


Figure 11. Thymocyte proliferation in response to increasing levels of lipopolysaccharide. The effect of varying levels of LPS on thymocyte proliferation at a constant suboptimal level (0.25 $\mu\text{g}/\text{ml}$.) of Con A are shown. Representative experiment of 3 experiments showing the mean $\pm$ standard deviations for quadruplicate cultures. Results expressed as cpm of ^3H thymidine uptake.

population to the depleted population was 2.27 ± 0.6 . The addition of 25 μ l/well of whole macrophage supernatant (prepared from 1×10^6 cells stimulated with 20 μ g/ml LPS and harvested at 2 hours) restored the response to that of the control. The purity of the macrophage cultures was confirmed by staining for lysozyme and acid phosphatase. Over 6 experiments, the mean of the ratio of the control response to the restored response was 1 ± 0.07 .

The restoration of the response with macrophage supernatant did not appear to be due to interleukin 1 as shown in Figure 12. Supernatant treated with phenylglyoxal to remove interleukin 1 activity was still capable of restoring the response to control levels. Similarly, the response was restored by the addition of macrophage supernatant, which had been partially purified (resulting in an Mr 36,000 factor from chromatography on Sephacryl S200) at a concentration which was active in the thymocyte viability assay.

Characterization of the thymocyte response.

Fractionation of the thymus cell suspension on a density gradient of Percoll confirmed that the buoyant fraction which contained mature thymocytes, macrophages, epithelial cells and non-epithelial dendritic cells, proliferated in response to Con A and 0.5 μ g of LPS. The dense fraction containing immature thymocytes and dense epithelial cells responded only weakly to Con A and LPS. Recombination of the two fractions restored the response to a level equivalent to that of whole thymus showing that no cells had been lost in the procedure. Figure 13 shows the

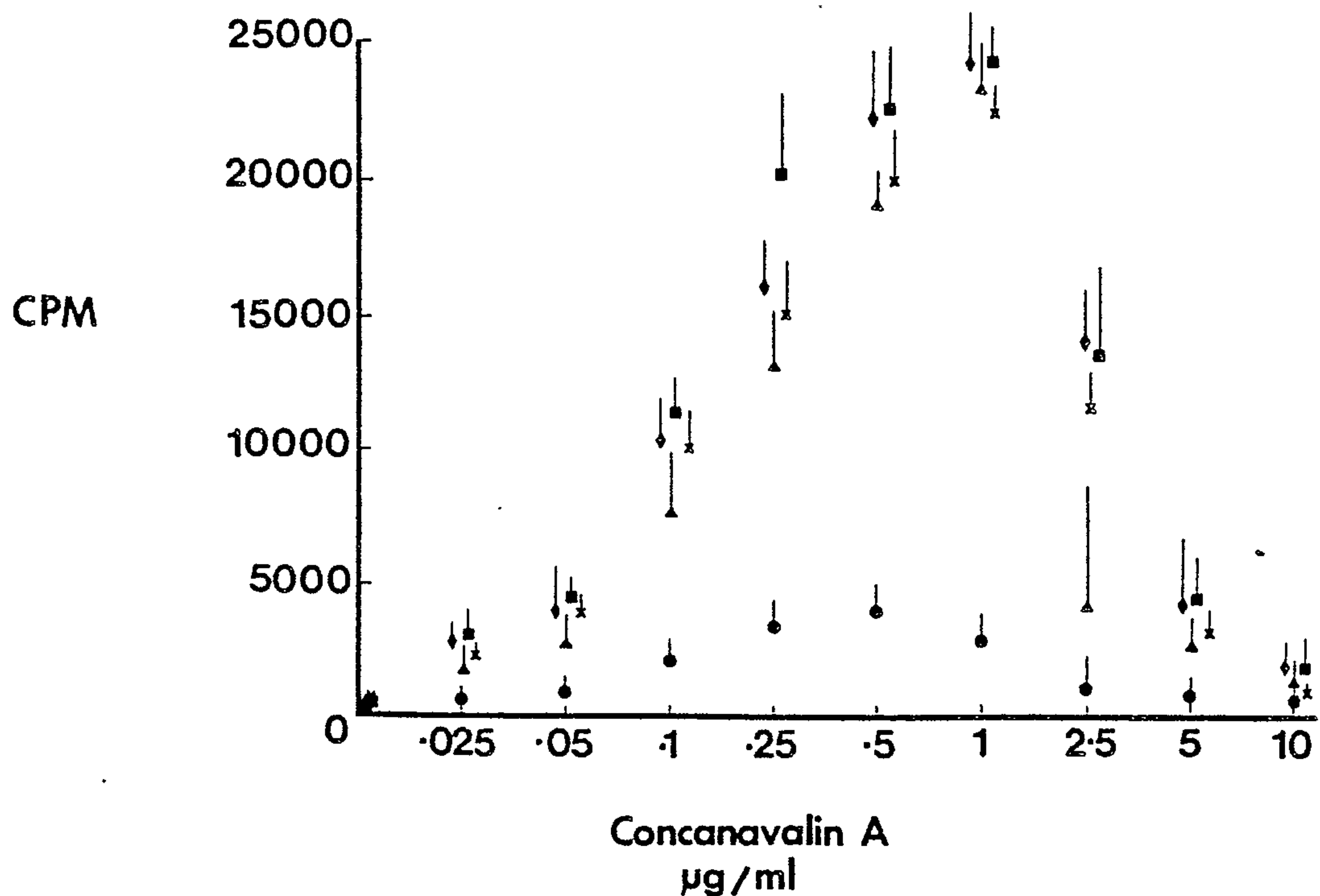


Figure 12. The effect of purified macrophage factor on thymocyte proliferation. Addition of macrophage supernatants which had been treated in one of three different ways restores the proliferative response of the adherent cell depleted population to that of a control population. Representative experiment of 3 experiments showing the mean $\pm$ standard deviations for quadruplicate cultures. Results expressed as cpm of ^3H thymidine uptake.

● adherent cell depleted population, ▲ a control, non-depleted population,
 ■ adherent cell depleted with whole macrophage supernatant, ◆ adherent cell
 depleted with whole macrophage supernatant treated with phenylglyoxal,
 x adherent cell depleted with partially purified macrophage factor.

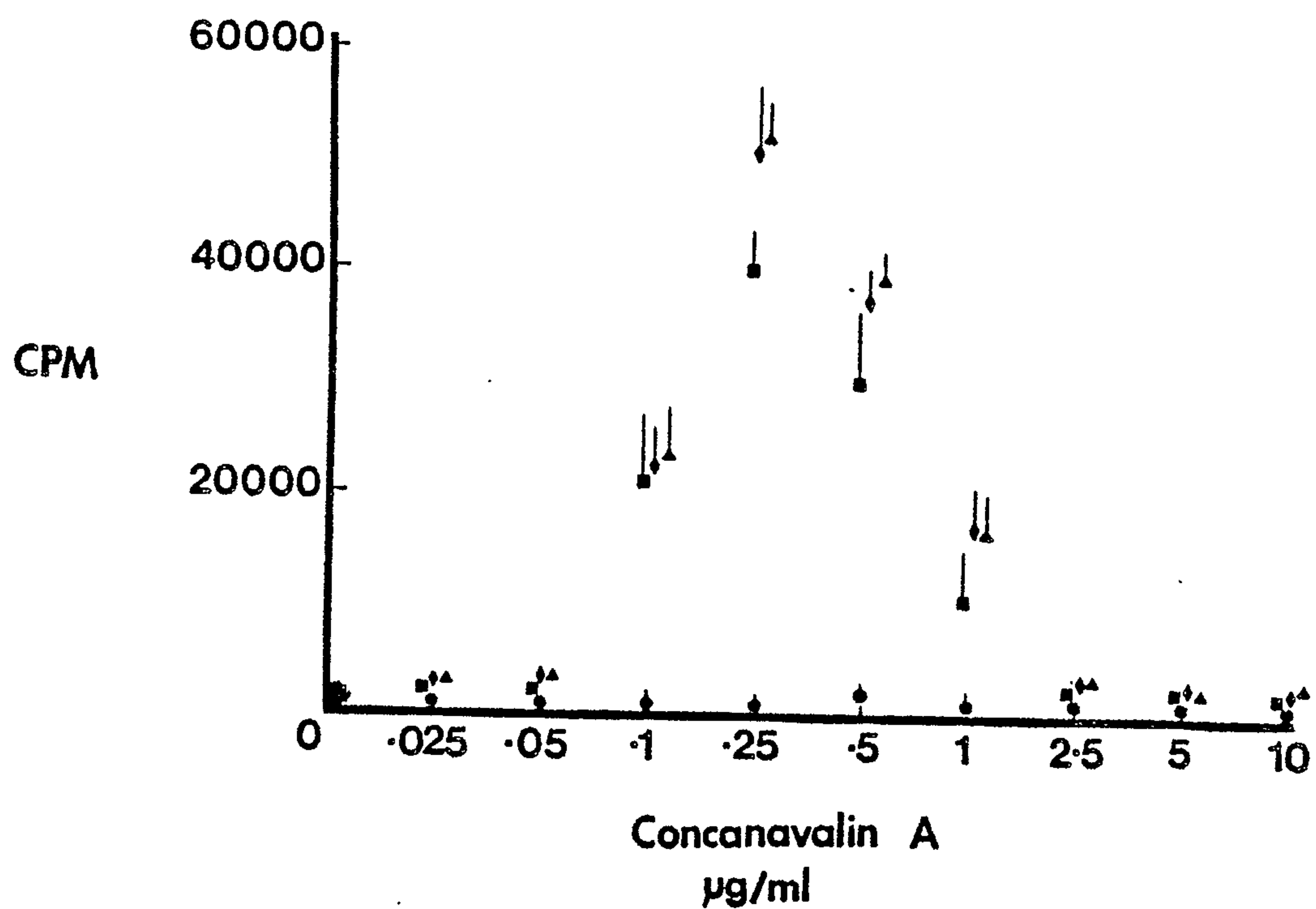


Figure 13. Characteristics of the thymocyte response. The response of divided, recombined and whole thymus to varying levels of Con A in the presence of 0.5 µg of LPS is shown. Representative experiment of 5 experiments showing the mean $\pm$ standard deviations for quadruplicate cultures. Results expressed as cpm of ^3H thymidine uptake. $\blacklozenge$ whole thymus, $\blacksquare$ buoyant fraction, $\bullet$ dense cell fraction, $\blacktriangle$ recombined fractions.

response of the buoyant, dense, recombined and whole fractions to Con A and LPS.

Enhancement of the proliferative response of immature thymocytes by a non-fractionated macrophage supernatant.

The dense thymic fraction was incubated at 1×10^6 cells/well with 2.5 μ g of MRCOX6 or MRCOX3. The proliferative response to Con A was measured. While MRCOX3 had only a slight effect, there was a substantial increase in proliferation with both MRCOX6 and two hour macrophage supernatant and an additive effect when the two treatments were combined as shown in Figure 14. This trend was reproduced over 8 experiments.

The effect of partially purified macrophage supernatant on dense immature thymocytes.

Incubation of the dense thymic cell fraction with partially purified macrophage supernatant (Mr 36,000 fraction from chromatography on Sephacryl S200) resulted in an increase in the proliferative response to Con A. This fraction was used at a concentration which demonstrated protective activity in the thymocyte viability assay and 3 different preparations were tested. All preparations showed a similar increase in proliferation as shown in Figure 15 and the effect was reproduced over 5 experiments.

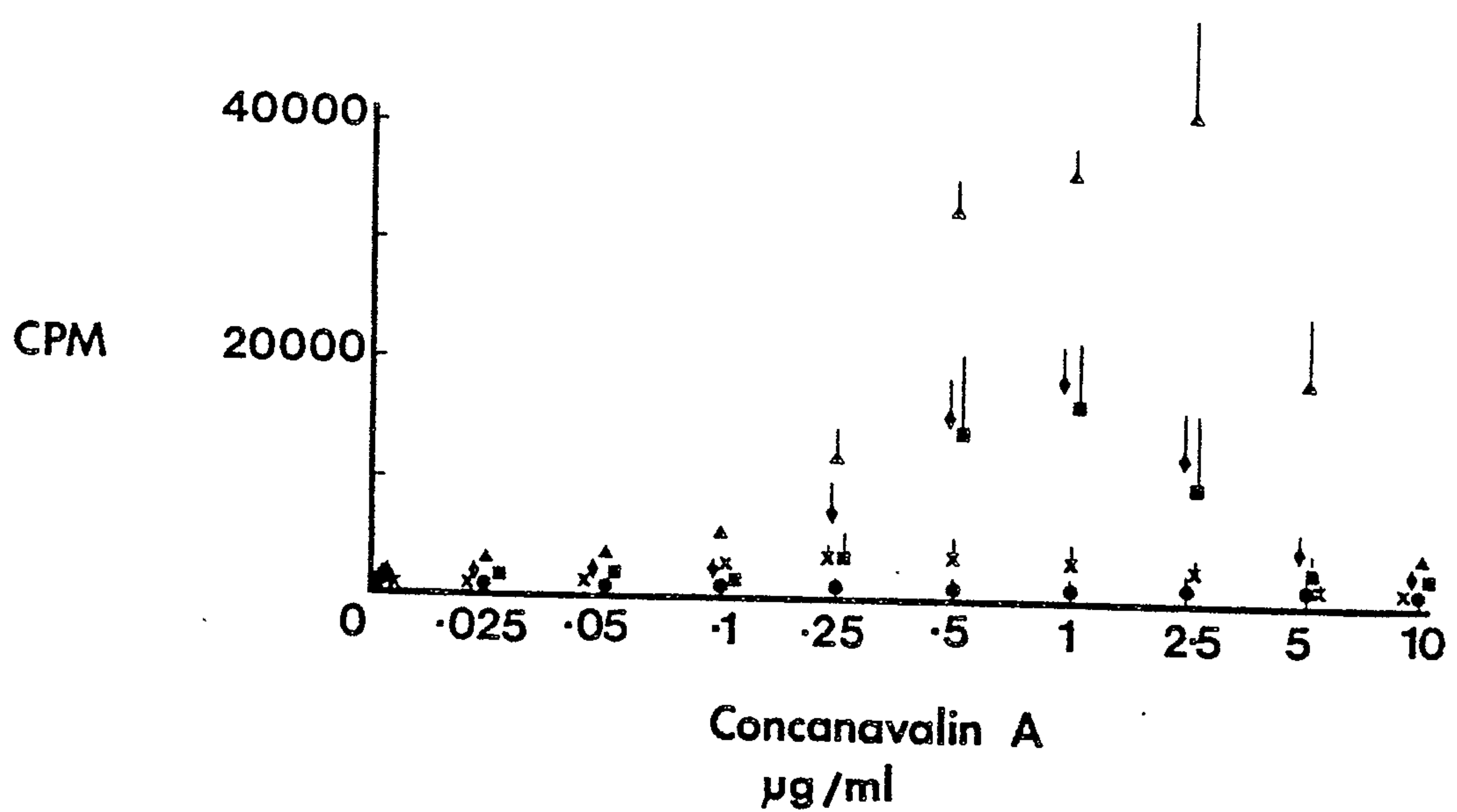


Figure 14. Enhanced proliferation of immature thymocytes in response to whole macrophage supernatant or an antibody to Ia. Dense immature thymocytes proliferating in response to either whole macrophage supernatant or an antibody to rat Ia (2.5 µg/ml of clone MRCOX6 or MRCOX3). Representative experiment of 8 experiments showing the mean $\pm$ standard deviations for quadruplicate cultures. Results expressed as cpm of ^3H thymidine uptake. ● dense cell fraction alone, ■ dense cell fraction + whole macrophage supernatant, ◆ dense cell fraction + antibody to Ia (MRCOX6), ▲ dense cell fraction + whole macrophage supernatant + antibody to Ia (MRCOX6) x dense cell fraction + antibody to Ia (MRCOX3)

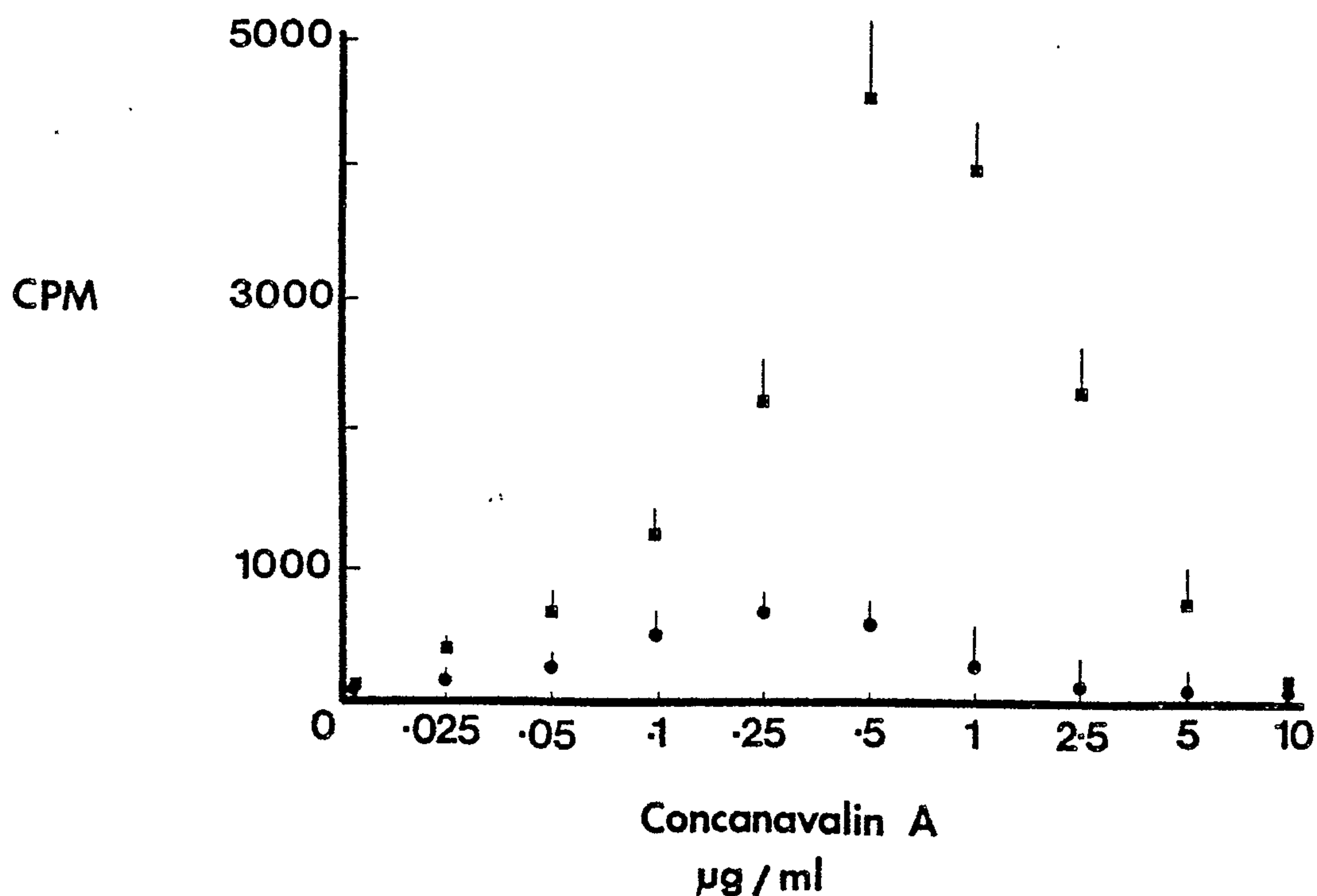


Figure 15. Enhanced proliferation of immature thymocytes in response to partially purified macrophage supernatant. The addition of partially purified macrophage supernatant to dense immature thymocytes results in an increase in proliferation over that of a control population. Pooled data for three preparations of purified Mr 36,000 monokine showing mean $\pm$ SEM Results expressed as cpm of ^3H thymidine uptake. ● control dense cell population, ■ immature dense thymocytes with partially purified macrophage supernatant (3 different preparations with equivalent activity in the thymocyte proliferation assay were used).

8.4 Discussion

The results of the present study indicate that macrophage products are responsible for the increased proliferative response of thymocytes to co-stimulation with Con A and microbial products. This data supports the efficient uptake of picogram quantities of a variety of bacterial components by macrophages which were determined to comprise less than 0.1% of the thymic cell suspension. The evidence for a role for macrophages in the augmentation of the proliferative response was provided by a diminished response following depletion of adherent cells and by the restoration of the activity by products from highly purified peritoneal macrophage cultures. An active macrophage component was shown to be the Mr 36,000 protein which did not have interleukin 1 activity as determined by a fibroblast proliferation assay.

The dense cell fraction which was shown previously to contain a mean of 87.2% thymocytes and 12.8% thymic epithelial cells did not respond to LPS in the presence of Con A. This fraction could, however, be induced to proliferate in the presence of the Mr 36,000 monokine and therefore contributed to the augmentation of proliferation of whole thymus in the presence of LPS. It was previously established that the Mr 36,000 monokine acts on dense thymic epithelial cells causing them to release products which maintain thymocyte viability. It has been demonstrated recently that human thymic epithelial cells are capable of providing an intrathymic source of interleukin 1 (Le et al., 1986)

which affects thymocyte proliferation. These authors also proposed that interleukin 1 produced by thymic epithelial cells might also have an important role in thymocyte maturation. In this context, the augmentation of the proliferative response of whole thymus to Con A which occurs in the presence of microbial products could be due to macrophages being stimulated to release the early Mr 36,000 monokine rather than interleukin 1 itself. In support of this, incubation of the monokine with phenylglyoxal, an arginine specific reagent which has been shown to destroy the activity of interleukin 1 (Klamfeldt, 1985 and Krakauer, 1985), failed to remove the stimulatory effect. This monokine would then act on the thymic epithelial cells stimulating them to release a number of factors which have the particular function of increasing the survival and maturation of immature, cortical thymocytes, leading to the participation of these cells in the proliferative response to Con A. One of the secretory products of the thymic epithelial cells could be interleukin 1 which could act at the level of thymocyte maturation as well as being a co-stimulant in proliferation. The potential for signal amplification in this cascade was illustrated in the previous chapter where it was calculated that the Mr 36,000 monokine from one peritoneal macrophage promoted the survival of 10^5 thymocytes.

Nitta et al., (1985) proposed two mechanisms for the enhanced DNA synthesis by mature thymocytes after co-stimulation with Con A and LPS, both of which required the presence of Ia-positive accessory cells. The first was based on interleukin 1 dependent interleukin 2 production where accessory cells

stimulated with LPS produced interleukin 1 which augmented the production of interleukin 2 by thymocytes stimulated with Con A. In the present experiments the augmentation of the proliferative response of whole thymus to Con A in the presence of LPS could be explained by this mechanism. However, the monokine did not appear to be interleukin 1. In the second mechanism, Nitta et al. proposed that LPS or other mitogens acted directly on thymocytes stimulated with Con A to enhance the production of interleukin 2. The latter proposal was demonstrated using C3H/HeJ mouse thymic cells which are non-responsive to LPS and which also failed to produce any detectable interleukin 1 activity. In support of this direct action of LPS on thymocytes, Le et al., (1987) have shown that this bacterial product can induce gamma-interferon production by human peripheral T lymphocytes in the presence of accessory cells. This could be inhibited by antibody specific for the class II histocompatibility antigen, HLADR indicating that this complex was essential in the process. The studies were inconclusive in determining whether interleukin 1 was fully responsible for the observed effects since interleukin 1 alone failed to induce detectable levels of gamma-interferon.

It has also been reported (Durum & Gershon, 1982 and Gilman et al., 1983) that some monoclonal antibodies specific for Ia can inhibit the production of interleukin 1 by accessory cells. The mechanism whereby these antibodies exert their effect is unknown. Gilman et al., used the monoclonal antibody OX4 which has the same specificity as the OX6 used in the present study. In contrast to Gilman's data, in the present experiments,

OX6 enhanced thymocyte proliferation when added to the dense cell fraction and this mimicked the proliferation seen in the presence of the Mr 36,000 factor from macrophages. The OX3 clone which did not protect thymocytes in the previous study, produced only a slight increase in the proliferative response. The additive effect of the antibody and macrophage factor was not examined in any detail in the present study but could be explained in one of two ways. It could simply be a dose additive effect leading to a doubling of the response or alternatively, the antibody and the macrophage factor could act on Ia positive thymic epithelial cells leading to the production of different secretion products. In the previous chapter it was reported that the Ia antibody, OX6 mimicks the protective effect of the Mr 36,000 monokine on thymocytes. Evidence was obtained that the monokine bound at Ia at a site close to that recognized by OX6. It is becoming increasingly well recognized that Ia molecules are involved in more than the cell-cell interaction of antigen presentation to T cells. In this context, a signal transducer function for Ia has been proposed (Palacios, 1985 and Cambier & Ransom, 1987).

There is a basis for extrapolating the findings of the present study to thymocyte maturation in situ. The studies of Raviola & Karnovsky (1972) in particular, have revealed the absence of a blood-thymus barrier in the post-capillary venules of the cortico-medullary junction. These vessels were shown to be sinusoidal in nature with macrophages and epithelial cells having access to the vascular contents. It seems plausible that at this site microbial products could trigger the cascade of reactions

detailed in the present study. This could lead to an increased survival and maturation of cortical thymocytes. An extension of this is a mechanism whereby trace amounts of bacterial products have an important role in the seeding of lymphoid organs with T lymphocytes. Evidence in support of this concept is provided from studies on germ-free animals where weak and delayed immune responses have been noted (Schwab, 1977). Of particular relevance is the work relating to cell mediated immunity. Ueda et al. (1975) reported a delay in the development of skin test reactivity to purified protein derivative in germ free mice following sensitization in Freund's complete adjuvant. Adoptive transfer experiments and in vitro studies of lymphokine production established that the defect occurred in the induction rather than the effector phase of immunity. In addition, spleen cells from germ free rats had a decreased response to polyclonal T cell mitogens when compared with their conventionally reared cohorts (Wells et al., 1979). Macdonald et al., (1979) did not detect any abnormality in the delayed hypersensitivity response in germ free mice following sensitization in Freund's complete adjuvant but they did note a reduced skin test response to intravenously administered sheep red blood cells. This could be explained by the transient nature of this form of cell mediated response (Lagrange et al., 1979) and therefore the requirement for a substantial population of responding cells. Overall, the findings in germ free animals are compatible with a limited seeding of the peripheral tissues with immunocompetent T cells. Persistent antigenic stimulation, as occurs in sensitization with Freund's adjuvant, would allow clonal proliferation to reach a stage where the expected level of

response occurs. While there is substantial evidence for the presence of immunocompetent T cells in germ-free animals, this could be explained by the low level of antigenic stimulation from bacterial products present in the food of these animals. This source of stimulation could also account for the varying levels of impairment of immune reactivity which have been reported.

Finally, there is evidence that the mono-association of particular bacterial species with germ free animals can restore immune reactivity to normal levels. Salmonella species (Macdonald et al., 1979) and Escherichia coli (Ueda et al., 1975) were effective in this regard whereas Bacteroides fragilis and Propionibacterium acnes (Wells et al., 1979) were not. This could be related to the potency of the lipopolysaccharides of the former species in stimulating the secretion of monokines (Newton, 1986).

CHAPTER NINE

CHARACTERISTICS OF THE PRODUCTION OF THE MONOKINE

9.1 Introduction

An Mr 36,000 monokine which is released in two hours by stimulated rat peritoneal macrophages was described in Chapter 7. Its effect is to protect dense immature thymocytes from inevitable loss of viability, in vitro by binding at Ia on thymic epithelial cells and further stimulating these cells to release a protective factor for the thymocytes. Mononuclear phagocytes can be stimulated to secrete an extensive and diverse range of products including interleukin 1 (Dinarello, 1984), colony stimulating factor (Galleli & Chedid, 1986), tumour necrosis factor (Carswell et al., 1975), prostaglandins (Humes et al., 1982) and neutrophil chemotactic factor (Snella, 1986). While it is recognized that some of these products interact and can be induced by other macrophage products (Philip & Epstein, 1986 and Broudy et al., 1986), a cohesive framework for their interaction and function within the immune system has yet to be established. In view of the fact that macrophages are capable of producing so many active molecules, the described effects of unfractionated supernatants are often the sum of many effects of interacting substances. There is evidence that different monokines have multiple and often overlapping activities (Le & Vilcek, 1987). In addition, they have a propensity to affect the activities of one

another (Libby et al., 1986), and these factors combined, complicate the study of monokine production.

Against this background, the aim of the present investigations was to provide more definitive data on the production of the described monokine. In relation to this, the time course for its production together with the effect of different stimuli at various doses was examined. In addition, the nature of the production of the macrophage factor was studied using three different protein synthesis inhibitors and trypsin treated and untreated lysed macrophage preparations. The effect of restimulation of cultures was examined in an attempt to increase the yield of the product. This is important in relation to the purification and further characterization of the monokine.

9.2 Materials and Methods

Materials

Animals: Inbred Wistar-Furth rats were obtained from the Blackburn Animal House, University of Sydney. They were bred to produce the four to eight week old animals which were used for this study. The rats were housed in cages which had wire mesh bottoms and were allowed water and commercial pellets ad libitum. Rats were killed using prolonged ether anaesthesia.

Reagents: Tissue culture media including RPMI 1640, foetal calf serum, penicillin and streptomycin and sodium bicarbonate were obtained from Flow Laboratories. Lipopolysaccharide from Salmonella enteriditis (LPS) and muramyl dipeptide (MDP),

trypsin (from bovine pancreas), prostaglandin E₂ and Trypan blue powder were all purchased from Sigma. Protein synthesis inhibitors, actinomycin D, cycloheximide and puromycin, as well as indomethacin, were also from Sigma. Dulbecco phosphate buffered saline tablets were from Oxoid and pyrogen-free water was obtained from Travenol Laboratories.

Plasticware: Lux 5350, 25 sq. cm tissue culture flasks were from Flow Laboratories.

Separation techniques: Percoll was purchased from Pharmacia and ultra filtration membranes were from Amicon Ltd.

Methods

Cell Preparation Procedures: RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum (FCS) and 100 U/ml penicillin and 100 µg/ml streptomycin was used for all cell preparation.

Thymus Cells: Thymus glands were dissected from four to eight week old Wistar-Furth rats. Thymuses were sliced and passed through a 0.5 mm pore size stainless steel wire mesh and the resulting suspension was fractionated on a colloidal silica density gradient (Salisbury et al., 1979). The dense fraction was taken, washed three times and used in the thymocyte viability assay at a density of 1×10^7 cells/ml. The thymocytes and epithelial cells in this fraction were identified using monoclonal antibodies as described in a previous chapter.

Macrophages: The macrophages from which the culture supernatants were prepared came from the peritoneal washings

of six to eight week old Wistar-Furth rats. Cells were collected in RPMI 1640 medium supplemented with 10% FCS and were seeded into tissue culture flasks at a density of 1×10^6 cells/ml (5×10^6 cells/flask). The cells were allowed to adhere for one hour at 37°C in 5% CO_2 in air, prior to washing five times with Dulbecco's phosphate buffered saline (PBS). The cells were then stimulated with either lipopolysaccharide (1-20 $\mu\text{g/ml}$) or muramyl dipeptide (0.5-2.5 $\mu\text{g/ml}$) and supernatants were collected at various time intervals between five minutes and forty-eight hours according to the experiment. In the case of the unstimulated macrophage supernatant, an identical procedure was followed without the addition of the stimulant and the supernatant was collected two hours after the replacement with serum free medium.

Lysed Macrophage Preparation: Macrophages were seeded into tissue culture flasks as described and were allowed to adhere. Following adherence, the cells were removed using a rubber policeman and the resulting suspension was frozen and thawed three times to cause lysis of the cells. The suspension was centrifuged to remove cellular particles and the supernatant was used in the viability assay.

Trypsin-Treated Lysed Macrophages: In order to remove any surface proteins from macrophages, cell cultures were incubated for thirty minutes at 37°C in the presence of 1 mg/ml of trypsin. The enzyme activity was arrested by the addition of 10% FCS, the cells were washed and then frozen and thawed as described previously. The resulting suspension was spun and the supernatant was taken.

Supernatants Prepared in the Presence of Protein Synthesis Inhibitors: Macrophage supernatants produced in the presence of inhibitors of protein synthesis were prepared as follows; The macrophages were allowed to adhere and were then washed to remove any traces of serum. The protein synthesis inhibitors were added at the same time as the LPS (20 µg/ml) in the following concentrations: actinomycin D - 1 µg/ml, (Craig & Kostura ,1983); puromycin - 100 µg/ml (Hand,1975); cycloheximide - 10 µg/ml (Rahmsdorf et al ,1986).

Supernatants from Restimulated Cultures: Supernatants were taken after two hours from cultures stimulated with 20 µg/ml LPS. At this time , the cultures were restimulated with 20 µg/ml LPS alone or in combination with 10^{-5} M indomethacin. The supernatants were collected at four hours and the cultures were restimulated. This procedure was repeated at six hours and the final supernatants were collected at eight hours.

Thymocyte Viability Assay: Dense thymocyte suspensions were divided into 1 ml aliquots containing 1×10^7 cells. Test supernatants were added as 50 µl samples to these cells and viability was assessed at hourly intervals by the exclusion of Trypan blue (0.4% solution). Viability was expressed as a percentage of the original cell number.

9.3 Results

The Effect of Two Stimulants and Time of Supernatant Collection

The titre of the activity was defined as the reciprocal of the maximum dilution of unfractionated macrophage supernatant which gave complete protection at four hours in the thymocyte viability assay. This applied to 50 μ l of supernatant added to 1×10^7 cells in a total volume of 1 ml. Varying concentrations of LPS and MDP were added to freshly established peritoneal macrophage cultures and the supernatant was collected after two, eight or twenty-four hours incubation and tested for activity in the thymocyte viability assay. The results are shown in Figure 16. For LPS, there was a proportionate increase in titre from 1 to 10 μ g LPS and then an abrupt increase from ten units at 10 μ g LPS to 500 units at 20 μ g LPS. There was no demonstrable activity at eight or at twenty-four hours after the addition of LPS. The response to MDP was quite different in both the titre achieved and in the persistence of the response so that for 2.5 μ g MDP although the titre was only one unit it persisted for at least twenty-four hours. Controls were set up to test the effect of the stimulants alone or in combination with the partially purified macrophage factor (see Appendix). Neither stimulant protected the thymocytes or affected the activity of the macrophage factor.

Time Course for the Release of the Monokine

The time course for the release of the macrophage factor was examined using 20 μ g/ml LPS as the stimulant. As shown in

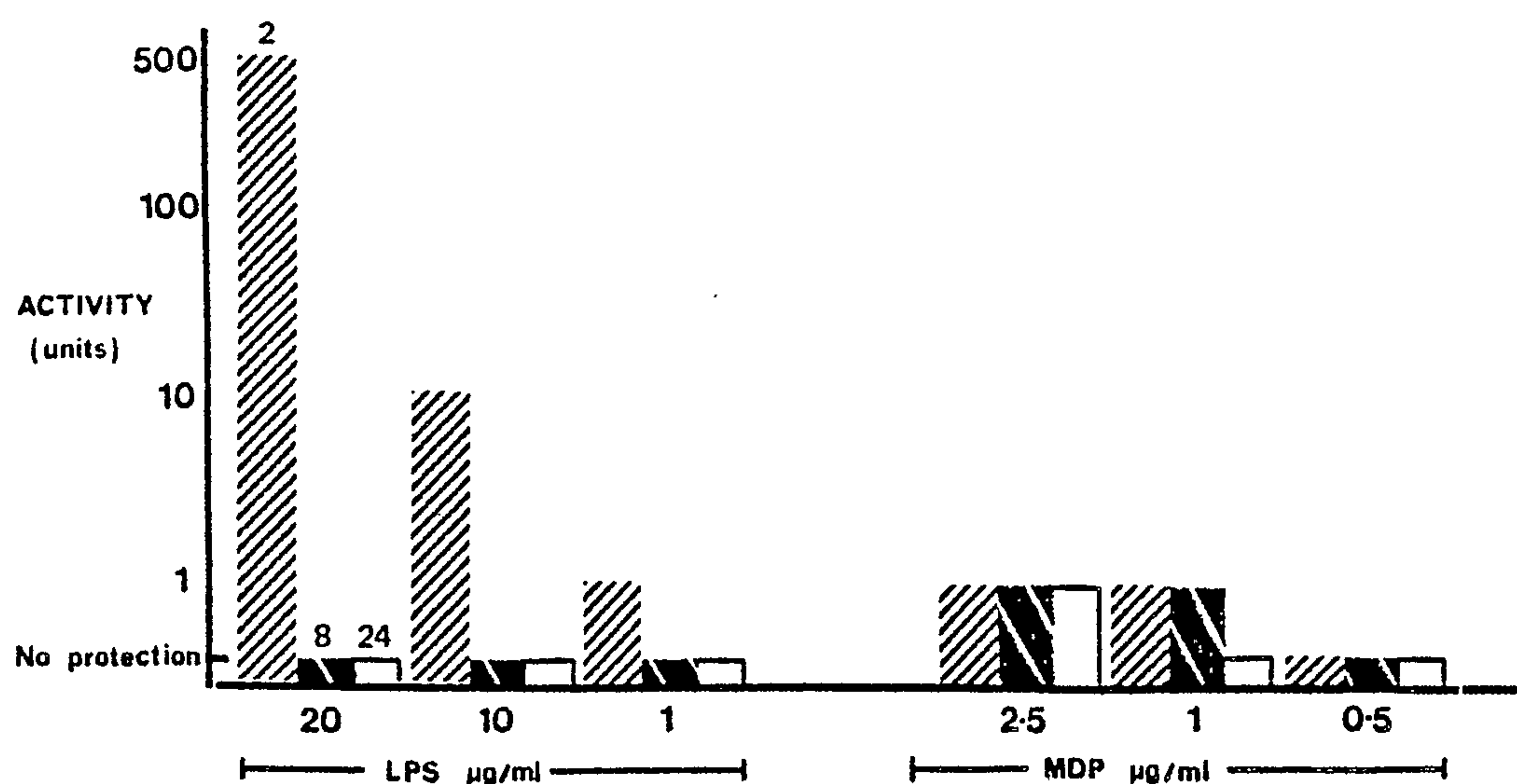


Figure 16. Production of the macrophage factor.

The production of the macrophage factor in response to varying levels of two different stimuli is shown. Production was determined by the titre resulting in 100% protection in the thymocyte viability assay. No protection refers to anything less than 100% protection. The supernatant was collected at three different times as shown;  2 hours,  8 hours,  24 hours. Results are the mean of four experiments.

Figure 17, low level activity was released from the macrophages within five minutes after the addition of the stimulant. After one hour the activity had increased ten-fold with the peak response of 500 units being reached at two hours. The activity decreased to 100 units at three hours and from four hours to forty-eight hours there was no demonstrable activity.

The Effect of Three Protein Synthesis Inhibitors

Inhibitors of protein synthesis were added to macrophage cultures at the same time as 20 μ g LPS and the supernatants were collected at time intervals up to two hours. They were dialysed to remove low molecular weight inhibitors and then tested for protective activity in the thymocyte viability assay. The results displayed in Figure 18 show that in the presence of each of the three protein synthesis inhibitors, protective activity was demonstrable in the culture supernatants at five and ten minutes after LPS challenge. By fifteen minutes, however, activity had been lost in the puromycin and cycloheximide treated cultures whereas it was still present in the cultures which had been incubated with actinomycin D. From thirty minutes to two hours, activity was lost in all of the treated cultures.

Distribution of the Factor

The rapid release of low titre macrophage factor from cells stimulated with LPS, together with the evidence for the existence of mRNA for this product, as demonstrated by the effects of actinomycin D, suggested that the factor might be present in normal cells. The distribution of the factor was

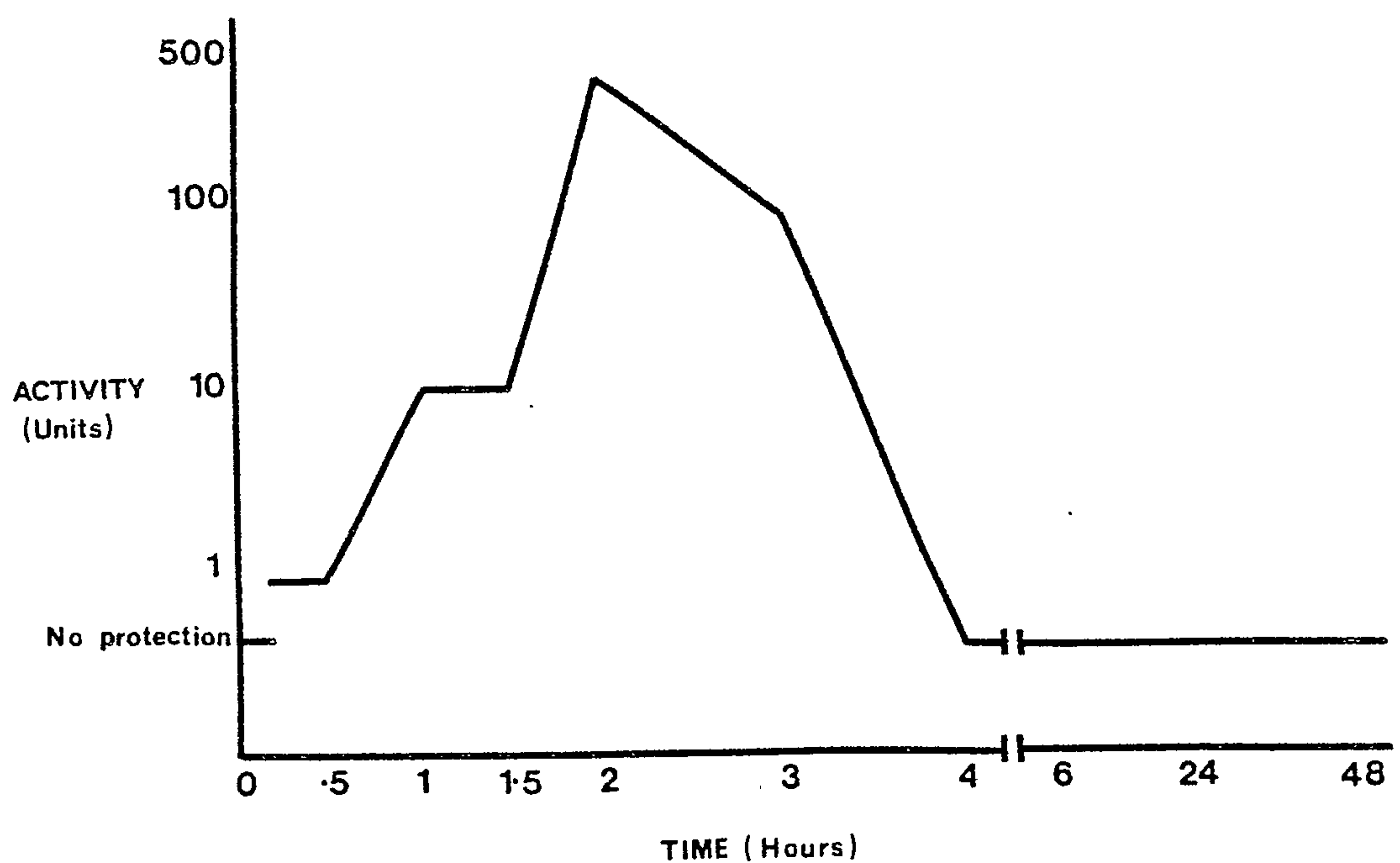


Figure 17. Time course for the production of the macrophage factor. Supernatants from macrophages stimulated with 20 $\mu\text{g/ml}$ LPS were collected at various time intervals after stimulation as shown. Production was expressed as the titre of macrophage factor resulting in 100% protection in the thymocyte viability assay. Any protection less than total was referred to as no protection. Representative experiment of two experiments.

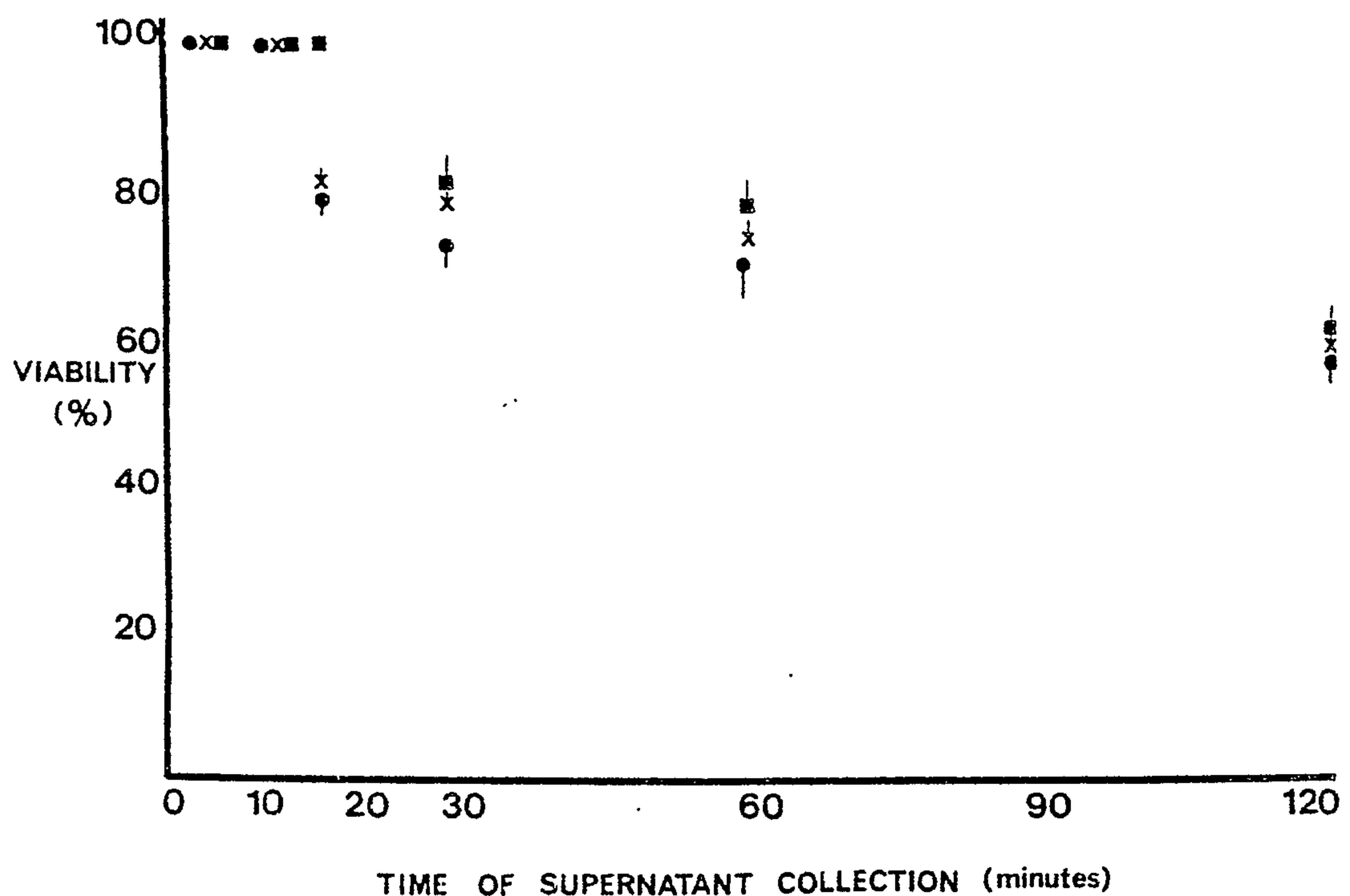


Figure 18. The effect of protein synthesis inhibitors on the production of the macrophage factor.

Three protein synthesis inhibitors, $\blacksquare$ actinomycin D ($1 \mu\text{g/ml}$), $\times$ cycloheximide ($10 \mu\text{g/ml}$) or $\bullet$ puromycin ($100 \mu\text{g/ml}$) were added to macrophage cultures simultaneously with LPS ($20 \mu\text{g/ml}$) and supernatants were collected at six intervals between five minutes and two hours for testing in the thymocyte viability assay. Protective activity of the macrophage factor at the various times is indicated by the percentage viability of the thymocytes at three hours. Results are the mean of three experiments $\pm$ SEM.

examined further by trypsinising the cells and preparing cell lysates as described in Methods. The data shown in Figure 19 indicate that lysates of unstimulated cells also contained protective activity. Trypsin treatment to hydrolyse surface proteins did not remove the protective effect of the cell lysates, indicating that the factor was cytosolic, rather than on the cell surface. Unstimulated macrophage supernatants failed to protect the thymocytes.

The Effect of Restimulation of Macrophage Cultures

The abrupt decrease in titre from a peak at two hours after stimulation to no detectable protective effect at four hours, was studied by examining the effects of restimulation with LPS at two, four and six hours of culture. The results are shown in Figure 20 and indicate that macrophages were anergic to further stimulation. In this context, it was noted that 10 nM of prostaglandin E₂ added together with LPS at 20 µg/ml to fresh macrophage cultures, totally removed the titre of activity at two hours. Indomethacin (10⁻⁵ M), added together with LPS to fresh cultures did not alter the peak titre at two hours, however, in the presence of this inhibitor of prostaglandin synthesis macrophages which were restimulated at four hours did release low titre activity at six hours of incubation. There was no effect of the presence of indomethacin, however, on restimulation at two hours or at six hours.

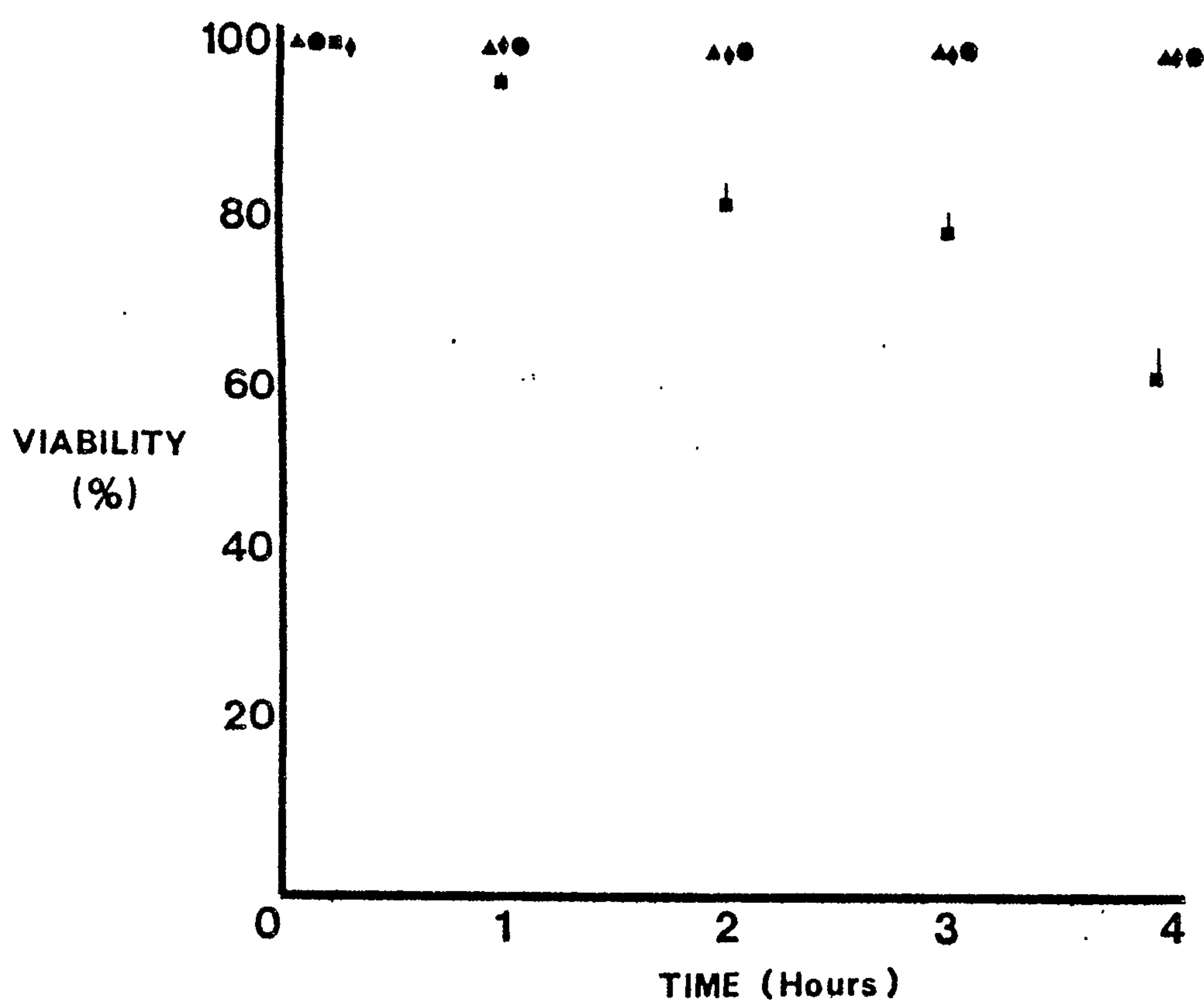


Figure 19. The effect of control supernatants on the production of the macrophage factor.

Three control supernatants, ▲ lysed macrophage supernatant, ◆ trypsin treated lysed macrophages and ■ unstimulated macrophage supernatants were compared to ● macrophage supernatant from macrophages stimulated with 20 µg/ml LPS. The percentage viability of the thymocytes over four hours is shown. Results are the mean of five experiments $\pm$ SEM.

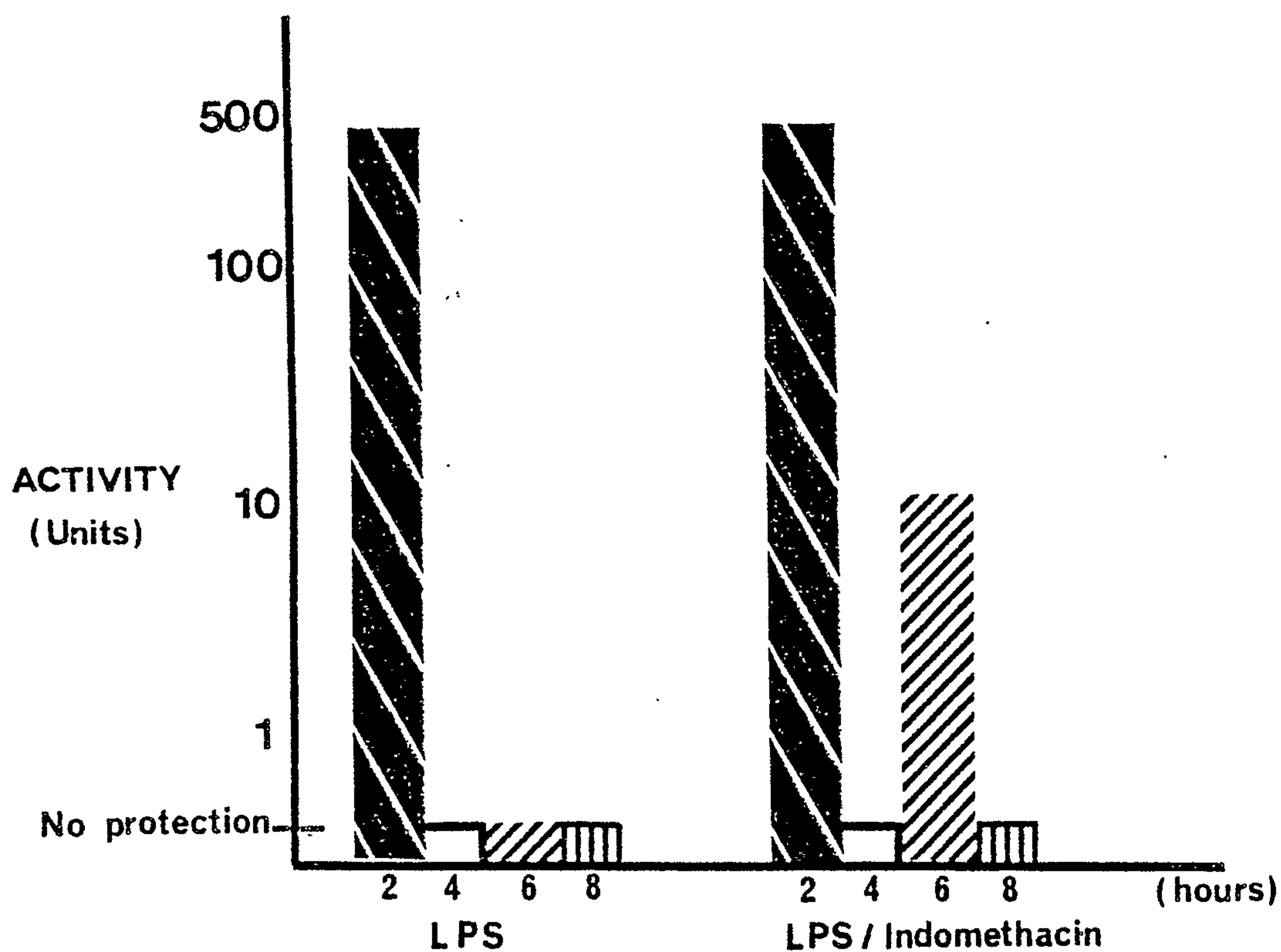


Figure 20. The effect of restimulation of macrophage cultures, with LPS, on the production of the macrophage factor.

Supernatants were collected at two hourly intervals and cultures were restimulated with either LPS only (20 $\mu\text{g/ml}$) or LPS (20 $\mu\text{g/ml}$) and indomethacin (10^{-5} M) in new medium. Production of the factor was expressed as the titre resulting in 100% protection in the thymocyte viability assay for supernatants collected at:  two hours,  four hours,  six hours and  eight hours after initial stimulation.

9.4 Discussion

The results of the present study indicate that an Mr 36,000 monokine is produced by rat peritoneal macrophages in response to either LPS or MDP. The yield as assessed by the number of units of protective activity in the thymocyte viability assay is, however, greatest in response to 20 µg/ml LPS when the supernatant is collected two hours after the cells are challenged. Increasing the culture time beyond two hours led to a decrease in the number of units of activity at three hours and a loss of protective activity beyond that time and up to forty-eight hours.

The early release of low level activity from five minutes onwards suggested that the factor was already present within the macrophages and was simply released. Lysates of unstimulated cells demonstrated protective activity in the thymocyte viability assay, however, this activity could not be titrated. Addition of protein synthesis inhibitors, actinomycin D, puromycin and cycloheximide to cultures at the same time as the LPS demonstrated that de novo protein synthesis was necessary for continued production of the macrophage factor. In contrast to cultures with LPS alone, protective activity could not be detected beyond ten minutes with puromycin and cycloheximide or after fifteen minutes with actinomycin D. Hydrolysis of surface proteins by trypsinisation, prior to washing and lysis of the cells failed to remove the protective activity indicating that the factor was not a surface protein on the macrophage.

It was postulated that the abrupt decrease in titre of the protective activity, after two hours, could be due to prostaglandin production by the macrophages. It has been shown, (Papiernik & Homo-Delarche, 1983) that thymic phagocytes in culture produce prostaglandin E₂ spontaneously but that this was greatly enhanced by the addition of LPS to the cultures. These authors also provided evidence that the addition of indomethacin to cultures decreased the production of prostaglandin in response to LPS, almost ten-fold. In the present study, the addition of 10⁻⁵ M indomethacin and LPS to cultures together with new medium at four hours, resulted in a further peak of protective activity which was not seen in cultures restimulated with LPS alone. The role of prostaglandins was further confirmed in this study where the addition of 10 nM prostaglandin E₂ to cultures at the same time as the LPS, totally removed the titre of activity at two hours. The later release of prostaglandins is supported by Hsueh, *et al.*, (1980) who showed that the production of prostaglandins by alveolar macrophages was not associated with either the attachment or the engulfment phases of phagocytosis but was concomitant with a much later event which occurred after the stimulus had been internalised. This was demonstrated using concanavalin A-treated red blood cells which attach to macrophages but are not engulfed and for which it was shown that the prostaglandin production was no different from a control group. In contrast, when anti-red blood cell antiserum was added to the culture medium, the red blood cells were internalised by the macrophages and prostaglandin production was shown to increase by sixty percent.

The time course for the production of this factor varied from that of interleukin 1 or lymphocyte activating factor in mice, humans and rats. Mizel, (1981) showed that mouse peritoneal macrophages stimulated with phorbol myristate acetate reach a peak level of production of interleukin 1 around twenty-four hours and this then plateaus. Similarly, Wood et al., (1983) collected supernatants from a Balb-c macrophage line, 2-3 days after the cells were stimulated with LPS (from Escherichia coli) in order to harvest lymphocyte activating factor for testing in the thymocyte proliferation assay. Human monocytes and macrophages stimulated with LPS show a maximum yield of lymphocyte activating factor at twenty-four hours with very little production prior to two hours as assessed by the ability of the supernatants to augment proliferation of mouse thymocytes in response to phytohaemagglutinin (Treves et al., 1983). Bird et al., (1985) prepared interleukin 1 from Sprague Dawley rat peritoneal macrophages by culturing the cells for forty-eight hours in the presence of 10 µg/ml LPS.

Examination of the time requirement for tumour necrosis factor production by LPS induction of human monocytes showed that cytotoxicity reached plateau values within two hours (Kornbluth & Edgington, 1986). The monocytes, however, needed to be primed two to three days prior to challenging with LPS, with an agent such as tubercle bacilli (Carswell et al., 1975 and Mestan et al., 1986). This was not necessary for the early production of the factor described in the present investigation. Beutler et al., (1985) isolated cachectin, now thought to be identical to tumour necrosis factor, (Vilcek et al., 1986), from a macrophage cell line

which had been stimulated 22 hours previously, with LPS from E. coli.

While it is recognized that macrophages will produce a large number of soluble factors in response to challenge with LPS, the early release of this factor in the absence of priming the cells, together with the abrupt decrease in production at two hours suggests that this factor is different from other well recognised monokines.

CHAPTER TEN

GENERAL DISCUSSION

The experiments described in this thesis were undertaken to examine the role and interaction of macrophages, T lymphocytes and epithelial cells in the pathogenesis of an oral mucosal disease, lichen planus. In the course of the early experiments, a rat thymic cell model was developed and this has provided a useful tool for studying such cellular interactions and, in addition, it has provided insights into thymic and macrophage biology.

It was observed that the viability, in vitro, of dense, immature rat thymocytes decreased markedly over a four hour incubation period. This was attributed to programmed cell death, that is apoptosis, on the basis of literature reports (Kerr et al., 1972, Wyllie, 1980 and Cohen & Duke, 1984), together with the finding in the present study that the viability of the thymocytes was maintained by the addition of puromycin. This provided evidence that protein synthesis was required for cell death and this is a known parameter of apoptosis. Confirmation of apoptotic death would, however, require study of DNA fragmentation profiles to examine for the characteristic cleavage by the calcium-dependent endonuclease (Cohen & Duke, 1984). The rapid rate of death observed in the present study is in accord with that found by Duke & Cohen, (1986), who observed apoptosis following interleukin 2 withdrawal from stimulated lymphocytes. It does,

however, pose the interesting question of the stage of DNA degradation at which the cell becomes irreversibly committed to death.

The addition of an Mr 36,000 monokine from cultured rat peritoneal macrophages, which had previously been stimulated with lipopolysaccharide, prevented these cells from dying. The early release of this factor together with preliminary results of its functional and physical properties suggest that it is different from the well characterized monokines. The macrophage factor was found to bind in competition with a monoclonal antibody directed against a common determinant on Ia but to bind non-competitively with a rat strain specific epitope on Ia. In preventing the inevitable cell death of immature thymocytes, it would appear that this monokine binds to the Ia protein complex on the cell surface of thymic epithelial cells causing them in turn to release an activity which is directly responsible for at least the temporary survival of thymocytes.

In addition, it was noted that the addition of picogram quantities of bacterial products to rat thymic cells in culture produced a doubling of the proliferative response of these cells to sub-optimal levels of concanavalin A. This effect could be prevented by the depletion of adherent cells which comprised less than 0.1% of the total population. The addition of the described whole macrophage supernatant or partially purified, Mr 36,000 monokine or of an antibody to a common determinant on Ia restored the proliferative response in adherent cell-depleted populations. Although the dense cell fraction from density gradient separation on Percoll did not respond to concanavalin A,

it could be induced to do so by the addition of the monokine. This provided evidence that the monokine could provide maturation and co-activation signals for at least some of the immature thymocytes as well as temporarily arresting the apoptotic process. This occurred either by its direct action on the thymocytes or alternatively, indirectly through thymic epithelial cells.

On this basis, it was proposed that bacterial products stimulate thymic macrophages to release the Mr 36,000 monokine which in turn stimulates the thymic epithelial cells to release products which promote the survival and maturation of immature thymocytes.

The results of these investigations have implications for the understanding of thymic biology and macrophage function, and in addition, they relate back to the initial question of the interaction of these cell types in chronic inflammatory oral mucosal conditions such as lichen planus.

10.1 Thymic Biology

There is a basis for extrapolating the findings of the present studies to thymic biology and in particular to thymocyte selection and maturation. The events that occur in the thymus and lead to the development of mature, differentiated and phenotypically distinct thymocytes can be considered, albeit simplistically, to occur in three stages. In the first stage, which occurs at about Day 11 of foetal development in mice (Jenkinson,

1981), the thymus is colonized by lymphoblasts from foetal liver. From birth onwards, this population is maintained by a self-renewing intrathymic pool of cells (Scollay et al., 1986) with an additional contribution from the bone marrow (Fowlkes et al., 1985 and Kyewski, 1987). Secondly, there is a selection of a small group of thymocytes which will go on to mature; the remainder will undergo apoptosis (De Waal Malefijt et al., 1986 and Kinnon et al., 1986). Thirdly, there is a further selection from the group of cells previously chosen to survive and these cells will go on to differentiate into cells with the potential to eventually seed the peripheral lymphoid organs. This raises several questions which will be considered in the context of the development of T helper/inducer cells.

Colonisation of the thymus with thymocyte precursors.

In relation to the first stage of thymocyte development, it is necessary to consider the phenotype of cells entering the thymus. There is evidence that thymic precursor cells that enter the thymus in foetal life are both CD4 negative and CD8 negative (Kingston et al., 1985 and Paterson & Williams, 1987). Postnatally, the thymocyte precursor population is renewed by cells from bone marrow, which are known to be Ia positive (Engleman et al., 1980) as well as CD4 and CD8 negative. Of importance is the stage at which CD4 negative thymocytes start to express CD4. This will be considered further, below. It has been demonstrated that the presence of Ia positive accessory cells is essential for the expression of CD4 (T4) by thymocytes (Blue et al., 1985 and Larocca et al., 1987). Furthermore, the addition of

monoclonal antibodies against HLA-DR inhibited the expression of CD4.

It has also been shown that a single precursor cell will recolonize an alymphoid thymic lobe in organ culture and produce phenotypically distinct T-cell populations (Kingston et al., 1985 and Williams et al., 1986). This provided evidence that the commitment to specificity of the T-cell occurred within the inductive environment of the thymus rather than the bone marrow. Penit (1986) supported the single lineage model for in vivo thymocyte maturation. In contrast, another study (Ezine et al., 1987), reported that although all possible phenotypic clones were represented from a single clonogenic precursor, there was an extreme bias towards subsets of cells expressing only L3T4 or Lyt-2. This raised two possibilities; the first and more unlikely, was that cells might be committed pre-thymically and the alternative was that positive or negative selection of distinct clones was related to a defined site within the thymus. The latter, however, posed the problem that the frequency of a given subset of cells could vary dependent on the time at which the site was analysed. As current technology does not permit analysis of both the location of the clone and the subset frequencies in the same population, this issue remains an enigma. Kyewski (1987) also favoured a thymic compartmentalization for the interaction of stromal cells and thymocytes but interpreted his results on the assumption that isolated lymphostromal complexes represented a direct correlate in vitro of specific cell-cell interactions in vivo. Similarly Nabarra & Andrianarison (1987) postulated that different types of

T cells matured in different regions of the thymus and in addition, there were several types of epithelial cells, each with a unique function in this role.

Induction of Thymocyte Apoptosis and Survival

Apoptosis. In the second stage of thymocyte development, the majority of uncommitted precursor cells are induced to apoptose but a small number of cells will survive. The mechanism for the induction of apoptosis and the selection of those cells chosen to survive are important questions. There is evidence that apoptosis of immature thymocytes occurs in the presence of near physiological concentrations of glucocorticoid hormones (Wyllie, 1980). Cohen & Duke, (1984) proposed that all thymocytes have the calcium and magnesium-dependent endonuclease that is ultimately responsible for apoptosis but that mature thymocytes lack the glucocorticoid-inducing mechanism for activating it. Arachidonate metabolites have been implicated in the induction of apoptosis although the evidence for their having a direct role was inconclusive (Taylor et al., 1985). The withdrawal of interleukin 2 from activated spleen cells and from an interleukin 2-dependent cell line, has also been demonstrated to induce apoptosis (Duke & Cohen, 1986). It can be concluded that while apoptosis of thymocytes within the thymus is a well recognized phenomenon, little is known about its induction.

Survival. The mechanisms governing the selection of cells that will survive as opposed to those that will die are unknown. Kinnon et al., (1986) attempted to implicate the activation of the alpha and beta chains of the T cell antigen receptor in this

process. From their studies, it was concluded that those cells that went on to die, did not do so because of a failure to rearrange or to transcribe T cell antigen receptor genes. In fact, it was shown that all thymocytes expressed transcripts of both chains but at differing levels. Immature precursor cells expressed lower levels of the alpha and beta chain mRNA than cells that were likely to be their descendants. Cells that were apparently committed to die (a population with low affinity for peanut agglutinin which had been enriched for Lyt-2 and L3T4 positive cells) had even higher levels of these transcripts than either of the other populations.

In a more recent study, Born et al., (1987) confirmed that the T cell antigen receptor played no role in the selection of immature thymocyte populations, for survival. Experiments using foetal thymus organ cultures demonstrated that antibodies to the T cell receptor neither triggered the death nor the proliferation of the immature thymocytes. It was postulated that the thymocyte was at this stage anergic to intracellular signalling. The T cell receptor was, however, implicated in thymocyte maintenance or selection at, or after a point at which they had entered a maturation pathway and this will be discussed later. The criteria for selection of cells that will survive or alternatively, apoptose, remains an enigma.

A role for bacterial products in the induction of the apoptotic process and in thymocyte survival.

On the basis of the results presented in this thesis, the following is proposed: The majority of thymic macrophages are

concentrated at the cortico-medullary junction, where a blood-thymus barrier is conspicuously absent (Raviola & Karnovsky, 1972), and these cells therefore have access to the vascular contents. Although the penetration of protein antigens into the thymus is a topic surrounded by controversy, it has been demonstrated to occur (Kyewski et al., 1984). The stimulation of these macrophages by microbial antigens could lead to the production of the Mr 36,000 monokine, described in this thesis. The macrophage factor would then bind to any cell that presented Ia on its surface, including thymic epithelial cells, the macrophages themselves and the Ia-positive thymocytes. In support of the production of this monokine within the thymus, data presented in this thesis indicated that picogram quantities of microbial products were effective in promoting thymocyte survival and maturation. It is also possible that circulating monokine derived from extrathymic sources could act directly on thymic elements or indirectly through a relay effect on the thymic macrophages. In this latter context it will be of interest to determine if the monokine stimulates its own production in Ia-positive macrophages. A caveat on the proposal is the possible anatomical restriction, that is, the distribution of cells and microenvironmental influences, in relation to the proposed interactions. A review of the literature on thymocyte maturation did not, however, resolve this question. An initial approach to this problem would be to use direct immunofluorescence on sections of thymus. The distribution of precursor cells could be studied by dual-labelling using rhodamine-labelled OX52 for the pan T cell and thymocyte marker and fluorescein-labelled OX6 for Ia. The distribution of

epithelial cells could be ascertained by staining for keratin using the affinity-purified rabbit immunoglobulin described in this thesis (Chapter 7).

Protection. The thymic epithelial cells would respond to the monokine by producing the factor that is directly responsible for the protection of thymocytes from death.

Preliminary experiments (see Appendix) have suggested that the thymic epithelial cell factor, when induced by the macrophage factor, has a molecular weight of around 320, 000. Induction of the thymic epithelial cell factor with MRCOX6 (the antibody to a common determinant on Ia), results in the release of a protein of molecular weight, 760,000. Further to this, the thymic epithelial cell factor induced by the antibody, but not that induced by the macrophage factor, could be absorbed by protein A-Sepharose. The large molecular weight of the protein together with the fact that the molecular weight of the product varied according to the method by which it was induced, suggested that the thymic epithelial cell factor might in fact be an Ia-ligand complex that is shed from the thymic epithelial cell. If this does occur, then the selection of thymocytes for which apoptosis is arrested, could be based on their ability to bind the Ia-ligand complex that is shed. This appears to be related to the expression of CD4 by the thymocytes since pre-incubation of immature thymocytes with anti-CD4 antibodies inhibits the observed protective effect of the monokine-induced thymic epithelial cell factor.

It was not possible to absorb the monokine with the monoclonal antibody, W3/25 to CD4, indicating that it is unlikely that this macrophage product is soluble CD4.

There is evidence presented in this thesis with the studies on thymocyte proliferation, that some of the thymocytes have matured. An immature thymocyte population which failed to proliferate in the presence of concanavalin A could be induced to do so when this monokine was added to cultures.

On this basis, the number of cells selected to survive and mature to this point, could be proportional to the production of the monokine and also to the number of Ia molecules available on thymic epithelial cells. It is also implied that the expression of CD4 by a thymocyte does not guarantee its survival.

Induction of Apoptosis in Thymocytes. As discussed previously, the mechanism of the induction of apoptosis in uncommitted thymocytes is unknown. A role for glucocorticoids has been proposed but it is difficult to perceive the degree of specificity that would be required, if these hormones were solely responsible. It would seem to be more rational if specific ligand-receptor interactions at the cell surface exerted a primary role in the control of the apoptotic mechanism. In support of this contention, there is recent evidence (Ucker, 1987) for cytotoxic lymphocytes inducing apoptosis in their target cells and this presumably involves specific receptor-ligand interactions.

Using the precedent of the effect of the monokine on thymic epithelial cells, it is proposed that the monokine could bind at Ia on precursor thymocytes and thereby activate these cells. The

activation of the apoptotic mechanism could be coupled with maturation events such as the expression of CD4. The binding of an Ia-ligand complex, released from either thymic epithelial cells or thymocytes, at CD4 could provide a temporary arrest of the apoptotic pathway. In support of the specificity of this interaction in halting programmed cell death, it was shown that neither the monoclonal antibody W3/25, against CD4 or MRCOX52, against the pan T cell and thymocyte marker could protect the thymocytes.

This proposal could be tested by isolating the Ia-positive thymocytes. The dense fraction on Percoll gradient separation was shown to consist of Ia-positive thymocytes, Ia-positive thymic epithelial cells and a major population of Ia-negative thymocytes. It should be possible to remove this latter population with an antibody/complement lysis technique using a monoclonal antibody to CD4. Preliminary results have shown that thymic epithelial cells adhere to plastic surfaces over twenty-four hours. It may be possible to improve adherence using fibronectin or a commercially available extracellular matrix to pre-coat tissue culture flasks. The non-adherent, Ia-positive thymocytes could then be exposed to the monokine either in the presence or absence of the monoclonal antibody W3/25 against CD4. The prediction to be tested is that in the presence of the antibody, the monokine will induce apoptotic death in some of the thymocytes, whereas in the absence of the antibody, the Ia-ligand complexes will bind at the newly expressed CD4 and protect the cells.

Selection of Thymocytes to Seed the Peripheral Lymphoid Organs

In the third stage of the differentiation of thymocytes, a further selection of cells occurs from a population that has temporarily been rescued from apoptotic death. At the same time, those cells with a high affinity for self, that is, those cells that would react in the periphery, directly with self MHC proteins in the absence of antigen, are depleted (Farr et al., 1985). The basis for this second and more precise process of selection is also unclear although two factors thought to be essential, are the direct contact of thymocytes with thymic epithelium and the elaboration of thymic hormones.

Direct contact of thymocytes with thymic epithelial cells. The association of thymocytes with Ia positive thymic epithelial cells appears to represent a relatively late event in cortical T cell differentiation, as deduced in experiments using radiation chimeras (Kyewski, 1987). The late occurrence of these interactions is in keeping with previous findings that imply a necessity to first generate a T cell repertoire, out of which, T cell clones with appropriate specificity could bind to Ia positive epithelial cells. It is possible that the interaction between thymocytes and epithelial cells that occurs, at this stage, represents what is described as a thymic nurse cell. Thymic nurse cells are thought to be epithelial cells which are completely surrounded by up to two hundred actively dividing thymocytes (Wekerle et al., 1980, Ritter et al., 1981, Kyewski & Kaplan, 1982 and De Waal Malefijt et al., 1986). There is some controversy as to whether the cells are actually enclosed within

the epithelial cell or whether it is merely an intimate association (Adkins, 1987). It has been proposed that the selection of thymocytes involves the interaction of the T-cell antigen receptor on thymocytes with the histocompatibility proteins on thymic epithelial cells (Farr et al., 1985). Of particular importance is the observation that Ia-positive thymic nurse cells reside in the cortex which is apparently secluded from blood-borne antigens via its specialized vasculature (Raviola & Karnovsky, 1972). This suggests a role for thymic epithelial cells in the selection of T cells via class II histocompatibility proteins, in the absence of nominal antigens.

The role of direct contact of thymocyte precursors and thymic epithelium at some stage of differentiation is suggested, firstly, by their migration into the thymus, which if thymic hormones alone were effective, would be unnecessary. Secondly, the migration of foetal liver cells to the thymus occurs at a time when the influence of thymic hormones is unable to achieve the maturation of T cells (Stutman, 1978). The binding of thymocytes to epithelial cells is thought to be the specific domain of thymic epithelial cells since epithelial cells from peripheral sites are ineffective (Singer et al., 1986 and Wolf Vollger et al., 1987). Interestingly, the observed binding between thymic epithelial cells and thymocytes could not be inhibited by antibodies to class I or II proteins. In relation to this, the same antibody was used in the studies of Singer et al., (1986) and Wolf Vollger et al., (1987). This suggested, in contrast to other studies, that Ia was not involved in the binding of thymocytes to thymic epithelial cells. Alternatively these results might be related to the

specificity of the antibody, which was demonstrated to be critically important in the experiments, in this thesis, where the binding site for the monokine could be blocked with MRCOX6 but not MRCOX3. If the antibody to Ia that was used was directed against a highly conserved determinant, it might block an interaction, however, if it was against a polymorphic determinant it might not interfere with the binding between thymocytes and thymic epithelial cells. Wolf Vollger et al., (1987) observed that an antibody to CD2 on thymocytes and LFA-3 on thymic epithelial cells were both capable of inhibiting the binding of the two cell types. CD2 is non-covalently linked to the T-cell antigen receptor and could therefore possibly interfere with binding that occurred via this molecule. The role of the LFA-3 molecule in the binding process is unknown although it has been shown to inhibit the binding of cytotoxic T cells to target cells and is, therefore, probably unrelated to the generation of a T helper subset of thymocytes within the thymus.

A later study (Denning et al., 1987) revealed that while binding was important, the generation of activated thymocytes was critically dependent on the number of thymic epithelial cells added to thymocyte cultures and in addition, required protein synthesis by epithelial cells.

The role of thymic hormones. The role of thymic humoral factors in the differentiation of T cells has been convincingly established (Kruisbeek et al., 1977, Beardsley et al., 1983, Nieburgs et al., 1985, Sztein et al., 1986 and Pfeifer et al., 1986) and many of these polypeptides have been isolated, characterized and sequenced. They have been attributed the function of inducing T

cell differentiation markers on thymocytes (Berrih et al., 1985) as well as promoting the production of interleukin 2 (Stzein et al., 1986) and increasing cyclic AMP production (Bach & Papiernik, 1981). Despite these advances, the exact stage at which of each of these factors is functional in thymocyte maturation and differentiation will remain an enigma until the precise sequence of events occurring in vivo in thymocyte maturation and differentiation is established.

The effect of thymic epithelial cell culture supernatants on thymocyte maturation in vitro has previously been described by Kruisbeek (1979). Beardsley et al. (1983) demonstrated that T-cell maturation could be induced by the supernatant from a cloned line of thymic epithelial cells. At about the same time, De Vries et al., (1983) showed that interleukin 1 could induce phenotypic differentiation of small thymocytes to a point at which they were capable of responding to interleukin 2.

More recently, human thymic epithelial cells have been shown to produce an interleukin 1-like factor which augmented the proliferation of C3H/HeJ mouse thymocytes to phytohaemagglutinin (Le et al., 1987). It had a number of biological and functional similarities to monocyte-derived interleukin 1. Prior to this, De Luca & Mizel (1986) had shown that a monoclonal antibody to interleukin 1 inhibited the development of normal T cells. In this context, they also demonstrated that an antibody to Ia had a similar effect which could be overcome by the addition of purified, recombinant interleukin 1.

It has been fairly conclusively established, that direct cell contact between thymocytes and epithelial cells, as well as cytokine production by thymic epithelial cells are required for the development of a functional T-cell repertoire but there are many questions which remain unanswered.

A role for signal transduction by Ia in the production of cytokines.

The following hypothesis for the triggering of cytokine production, by epithelial cells, is proposed (Figure 21) and could be tested experimentally: If selected thymocytes bound to the Ia molecules on thymic epithelial cells via their T cell antigen receptor as well via CD4, this could trigger the synthesis and/or release of thymic hormones from the epithelial cell. In this context, direct cell to cell contact between the T-cell receptor and the Ia/antigen complex on macrophages, has been shown to induce the production of membrane associated interleukin 1 (Weaver & Unanue, 1986). The proposed interaction might also promote the expression of interleukin 2 receptors and interleukin 2 release by the thymocytes. In this manner the interaction can be viewed as occurring in two directions at once, such that Ia is the ligand for the thymocyte while CD4 and the T cell antigen receptor are the ligands for the thymic epithelial cell. Meuer et al., (1984) have shown that the binding of a T-cell receptor can lead to the expression of another receptor and the secretion of its ligand. In the thymic environment the production of interleukin 2 by the thymocyte could lead to the proliferation of appropriate clones of selected T cells. The secretion of interleukin 1, as reported by Le et al., (1987) could provide the necessary

signal for the production of interleukin 2. The expression of the interleukin 2 receptor has been demonstrated in foetal thymuses, however, it was confined to thymocytes that were both CD4 and CD8 negative (Shimonkewitz et al., 1987). This may represent an earlier stage in thymocyte development because it was subsequently demonstrated that those cells that expressed the interleukin 2 receptor, did so, just prior to the expression of CD4 and CD8.

The proposed hypothesis could be tested by taking a dense immature population of thymocytes and inducing the temporary survival of all of them by adding the monokine at an appropriate concentration. The next step would be to selectively block the Ia, T-cell antigen or CD4 receptors, or combinations thereof, using monoclonal antibodies with appropriate specificities. The binding of T cells and epithelial cells could then be monitored using a thymic epithelial cell-thymocyte binding assay such as the one described by Singer et al., (1986). Born et al., (1987) have previously established the importance of the T-cell antigen receptor, at this later stage, using this blocking technique. In addition, the production of interleukin 1 by thymic epithelial cells could be measured using an interleukin 1-dependent cell line assay system. Interleukin 2 production could be monitored in a similar manner using an interleukin 2-dependent cell line.

Following on from the proposed hypothesis, if the interaction of the T-cell antigen receptor on the thymocyte and Ia on the thymic epithelial cell was of low affinity, there might be an

inadequate stimulus for the production of thymic hormones and those thymocytes would apoptose.

This raises a further but very important question which relates to the basis on which cells with high affinity for self, are depleted. It could be argued that the binding of those thymocytes with a very high affinity for Ia to thymic epithelial cells leads to the induction of an apoptotic mechanism which either overrides or negates the production of thymic hormones.

In summary, the monokine arrested the death of cortical thymocytes through an effect relayed via thymic epithelial cells. In the presence of thymic epithelial cells some of the thymocytes went on to mature to the extent that they proliferated in the presence of concanavalin A. The importance of the continuing presence of thymic epithelial cells in this maturation process was not examined in this thesis. This could be achieved, however, by protecting the isolated Ia-negative thymocytes (after the depletion of Ia-positive epithelial cells and precursor thymocytes) with the purified, high molecular weight thymic epithelial cell factor. The thymocytes could then be added to unstimulated thymic epithelial cells or could be incubated alone, or in the presence of whole supernatant from monokine-treated thymic epithelial cells. The thymocytes could then be studied for maturation markers such as the expression of the peanut agglutinin receptor as determined by binding of fluorescein-labelled peanut agglutinin and for responsiveness to mitogens.

Preliminary evidence was obtained that a specific ligand-receptor interaction arrested the death of cortical thymocytes of which, one hundred percent were committed to die. It is also proposed that apoptosis can be induced in susceptible cells by specific interactions at the cell surface. If this proved to be correct, then a comprehensive model for the role of the monokine in thymocyte commitment could be developed. Bacterial products acting within the thymus or at distant sites could induce the secretion of the monokine which has the dual role of inducing commitment in precursor thymocytes, to activate both apoptotic and differentiation pathways. The outcome at this early phase, and at a later stage of selection based on the binding of the T-cell antigen receptor by the thymic epithelial cell, would be seen as the net result of competing influences at the cell surface. In some cells apoptosis would be driven while in others, the apoptotic mechanism would be shut down and maturation would occur. The support for this model arising from studies on germ-free animals is discussed in Chapter 8 but it is of interest to note that the seeding of the lymphoid organs with competent T cells could be seen to occur in response to microbial signals, such as occurs following colonisation of the newborn and as a consequence of infection in the young animal. The continued seeding of the thymus with thymocyte precursors could prevent possible exhaustion of Ia-positive thymocytes following prolonged secretion of high levels of the monokine.

10.2 Macrophage Biology

Macrophages are the ancestral defence cells. They have a clearly defined function in phagocytosing and destroying microorganisms and in this process they are aided by receptors for complement and for immunoglobulins. The concept of the homeostatic function of macrophages has continued to broaden with the recognition of their key role in haemostatic processes and in iron metabolism, in addition to their inflammatory and immune functions. While in the context of wound healing, it is recognized that these cells have the capacity to mediate the turnover of extracellular matrix components by the secretion of hydrolytic enzymes and oxygen radicals, there has recently been a shift to the study of the role of cytokines released by these cells in both direct and indirect control of many aspects of macrophage biology. This is due in part to the multifaceted functions of molecules such as interleukin 1 and tumour necrosis factor and also to the enormous amplification of biological pathways which these molecules impart.

Macrophages, present as tissue histiocytes, are abundant in most tissues. In the inflammatory response these cells are supplemented locally by blood monocytes which migrate in response to a variety of chemotactic stimuli. In the context of the immune response, the expression of class II proteins at the cell surface is an essential requirement for co-operative interaction with T helper/inducer lymphocytes. This relates specifically to the capacity of the macrophage to present immunogenic peptides but also to the propensity of the cell to

secrete, or to express at the cell surface, cytokines such as interleukin 1.

Experiments using mice have indicated that macrophage expression of Ia is not constitutive but is under regulation and is transitory so that eventually, all Ia positive cells will become negative (Unanue & Allen, 1987). The ratio of Ia positive to Ia negative macrophages varies greatly among different tissues, for example, macrophages in the red pulp of the spleen are positive while those in the white pulp are negative. Peritoneal macrophages were reported as being mostly Ia negative in mice. In contrast, in the present studies it was found using a complement/antibody lysis technique that the majority of rat peritoneal macrophages were positive. The reason for the differences in the ratio of positive to negative cells in different tissues and different species has not been established. However, it is known that the local production of prostaglandins will decrease the basal level of Ia expression (Unanue & Allen, 1987). In this manner the macrophage can regulate its own expression of a key molecule.

As discussed in Chapter 4, the lymphokine, gamma-interferon can maintain or increase Ia expression, possibly by suppressing prostaglandin synthesis by the macrophages. Gamma-interferon may also synergize with tumour necrosis factor in promoting class II expression in these cells (Pujol-Borrell et al., 1987).

The importance of Ia expression in the context of the present discussion lies in the probable enhancement of cytokine secretion. In relation to this Palacios (1985) has proposed that Ia

positive monocytes secrete significantly higher levels of interleukin 1 than Ia negative monocytes. Similarly, Gilman et al., (1983) concluded that Ia positive macrophages were ten to fifteen fold more efficient producers of interleukin 1 than Ia negative macrophages. In this context, it is interesting to note that there are two sets of stimuli relevant to the production of interleukin 1. The first involves the direct stimulation of macrophages with microbes and their products and the second occurs via a CD4-positive T cell which will recognize a processed protein in association with Ia on the surface of a macrophage and induce it ,within a couple of hours, to produce membrane Il-1 (Kurt-Jones et al., 1986). It has been shown that both a T cell product and direct cell contact are involved in the process but the precise mechanism is not known.

In this way, a balance can be envisaged with the majority of the histiocytic macrophages being Ia-negative, prostaglandin secreting cells and poor producers of cytokines while newly recruited monocyte-like cells will be mainly Ia-positive and good producers of cytokines in response to a variety of stimuli. The presence of gamma-interferon secreted by activated T helper cells would serve to augment cytokine production by maintaining the Ia-positive population.

A proposed role for the Mr 36,000 monokine as a regulator of cytokine production.

From the discussion above, the question of a cause and effect relationship of Ia and cytokine production arises. In general terms, support for a signal transduction function of Ia

glycoproteins comes from the studies of Palacios (1985) and Cambier & Ransom, (1987) which were reviewed in Chapter 4. Palacios (1985) showed that the binding of selected monoclonal antibodies to Ia on human monocytes could trigger the production of interleukin 1. In relation to this, the Mr 36,000 monokine described in this thesis was shown to be a product of Ia-positive macrophages. Evidence was obtained that the messenger RNA and the protein were present in unstimulated cells and that the monokine was released within minutes after the addition of lipopolysaccharide to the cultures. The titre in the culture medium fell rapidly after two hours, possibly as a consequence of binding to Ia on the producing cells. The addition of prostaglandin E₂ to the culture inhibited the synthesis of the monokine while indomethacin enabled cultures to respond to a second challenge with lipopolysaccharide.

The association of the expression of Ia and cytokine production, together with reports that Ia can serve as a receptor in signalling cytokine production and the fact that the Mr 36,000 monokine is released very rapidly provide the observational basis for the hypothesis that this factor functions as an important regulatory agent.

This could be tested experimentally in the following manner:

The partially purified monokine could be added at a known titre to freshly seeded peritoneal macrophage cultures. Culture supernatants and cell lysates could be collected at various times and tested for activity, as determined by titre, in the thymocyte viability assay. Interleukin 1 titres could be determined by the augmentation of the proliferation of an interleukin 1-dependent

cell line and tumour necrosis factor could be measured by the killing of a susceptible cell line. Essential controls would include boiling to destroy the protein; this would provide a measure of contamination with lipopolysaccharide. In keeping with the findings in this thesis, F'ab fragments of the anti-Ia antibody OX6 could be used to block the binding of the monokine to Ia. The extent to which lipopolysaccharide serves to induce the secretion and release of the monokine, as a primary event in its effect on macrophages, could be evaluated by performing experiments in which Ia is blocked with F'ab OX6 prior to adding the bacterial product.

In a similar manner, the role of CD4 in a possible cascade reaction, could be evaluated by blocking with F'ab monoclonal antibodies to this determinant. The proposed sequence of events is outlined in Figure 22.

A related phenomenon has been reported for interleukin 1 in experiments where recombinant human interleukin 1 has been demonstrated to induce the production of interleukin 1 by cultured human vascular endothelial cells and smooth muscle cells although the response was more transient and rapid (Warner et al., 1987). Dinarello et al. (1987) have reported similar findings with human blood monocytes. The potential for amplification in disease processes is enormous but is equally well controlled by the interleukin 1-induced production of prostaglandins which reduces the expression of biologically active interleukin 1.

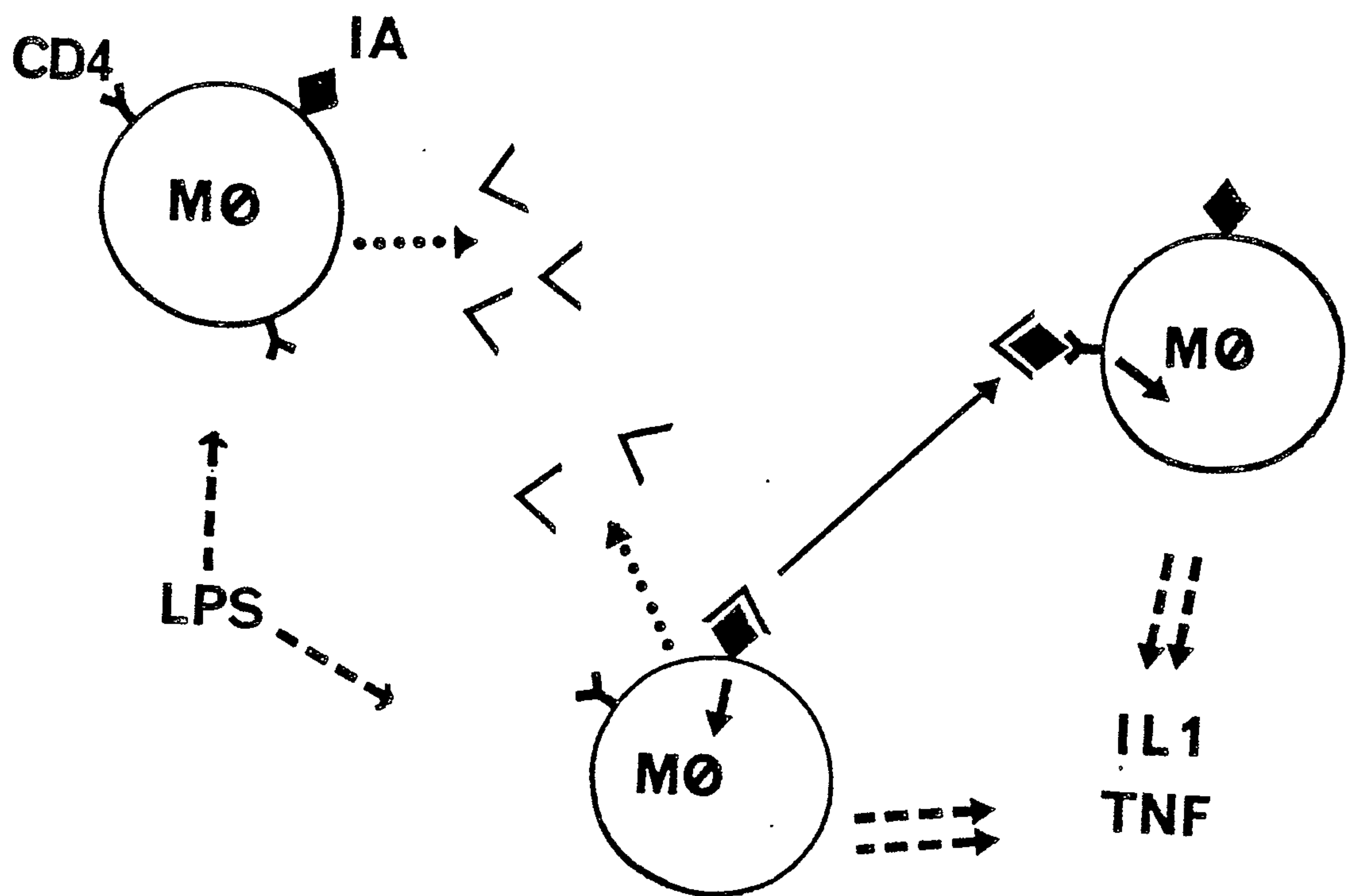


Figure 22. A hypothetical role for the monokine in stimulating autocrine activity of macrophages.

◆ Ia molecule, < macrophage factor, Y CD4 molecule, MØ - macrophage, IL 1 - interleukin 1, TNF - tumour necrosis factor, LPS - lipopolysaccharide.

It would also be of interest to determine whether purified, recombinant interleukin 1 and tumour necrosis factor can modulate the secretion of the Mr 36,000 monokine.

Based on the findings in this thesis, it is axiomatic that the Mr 36,000 monokine should bind to any cell which expresses surface Ia. In this way there is potential to modulate the functions of other cell types in addition to thymic epithelial cells and macrophages.

10.3 Mucosal Disease

The observed histological changes in lichen planus raise several questions about the pathogenesis of this and other oral mucosal diseases. The expression of HLA-DR by keratinocytes together with the predominance of T helper cells in the infiltrate provide support for a contact sensitivity type of reaction occurring in lichen planus. Cell-mediated immunity was thought at one time to be characterized by a constant and relatively simple morphological pattern. However, it is now appreciated that there is considerable diversity in morphologic features. Nonetheless, work on delayed hypersensitivity remains essentially at a descriptive, morphologic level (Dvorak et al., 1986) with little knowledge of what is driving the changes that are observed. In human cutaneous responses to tuberculin, the prototype of delayed hypersensitivity responses, it has been noted that there is epidermal proliferation and also that the phenotype of the keratinocytes changes from Ia negative to Ia positive (Kaplan et al., 1986). The expression of Ia was assumed to be induced by

gamma-interferon but its role is unknown. Similarly in lichen planus there is increased HLA-DR expression within the epidermis (Tjernlund, 1980, Lampert, 1984 and Regezi et al., 1985) and Volc-Platzer et al., (1984) have presented evidence for the synthesis of HLA-DR by keratinocytes.

There is growing acceptance of the important sentinel function of Ia-positive Langerhans cells within epithelium (Chapter 3). However, the abrupt change of the numerically predominant keratinocytes from Ia-negative to Ia-positive, naturally focusses attention on the functional significance of this new surface marker on these cells.

Ia antigens have also been detected on keratinocytes in graft-versus-host disease (Mason et al., 1981 and Lampert et al., 1982), the skin manifestations of which are regarded as being a cell-mediated immune reaction. Clinically and histologically, the presentation of graft-versus-host disease is similar if not identical to lichen planus (Saurat & Gluckman, 1977). In this situation, it is proposed that the expression of Ia by the keratinocytes may result in the recognition of alloantigen by the injected T lymphocytes used to induce the disease, thereby conferring on the epithelial cells an antigen presenting function.

The reason for the increased expression of class II antigens by the keratinocytes in lichen planus remains an enigma. It raises the question of whether the epithelial cells are functioning as antigen presenting cells for the infiltrating lymphocytes or alternatively, whether the Ia molecules on epithelial cells serve

a transducer function in relaying intercellular messages between cells.

An integral role for keratinocytes in immune responses is further supported by the finding that keratinocytes can secrete interleukin 1 (Luger et al., 1982, Sauder et al., 1982 and Mergenhagen, 1984). Interleukin 1 has also been detected in normal epidermis (Hauser et al., 1986) although the question of whether it is constitutive or induced by environmental stimuli remains unanswered.

A proposed role for the Mr 36,000 monokine in delayed hypersensitivity reactions.

The secretion of the monokine (Mr 36,000) by the macrophages in the inflammatory focus could result in the modulation of the functions of a number of cell types. The binding to Ia-positive keratinocytes could result in the increased synthesis and secretion of interleukin-1 by these cells. In addition, applying the analogy with thymic epithelial cells, the keratinocytes might shed a complex which protects in the thymocyte viability assay. This, in turn, would lead to the question of the role of complex binding at CD4 on activated T helper cells. T lymphocytes were shown to be susceptible to apoptosis when denied interleukin 2 (Duke & Cohen, 1986). It could be argued that for T cells, as it was for thymocytes, that competing interactions at the cell surface alternatively drive apoptosis or proliferation and secretion. On this basis, a high affinity binding of the T-cell antigen receptor would deliver an apoptotic signal whereas binding of interleukin 1 and of the putative Ia-ligand complex

would shut down apoptosis and stimulate the synthesis and secretion of lymphokines.

The effect of this monokine on keratinocytes could be tested experimentally. With the idea of pursuing this, keratinocyte cultures have been established from foetal or newborn rat skin using a trypsinisation procedure. Cultures were 96 to 100 % positive for an antibody to rat hair and foot callous keratin and could be grown from a single cell suspension on plastic microwell plates without a supporting matrix. This allowed large numbers of cultures to be established from a realistic and workable number of animals. It was also shown that the addition of a crude lymphokine supernatant to the keratinocytes induced Ia expression. This supernatant was assumed to contain gamma interferon but was by no means pure and possibly contained contaminants such as the monokine described in this thesis. In this manner, however, it would be possible to use recombinant rat gamma-interferon, to induce Ia expression in keratinocyte cultures and measure the potential of these cells to produce proteins such as interleukin 1 compared to control, non-Ia positive cells. An assay for interleukin 1 activity could be established using an interleukin 1 dependent cell line to avoid the problems associated with the thymocyte proliferation assay.

It would also be possible to study the direct effect of the partially purified monokine on keratinocytes in this system. Once Ia expression had been induced, it would be possible to examine the binding of the monokine to these cells by attempting to block the binding with the F'ab portion of MRCOX6. Similarly, the effect of MRCOX6, the antibody to the common determinant

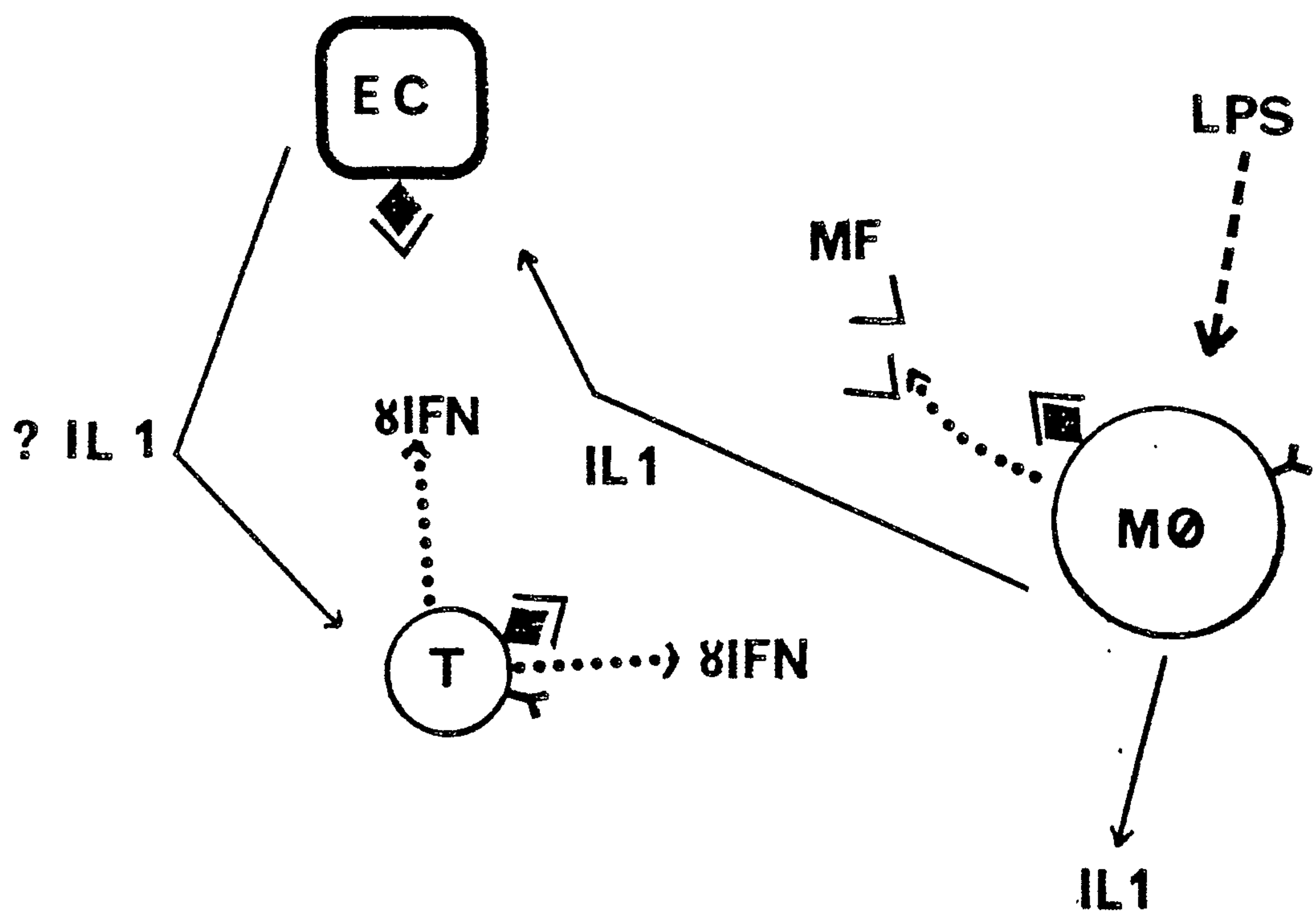
on Ia, in stimulating the synthesis of proteins such as interleukin 1 could be studied. It would be interesting to examine, using either MRCOX6 or the monokine, whether the production of the protective factor for thymocytes was the specific domain of thymic epithelial cells or whether it was common to keratinocytes as well. Preliminary studies have been carried out where 2 hour, LPS-stimulated macrophage supernatants have been added to keratinocyte cultures and this has revealed a modest increase in overall protein synthesis, determined using a Bradford protein assay (Bradford, 1976). These cultures had not been induced to express Ia which, on the basis of the proposed hypothesis, would significantly increase the potential for protein synthesis by epithelial cells. The non-specific production of protein by epithelial cells could be assessed more accurately by measuring the incorporation of tritiated leucine or alternatively, ³⁵S-labelled methionine, by keratinocytes which had been stimulated with the monokine, compared to control, non-stimulated cultures.

As postulated previously, if the macrophages presented Ia they would also have the potential to bind the monokine and perhaps be stimulated in an autocrine fashion to produce more of the monokine, thus providing an effective amplification for the system.

In addition, it is known that activated T lymphocytes express Ia and would, therefore, also have the potential for signal transduction. An interaction between Ia molecules on T cells and the monokine might, in addition to providing a source of shed Ia-ligands, provide a necessary trigger for the production of

lymphokines such as gamma interferon. In relation to this, Moretta et al., (1982) demonstrated strong inhibition of interleukin-2-dependent proliferation of a T cell line in the presence of a monoclonal antibody to a common determinant on Ia. A hypothetical outline for cell-mediated immune reactions is proposed in Figure 23.

The monokine, described in this thesis, has the potential to protect thymocytes from apoptosis in vitro, but in addition, may have implications in thymus biology in the selection of thymocytes to seed peripheral lymphoid organs. It may also be important in the autocrine activation of macrophages and the maintenance and selection of T cell populations in chronic inflammatory oral mucosal conditions such as lichen planus.



ε

Figure 23. Hypothetical outline of cell-mediated immunity.

■ Ia molecule, < macrophage factor, < CD4 molecule, MØ - macrophage, IL 1 - interleukin 1, γ IFN - gamma interferon, EC - epithelial cell, T - T cell, LPS - lipopolysaccharide.

APPENDIX

The experiments and results described in this section are preliminary investigations of areas that follow directly on, from the work presented in this thesis. This includes the partial purification of the macrophage factor and some preliminary data on the production and nature of the thymic epithelial cell factor.

Partial purification of the macrophage factor

The macrophage factor was prepared as described in Chapter 7. It was run over an XM 300 membrane, to remove the bulk of the lipopolysaccharide and then through a PM 10 membrane and the fraction above the membrane was retained and sterile filtered as described. The molecular weight was determined by gel filtration fractionation on Sephacryl S200 and the activity was localized to an Mr 36,000 fraction (Yield = 80% of loaded sample). The macrophage factor was further purified by ion exchange chromatography on DEAE Sephacel (Pharmacia). The active fraction (5ml) from Sephacryl S200 was loaded onto a DEAE Sephacel column (1.6 x 60cm) and the activity eluted from this column in 2x 2ml fractions at 120-124 ml after a gradient of 200 mls of 0.05 M Tris (pH 8.0) + 200mls of the same buffer with 0.5M NaCl was applied (Yield = 50% of loaded sample). The active fractions were pooled and sterile filtered and half of the active fraction was dialysed against 1M $(\text{NH}_4)_2\text{SO}_4$ at pH 7.0 in preparation for chromatography on Phenyl-Sepharose CL-4B (Pharmacia). A 7 ml sample was applied to 10 mls of Phenyl-

Sephacrose in a 0.9 cm diameter column with adaptor, the matrix having been equilibrated with 1M $(\text{NH}_4)_2\text{SO}_4$, (pH 7.0). The flow rate was 20 ml/hour and a 100 ml gradient of 1M $(\text{NH}_4)_2\text{SO}_4$, (pH 7.0) + 100mls H_2O was applied immediately after the addition of the sample and 2 ml fractions were collected. The activity was recovered in 2 fractions, which represented 2% of the total gradient (Yield = 27% of loaded sample).

Production of the thymic epithelial cell factor

The thymic epithelial cell factor was induced with either the macrophage factor or an antibody to a common determinant on Ia, as follows: Whole thymus cell suspensions were treated with a rat pan T cell marker, MRCOX52 and complement for thirty minutes at 37°C. The remaining cells were then fractionated on Percoll and the dense cell fraction which contained keratin-positive thymic epithelial cells was collected. These cells were divided into three aliquots and were cultured, in tissue culture flasks at a density of 1×10^8 cells/ml in RPMI 1640 medium. The first aliquot served as a control, the second aliquot was stimulated with 2.5 $\mu\text{g}/\text{ml}$ of MRCOX6 (the antibody to a common determinant on Ia) and the remaining suspension was stimulated with the macrophage factor (at dilution equivalent to 1/250 of the original macrophage supernatant). The cultures were incubated for three hours, after which the supernatants were collected. The resultant supernatants were tested in the thymocyte viability assay using a dense cell fraction depleted of Ia positive cells. The macrophage-induced thymic epithelial cell factor (MF-TECF) was active at a concentration of 1/5000 and the antibody-induced epithelial cell factor (MRCOX6-TECF) protected the

thymocytes at a dilution of 1/750 in the thymocyte viability assay. The control supernatant from unstimulated thymic epithelial cells was inactive in the thymocyte viability assay.

Physical properties of the thymic epithelial cell factor

The protective activity of the macrophage factor-thymic epithelial cell factor was destroyed by incubating the factor (at a 1/1000 dilution) with 1 mg/ml of trypsin. Activity was also destroyed by boiling the factor prior to testing it in the thymocyte viability assay, suggesting that it was probably protein.

The molecular weight of both MF-TECF and MRCOX6-TECF were determined by gel filtration fractionation on Sepharose CL-6B (Pharmacia). The results are shown in Figure 24. A column (100cm x 2.6 cm) was calibrated with ferritin (Mr 440,000), aldolase (Mr 158,000), albumin (Mr 68,000) and ovalbumin (Mr 43,000) in PBS with 0.1M NaCl and was run with a 1ml sample under total identical conditions. The MF-TECF eluted at a molecular weight of 320,000 and the MRCOX6-TECF eluted at 760,000.

A possible binding site for the MF-TECF was suggested by experiments using a monoclonal antibody to rat T helper cells which bound at CD4 (Sera Lab, Clone W3/25). Whole thymus suspensions were incubated with this antibody, Ia positive cells were removed and the remaining cells were fractionated on Percoll for use in the thymocyte viability assay. The addition of MF-TECF to this dense cell fraction failed to protect the

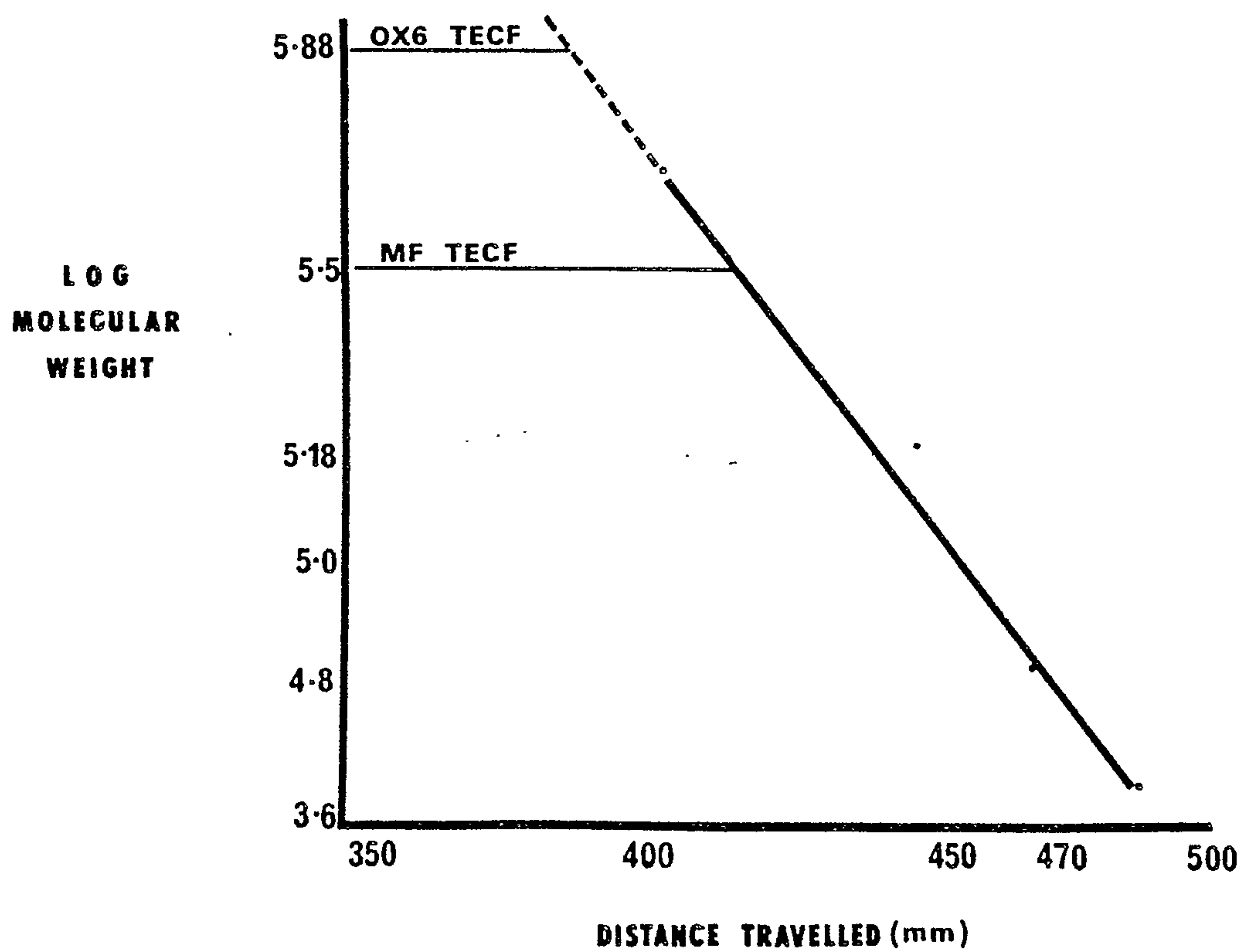


Figure 24. Molecular weight determination of the thymic epithelial cell factor using Sepharose CL-6B chromatography.

thymocytes, suggesting that the availability of CD4 molecules was important in this process.

Characteristics of the release of the thymic epithelial cell factor

The MF-TECF was prepared as previously described but supernatants were collected from cultures at various intervals between five minutes and three hours and tested in the thymocyte viability assay. As shown in Figure 25, low titres of activity are produced in the first fifteen minutes following which the titre increases steadily up to three hours. The addition of one of three protein synthesis inhibitors, actinomycin D, cycloheximide or puromycin had no effect on the production of MF-TECF, indicating that protein synthesis was not required.

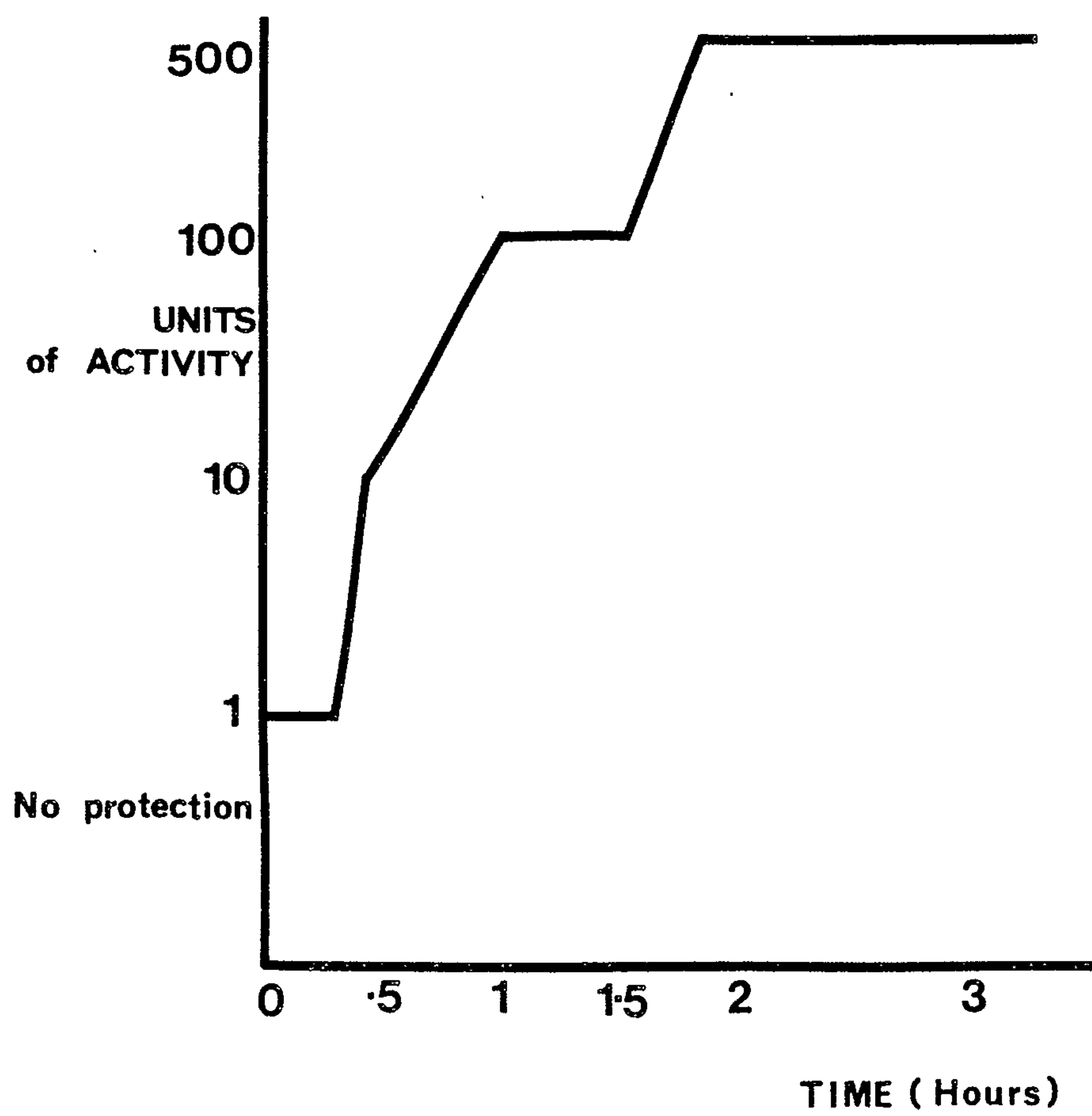


Figure 25. The time course for the production of the thymic epithelial cell factor.

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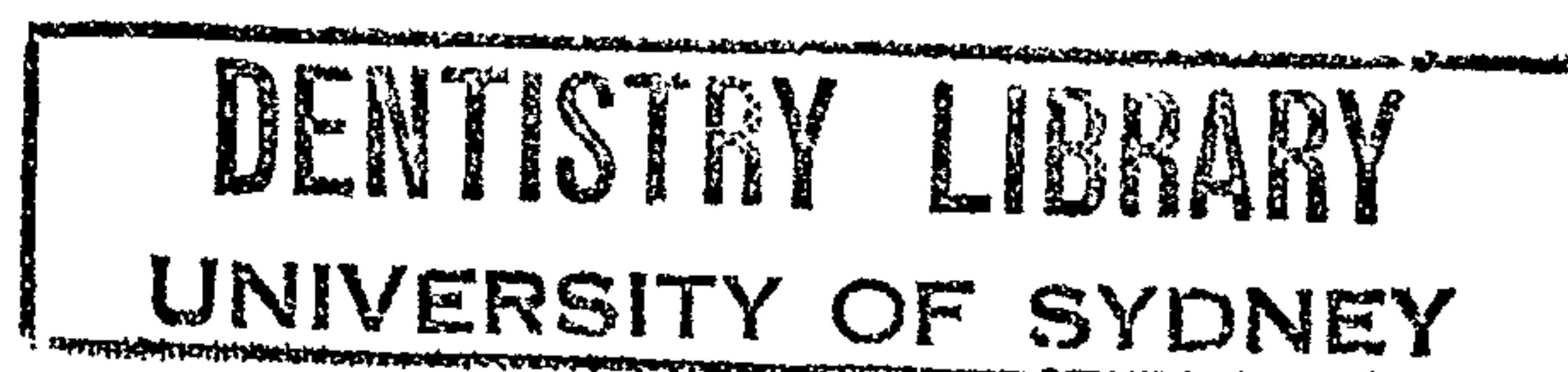
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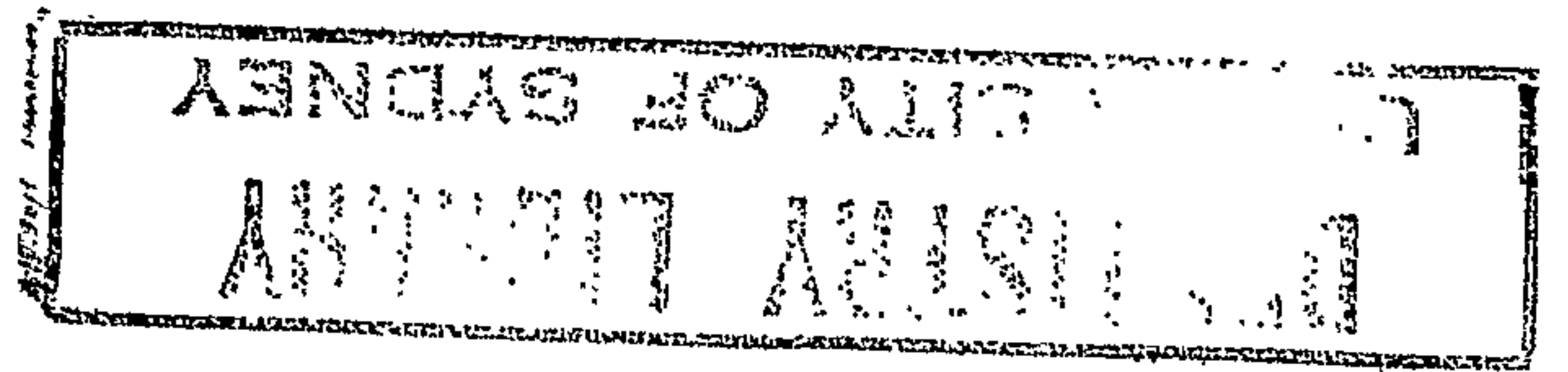
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ERRATA

Emendations are denoted by **£** in the text.

1. Page 22, line 6, "determinant" should be "antigen".
2. Page 25, last line, "H-A" should be "H-2".
3. Page 42, the second paragraph should be deleted as it repeats the first paragraph on page 41.
4. Page 44, line 14, "with activated T cells" should read "on activated T cells."
5. Page 44, last paragraph, the experiments of Muchmore et al., (1982), do not exclude steric hindrance as a mechanism in the antigen-specific proliferation of human lymphocytes, as is stated. The experiments imply that another mechanism exists for this process.
6. Page 45, last paragraph, "antibody-specific responses" should read "antigen-specific responses".
7. Page 48, line 6, "Ia receptor" is too specific and should read "Ia molecule".
8. Page 48, the experiments of Clement et al., (1986), although inconclusive, suggest that the HLA-DR molecule might be important in signal transduction.
9. Page 49, second paragraph, in the experiments of Corley et al., (1985), the ligand for Ia in B cell activation could be CD4.
10. Page 49, second last line, the antigen presenting cells are antigen-pulsed rather than antigen-stimulated.
11. Page 58, second paragraph, should read; "In this context, it is interesting to note the existence of a nude mutation in mice (Pantelouris, 1968) and a similar mutation in rats (Festing et al., 1978 and Douglas-Jones et al., 1985), both of which contain a small and highly abnormal thymus rudiment.

12. Page 63, second paragraph, line 7, "antigens" should be "proteins".
13. Page 74, the first sentence would be better supported by the following reference; Zinkernagel, R.M. and Doherty, P.C. (1974). Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature. 248, 701-702.
14. Page 146, line 7, "Balb-c" should be BALB/c.
15. Figure 23. A more appropriate title for the figure would be "Possible interactions in cell-mediated reactions" as there is no extraneous antigenic stimulation of T cells in the model presented and this is an essential element in the definition of cell-mediated immunity.