MECHANISM OF TUMOUR RESISTANCE IN SALMONELLA-IMMUNIZED MICE

Vincent J. La Posta, B.Sc. (Hons.) (Adelaide)

A thesis submitted for the degree of

Doctor of Philosophy

Department of Microbiology and Immunology,

The University of Adelaide,

Adelaide, South Australia.

July, 1983.

TABLE OF CONTENTS

ABSTRACT xiii

SIGNED STATEMENT xv

ACKNOWLEDGEMENTS xvi

ABBREVIATIONS USED IN THIS THESIS xvii

CHAPTER ONE

TUMOUR RESISTANCE INDUCED BY INFECTION WITH INTRACELLULAR BACTERIAL PARASITES

1.1 The role of immune responses in controlling the growth of tumours 1

1.2 The induction of non-specific tumour resistance by infection with microbial pathogens 4

1.3 Resistance to intracellular bacterial parasites 5

1.3.1 The carrier state 5

1.3.2 Requirement for activated macrophages to control IBP infections 6

1.3.3 Sensitized thymus-derived lymphocytes induced during IBP infections mediate macrophage activation 9

1.3.4 The life span and migratory characteristics of the lymphocytes generated during IBP infections 10

1.3.5 Newly formed protective lymphoblasts accumulate at sites of inflammation 12

1.4 The characteristics of tumour resistance induced by IBP 14

1.5 Biochemical markers for activated macrophages 17

1.6 The mechanism of macrophage activation *in vitro* 19

1.6.1 Role of soluble factors (MAF) 19

1.6.2 Macrophages must internalize MAF to be activated 20

1.6.3 Different macrophage subpopulations vary in sensitivity to MAF 21

1.6.4 The role of lipopolysaccharides in macrophage activation by MAF 22

1.6.5 Macrophage activation proceeds as a series of steps 23

1.6.6 Modulation of the activated state 25

1.6.7 Characterization of MAF 26

1.7 *In vitro* assays for the anti-tumour activity of macrophages 28

(a) Assays which rely on membrane rupture 28

(b) Assays which rely on nuclear disintegration 28

(c) Assays which measure the inhibition of cell division 29

(d) Assays which measure the detachment of adherent target cells 29

1.8 Nature of the cells killed by macrophages 30

1.9 Mechanism of tumour cytotoxicity by activated macrophages 31

1.9.1 Cytotoxicity requires binding of macrophages to tumour cells 31

1.9.2 The relationship of binding to cytolysis 33

1.10 The nature of the tumour-cell receptors on cytotoxic macrophages 34

1.10.1 Receptors on specifically-cytotoxic macrophages 34

1.10.2 Antibody-dependent cell-mediated cytotoxicity 35

1.10.3 Receptors on non-specifically cytotoxic, activated macrophages 37

1.11 The role of soluble cytotoxic factors secreted by macrophages 38

1.11.1 Effect of thymidine 38

1.11.2 Effect of arginase 39

1.11.3 Effect of serine proteases 41

1.11.4 Tumour necrosis factor 43

1.11.5 Oxygen Metabolites 43

1.12 Natural killer cells 47

1.12.1 Characteristics of NK cells 47

1.12.2 Evidence of a role for NK cells *in vivo* 49

1.12.3 The mechanism of NK cell-mediated lysis 50

1.13 Aims of this study 53

1.14 Summary 54

CHAPTER TWO

MATERIALS AND METHODS

2.1	Mice	55
2.2	Bacteria	55
2.3	Immunization of mice with 11RX	56
2.4	Preparation of 11RX antigen extract	56
2.5	Lipopolysaccharide	57
2.6	Anti-Thyl.2 antiserum	57
2.7	Media for preparation and culture of cell suspensions	57
2.8.1	Hanks' Balanced Salt Solution	57
2.8.2	Tissue culture media	57
2.9	Composition of gaseous phases for buffering _in vitro_ cell cultures	58
2.10	Trypan Blue for injection into mice	58
2.11	Cell counting	59
2.12	Differential Counting	59
2.13	Tumour Cell Lines	59
2.14	Immunization of mice with allogeneic tumour cells	60
2.15	Peritoneal Cells	60
2.16	Preparation of spleen and lymph node cell suspensions	60
2.17	Splenic lymphocytes	61
2.18	Blast cells	61
2.18.1	For use in _in vitro_ cytotoxicity assays, cold target inhibition assays and cell binding assays	61
2.18.2	For use in IL2 assay	61
2.19	Adherence of PC to Plastic	62
2.20	Preparation of PC which do not adhere to nylon wool	63
2.21	Preparation of PC which do not adhere to glass wool	63
2.22	Fractionation of PC on Percoll density gradients	63
2.22.1	Discontinuous density gradients	64

2.22.2 Continuous density gradients 64

2.23 Removing polymorphonuclear cells from cell suspensions 65

2.24 Radiolabelling of Cells 65

2.24.1 Labelling cells with ^{125}I 65

(i) *in vivo* 65

(ii) *in vitro* 66

2.24.2 Labelling cells with ^{51}Cr 66

2.24.3 Labelling cells with ^{3}H-TdR 67

2.25 The measurement of anti-tumour activity *in vivo* 67

2.25.1 Clearance of ^{125}I-EAT cells from the peritoneal cavity 67

2.25.2 Whole body retention of ^{125}I 68

2.26 *In vitro* cytotoxicity assay 69

2.27 Cold Target Inhibition of *in vitro* cytotoxicity 70

2.28 Killing of Tumour Cells Across Cell Impermeable Membranes 71

2.29 Cell-binding assay 71

2.30 Sugars used in an attempt to inhibit PC cytotoxicity 72

2.31 Testing lymphoid cells for the ability to release lymphokines 72

2.31.1 Induction of lymphokine release 72

2.31.2 Assays for lymphokines in culture supernatants 73

2.31.3 Interleukin 2 assays 73

2.31.4 Macrophage Activation Factor 74

2.32 Preparation of lymphokine standards 75

2.32.1 Macrophage Activation Factor 75

2.32.2 Preparation of Interleukin 2 standard 76

CHAPTER THREE

CHARACTERIZATION OF THE EFFECTOR CELLS RESPONSIBLE FOR TUMOUR RESISTANCE IN SALMONELLA ENTERITIDIS 11RX IMMUNIZED MICE

3.1 Introduction 78

3.2. The effect of trypan blue on the clearance of ^{125}I-EAT from mice Immunized with S. enteritidis 11RX 83

3.2.1 Short-term ip immunized mice 84

3.2.2 Long-term iv immunized mice recalled with 11RX antigen ip 85

3.2.3 Long-term ip immunized mice. 85

3.3. The cytolytic activity of PC from trypan blue-treated 11RX-immunized mice 85

3.3.1 Short-term 11RX immunized mice 86

3.3.2 Recalled, long-term iv-immunized mice 87

3.4 The *in vitro* activity of cytotoxic lymphocytes from allo-immunize mice injected with trypan blue 87

3.5 The cytotoxic activity of PC from short-term immunized mice after pretreatment *in vitro* with trypan blue 88

3.6 The *in vitro* cytotoxic activity of PC in the presence of trypan blue 89

3.7 Characterization of cytotoxic peritoneal cells 90

3.7.1 Adherence to Plastic 91

3.7.2 Adherence to nylon wool 92

3.8 Bouyant density fractionation of PC on discontinuous gradients 93

3.8.1 PC from recalled, iv immunized mice 94

3.8.2 PC from short-term ip immunized mice 94

3.9 Buoyant density fractionation of PC using continous gradients 95

3.10 Summary and conclusions 96

CHAPTER FOUR

THE INTERACTION OF ACTIVATED MACROPHAGES WITH NORMAL AND NEOPLASTIC CELLS

4.1 Introduction 97

4.2 The effect of varying the effector to target cells ratio on *in vitro* cytotoxicity 101

4.3	The role of soluble factors in the lysis of tumour cells by PC from 11RX-immunized mice	102
4.3.1	The effect of low cell density on *in vitro* cytolysis	102
4.3.2	Separation of tumour cells and PC by cell impermeable membranes	103
4.4	The effect of antibody on the cytolysis of ^{51}Cr-P815 cells by cytotoxic PC	103
4.5	Con-A activated splenic blast cells as targets for tumouricidal PC	106
4.5.1	Summary of experiments using ^{51}Cr-blast cells as target cells	106
4.5.2	Criticism of previous studies using Con A blast cells as target cells	107
4.5.3	^{125}I-blast cells are not lysed by PC from 11RX-immunized mice	108
4.6	The inhibition of cytolysis by unlabelled tumour cells	109
4.7	Con A activated-blast cells as cold target inhibitors of cytotlysis	111
4.7.1	Con A blast cells do not inhibit the lysis of ^{51}Cr-P815 cells	111
4.7.2	The lysis of ^{51}Cr-P815 cells by Con A blast cells is due to cytotoxic cells	112
4.7.3	Con A blast cells do not inhibit lysis of ^{51}Cr-EAT cells	113
4.8.	The ability of activated macrophages from 11RX-immunized mice to bind tumour cells	113
4.8.1	The effect of varying the concentration of P815 cells on binding to activated macrophage monolayers	114
4.8.2	The effect of varying the number of adherent cells on the binding of P815 cells	115
4.8.3	The effect of antibody on the binding of P815 cells to activated macrophages	115

4.8.4 Binding of P815 cells to normal macrophages and to macrophages obtained at different times after immunization 116

4.9 The lysis of tumour cells bound to adherent cytotoxic PC 117

4.9.1 Experimental design 118

4.9.2 The effect of varying the number of ^{51}Cr-P815 cells 118

4.9.3 The effect of varying the number of macrophages 119

4.10 The binding of normal cells to activated macrophage monolayers 120

4.11 The binding of Con A-activated blast cells to normal macrophages 122

4.12 Attempts to reconcile the differences between the results obtained from binding and cold target inhibition experiments 122

4.12.1 The effect of unlabelled P815 cells on the viability of ^{51}Cr-blast cells co-cultured with PC from11RX-immunized mice 123

4.12.2 Con A blast cells do not induce the release of cytotoxic factors from PC 123

4.12.3 The binding of Con A blast cells to macrophage monolayers is short-lived 124

4.13 The effect of simple sugars on the cytolysis of P815 cells by PC 125

4.14 Summary and conclusions 126

CHAPTER FIVE

THE LIFE SPAN OF LYMPHOCYTES MEDIATING THE RECALL OF TUMOUR RESISTANCE IN LONG-TERM 11RX-IMMUNIZED MICE

5.1 Introduction 128

5.2 Dependence of *in vivo* cytotoxicity on the number of spleen cells transferred 132

5.3 Systemic transfer of the ability to recall tumour resistance 133

5.4 The functional life Span of 11RX sensitized Lymphocytes after systemic transfer 135

5.5. The functional life span of sensitized lymphocytes after local transfer 136

5.5.1 Experiment 1: Decay of spleen cell activity after ip transfer 137

5.5.2 Experiment 2: Direct comparison of the decay of lymphocyte function after iv and ip transfer 138

5.5.3 Experiment 3: Comparison of the decay of spleen cell activity in recipients and donors 139

5.6 Summary and conclusions 140

CHAPTER SIX

LYMPHOKINE RELEASE DURING 11RX INFECTION

6.1 Introduction 142

6.2 The release of Interleukin 2 by 11RX-sensitized lymphoid cells 145

6.3 Requirement for T cells in the release of IL2 148

6.4 The anatomical distribution of lymphocytes releasing IL2 at various times after 11RX immunization 149

6.5 Is the inactivity of LNC and spleen cells due to the lack of antigen presenting cells? 150

6.5.1 Ability of normal PC to present antigen 150

6.5.2 Effect of adding normal PC on the ability of lymphoid cell suspensions to release IL2 151

(i) Lymph node cells 151

(ii) Spleen cell suspensions 151

6.6 Is the lack of IL2 secretion due to suppression 152

6.7 Preliminary attempts to detect MAF release 153

6.7.1 Activation of PC from long-term 11RX-immunized mice 153

6.7.2 Activation of macrophages by conditioned culture media 155

6.8 Development of an assay for MAF in culture supernatants 156

6.9 T cells are required for the release of MAF 157

6.10 The cytostatic activity of lymphokine-treated macrophages 158

6.11 Variability of the MAF assay 159

6.11.1 Lack of reproducibility of MAF assays using SMP1 159

6.12 Effect of varying time of incubation with ppi PC with conditioned culture supernatants 160

6.13 Effect of adding LPS and prolonging incubation time prior to harvesting 161

6.14 Cytotoxic activity of lymphokine-treated macrophages is consistently detected using long-term assays 163

6.15 Some cell populations appear to have cytotoxic activity when long-term assays are sampled with cell harvester 164

Experiment 1 165

Experiment 2 165

Experiments 3-5 165

6.16 The effect of delaying the addition of target cells on the cytotoxic activity of lymphokine-activated macrophages 167

6.17 The relationship between cytostasis and cytotoxicity 168

6.18 The relationship between IL2 and MAF release by lymphoid cells at different times after immunization with 11RX 170

6.19 Summary and conclusions 172

CHAPTER SEVEN

THE MECHANISM OF TUMOUR RESISTANCE IN 11RX IMMUNIZED MICE

7.1 Introduction 175

7.2 The nature of the effector cells responsible for tumour resistance in 11RX immunized mice. 175

7.2.1 The effect of trypan blue on cytotoxicity 176

7.2.2 Fractionation of cytotoxic PC on Percoll density gradients 179

7.2.3 Fractionation of cytotoxic PC into adherent and non-adherent subpopulations 181

7.2.4 The relative contribution of activated macrophages and NK cells to tumour resistance induced by 11RX infections 182

7.3 The requirement for cell contact in the lysis of tumour cells by PC 183

7.4 Effect of antibody on the cytolytic activity of PC 185

7.5 Relationship of the ability of activated macrophages to bind and lyse tumour cells 187

7.6 The susceptibility of Con A blast cells to lysis by activated macrophages 188

7.7 The recognition of tumour cells by activated macrophages 190

7.7.1 The results of cold target inhibition studies 191

(a) Activated macrophages recognize structures which are present on all types of tumour cells 191

(b) Activated macrophages do not interact with dividing T cells in the same way as they do with tumour cells 191

(c) Possible reasons why the findings of cold target inhibition experiments differ from those of Hamilton and Fishman 192

i Reutilization of label 193

ii The use of "normal" cell lines 193

iii Possible differences in the way various tumour cells interact with macrophages 194

iv Competition may not involve tumour-binding receptors on macrophages 195

7.7.2 The results of cell binding experiments 196

(a) Activated macrophages bind tumour cells and dividing and non-dividing lymphocytes with similar efficiency 196

(b) The binding of Con A blast cells to activated macrophages is transient 197

(c) Does binding of tumour cells and blast cells to activated macrophages require the same receptor? 198

7.7.3 The role of carbohydrates in the recognition of tumour cells by activated macrophages 199

7.8 Characterization of lymphocytes induced by immunization with 11RX 200

7.9 The functional life-span of lymphocytes mediating the recall of tumour resistance in 11RX immunized mice. 201

7.9.1 Short-lived lymphocytes are detected after iv transfer of spleen cells 202

7.9.2 Long-lived lymphocytes are detected after ip transfer of spleen cells 203

7.9.3 Relative contribution of long-lived and short-lived lymphocytes in recall of tumour resistance 204

7.9.4 The possible role of long-lived non-recirculating peritoneal lymphocytes in recalling tumour resistance 205

7.10 The release of lymphokines by lymphoid cells from 11RX-immunized mice 207

7.10.1 Differences between macrophage activation *in vitro* by lymphokines and activation *in vivo* by 11RX infection 208

7.10.2 Spleen cells and LNC release little or no IL2 210

7.10.3 The possible action of suppressor cells in controlling the *in vitro* release of IL2 and MAF by PC early after 11RX infection 211

7.10.4 The relationship between the lymphocytes which release MAF *in vitro* and those which confer protection *in vivo* 213

7.11 Summary, conclusions and future directions 214

PUBLICATIONS 217

BIBLIOGRAPHY 218

ABSTRACT

The experiments reported in this thesis investigated the tumour resistance induced by immunization of (BALB/cx C57BL/6)F_1 mice with a live vaccine of the intracellular bacterial parasite, *Salmonella enteritidis* 11RX (11RX). The aims were to define the cytotoxic effector cells, the mechanism by which they recognize tumour cells and to characterize the 11RX-sensitized T cells induced by 11RX infections which are involved in the generation of tumour resistance.

This study confirmed previous suggestions that activated macrophages are the principal cytotoxic cells present in the peritoneal cavity of 11RX-immunized mice. It was found that the lysis of tumour cells *in vitro* is dependent on contact between effector and target cells and that activated macrophages bind tumour cells more efficiently than do macrophages from normal mice. Unlabelled tumour cells could competitively and non-specifically inhibit the lysis of radioactively labelled tumour cells by peritoneal cells (PC) from 11RX-immunized mice.

Concanavalin A-activated splenic blast (blast) cells were compared to tumour cells for their ability to interact with activated macrophages. Blast cells were not lysed by PC from 11RX-immunized mice and did not inhibit the lysis of tumour cells *in vitro*. However, blast cells did bind to monolayers of activated macrophages and to a lesser degree to normal macrophages. The extent of blast cell binding to the two macrophage types was equal to that observed with tumour cells. Unlike the adherence of tumour cells to activated macrophages, the bond between blast cells and activated macrophages was labile and dissociated with incubation *in vitro* suggesting that the two types of interactions were qualitatively different.

Spleen cells from 11RX-immunized mice could transfer to naive recipients the ability to recall resistance to intraperitoneal (ip) tumour challenge upon ip injection of 11RX antigen extract. When the ip route was used to transfer spleen cells, high levels of ip tumour resistance could be recalled three weeks later.

Tumouricidal activity could be induced in inflammatory macrophages by culturing them in vitro with lymphoid cells from 11RX-immunized mice and 11RX antigen. The lymphokines, Interleukin 2 (IL2) and Macrophage Activation Factor (MAF) are released by 11RX-sensitized lymphoid cells when they are stimulated in vitro with 11RX antigen. Lymphoid cell populations differ in their ability to release these lymphokines and, furthermore, the ability to release MAF did not correlate directly with the release of IL2. Treatment of lymphoid cells with anti-Thy 1.2 antibody and complement abolished their ability to release the two lymphokines.

From these observations the following conclusions were drawn. The tumour resistance induced in mice by 11RX immunization is effected mainly by activated macrophages and is mediated by 11RX-sensitized T cells which are long-lived. These sensitized T cells can activate tumouricidal macrophages by releasing MAF when stimulated with bacterial antigens. The sensitized T cells which release MAF may be different from those which release IL2.

Lysis of tumour cells by activated macrophages requires the binding of target cells by effector cells which is mediated by surface structures common to tumour cells. The binding structures present on normal dividing blast cells appear to be different from those present on tumour cells. This may explain why blast cells are not killed by 11RX-activated macrophages.