MANIPULATION OF THE IMMUNOSTIMULATORY CAPACITY OF A HUMAN MYELOID LEUKAEMIA CELL LINE HL-60.

By Sean Michael Geary, B.Sc. Hons, Department of Haematology, I.M.V.S., Adelaide.

A degree submitted for the degree of Doctor of Philosophy, Faculty of Science at the University of Adelaide

November, 1993

ABSTRACT

The aim of this project was to determine the reason for the lack of ability of many myeloid leukaemic cell populations to stimulate allogeneic lymphocytes in mixed leucocyte culture (MLC), with a view to manipulating the immunogenicity of these cells for therapeutic purposes. The myelogenous leukaemic cell line, HL-60, was chosen as a model system since it has been reported that cells of this line can be induced, by a variety of agents, to differentiate along the granulocytic or monocytic pathways, with or without the acquisition of class II major histocompatability (MHC) antigens. It was predicted that at least some of these treatments would result in the acquisition of allostimulatory activity, and would thus allow analysis of the requirements for this activity.

HL-60 cells cultured with a physiological concentration (10 nM) of all-*trans* retinoic acid (RA) for 7 days (HL-60-R7) possessed an enhanced capacity to stimulate the proliferation of $CD2^{+}$-enriched lymphocytes derived from the peripheral blood of healthy human donors in MLC. The lymphocyte populations responding to HL-60R7 possessed NK activity (i.e. the ability to lyse K562 cells) and an enhanced capacity to lyse HL-60 and HL-60-R7 cells. Examination of the HL-60 cells cultured for 7 and 12 days with RA, carried out by means of enzyme histochemistry, cell surface marker analysis and morphology, revealed a somewhat heterogeneous population of cells with monocyte-like, neutrophil-like and other less readily definable cell types being present. The presence of monocyte-like cells was indicated by the cell surface expression of CD36 by a proportion of the cells and, although no cells expressed detectable levels of MHC class II molecules on day 7, by day 12 a small percentage (15%) were positive for low-medium levels of class II antigen. However morphological and cytochemical studies failed to indicate the emergence of a clearly identifiable population of mature cells.

Additional cell surface marker analysis revealed an up-regulation of CD11b and ICAM-1, along with a down-regulation of MHC class I molecules and transferrin receptor (CD71). Analysis of the levels of expression of mRNA for four immunologically relevant cytokines by HL-60 and HL-60-R7 cells was performed by the RNase protection assay, which revealed that both populations failed to express detectable levels of IL-6 mRNA and barely detectable levels of IL-1α and β mRNA, with the most prominent mRNA species being that coding for TNFα. The levels of expression of TNFα mRNA appeared to remain unchanged after R.A.-treatment.

The stimulatory activity of HL-60-R7 cells in MLC despite their failure to express detectable MHC class II antigens prompted examination of the nature of the responding lymphocytes. Two-colour immunofluorescence analysis using CD25 expression as an indicator of cell activation revealed that the predominant phenotype of the responding cells was $CD16^+$ and/or $CD8^+$, with $CD4^+$ lymphocytes being stimulated to a lesser extent. However, removal of the $CD4^+$ population significantly impaired the proliferative response to HL-60-R7 cells as did the addition of monoclonal antibodies specific for MHC class II antigen. In contrast, removal of MHC-class II^+ cells from the lymphocyte populations had no effect on the proliferative response. The addition of anti-TNFα almost completely abrogated the response to HL-60, HL-60-R7 and RC-2a cells, whereas anti-IL-1 and anti-IL-12 appeared to have no effect.

A wide variety of mostly myeloid cell lines were also tested for their cell surface marker expression as well as their immunostimulatory nature in MLC, where it was found that the majority of these cell lines were only weakly stimulatory.

Contents

Abstract i

Statement

Acknowledgements

Abbreviations

Chapter 1: Introduction

1.1 Leukaemia 1

1.2 Growth requirements for leukaemic cells 5

1.3 Leukaemic cell lines 6

1.3.1 General characteristics of leukaemic cell lines 6

1.3.2 HL-60 6

1.3.3 Differentiation of HL-60 and other leukaemic cell lines 8

1.3.4 The role of RA in the differentiation of HL-60 cells 9

1.4 T lymphocyte activation 11

1.4.1 T lymphocytes 11

1.4.2 Requirements for T lymphocyte activation 14

1.4.3 The biochemistry of T lymphocyte activation 18

1.4.4 Cytokines as second signals 20

1.4.5 The role of accessory molecules in T lymphocyte activation 22

1.5 Natural killer cells 28

1.5.1 Definition of natural killer cells 28

1.5.2 Mechanism of NK cell activation 31

1.6 Tumour immunology 36

1.6.1 The role of T lymphocytes in controlling cancer 36

1.6.2 The role of NK cells in controlling cancer 40

1.6.3 Therapeutic strategies for acute myeloid leukaemia 43

1.6.4 Aim 45

Chapter 2: Materials and methods

2.1 Tissue culture 47

2.1.1 Media 47

2.1.2 Maintenance of cell lines 48

2.1.3 Induction protocol for cell lines 48

2.1.4 Cryopreservation of cells 49

2.1.5 Thawing cells 49

2.2 Histochemical staining techniques 50

2.2.1 Preparation of cell smears 50

2.2.2 Staining for the presence of non-specific esterase and chloroacetate esterase 50

2.2.3 Toluidine blue staining 51

2.2.4 Luxol fast blue staining 51

2.2.5 Nuclear staining: the Diff quick method 52

2.3 Preparation of $CD2^+$-lymphocyte-enriched populations 52

2.4 Panning of lymphocytes 53

2.5 Cell sorting 54

2.6 Mixed leukocyte cultures 54

2.7 MLC inhibition assays 55

2.8 Thymocyte proliferation assay 56

2.9 Proliferation assay for IL-12 and anti-IL-12 activity 56

2.10 Cytotoxicity assay 57

2.11 Immunofluorescence and related techniques 58

2.11.1 Monoclonal antibodies - details 58

2.11.2 Indirect immunofluorescence 59

2.11.3 Direct immunofluorescence 60

2.11.4 Purification of mouse IgG monoclonal antibodies 60

2.11.5 Fluorescein-labelling of monoclonal antibodies 62

2.11.6 Enzyme-linked immunosorbent assay 63

2.12 Molecular biology 64

2.12.1 Small scale plasmid preparation 65

2.12.2 Large scale plasmid preparation 65

2.12.3 RNase protection assay 67

2.12.4 Details of plasmids containing cDNA inserts 70

Chapter 3: The effect of culturing HL-60 cells with all-*trans* retinoic acid

3.1 Introduction 74

3.2 Preliminary studies 75

3.3 The effect of varying the concentration of RA upon the proliferative capacity and viability of HL-60 cells 77

3.4 Morphology of RA-treated HL-60 cells 79

3.5 Staining for the presence of non-specific esterase 81

3.6 Staining for the presence of chloroacetate esterase 81

3.7 Toluidine blue and Luxol fast blue staining of RA-treated HL-60 cells 82

3.8 Analysis of cell surface antigen expression by RA-treated HL-60 cells 82

3.9 Discussion 89

Chapter 4: Characterization of the lymphocyte response to HL-60-R7 and RC-2a cells

4.1 Introduction 92

4.2 Phenotypic definition of $CD2^{+}$-lymphocyte-enriched populations prior to their introduction to the MLC 93

4.3 Analysis of LEP responding to HL-60-R7 and RC-2a cells 95

4.4 Determination of the proportion of lymphocytes expressing CD3 98

4.5 Detection of activated $CD16^{+}$ lymphocytes 99

4.6 Cytolytic activity of LEP derived from MLC 100

4.7 Investigation of the role of $CD4^+$ lymphocytes and IL-2 in the LEP responses to HL-60-R7 and RC-2a cells 101

4.8 The role of MHC class II molecules in the LEP responses to HL-60-R7 and RC-2a cells 103

4.9 The role of other molecules in the LEP responses to HL-60-R7 and RC-2acells 106

4.10 Discussion 107

Chapter 5: The role of cytokines in the lymphoproliferative responses to HL-60-R7 and RC-2a cells

5.1 Introduction 112

5.2 Expression of TNF-α mRNA by HL-60-R7 cells and other cell lines and the role of TNF-α in the MLC 114

5.3 Expression of IL-1α and IL-1β mRNA by HL-60-R7 cells and other cell lines and the role of IL-1 in the MLC 116

5.4 Expression of IL-6 mRNA by HL-60-R7 cells and other cell lines 117

5.5 Expression of stem cell factor mRNA by HL-60-R7 and RC-2a cells and the role of stem cell factor in the MLC 117

5.6 The role of IL-12 in the LEP response to HL-60-R7 and RC-2a cells 118

5.7 Discussion 119

Chapter 6: Analysis of the cell surface antigen expression and the immunostimulatory nature of a variety of cell lines

6.1 Introduction 121

6.2 IIF assays and MLC studies of a variety of cell lines 122

6.3 Discussion 123

Chapter 7: RA-resistant HL-60 cells

7.1 Emergence and characteristics of RA-resistant HL-60 cells 128

7.3 Discussion 131

Chapter 8: General discussion 132

Bibliography 140

Appendix 1: Supplementary results

A1.1 Fluoresceination of 3D3.3

A1.2 Determining the concentration of mAb used in MLC inhibition assays

Appendix 2: Reagents and apparati

A2.1 Reagents for non-specific and chloroacetate esterase staining

A2.2 Preparation of $CD2^+$-lymphocyte-enriched populations

A2.3 Indirect immunofluorescence

A2.4 Purification of mouse monoclonal antibodies

A2.5 Small scale preparation of plasmid DNA

A2.6 RNase protection assay

Appendix 3: Glossary of cytokines and cell surface markers