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INTERLEUKIN 1 PRODUCTION IN HEALTH AND DISEASE

BY

RENEÉ MITCHELL

Being a Dissertation presented in fulfilment of the requirement governing the Degree of Master of Science in The School of Medicine, University of the Witwatersrand

Johannesburg
1981

This is to certify that the Dissertation "Interleukin 1 Production in Health and Disease" presented for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg, is my own work and has not been presented at any other university.



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3. The research on which this Dissertation is based was commenced in January 1980 and completed in July 1981.

Dedication: To my mother and father, for their constant support and encouragement during my student years.

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ABBREVIATIONS

AB Serum	Human Allogeneic Serum
Ag	Antigen
AML	Acute Myeloid Leukaemia
AMML	Acute Myelo-Monocytic Leukaemia
BAF	B Cell Activating Factor
BCG	Bacillus Calmette and Guerin
BDF	B Cell Differentiating Factor
cAMP	Cyclic Adenosine Monophosphate
cc	Cubic Centimetre
cGMP	Cyclic Guanosine Monophosphate
CML	Chronic Myeloid Leukaemia
C3	Third Component of Complement
Con A	Concanavalin A
CPM	Counts per Minute
cRPMI	Complete Medium, that is, RPMI - 1640 Plus 10% AB Serum
CTL	Cytolytic
dBcGMP	dibutyryl Cyclic Guanine Monophosphate
DMSO	Dimethylsulphoxide
E. COLI	Escherichia Coli
FCS	Foetal Calf Serum
g	Gravity
G_o	Resting Period of Cell Cycle
G_1	First Growth Period of Cell Cycle
gr	Gramme
GRF	Genetically Related Factor
HHF	Human Helper Factor
HLA	Human Leukocyte Antigen

Ig	Immunoglobulin
IL - 1	Interleukin 1
IL - 2	INTERLEUKIN 2
LPF	Lymphocyte Potentiating Factor
LPS	Lipopolysaccharide
M	Molar
MAF	Macrophage Activating Factor
MCF	Macrophage Culture Fluid
MDP	Mycobacterial Muramyl Dipeptide
2ME	2 Mercapto - Ethanol
MER	Mycobacterial Methanol Extraction Residue
mg	Milligramme
MHC	Major Histocompatability Complex
ml	Millilitre
MLC	Mixed Lymphocyte Culture
MLR	Mixed Lymphocyte Reaction
mM	Millimolar
MN	Mononuclear Cells
Mϕ	Macrophage
$NaIO_4$	Sodium Periodate
NH_4 Cl	Ammonium Chloride
NMF	Non-Specific Macrophage Factor
PBS	Phosphate Buffered Saline
PHA	Phytohaemagglutinin
PMA	Phorbol Myristic Acetate
PNA	Peanut Agglutinin
PPD	Purified Protein Derivative
PWM	Pokeweed Mitogen
R - Cell	Responder Cell
RNA	Ribonuleic Acid

S	Synthetic Stage of Cell Cycle
S - Cell	Suppressor Cell
sIg	Surface Immunoglobulin
SLE	Systemic Lupus Erythematosus
TAF	T Cell Activating Factor
TCGF	T Cell Growth Factor (IL - 2)
TDF	Thymocyte Differentiating Factor
TL Ag	Thymic Leukaemia Antigen
TMP	Thymocyte Mitogenic Factor
µCi	Micro Curie
µg	Microgramme
WSA	Water Soluble Activity

LIST OF FIGURES

FIGURE		PAGE
1	Macrophage Maturation	2
2	Model for IL - 1 Production	23
3	Model for T Cell Activation	31
4	Model for B Cell Activation	33
5	Model for Cytotoxic T Cell Generation	35
6	IL - 1 (LAF) Production	39
7	Assay for IL - 1 (LAF)	41
8	Splenocyte & B Cell Preparation	87
9	IL - 1 (LAF) Induction of Helper Effect	89
10	Model for T Cell Activation	137

LIST OF GRAPHS

GRAPH		PAGE
1	Thymocyte Stimulation (CPM) by IL - 1 Supernatants	45
2	Thymocyte Stimulation by IL - 1 Supernatants (CPM) versus the concentration of LPS µg/ml required for optimum IL - 1 production	49
3	Thymocyte stimulation by IL - 1 Supernatants (CPM) versus time of LPS pulse (HRS) required for optimum IL - 1 production	52
4	Thymocyte stimulation (CPM) by IL - 1 Supernatants versus the adherent cell concentration (Cells/ml) required for optimum IL - 1 production	55
5	Thymocyte stimulation (CPM) by IL - 1 Supernatants versus the time (days) required for optimum IL - 1 production by adherent cells	58
6	Thymocyte stimulation (CPM) by IL - 1 Supernatants versus the time (days) required for IL - 1 to maximally enhance Thymocyte DNA synthesis	62
7	Thymocyte stimulation (CPM) by IL - 1 Supernatants versus the optimal number of Thymocytes (Cells/ml) required to assay lL- 1 Supernatants	65
8	Thymocyte stimulation (CPM) by IL - 1 Supernatants versus the dilution of IL - 1 activity	68
9	Thymocyte stimulation by IL - 1 Supernatants (Stimulation Index) of SLE patients and normal control	80

GRAPH PAGE

10 Thymocyte stimulation (CPM) by IL - 1 Supernatants and the effect on stimulation by Thymic Hormone pretreatment of the Thymocytes 117

11a Thymocyte stimulation (CPM) by IL - 1 Supernatants versus PHA concentration (μg/ml) - suboptimal doses 145

11b Thymocyte stimulation (CPM) by IL - 1 Supernatants versus PHA concentration (μg/ml) - optimal doses 146

12a Thymocyte stimulation (CPM) by IL - 1 Supernatants versus Con A concentration (μg/ml) - suboptimal doses 152

12b Thymocyte stimulation (CPM) by IL - 1 Supernatants versus Con A concentration (μg/ml) - optimal doses 153

13 Thymocyte stimulation (CPM) by IL - 1 Supernatants versus the concentration of Con A (μg/ml) and the effect of Thymic Macrophage depletion of Thymocytes on this stimulation 157

LIST OF TABLES

TABLE		PAGE
1a	IL - 1 Production by Normal Peripheral Blood Adherent Cells	43
1b	Means and SD of the Ten Experiments in Table 1a showing IL - 1 Production by Normal Peripheral Blood Adherent Cells	44
2a	LPS Concentration required for Optical IL - 1 Production	47
2b	Means & SD of the three Experiments in Table 2a showing LPS Concentration for Optimal IL - 1 Production	48
3a	LPS Pulsing Experiment	50
3b	Means & SD of the LPS Pulsing Experiments in Table 3a	51
4a	Adherent Cell (Monocyte) Concentration for Optimal IL - 1 Production	54
4b	Means & SD of the Experiments in Table 4a showing the Monocyte Concentration for Optimal IL - 1 Production	54
5a	Optimum time for IL - 1 Production	56
5b	Means & SD of the three IL - 1 Production Kinetic Experiments shown in Table 5a	57
6a	Time required for IL - 1 to act on Thymocytes	60

TABLE PAGE

6b Means & SD of the three Experiments in Table 6a showing the time required for IL - 1 to act on Thymocytes 61

7a Optimal number of Thymocytes required to Assay IL - 1 63

7b Means & SD of three Experiments in Table 7a showing the Optimal number of Thymocytes required to Assay IL - 1 64

8a Diluting out IL - 1 Activity 66

8b Mean & SD of the three experiments in Table 8a showing the Diluting out of IL - 1 Activity 67

9 IL - 1 production in SLE as compared to normal controls. 78

10 Suppressor Cell Assay showing if IL - 1 induces Suppressor Cells in the Thymocyte Population 93

11 Helper Cell Induction of B Cell Proliferation 95

12 Optimum time for Thymocyte Pretreatment with IL - 1 for useage in the Helper Cell Assay shown in Table 11 96

13 Optimum Thymocyte to B Cell Ratios for useage in the Helper Cell Assay shown in Table 11 98

14 Optimum time for Thymocyte & B Cell Coculture for useage in the Helper Cell Assay shown in Table 11 99

TABLE		PAGE
15	IL - 1 Pretreated Thymocyte Induction of Immunoglobulin Secreting Cells	100
16a	The effect of Thymic Hormones on Thymocytes as compared to that of IL - 1	110
16b	Mean & SD of the three experiments in Table 16a indicating the effect of Thymic Hormones on Thymocytes as compared to that of IL - 1	111
17a	The Coaction of IL - 1 & Thymic Hormones on Thymocytes	112
17b	Means & SD of the three experiments in Table 17a showing the Coaction of IL - 1 & Thymic Hormones on Thymocytes	113
18a	The effect of Thymic Hormone Pretreatment of Thymocytes on their response to IL - 1	115
18b	The mean & SD of the three experiments in Table 18a showing the effect of Thymic Hormone Pretreatment of Thymocytes on their response to IL - 1	116
19	Experiment indicating whether Thymic Hormones Induce Suppressor Cells in the Thymocyte population	118
20	Suppressor Cell Induction by Sodium Periodate in Mononuclear Cells	129
21a	The effect of Sodium Periodate Suppressor Cells on IL - 1 Production by Adherent Cells	130

TABLE		PAGE
21b	Mean & SD of the three experiments in Table 21a showing the effect of Sodium Periodate Suppressor Cells on IL - 1 Production by Adherent Cells	131
22	The Effect of Human Helper Factor on IL - 1 Production by Adherent Cells	233
23	The Effect of Hydrocortisone Pretreatment of Adherent Cells on IL - 1 Production by these Cells	135
24a	The Effect of IL - 1 on the Response of Thymocytes to Suboptimal doses of PHA	143
24b	The Mean & SD of the three Experiments in Table 24a showing the effect of IL - 1 on the Response of Thymocytes to Suboptimal doses of PHA	144
24c	The Effect of IL - 1 on the Response of Thymocytes to Optimal doses of PHA	148
24d	The Mean & SD of the three Experiments in Table 24c showing the Effect of IL - 1 on the Response of Thymocytes to Optimal doses of PHA	149
25a	The Effect of IL - 1 on the Response of Thymocytes to Suboptimal doses of Con A	150
25b	The Mean & SD of the three Experiments in Table 25a showing the Effect of IL - 1 on the Response of Thymocytes to Suboptimal doses of Con A	151

TABLE PAGE

25c The Effect of IL - 1 on the Response of Thymocytes to Optimal doses of CON A 154

25d The Mean & SD of the three Experiments in Table 25c showing the Effect of IL - 1 on the Response of Thymocytes to Optimal doses of CON A 155

26 The Effect of Adherent Cell Depletion of Thymocytes on their Response to CON A & the Effect of IL - 1 on the System. 156

SUMMARY

Interleukin 1 (IL - 1) is a macrophage factor that exerts control over T cell activation. The experimental work presented in this dissertation consists of studies on IL - 1 production by monocytes which can be used as an in vitro correlate for monocyte function, as well as the effect of IL - 1 on immature T cells, that is, thymocytes.

In accomplishing the studies presented in this dissertation, a two stage assay was employed. Firstly IL - 1 containing supernatants were produced by stimulated human peripheral blood adherent cells and secondly, the IL - 1 supernatants were assayed on mouse thymocyctes in which these supernatants caused mitogenesis.

A routine assay for IL - 1 containing supernatant production was established. It was found that the strongest IL - 1 supernatants were produced when 5×10^5 adherent cells per millilitre were cultured in the presence of 20 μg/ml LPS for 24 hours. The assay for IL - 1 was found to be most accurate when 10 μl of supernatant was cultured in the presence of 0.1 ml of 10×10^6/ml mouse thymocytes for 72 hours. Mitogenesis of the thymocytes was indicated by 3H - thymidine incorporation as measured by liquid scintillation counting.

IL - 1 production in a number of SLE patients was compared to that of normal controls by the above method. The production of IL - 1 in SLE patients was found to be within the range of the normal controls.

Studying the effects of IL - 1 on thymocytes it was found that IL - 1 caused thymocytes to develop into T helper cells as indicated by their ability to:-

(1) cause an increase in mitogenesis in coculture with B cell enriched splenocytes plus PWM

(2) cause an increase in immunoglobulin secreting cells when cocultured with sensitized splenocytes plus sensitizing antigen, as assayed by immunofluorescence.

Since IL - 1 is secreted by one cell type, namely the macrophage, and acts on another cell type, namely the immature or mature T cell its action was compared with that of thymic hormones thymopoietin, thymic humoral factor and thymosin. Thymic hormones did not cause mitogenesis in thymocytes as does IL - 1. However IL - 1 may have certain maturational effects on thymocytes similar to those of thymic hormones, although this has not been clearly defined.

The effect of sodium periodate induced suppressor cells, human helper factor, and hydrocortisone pretreatment of macrophages on IL - 1 production was studied. It was found that - (1) sodium periodate induced suppressor cells had no effect on IL - 1 production (2) human helper factor enhanced IL - 1 production and (3) hydrocortisone pretreatment of macrophages suppressed their secretion of IL - 1. This could be important in understanding the control mechanisms operative in the cell mediated and humoral immune responses.

Mitogen responsiveness of thymocytes in the presence or absence of IL - 1 was studied and indicated that, thymocytes alone were unresponsive to PHA but IL - 1 caused them to respond slightly to PHA. Thymocytes responded greatly to the mitogenic effects of CON A. In the presence of IL - 1 the responses to low doses of CON A were enhanced whereas the responses to high doses were depressed. Furthermore, if thymocytes were depleted of adherent cells, in the presence of IL - 1 the responses

to low doses of CON A remained enhanced whereas the responses to high doses were no longer depressed. This may be important in indicating the role of macrophages in controlling responses of immune cells to mitogens.

Consequently, the effect of IL - 1 on mature peripheral blood effector T cells and their functions, such helper and suppressor and cytotoxic cell induction and factor production by these cells is being studied. The view to these studies is the elucidation of the role of accessory cells and their products in the control of the immune response.

INDEX

	PAGE NO.
ACKNOWLEDGEMENTS	i
ABBREVIATIONS	ii - iv
LIST OF FIGURES	v
LIST OF GRAPHS	vi - vii
LIST OF TABLES	viii- xii
SUMMARY	xiii- xv
CHAPTER ONE	
1. GENERAL INTRODUCTION	1
1.1 Macrophages	1
1.1.1 Life History of Macrophages	1
1.1.2 Phylogeny	1
1.1.3 Ontogeny	3
1.1.4 Maturation	3
1.1.5 Structure	4
1.1.6 Metabolism	5
1.1.7 Activation	5
1.1.8 Function	11
1.2 Interleukin - 1	17
1.2.1 Production of IL - 1	17
1.2.1.1 Normal Cell Sources of IL - 1	17
1.2.1.2 Inducing Agents	18
1.2.1.3 Conditions for the Production of IL - 1	20
1.2.1.4 Mechanism of IL - 1 Production	20
1.2.1.5 IL - 1 Production by Macrophage Cell Lines	24
1.2.2 Purification and Physical Properties of IL-1	
1.2.2 Dialysis of IL - 1	
1.2.2.1 Purification of Crude IL - 1	24

PAGE NO.

1.2.2.3 Heat Stability and pH Sensitivity 26

1.2.2.4 Effect of Chemicals and Enzymes on IL - 1 Activity 27

1.2.3 Effects of IL - 1 28

1.2.3.1 Target Cells 28

1.2.3.2 Reaction of Target Cells 28

1.2.4 Models for IL - 1 Function 30

1.2.4.1 T Cell Activation 30

1.2.4.2 B Cell Activation 32

1.2.4.3 Cytotoxic T Cell Generation 34

CHAPTER TWO 36

2. SETTING UP OF A ROUTINE ASSAY FOR IL - 1 PRODUCTION AND ASSESSMENT 37

2.1 Introduction 37

2.2 Materials and Method 38

2.2.1 IL - 1 Supernatant Production 38

2.2.2 Assay for IL - 1 Action 40

2.2.2.1 Thymocyte Preparation 40

2.2.2.2 IL - 1 Supernatant Assay 40

2.3 Results 42

2.3.1 Experiments Indicating IL - 1 Production by Normal Peripheral Blood Adherent Cells 42

2.3.2 The Optimum Amount of LPS Required for Monocyte Stimulation 42

2.3.3 LPS Pulsing Experiments 46

2.3.4 The Optimum Adherent Cell Concentration Required for IL - 1 Production 46

2.3.5 The Optimum Time for IL - 1 Production by Monocytes 53

2.3.6 Time Taken for IL - 1 to Act on Mouse Thymocytes 53

2.3.7 The Optimum Number of Thymocytes Required To Assay IL - 1 59

2.3.8 The Effect of Diluting a Stock IL - 1 Containing Supernatant on Thymocyte Stimulation 59

2.4 Discussion 59

2.4.1 E. Coli Lipopolysaccharide Stimulated "Adherent Cells" Secrete IL - 1 Containing Supernatants 59

PAGE NO.

2.42 Action of IL - 1 on Thymocytes 73.

CHAPTER THREE

3. IL - 1 PRODUCTION IN PATIENTS WITH SLE AS COMPARED TO NORMAL CONTROLS 76

3.1 Introduction 76

3.2 Materials and Methods 77

3.2.1 Patients & Controls 77

3.2.2 Experimental Design 77

3.3 Results 79

3.4 Discussion 79

CHAPTER FOUR 83

4. THE EFFECT OF IL - 1 CONTAINING SUPERNATANTS ON THYMOCYTES 84

4.1 Introduction 84

4.2 Materials and Methods 85

4.2.1 Suppressor Assay 85

4.2.2 B Cell Proliferative Response as an Assay For Helper Cells 86

4.2.3 Immunofluorescent Studies to Assess the Number of Immunoglobulin Secreting Cells Stimulated by Helper Cells Induced by IL - 1 Pretreated Thymocytes 90

4.2.3.1 Cell Culture Preparations 90

4.2.3.1 Preparation and Staining of Slides 91

4.3 Results 91

4.3.1 Results Indicating Whether IL - 1 Induces Suppressor Cells in the Thymocyte Population 91

4.3.2 Helper Cell Assay Results 92

4.3.2.1 Helper Cell Proliferative Assay 92

4.3.2.2 Optimal Time for Preculture of Thymocytes with IL - 1 Supernatants to Stimulate Helper Cell Development 94

4.3.2.3 Optimal Cell Ratios for the Helper Cell Assay 94

4.3.2.4 Optimal Culture Time for the Coculture of Pretreated Thymocytes Plus B Cells Plus PWM to Show the Helper Ability of Thymocytes 97

4.3.3 IL - 1 Induction of Thymocyte Helper Cells as Shown by Their Causing the Development of Immunoglobulin Secreting Cells 97

PAGE NO.

4.4 Discussion 101

CHAPTER FIVE 104

5. IL - 1 CONTAINING SUPERNATANT ACTION ON THYMOCYTES AS COMPARED TO THE ACTION OF THYMIC HORMONES 105

5.1 Introduction 105

5.2 Materials and Methods 105

5.2.1 To Investigate Whether Thymic Hormones Have a Similar Effect to IL - 1 Supernatants on Thymocyte Mitogenesis 105

5.2.2 To Investigate Whether Thymic Hormones and IL - 1 Supernatants Act Synergistically on Thymocytes 106

5.2.3 To Investigate Whether Preincubation of Thymocytes with Thymic Hormones Has Any Effect on the Response of These Cells to IL - 1 Containing Supernatants 107

5.2.4 To Investigate Whether Thymic Hormones Stimulate the Development of Suppressor Cells in Thymocyte Populations 108

5.3 Results 109

5.3.1 Results of Experiments to Investigate the Effects of Thymic Hormones on Mitogenesis of Thymocytes 109

5.3.2 Results to Indicate Whether Thymic Hormones and IL - 1 Supernatants Act Synergistically on Thymocytes 109

5.3.3 Results to Indicate Whether Pretreatment of Thymocytes with Thymic Hormones Results in a Reduction in their Response to IL - 1 114

5.3.4 Results Indicating Whether Thymic Hormone Pretreatment of Thymocytes Results in the Development of a Suppressor Cell Subset in the Thymocyte Population 114

5.4 Discussion 119

CHAPTER SIX 123

6. THE EFFECT OF SUPPRESSOR CELLS, HELPER FACTOR AND HYDROCORTISONE ON IL - 1 PRODUCTION 124

6.1 Introduction 124

6.2 Materials and Methods 124

6.2.1 The Effect of Suppressor Cells (Sodium Periodate Induced) on IL - 1 Production by Macrophages 124

PAGE NO.

6.2.1.1 Production of Sodium Periodate Suppressor Cells 125

6.2.1.2 Testing for the Production of Suppressor Cells by Suppression of 3H-Thymidine Incorporation 125

6.2.1.3 The Effect of Suppressor Cells on IL - 1 Supernatant Production 126

6.2.2 The Effect of Helper Factor on IL - 1 Production by Macrophages 126

6.2.2.1 Helper Factor Production 126

6.2.2.2 The Effect of Helper Factor on IL - 1 Production by Adherent Cells 127

6.2.3 The Effect of Cortisone Pretreatment of Adherent Cells on IL - 1 Production 127

6.2.3.1 Hydrocortisone Pretreatment of Adherent Cells 127

6.2.3.2 IL - 1 Production by Hydrocortisone Pretreated Adherent Cells 127

6.3 Results 128

6.3.1 The Effect of Sodium Periodate Suppressor Cells on IL - 1 Supernatant Production by Macrophages 128

6.3.1.1 Results Showing the Induction of Suppressor Cells by $NaIO_4$ 128

6.3.1.2 Results Showing the Effect of $NaIO_4$ Suppressor Cells on IL - 1 Supernatant Production by Macrophages 128

6.3.2 The Effect of HHF on IL - 1 Production by Adherent Cells 128

6.3.3 The Effect of Hydrocortisone Pretreatment of Adherent Cells on Their IL - 1 Production 132

6.4 Discussion 134

6.4.1 Suppressor Cell Effect on IL - 1 Production 134

6.4.2 HHF Effect on IL - 1 Production 134

6.4.3 Hydrocortisone Pretreatment of Adherent Cells on their IL - 1 Production 136

CHAPTER SEVEN 140

7. MACROPHAGES, MITOGENS AND IL - 1 141

7.1 Introduction 141

7.2 Materials and Methods 141

7.2.1 The Effect of Mitogens CON A and PHA on Thymocytes and Modulation of These Effects by IL - 1 141

PAGE NO.

7.2.2 The Effect of Adherent Cell Depletion of Thymocytes on their response to CON A and PHA and Modulation of the Response by IL - 1 142

7.3 Results 142

7.3.1 The Effect of IL - 1 on the Response of the Thymocytes to CON A and PHA 142

7.3.1.1 Suboptimal Doses 142

7.3.1.2 Optimal Doses 142

7.3.2 Con A Responses 147

7.3.2.1 Suboptimal Doses 147

7.3.2.2 Optimal Doses 147

7.3.3 The Effect of Adherent Cell Depletion of Thymocytes on their Response to CON A including the effect of IL - 1 on the system 147

7.4 Discussion 158

7.4.1 Thymocyte Response to Mitogens 158

7.4.2 IL - 1 Mitogens and Thymocyte responses 159

7.4.3 Thymic Macropages, their effect on Thymocyte responses to Mitogens and Modulation by IL - 1 161

CHAPTER EIGHT 163

8. Appendices and References 164

8.1 Appendix 164

8.1.1 Sources of Materials 164

8.1.2 Sources of Equipment

8.2 References

1. GENERAL INTRODUCTION

1.1 Macrophages

1.1.1 Life history of macrophages

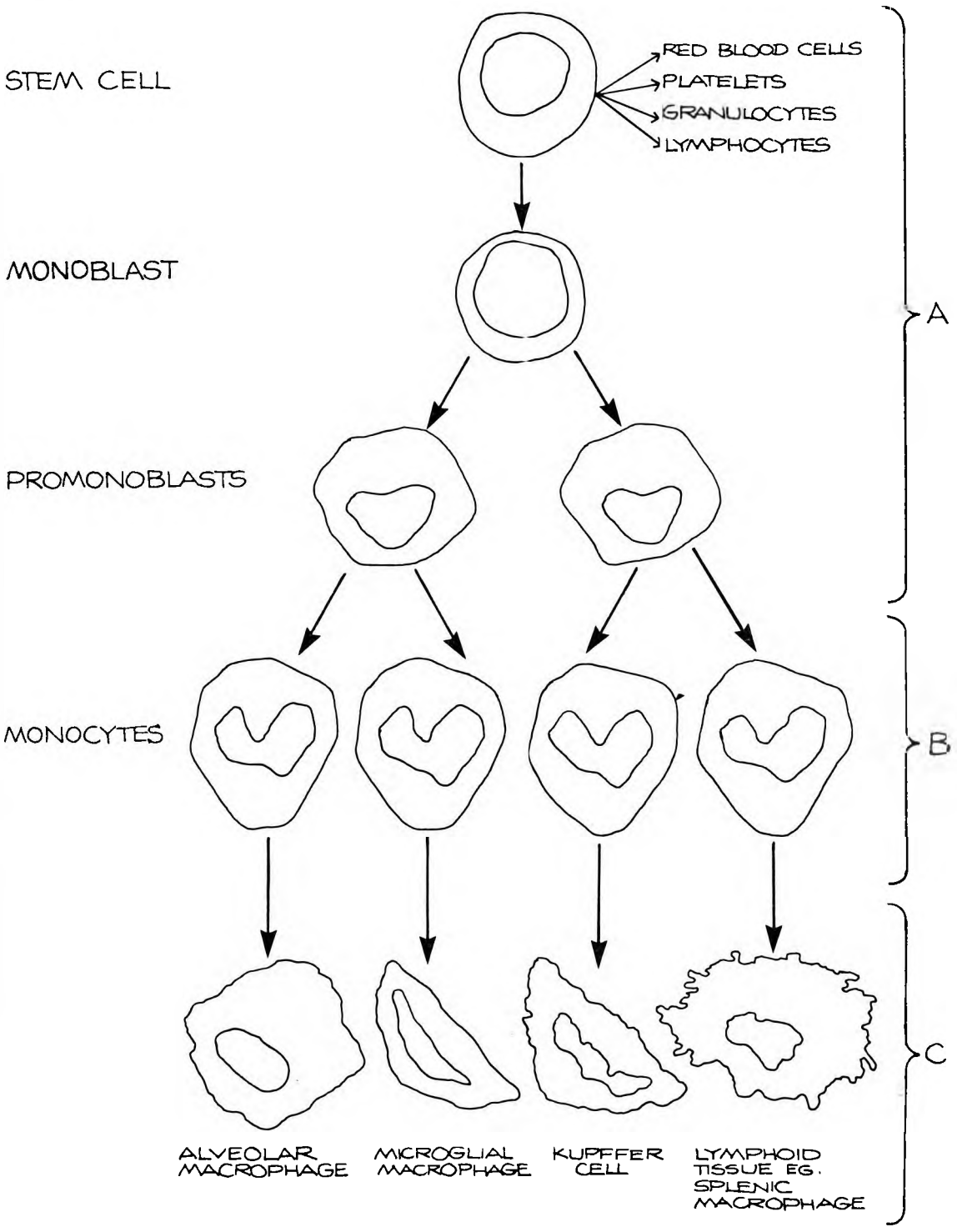
As early as the 1800's the macrophage was postulated to be an essential element in host defence (Metchnikoff, 1884; Virchow, 1885). "The Theory of Phagocytes" was formulated by Metchnikoff (Metchnikoff, 1905) based on the observation that amoeboid cells of mesodermal origin have the ability to engulf foreign matter. Such cells have been observed throughout the phylogenetic scale and studied in vertebrates as well as invertebrates (Albrecht and Hong, 1976). For a number of years the role of the macrophage in both cellular and humoral immunity was largely ignored. However, recently, with the renewed interests and major advances in cell mediated immunity, and its role in acquired cellular resistance, tumor immunology, transplantation immunology, as well as numerous other immunological processes, the complex and critical role of the macrophage as the central controlling cell in both cell mediated and humoral immunity has been made increasingly evident.

1.1.2 Phylogeny

The macrophage serves both in defence mechanisms and in the digestive process in early arising species, but becomes mainly concerned with host defences as the phylogenetic tree is ascended. Certain invertebrates as well as lower vertebrates, such as, the hagfish, show an essentially non-specific macrophage dependent immune response, while slightly more advanced vertebrates, such as, the lamprey, guitarfish and haddelfish, begin to exhibit

FIG. 1: MACROPHAGE MATURATION

A BONE MARROW
B: BLOOD
C PERIPHERAL TISSUES



specificity and rudimentary memory in their immune responses (Good and Papermaster, 1964; Anderson and Mattiesen, 1966). The intricate immune system of mammals has evolved from these earlier forms with the addition of other constituents such as, lymphocytes and numerous others, while still retaining the macrophage as the central cell, controlling both afferent and efferent limbs of the immune response.

1.1.3 Ontogeny

Macrophages are observed relatively early in embryonic development (Albrecht and Hong, 1976) and are known to play a role in tissue resorption in association with tissue remodelling in embryonic development, having developed their synthetic and secretory functions earlier (Anderson and Mattiesen , 1966). Studies in animal models have shown an increase in macrophage function throughout foetal and neonatal life. Furthermore, at birth a large number of macrophage functions appear to be underdeveloped or immature (Reade, 1968).

1.1.4 Maturation

Macrophages arise chiefly from bone-marrow stem cells from which the monoblast, promonocyte and finally monocyte develops. The monocytes circulate briefly in the blood and then migrate into various tissues where they mature both functionally and morphologically to become a typical tissue macrophage (Van Furth and Cohn, 1968) (see Figure I). It should be noted that as the macrophage matures its phagocytic ability increases, it acquires more surface receptors and greater endocytic capacity than the promonocyte or monocyte which only has C3 and Fc receptors but few others (Unanue, 1979). Moreover, localized

tissue macrophages such as those in the peritoneal cavity and lung proliferate when activated by non-specific or specific antigenic stimuli, and inducer substances produced by fibroblasts or stimulated lymphocytes (Forbes, 1966; Ichikauea et al., 1966; Hadden et al., 1975). There is also experimental data indicating that cells having the morphology of small lymphocytes differentiate into macrophages following prolonged reticuloendothelial stimulation or in response to polymorphonuclear secretory products as well as their degradation products (Elves, 1967; Gough et al., 1965). The lifespan of various macrophages in vivo varies greatly, but may be many months (Albrecht and Hong, 1976). Mature mouse peritoneal macrophages have been sustained in vitro for several months (Chang and Andersen, 1974) and macrophage cell lines have been established.

1.1.5 Structure

The morphology of macrophages found in different tissues varies, but the general structure of macrophages when grown in vitro is as follows:-

The nucleus is oval or kidney shaped, eccentrically situated and surrounded by a well defined centrosphere containing secondary lysosomes and endocytic vesicles. The cytoplasmic components consist of rod shaped mitochondria which are often clustered near the centrosphere, but are also scattered in the cytoplasm; numerous types of vesicles; rough endoplasmic reticulum; ribosomes and polyribosomes (Nicols and Bainton, 1975; Cohn, 1975). Microtubules, which appear to originate at the centrioles, radiate throughout the cytoplasm providing rigidity and cellular organization. Pinocytotic vesicles from

the periphery seem to move along the microtubules. The macrophage surface consists of numerous undulating ruffled membranes, ridges, flaps and microprocesses (Carrel and Ebeling, 1926; Albrecht et al., 1972).

1.1.6 Metabolism

The energy source of the macrophage is via both anaerobic and aerobic respiration. Depending on the type of macrophage, either one or the other energy source may predominate (Oren et al., 1973). A wide range of enzyme activities have been found in macrophages of different locations and different species (Pearsall and Weiser, 1970). Some of these include lysozyme, acid phosphatases, ribonucleases, esterases, lipases, dehydrogenases, proteases and proteinases. The quantity of enzyme present varies depending on the state of activation of the macrophage (Cohn, 1975; Wahl et al., 1975; Werb and Gordon, 1975a; Werb and Gordon, 1975b). Generally the enzyme activity is lower in the blood monocyte than in the more mature tissue macrophage, however, there are a few exceptions (Pearsall and Weiser, 1970; Van Furth, 1974).

1.1.7 Macrophage activation

Macrophages are said to be "activated" when they show enhanced bactericidal properties and increased cytotoxic activity against target cells. On the other hand, "stimulated" macrophages show less marked functional changes that do not include bactericidal or cytocidal effects (Ögmundsdóttir and Weir, 1980).

1.1.7(1) Agents "stimulating" macrophages

Such agents include mineral oil, thioglycollate, casein, proteose peptone broth and glycogen. Morphological changes that occur when macrophages are stimulated include an increase in:- size; extent of spreading; membrane ruffling; pseudopodia prominence; cytoplasmic granules and vacuoles (Hirsch and Fedorko, 1970; Carr and Carr 1970). An important characteristic of stimulated macrophages is that they can be activated by agents that normally do not bring about activation (Christie and Bomford, 1975).

1.1.7(2) Agents "activating" macrophages

Activating agents include:

(a) Pathogens such as mycobacteria, listeria, brucella (Mackaness, 1970; North, 1969; Ruskin et al., 1969; Hibbs et al., 1972), toxoplasma, trypanosomes and besnoilia (Ruskin et al., 1969; Hibbs et al., 1972; Remington et al., 1972; McLeod and Remington, 1977), as well as trichinella spiralis (Wing et al., 1977). In the before mentioned cases activation is dependent upon T lymphocytes sensitized with the activating agent. The resultant activation is however non-specific. Macrophages can be activated in vitro by exposure to sensitized T lymphocytes in the presence of sensitizing antigen (Simon and Sheagren, 1971, 1972; Krahenbuhl and Remington, 1971) or supernatants of such lymphocytes (Fowles et al., 1973; Davis and Remold, 1976).

(b) Polyanions viz. double stranded RNA, bacterial endotoxin and pyran copolymer (Evans and Alexander, 1976; Baird and Kaplan, 1975; Schultz et al., 1977a and b). Prolonged culture with mycobacteria and mycobacterial extracts and Group A Streptococcal cell wall activates macrophages (Bruley-Rosset et al., 1976; Schwab and Smialowicz, 1975). The above substances do not require lymphocytes for their activating ability and it is queried whether the action is mediated through alternate complement pathway components or interferon, both of which have macrophage activating ability (Schorlemmer and Allison, 1976; Ferluga et al., 1978; Schultz et al., 1977a and b).

(c) Immunoglobulin, either in the form of immune complexes or aggregated immunoglobulin, render macrophages non-specifically cytotoxic (Evans, 1975; Evans and Alexander, 1976).

1.1.7(3) Mechanisms of macrophage activation

1.1.7(3).1. Binding of activating agents to macrophage plasma membranes:- As discussed before there are three categories of activating agents viz. components of immunoglobulin and complement, products of activated lymphocytes, and non-immunological agents.

(a) Binding of immunoglobulin and complement:- Macrophage plasma membranes have specific receptors for the Fc portion of immunoglobulin. The Fc receptor also binds C reactive protein (Mortensen and Duszkievicz, 1977). The macrophage

receptor for complement is separate from the Fc receptor. Human monocytes can bind opsonized particles coated with C3b, C3d and C4 (Ross and Polley, 1975). The complement receptor only mediates phagocytosis if the macrophage is stimulated or activated (Bianco, et al, 1975; Morland and Kaplan, 1977).

(b) Binding of lymphocyte products :-

A receptor probably exists and is thought to be either a glycoprotein (Remold, 1973; Rocklin, 1976) or glycolipid (Higgins et al., 1978).

(c) Binding of non-immunological activating agents:- These substances act on the macrophage membrane without the intervention of the specific immune response. Little is known concerning the nature of binding, or receptors for these products. A number of theories as to their mode of action do exist and are as follows:-

(i) The physicochemical interaction depending on the hydrophobicity of the binding agent (van Oss and Gillman, 1972a and b; Wilkinson, 1976; Tagesson, et al, 1977). It has been suggested that membrane lipids are important in the binding of hydrophobic molecules (Wilkinson, 1975, 1976) such as casein, endotoxin activated serum or denatured albumin.

(ii) Concerning synthetic chemotactic agents. There are specific receptor sites on macrophages. The binding is irreversible and has chemical specificity. These substances are similar to the chemotactic substances derived from bacteria and it is therefore suggested that a general recognition mechanism exists for these products characteristic for prokaryotic cells (Schiffman, et al, 1975); Williams et al., 1977).

(iii) Binding could possibly involve surface glycoproteins (Bona, et al., 1968; Rabinovitch, 1967) as well as cell surface negative charges. A decrease in the negative cell surface charge increases phagocytosis and vice-versa (Nagura, et al., 1977). The process also involves divalent cations (Wilkins and Bangham, 1964) and could result from a bridging between hydroxyl groups in membrane glycoprotein and some component of the activating agent via ions.

1.1.7(3).2. Stimulus-response coupling in macrophage activation

(a) Signal transmission in cellular stimulation.

The metabolic stimulation of a cell by interaction with a drug or hormone with the plasma membrane is initiated by the transmission of an activating signal across the membrane. The process involves a second

messenger viz. cAMP. When signal transmission is mediated via a cAMP increase, the hormone receptor itself may be part of adenylcyclase which catalyzes the formation of cAMP, or binding may be as a result of a complex forming between the receptor and enzyme leading to enzyme activation (Rimon et al., 1978). The increase in cAMP leads to a cascade of enzymatic reactions (Robison et al., 1968; Catt and Dufau, 1977). cAMP can mediate stimulation and inhibition in the immune response and its actions are often opposed by cGMP. Cells stimulated by hormones have an advanced turnover of the minor membrane lipid phosphatidylinositol, which may induce Ca^{++} influx required for an increase in cGMP but not cAMP (Michell et al., 1977). In the case of macrophage activation, cGMP appears to be the second messenger, which is feasible, since cGMP promotes a number of immune functions whereas cAMP appears to inhibit these same functions (Ögmundsdóttir and Weir, 1980).

(b) Surface interactions in macrophage activation

When macrophage activation follows interaction with lymphocytes, lymphokines, immunoglobulin or complement, it is probably mediated by binding to specific receptors. It is however unlikely that specific receptors exist for all directly acting polyanions. The possibility exists that some reagents cross-react with receptors for physiological mediators (Ögmundsdóttir and Weir, 1980).

1.1.8 Macrophage function

The macrophage seems to be the central regulatory cell in the immune response. Classically the macrophage is regarded as the "phagocyte", but they are also highly secretory cells, both functions being closely related, that is, phagocytosis tends to stimulate secretion (Unanue, 1979). The role of the macrophage as a lymphocyte regulatory cell is most significant in the immune response and will be discussed further.

1.1.8.1 Macrophage - lymphocyte interactions

To understand the complex association between macrophages and lymphocytes it is necessary to briefly preview the phagocytic process. Phagocytosis consists basically of two steps (1) attachment - here the macrophage, by means of its membrane receptors, binds the antigen, and (2) interiorization - which consists of invagination of the cell membrane resulting in the formation of an endocytic vesicle surrounding the antigenic particle. The endocytic vesicle moves into the cell, becomes attached to a lysosome which releases its enzymatic contents into the vesicle, thereby digesting the foreign particle. Phagocytosis is aided if the foreign antigen is coated with antibody or C3, because it then binds to the macrophage membrane via its Fc or C3 receptor. Presentation of antigen by macrophages can occur in one of two ways. Firstly, a few molecules of antigen may remain surface bound, although the bulk of the molecules become internalized (Unanue et al., 1969; Unanue and Cerottini, 1970). Secondly, a few molecules of partially degraded antigen may be released from the macrophage by exocytosis and be found adhering to the membrane (Cruchard and Unanue, 1971; Calderon and

Unanue, 1974). Either of these antigen forms can then be recognized by immune lymphocytes. It should be further noted that the surface bound antigen must in some way be associated with major histocompatability complex (MHC) gene products before being able to be recognised by T lymphocytes (Erb and Feldmann, 1975; Rosenthal and Shevach, 1973; Barcenski and Rosenthal, 1977; Swartz and Paul, 1976; Pierce et al., 1976). It is not yet known whether MHC gene products regulate a step in antigen processing or whether the antigen becomes involved in a physical association with the MHC product or both (Unanue, 1978). Furthermore, it seems that only a certain subset of macrophages namely the Ia positive macrophage (in the case of the mouse) are involved in antigen presentation (Rosenthal and Shevach, 1973).

On the basis of the before-mentioned, two alternative mechanisms for macrophage-bound antigen interaction with lymphocytes have been proposed. Firstly, macrophages present antigen through membrane-bound molecules that are recognised by the B cell, since B cells are known to recognize native antigen molecules (Sela et al., 1967). Secondly, the T cell recognizes antigen that has been processed through an MHC controlled step (Unanue, 1978). The T cell then responds, proliferates and/or secretes mediators that influence other T cells, B cells or macrophages. It has also been hypothesized that in the case of T-B cell collaboration, which takes place effectively with macrophage associated antigen under no MHC control, it is the B cell that first recognizes the native antigen on the macrophage cell surface. The B cell then processes the antigen and presents it to the T cell. The B cell handling the antigen is under the genetic control of the MHC (Katz et al., 1973; Schreiner and

Unanue, 1976). Therefore, it seems that T cells can recognize antigen processed either by macrophages or B cells, but the macrophages recognize many antigens whereas the B cell recognizes only those with specificity for its surface antigen (Unanue, 1976).

In conclusion it appears that interactions between macrophages and lymphocytes takes place in two stages. In the first instance, Ia positive macrophages bind antigen and present it to T cells which become activated and secrete lymphocytic mediators viz. chemoattractants (attract fresh macrophages from the bond marrow) and (MAF) macrophage activating factor (induces microbicidal activity). These T cell products activate macrophages, which can be either Ia^+ or Ia^-, to kill the pathogen (Unanue, 1978).

1.1.8.2 The macrophage as a secretory cell

A number of molecules that have profound effects on lymphocytes and their functions, are secreted by macrophages. The types of molecules secreted by macrophages depend largely on the state of activation of the macrophage (Unanue, 1976). Molecules secreted by macrophages include: complement proteins, lysosomal enzymes, neutral proteases, interferon, lysozyme, plasminogen activator, antigen fragments and a number of less well defined factors which appear to regulate lymphocytes (Unanue, 1978). The lymphoregulatory molecules secreted by macrophages are numerous and there is much controversy concerning the different names given by various groups of workers to macrophage factors, that are probably similar in function. Recently, the following non-specific "Lymphocyte activating" factors produced by

macrophages and acting on T cells or their precursors have been defined (Diamantstein et al., 1979):

(a) The genetically related factor (GRF) contains macrophage Ia and a fragment of antigen (Erb and Feldmann, 1975; Erb et al., 1976) and probably mediates a recognition event that occurs on the macrophage surface. Futhermore, it acts on T_1 cells converting them to T_2 helper cells (Erb et al., 1977). The molecular weight of GRF is 55 000 daltons.

(b) The thymocyte mitogenic protein (TMP) also known as lymphocyte activating factor (LAF) is directly mitogenic for thymocytes and to a lesser extent peripheral T cells. It has a molecular weight of about 15 000 daltons (Unanue, 1978; Rosenstreich and Mizel, 1978; Blyden and Handschumacher, 1977; Claesson et al., 1977). There is also a large molecular weight (50 000 - 85 000 dalton) form of LAF (Blyden and Handschumacher, 1977; Lachman et al., 1977; Wood et al., 1976). It is not species specific.

(c) T cell activating factor (TAF)

It is not mitogenic, but potentiates the actions of antigens or mitogens such as PHA on macrophage-depleted peripheral T cells. It acts on cells in the G_1 phase probably by causing an increase in intracellular cGMP (Katz et al., 1978). The molecular weight of TAF is greater than 50 000 daltons. Macrophages also secrete transferrin (Tormey and Mueller, 1972) and its similarity in structure and action to TAF could indicate that TAF is

in fact transferrin. Both TMP (LAF) and TAF are produced by macrophages stimulated by phagocytosis, endotoxin or T cell factors, and are also produced by a variety of continuous macrophage-like cell lines which are appropriately stimulated (Lachman et al., 1977). There is, however, some controversy as to whether the P388D line actually produces TAF (Rosenstreich and Mizel, 1978).

(d) Lymphocyte potentiating factor (LPF)

It acts on cells in the G_1 phase and has a molecular weight of 1 000 daltons (Masuzawa et al., 1978). Its similarity to TAF indicates that it could in fact be an active fragment of the larger TAF molecule.

(e) Thymocyte differentiating factor (TDF)

This factor acts rapidly on thymocytes to alter their surface markers viz. to lose thymic leukaemia antigen and increase H-2 antigen and allow them to become responsive in a mixed lymphocyte culture (Unanue, 1978). It has a molecular weight of about 10 000 daltons and resembles thymic hormones in its action (Diamantstein et al., 1979).

Several other factors assayed by different techniques seem to be similar to one of the above, based on function and/or physicochemical analysis. A factor known as NMF (Erb et al., 1977), assayed by the plaque-forming response to sheep red blood cells, has been equated with TAF.

Macrophages also produce factor(s) that appear to act directly on B cells and/or their precursors stimulating differentiation and/or proliferation. This factor(s) has been designated B cell activating factor (BAF) (Wood et al., 1976) and has a molecular weight of approximately 18 000 daltons. Others regard BAF as a heterogeneous group of molecules, the effects of which are probably due to enzymatic activities (Diamantstein et al., 1979). BAF has recently been indicated as being identical to LAF (Mizel and Farrar, 1979). Less well characterised is a factor which causes angiogenesis (Polverini et al., 1977) and another that produces lymphocyte chemotaxis (Ward et al., 1977).

Macrophages, as well as secreting lympho-stimulatory molecules also secrete inhibitory molecules, some of which are nucleosides (Chen et al., 1977; Stadecker et al., 1977; Nelson, 1973; Waldmann and Gottlieb, 1973; Ulrich, 1974; Kasahara and Shiori-Nakano, 1976; Calderon et al., 1974). These nucleosides tend to inhibit tritiated thymidine incorporation into dividing cells (Calderon et al., 1976). Nucleosides secreted by macrophages include thymidine because it lacks thymidine kinase (Chan et al., 1974) as well as guanosine and adenosine (Stadecker et al., 1977).

The above support the hypothesis that the macrophage plays a role in antigen-driven differentiation of lymphocytes through antigen processing and secretory functions during phagocytosis. The lymphocyte-proliferating effect of macrophage secretory products TMF, TAF and perhaps BDF partly explains macrophage function in enhancing lymphocyte response to antigen. Furthermore, through the secretion of TDF the macrophage appears to participate in antigen-independent differentiation of the

lymphoid cell line. Briefly, the T stem cell originates in the bone marrow as the prothymocyte which migrates to the thymus. Here it differentiates into the immature thymocyte under the inductive influence of the thymic microenvironment. As the thymocyte matures it migrates from the cortex to the medulla. Monocytes and macrophages are found at the cortico-medullary junction, and secretion products viz. TDF from these cells probably act on these cells to mature them further, after they have been acted on by thymic hormones (Unanue, 1979).

1.2. Interleukin - 1 (IL - 1)

1.2.1 Production of IL - 1

1.2.1.1 Normal cell sources of IL - 1

IL - 1 is produced by adherent rather than non adherent cells (Gery and Waksman, 1972; Gery and Handschumacher, 1974). Its activity has been produced from human (Gery et al., 1971, 1972), mouse (Gery and Waksman, 1972), rat (Hoessli et al., 1977), guinea-pig (Rosenstreich et al., 1976), monkey (Blyden and Handschumacher, 1977), rabbit (Ulrich, 1977) and dog (Blyden and Handschumacher, 1977) adherent cells. Cells from the peripheral blood, spleen, peritoneal and pulmonary alveolar regions (Blyden and Handschumacher, 1977; Ulrich 1977; Gery and Waksman, 1972) as well as mouse bone marrow and thymus cells (Gery and Waksman, 1972) have been shown to produce IL - 1 in various amounts. It should be noted that the adherent cells producing IL - 1 are mononuclear phagocytes (macrophages), since little if any is made by adherent B cells (Calderon et al., 1975), and macrophage cell lines produce IL - 1 in the absence of B cells (Oppenheim et al.,

1979). Furthermore, adherent T cells do not produce IL - 1 since treatment of adherent cells with anti-theta serum and complement (Calderon et al., 1975) does not prevent IL - 1 production, and nude mice spleen cells produce IL - 1 (Hoessli and Waksman, 1976).

1.2.1.2 Inducing agents

IL - 1 activity is measured by its ability to stimulate thymocyte mitogenesis (Oppenheim et al., 1979). Agents that stimulate IL - 1 production can be divided into two classes viz. those that stimulate macrophages directly and those that activate lymphocytes which in turn activate the macrophage. The mode of action of a stimulant is difficult to determine since some lymphocyte mitogens may also directly stimulate the macrophage (Oppenheim et al., 1979).

Stimulants of IL - 1 production include: B cell mitogens such as bacterial endotoxin [LPS] (Gery and Waksman, 1972; Unanue et al., 1976a; Blyden and Handschumacher, 1977; Lachman et al., 1976), purified protein derivative [PPD] (Mokyr and Mitchell, 1975), pyran copolymer (Meltzer and Oppenheim, 1977) and mycobacterial glycopeptides (Rook and Stewart-Tull, 1976). These stimulants act directly on macrophages since they are active in lymphocyte-depleted adherent cell cultures (Oppenheim et al., 1979). Phorbol myristic acetate [PMA] is a T cell mitogen which can activate macrophages directly in man (Mizel et al., 1978c). Some mitogens however indirectly stimulate macrophages, since mitogen induced lymphokines (Meltzer and Oppenheim, 1977; Mizel et al., 1978a) and MAF-containing supernatants from cultured lymphocytes are potent stimulators of IL - 1 (Oppenheim et al.,

1979). T cell mitogens that indirectly activate macrophages by activating T cells, include sodium periodate [$NaIO_4$] (Novogrodsky and Gery, 1972), phytohaemagglutinin [PHA] (Gery et al., 1972) and concanavalin A [CON A] (Gery et al., 1972). Alloantigens (Koopman et al., 1977) and antigens most likely stimulate macrophages indirectly through sensitized or activated T cells (Unanue et al., 1976b; Mizel et al., 1978a). Certain agents that can induce phagocytosis also stimulate IL - 1 release, namely, beryllium salts and barium sulphate (Unanue et al., 1976c; Blyden and Handschumacher, 1977), bacteria, polystyrene, latex particles, antigen/antibody complexes (Blyden and Handschumacher, 1977), irradiation (Geiger et al., 1973), CON A 1972), MAF (Meltzer and Oppenheim, 1977) and adjuvants including LPS (Gery and Waksman, 1972), BCG (Mitchell et al., 1973), mycobacterial muramyl dipeptide [MDP], mycobacterial methanol extraction residue [MER], mycobacterial water soluble activity [WSA] and mycobacterial peptidoglycolipid fraction (Rook and Stewart-Tull, 1976; Oppenheim et al., 1979) as well as the mixed lymphocyte reaction [MLR] (Koopman et al., 1977). It should be noted that not all inducers of phagocytosis induce IL - 1 production. Substances such as thioglycolate and mineral oil do not . (Gery and Wieber, 1975; Meltzer and Oppenheim, 1977).

Cyclic GMP (Dimantstein and Ulmer, 1976) and stimulators of guanyl cyclase such as phorbol myristic acetate [PMA], serotonin and methysergid as well as leukocyte extracts containing transfer factor (Sandler et al., 1975; Togawa et al., 1979a) and vitamin C (Blyden and Handschumacher, 1977), increase IL - 1 production.

Miscellaneous stimulants of IL - 1 production include DMSO (Blyden and Handschumacher, 1977), colchicine and mycostatin (Wood and Cameron, 1976).

1.2.1.3 Conditions for the production of IL - 1

IL - 1 can be produced from the mononuclear cell fraction or adherent cell fraction in serum-containing rather than serum-free medium. Human mononuclear cell cultures fail to produce IL - 1 in the absence of serum (Lachman et al., 1976), although rat and mouse peritoneal adherent cells (Hoessli et al., 1977) will produce IL - 1 in serum-free media supplemented with 2-mercaptoethanol [2ME].

In humans the optimal cell concentration for IL - 1 production is 3 - 10 x 10^6 mononuclear cells per millilitre (Oppenheim et al., 1979) or lower. At higher cell concentrations IL - 1 production tends to decrease either due to contact inhibition or the production of an inhibitor (Oppenheim et al., 1979). An inhibitor has as yet not been identified.

IL - 1 appears in the culture supernatants of human leukocytes within 6 hours and reaches peak biological activity within 24 - 48 hours (Blyden and Handschumacher, 1977). In the case of murine cells the peak is reached in 12 - 72 hours (Hoessli and Waksman, 1976). Continuous exposure of cells to stimulants leads to greater IL - 1 production than pulsing with stimulants (Oppenheim et al., 1979).

1.2.1.4 Mechanism of IL - 1 production

Little is known about the mechanism of IL - 1 production. It has been shown that irradiation enhances IL - 1 production

(Geiger et al., 1973). However, its production is blocked by metabolic inhibitors, thereby suggesting that IL - 1 is actively synthesized and secreted by stimulated cells rather than simply being passively released (Blyden and Handschumacher, 1977). Furthermore colchicine, which depolymerizes microtubules, induces IL - 1 production indicating that IL - 1 may be generated independently of exocytosis of lysosomal enzymes (Oppenheim et al., 1979).

Evidence indicates that activated macrophages secrete more IL - 1 than resting cells (Mokyr and Mitchell, 1975). However, only certain types of macrophage activation stimulate IL - 1 secretion (Unanue et al., 1976b), while others do not (Gery and Wiener, 1975). Furthermore, it has been shown that both direct stimulation of macrophages, as well as indirect effects of lectins and T-dependent antigens mediated by contact with activated lymphocytes and lymphokines, may lead to IL - 1 production which, in turn, can lead to rapid participation of greater numbers of lymphocytes and lymphokines (Ononue, 1976).

Experimental evidence has lead to the following hypothesis for IL - 1 synthesis and secretion (Unanue, 1978) [see Figure 2].

The stimulus leads to a brief formation of a pool of precursor IL - 1 (1) since the addition of a protein synthesis inhibitor at the onset of stimulation inhibits IL - 1 production. The precursor IL - 1, in a way that does not involve protein synthesis converts to active IL - 1(2). Some IL - 1 is then degraded (3) while the rest is released (4), since the decline in cytoplasmic IL - 1 does not correlate with its release from the cell. The control protein acts at stages (2) and/or (3).

When no control protein is made, the pool of precursor IL - 1 continues to convert to the active product. The fact that highly activated macrophages show less cellular and secreted IL - 1, may be a reflection of an increased content of the control protein. Phagocytosis, which stimulates IL - 1 production and secretion, may act by increasing the rates of steps (1) and (4).

Experimental evidence to explain the above hypothesis is as follows:-

Cycloheximide added at the onset of culture stops synthesis of IL - 1, but when added 1 - 2 hours after onset results in an increase in secretion. In these instances IL - 1 in the cell did not decline, yet secretion increased. It is therefore postulated that the secretion of IL - 1 involves a control protein. By preventing synthesis of the control protein, IL - 1 persists in the cell and is secreted. The persistence of IL - 1 in the cell and its increased secretion in the continuous presence of inhibitors of protein synthesis puts forward the theory that IL - 1 exists in two forms, a precursor and an active form, and the change from precursor to active forms does not involve protein synthesis.

A MODEL FOR IL - 1 PRODUCTION

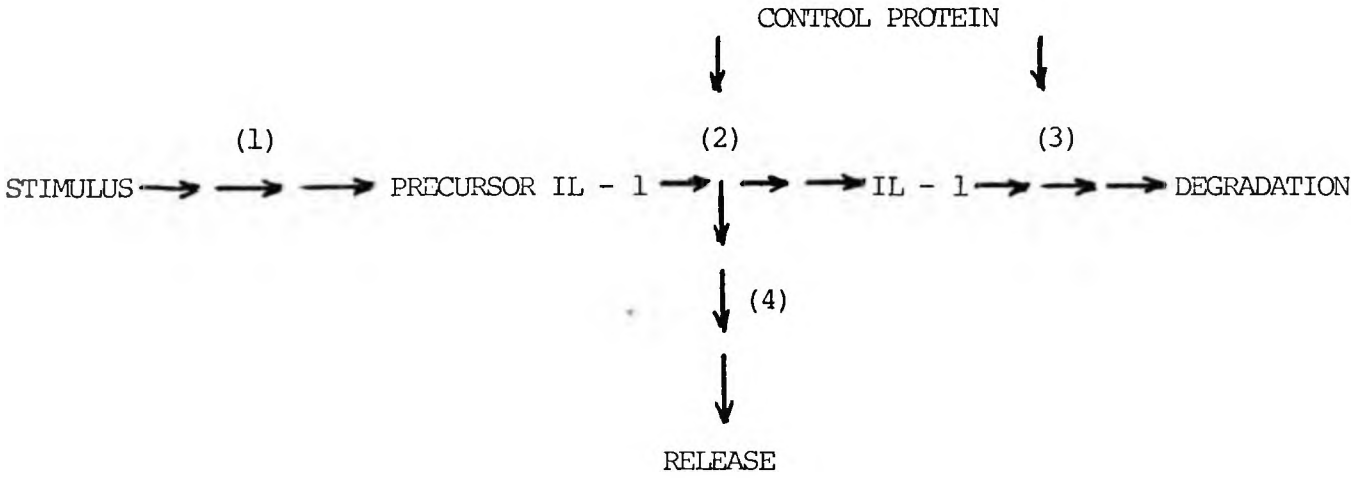


FIGURE 2

1.2.1.5 IL - 1 production by macrophage cell lines

Several murine macrophage cell lines have been shown to produce IL - 1 on stimulation (Lachman et al., 1976; Hacker et al., 1977). $P388D_1$, J774 PU5 and WEHI are three macrophage cell lines which on stimulation with "crude" LPS produce a soluble factor which is mitogenic for thymocytes and has a molecular weight of 50 - 80 000 daltons, whereas IL - 1 produced by normal adherent cells has a molecular weight of 15 000 daltons (Gery and Handschumacher, 1974; Koopman et al., 1977). "Purified" LPS however causes cell line macrophages to secrete a 15 000 dalton moiety that is functionally and chemically equivalent to IL - 1 (Mizel et al., 1978b). PHA-activated T cells enhance IL - production by $P388D_1$ cell lines (Mizel et al., 1978b). This enhancement requires cell contact (Unanue et al., 1976b; Mizel et al., 1978a) as well as viable T cells (Oppenheim et al., 1979). PMA also stimulates IL - 1 production by $P388D_1$ cell lines (Mizel et al., 1978c). The following agents do not generate significant IL - 1 activity from $P388D_1$ cell lines:- cGMP, dBcGMP, PHA, CON A and serotonin (Oppenheim et al., 1979). MDP stimulates $P388D_1$ cells to produce small, but significant, amounts of IL - 1 (Oppenheim et al., 1979). Since cell lines represent homogeneous populations of cells, they are excellent for the evaluation of stimulants of IL - 1, their mode of action as well as the production of large pools of IL - 1 required for biochemical analysis (Oppenheim et al., 1979).

1.2.2 Purification and physical properties of IL - 1

1.2.2.1 Dialysis of IL - 1

Dialysis does not affect the activity of crude IL - 1 prepared in the presence of serum. Some loss of activity is however seen when crude IL - 1 without serum is dialyzed (Gery and Handschumacher, 1974).

1.2.2.2 Purification of crude IL - 1

As determined by gel-filtration chromatography, the majority of IL - 1 activity produced by normal human and mouse macrophages stimulated by various agents, is found in forms that have molecular weights between 5 000 and 20 000, most being closer to 15 000 daltons in human (Gery and Handschumacher 1974; Calderon et al., 1975; Koopman et al., 1977; Blyden and Handschumacher, 1977; Economou and Shin, 1978; Lachman et al., 1977; Togawa et al., 1979a) and between 15 000 and 45 000 daltons in murine forms. Higher molecular weight forms of IL - 1, (greater than 35 000,) have been observed in both man (Gery and Handschumacher, 2974; Blyden and Handschumacher, 1977; Wood et al., 1976) and mice (Wood and Gaul, 1974; Blyden and Handschumacher, 1977; Togawa et al., 1979; Beller and Unanue, 1977). This larger moeity of IL - 1 seems to be due to IL - 1 binding to serum components (Oppenheim et al., 1979) or IL - 1 is a heterogeneous group of substances (Farrar and Koopman, 1979). By gel filtration chromatography crude human and murine IL - 1 can be purified approximately 500 fold giving a yield of thirty to sixty percent (Blyden and Handschumacher, 1977). Crude IL - 1 derived from the macrophage cell line $P388D_1$ yields activity of molecular weight 16 000 daltons when purified on gel filtration columns. P $388D_1$ derived IL - 1 can be purified approximately 20 000-fold with a seven percent yield using ammonium sulphate precipitation, DEAE cellulose, sephacryl S200 and phenyl-sepharose

chromatography followed by electrophoresis on ten percent polyacrylamide gels (Mizel, 1979). Furthermore, when $P388D_1$ -derived IL - 1 is chromatographed on DEAE cellulose following gel filtration chromatography, the IL - 1 is resolved into three to four charge species, the number of ions being dependent on the IL - 1 inducer (Mizel, 1979).

1.2.2.3 Heat stability and pH sensitivity

IL - 1 derived from mouse peritoneal cells is stable between pH 5,5 - 10,5, however at lower or higher values there is some reduction in activity (Gery and Handschumacher, 1974). Human derived IL - 1 gives similar results (Oppenheim et al., 1979). $P388D_1$-derived IL - 1 is stable in distilled water and over a pH range of 4 - 9 (Mizel, 1979).

Partially purified human and mouse derived IL - 1 is stable for two months at -20°C, ten days at 4°C and for 14 hours at 37°C (Blyden and Handschumacher, 1977; Gery and Handschumacher, 1974). Higher temperatures or longer periods of time at certain temperatures reduce IL - 1's biological activity. For example, heating IL - 1 for ten minutes at 60°C results in a three-quarter decrease in activity, and further heating at 80°C further reduces IL - 1 activity of IL - 1 prepared in the absence of serum, and completely eliminates the activity of IL - 1 prepared in the presence of serum (Gery and Handschumacher, 1974). This suggests that heating of IL - 1 prepared in the presence of serum results in the production of an inhibitory factor. The fact that normal serum heated to 80°C inhibits the responses of thymocytes to IL - 1 or CON A supports this hypothesis (Gery and Handschumacher, 1974). Crude IL - 1 can be stored at -10°C for 3 months (Blyden

and Handschumacher, 1977).

1.2.2.4 The effect of certain chemicals and enzymes on IL - 1 activity

Partially purified human IL - 1 activity is unaffected by treatment with sodium periodate and iodoacetate which suggests that if carbohydrate or sulfhydryl residues are present in IL - 1, they are not essential for its activity (Blyden and Handschumacher, 1977). Human IL - 1 has been found to be highly susceptible to inactivation by proteolytic enzymes such as chymotrypsin (Blyden and Handschumacher, 1977; Calderon et al., 1975). Furthermore, some workers report that IL - 1 does not appear to have protease activity, since inhibitors of serine proteases do not affect IL - 1 activity (Blyden and Handschumacher, 1977; Unanue, 1978). Others, however, have found that $P388D_1$-derived IL - 1 activity is greatly reduced by proteases containing endo- and ectopeptidases, but is only weakly affected by chymotrypsin, trypsin and papain, three endopeptidases (Mizel, 1979). Murine peritoneal exudate IL - 1 is also resistant to trypsin and papain (Calderon et al., 1975). $P388D_1$-derived IL - 1 furthermore is resistant to irreversible denaturation by urea, to reduction and alkylation and to two molar sodium chloride (NaCl) (Mizel, 1979).

Recently it has been suggested that IL - 1 may represent the combined action of several enzymes which involve protease action. Also, IL - 1 activity has been shown to parallel that of carboxypeptidase B (Katz et al., 1980).

1.2.3 Effects of IL - 1

1.2.3.1 Target cells

T cells are the immediate targets of IL - 1 and not B cells (Unanue et al., 1976a), but IL - 1 activated T cells can stimulate B cells to produce antibody (Oppenheim et al., 1979). A discrepancy concerning the above statement has been found, since it has been reported that IL - 1 containing supernatants promote proliferation of a B cell line (Namba and Hanaoka, 1974). However the factor involved in the latter has a molecular weight of 50 000 and therefore may not be chemically related to IL - 1. The effect of IL - 1 on mouse thymocyte DNA synthesis is marked and forms the basis of the bioassay for IL - 1 activity. The thymocyte proliferative response to IL - 1 is detectable at 36 - 48 hours and peaks at 72 - 90 hours (Oppenheim et al., 1979). It has been reported that IL - 1 has a greater mitogenic effect on the more mature, cortisone resistant cells than on untreated thymocytes (Glyden and Handschumacher, 1977). Results in contrast with this observation are that the mouse thymocyte proliferative response decreases after ten weeks of age when the ratio of cortisone-resistant to sensitive thymocytes has increased (Blyden and Handschumacher, 1977), and that the reactivity of untreated cells to the IL - 1 is greater than that of treated cells (Oppenheim et al., 1979). Probably both subpopulations of thymocytes can proliferate actively in response to IL - 1 (Gery et al., 1972).

1.2.3.2 Reaction of target cells

IL - 1 produces a number of effects on cells other than

cell proliferation, such as:

(a) Substitute for macrophages in mitogen and antigen-induced T cell activation (Rosenstreich et al., 1976; Hoffman and Dutton, 1971; Erb and Feldmann, 1975). The latter applies to particulate antigen but could also apply to soluble antigen although such a reaction requires macrophage and histocompatible T cell interaction. In this case IL - 1 could participate in the macrophage-T cell interaction since IL - 1 is produced in greater amounts in a mixed lymphocyte culture (MLC) when lymphocytes are HLA-identical (Farr et al., 1977).

(b) Induce antibody production (plaque forming cell response to sheep red blood cells) in nude mice spleen cells. This could be due to IL - 1 acting directly on the B cells or could be due to helper cell induction of T cells which then promote B cell antibody responses (Koopman et al., 1977).

(c) Promote T cell lymphokine production (Young and McIvor, 1980).

(d) Induce T cell differentiation. IL - 1 containing supernatants have been found to potentiate mixed lymphocyte reaction (MLR) effectiveness of both stimulator and responder thymocyte populations (Oppenheim et al., 1976). Also, the type of reaction of a T cell, such as lymphokine production or proliferation, seems to depend in part on the maturity of the T cell (Gery et al., 1972; Calderon et al., 1975).

(e) Induce cytotoxic T cell induction from peripheral T cells and thymocytes (Wagner et al., 1980).

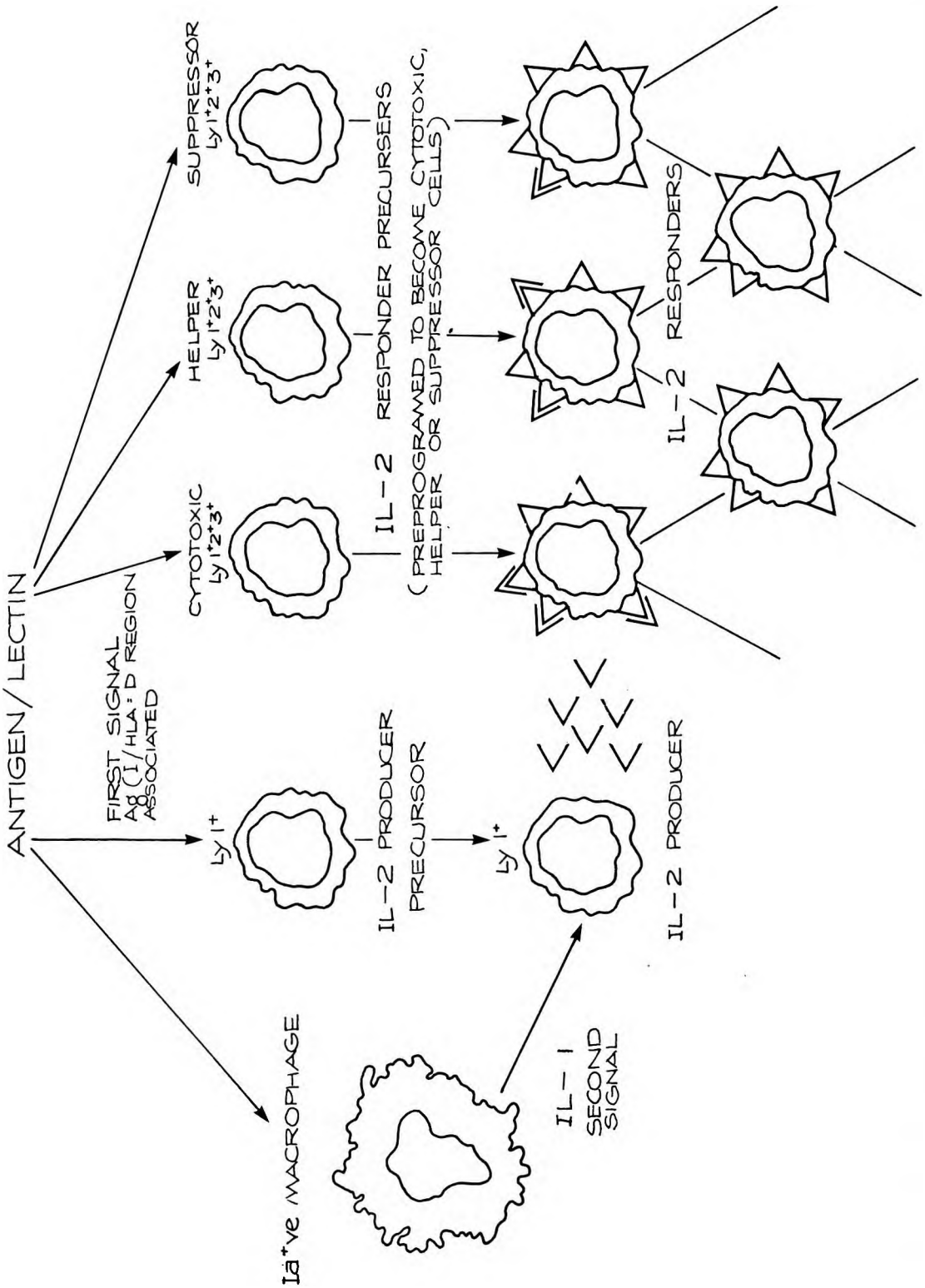
(f) Prevents the in vitro myelopoietic depressive effects of glucocorticoids. It has been found that Dexamethasone suppresses the formation of granulocyte/macrophage colonies by murine bone marrow cells cultured with L cell derived colony stimulating factors, but has no effect when macrophage cell lines are used as the source of colony stimulating factor. This difference is attributed to IL - 1 in the macrophage supernatant, which suggests that, granulocyte/macrophage progenitor cells are direct targets of IL - 1. Furthermore, protection of granulocyte/macrophage colony formation from Dexamethasone suppression can be used to assay IL - 1. Comparative studies indicate this assay to be ten or more times more sensitive than the conventional LAF assay. (Mishell et al.1981).

1.2.4 Models for IL - 1 function

1.2.4.1 T cell activation (see Figure 3)

T cell proliferation involves three cell types and two soluble mediators, a monokine and a lymphokine. Stimulation by antigen or lectin activates Ia positive macrophages (Werkerle et al., 1980) to produce IL - 1. IL - 1 then acts as a second signal in conjunction with I region (HLA-D region) associated antigen, the first signal, causing IL - 2 producer precursors to develop into IL - 2 producer cells. Thereafter IL - 2, a non-specific mediator, acts on IL - 2 responder cells which are Ly 1,2,3 cells which have developed receptors for IL - 2 under the

FIG. 3: T CELL ACTIVATION



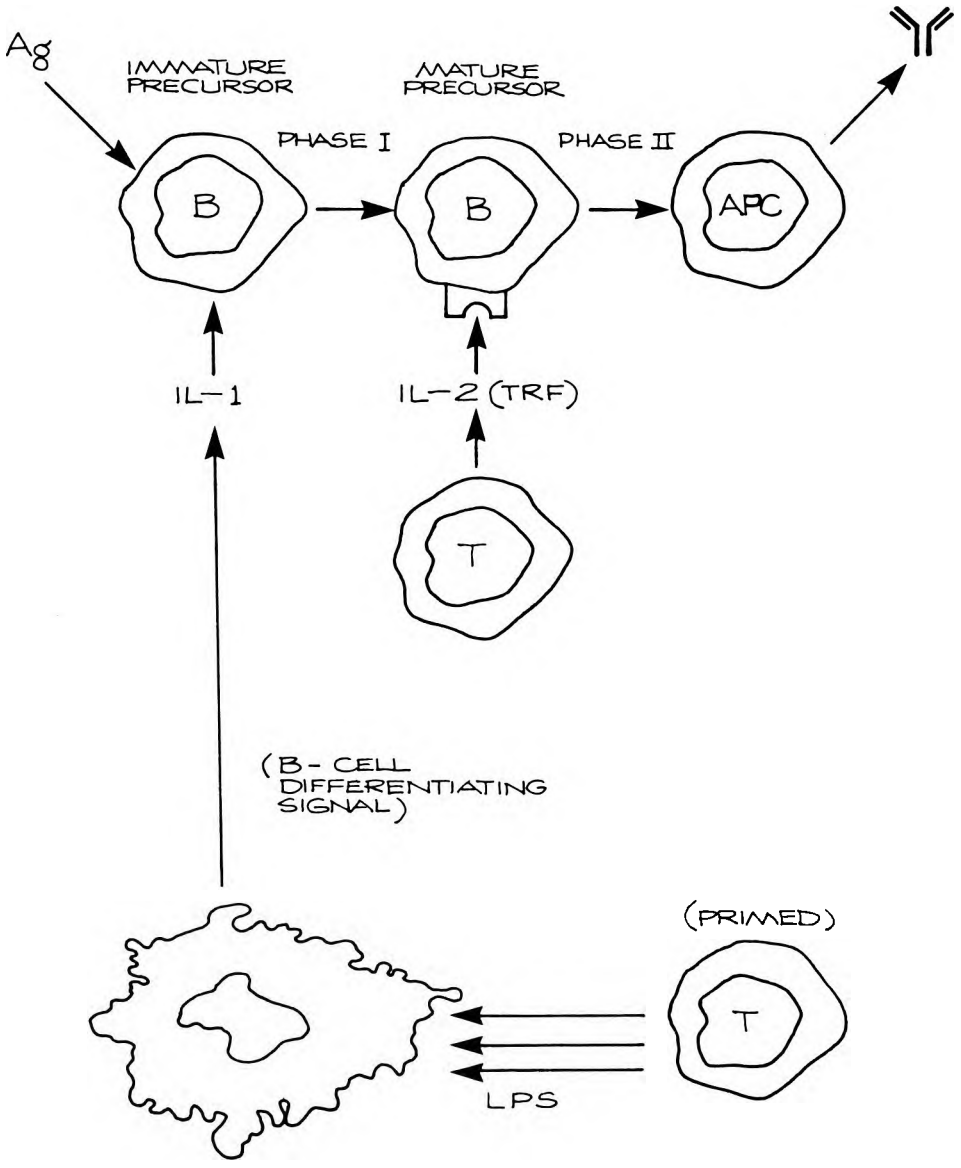
influence of K/D region associated antigen or lectin. These responder cells then proliferate, developing into cytotoxic (Ly 2^+3^+) cells, suppressor (Ly 2^+3^+) cells or helper (Ly 1^+) T cells (Altman, 1981; Wagner et al., 1980; Smith, 1980; Paetkau et al., 1980).

1.2.4.2 A model for B-cell activation in the humoral response (Figure 4)

(a) Particulate antigen

Induction of a humoral response to particulate antigen is initiated by the interaction of antigen with the B cell. This interaction converts resting B cells into cycling B cells (Dutton, 1975; Schimpl and Wecker, 1975). A small fraction of B cells spontaneously interact with T helper cells or their product IL - 2, and convert into antibody producing cells. The majority of antigen-reactive B cell precursors would require an additional differentiation signal provided in the form of IL - 1. This is secreted by macrophages under the appropriate stimulation which would occur in the presence of activated T helper cells but not in the presence of TRF(IL - 2) or non-activated T cells (Hoffmann, 1980). It is known that IL - 1 induces early B cell markers such as surface immunoglobulin, (Ig), Ia and complement receptors (Hoffmann et al., 1978). Furthermore the ability of B cells to cooperate with T helper cells occurs later in development about the time at which B cells express complement receptors, which coincides with the expression of surface IgD (Hoffmann et al., 1976).

FIG. 4: B CELL ACTIVATION



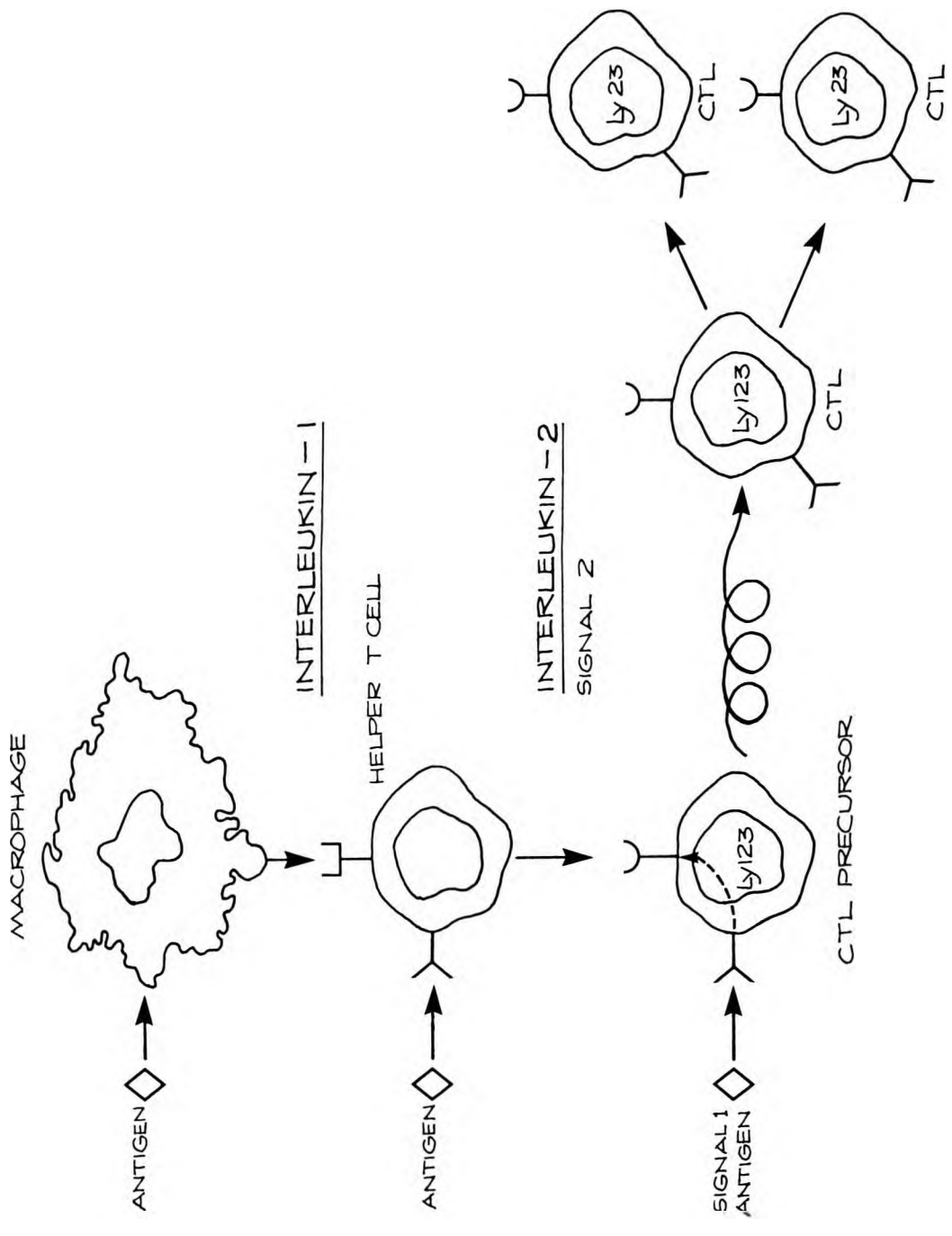
(b) Soluble antigen

The initiation of an immune response to soluble antigen is more complicated. It seems to require two distinct types of T helper cells (Sierkosz et al., 1979). One T helper cell is involved in the initiation of B cell proliferation and acts in an antigen specific manner. Its activity cannot be replaced by IL - 2. The second type of helper cell is required for the response to soluble and cell-bound antigen. The activity can be replaced by IL - 2 (Sierkosz et al., 1979). Therefore the major difference in the plaque cell forming response between soluble and cell-bound antigen is perhaps that the former requires a special T cell to initiate the B cell response, whereas the latter interacts with precursor B cells effectively in an immunogenic fashion without T cell help, and depends on T cells only for conversion of B cells into antibody secreting cells (Hoffmann, 1979).

1.2.4.3 Cytotoxic T cell generation (See Figure 5)

Adherent cell (macrophage) derived IL - 1 plus antigen is required to activate clonally distributed Ly 1 T helper cells. Once primed, antigen alone or metabolically inactivated allogeneic cells can restimulate T helper cells. Antigen specific (re)stimulation is a prerequisite for IL - 2 release by Ly 1 T cells. The helper effect mediated by IL - 2 is non-specific. After interaction with antigen, clonally distributed cytotoxic lymphocyte (CTL) precursors develop sensitivity to IL - 2, which acts as a physiological mitogen thus controlling clonal expansion of antigen reactive CTL precursors (Wagner et al., 1980).

FIG. 5 : CYTOTOXIC T CELL GENERATION



CHAPTER 2

2. SETTING UP OF A ROUTINE ASSAY FOR IL - 1 PRODUCTION AND ASSESSMENT

2.1 Introduction

The initiation of the immune response to a foreign body requires cellular interaction between various subsets of macrophages, T cells and B cells. Macrophages seem to play a central controlling role in these interactions which may involve direct cell contact and/or the release of soluble mediators. Soluble mediators released by macrophages may be stimulatory in nature (Gery et al., 1972; Gery and Waksman, 1972) or suppressive and cytotoxic (Unanue, 1978; Unanue, 1976; Allison, 1978; Waksman, 1978).

Deficiencies in the immune response are numerous and the causes multifactorial. Some deficiency states could result from defective, or lack of, secretion of a single monokine secreted by adherent cells. For this reason this chapter deals with the establishment of a routine assay for a factor secreted by lipopolysaccharide stimulated adherent cells, recently designated Interleukin-1 [IL-1] (Mizel and Farrar, 1979). This monokine was originally described by Gery and co-workers (Gery et al., 1971; Gery et al., 1972; Gery and Waksman, 1972; Gery and Handschumacher, 1974) under the name "Lymphocyte Activating Factor" [LAF]. This factor has been described by various groups of workers over the years, who have assigned different names and biological functions to the same molecule. Acronyms include:

i) Mitogenic protein [MP] (Unanue and Kiely, 1977).

ii) Helper-peak-1 [HP-1] (Koopman et al., 1978)

iii) T-Cell Replacing factor III [TRF-111] (Watons, 1973).

iv) T-Cell Replacing factor$_{M\emptyset}$ [TRF$_{M\emptyset}$] (Hoffmann and Watson, 1979).

v) B cell activating factor [BAF] (Wood, 1979).

vi) B cell differentiation factor [BDF] (Hoffmann et al., 1979).

2.2 Materials and methods

2.2.1 Interleukin-I (IL - 1) supernatant production (see Figure 6)

Venous blood obtained from normal human donors by veno-puncture was mixed with preservative free heparin (10 units per millilitre). The whole blood was separated by centrifugation on a Ficoll-hypaque density gradient as described by Boyum (1968). The mononuclear cell fraction at the plasma-ficoll interphase was collected, washed and adjusted to a concentration of approximately 2,5 x 10^6 cells/ml in complete medium. 30 ml of mononuclear cells were incubated in 140 x 15 mm glass petri dishes for 4 - 24 hours at 37°C in a humidified CO_2 incubator. The non-adherent cells were then decanted and the remaining adherent cells washed three times. The adherent cells were then removed from the glass plates by treatment with EDTA for 15 minutes at 37°C, washed three times and suspended in complete medium. The purity of the adherent cells was found to be between 70 - 80 percent monocytes as assessed by the ingestion of yeast particles. Monocytes (adherent cells) were then cultured at concentrations of 1 x 10^5 to 1 x 10^6 cells/ml with Ecoli lipopolysaccharide (LPS) at concentrations of 0 to 40 µg/ml in tissue culture flasks in a

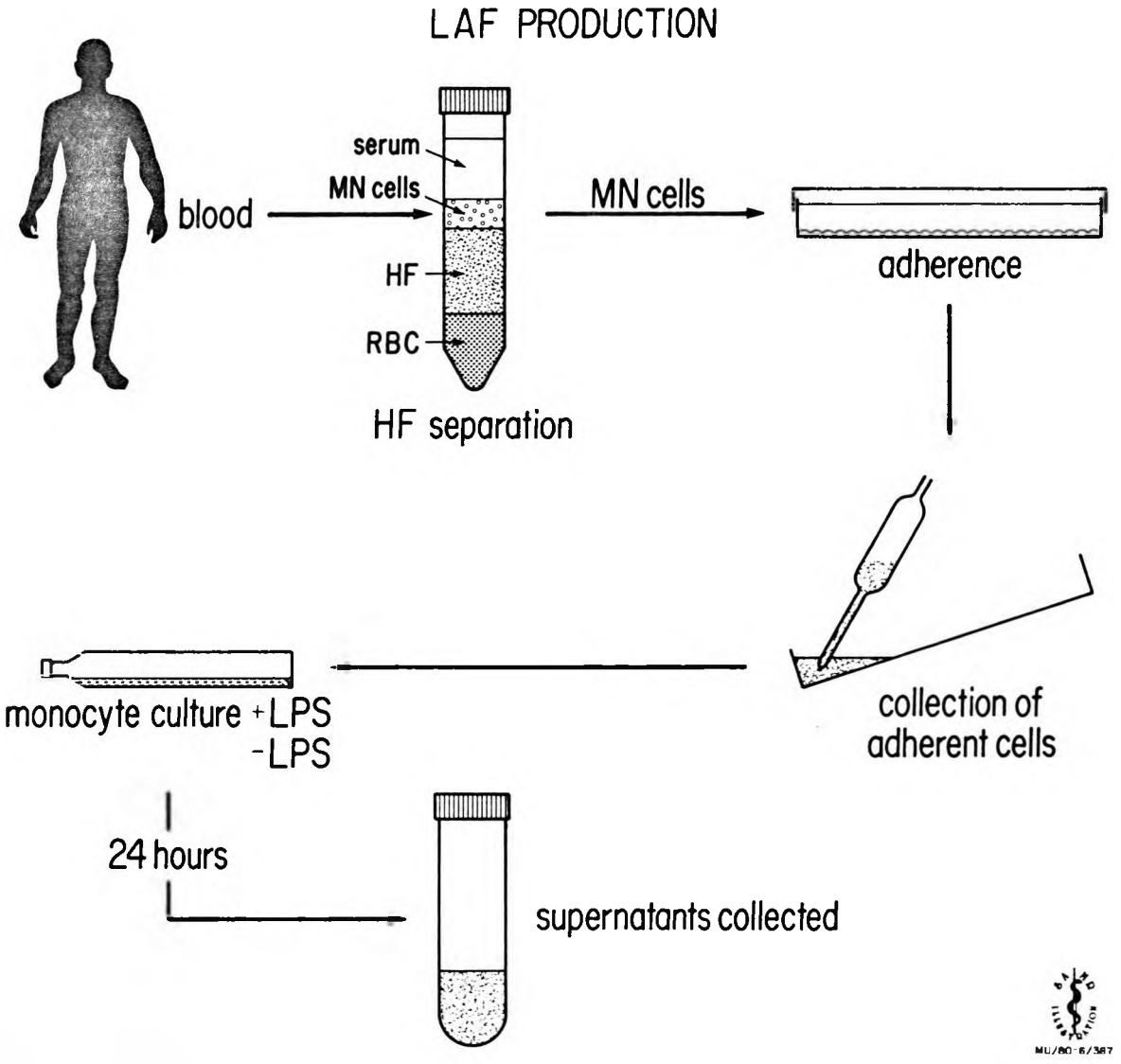

LAF PRODUCTION
blood
serum
MN cells
HF
RBC
HF separation
MN cells
adherence
collection of
adherent cells
monocyte culture +LPS
-LPS
24 hours
supernatants collected

Figure 6

37°C humidified 5% CO_2 incubator for 24, 48, 72, 96 and 168 hours. Controls were set up in the same manner as the tests but the LPS was added only after the culture period. The supernatants were then collected, centrifuged to remove cellular debris and stored at -20°C.

2.2.2 Assay for Interleukin-I action (see Figure 7)

2.2.2.1 Thymocyte preparation

Mouse thymuses were the source of cells for the Interleukin-1 (IL - 1) assay. Thymuses from male or female BALB/C mice (SAIMR Animal House) 6 - 8 weeks old were asceptically removed and teased apart in complete medium. The suspension of cells was allowed to settle for 5 minutes and the thymocytes decanted from the settled debris. The cells were then washed three times and suspended in complete medium at a concentration of 10 x 10^6/ml. The viability of the cells was evaluated by the trypan blue exclusion technique and found to be greater than 90%.

2.2.2.2 Interleukin-1 supernatant assay

0,1 ml of thymocytes at concentrations of 1 x 10^6 - 20 x 10^6/ml were incubated with 0,01 to 0,1 ml of IL - 1 containing supernatant in round bottomed tissue culture plates in triplicate for 24, 48, 72, 96, 120, 144 and 168 hours in a 37°C humidified 5% CO_2 incubator. 0,5 µCi of [methyl ^3H] thymidine was added for the last 6 hours of culture. The cells were then collected on glass fibre filters using an automatic cell harvester (Mash II) and thymidine incorporation was assessed by liquid scintillation counting. The above assay was controlled by incubating thymocytes with medium, lipopolysaccharide and

ASSAY FOR LAF

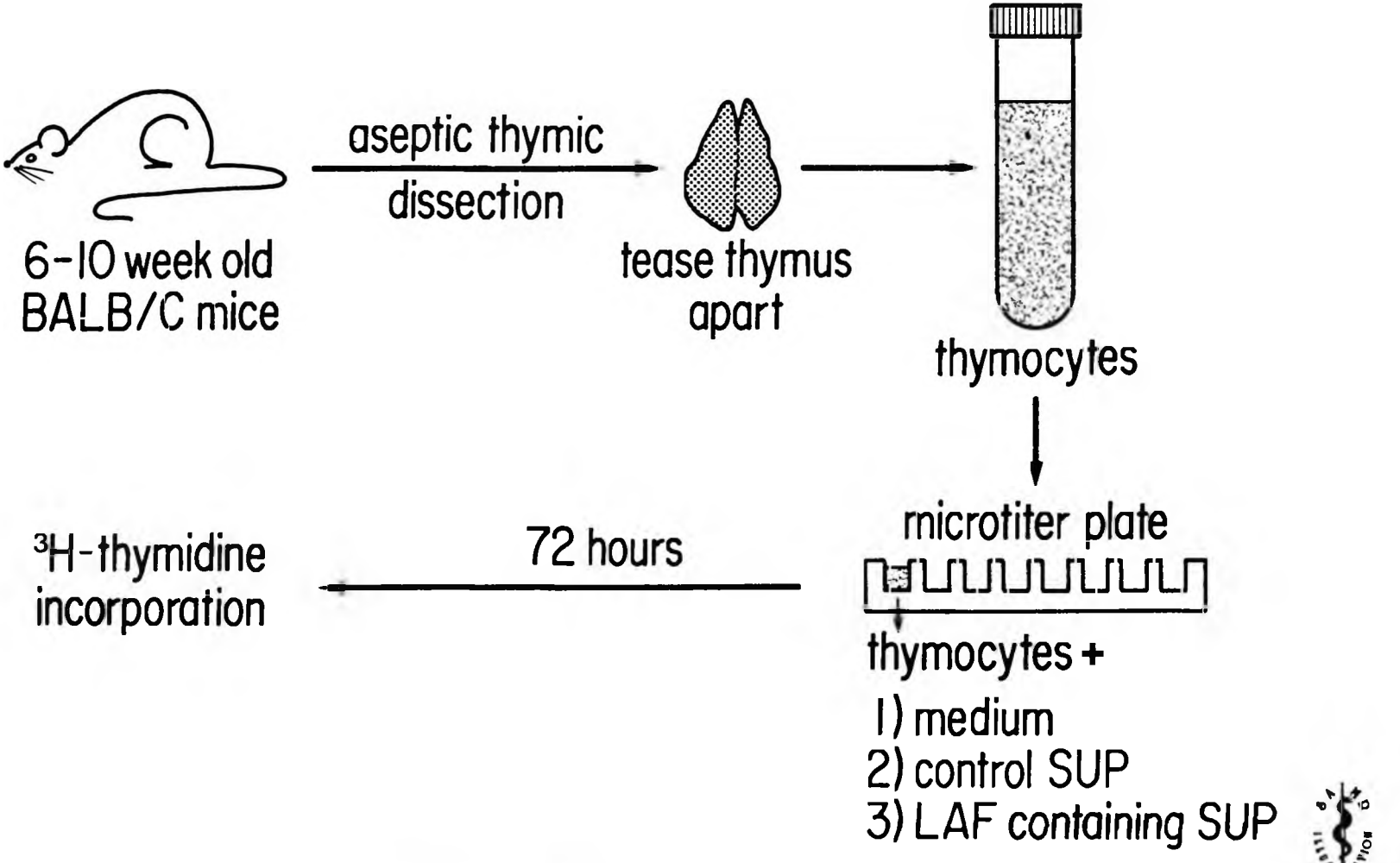


Figure 7

control supernatants and treating these cultures in the same manner as the test cultures. Experiments were carried out in triplicate and the results presented as the means of the triplicate cultures.

2.3 Results

2.3.1 Experiments indicating Interleukin-1 (IL - 1) production by normal peripheral blood adherent cells

5×10^5/ml monocytes were cultured in the presence or absence of 20 μg/ml LPS for 24 hours. Culture supernatants were then assayed for IL - 1 activity on mouse thymocytes as described in point 2.222. Appropriate controls were set up to monitor the experiments. The results are presented in Tables 1a and b and Graph 1.

The results in Tables 1a and b, and Graph 1, indicate that small amounts of IL - 1 and, in some cases, relatively large amounts are secreted spontaneously. However, LPS activates monocytes causing a large increase in IL - 1 output. Furthermore, LPS itself, does not appear to have a significant mitogenic effect on mouse thymocytes.

2.3.2 The optimum amount of LPS required for monocyte stimulation

5×10^5/ml monocytes were incubated with varying concentrations of LPS for 24 hours. The supernatants were then collected and assayed on mouse thymocytes. Results obtained are shown in Tables 2a and b and Graph 2.

The results show that LPS greatly enhances Interleukin - 1 production by monocytes. Furthermore, the data indicates that

Experiment No.	Thymocytes plus medium	Thymocytes plus LPS	Thymocytes plus control supernatant	Thymocytes plus IL-1 supernatant
1	109,7	862,0	2 740,2	12 290,0
2	395,7	748,7	4 380,7	12 564,3
3	426,7	588,0	1 031,3	8 362,0
4	125,0	598,4	2 041,9	13 390,9
5	158,4	784,3	1 005,0	9 081,5
6	168,0	353,3	3 306,7	13 037,3
7	168,0	353,3	3 346,0	11 714,7
8	168,0	353,3	1 257,3	8 634,0
9	396,7	500,0	1 558,7	9 380,7
10	426,5	1 339,5	1 794,0	9 200,0

Figure 1a

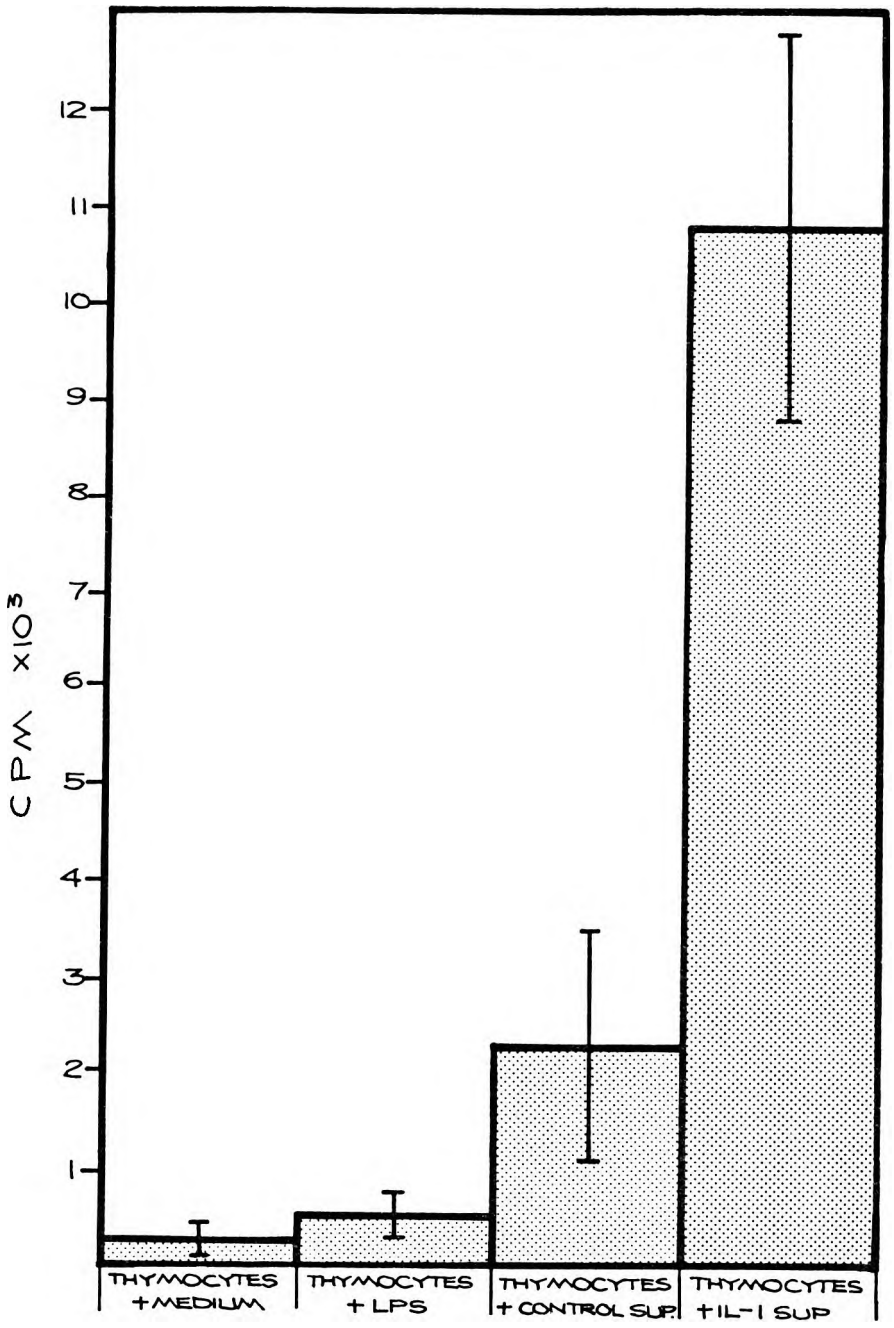
IL - 1 PRODUCTION BY NORMAL PERIPLHERAL BLOOD ADHERENT CELLS

Thymocytes plus medium	Thymocytes plus LPS	Thymocytes plus control supernatant	Thymocytes plus IL-1 supernatant
245,3 (± 137)	548,1 (± 199,5)	2 246,2 (± 1 147,3)	10 765,5 (± 2 001,1)

TABLE 1b.

Means and SD of the ten experiments in Table 1a. showing IL - 1 production

THYMOCYTE STIMULATION (CPM) BY IL - 1 SUPERNATANTS



GRAPH 1.

IL - 1 production is mostly enhanced employing an LPS concentration of between 10 and 20 µg/ml. The graph begins to plateau above LPS concentrations of 20 µg/ml indicating a toxic effect of LPS on the monocyte cultures.

2.3.3 Lipopolysaccharide pulsing experiments

Monocytes (adherent cells) were incubated at a concentration of 5×10^5 cells/ml with and without 20 µg/ml LPS for 2, 4, 6 or 24 hours. After 2, 4 and 6 hours the supernatants were decanted and the culture plates washed three times. Thereafter they were set up in fresh medium for a further 24 hours. After this time interval the supernatants were collected and assayed for the presence of IL - 1. The results are shown in Tables 3a and b, and Graph 3. As will be noted from these tables and graph, only small amounts of IL - 1 are produced when monocytes are pulsed with LPS. The greatest stimulation of IL - 1 production occurs when LPS is present for the entire culture period.

2.3.4 The optimum monocyte (adherent cell) concentration required for IL - 1 production

Varying concentrations of monocytes were incubated in the presence and absence of LPS (20 µg/ml) for 24 hours. The supernatants were then collected and assayed for IL - 1 production as described previously. Tables 4a and b, and Graph 4, show the results obtained. As indicated in these tables and graph the range of monocyte concentrations over which IL - 1 production is optimal is $2,5 \times 10^5$ monocytes/ml to 5×10^5 monocytes/ml. The reason why IL - 1 production does not increase linearly with an increase in cell concentration is unclear, but is probably due to the cell crowding in the culture vessel as well as limited

TABLE 2a.

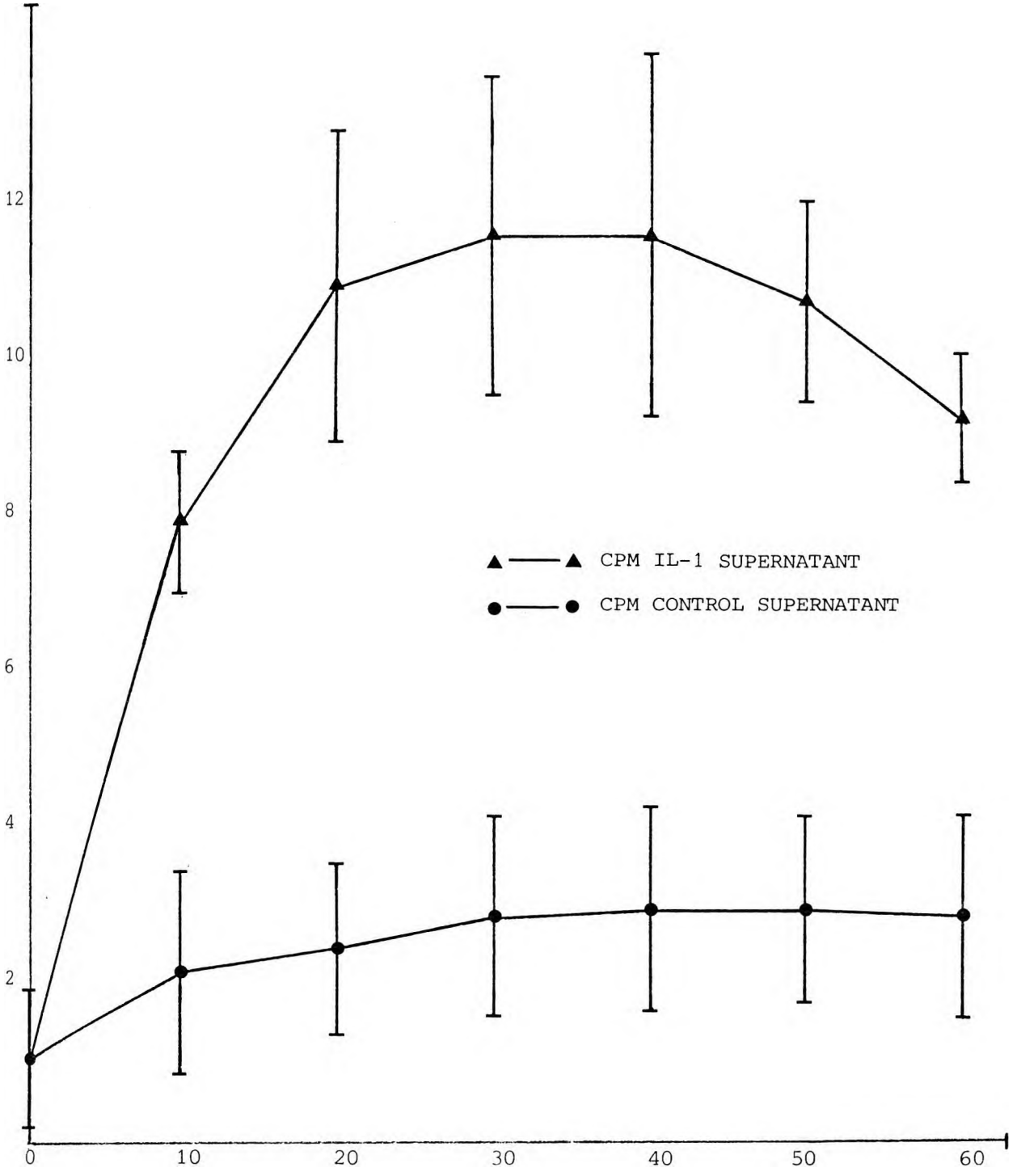
LPS CONCENTRATIONS REQUIRED FOR OPTIMAL IL - 1 PRODUCTION

Experiment No.	LPS concentration µg/ml	CPM control	CPM test	Stimulation (Test/Control)
1	0	235,0	235,0	1
	10	1 327,1	8 007,7	6,0
	20	1 592,5	9 915,4	6,2
	30	1 650,0	10 425,0	6,3
	40	1 735,7	11 014,1	6,3
	50	1 700,0	10 750,0	6,3
	60	1 600,5	9 800,0	6,1
2	0	2 024,3	2 024,3	1
	10	3 645,8	7 050,3	1,9
	20	3 695,5	9 660,2	2,6
	30	4 316,6	10 640,7	2,5
	40	4 435,3	9 836,2	2,2
	50	4 198,2	9 500,6	2,2
	60	4 103,7	8 362,3	2,0
3	0	1 001,6	1 001,6	1
	10	1 588,9	8 897,3	5,6
	20	2 168,8	13 468,3	6,2
	30	2 651,6	14 105,1	5,3
	40	2 798,2	14 331,1	5,1
	50	2 765,6	12 202,5	4,4
	60	3 112,8	9 746,7	3,1

TABLE 2b.

LPS concentration µg/ml	CPM control	Standard deviation	CPM test	Standard deviation
0	1 087,0	± 897,7	1 087,0	± 897,7
10	2 187,3	± 1 269,9	7 985,1	± 923,7
20	2 485,6	± 1 086,7	11 014,6	± 2 128,8
30	2 872,7	± 1 347,0	11 723,6	± 2 065,3
40	2 989,7	± 1 297,1	11 727,1	± 2 330,7
50	2 967,0	± 1 253,6	10 817,7	± 1 352,2
60	2 939,0	± 1 260,6	9 303,0	± 815,1

Means of the three experiments in Table 2a showing the concentration of LPS required for optimal IL - 1 production



IL-1 STIMULATION OF THYMOCYTES (CPM) VERSUS LPS CONCENTRATION REQUIRED FOR OPTIMAL IL-1 PRODUCTION.

GRAPH NO. 2.

TABLE 3a.

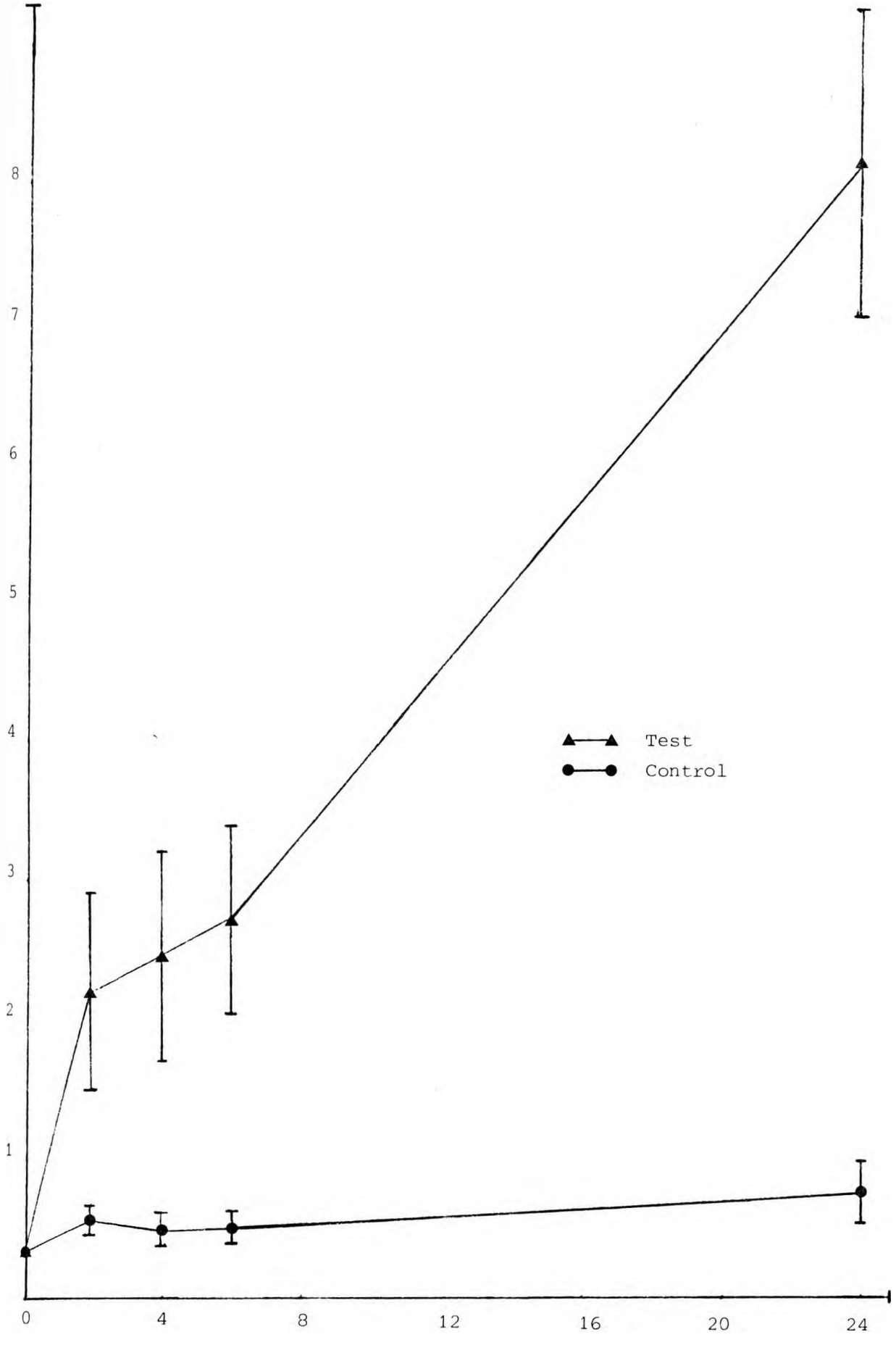
LPS PULSING EXPERIMENT

Experiment No.	Time (hours) of LPS pulse	CPM control	CPM test
1	0	309,7	309,7
	2	508,7	1 867,9
	4	523,1	1 989,8
	6	628,6	2 378,9
	24	952,2	7 428,8
2	0	309,7	309,7
	2	642,8	1 663,5
	4	485,3	1 819,9
	6	417,2	2 202,4
	24	738,0	9 449,2
3	0	309,7	309,7
	2	530,2	2 989,1
	4	350,0	3 308,7
	6	401,7	3 562,2
	24	570,5	7 587,1

TABLE 3b.

Means of the three LPS pulsing experiments in Table 3a.

Time (hours) of LPS pulse	CPM control	± SD	CPM test	± SD
0	309,7	0	309,7	0
2	560,6	± 72	2 173,5	± 713,7
4	452,8	± 91	2 372,8	± 814,9
6	482,5	± 126,8	2 714,5	± 739,4
24	753,6	± 191,3	8 155,0	± 1 123,6



THE GRAPH OF IL-1 PRODUCTION (CPM) VERSUS THE TIME REQUIRED FOR LPS TO BE PRESENT IN THE CULTURES TO STIMULATE OPTIMAL IL-1 PRODUCTION.

GRAPH NO. 3.

nutrients in the culture fluid.

2.3.5 The optimum time for Interleukin-1 production by monocytes

5×10^5/ml monocytes were cultured in the presence and absence of 20 µg/ml LPS for various time periods. The supernatants were then collected and assayed for IL - 1 production. Tables 5a and b, and Graph 5, indicate the results obtained. These tables and graph indicate that IL - 1 secretion occurs optimally between days 1 and 2, that is 24 - 48 hours of culture. The fact that IL - 1 production activity falls off after 48 hours could be due to the production of an inhibitor by the monocytes themselves.

2.3.6 Time taken for IL - 1 to act on mouse thymocytes (i.e. the number of days of culture for IL - 1 to enhance DNA synthesis in thymocytes maximally)

IL - 1 containing supernatants and control supernatants were added to thymocytes (10×10^6/ml) at time 0. Thereafter cultures were harvested at different times after the addition of tritiated thymidine for the final six hours of culture. Results are shown in Tables 6a and b, and Graph 6. These tables and graph indicate that IL - 1 begins to stimulate thymocyte DNA synthesis on day two reaching a maximum on day three. Thereafter IL - 1 does not further stimulate thymocyte DNA synthesis. By day seven the count is extremely low probably because nutrients have been consumed in the culture medium and the cells are no longer active.

TABLE 4a.

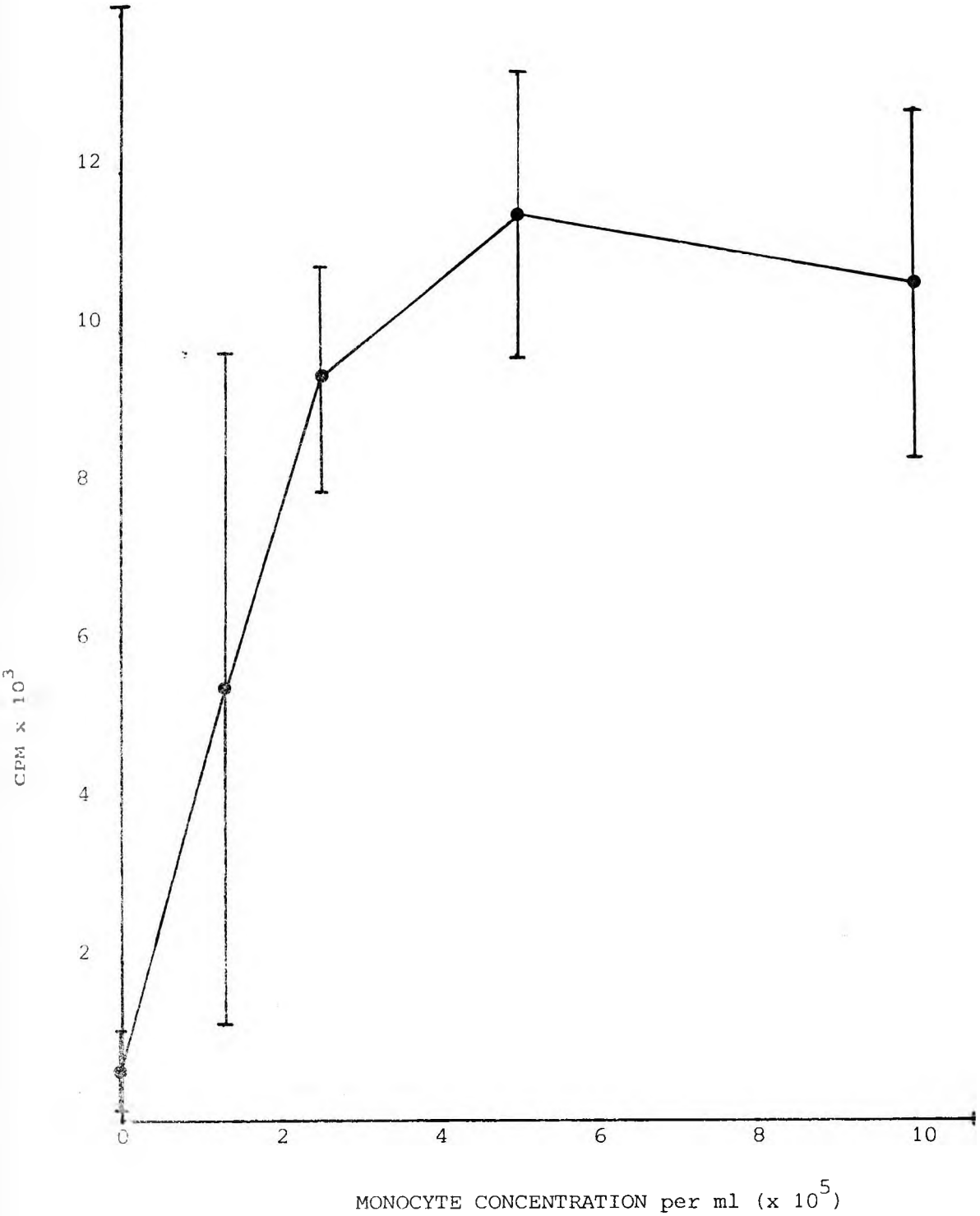
MONOCYTE CONCENTRATION FOR OPTIMAL IL - 1 PRODUCTION

Experiment No.	CPM test minus CPM control				
	0 monocytes/ml	1,25 x 10^5/ml	2,5 x 10^5/ml	5 x 10^5/ml	1 x 10^6/ml
1	1 218,4	3 235,2	9 143,5	11 349,0	10 615,3
2	212,5	2 859,4	8 081,3	9 730,6	8,444,7
3	317,0	10 250,0	10 892,0	13 282,0	12 801,0

TABLE 4b.

Means of the three experiments shown in Table 4a indicating monocyte concentration for optimal IL - 1 production

Monocyte concentration	0	1,25 x 10^5/ml	2,5 x 10^5/ml	5 x 10^5/ml	1 x 10^6/ml
CPM	582,6	5 448,2	9 372,3	11 453,9	10 620,3
Standard deviation	± 553,1	± 4 162,7	± 1 419,2	± 1 778,0	± 2 178,1



THE GRAPH OF IL-1 PRODUCTION (CPM) VERSUS THE NUMBER OF MONOCYTES REQUIRED FOR OPTIMAL IL-1 PRODUCTION.

GRAPH NO. 4.

TABLE 5a.

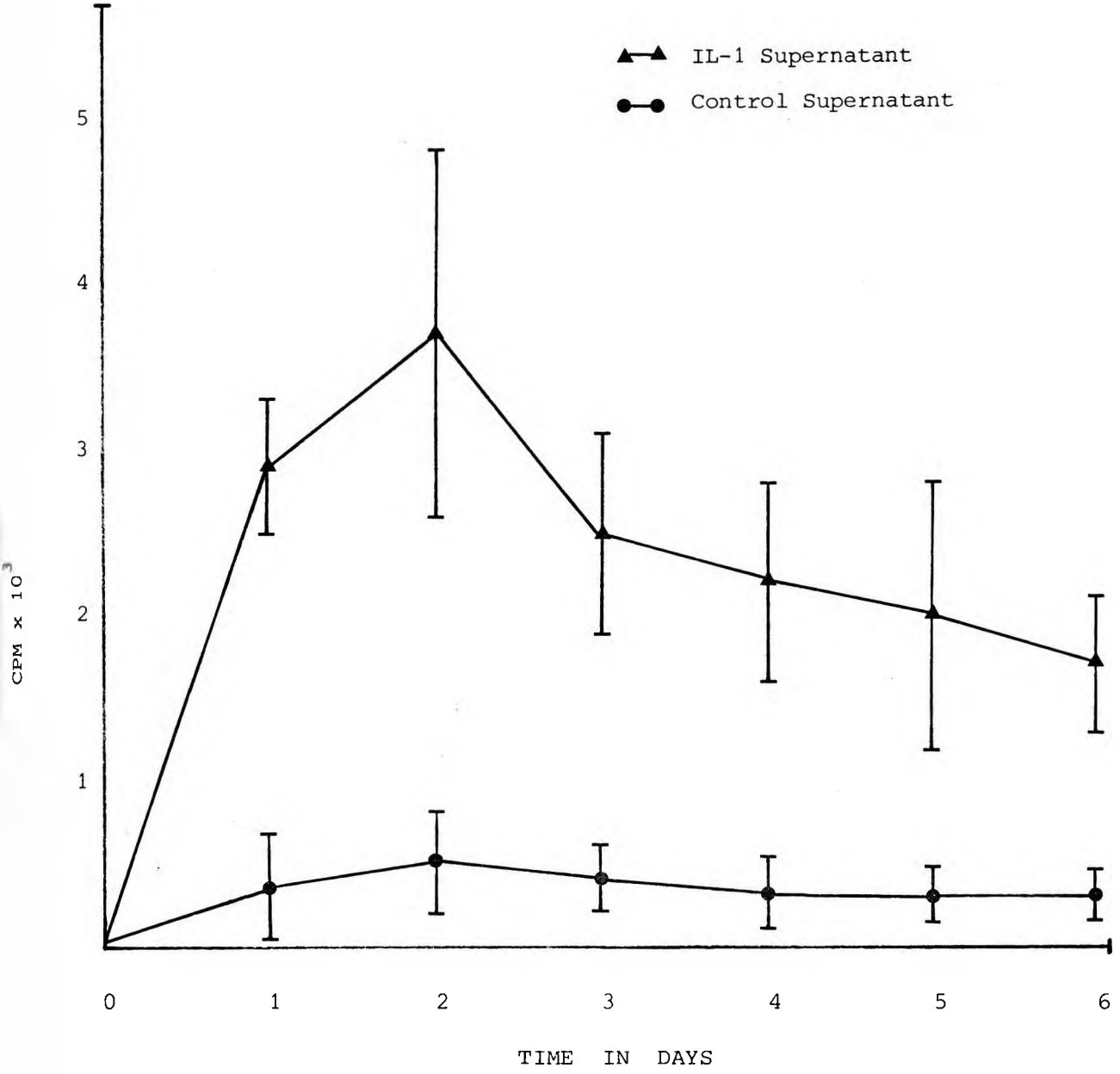
OPTIMUM TIME FOR IL - 1 PRODUCTION

Experiment No.	Time in days	CPM control	CPM test
1	1	161,4	3 293,2
	2	378,7	4 901,8
	3	350,0	3 200,1
	4	281,9	2 958,9
	5	260,7	2 900,4
	6	250,3	1 969,0
2	1	700,3	3 009,3
	2	859,9	3 362,9
	3	625,3	2 225,2
	4	501,4	1 750,8
	5	475,4	1 296,2
	6	426,1	1 191,4
3	1	205,5	2 504,9
	2	290,9	2 842,1
	3	197,0	2 167,9
	4	150,0	2 000,5
	5	149,3	1 908,4
	6	149,5	1,885,8

TABLE 5b.

Means of the three IL-1 production kinetic experiments in Table 5a.

Mean (days)	CPM control	Standard deviation	CPM test	Standard deviation
1	355,7	± 299,2	2 935,8	± 399,3
2	509,8	± 306,3	3 702,3	± 1 071,0
3	390,8	± 217,0	2 531,1	± 580,1
4	311,1	± 177,5	2 236,7	± 637,8
5	295,1	± 165,8	2 035,0	± 809,6
6	275,3	± 140,0	1 682,1	± 427,0



GRAPH OF IL-1 PRODUCTION (CPM) VERSUS THE TIME REQUIRED FOR OPTIMUM IL-1 SECRETION.

GRAPH NO. 5.

2.3.7 The optimum number of thymocytes required to assay IL - 1

Thymocytes at varying concentrations were added to a fixed volume of Interleukin-1 containing supernatant and the appropriate controls. The cultures were incubated for three days and the stimulation of thymocyte DNA synthesis measured as described previously. The results are indicated in Tables 7a and b, and Graph 7. These results show that 5-20 x 10^6 cells/ml is the optimum cell concentraion to use in order to assay IL - 1.

2.3.8 The effect of diluting a stock IL - 1 containing supernatant on thymocyte stimulation

IL - 1 containing supernatants and the appropriate controls were diluted to various concentrations of the stock supernatant and tested for their ability to stimulate mouse thymocytes as described in point 2.2.2. Tables 8a and b, and Graph 8, give the results obtained. The results shown in these tables and graph indicate that Interleukin-1 action can be diluted out, but, even at dilutions of 1:32 Interleukin-1 action is still detectable. The action of Interleukin-1 seems hyperbolic, i.e. its action tends towards zero when diluted.

2.4 Discussion

2.4.1 *E coli* lipopolysaccharide stimulated "adherent cells" secrete IL - 1 containing supernatants

In this thesis IL - 1 has been produced by human peripheral blood adherent cells stimulated by *E. coli* lipopolysaccharide. It has been found that IL - 1 is produced by adherent rather than non-adherent mouse, rat and human cells (Gery and

TABLE 6a.

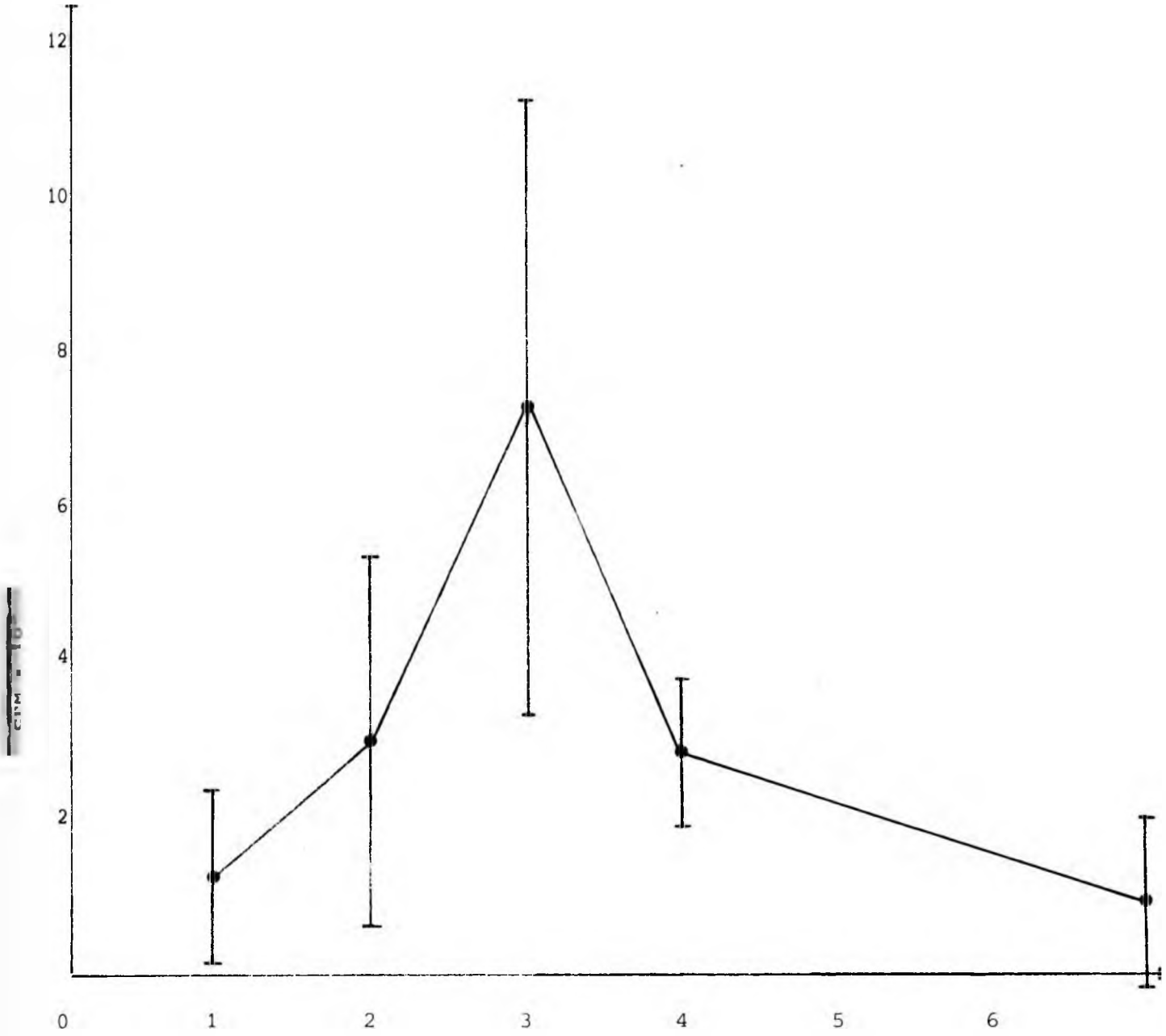
TIME REQUIRED FOR IL - 1 TO ACT ON THYMOCYTES

Experiment No.	Time of incubation (days)	CPM control	CPM test	CPM test - CPM control
1	1	321,9	603,9	282,0
	2	435,3	1 769,2	1 333,9
	3	1 026,7	5 323,5	4 296,8
	4	1 437,0	5 170,0	3 733,0
	7	67,7	132,9	65,2
2	1	1 101,7	3 735,6	2 633,9
	2	1 002,0	6 784,5	5 782,5
	3	902,3	12 794,3	11,892,0
	4	702,5	3 713,8	3 011,3
	7	453,3	2 512,4	2 059,1
3	1	1 127,3	2 231,5	1 104,2
	2	2 159,4	4 149,8	1 990,4
	3	510,0	6 328,0	5 818,0
	4	383,3	2 370,6	1 987,3
	7	269,1	720,2	451,1

TABLE 6b.

Mean of the results indicated in Table 6a.
for the time required for IL - 1 to act on thymocytes.

Time of culture (days)	CPM test minus control	Standard deviation
1	1 340,0	± 1 193,5
2	3 035,6	+ 2 401,4
3	7 335,6	± 4 018,6
4	2 910,5	± 877,2
7	858,5	± 1 057,5



THE GRAPH OF IL-1 ACTION (CPM) VERSUS THE TIME REQUIRED FOR ITS STIMULATION OF THYMOCYTES.

GRAPH NO. 6.

TABLE 7a.

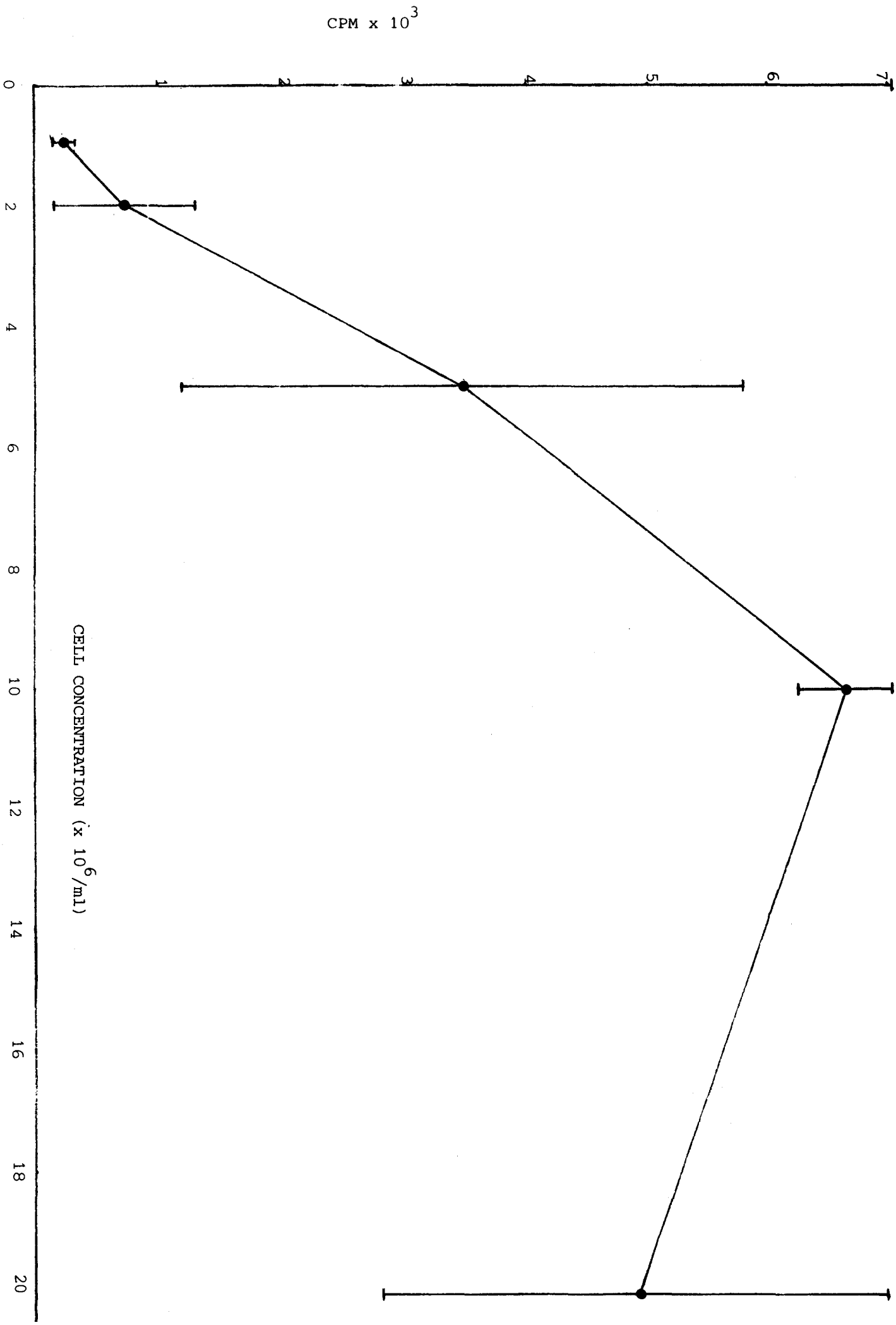
OPTIMAL NUMBER OF THYMOCYTES REQUIRED TO ASSAY IL - 1

Experiment No.	Thymocyte concentration/ml	CPM control supernatant	CPM test supernatant	CPM test - CPM control
1	1×10^6	56,6	316,7	260,1
	2×10^6	70,0	838,0	768,0
	5×10^6	243,6	3 833,9	3 590,3
	10×10^6	670,1	7 209,7	6 539,6
	20×10^6	1 130,4	6 318,7	5 188,3
2	1×10^6	427,8	597,8	170,0
	2×10^6	279,5	362,8	83,3
	5×10^6	348,2	1 038,1	689,9
	10×10^6	856,8	7 204,3	6 347,5
	20×10^6	3 418,8	11 167,7	7 748,9
3	1×10^6	295,8	513,8	218,0
	2×10^6	236,1	1 540,1	1 304,0
	5×10^6	2 657,4	8 919,1	6 261,7
	10×10^6	2 590,7	9 682,7	7 092,0
	20×10^6	3 367,9	5 341,6	1 973,7

TABLE 7b. OPTIMAL NUMBER OF THYMOCYTES REQUIRED TO ASSAY IL - 1

Means of the three experiments shown in Table 7a.

Thymocyte concentration/ml	CPM test - CPM control	± SD
1×10^6	216,0	± 45,1
2×10^6	718,4	± 611,8
5×10^6	3 514,0	± 2 786,7
10×10^6	6 659,7	± 386,5
20×10^6	4 970,3	± 2 893,8



THE GRAPH OF THYMOCYTE STIMULATION BY IL-1 SUPERNATANTS (CPM x 10^3) VERSUS THE THYMOCYTE CONCENTRATION (x10^6/ml)

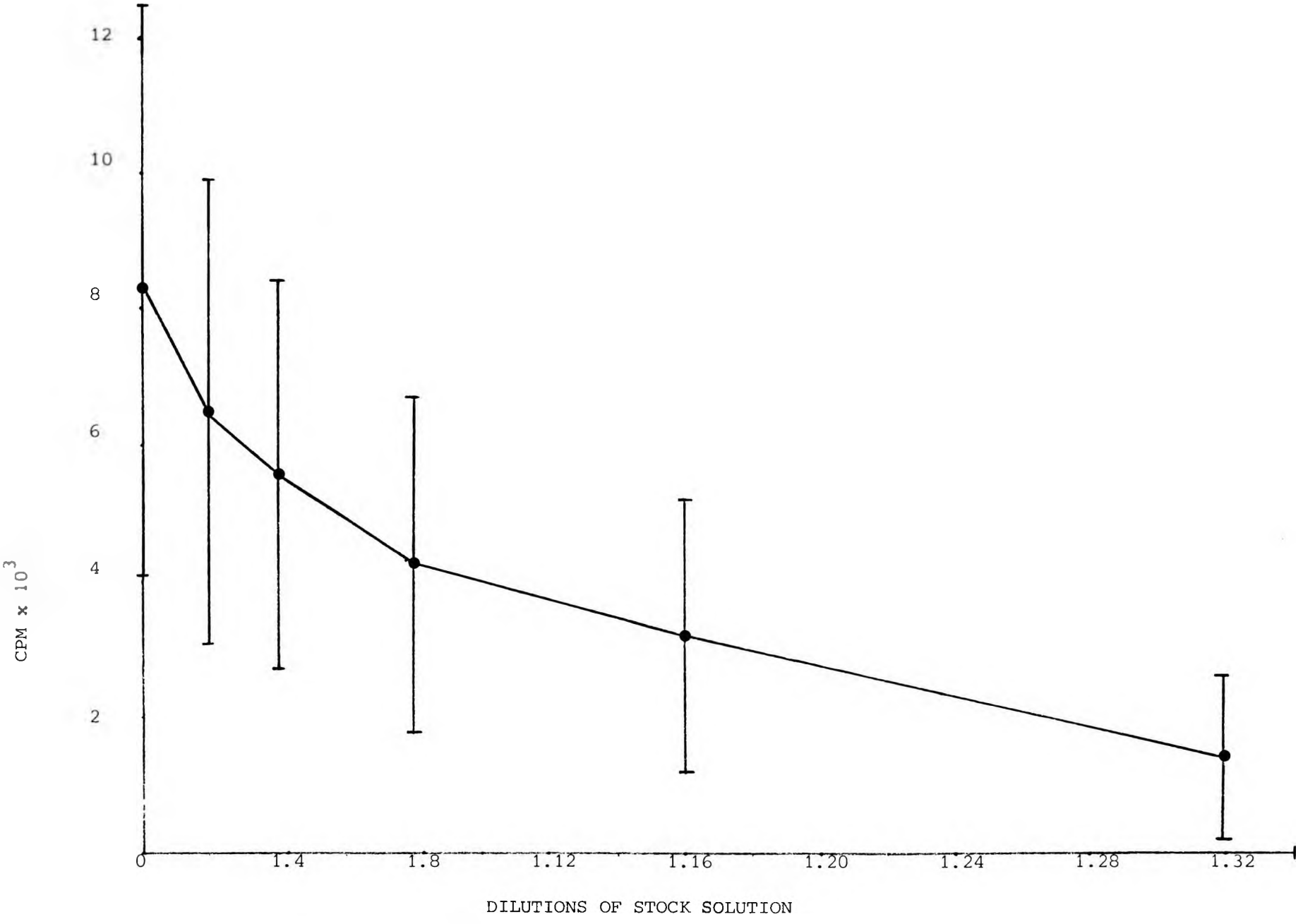
TABLE 8a. DILUTING OUT OF IL - 1 ACTIVITY

Experiment No.	Dilution of stock	CPM control	CPM test	CPM test - Control
1	0	3 495,0	11 152,5	7 657,5
	1:2	2 337,0	8 828,0	6 491,0
	1:4	2 305,5	8,153,5	5 848,0
	1:8	2 047,5	7 455,5	5 408,0
	1:16	1 666,8	5 049,0	3 382,1
	1:32	1 170,1	3 841,0	2 670,9
2	0	3 026,2	15 831,6	12 805,4
	1:2	1 379,0	11 209,9	9 830,9
	1:4	1 018,5	9 998,7	8 980,2
	1:8	1 225,0	7 151,5	5 926,5
	1:16	647,5	5 835,0	5 187,5
	1:32	353,4	1 705,4	1 352,0
3	0	1 121,4	5 493,2	4 371,8
	1:2	837,8	3 935,7	3 097,9
	1:4	658,8	2 622,6	1 963,8
	1:8	518,2	1 977,6	1 459,4
	1:16	486,4	1 631,1	1 144,7
	1:32	364,3	575,1	210,8

TABLE 8b. DILUTING OUT OF IL - 1 ACTIVITY

Means of the three experiments shown in Table 8a.

Dilution of stock	CPM test - Control	Standard deviation
0	8 278,2	± 4 250,9
1:2	6 473,3	+ 3 366,5
1:4	5 597,3	± 1 786,1
1:8	4 264,6	± 2 443,2
1:16	3 238,1	+ 2 025,2
1:32	1 411,2	± 1 231,1



THE GRAPH OF THE STIMULATION BY DILUTIONS OF STOCK IL-1 CONTAINING SUPERNATANTS (CPM) VERSUS DILUTIONS OF THE STOCK SOLUTION.

Waksman, 1972). Support of this statement is provided by the facts that, firstly, IL - 1 is secreted by mouse peritoneal cells even after treatment with anti-theta serum and complement to eliminate T cells (Calderon et al., 1975). Secondly, T cell depleted mouse spleen cells produce IL - 1 (Hoessli and Waksman, 1976), and finally it is elaborated to a greater degree by peritoneal cells from nude than from normal mice (Meltzer and Oppenheim, 1977). It seems unlikely that contaminating adherent B cells are involved in IL - 1 secretion since in this work the purity of macrophages (monocytes) by yeast ingestion was shown to be greater than 80% and by morphology greater than 90%. Also it has been found that IL - 1 is secreted by macrophage cell lines that contain no contaminating B cells (Calderon et al., 1975). Therefore experimental evidence conclusively indicates that IL - 1 is produced by macrophages. The number of adherent cells required for optimal IL - 1 production in this system was found to be from $2,5 \times 10^5 - 5 \times 10^5$/ml. At higher concentrations IL - 1 production seemed to be inhibited. This inhibition could be due to a number of factors:

i) The size of the culture vessel could be too small for large cell numbers, thereby resulting in overcrowding, rapid useage of nutrient, cell death and decreased IL - 1 production.

ii) At high cell concentrations an inhibitory factor could be produced limiting IL - 1 production.

Bacterial lipopolysaccharide is largely considered an immune adjuvant. The effects that LPS exert on the immune response are complex, (Hoffmann and Gilbert 1980) since under varying conditions LPS can act to either enhance or suppress the various

arms of the immune response (Franzl and McMasters, 1968; Hoffmann et al., 1977). IL - 1 is a possible mediator of LPS adjuvancy (Mizel, 1980) since LPS is a potent stimulator of IL - 1 production by macrophages and IL - 1 plays an important role in macrophage mediated T cell activation. However, the mode of action of bacterial lipopolysaccharide on the immune response is largely unknown but the following theories have been put forward based on experimental evidence:-

E. coli lipopolysaccharide is a non-immunological macrophage activating agent which acts directly on the macrophage and not by causing the release of macrophage activating agents by T cells (Ögmundsdóttir and Weir, 1980). It binds to the macrophage (MØ) plasma membrane by the macrophage lectin-like receptors that are specific for sugars present in bacterial cell walls (Ögmundsdóttir and Weir, 1976; Freimer et al., 1978). Binding of endotoxin is probably easily mediated by a non-specific carbohydrate-mediated recognition mechanism by virtue of its polysaccharide chain. Such binding will not result in full macrophage activation (Ögmundsdóttir and Weir, 1979). The lipid A fraction of endotoxin has the full activating power of the larger molecule (Alexander and Evans, 1971; Wahl et al., 1974). Since lipid A is a glycolipid (Lüderitz et al., 1973) it might still attach to carbohydrate binding sites, but the lipid moiety seems essential for its action. E. coli lipopolysaccharide could act in a similar manner to cholera toxin on gut musocal cells. Cholera toxin binds to the cell surface by one subunit of its molecule and the part of the molecule mediating the toxic effect is thought to be inserted subsequently into the lipid bilayer of the plasma membrane (Bennett, et al., 1975; Cuatrecasas et al., 1976).

This also suggests the importance of plasma membrane mobility in receptor mediated stimulation of cells, enabling reversible linkage of a surface receptor and the membrane enzymes involved in the transmission of signals across the membrane (Cautracasas, 1974; Bennett et al., 1975). Concerning signal transmission in macrophage stimulation - it is known that the binding of drugs or hormones to cell membranes results in cell stimulation by the transmission of an activating signal across the cell membrane. This process involves a second messenger that is either cAMP or cGMP. An increase in intracellular second messenger is probably either caused by the complexing of the catalytic enzyme with the receptor on the binding of the first messenger, that is the drug or hormone, or the first messenger receptor itself may be part of the catalytic enzyme resulting in enzyme activation upon binding of the first messenger (Rimon et al., 1978). An increase in the second messenger results in an enzymatic cascade (Robison, et al, 1968; Catt and Dufau, 1977). In the case of macrophage activation there is an increase in the second messenger cGMP (Sandler et al., 1975a, b and c) but not cAMP. This could be significant since it is known that increases in cAMP inhibit several aspects of the immune and inflammatory response (Henney and Lichtenstein, 1971; Bourne et al., 1974) whilst cGMP is stimulatory (Watson, et al, 1973; Watson, 1975; Weissmann et al., 1975; Bourne et al., 1971; Estensen et al., 1973).

Experimentally this project has shown that LPS greatly enhances IL - 1 production by human peripheral blood adherent cells by activating the adherent cells as compared to adherent cells not treated with LPS. Furthermore, the optimal dose of LPS required for maximal stimulation appears to be 10 - 20 μg/ml,

thereafter, the LPS retards IL - 1 production. This retardation could be due to the following:-

i) LPS becoming toxic to the system.

ii) LPS at higher concentrations acting as an immuno-inhibitor instead of stimulant.

Finally for optimal IL - 1 secretion by adherent cells LPS was required for the entire culture period. Pulsing the cells with LPS did not lead to optimal IL - 1 secretion. The reasons for this phenomenon could be as follows:-

i) Stimulation of adherent cells by LPS requires long periods and pulsing with LPS between 2 and 6 hours is not sufficient to activate the adherent cells.

ii) LPS is weakly bound to the adherent cell plasma membrane and washing of the cells may remove the bound LPS thereby inhibiting the activation process. Similar results have been found by other workers regarding LPS presence for IL - 1 production (Meltzer and Oppenheim, 1977).

The time required for LPS activated adherent cells to produce optimal amounts of IL - 1 was found to be 24 - 48 hours in this study. Other workers have made similar observations (Blyden and Handschumacher, 1977). After 48 hours IL - 1 production tends to be retarded and even falls. This could be due to the production of a control factor which degrades IL - 1 in the cell as it is being produced or controls IL - 1 production. The involvement of a control proten in IL - 1 production has been hypothesized (Unanue, 1978). This explanation is feasible

because in the initiation of the immune response only certain amounts of IL - 1 are required and there must be some mechanism to switch off IL - 1 production by activated adherent cells. Maximal IL - 1 production probably only occurs after 24-48 hours because macrophage (MØ) activation by LPS and synthesis of protein moieties of IL - 1 are probably only completed after this time period.

2.4.2 Action of IL - 1 on thymocytes

IL - 1 is assayed on mouse thymocytes in which it causes proliferation as is indicated by an increase in thymidine uptake by these cells. The time taken for human IL - 1 to cause optimal proliferation of thymocytes is 3 days as is indicated by experimental results in this dissertation. The response however begins within 36 - 48 hours. Other workers have shown similar results (Oppenheim, et al, 1979). When using murine IL - 1, the time for optimal thymocyte stimulation varies slightly from the above (Kierszenbaum and Waksman, 1977). It has been observed that IL - 1 has greater mitogenic effects on the more mature, cortisone-resistant cells (Blyden and Handschumacher, 1977). This data however is in contrast with the fact that IL - 1 action on thymocytes decreases after the age of 10 weeks (data not shown) when the ratio of cortisone-resistant to sensitive thymocytes has increased. Furthermore, other workers have shown that both subpopulations of thymocytes are responsive (Gery et al., 1972) and that the proliferative response of untreated thymocytes is greater than that of cortisone-resistant subpopulations (Oppenheim, et al, 1979). This contrasting evidence could be explained as follows:-

It has been shown that only actively cycling cells can respond to macrophage culture fluids (MCF) (Beller and Unanue, 1979) which contain IL - 1. Apparently the response of thymocytes to MCF is based on a dual signal mechanism (Togawa, et al, 1979b). The first signal is probably a characteristic inherent in a subpopulation of cycling cells, for example cyclic nucleotide levels, and this first signal allows the cell to respond to some factor, probably IL - 1, in MCF and thereby start proliferating. Therefore, the varying responses of different thymocyte subpopulations to IL - 1 could be due to varying numbers of cycling cells in the thymocyte subpopulation at that particular time. Workers have also shown that non-cycling thymocytes can be made responsive to IL - 1 after a lectin pulse, which restores optimal responsiveness to MCF when given on day 2 or 3, but not day 1 (Beller and Unanue, 1979). This could explain why thymocyte proliferation is most marked on day 3 in the results presented in this dissertation, i.e. the thymocytes may have stopped cycling and the LPS present in the IL - 1 preparation may stimulate the cells to cycle on day 2 and 3 thereby allowing them to respond optimally to IL - 1 on day 3. Furthermore, it may also explain why such high concentrations of thymocytes are required to achieve optimal stimulation by IL - 1, i.e., the greater the cell number, 10 - 15 x 10^6/ml, the greater the number of naturally cycling or induceable thymocytes there are to respond to IL - 1.

CHAPTER 3

3. IL - 1 PRODUCTION IN PATIENTS WITH SLE AS COMPARED TO NORMAL CONTROLS

3.1 Introduction

IL - 1 production is a relatively easy test for the testing of macrophage secretory function. It has been found that IL - 1 production is depressed in a number of disease states of man and mouse (Oppenheim et al., 1979). IL - 1 production is depressed in patients with tumour loads (Leikin et al., 1978). In these people both monocytic and lymphocytic functions are depressed which is probably as a result of the tumour load rather than a direct cause of the disease. Proof of this is that carcinoma patients in remission have normal IL - 1 production. Anergic patients with Hodgkins's disease and tuberculosis have decreased IL - 1 production although their number of circulating monocytes is usually raised (Scheichter et al., 1977). In malaria infections induced in mice,IL - 1 secretion processes from hypersecretion to hyposecretion (Wyler et al., 1979). Leukaemia patients with AML and AMML secrete large quantities of IL - 1, whereas those with CML and AML and AMML with few monocytes do not release significant amounts of IL - 1 (Lachman et al., 1978).

Because the macrophage appears to be the central cell in controlling the immune response it seemed interesting to study IL - 1 secretion in diseases where control of the immune response is aberrant and in which macrophage function may be abnormal. One such disease is "Systemic Lupus Erythematosus" (SLE) and for this reason IL - 1 secretion was studied in a number of SLE cases and compared to normal IL - 1 secretion.

3.2 Material and methods

3.2.1 Patients and controls

The study group comprised ten patients with active Systemic Lupus Erythematosis (SLE). Their ages ranged from 15 to 48 years and all had, at some time, manifested marked disease activity as evidenced by low serum complement levels, positive anti-nuclear and anti-DNA antibody levels. All patients satisfied the ARA preliminary criteria for the diagnosis of SLE. Eight of the ten patients were on treatment with varying doses of corticosteroids when tested. The control group consisted of healthy laboratory workers.

3.2.2 Experimental design

From the results in Chapter 2 a routine assay was set up for testing IL - 1 production in patients. The assay was essentially the same as the one described in Chapter 2 except that:

1) 5×10^5 monocytes/ml were used for IL - 1 supernatant production.
2) The concentration of LPS used to stimulate monocytes was 20 μg/ml.
3) IL - 1 supernatant production was allowed to occur by culturing monocytes for 24 hours.
4) IL - 1 supernatants were assayed on 10×10^6 thymocytes/ml and were cultured for a period of 72 hours.
5) Patients results were compared to those of normal individuals.

TABLE 9. IL - 1 PRODUCTION IN SLE AS COMPARED TO NORMAL CONTROLS

Group	Mean	Standard deviation	Range
Normal controls	5,2	± 5,1	1,5 - 14,2
SLE patients	4,3	± 4,0	1,4 - 14,4

3.3 Results

The results of IL - 1 production by the adherent cells (monocytes) of ten SLE patients was compared to that of the monocytes of a normal control group. The results are shown in Table 9 and Graph 9. Results are represented as a stimulation index which is counts per minute (cpm) of the test (IL - 1 containing) supernatant divided by cpm of the control supernatant.

The results presented in Table 9 and Graph 9 indicate that IL - 1 production by individuals with SLE falls into the normal range of production by normal people.

3.4 Discussion

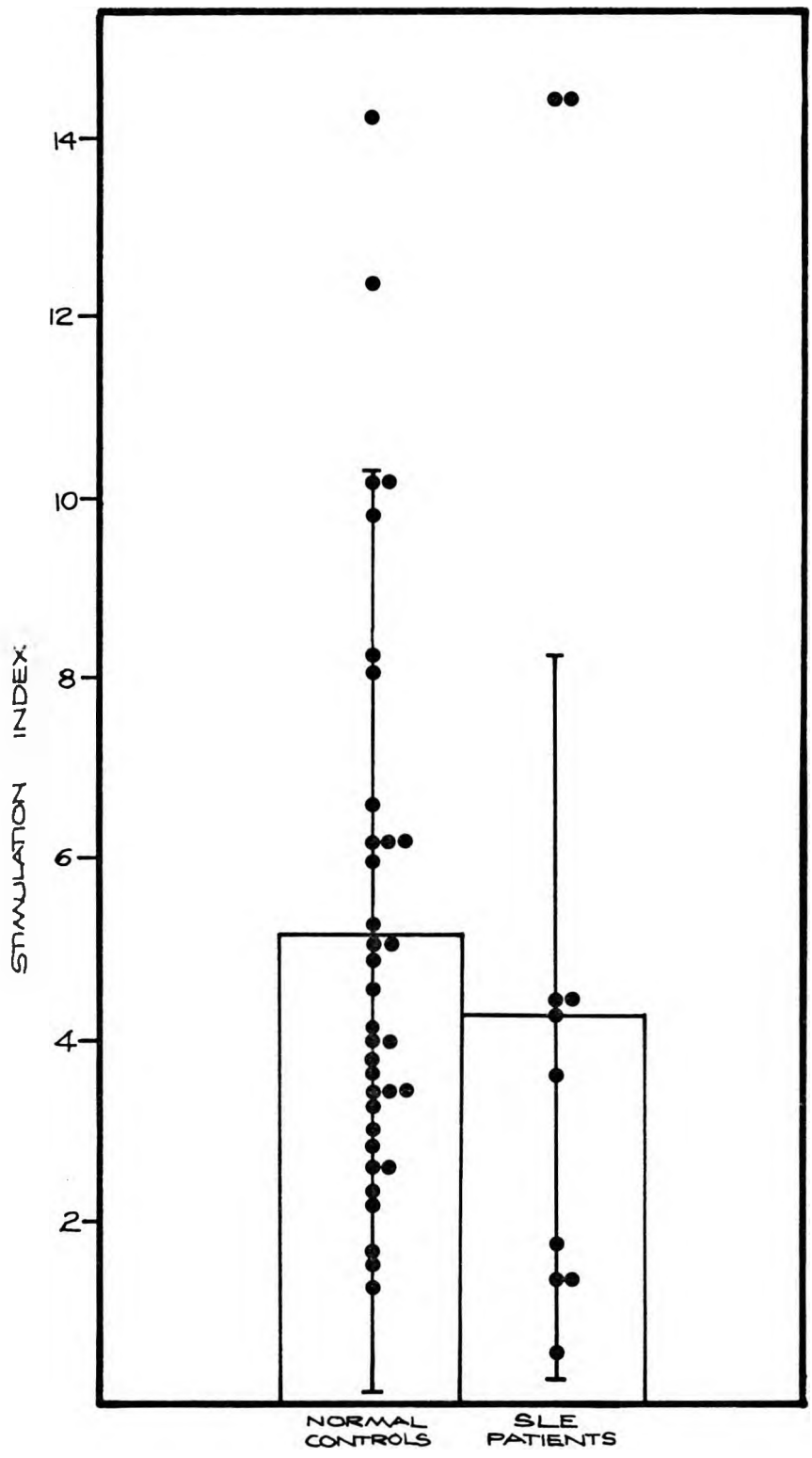
SLE is a multisystem autoimmune disease of unknown aetiology characterised by the production of large quantities of autoantibodies. Viral, genetic, hormonal and immunologic factors have been implicated in the pathogenesis of the disease (Bach and Dardenne, 1972; Christian et al., 1975; Panem et al., 1976; Budman et al., 1977). However, whether the disease is due to a virus, hormonal or immunological disorder, the ultimate effect seems to be on the immune system. Immunological abnormalities found in patients with active SLE include:

1) Impaired proliferation of lymphoid cells in response to mitogens, antigens and allogeneic cells (Rosenthal and Franklin, 1975).

2) Hyporesponsiveness to intradermal injection of antigen (Baum and Ziff, 1969).

3) Impaired primary antibody responses (Horwitz and Cousar, 1975).

THYMOCYTE STIMULATION BY IL - 1 SUPERNATANTS (STIMULATION INDEX) OF SLE PATIENTS AND NORMAL CONTROLS



GRAPH 9.

4) Excess production of autoantibodies to a variety of tissues including T lymphocytes, probably T suppressor cells, as a result of B cell hyperactivity (Kunkel and Tan, 1964; Lies et al., 1973; Wernet and Kunkel, 1973). The cause of the B cell hyperactivity and resulting autoantibody production is largely unknown, but has been explained by many workers to be as a result of suppressor T cell absence or malfunction (Sakane et al., 1978, 1979; Krakauer et al., 1980). Explanations for the suppressor cell defect include selective viral infection (Krakauer et al., 1980), or the production of antibodies to suppressor cells or their precursors resulting in their inactivation (Glinski et al., 1976; Sagawa and Adbou, 1979; Sakane et al., 1979). It has been hypothesized that the suppressor cell defect results in autoantibody production, including an antibody to suppressor cells which causes further suppressor cell malfunction therefore resulting in a self-perpetuating cycle (Krakauer et al., 1980).

The macrophage is said to be the central controlling cell in the immune response and could therefore theoretically be involved in the pathogenesis of SLE. As discussed in General Introduction, page 30 macrophages stimulated by foreign substances secrete IL - 1 which in turn acts on helper cells together with antigen thereby activating them. The activated helper cells then secrete IL - 2 which, together with antigen, stimulates the proliferation of T suppressor, helper and cytotoxic progenitor cells to become effector cells. T helper effectors then act on B cells causing them to develop into antibody secreting plasma cells (Smith, 1980). Now, if macrophages of patients with SLE are continually stimulated by, for example, immune complexes, which are known to be present in SLE sera in increased amounts (Kavai et al., 1977) then there will be an excess of IL - 1 secretion ultimately resulting in an excess production of antibody. On the other hand the macrophages may be secreting IL - 1 in normal amounts, but excessive IL - 2 may be secreted by T cells due to an imbalance in control mechanisms - possibly a defect in T suppressor cells specific for IL - 2

secretor or precursor cells. The latter seems to be the more likely hypothesis since in this thesis it has been shown that the peripheral blood adherent cells of SLE patients secrete IL - 1 similarly to those of normal controls. It is however extremely difficult to test macrophage function of SLE patients, since the majority are on high doses of steroids which tend to alter their immune status, since steroids are targets for macrophages and T cells (Ralph, 1973; Vann, 1974; Lee et al., 1975; Vassalli et al., 1976; Mishell et al., 1977; Ralph et al., Werb et al., 1978). Therefore, extensive study in this field is required on untreated patients, in which case it might be found that excess IL - 1 secretion is operative in the pathogenesis of the disease.

It should be noted that macrophage defects have been described in SLE. These include:

1) A rapid turnover of macrophage Fc receptors (Kavai et al., 1980).

2) Impaired phagocytosis by macrophages (Landry, 1977).

3) Lack of secretion of a macrophage substance which appears to stimulate the development of a suppressor cell population (Wadee et al., 1981).

However, whether the above defects of macrophages in SLE have any effect on IL - 1 secretion by these cells is as yet unknown.

CHAPTER 4

4. THE EFFECT OF IL - 1 CONTAINING SUPERNATANTS ON THYMOCYTES

4.1 Introduction

For an in-depth understanding of immune deficiency states, it is necessary to understand and define the cellular interactions which programme, as well as implement, lymphoid maturation. Of particular interest is T cell maturation. The thymic epithelial cell of the thymic reticulum is considered of great significance in this process. The epitehlium is said to be a source of thymic hormones which are concerned with the various stages of thymocyte differentiation (Kruisbeek et al., 1977: Dardenne et al., 1974). The non-lymphoid reticulum has furthermore been implicated in allowing the thymocyte to recognize "self" H-2 antigen (Zinkernagel, 1978). The thymic reticulum moreover is heterogeneous, being composed of macrophages as well as epithelial cells and fibroblasts (Wekerle et al., 1973; Hoefsmit and Gerver, 1975). Thymic macrophages, via secretion of lymphoregulatory molecules, may play a central role in T lymphocyte maturation.

For the above reasons a study was embarked upon to investigate the possible effects of lipopolysaccharide-stimulated adherent cell IL - 1 production on the differentiation of thymocytes to various types of mature T cells. Other workers have shown effects of macrophage culture fluids on thymocyte maturation, such as, changes in membrane properties and responsiveness in MLC (Beller and Unanue 1977, 1980), as well as the maturation of thymocytes to T helper cells (Young and McIvor, 1980). This study has shown that IL - 1 containing supernatants induce the formation of helper cells in the thymocyte population

as is indicated by an increase in the proliferative responses, as well as antibody production by B cells in the presence of IL - 1 pretreated thymocytes.

4.2 Materials and methods

4.2.1 Suppressor assay

The purpose of this experiment was to see if IL - 1 containing supernatants induced suppressor cell (S-cell) production in a population of mouse thymocytes. The assay was carried out as follows:-

Stage one - activation of mouse thymocytes by IL - 1 containing supernatants (IL - 1 supernatants)

Mouse thymocytes were collected as described under point 2.2.2.1, and suspended to a concentration of 10 x 10^6/ml in cRPMI. 2,5 ml aliquots were cultured in tissue culture tubes with 0,25 aliquots of IL - 1 supernatant or control supernatants for 72 hours at 37°C in a humidified 5% CO_2 incubator. At the end of the culture period the cells were washed three times, irradiated at 3 000 rads, washed once more and resuspended to a concentration of 1 x 10^6/ml in complete medium cRPMI.

Stage two - assay for suppressor activity

Responder cells (R-cells) were either syngeneic or allogeneic mouse splenocytes which were collected as follows:- Spleens of BALB/c mice were removed aseptically and teased apart in medium. The suspension of cells was allowed to settle for 5 - 10 minutes and the splenocytes were decanted from the settled debris. The cells were washed twice and mixed with NH_4Cl for 10 minutes at 4°C to lyze the red blood cells. They were then

centrifuged at 100 - 150 g for 5 - 10 minutes to allow the splenocytes to settle as a pellet and the red blood cell membranes to remain in the supernatant. The cells were washed twice more and suspended in cRPMI to a concentration of 1 x 10^6/ml. 0,1 ml aliquots of R-cells were mixed with equal aliquots of IL - 1 supernatant or control supernatant pretreated thymocytes in round bottomed microtitre plates. The cultures were stimulated with 20 µg/ml PHA. The cultures were incubated at 37°C for 72 hours in 5% CO_2 humidified air. 0,5 µCi of (methyl 3H) thymidine was added to each culture well for the last six hours of culture and the cells were then harvested by a multiple automatic harvester. Incorporation of labelled thymidine into the cells was measured by liquid scintillation counting. The experiments were carried out in triplicate and the results represented as the means.

4.2.2 B cell proliferative response as an assay for helper cells

Results obtained in the suppressor cell experiments (see Section 4.3.1) indicated that IL - 1 supernatants did not induce the formation of suppressor cells in thymocyte population. However, preliminary evidence indicated a helper effect and this was further investigated using the helper cell assay.

Stage one - B cell separation

The first part of this technique involved B cell separation from splenocytes (see Figure 8). Spleens were asceptically removed from BALB/c mice and treated as is described in stage two of the suppressor assay except that the pure splenocytes were suspended to a concentration of about 15 - 50

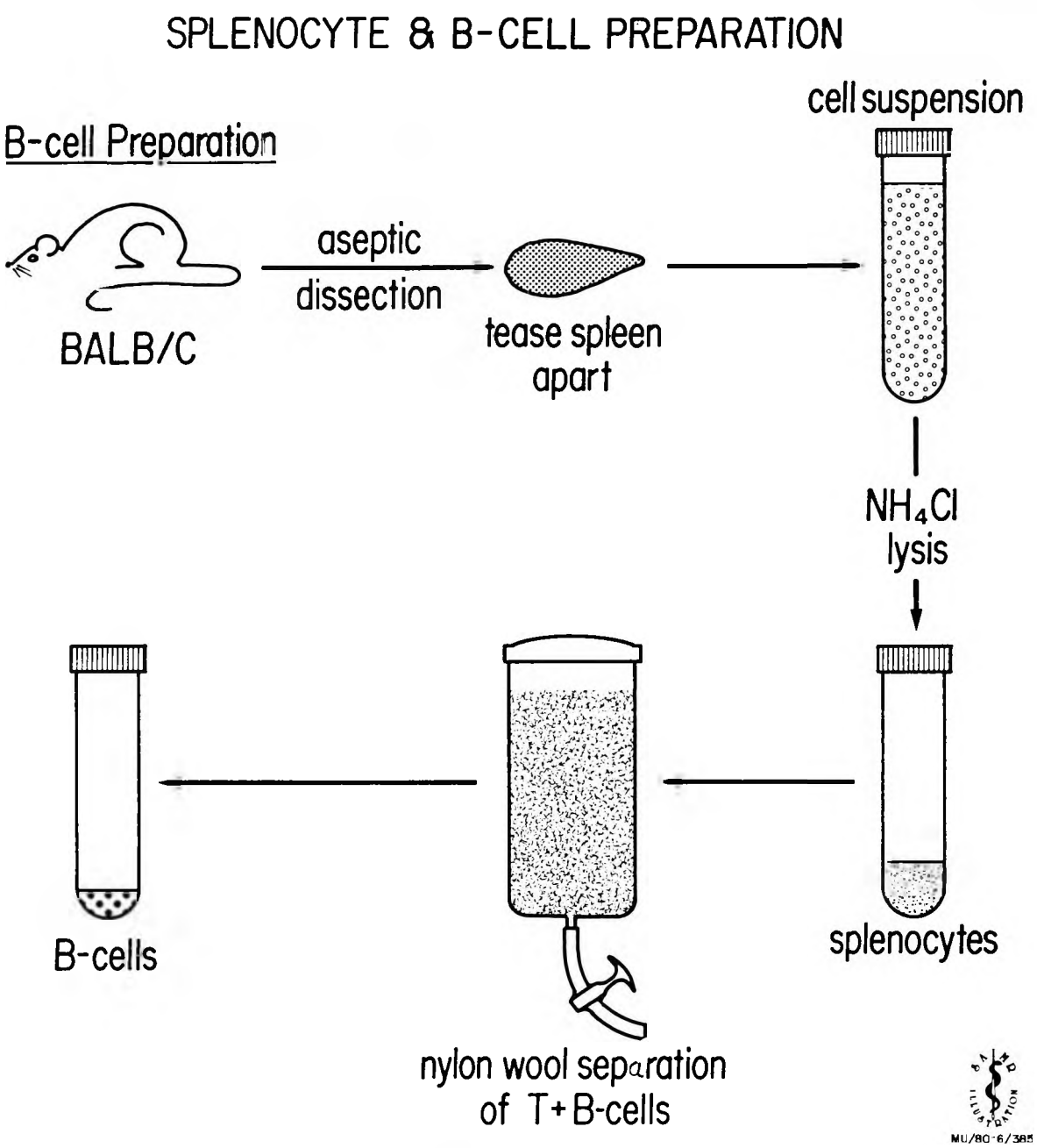

SPLENOCYTE & B-CELL PREPARATION
B-cell Preparation
cell suspension
aseptic
dissection
BALB/C
tease spleen
apart
NH4Cl
lysis
B-cells
splenocytes
nylon wool separation
of T+B-cells
MU/80-6/385

Figure 8

cells/ml in warmed RPMI + 5% FCS. The splenocytes were separated on a nylon wool column as follows:- 0,7 g of sterile nylon wool was teased apart under media (heated RPMI) in a large petri dish with forceps until there was no resistance. The saturated nylon wool was added little by little to a 5 cc syringe "column". The column was washed with 25 cc of heated RPMI (allowing a moderate drip rate). Then 10 cc of warmed RPMI + 5% FCS was added, being careful not to allow the level of the media to drop below the top of the column. A stopcock was attached to the bottom of the syringe and the top was covered with parafilm and the column incubated for one hour at 37°C. The splenocyte suspension was then added slowly to the top of the column controlling the drip rate of the eluted media with the stopcock. When all the cells were added the stopcock was closed and the column incubated at 37°C for one hour. Thereafter 12,5 ml of medium was dripped very slowly into the column and the slowly eluted non-adherent cells (T cells) collected. The column was then washed with 50 cc of warmed RPMI 5% FCS, allowing a fast drip at the eluting end of the column. The barrel of the column was then tapped vigorously several times, the stopcock opened and the plunger inserted into the syringe, pushing the nylon wool until tightly packed. More medium was then added and the plunging process repeated three times. The eluted material contained the adherent cells (B cells and macrophages). The purity of the B cells so obtained was tested by surface immunofluorescence and found to be 60% or greater.

Stage two - IL - 1 induction of the helper effect (see Figure 9)

Mouse thymocytes were collected as described under

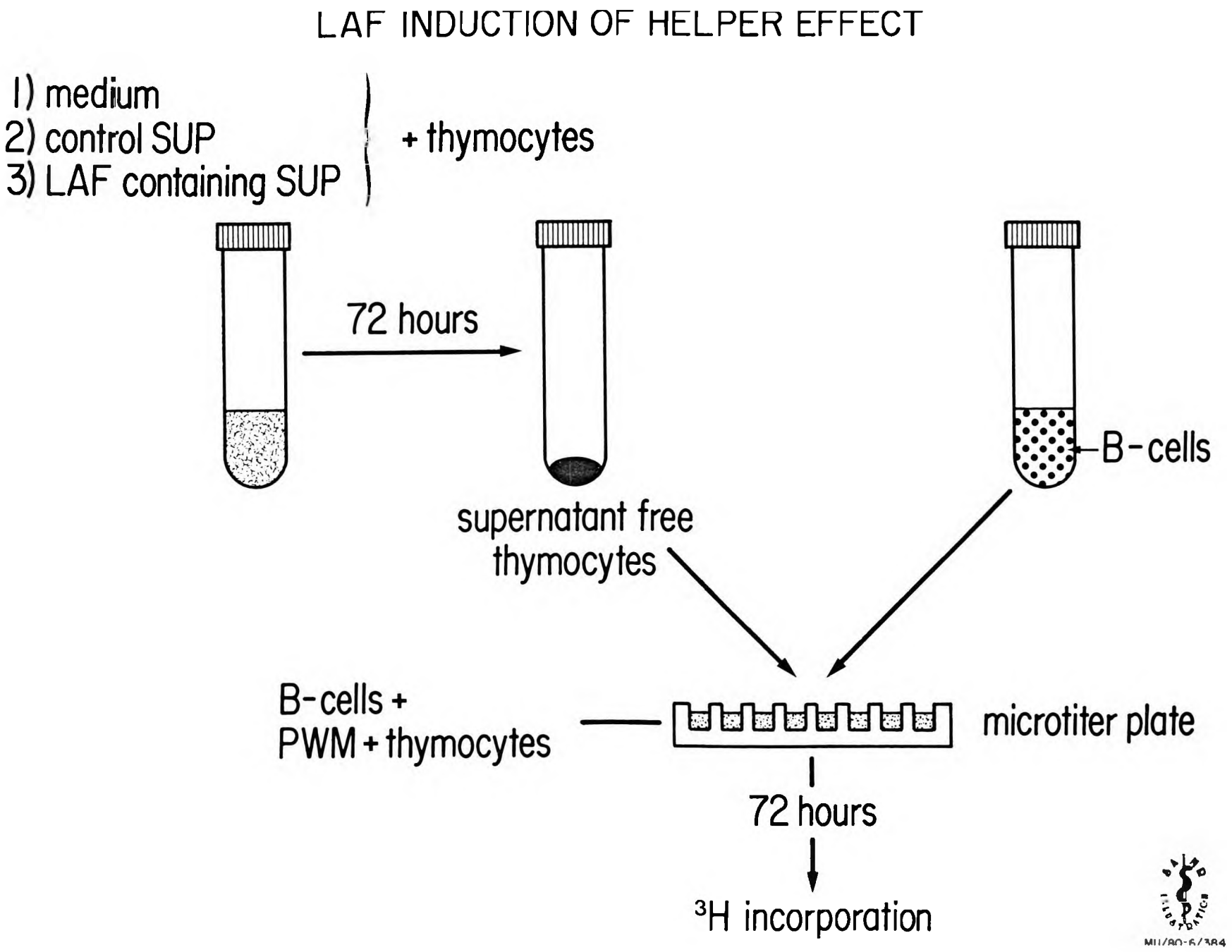

LAF INDUCTION OF HELPER EFFECT
1) medium
2) control SUP
3) LAF containing SUP
+ thymocytes
72 hours
B-cells
supernatant free thymocytes
B-cells +
PWM + thymocytes
microtiter plate
72 hours
3H incorporation

Figure 9

point 2.2.2.1, and suspended to a concentration of 10 x 10^6/ml in cRPMI. 2,5 ml aliquots were cultured in tissue culture tubes with 0,25 ml IL - 1 supernatant or control supernatant for 24, 48 or 72 hours. The cells were then washed 3 times and resuspended in cRPMI at a concentration of 2 x 10^6/ml. 0,1 ml aliquots of B cells and equal aliquots of pretreated thymocytes at ratios of 1:1, 2:1 and 1:2 were cultured in round bottomed microtitre plates in the presence or absence of 20 µl PWM (1:10 dilution of stock) for 72 or 120 hours in a 37°C, 5% CO_2 humidified incubator. 0,5 µCi of (methyl 3H) thymidine was added to each culture well for the last six hours of incubation and the cells harvested. Incorporation of labelled thymidine was measured by liquid scintillation counting. The cultures were set up in triplicate and the results represented as means.

4.2.3 Immunofluorescent studies to assess the number of immunoglobulin secreting cells stimulated by IL - 1 induction of helper cells in thymocytes

4.2.3.1 Cell culture preparations

BALB/C mice were injected with 0.2 ml of a 1% solution of sheep red blood cells (SRBC) in their lateral tail veins. After three days the mice were sacrificed, the spleens removed and treated as described under point 4.2.1. 0.5 ml of spleen cells (30 x 10^6/ml) were cocultured with 0.5 ml of thymocytes (8 x 10^6/ml), pretreated with control or IL - 1 supernatants for 24 hours, plus 50 µl of 1% SRBC in petri - dishes. The cultures were set up in duplicate and incubated for 3 days in a 37°C 5% CO_2 humidified incubator. The cells were fed with 0.1 ml of nutrient broth and 20 µl of FCS each day. After three days

the cells from the cultures were harvested and washed in PBS.

4.2.3.2 Preparation and Staining of slides for immuno-fluorescent studies

Slides were prepared by placing one drop of cell suspension on a 10 mm diameter round coverslip which had been lodged at the bottom of a 50 ml falcon tube with a hole punctured in the base. The tubes were then centrifuged at 100 x g for 3 mins. This caused the cells to stick to the coverslip and allowed the excess fluid to drain from the tubes. Fixation was then accomplished by means of an acetic acid - ethanol solution for 15 min. at -20°C. After fixation the cells were not allowed to dry. The coverslips were then washed for 15 min. at + 4°C in PBS with three changes of buffer. The washed coverslips were then drained, put in a moist chamber and covered with fluorescent conjugated horse anti-mouse immunoglobulin to which rhodamine labelled anti - albumin had been added as a counterstain. After incubation at room temperature for 30 min. the coverslips were washed in PBS for 15 min. They were then dried and inverted onto glass slides onto which a drop of buffered glycerol had been placed. The slides were then examined under a Reichel Zetopan fluorescence microscope using transmitted light.

4.3 Results

4.3.1 Results indicating whether IL - 1 induces suppressor cells in the thymocyte population (see Table 10).

Thymocytes were pretreated with control or IL - 1 containing supernatants for 72 hours. The cells were then washed, irradiated and cocultured with splenocytes in the presence or absence of PHA in order to assess suppressor cell activity.

As is indicated in Table 10, IL - 1 does not induce suppressor cell development in the thymocyte population; but instead, appears to activate helper cells in this thymic population. It should be noted that the experiments had to be expressed individually and not compared as a group because the different experiments were carried out using different batches of crude IL - 1. Each batch was prepared from the monocytes of a different donor and therefore varied greatly in the amount of IL - 1 produced, which could not be standardized. For this reason all the results in Section 4.3 are presented as individual experiments.

4.3.2 Helper cell assay results

(see Tables 11, 12, 13 and 14).

4.3.2.1 Helper cell assay results
(see Table 11)

Thymocytes were pretreated with control and IL - 1 containing supernatants for 72 hours, washed and cocultured with B cell enriched splenocytes at a ratio of 1:1 in the presence and absence of PWM for three days.

The results shown in Table 11 indicate that IL - 1 containing supernatants stimulate thymocytes to become helper cells. These cells in turn help B cells to undergo proliferation in the presence of poke-weed mitogen (PWM). It should be noted that the control supernatants also seem to stimulate helper activity, although to a lesser degree than the IL - 1 containing supernatants. This is because monocytes spontaneously secrete small amounts of IL - 1, but, IL - 1 secretion is greatly enhanced

TABLE 10.

SUPPRESSOR CELL ASSAY

Experiment No.	CPM Splenocytes + PHA (20 ug/ml)	CPM Medium pretreated thymocytes + splenocytes + PHA (20 ug/ml	CPM Control supernatant pretreated thymocytes + splenocytes + PHA (20 µg/ml)	CPM IL-1 supernatant pretreated thymocytes + splenocytes + PHA (20 µg/ml)	Percentage help = $\frac{\text{CPM test - CPM control}}{\text{CPM control}}$ x 100
1	3 891,1	2 749,6	7 644,4	13 573,2	77,6%
2	3 297,1	2 364,4	7 443,2	10 176,4	36,7%
3	6 813,0	7 114,7	13 444,0	20 700,0	54,0%
4	12 063,9	13 529,9	15 540,4	25 144,4	61,8%
5	12 968,7	13 941,5	29 851,6	39 624,8	32,7%

by LPS. Furthermore, as is shown, the helper effect is not due to LPS, although LPS is known to stimulate B cells. Experimental results not shown here indicate that thymocytes alone or pre-treated thymocytes do not respond to PWM, and B cell enriched splenocytes respond slightly, but not significantly, depending on the degree of T cell contamination in B cell preparations.

4.3.2.2 Optimal time for preculture of thymocytes with IL - 1 supernatants to stimulate helper cell development (see Table 12).

Thymocytes were preincubated with IL - 1 containing- and control supernatants for 24, 48 or 72 hours. The thymocytes were then washed, the viability corrected for and cocultured with B cells in the presence and absence of poke-weed mitogen (PWM).

The results shown in Table 12 indicate that IL - 1 containing supernatants induce helper cells in the thymocyte population following 24, 48 and 72 hour preincubations of the thymocytes with IL - 1. The difference in the help achieved during the different preincubation times varies very little.

4.3.2.3 Optimal cell ratios for the helper cell assay (see Table 13)

Thymocytes were preincubated for 72 hours with test and control supernatants. The thymocytes were then washed and cocultured with B cells plus PWM at ratios of thymocytes to B cells of 1:1, 2:1 and 1:2.

The results shown in Table 13 indicate that a thymocyte to B cell ratio of 2:1 provides the greatest help. A thymocyte to B cell ratio of 1:1 also provides significant help.

TABLE 11. HELPER CELL INDUCTION OF B CELL PROLIFERATION

Experiment No.	CPM Medium pretreated thymocytes + B cells + PWM	CPM LPS pretreated thymocytes + B cells + PWM	CPM Control supernatant pretreated thymocytes + B cells + PWM	CPM IL-1 pretreated thymocytes + B cells + PWM	Help = CPM test / CPM control
1	4 016,3	9 444,0	14 219,0	18 898,0	1,33 X
2	272,9	1 798,4	1 773,0	10 795,4	6,09 X
3	904,0	4 131,3	11 639,3	20 096,7	1,73 X
4	793,9	6 046,9	5 206,5	14 478,7	2,78 X
5	396,2	932,1	8 055,0	15 587,3	1,93 X

TABLE 12.

OPTIMUM TIME FOR THYMOCYTE PRETREATMENT WITH IL - 1 FOR USEAGE IN THE HELPER ASSAY SHOWN IN TABLE 11

Experimental No.	Time of preculture (hours)	CPM Medium pretreated thymocytes + B cells + PWM	CPM LPS pretreated thymocytes + B cells + PWM	CPM Control supernatant pretreated thymocytes + B cells + PWM	CPM IL-1 supernatant pretreated thymocytes + B cells + PWM	Help = CPM test / CPM control
1	24	4 632,7	11 508,0	12 144,0	22 719,3	1,87 X
	48	5 660,0	17 954,0	16 196,0	28 913,0	1,78 X
	72	5 708,0	5 979,3	13 487,0	21 944,7	1,63 X
2	24	8 249,6	10 866,1	7 679,4	15 645,9	2,04 X
	48	2 717,6	4 221,2	5 062,8	14 222,8	2,81 X
	72	1 348,1	4 926,0	5 206,5	14 478,7	2,78 X

4.3.2.4 Optimal culture time for the coculture of pretreated thymocytes plus B cells plus PWM to show the helper ability of the thymocytes (see Table 14)

Thymocytes were pretreated with control and IL - 1 containing supernatants for 72 hours, washed and cocultured with B cells plus PWM for three or five days. Results are shown in Table 14.

The results indicate that the helper effect is evident after three and five days coculture of thymocytes with B cells and PWM. This experiment was carried out since it is said that PWM takes five days to act optimally in culture. In Experiment 2, the medium pretreated thymocytes plus B cells plus PWM gave high results because of a large number of contaminating T cells in the B cell enriched preparation.

4.3.3 IL - 1 Pretreated thymocyte induction of immunoglobulin secreting cells in the thymocyte population

Splenocytes obtained from sRBC sensitized mice were cocultured with control and IL - 1 pretreated thymocytes plus sRBC. After three days the cultures were harvested and the cells obtained stained with horse anti - mouse immunoglobulin for the detection of plasma cells. The results shown in Table 15 indicate that IL - 1 pretreated thymocytes cause an increase in immunoglobulin secreting cells as is indicated by the greater number of fluorescent cells seen in these cultures as compared to the controls. This is either due to the IL - 1 inducing helper cells in the thymocyte population which in turn help B cells become immunoglobulin secreting plasma cells or the IL - 1 pretreated thymocyte activates a T cell in the splenocyte population to

TABLE 13. OPTIMUM THYMOCYTE TO B CELL RATIOS FOR USEAGE IN THE HELPER ASSAY SHOWN IN TABLE 11

Cell numbers		CPM medium pretreated thymocytes + B cells + PWM	CPM LPS pretreated thymocytes + B cells + PWM	CPM control supernatant pretreated thymocytes + B cells + PWM	CPM IL-1 supernatant pretreated thymocytes + B cells + PWM	Help = $\frac{\text{CPM test}}{\text{CPM control}}$
Thymocytes	B cells					
2×10^6/ml	2×10^6/ml	272,9	1 798,4	1 773,0	10 795,4	6,09 X
2×10^6/ml	1×10^6/ml	182,9	409,8	625,6	8 525,4	13,63 X
1×10^6/ml	2×10^6ml	502,1	1 339,7	1 452,8	6 655,5	4,58 X
1×10^6/ml	1×10^6/ml	106,8	316,5	563,2	4 230,2	7,5 X

TABLE 14. OPTIMUM TIME FOR THYMOCYTE AND B CELL COCULTURE FOR USEAGE IN THE HELPER ASSAY SHOWN IN TABLE 11

Experiment No.	Time of culture (days)	CPM Medium pretreated thymocytes + B cells + PWM	CPM LPS pretreated thymocytes + B cells + PWM	CPM Control supernatant pretreated thymocytes + B cells + PWM	CPM IL-1 supernatant pretreated thymocytes + B cells + PWM	Help = CPM test / CPM control
1	3	793,9	6 046,9	5 206,5	15 587,3	2,99 x
	5	1 838,5	17 094,6	18 451,3	25 128,0	1,36 x
2	3	10 204,0	15 631,7	20 406,7	25 085,7	1,23 x
	5	13 847,9	16 634,0	22 315,0	31 104,6	1,39 x

TABLE 15

IL - 1 PRETREATED THYMOCYTE INDUCTION OF IMMUNO-GLOBULIN SECRETING CELLS

	Experiment Number	1	2	3
% FLUORESCENT CELLS	Medium Pretreated Thymocytes + Sensitized Splenocytes + sRBC	13	18	14
	LPS Pretreated Thymocytes + Sensitized Splenocytes + sRBC	18	22	17
	Control Supernatant Pretreated Thymocytes + Sensitized Splenocytes + sRBC	24	20	20
	IL - 1 Supernatant Pretreated Thymocytes + Sensitized Splenocytes + sRBC	40	47	38

become a T helper cell which in turn aids the B cell to become a plasma cell.

Ideally, the splenocyte population should be separated and the B cell enriched population used, in which case the mechanism operative could be clearly elucidated. However, in the initial experiments (see Tables 11 - 14) in which it was shown that IL - 1 pretreated thymocytes caused an increase in PWM driven B cell mitogenesis B cell enriched populations were used. Therefore it seems more likely that the former mechanism mentioned is operative in these experiments.

It should be noted that the greater number of plasma cells seen in cultures containing LPS and control supernatant pretreated thymocytes as compared to those containing medium pretreated thymocytes is likely to be due to the adjuvant effect of the LPS that may have bound to the thymocytes, on the splenocyte population as well as the effect of the small amounts of control spontaneously secreted IL - 1 that is found in the supernatant on the thymocytes.

4.4 Discussion

The pathway of differentiation of progenitor T cells in the thymus is a complex process thought to involve numerous factors such as thymic hormones as well as products of thymic macrophages. In this thesis it has been shown that IL - 1 causes the development of T helper cells in the thymocyte population. How IL - 1 achieves this is unknown, but a number of theories can be applied to this process.

Classical models of T cell differentiation indicate that cortical thymocytes are immunoincompetent and give rise to

medullary thymocytes which, after maturation, constitute the pool of cells from which peripheral T cells arise. Experimental results of certain researchers are in conflict with the above since it has been found that 1) cortical (Ly 123, PNA^+) thymocytes are not immunoincompetent but express on the level of cytotoxic lymphocyte (CTL) precursors a repertoire and frequency comparable with that of peripheral T cells. Furthermore, it has been found that in vitro, phenotypical immune competence of cortical thymocytes becomes manifest in the presence of IL - 2, a mediator of help derived from Ly 1 T helper cells. It has therefore been suggested that Ly 1 T helper cells or IL - 2 (their product) may play a central role in the triggering and clonal expansion of T cells associated with cytotoxic effector functions as well as T cell differentiation (Zinkernagel and Doherty, 1979). For this reason it is postulated that during thymus ontogeny there is an early phase in which the majority of "lymphocyte-like" cells express the Ly 1 phenotype, a phenotype previously associated with only mature immunocompetent T cells. After the appearance of the Ly 1 thymic cells an increase in Ly 123 cells can be observed. This indicates that the Ly 1 cells appear first and may be instrumental in the development of prothymocytes to Ly 123 thymocytes. If prothymocytes already express this receptor repertoire and develop sensitivity to the effect of IL - 2 after contact with antigen, then intrathymic Ly 1 T helper cell derived IL - 2 could be the driving force for clonal expression of clonally distributed prothymocytes developing in the thymic cortex. Accordingly, postthymic Ly 123 Ag reactive T cells are exported from the thymus (Stutman and Shen, 1979). Within the periphery Ly 123 T cells represent the common

precursor cell pool of the functional heterogeneous T cell family which, under the influence of Ag plus Ly 1 T helper cell derived IL - 2, will differentiate in Ly 1 or Ly 23 cells. In this way a feedback mechanism is envisaged whereby the presence of Ag plus Ly 1 helper cells are able to recruit other Ly 1 T cells from the Ly 123 pool.

Presuming the above theory to be operative in vivo the role of IL - 1 in the thymocyte developmental process can be explained as follows:- If during early ontogeny the thymus is rich in Ly 1 T helper cells producing IL - 2, then IL - 1 must be present to act as a second signal in Ly 1 T cell activation for IL - 2 production, the first signal perhaps being a thymic hormone (see general introduction, point 1.241). The thymic reticulum is heterogeneous, being composed of thymocytes, macrophages and epithelial cells. Thymic macrophages could therefore theoretically be the source of IL - 1 which in turn activates Ly 1 T helper cells and therefore initiates the thymocyte maturation process (Wagner et al., 1980).

CHAPTER 5

5. IL - 1 CONTAINING SUPERNATANT ACTION ON THYMOCYTES AS COMPARED TO THE ACTION OF THYMIC HORMONES

5.1 Introduction

IL - 1 is a substance known to be secreted by macrophages. Furthermore thymic macrophages secrete IL - 1 in small amounts (Gery and Waksman, 1972). The thymus also secretes a number of thymic hormones which have profound effects on T cell maturation and differentiation (Bach et al., 1979) and IL - 1 is thought to be involved in this process as well (Oppenheim, et al., 1979; Beller and Unanue, 1980). Therefore it is reasonable to presume that IL - 1 has a possible thymic hormone-like action and is itself possibly a hormone, since it is produced by one cell type, namely the macrophage and acts on another cell type either locally, that is the thymocyte, or at a distance, the peripheral T cell. For this reason the effect of various thymic hormones, viz. thymic humoral factor (THF), thymopoietin and thymosin on thymocyte mitogenesis was compared to that of IL - 1 on these cells.

The results obtained in this project are inconclusive and further in-depth studies of purified characterized thymic hormones and purified IL - 1 are required to resolve this hypothesis.

5.2 Materials and methods

5.2.1 To investigate whether thymic hormones have a similar effect to IL - 1 supernatants on thymocyte mitogenesis

Thymocytes obtained and prepared as described in point 2.221 were suspended to a concentration of 10×10^6/ml. 0,1 ml of thymocytes

were cultured in round bottomed microtitre plates in triplicate in the presence of:

Medium 0,01 ml,
lipopolysaccharide 2 µg/ml,
control supernatant, stock,
IL - 1 containing supernatant, stock,
thymic humoral factor (THF) 20 µg/ml and 40 µg/ml,
thymopoietin 0,1 µg/ml and 1 µg/ml,
thymosin 100 µg/ml,

for 72 hours in a 37°C, 5%, CO_2 humidified incubator. 0,5 µCi (methyl ^3H) thymidine was added for the last six hours of culture. The cells were then harvested and the incorporation of thymidine measured by liquid scintillation counting. Results were calculated as the means of the triplicate cultures.

5.2.2 To investigate whether thymic hormones and IL - 1 supernatants act synergistically on thymocytes

Thymocytes obtained and prepared as described in point 2.221 were suspended to a concentration of 10 x 10^6/ml. 0,1 ml of thymocytes were cultured in round bottomed microtitre plates in triplicate in the presence of:

Medium plus	medium, THF 20 µg/ml and 40 µg/ml, thymopoietin 0,1 µg/ml and 1 µg/ml, thymosin 100 µg/ml.
Lipopolysaccharide plus	medium, THF 20 µg/ml and 40 µg/ml, thymopoietin 0,1 µg/ml and 1 µg/ml, thymosin 100 µg/ml.

Control supernatant plus medium,
THF 20 µg/ml and 40 µg/ml,
thymopoietin 0,1 µg/ml and 1 µg/ml,
thymosin 100 µg/ml.

IL - 1 containing supernatant plus medium,
THF 20 µg/ml and 40 µg/ml,
thymopoietin 0,1 µg/ml and 1 µg/ml,
thymosin 100 µg/ml.

The cultures were then treated as described in 5.2.1

5.2.3 To investigate whether preincubation of thymocytes with thymic hormones has any effect on the response of these cells to IL - 1 containing supernatants

3 ml of thymocytes at a concentration of 10 x 10^6/ml were cultured in tissue culture tubes at 37°C in a humidified 5%, CO_2 incubator in the presence of:

Medium,
THF 20 µg/ml and 40 µg/ml
thymopoietin 0,1 µg/ml and 1 µg/ml,
thymosin 100 µg/ml,

for 24 hours. The cells were then washed 3 times, counted, viability corrected for and resuspended in complete medium at a concentration of 10 x 10^6/ml. 0,1 ml of thymic hormone pre-treated thymocytes were then cultured in round bottomed microtitre plates in triplicate in the presence of:

Medium,
LPS,
control supernatant,
IL - 1 containing supernatant,

for 72° hours in a 37°C humidified 5%, CO_2 incubator. The cultures were then treated as described in 5.2.1. Results obtained in this experiment, see tables 18a and b and point 5.33, led to the hypothesis that thymic hormones cause the development of suppressor cells in the thymocyte population. For this reason a suppressor assay was carried out as described in 5.2.4.

5.2.4 To investigate whether thymic hormones stimulate the development of suppressor cells in thymocyte populations

Thymocytes were precultured in the presence of thymic hormones as described in 5.2.3. After preculture, the thymocytes were washed 3 times, counted, the viability corrected for, and re-suspended to a concentration of 2 x 10^6/ml. Splenocytes prepared as described in point 4.2.1 were suspended in complete medium to a concentration of 2 x 10^6/ml. The following microcultures were then set up in round bottomed culture plates in triplicate:-

Medium pretreated thymocytes 0,1 ml plus	medium 0,1 ml, or splenocytes 0,1 ml.	Plus/minus PHA 10 µg/ml.
THF 40 µg/ml pretreated thymocytes plus	medium, or splenocytes.	
Thymopoietin 1 µg/ml pretreated thymocytes plus	medium, or splenocytes.	
Thymosin 100 µg/ml pretreated thymocytes plus	medium, or splenocytes.	

The cultures were then treated as described in 5.2.1

5.3 Results

5.3.1 Results of experiments to investigate the effects of thymic hormones on mitogenesis of thymocytes

Briefly, thymocytes were cultured in the presence and absence of IL - 1 supernatant and the mitogenesis obtained compared to that when thymocytes were cultured in the presence of thymic hormones. The results are shown in Tables 16a and b. The results indicate that thymic hormones do not cause mitogenesis and therefore do not emulate IL - 1 in their action on thymocytes. However, IL - 1 may mature thymocytes as does thymic hormone by causing the development of markers and receptors on the cells.

5.3.2 Results to indicate whether thymic hormones and IL - 1 supernatants act synergistically on thymocytes

Thymocytes were cultured with and without IL - 1 in the presence or absence of thymic hormones. The resultant mitogenesis of the various cultures was compared. The results are given in Tables 17a and b. The results indicate that IL - 1 and thymic hormones do not act synergistically. However, in the presence of thymic hormones the response of thymocytes to IL - 1 is reduced by an average of 39%. This could mean that either thymic hormones mature thymocytes, therefore reducing their ability to respond to IL - 1 as IL - 1 only acts on immature cells, or thymic hormones cause the development of a suppressor cell sub-population in thymocytes. The experiments carried out to test these hypotheses are given in 5.3.3 and 5.3.4 and the results indicated in Tables 18a and b, 19 and Graph 10.

TABLE 16a THE EFFECT OF THYMIC HORMONES ON THYMOCYTES AS COMPARED TO THAT OF IL - 1

System	CPM Experiment 1	CPM Experiment 2	CPM Experiment 3
Thymocytes + medium	91,1	514,7	248,9
Thymocytes + LPS	1 346,3	465,3	260,0
Thymocytes + control supernatant	705,3	1 031,3	351,9
Thymocytes + IL-1 supernatant	5 782,5	9 756,3	5 949,4
Thymocytes + THF 20 µg/ml	108,5	457,3	157,6
Thymocytes + THF 40 µg/ml	98,7	536,0	149,1
Thymocytes + thymopoietin 0,1 µg/ml.	67,3	758,7	295,0
Thymocytes + thymopoietin 1,0 µg/ml	92,7	650,7	151,7
Thymocytes + thymosin 100 µg/ml.	121,7	588,0	344,7

TABLE 16b. Means of the three experiments shown in Table 16a indicating the effect of thymic hormones on thymocytes as compared to that of IL - 1

System	CPM	Standard deviation
Thymocytes + medium	284,9	± 214,1
Thymocytes + LPS	690,5	± 577,1
Thymocytes + control supernatant	696,1	± 339,8
Thymocytes + IL-1 supernatant	7 162,7	± 2 247,6
Thymocytes + THF 20 µg/ml	241,1	± 188,8
Thymocytes + THF 40 µg/ml	261,3	± 239,3
Thymocytes + thymopoietin 0,1 µg/ml	373,7	± 352,3
Thymocytes + thymopoietin 1,0 µg/ml	298,4	± 306,5
Thymocytes + thymosin 100 µg/ml	351,5	± 233,2

TABLE 17a THE COACTION OF IL - 1 AND THYMIC HORMONES ON THYMOCYTES

Experiment No.	System	CPM Medium	CPM THF 20 µg/ml	CPM THF 40 µg/ml	CPM Thymopoietin 0,1 µg/ml	CPM Thymopoietin 1,0 µg/ml	CPM Thymosin 100 µg/ml
1	Thymocytes + medium	281,0	267,5	794,5	679,5	832,0	1 290,0
	Thymocytes + LPS	921,0	691,5	950,0	1 104,0	1 465,0	1 019,5
	Thymocytes + control supernatant	3 274,0	4 244,5	3 133,0	4 704,0	5 479,0	3 751,5
	Thymocytes + IL-1 supernatant	16 543,5	17 672,5	16 205,0	13 024,5	15 222,5	13 369,5
2	Thymocytes + medium	808,0	945,5	422,0	293,5	599,0	481,0
	Thymocytes + LPS	1 496,5	1 690,0	657,0	658,5	595,0	765,0
	Thymocytes + control supernatant	4 059,0	5 244,5	5 357,5	3 318,0	3 122,0	3 011,5
	Thymocytes + IL-1 supernatant	16 109,0	12 105,0	10 664,0	13 221,5	11 533,0	11 125,5
3	Thymocytes + medium	1 032,0	2 003,0	2 312,0	2 014,5	1 424,5	1 540,5
	Thymocytes + LPS	968,5	2 297,0	3 685,0	1 442,5	2 579,5	2 080,0
	Thymocytes + control supernatant	2 669,5	4 087,0	4 375,0	2 576,5	2 759,5	3 747,0
	Thymocytes + IL-1 supernatant	13 710,5	8 450,0	6 349,0	5 797,0	7 548,0	7 521,5

TABLE 17b Means of the three experiments represented in Table 17a showing the coaction of IL - 1 and thymic hormones on thymocytes.

System	CPM medium	CPM THF 20 µg/ml	CPM THF 40 µg/ml	CPM Thymopoietin 0,1 µg/ml	CPM Thymopoietin 1 µg/ml	CPM Thymosin 100µg/ml
Thymocytes + medium	707,0 (± 385,5)	1 072,0 (± 874,6)	1 176,2 (±1 001,1)	995,8 (± 993,6)	951,8 (± 425,6)	1 103,8 (± 553,7)
Thymocytes + LPS	1 128,7 (± 319,4)	1 559,5 (± 810,7)	1 764,0 (±1 670,1)	1 068,3 (± 393,2)	1 546,5 (± 994,8)	1 288,2 (± 697,4)
Thymocytes + control supernatant	3 334,2 (± 696,7)	4 525,3 (± 627,8)	4 288,5 (±1 114,8)	3 532,8 (±1 079,9)	3 786,8 (±1 476,6)	3 503,3 (± 425,9)
Thymocytes + IL-1 supernatant	15 454,3 (±1 525,7)	12 742,5 (±4 644,2)	11 072,7 (±4 940,7)	10 681,0 (±4 230,8)	11 434,5 (±3 838,2)	10 672,2 (±2 950,2)

5.3.3 Results to indicate whether pretreatment of thymocytes with thymic hormones results in a reduction in their response to IL - 1

Thymocytes were pretreated with thymic hormones. The cells were then washed and cultured in the presence of IL - 1 containing supernatants and controls. The results are shown in Tables 18a and b and Graph 10. The results in Graph 10 show the suppression of thymocyte response to IL - 1 by thymic hormones to be an average of 33%. This indicates that IL - 1 and thymic hormones are not acting as antagonists, but that thymic hormones are either maturing thymocytes and therefore rendering them less responsive to IL - 1, or are inducing a subpopulation of suppressor cells in the thymocyte population.

5.3.4 Results indicating whether thymic hormone pretreatment of thymocytes results in the development of a suppressor cell subset in the thymocyte population

Thymocytes were pretreated with thymic hormones. The cells were then washed, irradiated and cultured with splenocytes in the presence or absence of PHA to assay for suppressor cell development. The results are shown in Table 19 and indicate that thymic hormones do not induce a suppressor cell subset in thymocytes. Therefore, the effect of thymic hormones on thymocytes is to mature them and make them less responsive to IL - 1 activation. Furthermore, it seems that thymocytes are naturally suppressive and that thymic hormones reduce this suppressive effect as is seen by the fact that spleen cells plus medium pretreated thymocytes plus PHA give lower counts than spleen cells alone plus PHA and that thymic hormone pretreatment increases the counts.

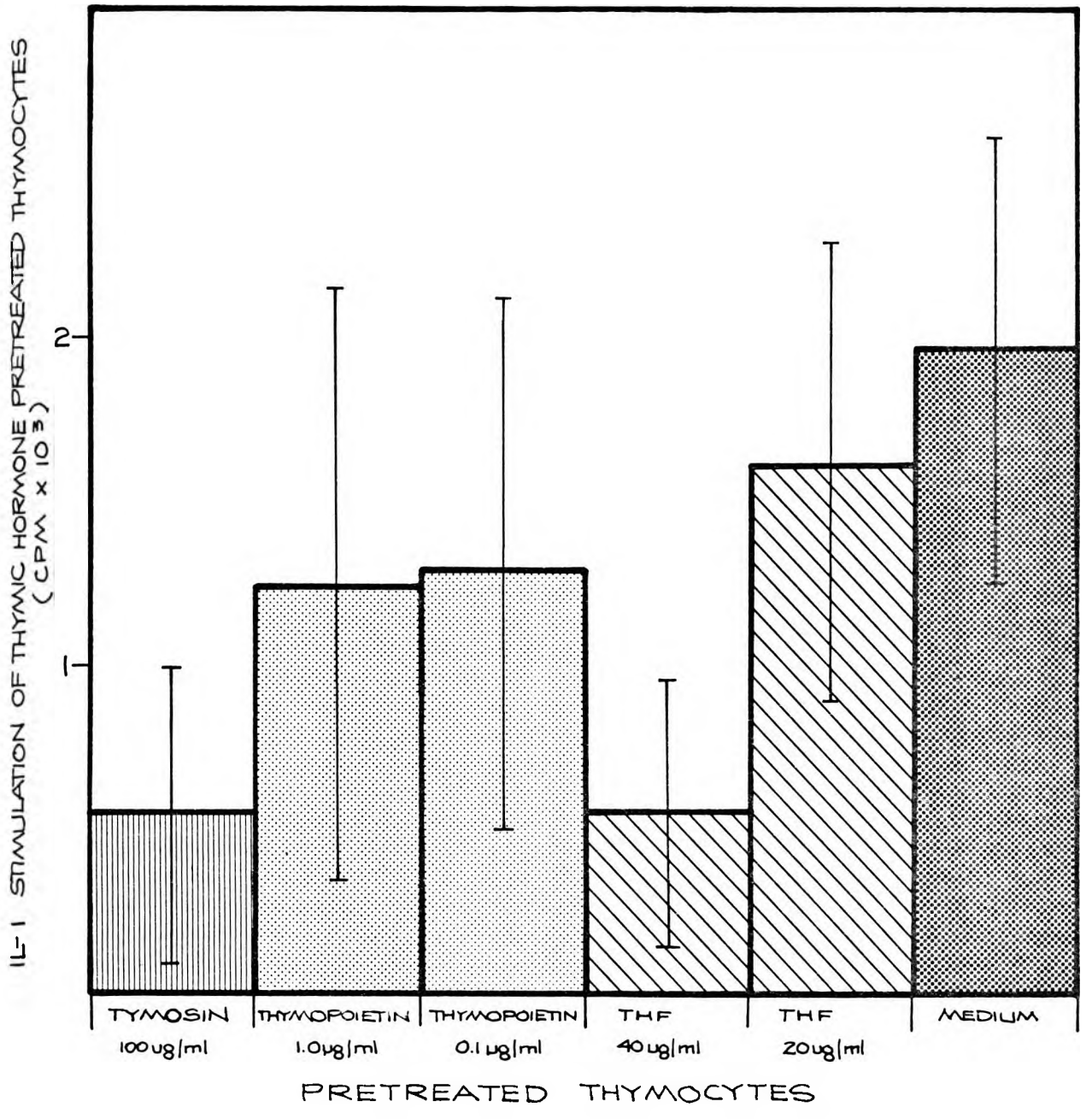
TABLE 18a THE EFFECT OF THYMIC HORMONE PRETREATMENT OF THYMOCYTES ON THEIR RESPONSE TO IL - 1

Experiment No.	System	CPM Medium pretreated thymocytes	CPM THF 20 µg/ml pretreated thymocytes	CPM THF 40 µg/ml pretreated thymocytes	CPM Thymopoietin 0,1 µg/ml pretreated thymocytes	CPM Thymopoietin 1 µg/ml pretreated thymocytes	CPM Thymosin 100 µg/ml pretreated thymocytes
1	Medium	352,0	378,0	316,0	448,0	391,3	371,3
	LPS	478,7	544,0	521,3	477,0	662,0	690,0
	Control supernatant	864,0	680,0	660,6	534,0	861,3	806,0
	IL-1 supernatant	3 707,3	3 720,0	1 482,0	2 878,0	3 326,0	1 430,7
2	Medium	545,3	507,3	289,0	416,0	468,0	493,3
	LPS	787,7	755,0	491,0	499,0	663,0	713,0
	Control supernatant	878,0	762,3	545,7	487,3	766,0	821,0
	IL-1 supernatant	2 091,0	1 141,0	624,0	1 011,5	1 213,0	864,0
3	Medium	365,7	405,7	386,7	460,7	544,7	392,3
	LPS	580,0	537,3	512,7	479,7	703,0	706,7
	Control supernatant	653,7	575,0	737,5	722,3	1 137,0	897,0
	IL-1 supernatant	2 503,0	2 031,0	1 513,5	1 743,5	1 961,7	1 867,0

TABLE 18b Means of the experiments indicated in Table 18a showing the effect of thymic hormone pretreatment of thymocytes on their response to IL - 1.

System	CPM Medium pretreated thymocytes	CPM THF 20 µg/ml pretreated thymocytes	CPM THF 40 µg/ml pretreated thymocytes	CPM Thymopoietin 0,1 µg/ml pretreated thymocytes	CPM Thymopoietin 1,0 µg/ml pretreated thymocytes	CPM Thymosin 100 µg/ml pretreated thymocytes
Medium	421,0 (±107,9)	430,3 (± 68,1)	330,6 (± 50,4)	441,6 (± 23,0)	468,0 (± 76,7)	419,0 (± 65,2)
LPS	615,5 (±157,5)	612,1 (± 123,8)	511,7 (± 15,6)	485,3 (± 12,0)	676,0 (± 23,4)	703,2 (± 11,9)
Control supernatant	798,6 (±125,6)	672,4 (± 93,9)	647,9 (± 96,5)	581,2 (±124,4)	921,4 (± 192,7)	841,3 (± 48,8)
IL-1 supernatant	2 767,1 (±839,7)	2 297,3 (±1 310,0)	1 206,5 (±504,7)	1 877,7 (±940,4)	2 166,9 (±1 071,3)	1 387,2 (±502,9)

THYMOCYTE STIMULATION BY IL - 1 SUPERNATANTS (CPM)
AND THE EFFECT ON STIMULATION BY THYMIC HORMONE
PRETREATMENT OF THYMOCYTES.



GRAPH 10.

TABLE 19 EXPERIMENT INDICATING WHETHER THYMIC HORMONES INDUCE SUPPRESSOR CELLS IN THE THYMOCYTE POPULATION

System	CPM Medium pretreated thymocytes	CPM THF 10 µg/ml pretreated thymocytes	CPM Thymopoietin 10 µg/ml pretreated thymocytes	CPM Thymosin 100 µg/ml pretreated thymocytes	CPM No thymocytes (Medium only)
Medium	83,7	175,0	419,3	229,7	-
PHA 10 µg/ml	174,0	402,7	159,0	162,0	-
Spleen cells	113,0	368,0	196,0	218,7	218,7
Spleen cells + PHA 10 µg/ml	4 227,3	5 411,0	6 782,0	7 557,3	9 254,0

5.4 Discussion

As shown in the results THF, thymopoietin and thymosin did not cause proliferation in mouse thymocytes and therefore do not share the mitogenic effect of IL - 1 on thymocytes. Furthermore, pretreatment of thymocytes with thymic hormones resulted in a decrease in the proliferative response of these cells to IL - 1, which was not as a result of T suppressor cell induction by the thymic hormones and is therefore probably due to a maturational effect, resulting in decreased responsiveness of thymocytes to IL - 1. It is known that as mice age (above 10 weeks) the responsiveness of their thymocytes to IL - 1 decreases (Blyden and Handschumacher, 1977).

The above results are inconclusive and further studies are indicated to prove whether IL - 1 has a thymic hormone-like action. Experimentation has shown that a number of the effects of of IL - 1 on thymocytes and T cells parallel those of thymic hormones on these same cells. Consider the following:-

(i) Surface receptors/Ag

Most thymic extracts induce the appearance and/or changes in T cell differentiation antigens viz. theta Ag, Ly 123 Ag, TL Ag and xenogeneic Ag (Bach et al., 1971). They also all induce receptors for sheep red blood cells (Bach et al., 1971; Incefy et al., 1979). IL - 1 has been shown to cause an increase in "Ia-like" receptors but not H-2K/D on Ly 1 T cells (Lonai & Steinman, 1977). Both the former and latter receptors and/or surface Ag are associated with T cell maturation.

(ii) Potentiation of T lymphocyte functions

Thymic hormones increase the response of (mature and immature) T cells to T cell lectins PHA and CON A (Yakir et al., 1978; Low et al., 1979; Trainin et al., 1979). IL - 1 potentiates the effect of mitogens PHA and CON A on DNA synthesis by mouse thymocytes (Oppenheim, et al, 1976). Both the former and latter appear to be due to the maturation effects of thymic hormones and IL - 1 on T cells respectively, since the response of T cells to mitogens is enhanced as the cell matures. Furthermore, in the presence of IL - 1 allogeneic thymocytes develop the ability to have a bidirectional MLC (Oppenheim, et al., 1976) and thymic extracts increase the MLC capacity of null cells (Low et al., 1979) and increase T cell competence in MLC's of neonatally thymectomized mice (Yakir, et al., 1978). Other T cells functions that are induced or increased by IL - 1 and thymic extract alike, include the antibody response to sheep red blood cells (Yakir, et al.. 1978; Hoffmann and Dutton, 1979) and increased killing of tumour cells (Bach, 1977; Bach et al., 1979; Yakir, et al., 1978; Low et al., 1979; Cameron and Churchill, 1980.)

(iii) Development of T cell subsets

IL - 1 is known to activate Ly 1 T helper cells (Hoffmann, 1980; Wagner et al., 1980; Paetkau et al., 1980; Smith, 1980) and/or cause their development/maturation from thymocytes (Young and McIvor, 1980). It is probably through the activation of the Ly 1 T helper cell that IL - 1 augments all the T cell functions discussed above.

Thymic hormones, depending on their type, have been shown to cause the maturation of different T cell subsets. For example, thymosin α_1 causes the maturation of T helper cells, and α_7 that of T_S cells (Low et al., 1979) and FTS tends to induce the formation of T helper cells (Bach et al., 1979). Furthermore, in this project it has been shown that thymocytes appear to be naturally suppressive and that IL - 1 abrogates this suppressor activity in thymocytes (see page 118 of results). Other workers have shown that immature T cells (thymocytes) are suppressive (Folch and Waksman, 1974; Small, 1977) and that THF abrogates this suppressive effect in thymocytes.

(iv) Changes in cyclic nucleotide levels

Evidence implicates cyclic nucleotides cGMP and cAMP as mediators of hormonal action (Hadden et al., 1979). In general cGMP is associated with the promotion of immune functions such as proliferation and cAMP with inhibition (Hadden, 1977) and with promotion of differentiation. The natural inducers of thymic differentiation are thymic hormones which could act by increasing thymocyte cAMP. However, the only thymic hormone shown to increase cellular cAMP is THF (Bach et al., 1979). The role of cyclic nucleotides in intrathymic development of lymphocytes is poorly understood. cAMP + Mg^+ - Ca^{2+} promotes the thymocyte to flow from the mid-cycle point (G_1 - S boundary) through to mitosis. cGMP + Ca^{2+} - Mg^+ also does this (MacManus et al., 1975; Morgan et al., 1977). An explanation for this paradox could be as

follows:- The different lymphocyte subsets are developed at the level of the thymus and perhaps each manipulation selects for a specific T cell subset, for example, T helper cell versus T suppressor cell and mitogen versus allogeneic responder. This hypothesis predicts that thymic hormones act via cAMP as well as cGMP in regulating lymphocyte subset differentiation. It should be noted that IL - 1 acts near or at the mid-cycle point to bring about thymocyte mitogenesis in relation to an increase in cGMP (Wagshal and Waksman, 1978; Katz et al., 1978). Considering the aforementioned parallel in the maturational effects of IL - 1 and thymic extracts on thymocytes (T cells), it seems reasonable to hypothesize that IL - 1 has a hormonal effect probably inducing T cell maturation both in the thymus as well as the periphery through its secretion by thymic macrophages and peripheral blood and/or tissue monocytes respectively.

CHAPTER 6

6. THE EFFECT OF SUPPRESSOR CELLS, HELPER FACTOR AND CORTISONE ON IL - 1 PRODUCTION

6.1 Introduction

There are numerous regulatory mechanisms involved in the control of the immune response. Helper mechanisms enhance the immune response and suppressor mechanisms inhibit it. A fine balance between helper and suppressor mechanisms has to exist to result in an optimum response. The inhibition, or, switching off of an immune response, is probably controlled by T suppressor cells and/or suppressor products secreted by macrophages. On the other hand, the stimulation of the immune response is more than likely dependent on helper products secreted by macrophages and T helper cells.

The idea of the studies described in this chapter was to assess whether a factor secreted by PWM-activated T cells, namely, human helper factor (Saxon and Stevens, 1979) would enhance IL - 1 secretion by macrophages and if sodium periodate induced suppressor cells (Lomnitzer and Rabson, in press) had the ability to "switch off" IL - 1 production. The effect of cortisone on IL - 1 production was also studied to indicate whether the immunosuppresive effects of cortisone were due to their effect on macrophage function as measured by IL - 1 secretion.

6.2 Materials and methods

6.2.1 The effect of suppressor cells (sodium periodate induced) on IL - 1 production by macrophages

6.2.1.1 Production of sodium periodate suppressor cells

This was carried out by the method of Lomnitzer and Rabson (personal communication). Briefly mononuclear cells (MN) were obtained from the peripheral blood of healthy human donors by Ficoll-Hypaque density gradient centrifugation. The cells were washed twice in RPMI-1640, once in phosphate buffered saline (PBS) and were resuspended in PBS to a concentration of 10×10^6/ml. 10×10^6 MN/ml in PBS were incubated in the presence and absence of 2,0 mM $NaIO_4$ at 0°C for 30 minutes. Following incubation, the cells were washed three times in complete RPMI and resuspended to a concentration of 2×10^6 MN/ml. 3 ml. aliquots were then incubated for 72 hours at 37°C in 5% CO_2 in air. At the end of the culture period, the cells were washed twice in RPMI, irradiated with 2 000 rads and resuspended to 1×10^6 cells/ml in complete RPMI.

6.2.1.2 Testing for the production of suppressor cells by suppression of 3H-thymidine incorporation

0,1 ml of $NaIO_4$ pretreated or control cells were added to 0,1 ml of 1×10^6/ml fresh allogeneic or autologous cells in round bottom microtitre plates. The cell cultures were incubated in the presence and absence of 10 µg/ml PHA. The cultures were incubated for 96 hours. 0,5 µCi ^{3}H-thymidine was added for the last six hours of culture and the cells harvested with a multiple automated harvester and ^{3}H-thymidine incorporation assessed by liquid scintillation counting. Experiments were performed in triplicate and results expressed as mean CPM.

Suppression was calculated as follows:-

$$\% \text{ suppression} = \frac{B - A}{B} \times 100$$

where A is the proliferative response of the responder cells to which $NaIO_4$ treated cells were added and B is the proliferative response of the responder cells to which control cells were added.

6.2.1.3 The effect of suppressor cells on IL - 1 supernatant production

Adherent cells were obtained from whole blood as described in 2.2.1 and the suppressor cells produced as described in 6.2.1.1. 5×10^5/ml adherent cells were cocultured with suppressor cells or control cells at different concentrations in the presence or absence of LPS 20 μg/ml in round petri-dishes for 24 hours at 37°C in 5%, CO_2 in air. The supernatants were then collected and assayed for IL - 1 activity as described in 2.2.2.2.

6.2.2 The effect of helper factor on IL - 1 production by macrophages

6.2.2.1 Helper factor production

Mononuclear cells were obtained from the peripheral blood of healthy human donors by Ficoll-Hypaque density gradient centrifugation. The cells were washed three times in RPMI-1640 and were resuspended in complete RPMI to a concentration of 2×10^6 MN/ml. 3 ml of 2×10^6 MN/ml were cultured in tissue culture tubes in the presence and absence of pokeweed mitogen (PWM) for 24 hours at 37°C in 5%, CO_2 in air. Thereafter the supernatants were collected after centrifugation and stored at

-20°C. Controls consisted of MN cells cultured without PWM to which PWM was added after the culture period.

6.2.2.2 The effect of HF on IL - 1 production by adherent cells

Adherent cells were obtained from whole blood of normal human donors as described in 2.2.1. 5×10^5 adherent cells per millilitre were cocultured in petri dishes with helper factor and control factor supernatants in the presence and absence of LPS for 24 hours at 37°C in 5%, CO_2 in air. After 24 hours the supernatants were harvested and assayed for IL - 1 production as described in 2.2.2.2.

6.2.3. The effect of cortisone pretreatment of adherent cells on IL - 1 production

6.2.3.1 Cortisone pretreatment of adherent cells

Adherent cells were obtained from whole blood of normal human donors as described in 2.2.1. 1×10^6 adherent cells per millilitre were cultured in culture flasks in the presence or absence of hydrocortisone (10^{-5} m) for 24 hours. The adherent cell layers were then washed three times with warm RPMI.

6.2.3.2 IL - 1 production by cortisone pretreated adherent cells

Adherent cells pretreated as described in 6.2.3.1 were then cultured for a further 24 hours in the presence and absence of LPS (20 μg/ml). The supernatants were then harvested and assayed for IL - 1 production as described in 2.2.2.2.

6.3 Results

6.3.1 The effect of sodium periodate ($NaIO_4$) suppressor cells on IL - 1 supernatant production by macrophages (see Tables 20 and 21a and b)

6.3.1.1 Results showing the induction of suppressor cells by $NaIO_4$

Mononuclear cells were pretreated for half an hour with or without $NaIO_4$. They were then washed and cultured for 72 hours. Thereafter the cells were irradiated and cocultured with fresh mononuclear cells in the presence or absence of PHA. The results are indicated in Table 20. The results show that $NaIO_4$ induces the formation of suppressor cells in the mononuclear cell population.

6.3.1.2 Results showing the effect of $NaIO_4$ suppressor cells on IL - 1 supernatant production by macrophages

Macrophages (adherent cells) were cultured for 24 hours in the presence or absence of LPS with or without $NaIO_4$ suppressor cells. The supernatants were collected and assayed for IL - 1 activity on mouse thymocytes. The results are shown in Tables 21a and b.

The results indicate that sodium periodate suppressor cells have no effect on IL - 1 production by macrophages since IL - 1 produced in the presence of control cells and suppressor cells give similar results.

6.3.2 The effect of HHF on IL - 1 production by adherent cells

TABLE 20. SUPPRESSOR CELL INDUCTION BY SODIUM PERIODATE

Experiment No.	System	CPM	Suppression
1	Control	42 582,5	46%
	Test	22 830,0	
2	Control	39 958,7	34%
	Test	26 230,7	
3	Control	54 271,5	48%
	Test	28 155,7	

Key:

Control - Irradiated control cells + responder cells + PHA (10 µg/ml).

Test - Irradiated suppressor cells + responder cells + PHA (10 µg/ml).

TABLE 21a.

THE EFFECT OF SODIUM PERIODATE INDUCED SUPPRESSOR CELLS ON IL - 1 PRODUCTION

Experiment No.	IL-1 (MØ:LPS) + thymocytes (CPM)	IL-1 (MØ:control:LPS) + thymocytes (CPM)	IL-1 (MØ:S-cells:LPS) + thymocytes (CPM)
1	10 664,0	8 358,0	7 566,0
2	8 389,0	7 573,0	7 353,0
3	6 013,1	4 680,2	4 931,3

Key:

IL-1 (MØ:LPS) ——> IL-1 made by incubating 5 x 10^5 MØ/ml with 20 µg/ml LPS.

IL-1 (MØ:control:LPS) ——> IL-1 made by incubating 5 x 10^5 MØ/ml and 1 x 10^6ml sodium periodate control cells with 20 µg/ml LPS

IL-1 (MØ:S-cells:LPS) ——> IL-1 made by culturing 5 x 10^5 MØ/ml + 1 x 10^6/ml sodium periodate suppressor cells with 20 µg/ml LPS.

TABLE 21b. Means and standard deviations of the three experiments represented in Table 21a showing the effect of sodium periodate induced suppressor cells on IL - 1 production.

IL-1 (MØ + LPS) + thymocytes (CPM)	± SD	IL-1 (MØ + control cells + LPS) + thymocytes (CPM)	± SD	IL-1 (MØ + S-cells + LPS) + thymocytes (CPM)	± SD
8 355,4	± 2 325,6	6 870,3	± 1 936,9	6 616,8	± 1 463,5

IL - 1 and control supernatants produced in the presence and absence of HHF and control supernatants were assayed on mouse thymocytes. The direct effect of HHF and control supernatants as well as PWM on thymocytes were controlled for. The results are shown in Table 22.

The effect of PWM as well as helper factor and helper factor control supernatants directly on thymocytes, was monitored and in all cases caused no direct thymocyte stimulation.

As is shown in the results helper factor does stimulate macrophages to produce IL - 1, although in smaller quantities than LPS. Furthermore, LPS and helper factor do not appear to act synergistically probably because LPS binds to the macrophage preventing HHF acting on it. It should also be noted that the effect of PWM on macrophage stimulation was found to be negligible, that is, PWM did not cause macrophages to secrete IL - 1. Furthermore, it was also found that PWM did not cause mitogenesis in thymocytes.

6.3.3 Results showing the effect of hydrocortisone pretreatment of adherent cells on their IL - 1 production

IL - 1 and control supernatants produced from adherent cells pretreated with or without hydrocortisone were assayed on mouse thymocytes. The results obtained are shown in Table 23. Results indicate that hydrocortisone pretreatment of adherent cells results in a decrease in their IL - 1 production as compared to controls. This decrease occurs both in the spontaneous IL - 1 production as well as in the LPS stimulated IL - 1 production.

TABLE 22. THE EFFECT OF HUMAN HELPER FACTOR ON IL - 1 PRODUCTION BY MACROPHAGES

Experiment No.	Unstimulated MØ supernatant + thymocytes (CPM)	LPS stimulated MØ supernatant + thymocytes (CPM)	Control supernatant stimulated MØ supernatant + thymocytes (CPM)	Control supernatant + LPS stimulated MØ supernatant + thymocytes (CPM)	HF supernatant stimulated MØ supernatant + thymocytes (CPM)	HF supernatant + LPS stimulated MØ supernatant + thymocytes (CPM)
1	3 138,1	83 181,7	3 463,3	78 863,3	67 242,2	80 020,6
2	3 138,1	83 181,7	7 408,7	98 107,4	12 684,4	79 215,3
3	3 138,1	83 181,7	8 761,8	94 974,5	34 475,6	83 459,4

It should be noted that in experiment number 3 Table 23 spontaneous IL - 1 release by adherent cells was equivalent to LPS stimulated IL - 1 release. However, the hydrocortisone suppressive effect is still present.

6.4 Discussion

6.4.1 Suppressor cell effect on IL - 1 production

In this thesis it was found that suppressor cells had no effect on IL - 1 production by adherent cells. Considering the model of T cell activation (see Figure 10):-

The idea behind this experimentation was to show whether the T suppressor cells shown in the Figure 10 initiated their suppression by acting on the macrophage and inhibiting its secretion of IL - 1. Results indicate this not to be so. Recently it has been shown that the activated Ly 2 suppressor cells can feed back to inactivate the IL - 2 producer precursor or the IL - 2 producer itself, that is, the Ly 1 T helper cell (Eardley, 1981). Others have indicated that T suppressor cells act by passively absorbing IL - 2 (Smith, 1980). Also an inhibitor of IL - 2 has been found in mouse serum (Wagner et al., 1980) and this factor could be produced by T suppressor cells. However, manipulation of the system in this project could still show that T suppressor cells also act by inhibiting IL - 1 production by macrophages.

6.4.2 Human helper factor (HHF) effect on IL - 1 production

In this project HHF was shown to stimulate IL - 1 secretion of macrophages but not to act synergistically with LPS in stimulating IL - 1 production. Human helper factor is a soluble

TABLE 23.

Experiment Number	1	2	3
CPM Medium + Thymocytes	186.7	1804.3	205.0
CPM Adherent Cell Supernatant + Thymocytes	7 127.5	6 900.5	6 849.7
CPM Hydrocortisone Pretreated Adherent Cell Supernatant + Thymocytes	5 048.3	2 788.3	3 699.7
CPM Adherent Cell Control Supernatant + Thymocytes	12 020.7	9 133.3	12 624.7
CPM Hydrocortisone Pretreated Adherent Cell Control Supernatant + Thymocytes	5 391.3	7 792.7	7 183.7
CPM Adherent Cell IL - 1 Supernatant + Thymocytes	23 969.7	13 645.0	12 327.0
CPM Hydrocortisone Pretreated Adherent Cell IL - 1 Supernatant + Thymocytes	9 844.0	9 203.0	7 705.0

THE EFFECT OF HYDROCORTISONE PRETREATMENT OF ADHERENT CELLS ON THEIR IL - 1 PRODUCTION

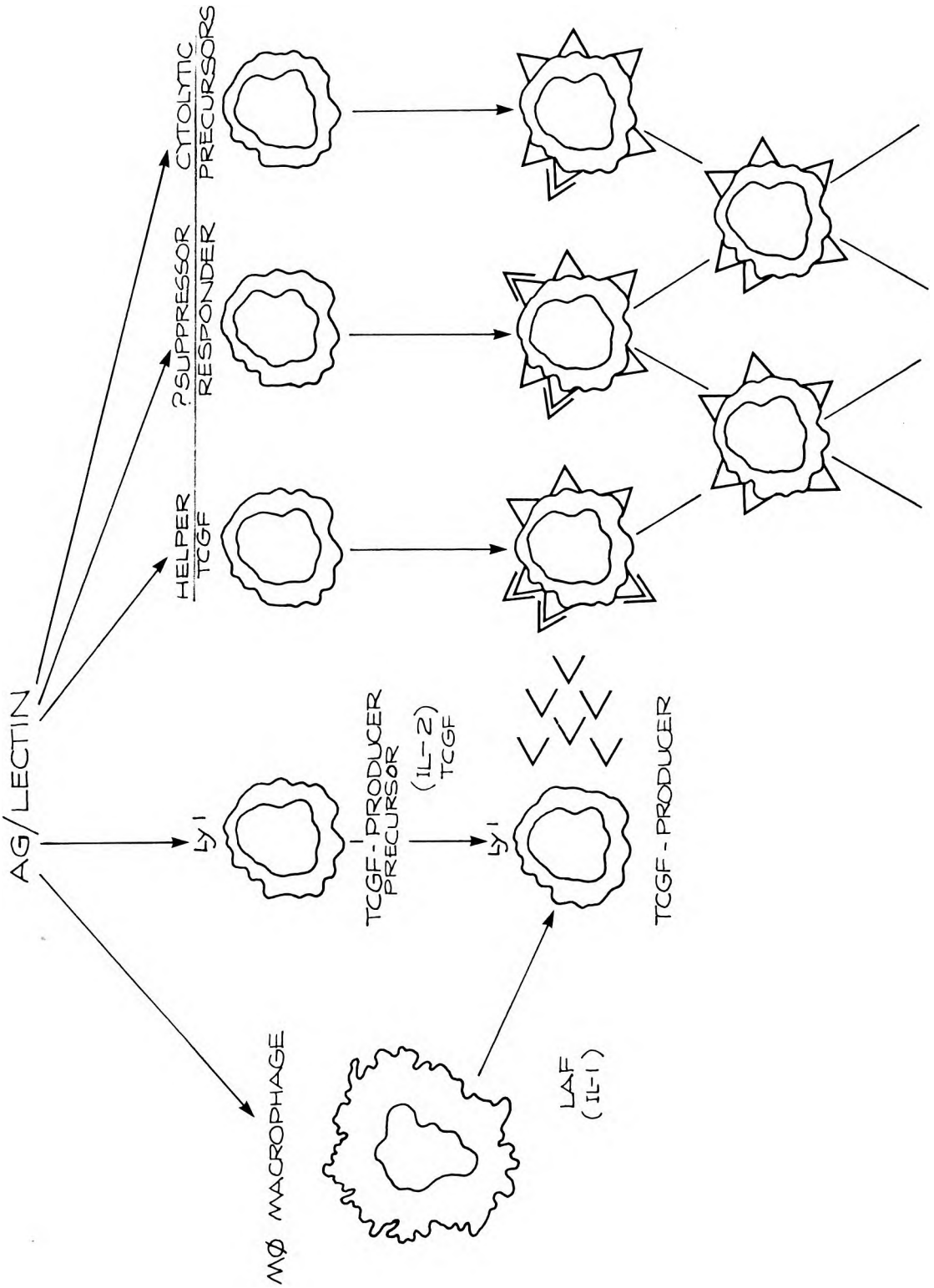
factor(s) derived from pokeweed mitogen stimulated human T helper lymphocytes. It is protein in nature, bears "Ia-like" determinants and will partially substitute for helper T lymphocytes in activating B lymphocytes to produce immunoglobulin (Saxon and Stevens, 1979). It is not surprising that HHF stimulates IL - 1 production by macrophages since it has been shown that T cell mitogens that presumably indirectly activate macrophages through activated T cells such as sodium periodate, phytohaemagglutinin and concanavalin A result in IL - 1 production (Novogrodsky and Gery, 1972 Gery et al., 1971). Furthermore, activated T cells (Unanue et al., 1976b; Mizel et al., 1978a), as well as T cell factors (MAF) (Meltzer and Oppenheim, 1977; Mizel et al., 1978a) also result in macrophage secretion of IL - 1.

The lack of synergy between HHF and LPS on IL - 1 production by macrophages could be as a result of competitive binding between LPS and HHF. There will only be a finite number of receptors on the macrophage and if they are saturated no further simulation of the cell can occur.

6.4.3 The effect of cortisone pretreatment of adherent cells on IL - 1 production.

Corticosteroids are known to have diverse effects on various facets of the immune response both in vivo and in vitro. The effect of glucocorticoids on various cell types appears to be mediated by the binding of the drug to receptors on the cells which then results in the triggering of a complex series of intracellular events (O'Malley 1971, Baxter and Forsham 1972, Thompson and Lippman, 1974).

FIG. 10: T CELL ACTIVATION



Although glucocorticoids have profound effects on cell traffic (Fauci 1979), in this thesis the effect of glucocorticoids on cell function is of more interest. Almost every facet of lymphocyte function such as cell activation, proliferation, differentiation, mediator production and release, response to mediators, antigen recognition, cytotoxic effector functions, surface markers, receptor function and many other have been shown to be suppressed to a greater or lesser degree by corticosteroids (Fauci 1979). The exact mechanism of suppression via corticocorticoids is greatly contested. It has, however, been shown that the functional capabilites of the monocyte appear to be relatively sensitive to pharmacological doses of corticosteroids as compared to lymphocytes. In this thesis IL - 1 production has been shown to be suppressed by hydrocortisone. Considering the recent model of T cell activation : In this model antigen or mitogen stimulates monocytes to secrete IL - 1, which in turn stimulates T helper cell development in conjunction with antigen or mitogen The T helper cells then secrete IL - 2 which together with antigen or mitogen activates effector T cells, that is, helper cells, suppressor cells, cytotoxic cells and lymphokine producing cells. In this case corticosteroids acting on the monocyte and suppressing its release of IL - 1 could result in the suppression of the entire cellular immune response. The mechanism operative in this suppression has not been elucidated, but, a number of theories can be put forward:

(1) It is known that corticosteroids bind to intracellular protein receptors (O'Malley 1971, Baxter & Forsham 1972, Thompson and Lippman 1974) and that the complex of receptor and hormone somehow initiate a series of cellular

events responsible for the effect of the corticosteroid. In the case of hydrocortisone the combination of hormone and receptor could in some way be leading to cellular events which inhibit protein synthesis and therefore IL - 1 production. Another possibility is that hydrocortisone causes the synthesis of other immunologically active proteins but "switches off" that of IL - 1.

(2) In the case of LPS stimulation of IL - 1 secretion, hydrocortisone could be binding to the LPS receptor site on the macrophage thereby preventing its activation. This mechanism however does not explain hydrocortisone pretreatment suppressing spontaneous IL - 1 release.

(3) Hydrocortisone could furthermore be interfering with the release of IL - 1 from the macrophage. In this case IL - 1 would be synthesized and stored in the cell, perhaps in a form similar to a lysosome. It is known that corticosteroids stabilize lysosomal membranes (Weissmann and Thomas 1963) and prevent the release of lysosomal contents from certain cell types (Wright and Malawista 1973, Ignarro 1977).

CHAPTER 7

7. MACROPHAGES, MITOGENS AND IL - 1

7.1 Introduction

Lymphoid cells can be stimulated in vitro to undergo blast transformation and synthesize DNA by mitogens such as PHA and CON A. The response of lymphoid cells to the various mitogens depends on the degree of maturation of the cells. Generally as cells mature they become more responsive to mitogenic stimulation probably because of the development of receptors for mitogen stimulated cell activation. Furthermore, there is probably heterogeneity among lymphocyte populations in terms of their relative responses to PHA and CON A (Stobo et al., 1972).

In this project the responses of thymocytes to mitogens CON A and PHA were studied, as well as the effect of these mitogens on the response of thymocytes to IL - 1. Furthermore, the function of the macrophage in the thymocyte population was studied with respect to its effect on the responses of thymocytes to the abovementioned mitogens and the use of IL - 1 in replacing macrophages in this response.

7.2 Materials and methods

7.2.1 The effect of mitogens (CON A and PHA) on thymocytes and modulation of these effects by IL - 1

Thymocytes were obtained as described in point 2.2.2.1. 0,1 ml of 10 x 10^6 thymocytes/ml were cultured in round bottomed microtitre plates in the presence or absence of IL - 1, with or without PHA or CON A. The cultures were set up in triplicate and incubated for 72 hours in 5%, CO_2 humidified air. 0,5 µCi of 3H-thymidine was added for the last six hours

of culture, the cells harvested and thymidine incorporation measured by liquid scintillation counting. The results are given as means of the triplicate cultures.

7.2.2 The effect of adherent cell depletion of thymocytes on the response of thymocytes to CON A and PHA, and modulation of the response by IL - 1

Thymocytes obtained as described before were adhered in cRPMI in 75 cm^3 culture flasks for 24 hours to deplete the cells of thymic macrophages. Thereafter the non-adherent cells were decanted, washed and resuspended in cRPMI at a concentration of 10 x 10^6/ml. Non macrophage depleted thymocytes were stored overnight in the cold. Cultures were set up as described in 7.2.1 using adherent cell (macrophage) depleted and whole thymocytes.

7.3 Results

7.3.1 The effect of IL - 1 on the response of thymocytes to mitogens CON A and PHA

7.3.1.1 Suboptimal doses

Thymocytes were cultured in the presence or absence of PHA (0 - 3 μg/ml) with or without IL - 1. The results are shown in Tables 24a and b and Graph 11a.

7.3.1.2 Optimal doses

Thymocytes were cultured in the presence or absence of PHA (0 - 20 μg/ml) with or without IL - 1. The results are shown inin Tables 24c and d and Graph 11b.

The results in Tables 24a to d and Graphs 11a and b.

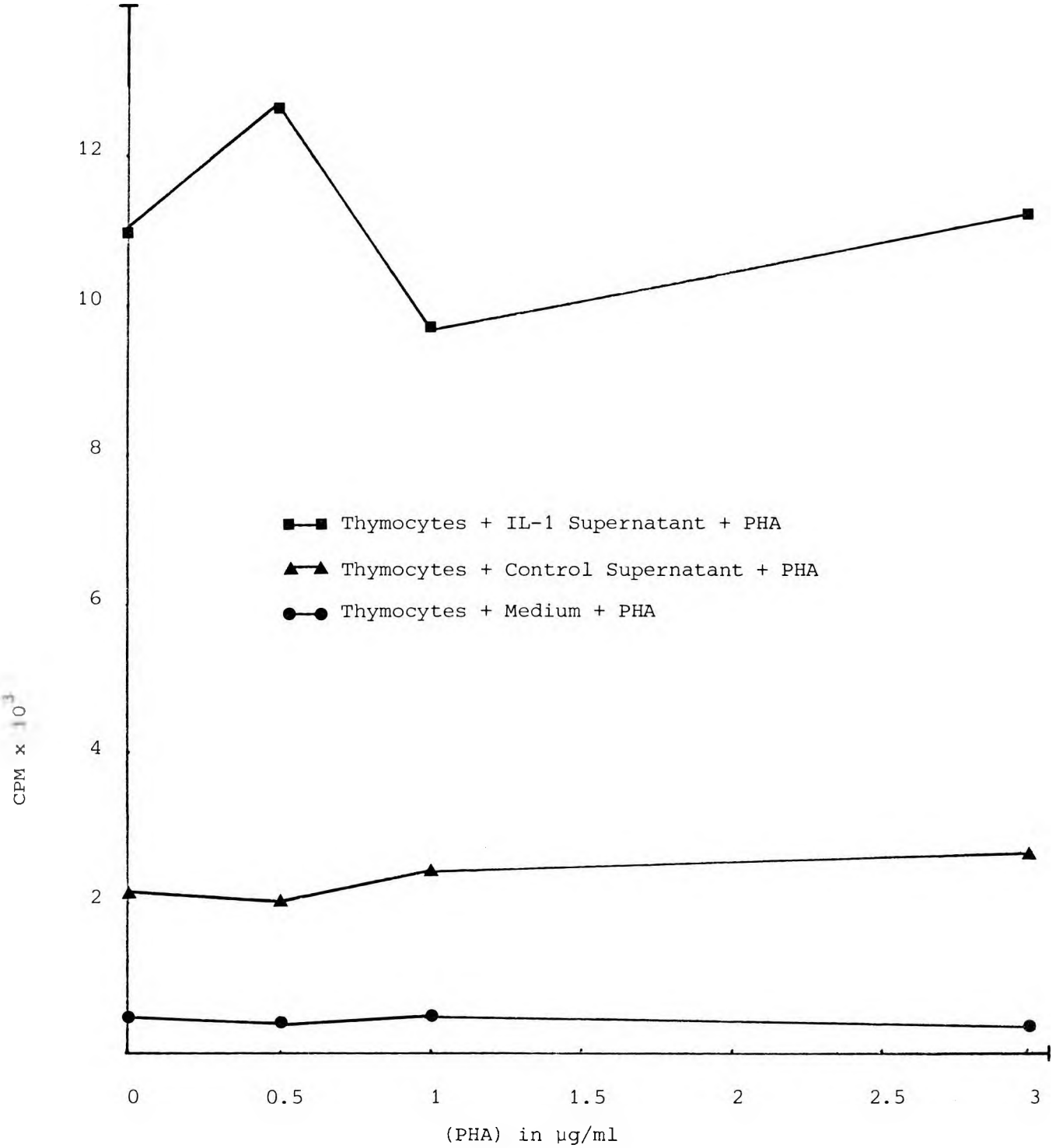
TABLE 24a.

THE EFFECT OF IL - 1 ON THE RESPONSE OF THYMOCYTES TO SUBOPTIMAL DOSES OF PHA

Experiment No.	Concentration of PHA µg/ml	CPM thymocytes + medium	CPM thymocytes + control supernatant	CPM thymocytes + IL-1 supernatant
1	0	426,5	1 794,0	9 200,0
	0,5	307,5	1 651,5	11 113,0
	1,0	431,0	3 031,0	8 127,9
	3,0	263,0	3 229,5	11 346,0
2	0	251,5	1 633,0	9 072,0
	0,5	322,0	1 639,0	10 119,0
	1,0	462,0	1 481,5	9 506,0
	3,0	288,0	2 005,5	9 584,0
3	0	878,6	2 981,5	14 501,5
	0,5	493,5	2 798,5	16 762,0
	1,0	433,2	2 768,0	11 614,0
	3,0	430,7	2 693,5	12 781,0

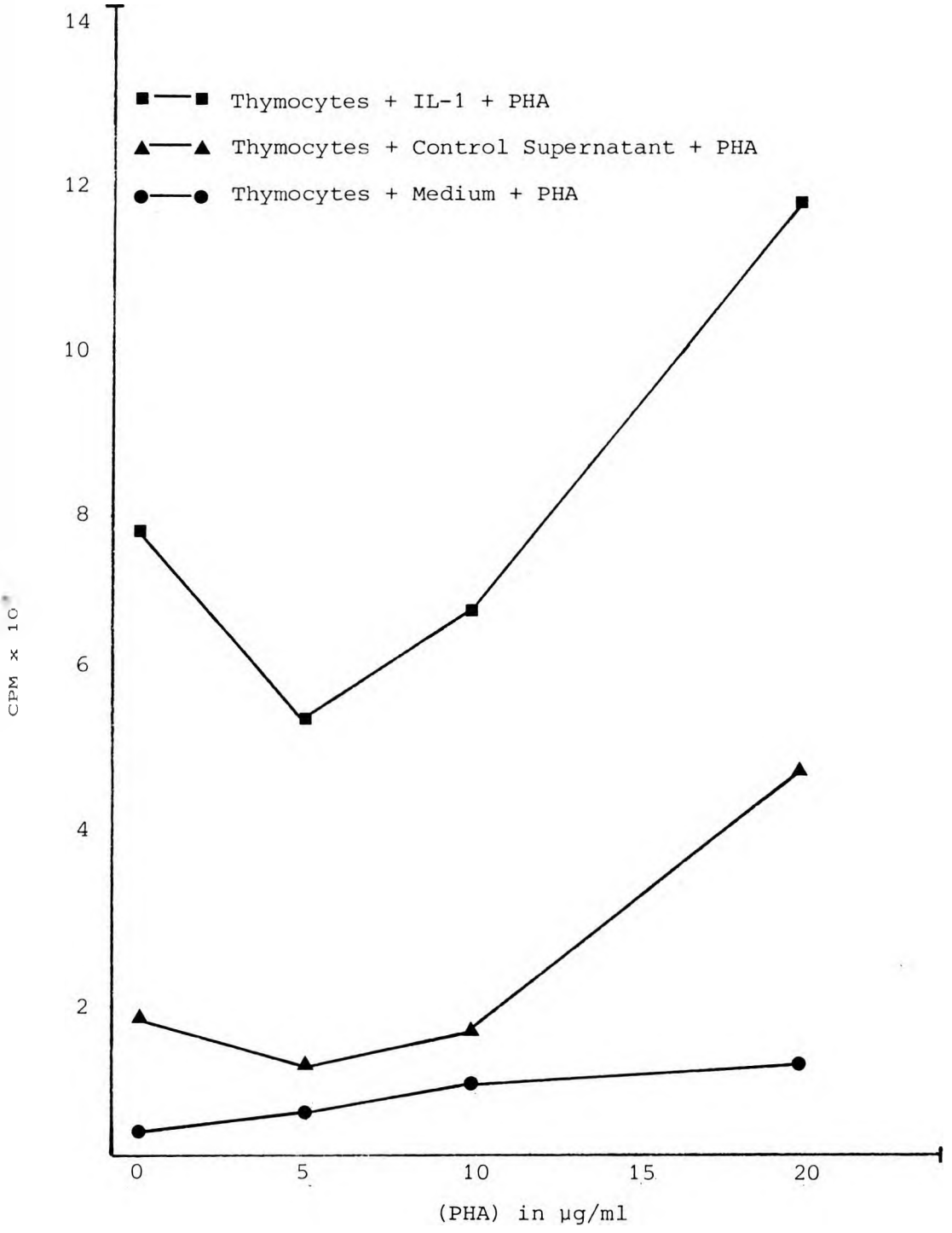
TABLE 24b. Means and standard deviations of the three experiments shown in Table 24a showing the effect of IL - 1 on the response of thymocytes to suboptimal doses of PHA

Concentration of PHA µg/ml	CPM thymocytes + medium	CPM thymocytes + control supernatant	CPM thymocytes + IL-1 supernatant
0	518,9 (±323,6)	2 136,2 (±736,5)	10 924,5 (±3 098,4)
0,5	421,0 (±103,4)	2 029,7 (±665,9)	12 664,7 (±3 583,0)
1,0	442,1 (± 17,3)	2 426,8 (±829,2)	9 749,3 (±1 750,9)
3,0	318,0 (± 90,1)	2 642,8 (±613,6)	11 237,0 (±1 601,3)



THE GRAPH OF STIMULATION OF THYMOCYTES (CPM) VERSUS PHA CONCENTRATION (µg/ml)

GRAPH NO. 11a



THE GRAPH OF STIMULATION OF THYMOCYTES (CPM) VERSUS PHA CONCENTRATION (µg/ml)

GRAPH NO. 11b

indicate that thymocytes are naturally unresponsive to PHA. Treatment of thymocytes with IL - 1 allows the thymocytes to respond slightly to PHA, that is, it slightly potentiates the response of thymocytes to IL - 1.

7.3.2 CON A responses

7.3.2.1 Suboptimal doses

Thymocytes were cultured in the presence or absence of CON A (0 - 3 μg/ml with or without IL - 1. The results are shown in Tables 25a and b and Graph 12a.

7.3.2.2 Optimal doses

Thymocytes were cultured in the presence of absence of CON A (0 - 20 μg/ml) with or without IL - 1. The results are shown in Tables 25c and d and Graph 12b.

The results shown in Tables 25a to d and Graphs 12a and b indicate that thymocytes are naturally responsive to the mitogenic effects of CON A. Furthermore CON A in suboptimal doses potentiates the effect of IL - 1 on thymocytes, but at higher concentrations inhibits this effect.

7.3.3 The effect of adherent cell depletion of thymocytes on their response to CON A including the effect of IL - 1 on the system

Thymocytes, adherent cell depleted or unseparated, were cultured in the presence or absence of CON A (0 - 20 μg/ml) with and without IL - 1. The results obtained appear in Table 26 and Graph 13 and are the means of three individual experiments.

TABLE 24c THE EFFECT OF IL - 1 ON THE RESPONSE OF THYMOCYTES TO OPTIMAL DOSES OF PHA

Experiment No.	Concentration of PHA µg/ml	CPM thymocytes + medium	CPM thymocytes + control supernatant	CPM thymocytes + IL-1 supernatant
1	0	221,3	3 184,9	11 001,3
	5	582,1	2 587,3	10 357,8
	10	674,8	3 151,7	10 909,0
	20	2 459,1	7 614,0	14 817,3
2	0	878,6	596,3	2 900,3
	5	485,2	799,5	2 811,2
	10	512,2	599,4	4 894,4
	20	622,1	3 918,2	8 980,9
3	0	316,7	1 558,7	9 380,7
	5	1 088,0	468,0	3 191,3
	10	1 914,7	1 199,0	4 578,7
	20	930,9	3 157,3	11 351,3

TABLE 24d The means and standard deviations of the three experiments shown in Table 24c. showing the effect of IL - 1 on the response of thymocytes to optimal doses of PHA.

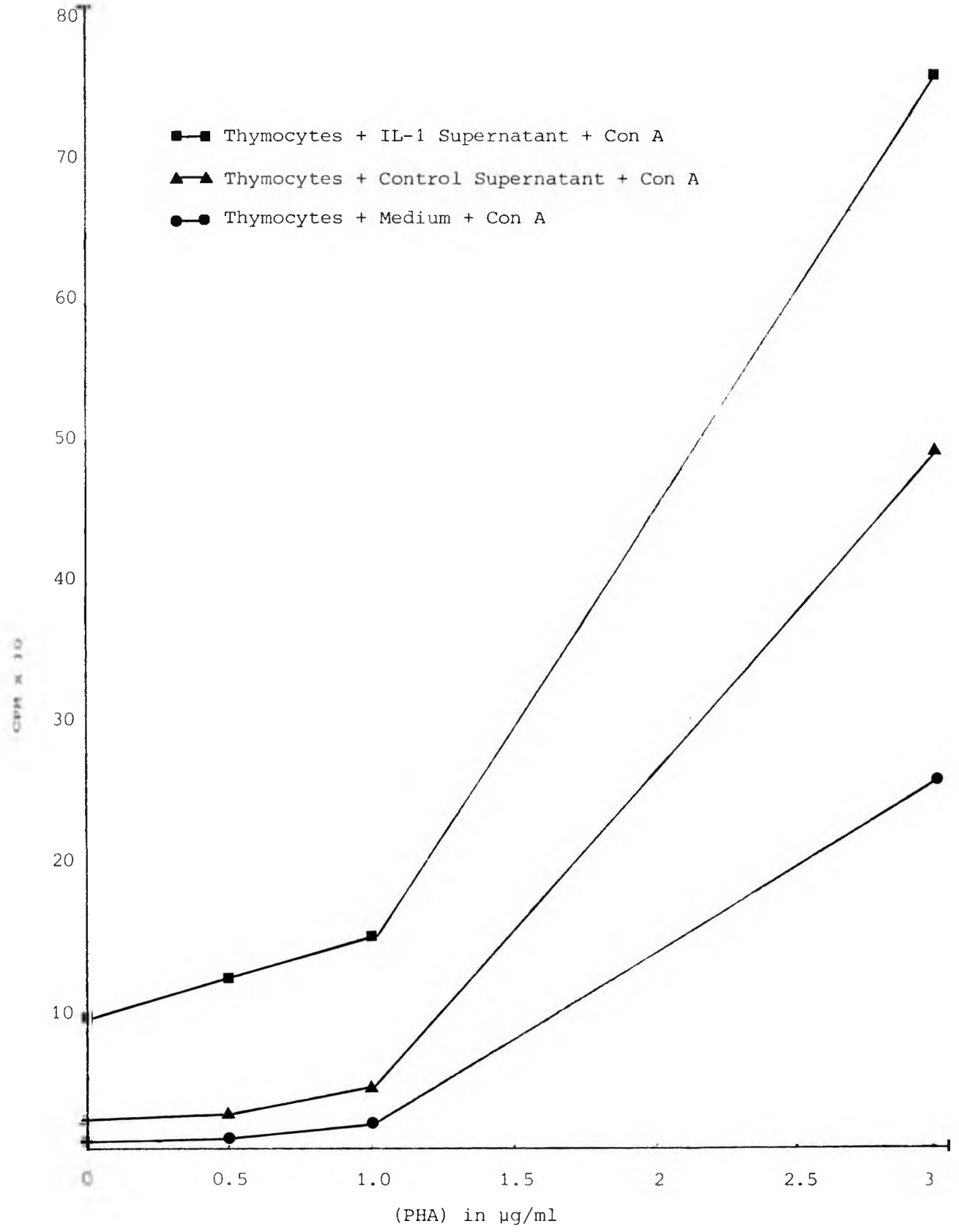
Concentration of PHA µg/ml	CPM thymocytes + medium	CPM thymocytes + control supernatant	CPM thymocytes + IL-1 supernatant
0	472,2 (±355,2)	1 780,0 (±1 308,4)	7 760,8 (±4 286,6)
5	718,4 (±323,7)	1 284,9 (±1 140,0)	5 453,4 (±4 251,6)
10	1 033,9 (±767,1)	1 650,0 (±1 334,6)	6 794,0 (±3 567,2)
20	1 337,4 (±983,6)	4 896,5 (±2 384,0)	11 716,5 (±2 935,3)

TABLE 25a THE EFFECT OF IL - 1 ON THE RESPONSE OF THYMOCYTES TO SUBOPTIMAL DOSES OF CON A.

Experiment No.	Concentration of CON A µg/ml	CPM thymocytes + medium	CPM thymocytes + control supernatant	CPM thymocytes + IL-1 supernatant
1	0	185,5	2 906,5	8 801,0
	0,5	600,5	2 549,5	11 181,0
	1,0	3 293,0	4 783,5	14 874,0
	3,0	13 724,0	25 858,0	56 750,5
2	0	416,0	1 591,0	11 902,0
	0,5	819,5	3 027,0	14 738,0
	1,0	680,5	4 321,5	14 299,5
	3,0	30 893,5	101 968,5	106 897,5
3	0	401,6	995,4	6 788,8
	0,5	504,0	884,0	10 201,1
	1,0	740,0	3 702,5	15 581,9
	3,0	32 865,3	20 703,9	64 338,0

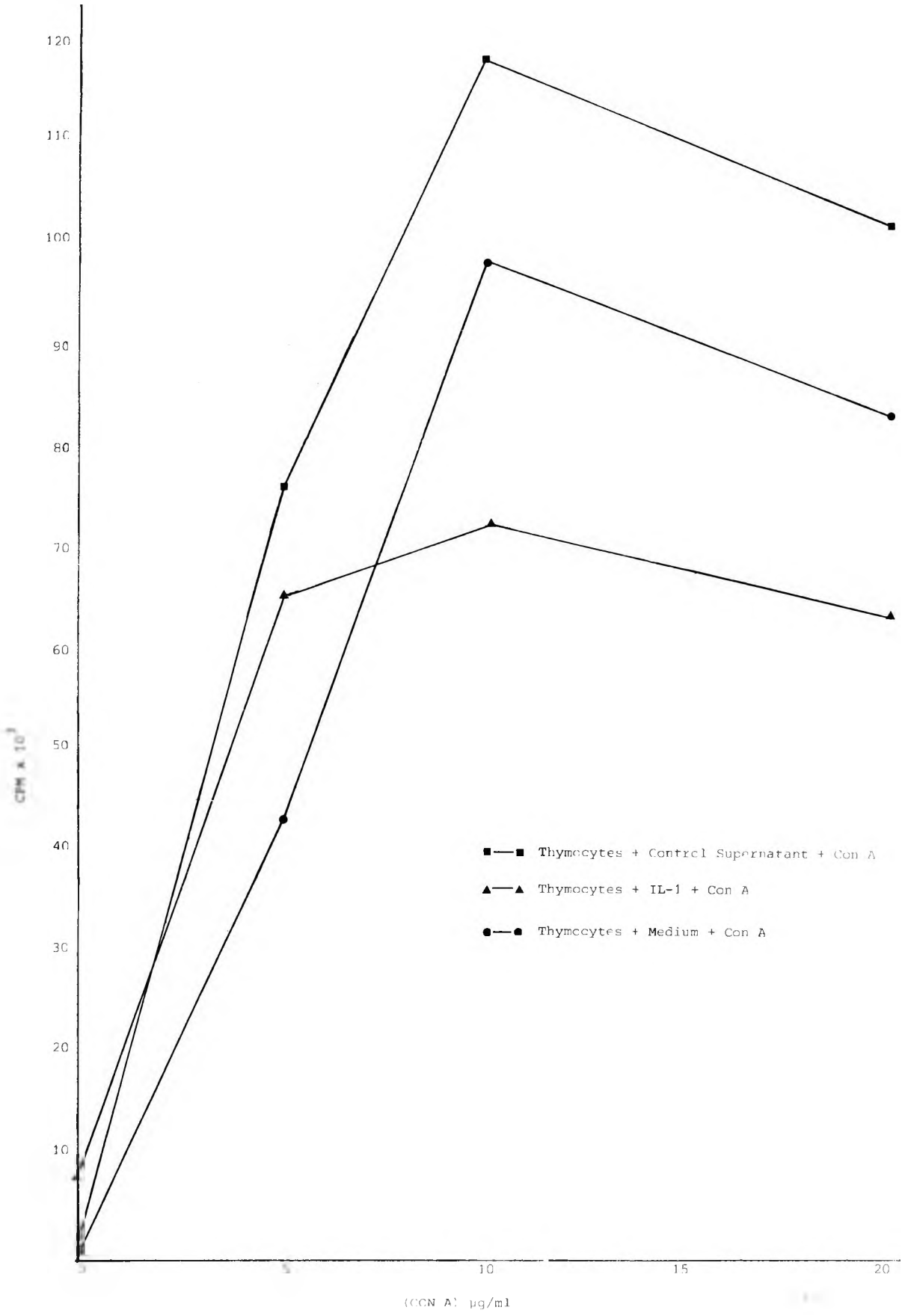
TABLE 25b The means and standard deviations of the three experiments shown in Table 25a. showing the effect of IL - 1 on the response of thymocytes to suboptimal doses of CON A.

Concentration of CON A µg/ml	CPM thymocytes + medium	CPM thymocytes + control supernatant	CPM thymocytes + IL-1 supernatant
0	334,4 (± 129,1)	1 831,0 (± 977,9)	9 163,9 (± 2 575,8)
0,5	641,3 (± 161,7)	2 153,5 (± 1 125,0)	12 040,0 (± 2 387,3)
1,0	1 571,2 (± 1 491,4)	4 269,2 (± 542,4	14 918,5 (± 642,3)
3,0	25 827,6 (±10 528,3)	49 510,1 (±45 503,3)	75 995,3 (±27 029,6)



THE GRAPH OF THYMOCYTE STIMULATION (CPM) VERSUS CON A CONCENTRATION (µg/ml)

GRAPH NO. 12a



THE GRAPH OF THYMOCYTE STIMULATION (CPM) VERSUS CON A CONCENTRATION (µg/ml)

GRAPH NO 12b

TABLE 25c THE EFFECT OF IL - 1 ON THE RESPONSE OF THYMOCYTES TO OPTIMAL DOSES OF CON A.

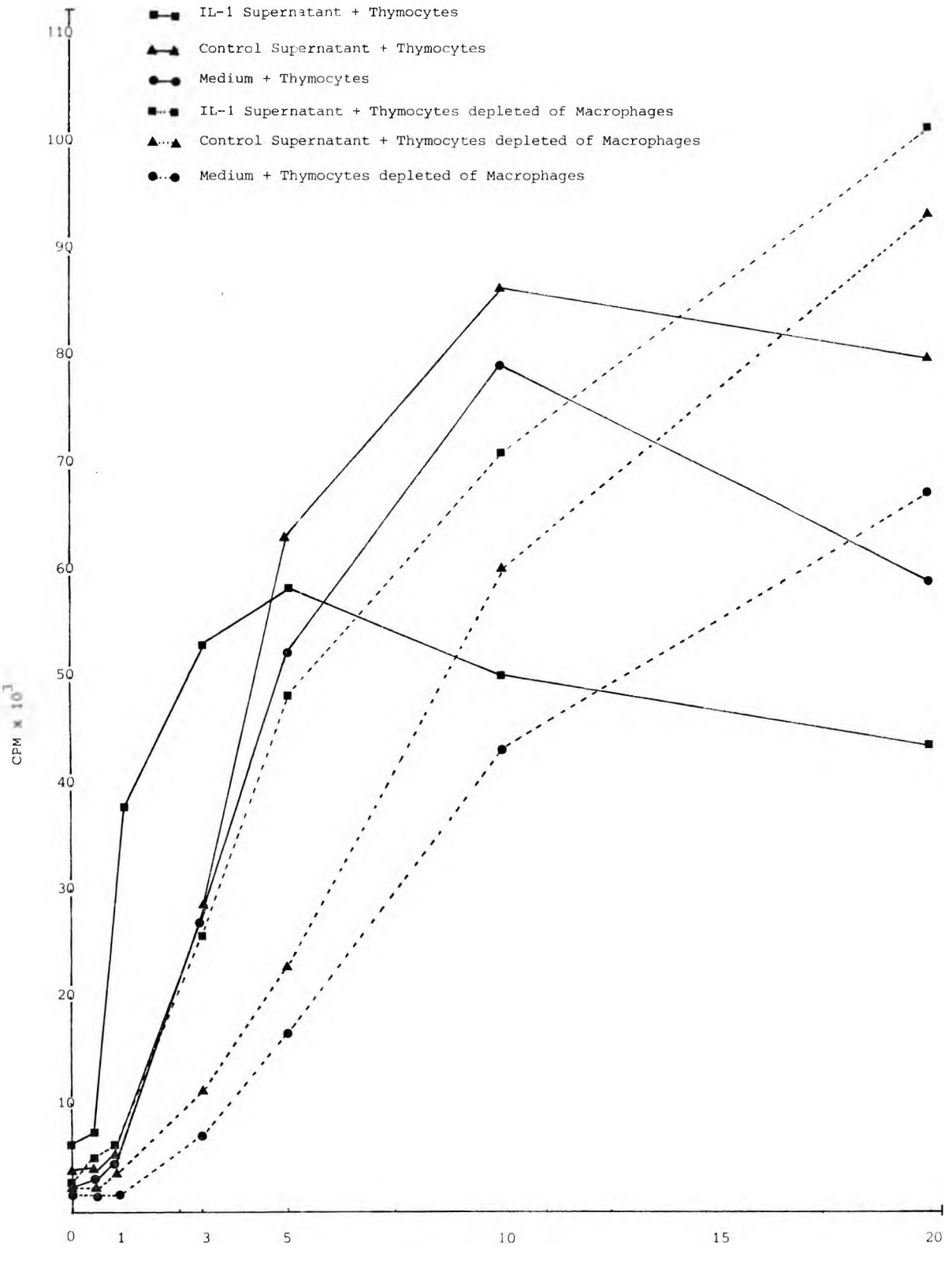
Experiment No.	Concentration of CON A µg/ml	CPM thymocytes + medium	CPM thymocytes + control supernatant	CPM thymocytes + IL-1 supernatant
1	0	396,7	1 558,7	9 380,7
	5	21 968,0	80 913,3	82 023,3
	10	127 331,3	145 086,0	136 012,7
	20	115 589,3	136 551,3	124 398,0
2	0	549,3	1 830,7	5 546,7
	5	31 005,3	38 205,3	45 612,0
	10	42 640,7	34 776,0	26 836,7
	20	27 044,0	23 232,0	19 236,7
3	0	401,6	995,4	6 788,8
	5	75 870,6	98 679,7	66 717,4
	10	123 661,5	175 434,1	53 540,2
	20	107 216,9	146 676,4	46 783,3

TABLE 25d The means and standard deviations of the three experiments shown in Table 25c showing the effect of IL - 1 on the response of thymocytes to optimal doses of CON A.

Concentration of CON A ug/ml	CPM thymocytes + medium	CPM thymocytes + control supernatant	CPM thymocytes + IL-1 supernatant
0	449,2 (± 86,7)	1 461,6 (± 426,0)	7 238,7 (± 1 956,2)
5	42 948,0 (±28 867,7)	75 599,4 (±31 082,6)	64 750,9 (±18 277,3)
10	97 877,8 (±47 872,0)	118 432,0 (±74 020,3)	72 129,9 (±56 912,5)
20	83 283,4 (±48 884,3)	102 153,2 (±68 535,0)	63 472,7 (±54 531,0)

TABLE 26 THE EFFECT OF ADHERENT CELL DEPLETION OF THYMOCYTES ON THEIR RESPONSE TO CON A AND THE EFFECT OF IL - 1 ON THE SYSTEM (MEAN OF THREE EXPERIMENTS)

(CON A) µg/ml	CPM MEDIUM		CPM CONTROL SUPERNATANT		CPM IL-1 SUPERNATANT	
	Thymocytes minus macrophages	Whole thymocytes	Thymocytes minus macrophages	Whole thymocytes	Thymocytes minus macrophages	Whole thymocytes
0	1 363,2	1 231,1	2 016,6	1 787,2	2 836,7	6 248,7
0,5	1 526,1	1 212,2	2 142,7	1 939,6	5 413,5	7 488,3
1,0	1 391,9	1 719,3	3 389,4	5 780,2	6 944,0	30 684,2
3,0	7 136,9	26 161,6	11 282,4	25 933,1	25 696,4	53 520,0
5,0	16 884,9	52 940,1	23 134,7	63 424,6	48 939,2	58 576,2
10,0	43 795,0	79 711,0	60 515,9	86 541,7	71 113,2	50 586,7
20,0	67 607,8	59 837,7	93 810,5	80 604,2	102 142,2	44 121,9



THE GRAPH OF THYMOCYTE STIMULATION (CPM) VERSUS CON A CONCENTRATION (µg/ml)

GRAPH NO. 13.

The results show, once again, that low concentrations of CON A potentiate the response of thymocytes to IL - 1 and high concentrations inhibit this same response. Furthermore, when thymocytes are depleted of macrophages this inhibitory effect of CON A is abrogated, indicating that the macrophage plays an important role in controlling the response of thymocytes to mitogens.

7.4 Discussion

7.4.1 Thymocyte response to mitogens

This project has shown that thymocytes are unresponsive to PHA. However, they respond extensively to CON A. Other workers have obtained similar results (Boyle, 1968; Takiguchi et al., 1971; Stobo et al., 1972). It has been found that thymic cells bind CON A and are stimulated, but bind PHA with only slight resultant activation. This suggests that activation of thymocytes by mitogens requires more than the binding of the mitogen to the cell - perhaps there is a specificity in the activation process due to a linkage between mitogen binding receptors and a cellular activation mechanism. Such a linkage could be postulated to exist for CON A receptors on thymocytes but not for PHA receptors (Stobo et al., 1972). Furthermore, the development of such a linkage could occur during the maturational process.

Recently it has been shown that two steps are necessary for T cell triggering. Firstly, the expression of growth receptors by T lymphocytes and secondly, the production of T cell growth factors in cultures containing both T cells and accessory cells. CON A was found to be able to induce both

responses, but is primarily a "step 2" ligand especially at low concentrations. PHA on the other hand is a potent "step 1" ligand but is limited with respect to "step 2" activity (Larsson and Coutinho, 1980). This could explain why thymocytes respond well to CON A but not to PHA.

7.4.2 IL - 1 , mitogens and thymocyte responses

In this thesis it was also shown that PHA slightly potentiates the effect of IL - 1 on thymocytes and that IL - 1 matures thymocytes so that they become slightly responsive to stimulation by PHA. Furthermore, at low concentrations, CON A enhances the effect of IL - 1 on thymocytes and at high concentrations this effect is inhibited, or, treatment with IL - 1 allows thymocytes to respond to a greater degree to suboptimal concentrations of CON A. An explanation for the above is as follows:-

PHA stimulates only the most immunocompotent medullary thymocytes which constitute only a small proportion of the thymocyte population, whereas CON A also stimulates a large proportion of the immunoincompetent cortical thymocyte population (Stobo and Paul, 1973). Others have also found that the effect of IL - 1 on DNA synthesis of thymocytes is biphasic - it increases low responses and diminishes high responses to CON A and increases thymocyte responses to PHA (Oppenheim, et al., 1976). Furthermore, these workers also found that at low concentrations of thymocytes both low CON A and PHA responses were increased by IL - 1, whereas at higher cell concentrations greater CON A responses were obtained and were decreased by IL - 1 whereas the PHA response continued to be elevated

(Oppenheim, et al., 1976). Therefore IL - 1 induced a shift in the ratio of CON A to PHA, which has been observed to be associated with increasing thymocyte development and maturation (Stobo and Paul, 1973). This statement is in keeping with the theory that IL - 1 has the ability to mature thymocytes.

IL - 1 supernatants have also been found to induce rapid transient increases in the intrathymocyte activity of adenyl cyclase and cAMP levels, whereas the mitogens CON A and PHA fail to do this (Oppenheim, et al., 1976). Generally it has been observed that elevation of cAMP promoted differentiation and inhibits cell proliferation (Watson, 1975; Diamantstein and Ulmer, 1975 a and b). Others have found that IL - 1, after a short period, increases thymocyte cGMP (Katz, et al., 1978; Oppenheim et al., unpublished observations). Considering the above information it could be hypothesized that IL - 1 initially causes an increase in cAMP which induces the prerequisite degree of differentiation in the more immature subpopulation of thymocytes so that they become capable of responding to mitogens. The IL - 1 or IL - 1 plus mitogen cause a change in the cAMP: cGMP ratio resulting in a relative increase in cGMP which favours cell proliferation.

Another theory as to how IL - 1 potentiates the effects of mitogens on thymocytes is via the T - cell activation model involving IL - 1 and IL - 2 (Smith, 1980). In this case IL - 1 acts on thymocytes maturing them to T helper cells which in the presence of lectin and IL - 1 secrete IL - 2, which then activates the more mature thymocytes causing them to proliferate in response to mitogen.

7.4.3 Thymic macrophages, their effect on thymocyte responses to mitogens and modulation by IL - 1

A further finding in this project is that adherent cell depletion of thymocyte populations results in a decrease in their response to CON A at low concentrations of CON A as compared to the response of unfractionated thymocytes. Furthermore, the decrease in the response of thymocytes to high CON A concentrations is not seen in the adherent cell depleted population. Furthermore, IL - 1 partially, or, in some cases completely, restores the response of adherent cell depleted thymocytes to CON A, but does not cause the decrease in the response at high CON A concentrations. These results indicate that the thymic adherent cell is important in controlling the response of thymocytes to the mitogen CON A.

Recently it has been shown that at least a subpopulation of thymocytes can be activated (G_o-G_1 shift) in the absence of macrophages, although the response is lower than in the whole thymocyte population. Furthermore, the activation process of macrophage-free thymocytes has been shown to be improved by 2-ME, indicating that macrophages play a physiological role in the environment of lymphocytes. The proliferative response (G_1-S shift) is optimized when IL - 2 is present and IL - 1 can partially substitute IL - 2, which supports the current hypothesis that IL - 2 production by T cells is induced by the macrophage product IL - 1. IL - 1 might interact with lymphocytes in the G_1-phase (Waksman and Waksshal, 1978; Stadler, et al., 1980) but it is not known whether macrophages or monokines (IL - 1) are required for the activation process (G_o-G_1 shift) and if

the IL - 2 is acting on the G_1-phase (G_1-S shift) thereby causing proliferation. It has also been found that macrophages are not as efficient as IL - 1 in the activation process and this could be related to the macrophages exhibiting suppressor activity via prostaglandin secretion (Bettens, et al., in press). The latter could explain the decrease in the response of whole thymocytes to high CON A concentrations which is not found in macrophage depleted thymocytes to which IL - 1 has been added.

CHAPTER 8

8. APPENDICES AND REFERENCES

8.1. Appendix

8.1.1. Sources of Materials

Amino-acids, minimal essential medium essential (X50)	Difco, Detroit, Michigan, USA.
Amino-acids, minimal essential medium nonessential (X100)	Difco, Detroit, Michigan, USA.
Ammonium Chloride (NH_4Cl)	Merck, Darmstadt, Germany
Chemo-Fluor	Packard Instrument Company, Downer Grove, Illinois, USA
Concanavalin-A	Wellcome Reagents Ltd. Beckenham, England
Dextrose	Protea Laboratory Services Johannesburg, South Africa
Endotoxin, Bacterial Lipopoly-saccharide (E.Coli 0127:B8)	Difco, Detroit, Michigan, USA
Ethanol	Merck, Darmstadt, Germany
Ficoll	Pharmacia, Uppsala, Sweden
Fluorescein Conjugated Horse Anti-Mouse Immunoglobulin	Difco, Laboratories, Detroit Michigan USA
Glutamine (L)	Merck, Darmstadt, Germany
Hanks Balanced Salt Solution	Wellcome Reagents Ltd., Beckenham, England.
Heparin, Preservative Free	Evans Medical, Liverpool, England
Hydrochloric Acid	Mallinckrod Chemical Works St Louis, Missouri, USA
Hydrocortisone	Sigma Chemical Company, St Louis Missouri, USA
Hypaque	Winthrop Laboratories, Durban, South Africa
Medium, Minimal Essential	Wellcome Reagents Ltd, Beckenham, England
Medium, RPMI 1640	Polio Research Institute Johannesburg, South Africa

Mercapto-Ethanol (2ME)	Sigma Chemical Company, St Louis, Missouri, USA
Phosphate Buffered Saline (PBS)	Behring Institute, Behringwerker, Marburg Lahn, West Germany
Phytohaemagglutinin (PHA) Reagent Grade	Wellcome Reagents Ltd, Beckenham, England
Poke-Weed Mitogen	Gibco, Grand Island, New York, USA.
Rhodamine Conjugated Anti-Albumin	Difco Laboratories, Detroit, Michigan, USA
Serum, Fetal Calf	Wellcome Reagents Ltd, Beckenham, England
Serum, Human AB	Polio Research Institute, Johannesburg, South Africa
Sheep Red Blood Cells (sRBC)	Johannesburg Abattoir, South Africa
Sodium Bicarbonate (7,5%)	Merck, Darmstadt, Germany
Sodium Chloride	Hopkin and Williams Pty (Ltd) Johannesburg, South Africa
Sodium Periodate	Merck, Darmstadt, Germany
Sodium Pyruvate	Merck, Darmstadt, Germany
Thymic Humoral Factor (THF)	Weizman Institute Israel
Thymopoietin	Ortho Pharmaceutical Corporation Raritan, NI
Thymosin	Ortho Pharmaceutical Corporation Raritan, NI
Tritiated Thymidine (Methyl - ^{3}H) 17 Ci/mmol	Radio Chemical Centre, Amersham, England
Versene	Wellcome Reagents Ltd Beckenham, England

8.1.2 Sources of Equipment

Centrifuge Tubes (15 & 50 ml) (No. 2095 & 2070)	Falcon Plastics Division of Becton, Dickinson & Co., Cockeysville MD, USA
Microtitre Plates, Round Bottomed	Greiner, Nürtingen, Germany

Millipore Filters, Type GS 0.22 μM	Millipore Corporation, Bedford, Massachusettes, USA
Multiple Automatic Harvester (MashII)	Microbiological Associates Bethesda, Maryland, USA
Nylon Fibre, LP - 1, Leuco-Pak Leukocyte Filter	Fenwal Laboratories, Morton Grove, Illinois, USA
Sterile Pipettes (1 + 10 ml)	Falcon Plastics Division, Oxnard, California
Scintillation Vials	Packard Instrument Company, Downer Grove, Illinois, USA
Syringes, Plastic Disposable (1 - 50 ml)	Monoplast, Johannesburg, South Africa
Tissue Culture Dishes (60 x 15 mm and 35 x 10 mm)	Lux Scientific Corporation, Newburg Park, California
Tissue Culture Flasks ($25\ cm^2$, $75\ cm^2$)	Falcon Plastics, Oxnard, California, USA
Tissue Culture Tubes (16 x 125mm, 17 x 100mm)	Falcon Plastics, Oxnard California, USA
Tri-Carb Liquid Scintillation Counter, Model 3390	Packard Instrument Company, Downer Grove, Illinois, USA

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