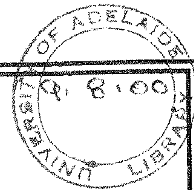

Engineering Antibodies for Use in Leukemia and Lymphoma Therapy

Penelope Jane Adamson B.Biotech. (Hons)

Child Health Research Institute

Department of Paediatrics

Faculty of Medicine

University of Adelaide

South Australia

A thesis submitted for the degree of Doctor of Philosophy

March, 2000

Table of Contents

Table of Contents ii

Table of Figures x

Table of Tables xiii

Summary xv

Declaration xvii

Acknowledgements xviii

Publications Arising From This thesis xix

Abbreviations xx

Chapter 1 - *Introduction* 1

1.1 Summary 2
1.2 B Cell Malignancy 2
1.3 Antibodies 3
1.4 The Antibody Response 5
1.5 Hybridoma Technology and Monoclonal Antibodies 6
1.6 Limitations of Murine Hybridoma Antibodies 7
1.7 Human Anti Mouse Antibody (HAMA) Response 7
1.8 Epstein Barr Virus Transformation 9
1.9 Chimeric Monoclonal Antibodies 10
1.10 CDR Grafting and Humanised Monoclonal Antibodies 11
1.11 Single Domain Antibody Fragments 12

1.12 Fv Antibody Fragments 13

1.13 Single Chain Fv Antibody Fragments 14

1.14 Fab Antibody Fragments 14

1.15 $F(ab)_2$ Antibody Fragments 15

1.16 Multimeric Antibodies and Bispecific Antibody Fragments 16

1.17 Phage Display Technology 17

1.18 Ribosomal Protein Display 20

1.19 Bacterial Protein Display 20

1.20 Human Antibody Repertoires in SCID Mice 21

1.21 Antibody Conjugates 23

Immunotoxins 23

Radioimmunotherapy 24

Drug Conjugates 25

Other Conjugates 25

1.22 Cell Surface Antigens 26

1.23 CD19 26

1.24 CD20 29

1.25 Thesis Plan 32

Chapter 2 - *General Methods* 34

2.1 Summary 35

2.2 Materials and Methods 35

2.2.1 General Media and Solutions 35

2.2.2 Bacterial Culture Media, Solutions and Techniques 36

2.2.3 Tissue Culture Media, Solutions and Techniques 40

2.2.4 Molecular Biology Solutions and Techniques 43

2.2.5 Protein Screening Assays and Solutions 50

2.2.6 List of Suppliers 55

Chapter 3 - ***Construction of a Transfected Cell Line Expressing Soluble Human CD19 Protein and Production of a Synthetic Peptide of Human CD20*** 56

3.1 Summary 57

3.2 Introduction 58

3.3 Materials and Methods 60

3.3.1 CD20 Peptide 60

3.3.2 CD20 ELISA 60

3.3.3 CD19 60

3.3.4 Murine IgG Plasmid 63

3.3.5 Expression Vectors 63

3.3.6 Primers 63

3.3.7 Cell Lines and Maintenance 67

3.3.8 Transfection 67

3.3.9 Slot Blot Assay 68

3.3.10 cDNA Generation for Screening of Clones 68

3.3.11 PCR Screening of DNA and mRNA Extractions 68

3.4 Methods and Results 70

3.4.1 Analysis of the CD20 Peptide 70

3.4.2 Production of Recombinant Soluble CD19 Protein in a Mammalian Expression System (Reaction 1) 70

3.4.3 Production of Recombinant Soluble CD19 Protein in a Mammalian Expression System (Reaction 2) 72

3.4.4 Production of Recombinant Soluble CD19 Protein in a Mammalian Expression System (Reaction 3) 74

3.4.5 Production of Recombinant Soluble CD19 Protein fused to a Murine Fc Domain 76

3.5 Discussion 85

Chapter 4 - *Production of a Transfected Cell Line Expressing Membrane Bound Human CD19* 88

4.1 Summary 89

4.2 Introduction 90

4.3 Materials and Methods 92

4.3.1 Cell Lines and Maintenance 92

4.3.2 Digestion of the CD19 Gene 92

4.3.3 Ligation of CD19 into pIRES1neo 92

4.3.4 Screening Colonies for CD19 93

4.3.5 Flow Cytometry 93

4.3.6 Cloning of the Membrane Bound CD19 Transfectant 93

4.3.7 Comparison of Reactivity of FMC63 as a CD19 Antibody 94

4.4 Results 95

4.4.1 Production of Recombinant Membrane Bound CD19 Protein 95

4.4.2 Screening CD19 Transfected Cells for CD19 Expression Using Flow Cytometry 95

4.4.3 Cloning the Transfected Culture Expressing CD19 98

4.4.4 Comparison of FMC63 to Commercial Anti Human CD19 Antibodies 98

4.5 Discussion 100

Chapter 5 - *Screening Patient Samples for Antibody Against CD19 and CD20* 102

5.1 Summary 103

5.2 Introduction 104

5.3 Materials and Methods 108

5.3.1 Patient and Control Plasma and Serum Samples 108

5.3.2 ELISA 108

5.3.3 Flow Cytometry 108

5.3.4 Statistics 109

5.4 Results 110

5.4.1 ELISA 110

Comparison of Patient Samples to Control Samples 110

Identification of Outliers 110

5.4.2 Flow Cytometry 115

Identification of Outliers 115

5.5 Discussion 120

Chapter 6 - *Construction of a Phage Display Antibody Library* 122

6.1 Summary 123

6.2 Introduction 124

6.3 Materials and Methods 127

6.3.1 pAbClone Vector 127

6.3.2 Generation of cDNA 127

6.3.3 PCR 127

6.3.4 Digestion of Light Chains and pAbClone Vector 130

6.3.5 Ligation of Light Chains into Vector 131

6.3.6 Transformation into XL1-Blue *E. coli* 131

6.3.7 Screening of Colonies for Light Chain Inserts 131

6.3.8 Digestion of Heavy Chains and Light Chain/pAbClone 132

6.3.9 Ligation of Heavy Chains into Vector 132

6.3.10 Transformation into XL1-Blue *E. coli* and Screening 132

6.3.11 The "Einstein" Library 132

6.3.12 Library Preparation 133

6.3.13 Library Panning 133

6.3.14 Phage Titration 134

6.4 Results 135

6.4.1 Construction of a Fab Phage Display Library 135

6.4.2 Library Panning 135

6.5 Discussion 139

Chapter 7 - *Construction and Analysis of a scFv Antibody Against CD20* 141

7.1 Summary 142

7.2 Introduction 144

7.2.1 Antibody Fragments in Medicine 144

7.2.2 scFv Preparation from Hybridomas 146

7.3 Materials 148

7.3.1 pAK Vectors 148

7.3.2 Sequencing Primers 148

7.3.3 Cell Lines and Maintenance 148

7.4 Methods and Results 151

7.4.1 CD20 Parent Hybridoma 151

7.4.2 Construction of a scFv Antibody Against CD20 151

7.4.3 ELISA 159

7.4.4 Cellular Specificity of CD20 scFv 159

7.4.5 Methods for Improving scFv Production 169

7.4.6 Determination of Optimal Glucose Concentration for Production of scFv 171

7.4.7 Scale up of Protein Production Using Fermentation 173

7.4.8 Purification of scFv 173

7.4.9 Determination of Protein Concentration 178

7.4.10 Blocking and Competition Assays Using Flow Cytometry 178

7.4.11 Enzyme Treatment 179

7.4.12 BIAcore Studies 182

7.4.13 scFv Computer Modelling 182

7.4.14 Comparison of the Modelling to Affinity Measurements 186

7.5 Discussion 188

Chapter 8 - ***Construction of a Fab Antibody Against CD20*** 191

8.1 Summary 192

8.2 Introduction 193

8.3 Materials 196

8.3.1 pComb3H Vector 196

8.3.2 Fab Heavy and Light Chain Primers 196

8.4 Methods and Results 199

8.4.1 Construction of the CD20 Fab 199

8.4.2 Production and Screening of Soluble Fab 203

8.4.3 Functional Testing of Fab Using Flow Cytometry 203

8.5 Discussion 209

Chapter 9 - *General Discussion* 210

Appendix 218

Appendix 1 - Patient and Control Plasma and Serum Samples 219

A1.1 Patient Plasma Samples 219

A1.2 Control Plasma Samples 220

A1.3 Patient Serum Samples 221

A1.4 Control Serum Samples 222

Appendix 2 - Statistical Equations 223

A2.1 Quartiles 223

A2.2 The Interquartile Range 223

A2.3 Determining Outliers Using the Upper Limit 223

References 224

Summary

Cancer is one of the major causes of death in the Western world. The treatments currently in place fall short of ideal and this has necessitated the continued search for new, more effective remedies. One of the most promising of these new therapies has been the development of monoclonal antibodies to target treatment to a specific cell type or disease. Monoclonal antibodies were developed in 1975 by Kohler and Milstein and have been used in applications which have allowed detailed studies of the immune system and other systems in the human body. The use of these antibodies in humans has raised some interesting problems which need to be addressed before further treatment. The biggest concern has been the production of human anti mouse antibody (HAMA) responses to therapeutic antibodies of murine origin. This has led to many developments in the antibody engineering field, including the production of chimeric antibodies, humanisation of antibodies by CDR grafting or antibody resurfacing, and the production of antibody fragments by removing the regions not necessary for antibody binding. This thesis describes some of the methods used for the production of therapeutic antibodies, including phage display technology and the production of both anti CD20 scFv and Fab fragments from an anti CD20 hybridoma.

A phage display antibody library was derived from tonsil lymphocytes and a library of small size was produced. This library was not used for antigen panning. The two libraries, "Einstein" Kappa and Lambda, used for screening against CD20 peptide did not yield antibodies to CD20.

The aim was to then produce a library from the bone marrow of a patient in remission with a leukemia or lymphoma. To test the hypothesis that patients with a cancer will produce autoantibodies to the antigens on the surface of the cancerous cells, serum and plasma from cancer patients and patients with auto immune disease were tested for the presence of anti-CD19 or anti-CD20 antibodies. This work showed that autoantibodies to these antigens are not exclusively produced by patients with disease and not all patients produce high titres of these antibodies. These results suggest that if library construction is to be biased towards a particular antigen then screening of individuals serum or plasma, not just those with cancer, should be carried out.

Libraries of antibodies are most commonly screened against pure antigen. The CD20 antigen was produced by synthesising the 45aa loop which protrudes from the cell surface. An ELISA was developed which showed that many CD20 antibodies bind to the synthetic peptide.

Attempts were made to produce the extracellular region of the CD19 antigen in a mammalian expression system. Using a number of methods, soluble recombinant CD19 antigen could not be produced effectively. As an alternative the full length CD19 sequence was cloned into pIRES1neo and expressed in Chinese Hamster Ovary (CHO-K1) cells as a membrane bound protein. A variety of commercial antibodies bound to the CD19 transfected cell line but not to untransfected CHO-K1 cells in flow cytometry.

The anti-CD20 hybridoma, HB13d, was used to produce a single chain Fv fragment. The scFv was shown to bind strongly to CD20 in flow cytometry, ELISA and plasmon resonance. Preliminary experiments described a Fab fragment from the CD20 hybridoma.

The CD20 antigen has been shown to be a suitable target for the therapy of B lymphocyte disease with at least one antibody, Rituximab (an anti-CD20 chimeric antibody), approved for use in the treatment of Non Hodgkin's lymphoma. This shows potential for the anti-CD20 scFv in the treatment of B cell disease. The studies presented in this thesis could provide the basis for second-generation therapeutic agents.