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Antigen identification in paraneoplastic and post-infectious neurological disorders.

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By

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Abbreviations:

2D	Two dimensional
ABN	Association of British neurologists
ADEM	Acute disseminated encephalomyelitis
AMF	Autocrine motility factor
ASF	Ammonium sulphate fractionation
ASOT	Anti-streptolysin O titre
AU	Absorbance units
BNSU	British neurological surveillance unit
BSA	Bovine serum albumin
BD	Brainstem dysfunction
CAR	Cancer associated retinopathy
CD	Cerebellar degeneration
Cdr	Cerebellar degeneration related
CNS	Central nervous system
CSF	Cerebrospinal fluid
DIC	Disseminated intravascular coagulation
DRG	Dorsal root ganglia
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EL	Encephalitis lethargica
ELAV	Embroyonic lethal abnormal vision
ELISA	Enzyme linked immunsorbent assay
FPLC	Fluid phase liquid chromatography

GABHS	Group A β-haemolytic streptococcus
GBS	Guillain-Barré syndrome
GPI	Glucose-6-phosphate isomerase
HD	Hodgkin's disease
HRP	Horseradish peroxidase
IEF	Isoelectric focussing
IEX	Ion exchange
IMAC	Immobilised metal chelate affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVIg	Intavenous immunoglobulin
LDS	Lithium dodecyl sulphate
LE	Limbic encephalitis
LEMS	Lambert-Eaton myasthenic syndrome
MCS	Multiple cloning site
MF	Maturation factor
MFS	Miller-Fischer syndrome
MG	Myasthenia gravis
NB	Neuroblastoma
NHS	N-hydroxysucinnimide
NLK	Neuroleukin
NSCLC	Non-small cell lung cancer
OCD	Obsessive compulsive disorder
OD	Optical density
OM	Opsoclonus-myoclonus

PAGE	Polyacrylamide gel electrophoresis
PANDAS	Paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection.
PBS	pBluescript
PCD	Paraneoplastic cerebellar degeneration
PCR	Polymerase chain reaction
PEM	Paraneoplastic encephalomyelitis
PIOM	Post-infectious opsoclonus-myoclonus
PLE	Paraneoplastic limbic encephalitis
PND	Paraneoplastic neurological disorder
PNS	Peripheral nervous system
POM	Paraneoplastic opsoclonus-myoclonus
PYK	Pyruvate kinase
RF	Rheumatic fever
rNLK	Recombinant neuroleukin
RT-PCR	Reverse transcriptase polymerase chain reaction
SC	Sydenham's chorea
SCLC	Small cell lung cancer
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEREX	Serological identification of antigens by recombinant expression cloning.
SLE	Systemic lupus erythematosis
SSN	Subacute sensory neuronopathy

TBS	Tris-buffered saline
TFNR	Transcription factor-like nuclear regulator
TMB	3,3′,5,5′-Tetramethylbenzidine
TS	Tourette's syndrome
UCL	University College London
VGCC	Voltage gated calcium channel

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Abstract.

Anti-neuronal antibodies have been conclusively shown to be pathogenic in a handful of neurological disorders but their value in diagnosis is undeniable. Nevertheless, there are instances where diseases thought have an immunological component do not have clear antibody responses associated with them.

This thesis aimed to identify anti-neuronal antibodies and characterise their antigens in paraneoplastic and post-infectious neurological disorders. With regard to the latter, interest in the occurrence of anti-neuronal responses in post-streptococcal neurological disorders has culminated in the identification of four candidate auto-antigens. Recombinant forms of these proteins were produced and the frequency of an antibody response in patients determined. In addition, the antigen recognised by antibodies in the serum of patients with post-infectious opsoclonus-myoclonus was characterised using protein purification techniques and the frequency of an antibody response determined. Finally, a bacteriophage expression library was employed to study a novel antibody in a patient with paraneoplastic disease.

Our findings were unable to provide support for an antibody response against the candidate auto-antigens in post-streptococcal disease. However we were able to characterise two target antigens, one in post-streptococcal opsoclonus-myoclonus and one in paraneoplastic neurological disease. Both antigens are thought to have specific roles in the nervous system and have provided interesting opportunities for further research into there roles in neuronal, and in the case of paraneoplastic disease, tumour biology. Further investigation is required to determine the importance of the antibody response in both post-infectious and paraneoplastic disease.

Chapter 1: Introduction. **23**

1.1 Paraneoplastic Syndromes. 23

1.1.1 Pathological Mechanisms. 24

1.1.2 Classification of Paraneoplastic Disease by System. 24

1.2 Paraneoplastic Neurological Disorders. 26

1.2.1 Paraneoplastic Encephalomyelitis/Sub-acute Sensory Neuronopathy. 28

1.2.2 Paraneoplastic Cerebellar Degeneration. 30

1.2.3 Paraneoplastic Limbic encephalitis. 33

1.2.4 Paraneoplastic Movement Disorders. 34

1.2.5 Lambert Eaton Myesthenic Syndrome. 37

1.3 Antibodies and the Immune Response to Malignant Disease. 38

1.4 Anti-Neuronal Antibodies. 39

1.4.1 Anti-Hu Antibodies. 41

1.4.2 Anti-Yo Antibodies. 43

1.4.3 Anti-Ma Antibodies. 44

1.4.4 Anti-Ri Antibodies. 46

1.4.5 Anti-CV2/ CRMP-5 Antibodies. 46

1.4.6 What Are We Missing? 48

1.5 Pathogenesis of Paraneoplastic Neurological Disease. 50

1.6 Anti-tumour immunity in PND 53

1.7 Post-Infectious Neurological Diseases. 54

1.7.1 Paraneoplastic and Post-infectious Disease. 54

1.7.2 Breakdown of Tolerance and Molecular Mimicry. 55

1.7.3 Evidence for MolecularMimicry as a Cause of NeurologicalDisease. 56

1.8 Post-Streptococcal Diseases of the CNS. 57
1.8.1 Sydenham's Chorea. 59
1.8.2 Paediatric Autoimmune Neurophsychiatric Disorders Associated With Streptococcal infection (PANDAS). 60
1.8.3 Acute Disseminated Encephalomyelitis (ADEM) 61
1.8.4 Tourette's Syndrome 62
1.8.5 Encephalitis Lethargica. 63
1.9 Anti-Neuronal Antibodies in Post-Streptococcal Diseases of the CNS . 64
1.10 Putative Antigens in Post-streptococcal Diseases of the CNS 66
1.11 Post-Infectious Opsoclonus-myoclonus. 69
1.12 Summary and Aims 71

Chapter 2: Production of putative antigens recognised by antibodies of patients with post-infectious neurological disease. 73

2.1 Introduction. 73
2.1.1 Cloning cDNA. 74
2.1.2 Primer Design. 75
2.1.3 Bacterial Expression Vectors. 75
2.1.4 pRSET. 76
2.1.5 Purification of Recombinant Proteins. 77
2.2 Methods. 79
2.2.1 Preparation of Lymphocytes for mRNA Isolation. 79
2.2.2 Preparation of Brain Tissue for mRNA Extraction. 80
2.2.3 Total mRNA Isolation. 80
2.2.4 RT-PCR Amplification of α-Enolase and Aldolase C. 81

2.2.5 The Pyruvate Kinase and γ-Enolase I.M.A.G.E Clones. 83

2.2.6 PCR Amplification of Pyruvate Kkinase and γ-Enolase. 83

2.2.7 Preparation for Expression of Recombinant Proteins..... 84

2.2.8 Expression of recombinant antigens..... 85

2.2.9 Purification of Recombinant Antigens. 85

2.2.10 Patients and Controls. 86

2.2.11 Antibody Detection Using Recombinant Protein Immunoblots. 87

2.3 Results. 87

2.3.1 Cloning and Expression of Human α-Enolase. 87

2.3.2 Cloning and Expression of Human Aldolase C. 90

2.3.3 Cloning and Expression of Pyruvate Kinase..... 93

2.3.4 Cloning and Expression of Recombinant γ-Enolase from I.M.A.G.E Clone 3629603 94

2.3.5 Purification of recombinant proteins. 95

2.3.6 Reactivity of Patients Serum with Recombinant Putative Antigens. .. 96

2.4 Discussion. 98

2.4.1 Cloning of Human Aldolase C, α-Enolase and PYK. 99

2.4.2 Cloning and Expression of Recombinant γ-Enolase. 100

2.4.3 The Purification of Recombinant Proteins..... 101

2.4.4 Recognition of Recombinant Protein by Patients' Sera. 102

Chapter 3: Characterisation of a 56 kDa antigen recognised by antibodies in the serum of patients with opsoclonus-myoclonus. 106

3.1 Introduction. 106

3.1.1 Ammonium Sulphate Fractionation. 107

3.1.2 Ion Exchange Chromatography 107
3.2 Methods 108
3.2.1 Patients with Opsoclonus-myoclonus 109
3.2.2 Control Patients 109
3.2.3 Sample Preparation 111
3.2.4 Ammonium Sulphate Fractionation 111
3.2.5 2D Gel Electrophoresis 111
3.2.6 Ion Exchange Chromatography 113
3.2.7 Mass Spectrometry 114
3.2.8 Production of Neuroleukin Specific Antibodies 115
3.2.9 NLK Detection Using Cultured Neurones 115
3.2.10 Production of Recombinant Neuroleukin 117
3.2.11 Expression and Purification of Recombinant NLK 118
3.2.12 Detection of Anti-NLK Antibodies 118
3.2.13 Determination of Sequence Homology 118
3.2.14 Anti-His. Aantigen and Detection Antibody Concentration for rNLK ELISA 118
3.2.15 Determining Factors Responsible for Background Signal 121
3.2.16 Comparison of Blocking Agents 121
3.2.17 Comparison of Crude Bacterial Homogenates and His-Purified rNLK122
3.2.18 Use of Antigen Coated Wells 122
3.3 Results 123
3.3.1 Case History One 123
3.3.2 Case History Two 124
3.3.3 Anti-neuronal Antibody Detection 125

3.3.4 Identification of the 56 kDa Auto-antigen. 126
3.3.5 Comparison of Human NLK and Streptococcal Glucose-6-phosphate Isomerase (GPI). 128
3.3.6 RT-PCR Amplification of Human Neuroleukin. 128
3.3.7 Analysis of Ligated pRSETB 129
3.3.8 Sequencing of Successfully Ligated pRSETB 130
3.3.9 Production and Purification of Recombinant Neuroleukin. 130
3.3.10 Probing Purified Neuroleukin with Patients serum 130
3.3.11 Probing rNLK with Sera from a Cohort of OM Patients. 131
3.3.12 Immunocytochemistry 133
3.3.13 The His Capture ELISA. 134
3.3.14 Determination of Detector Antibody Concentration. 136
3.3.15 Determining Factors Responsible for Background Signal 138
3.3.16 Comparison of Blocking Agents. 139
3.3.17 Comparison of Crude Bacterial Homogenates and IMAC Purified rNLK 140
3.3.18 Use of Antigen Coated Wells. 141
3.3.19 Anti-NLK Antibodies in the CSF of Adults with Paraneoplastic Opsoclonus-myoclonus. 144
3.4 Discussion. 145
3.4.1 The Index cases. 145
3.4.2 Protein Purification and Identification. 145
3.4.3 Cohort Findings: Immunoblotting. 146
3.4.4 Cohort Findings: rNLK ELISA. 147
3.4.5 NLK. 148

3.4.6 NLK: A Reasonable Antigenic Target? 150

3.4.7 NLK: A Unifying Antigenic Target? 153

3.4.8 Areas for Future Research. 155

Chapter 4: Identification of a patient with a paraneoplastic syndrome and novel anti-neuronal antibodies 159

4.1 Introduction. 159

4.2 Methods 159

4.2.1 Paraneoplastic patients 159

4.2.2 Sample Preparation, Ammonium Sulphate Fractionation and 2D Electrophoresis. 161

4.2.3 Ion Exchange Chromatography 161

4.3 Results. 162

4.3.1 Identified Patients, Underlying Tumours and Diagnosis of PND. 162

4.3.2 Anti-neuronal Antibody Detection. 163

4.3.3 Details of Patient with Miscellaneous Anti-97 kDa Antibodies 164

4.3.4 Ammonium Sulphate Fractionation. 166

4.3.5 2D Gel Electrophoresis. 168

4.3.6 IEX Chromatography 169

4.4 Discussion. 171

4.4.1 Anti-Neuronal Antibody Results. 171

4.4.2 The Index Case 172

4.4.3 Antigen Purification by Ammonium Sulphate Fractionation, 2D Gel Electrophoresis and IEX Chromatography. 174

Chapter 5: Characterisation of the 97 kDa neuronal antigen by immunoaffinity chromatography. 177

5.1 Introduction. 177

5.1.1 Immunoaffinity Chromatography. 177

5.1.2 Pre-Activated N-hydroxysucinnimide Columns Matrices 179

5.2 Methods 180

5.2.1 Purification of IgG from Serum. 180

5.2.2 Coupling Purified IgG to the NHS Activated Column. 181

5.2.3 Isolation of Antigen from Cerebellar Homogenate. 182

5.2.4 Analysis of Eluted Proteins. 182

5.3 Results. 183

5.3.1 Isolation of IgG from Patients Serum. 183

5.3.2 Antigen Isolation from Cerebellar Homogenate. 184

5.3.3 Mass Spectrometry. 186

5.4 Discussion. 187

5.4.1 Antigen Purification by Immunoaffinity Chromatography. 187

5.4.2 Investigation of Potential Auto-antigens. 189

Chapter 6: Characterisation of the 97 kDa antigen by means of screening a bacteriophage expression library. 191

6.1 Introduction. 191

6.1.1 Bacteriophage Lambda. 191

6.1.2 Early libraries. 192

6.1.3 Coliphage. 193

6.1.4 λZAP. 194

6.1.5 pBluescript. 195

6.1.6 Construction of A λZAP Library. 196

6.1.7 Screening a Bacteriophage Library. 196

6.2 Methods 198

6.2.1 Cancer Controls. 198

6.2.2 Multiple Sclerosis Controls. 198

6.2.3 cDNA Library. 198

6.2.4 Preparation of XL1-blue MRF' 198

6.2.5 Titrating and Testing for cDNA Insertion. 199

6.2.6 Infection and Plating of XL1 blue for screening. 199

6.2.7 Probing Nitrocellulose Membranes. 200

6.2.8 Subsequent Screens. 201

6.2.9 Testing Selected Phage Using Control Serum. 201

6.2.10 pBluescript Excision. 203

6.2.11 Restriction Analysis and Sequencing of pBluescript. 203

6.2.12 Expression and Detection of Protein From pBluescript 20A. 204

6.2.13 Removal of Anti-E.coli Antibodies from Patients Serum. 205

6.2.14 Subcloning of 20A Into pRSETB. 205

6.2.15 Subcloning of 20A Into pRSETC. 206

6.2.16 Sequencing and Protein Prediction. 206

6.2.17 Detection of Expression of His-Tagged Proteins From pRSETB20A. 207

6.2.18 Comparison of Reactivity of Serum with Crude Bacterial Homogenates of pRSETB20A and pRSETC20A. 207

6.2.19 Avoiding Proteolysis of Recombinant Protein pRSETC20A: 208

6.3 Results. 210

6.3.1 Antigen Identification Using Patient's Serum to Screen a Bacteriophage Expression Library. 210

6.3.2 Control Reactivity with Selected Phage. 210

6.3.3 Case Details of Anti-Yo Positive Patient Reactive Against Phage 20A 215

6.3.4 Sequencing pBluescript 20A, B, C. 216

6.3.5 Reactivity of Patients Serum with Proteins Produced from pBS20A 217

6.3.6 Subcloning of cDNA 20A Into the Bacterial Expression Vectors pRSETB: 218

6.3.7 Subcloning of cDNA 20A Into the Bacterial Expression Vectors pRSETC 220

6.3.8 Reactivity of Patients Serum with Homogenates of pRSETB20A and pRSETC20A: 221

6.3.9 Timed Expression of Recombinant Protein From pRSETC20A Confirms Degradation. 223

6.3.10 Reducing proteolytic degradation of recombinant protein 20A..... 224

6.4 Discussion. 225

6.4.1 Screening the Library: Technical Considerations. 225

6.4.2 Screening with Patients Serum. 227

6.4.3 The Reactive Patients. 228

6.4.4 Expression of Protein 20A. 229

6.4.5 The Protein. 231

6.4.6 TFNR as a Candidate Antigen in Paraneoplastic Neurological Disease. 232

6.4.7 Areas for Further Investigation. 233

Chapter 7: Summary of findings, areas for further investigation and conclusions..... 235

7.1 *Antibodies Against Putative Targets in Post-infectious Neurological Disease.* 235

7.2 *Anti-neuroleukin Antibodies and Opsoclonus-myoclonus.* 235

7.3 *Antigen Identification in Paraneoplastic Disease.* 236

7.4 *Identification of a Target Antigen by Screening a Bacteriophage Display Library.* 237

7.5 *Conclusion.* 239

Chapter 8: Methods..... 241

8.1 Protein Techniques. 241

8.1.1 Polyacrylamide Gel Electrophoresis and Immunoblotting. 241

8.1.2 Colloidal Blue Staining. 242

8.1.3 Silver Staining. 242

8.1.4 Immunoblotting. 242

8.1.5 Enhanced Chemiluminescence. 243

8.1.6 Chromogenic Development. 244

8.1.7 Buffer Exchange Method. 244

8.1.8 Total Protein Method. 244

8.2 Molecular Biology Techniques. 245

8.2.1 Restriction Enzyme Digestions. 245

8.2.2 Agarose Gel Electrophoresis. 245

8.2.3 Gel Extraction. 245

8.2.4 Digestion and Ligation of RT-PCR and PCR Products. 246

8.2.5 Plasmid Extraction 247

8.2.6 DNA Sequencing 247

8.2.7 Production of Chemically Competent TOP10F' 248

8.2.8 Transformation of Chemically Competent TOP10F' 249

8.2.9 Transformation of BL21 (DE3) pLysS 249

8.2.10 Induction of Recombinant Protein Production 250

8.2.11 Preparation of Crude Bacterial Homogenates 250

8.2.12 IMAC Purification of His-tagged Recombinant Protein 250

8.2.13 Confirmation of Recombinant Protein Expression 251

References: **252**

Appendix 1: Materials, reagents and suppliers **276**

Appendix 2: Publications **282**

Table of tables:

Table 1-1: Paraneoplastic Diseases in Human Classified by System..... 25

Table 1-2: Paraneoplastic neurological diseases, associated symptoms and tumours. .. 26

Table 1-3: Well-described anti-neuronal antibodies found in assoication with PND. ... 41

Table 1-4: Clinical findings, streptococcal association and anti-neuronal antibodies in proposed post-streptococcal neuropsychiatric diseases..... 58

Table 1-5: Comparison of two cohorts of post-infectious OM..... 70

Table 2-1: mRNA/cDNA source, primers and predicted length of cDNA encoding the target antigens following RT-PCR or PCR..... 82

Table 2-2: Reaction conditions for the RT-PCR amplification of α-Enolase and aldolase C..... 83

Table 2-3: PCR reaction conditions for the amplification of PYK, γ-Enolase and truncated γ-Enolase..... 84

Table 2-4:Patient and control serum samples used for antibody detection..... 86

Table 3-1: Clinical details of control serum and CSF (n = 54)..... 110

Table 3-2: Paediatric OM patient details and investigation results for anti-NLK antibodies..... 132

Table 3-3: Criteria satisfied by NLK and anti-NLK antibodies as pathogenic factors in OM..... 150

Table 3-4: Criteria satisfied for anti-NLK antibodies in molecular mimicry..... 151

Table 4-1: Tumour associations in patients with positive serum anti-neuronal antibodies. 163

Table 6-1: Control serum tested against phage 20A..... 213

Table of figures:

Figure 1-1: Production of cross-reactive anti-neuronal antibodies in post-infectious and paraneoplastic disease. 55

Figure 2-1: The pRSET bacterial expression vector. 77

Figure 2-2: RT-PCR amplification of human α-Enolase 88

Figure 2-3: Selection of ligated pRSETαAENO. 89

Figure 2-4: Detecion of recombinant α-Enolase in bacterial homogenate. 89

Figure 2-5: RT-PCR amplification of human aldolase C. 91

Figure 2-6: Restriction enzyme analysis of transfected colonies using HindIII. 91

Figure 2-7: Detection of aldolase C in crude bacterial homogenates. 92

Figure 2-8: Purifcation of recombinant antigens. 95

Figure 2-9: Recombinant detection using patients serum. 96

Figure 2-10: Reactivity of patient and control serum with recombinant antigens. 98

Figure 3-1: Antibodies against a 56 kDa protein in serum and CSF of OM patients... 125

Figure 3-2: 2D gel electrophoresis of neuronal proteins following ammonium sulphate fractionation (40-60% saturation). 126

Figure 3-3: Two rounds of ion exchange chromatography were used to purify the 56 kDa antigen. 127

Figure 3-4: Selection of pRSETBNLK. 129

Figure 3-5: Immnuoblot using patients serum and CSF. 133

Figure 3-6: Immunocytochemistry. 134

Figure 3-7: Investigating the use of a capture antibody (anti-His) ELISA for the detection of anti-NLK antibodies. 135

Figure 3-8: Determination of optimal detector antibody dilution. 136

Figure 3-9: Initial NLK ELISA using patients serum. 137

Figure 3-10: Determination of factors contributing to background signal in rNLK ELISA. 139

Figure 3-11: Comparison of BSA and rabbit serum as blocking agents. 140

Figure 3-12: Comparison of crude homogenates of *E. coli* pRSETBNLK and purified rNLK in the NLK ELISA..... 141

Figure 3-13: Final rNLK ELISA. 143

Figure 3-14: Detection of anti-NLK antibodies in the CSF of patients with OM..... 144

Figure 4-1: [18F]fluoro-2-deoxyglucose-PET scan. 165

Figure 4-2: Evidence for an anti-neuronal response against a 97 kDa neuronal protein. 166

Figure 4-3: Ammonium sulphate fractionation of pig cerebellum (wide fractions). ... 167

Figure 4-4: Ammonium sulphate fractionation of pig cerebellum (narrow fractions). 168

Figure 4-5: 2D gel electrophoresis of cerebellar proteins precipitated at 20 % ammonium sulphate saturation..... 170

Figure 4-6: IEX purification of the 97 kDa antigen. 171

Figure 5-1: The principle of affinity chromatography..... 178

Figure 5-2: Protein binding by NHS activated chromatography column. 180

Figure 5-3: Purification of IgG from patients serum..... 183

Figure 5-4: Immunoaffinty purification of the 97 kDa antigen. 185

Figure 5-5: Peptide matches with Amino acid sequence of F3/F11/Contactin precursor. 186

Figure 5-6: Detection of contactin in cerebellar homogenate..... 187

Figure 6-1: Screening a bacteriophage expression library..... 197

Figure 6-2: Screening for and selection of positive plaques..... 211

Figure 6-3: Testing control serum against phage 20A (stage one)..... 212

Figure 6-4:Testing control serum against phage 20A (stage two) 214

Figure 6-5: DNA and amino acid sequence of the TFNR cDNA inserted into pBS20A. 217

Figure 6-6:DNA and amino acid sequence following subcloning of TFNR from pBS20A into the pRSETB bacterial expression vector. 218

Figure 6-7: Reactivity of patients serum and anti-His antibodies against post-IMAC proteins from pRSETB20A and pRSETB. 220

Figure 6-8: DNA and amino acid sequence following subcloning of TFNR cDNA frompBS20A into the pRSETC bacterial expression vector 221

Figure 6-9: Comparison of reactivity of patients serum with pRSETB20A and pRSETC20A 223

Figure 6-10: Timed protein expression from pRSETC20A 224

1 Chapter 1: Introduction.

1.1 Paraneoplastic Syndromes.

The paraneoplastic syndromes are a group of pathological conditions that are associated with the presence of an underlying malignancy but that are not a result of either local infiltration or metastatic spread of the cancer. Paraneoplastic syndromes are estimated to occur in 5% of cancer patients, but their incidence varies depending upon the tumour type, stage and diagnostic criteria (Smith and de Boer, 2002).

Paraneoplastic syndromes are of importance because they provide an insight into disease activity and prognosis and their treatment may improve the quality of life of the patient. In addition they are of diagnostic importance because they are commonly the presenting feature of an underlying malignancy. Importantly, research into the pathological mechanisms of paraneoplastic syndromes may improve our understanding of tumour biology and point towards rational strategies for therapeutic intervention.

To be classified as a paraneoplastic syndrome four criteria have been proposed (Smith and de Boer, 2002):

1: There must be a direct association between the syndrome and the presence of a tumour.

2: An agent i.e. a hormone or antibody should be detectable in the tumour, circulation and affected organ.

3: Treatment of the tumour should result in a reduction of the levels of the agent.

4: The tumour cells should produce the agent *in vivo*.

The final criterion may not be applicable to all paraneoplastic syndromes. For example, when the agent is an antibody produced as a result of the immune response to malignant tissue the agent itself is not produced by the tumour.

1.1.1 Pathological Mechanisms.

In many instances the causes of paraneoplastic syndromes are not fully understood and vary according to the type of tumour. However two broad pathological mechanisms have been proposed.

The first mechanism is the inappropriate secretion of hormones or other biologically active molecules from the normal source ('eutopic' secretion) or the tumour tissue ('ectopic' secretion). Paraneoplastic syndromes resulting from inappropriate secretion include Cushing's syndrome and hypercalcaemia of malignancy.

The second pathological mechanism is proposed to be an auto-immune mechanism in which effector cells or antibodies directed against malignant cells cross-react with healthy tissue. This mechanism is thought to account for some paraneoplastic diseases affecting the nervous, haematological, musculoskeletal and renal systems.

1.1.2 Classification of Paraneoplastic Disease by System.

Paraneoplastic syndromes can be classified according to the target tissue. Systemic effects of malignant disease include fever, fatigue and cachexia and have been reported to occur in as many as 80 % of patients with cancer (Albrecht and Canada, 1996; Vogelzang *et al*, 1997). A list of target tissues and paraneoplastic syndromes is given in Table 1-1. A more detailed table of the well-defined paraneoplastic disorders of the

nervous system is provided in Table 1-2.

Table 1-1: Paraneoplastic Diseases in Human Classified by System.

System	*Target Tissue*	*Paraneoplastic Syndrome*
Systemic	Systemic	Fever
		Cachexia
		Fatigue
	Connective tissue	SLE
		Sjögren's syndrome
		Scleroderma
	Articular	Arthritis
Haematological	Red cells	Aneamia of chronic disease
		Haemolytic anaemia
		Red cell aplasia
	White cells	Neutrophilia[1]
		Neutropenia[2]
		Eosinophilia
	Platelets	Thrombocytosis[1]
		Thrombocytopeni[2]
		Thromboembolic disease
Renal	Glomerular disorders	Membranous nephropathy
	Tubular disorders	Tubular nephropathy
Skin	Melanocytes	Acanthosis nigricans
		Sweet's syndrome
	Dermis	Bullous pemphigoid
		Dermatitis herpetiformis
		Dermatomyositis[3]

1: Neutrophilia/ thrombocytosis may result from growth factor secretion from malignant tissue
2: Neutropenia/ thrombocytopenia may occur following activation of an autoimmune mechanism.
3: Dermatomyocytosis also affects muscle tissue.

Table 1-2: Paraneoplastic neurological diseases, associated symptoms and tumours.

PND	*Symptoms*	*Mean Age of Onset*	*Male: Female*	*Tumour Association*
PEM SSN	SSN, LE, PCD, brainstem dusfunction.	60	3:1	SCLC
PCD				
Anti-Yo	Nystagmus ocular disturbance,	54-61		Ovarian, breast, uterus
HD	Ayaxia, Dysarthria	44	6:1	HD
LE	Subacute amnesic syndrome, affective disorders, seizures, hallucinations, paranoid delusions.	55	1.2:1	Testicular tumours
OM				
Adult	Chaotic eye movements, myoclonus,	66	6:1	Breast and SCLC (Adult)
Pediatric	ataxia.	6-36 months		Neuroblastoma (Pediatric)
LEMS	Limb weakness, ptosis, autonomic disturbance.	62	4.3:1	SCLC

PEM: paraneoplastic encephalomyelitis, SSN: subacute sensory neuronopathy, LEMS: Lambert Eaton myasthenic syndrome, OM: opsoclonus/ myoclonus, LE: limbic encephalitis, PCD: paraneoplastic cerebeller degeneration, HD: Hodgkin's disease, SCLC: Small cell lung cancer.

1.2 Paraneoplastic Neurological Disorders.

Paraneoplastic neurological disorders (PND) encompass a wide range of disorders affecting any part of the central or peripheral nervous system. The widely referenced work of Henson and Urich (1982) describes a number of clinico-pathological entities associated with tumours. These include encephalomyelitis, cortical cerebellar degeneration, peripheral neuropathy, muscular and neuromuscular disorders, and a miscellaneous group including opsoclonus-myoclonus and optic neuritis. Today a greater number of neurological diseases are thought to be paraneoplastic.

The frequency with which PND occurs is largely dependent on the methods employed in the detection of a neurological deficit and the definition of the disease. Elrington *et al* (1991) conducted a systematic examination of 150 patients with small cell lung cancer (SCLC) and found evidence of autonomic dysfunction in 44 % of patients while Lipton *et al* found deficits in peripheral nerve function in 43% of patients using quantitative thermal threshold testing (Lipton *et al*, 1987). Proximal weakness with one or more diminished deep tendon reflexes has been reported in association with lung cancer in 16 % of patients (Croft and Wilkinson, 1965).

In many cases muscle weakness and peripheral neuropathy could be secondary to the weight loss and nutritional disturbances frequently experienced by cancer patients (Hawley *et al*, 1980). As a result it is unclear what percentage of these disorders are truly paraneoplastic.

With the exceptions of Lambert-Eaton myasthenic syndrome (LEMS), which affects 3 % of patients with SCLC (Elrington *et al*, 1991) and myasthenia gravis (MG), which affects 15 % of patients with thymoma (Levy *et al*, 1998), distinct PND rather than mild generalised peripheral and autonomic neuropathy are rare. Reports on the estimated incidence vary. For example, paraneoplastic cerebellar degeneration (PCD) has a reported incidence ranging from 0.6 % (Croft & Wilkinson, 1965) to 26 % of patients (Wessel & Smith, 1988). These figures are derived from studies involving patients with clearly defined tumours. More recent reports suggest that the occurrence of clinically significant PND in patients with any cancer is approximately 0.01 % (Darnell and Posner, 2003).

In his book, Posner (1995) described some common characteristics shared by PND:

1: In many cases the onset of the neurological disorder is sub-acute.

2: The neurological disease precedes the identification of the underlying tumour.

3: When a tumour is discovered it is frequently small, non-metastatic and indolently growing.

The potential reasons for these findings will be discussed. What follows is an introduction to specific PNDs, the common underlying tumours and, where appropriate the association with anti-neuronal antibodies.

1.2.1 Paraneoplastic Encephalomyelitis/Sub-acute Sensory Neuronopathy.

Henson originally described 'encephalomyelitis with carcinoma' in patients with clinical signs of dysfunction of the nervous system who at autopsy had inflammatory involvement of the brain, brainstem, spinal cord, and dorsal root ganglia (DRG) (Henson *et al*, 1965). Today the term 'encephalomyelitis with carcinoma' has been replaced by paraneoplastic encephalomyelitis/ sub-acute sensory neuronopathy (PEM/SSN). This definition is used to encompass a number of discrete clinical entities such as paraneoplastic cerebellar degeneration, limbic encephalitis, brainstem dysfunction and autonomic neuropathy.

SCLC is the commonest tumour associated with PEM occurring in 74-83 % of patients (Dalmau *et al*, 1992; Graus *et al*, 2001). The disease is rare affecting less than 1 % of patients with SCLC (Elrington *et al*, 1991; Henson & Urich H 1982). Its incidence

peaks around 60 years of age reflecting the epidemiology of the underlying malignancy. In most cases the onset of neurological disease will prompt the search for an underlying cancer.

The commonest presenting feature of PEM/SSN is SSN, which is also the predominant neurological symptom during the course of the disease (Dalmau *et al*, 1992; Chalk *et al*, 1992). Graus described sensory neuropathy as the major neurological feature in 200 patients with PEM (Graus *et al*, 2001). Early symptoms include a patchy numbness often involving the face, trunk or proximal limbs, which progresses to involve all limbs, dysesthesia and severe aching or lancinating pain.

Despite the predominance of a single syndrome, the clinical picture commonly evolves as inflammation spreads to involve different areas of the nervous system. In the series of 71 patients studied by Dalmau *et al* only 27 % had uni-focal involvement of the nervous system throughout the course of the disease (20% sensory syndrome, 1% brainstem dysfunction, 6% limbic encephalitis) (Dalmau *et al*, 1992).

Microscopic examination of the nervous system of patients with PEM demonstrates neuronal degeneration and gliosis in multiple areas of the nervous system. The neuronal degeneration is frequently accompanied by a perivascular and parenchymal inflammatory infiltrate composed mainly of mononuclear cells (Scaravilli *et al*, 1999). Immunohistochemical analysis has shown that the parenchymal infiltrates are composed largely of CD8 positive T cells (Jean *et al*, 1994; Graus *et al*, 1990). Aggregation of B cells has not been positively demonstrated, but direct immuno-fluorescence to detect immunoglobulin has found evidence of IgG deposition in areas of inflammation. The

inflammatory infiltrate in the perivascular space is composed of B cells, macrophages and $CD4^+$ but not $CD8^+$ T cells.

The inflammatory infiltrates found within the DRG are different. They are composed of both $CD4^+$ and $CD8^+$ T cells, with B cells found in the stroma and surrounding the DRG neurones. Proliferation of satellite cells results in the formation of Nageotte nodules, which later predominate as the inflammation subsides (Wanschitz *et al*, 1997). IgG deposition is also found in the neurones of the DRG particularly at the peripheries close to satellite cells, but also showing staining of the nucleus and cytoplasm (Graus *et al*, 1990).

The prognosis of patients with PEM is poor, the median survival time being 11.8 months (Graus *et al*, 2001). Studies of tumours from affected patients have highlighted the limited spread of the underlying malignant disease implying that the death is most commonly a result of neurological complications such as respiratory failure or autonomic dysfunction leading to cardiac arrest.

1.2.2 Paraneoplastic Cerebellar Degeneration.

The occurrence of PCD, presenting as ocular disturbance, ataxia and dysarthria, and its association with malignant disease was first recognised in 1951. The condition is rare affecting less than 1 % of patients with cancer (Croft & Wilkinson, 1965) but has been reported in association with a range of tumours. The most common of these are SCLC, gynaecological cancers and Hodgkin's disease (HD). These malignancies underlie 90 % of cases.

PCD can occur as a as part of PEM. Neuronal loss and gliosis may be found in all areas of the cerebellar tissue and is often accompanied by patchy inflammatory change in other areas of the nervous system. However PCD also stands as an individual syndrome distinct from PEM with different pathological changes. Macroscopic examination of the nervous system of patients with non-PEM PCD may reveal a diffuse cerebellar atrophy (Scaravilli *et al*, 1999), however the most striking change is found on microscopic examination of the cerebellum which reveals complete loss of Purkinje cells (Posner, 1995; Verschuuren *et al*, 1996). Mild inflammatory change has been shown to accompany Purkinje cell loss in a few cases, but is thought to represent the active process of Purkinje cell damage which regresses once the Purkinje cells have been destroyed. In addition non-PEM PCD shows sparing of the deep cerebellar nuclei (Henson & Urich, 1982). The pathological picture described above is consistent between two subtypes of non-PEM PCD ie PCD associated with anti-Yo antibodies and PCD associated with HD.

1.2.2.1 PCD associated with anti-Yo antibodies.

PCD can be subclassified according to the detection of anti-Yo antibodies in the patients serum (See section 1.4.2). Anti-Yo positive patients are predominantly female aged between 54 and 61 years with an underlying cancer of the breast, ovarian or other gynaecological tissue (Peterson *et al*, 1992). The onset of the cerebellar symptoms is acute or sub-acute, with truncal and gait ataxia appearing over a course of days to weeks (Anderson *et al*, 1988) and preceding the diagnosis of the underlying tumour in 61-77 % of patients. The neurological deficit progresses over the following weeks before stabilising, often leaving the patient with severe disability.

Despite the lasting neurological deficit, progression of the underlying cancer is the commonest cause of death in these patients (Rojas *et al*, 2000). Although the primary tumours are usually smaller in patients suffering from PCD compared to neurologically intact cancer controls the tumours have generally infiltrated local lymph nodes or extended to involve adjacent tissues at the time of presentation. Such metastasis or local invasion drastically decreases the possibility of tumour control (Hetzel *et al*, 1990). In addition there is a striking difference between the median survival time of patients with PCD associated with breast and gynaecological tumours being 100 and 22 months for each tumour respectively (Rojas *et al*, 2000).

Although the development of anti-Yo PCD is almost exclusively restricted to women, male cases do occur. To date there have been four reported cases of men with anti-Yo PCD who after investigation were found to have malignant disease. Adenocarcinomas of the parotid gland, oesophagus and stomach were identified in three patients after the onset of neurological disease prompted intensive investigation. In a fourth, adenocarcinoma in a single lymph node was identified but the primary tumour eluded detection (Barnett *et al*, 2001; Krakauer *et al*, 1996; Meglic *et al*, 2001; Felician *et al*, 1995).

1.2.2.2 Paraneoplastic Cerebellar Degeneration and Hodgkin's Disease.

Although cases of PCD associated with HD are rare they can be considered to represent a second useful sub-classification. The pathological picture in these cases is identical to that described in the anti-Yo positive patients. However, the study by Hammack *et al* (1992) of the largest cohort of HD associated PCD has highlighted some differences

between this group and the anti-Yo positive patients. PCD associated with HD was found to occur more frequently in men and affected patients of a younger age than those with anti-Yo positive PCD. In addition, the diagnosis of lymphoma preceded the onset of PCD in 80 % of patients. The differences between HD and anti-Yo associated PCD are most probably a consequence of the differences in the underlying malignancies, with the sex and age bias reflecting the demographics of the tumour. The diagnosis of the underlying malignancy in advance of the onset of PCD is likely to be a result of the overt nature of HD. Stabilisation and even recovery from HD associated PCD has been reported.

1.2.3 Paraneoplastic Limbic encephalitis.

Just as PCD can be a manifestation of the multi-focal inflammatory changes of PEM or the result of distinct pathological changes, so too can paraneoplastic limbic encephalitis (PLE). PLE affects both men and women equally most commonly in the fifth decade of life, but has been reported in patients ranging from 11 to 75 years of agen (Gultekin *et al*, 2000). A number of tumour associations have been described (Dropcho, 1998) the most common being SCLC (50%), testicular (20%) and breast (8%) cancers (Gultekin, *et al*, 2000). The nature of these tumours accounts for the wide range in the ages of people affected. PLE commonly presents prior to the identification of the underlying malignancy with a sub-acute amnesic syndrome, an affective disorders composed of depression, anxiety and personality changes, seizures hallucinations or paranoid delusions.

Neuropathological changes typical of those described in PEM are found most consistently in the amygdala and hippocampus while involvement of other areas such as the cingulate gyrus or insular cortex may occur.

In the cohort studied by Gultekin *et al* (2000) PLE was associated with a poor neurological outcome particularly if the disease was associated with anti-Hu or anti-Ma2 antibodies (see sections 1.4.1and 1.4.3). It was also observed that while improvement in the neurological symptoms was uncommon the chances of it occurring were increased by the use of therapies directed against the underlying tumour.

1.2.4 Paraneoplastic Movement Disorders.

Paraneoplastic chorea is rare but has been documented in association with SCLC (Albin *et al*, 1988), Hodgkin's (Batchelor *et al*, 1998) and non-Hodgkin's lymphoma (Nuti *et al*, 2000) among others.

One of the best-described paraneoplastic movement disorders is opsoclonus-myoclonus (OM). This disorder is characterised by involuntary saccades of the eyes in all directions usually associated with myoclonus affecting the trunk, limbs and head. The disorder can be conveniently subdivided into adult and paediatric OM.

Adult OM may be the presenting feature of an occult malignancy (paraneoplastic opsoclonus-myoclonus, POM), usually SCLC, but may also occur in the absence of identified malignancy or following infection (post-infectious OM (PIOM), see section 1.11) (Verma anf Brozman, 2002; Imtiaz and Vora, 1999). One review of 58 patients with OM suggested that only 11 (19 %) were paraneoplastic (Digre, 1986).

In another series of 14 patients with clearly defined POM the mean age of onset was 66 years (range 53-75) with a striking male predominance (M: F, 6:1). As with other PND the onset of neurological disease frequently presents prior to the discovery of the underlying tumour (Bataller *et al*, 2001). The prognosis for paraneoplastic OM is poor. 7/11 of the cases reviewed by Digre (1986) died shortly after the onset of the neurological disease with the longest survival being 8 months. In the remainder the syndrome stabilised but never completely resolved. In most cases death was thought to be caused by the progression of the underlying tumour.

Paediatric POM commonly occurs between 6 and 36 months with females affected more frequently than males (Sakai *et al*, 1990). The disease is associated with an underlying neuroblastoma in 50 % of cases (Caviness *et al*, 1995), however since neuroblastoma has a tendency for spontaneous regression the frequency of paediatric POM can not be determined. As with adult OM some case are thought to be post-infectious in nature. In common with other PND, the neurological syndrome frequently precedes the detection of the underlying malignancy, which is often limited to the thoracic cavity. The neurological syndrome may show improvement with the initiation of immunomodulatory and surgical intervention but as many as 63 % of patients will be left with significant neurological deficit (Rudnick *et al*, 2001).

Pathological investigations of the nervous system in OM are interesting due to the conflicting nature of the reports. CT and MRI scans are frequently normal and are consistent with the lack of pathological change detected within the nervous system. It was once thought that the omnipause neurones were disrupted in OM, but pathological

specimens often show no change, while animal models with lesions to the omnipause neurons do not have OM (Posner, 1995a). Distinct areas of inflammation are often not present but when they do occur the areas of the nervous system involved vary. A few reports suggest changes affecting the Purkinje cells similar to those observed in cases of PCD however this is not a consistent finding.

Well-defined anti-neuronal antibodies including anti-Hu (Hersh *et al*, 1994) and anti-Ri (Prestigiacomo *et al*, 2001) have been associated with adult OM. More recent reports suggest a heterogeneous immune response occurs in which antibodies are directed against various antigens within the nervous system (Bataller *et al*, 2003).
Similarly anti-Hu antibodies have been detected in 16 % of patients with paediatric POM (Antunes *et al*, 2000) but are by no means the rule. Pranzatelli *et al* (2002) found no evidence of anti-Hu, Yo or Ri activity in 18 children with paraneoplastic POM. Connolly *et al* (1997) found anti-neuronal antibodies in nine patients with POM and described binding to the 210 kDa subunit of neurofilament among other antigens. Plioplys *et al* (1989) described cerebellar specific reactivity with 27, 35 and 62 kDa antigens. It is clear that a specific serological marker of adult or paediatric POM has not been described.

Reports regarding the benefit of immunomodulatory therapies to treat adult POM are inconsistent. Protein A column therapy has been reported to be beneficial in one case (Nitschke and Dropcho, 1995). In Batallers report 4/10 patients with breast cancer who did not receive surgical intervention died in spite of immunomodulatory therapies (plasmapherisis, IVIG, steroids). However such intervention may have the effect of dampening anti-tumour immunity. The lack of consistent pathological findings

indicating an immune mediated process and the lack of consistent detection of anti-neuronal antibodies means that there is no clear cause for this disorder.

1.2.5 Lambert Eaton Myesthenic Syndrome.

Lambert Eaton Myesthenic Syndrome is the most frequently occurring paraneoplastic neurological syndrome affecting 2-3 % patients with SCLC (Elrington *et al*, 1991) and is one of the few PND in which the associated antibodies have been proven to have pathological effect (see below). 60 % of patients who present with the syndrome are found to have an underlying malignancy on investigation which is almost always SCLC (O'Neill *et al*, 1988). The syndrome is characterised by proximal lower limb weakness, depression of deep tendon reflexes (which may be augmented following sustained contraction), autonomic disturbance (dry mouth, impotence, constipation) and ptosis.

Neurotransmission at the neuromuscular junction requires an influx of Calcium ions into the pre-synaptic neurone through voltage gated calcium channels (VGCC). The clinical picture described in LEMS is a consequence of impaired calcium flux due to the presence of anti-VGCC antibodies. It is though that in paraneoplastic LEMS the stimulus for this antibody production is the expression of VGCC on the surface of SCLC cells.

LEMS is debilitating but rarely fatal. In contrast to PND affecting the central nervous system (CNS), LEMS is responsive to treatment. Plasmapheresis or immunosuppression to lower the titre of circulating antibodies, mobilisation of calcium using guanidine hydrochloride or targeting potassium channels may be beneficial. In some cases

removal of the underlying tumour may reduce symptoms presumably because the antigenic stimulus for antibody production has been removed.

1.3 Antibodies and the Immune Response to Malignant Disease.

In humans the natural history of malignant disease represents a balance between an effective anti-tumour immune response and its evasion by tumour tissue. Evidence for this immune response is provided by the histological examination of tumours which commonly allows the identification of infiltrating lymphocytes, macrophages and NK cells. In addition tumour specific cytotoxic lymphocytes can be isolated from the peripheral blood of patients with various types of cancer.

The evidence for the role of B cell activity in the anti-tumour immune response is less compelling than that reported for the cellular response. In fact in some cases the occurrence of an active antibody response to the tumour may be associated with a poor prognosis indicating a failure of the immune system to control the malignant disease (Taylor and Gercel-Taylor, 1998).

Since tumours represent a heterogeneous population of cells it is not surprising that patients display a variety of responses against various tumour antigens. Probing cellular homogenates of cancer cell lines with antibodies from patients' sera reveals the diversity of the immune response. Using this approach Bazhin *et al* (Bazhin *et al*, 2003) demonstrated that a variety of antigens were commonly recognised by antibodies in the sera of patients with SCLC.

One method employed to asses B cell responses to malignancy is that of the SEREX technique (serological identification of antigens by recombinant expression cloning). This approach, which involves the construction of cDNA libraries from tumour tissue, has lead to the identification of a multitude of tumour-associated antigens (Krebs *et al*, 2003. While B cell responses occur, care must be taken to distinguish those responses that are a direct result of anti-tumour immunity and those that are part of the 'normal' antibody response to self antigens (Avrameas and Ternynck, 1995).

While the antibody response to tumour antigens is diverse, certain responses are common and may provide a means to define clinical phenotypes, diagnose underlying disease and act as prognostic indicators. For example anti-p53 antibodies are detected with a frequency of 9 % in patients with breast cancer (Crawford *et al*, 1982), and 2 % of patients with cancer of the prostate (Brandt-Rauf, 1997).

As has been alluded to, some anti-tumour responses result in the production of antibodies which cross-react with tumour and neuronal tissue. Applying the SEREX technique to the screening of libraries constructed from neuronal tissue allows a way to 'track back' from the neuronal tissue to allow the identification of potentially interesting antigens expressed by tumour tissue. Such an approach reduces the number of antibodies with the potential to react with antigens in the library since anti-neuronal responses are rare.

1.4 Anti-Neuronal Antibodies.

Since Wilkinson and Zeromski first documented the existence of 'antibodies against neurones in sensory carcinomatous neuropathy' in 1965 (Wilkinson and Zeromski) a

number of anti-neuronal antibodies have been identified and shown to occur in the serum and cerebrospinal fluid (CSF) of patients affected by PND (Table 1-3). The role of these antibodies in the pathogenesis of PND has been proven in only a few diseases, while in others their pathological significance remains a matter of debate.

Despite this, the association between antibody, PND and underlying tumour provides a useful diagnostic tool. Once anti-neuronal antibodies have been detected the search for an underlying tumour can be concentrated into a few defined areas.

Characterisation of the target antigen by application of the SEREX technique has in some cases identified previously unrecognised proteins, allowing speculation into their roles in normal tissue while providing valuable insights into tumour biology and anti-tumour immunity. An introduction to the common anti-neuronal antibodies is presented in Table 1-3.

Table 1-3: Well-described anti-neuronal antibodies found in assoication with PND.

Antibody	*Associated PND*	*Immunohistochemistry*	*Immunoblotting*	*Antigen and function*
Hu	PEM/ SSN	Neuronal nuclei. Nucleolus spared.	38-40 kDa band	Hu: Neuron specific RNA or DNA binding protein. Role in RNA processing in neurones.
Yo	PCD	Purkinje cell cytoplasm and axons. Coarse granular staining.	34 and 62 kD band from extracts of breast and ovary tumours.	CDR34 and CDR62: Involved in DNA binding and gene transcription.
Ma1	CD/ BD/ MW	subnuclear elements of neurones	37 and 40 kDa	Unknown function
Ma2	LE	Nucleus, perikarion	40 kD	Unknown function
Ri	OM	All nuclei of CNS	55 kDa	Ri: RNA binding protein.
CV2	CD/ SMN/ uveitis	Cytoplasm of oligodendrocytes	66 kDa	ULIP/ CRMP: Uncertain
VGCC	LEMS	Presynaptic	Not detectable	VGCC: ACh release
Tr	PCD	Purkinje cell cytoplasm. molecular layer	Negative immunblot	Not known

CD: cerebellar dysfunction, BD: brainstem dysfunction, MW: motor weakness, SMN: Sensorimotor neuropathy, PEM: paraneoplastic encephalomyelitis, SSN: subacute sensory neuronopathy, LEMS: Lambert Eaton myasthenic syndrome, LE: limbic encephalitis, PCD: paraneoplastic cerebeller degeneration.

1.4.1 Anti-Hu Antibodies.

The antibodies first described by Wilkinson and Zeromski were probably the same as those identified in four patients with both SSN and SCLC by Graus *et al* (Graus *et al*, 1986). Using a combination of immunohistochemistry and immunoblotting techniques this antibody has been well characterised, and designated anti-Hu after the patient in which the antibody was first described.

Immunohistochemical staining of neuronal tissue with anti-Hu positive sera produces strong staining of all CNS and peripheral nervous system (PNS) neuronal nuclei with a striking sparing of the nucleolus, and a faint granular staining of the cytoplasm.

Incubation of sera containing anti-Hu antibodies with systemic tissue does not produce a reaction. Immunoblotting of nuclear or crude neuronal extracts reveals the antibody reacts with series of proteins between 35 to 40 kDa. By eluting reactive antibodies from this region of the immunoblot Graus *et al* (Graus *et al*, 1986) were able to show that this anti-neuronal antibody cross-reacted with the patient's tumour.

The anti-Hu antibody is a highly specific marker of PEM/ SSN. Molinuevo quoted a specificity and sensitivity of 99 % and 82 % respectively for the anit-Hu antibody as a marker of SSN (Molinuevo *et al*, 1998). In addition the anti-Hu antibody is a useful marker of underlying malignancy. Approximately 78 % of patients with paraneoplastic disease and demonstrable anti-Hu antibodies have SCLC as the underlying tumour (Molinuevo *et al*, 1998; Dalmau *et al*, 1992). Only rarely is a tumour not found (Dalmau *et al*, 1992). Initially it was thought that the anti-Hu antibody did not occur in neurologically normal SCLC patients (Anderson *et al*, 1988). It has since been shown that anti-Hu antibodies can be detected in 16-25.5 % of neurologically intact patients with SCLC, although at a lower titre than in those patients with PEM/SSN (Dalmau *et al*, 1990; Monstad *et al*, 2004).

To date there are few reports of 'false positive' patients. In these cases it is thought that a tumour was present, but small and easily missed at autopsy. In addition Sjögren's syndrome may be complicated by a sub-acute sensory neuropathy clinically indistinguishable from paraneoplastic SSN with antibodies against a 38 to 40 kDa protein from neuronal tissue (Sillevis-Smitt *et al*, 1996; Moll *et al*, 1994). These false positives can be avoided either by routinely testing for anti-neuronal antibodies by both

histochemical and immunoblotting methods or by screening patients against recombinant antigen.

Cloning of the Hu antigen was achieved by Szabo *et al* in 1991. Using anti-Hu sera this group successfully screened a cerebellar Lambda ZAP cDNA expression library to identify two different but related genes that were subsequently designated HuD and HuC. These genes encode members of a highly conserved family of RNA binding proteins which has been expanded to include Hel-N1 and HuR (Nabors *et al*, 1998). The proteins are homologous to the embryonic lethal abnormal vision (ELAV) protein of Drosophilia, a protein required for the development and maintenance of the fly nervous system however its precise role in the human nervous system or tumour tissue remains unknown.

1.4.2 Anti-Yo Antibodies.

As described previously (see section 1.2.2.1), a subgroup of patients with PCD has been found to have anti-neuronal antibodies in their sera. Greenlee and Brashear (1983) and later Jaeckle *et al* (1985) described the presence of an antibody directed against the cytoplasm of Purkinje cells in patients with ovarian cancer. The anti-Purkinje cell antibodies stain the cytoplasm and proximal dendrites of the Purkinje cell layer with faint staining of the neurones of the molecular and granular layers, but do not stain neurones from other areas of the nervous system. Immunoblots of Purkinje cell extracts reveals that anti-Yo sera reacts consistently with a two antigens with a molecular weight of 34 kDa and 62 kDa.

Anti-Yo PCD sera has been used to clone DNA encoding the cerebellar degeneration related (Cdr) proteins. Cdr1 the gene that encodes the 34 kDa protein has been cloned (Dropcho *et al*, 1987) and mapped to the long arm of the X chromosome (Chen *et al*, 1990). The predicted amino acid sequence of this protein reveals an unusual structure composed of hexapeptide repeats making up 91 % of the total protein. As yet the function of this protein is unknown. Using cDNA expression libraries the 62 kDa antigen (subsequently designated cdr2) was cloned simultaneously by Sakai (Sakai *et al*, 1990) and Fathallah-Shaykh (1991) and mapped to chromosome 16. The encoded protein was shown to contain a leucine zipper and zinc finger motif and is speculated to play a role in gene expression. Corradi *et al* (1997) demonstrated that cdr2 mRNA is widely expressed in multiple systemic tissues, but its expression, regulated by a post-transcriptional mechanism, is restricted solely to the testis and the brain, both classically regarded as immunoprivileged sites.

Early reports suggested that cdr2 was only expressed by the tumours of those patients with anti-Yo associated PCD (Furneaux *et al*, 1990). However, more recently expression of cdr2 has been confirmed in a significant number of gynaecological tumours from neurologically normal patients (Darnell *et al*, 2000).

1.4.3 Anti-Ma Antibodies.

Dalmau *et al* (Dalmau *et al*, 1999) first described anti-Ma1 antibodies in the sera of patients with suspected PND. The antibodies were found in patients with brainstem and cerebellar dysfunction or motor weakness. Underlying tumours of the parotid gland, breast, lung or colon were found. These findings were obtained from a small group of patients, however it is though that anti-Ma1 antibodies are reliable markers of PND

involving the brainstem or cerebellum. The use of these antibodies in predicting the underlying malignancy is more uncertain. Each patient from the original paper suffered from different malignant disease. More recent studies have failed to highlight an overwhelming tumour association (Rosenfeld *et al*, 2001).

Staining of human tissue sections with anti-Ma sera reveals exclusive reactivity with subnuclear elements of neurones and testicular germ cells. Probing immunoblots of neuronal extracts shows that the antibodies react with 37 and 40 kDa bands and also react with the associated malignancy. As with other anti-neuronal antibodies, the 37 kDa target antigen (Ma1) has been cloned from a bacteriophage expression library. The function of this protein, the expression of which is restricted to brain and testicular tissue, is unknown, and there is no homology shown between known proteins or sequence motifs.

Serum from some patients with PLE, accompanied in some cases by brainstem or cerebellar dysfunction, and testicular tumours was found to contain anti-neuronal antibodies against a 40 kDa protein. The antigenic target was found to share significant homology with Ma1, the antibodies and antigen were therefore designated anti-Ma2 and Ma2 respectively. Immunohistochemical staining of neuronal sections shows reactivity with subnuclear and cytoplasmic structures in all neurones. Normal expression of Ma2 is limited only to brain and testicular tumours. As with Ma1 the function of this protein is unknown.

1.4.4 Anti-Ri Antibodies.

The anti-Ri antibody was first described in a patient with breast cancer and opsoclonus-myoclonus (Budde-Steffen *et al*, 1988). The antibody provides an identical histochemical picture to that produced by anti-Hu serum with the exception that anti-Ri serum does not react with nervous tissue from the PNS. Immunoblots of extracts of cortical neurones show that the antibody reacts with two proteins, one of 55 kDa and one of 80 kDa. The antibodies eluted from these regions react specifically with neoplastic tissue taken from the patients but not from neurologically normal tumour controls (Luque *et al*, 1991).

The gene encoding the antigen has been cloned and designated Nova-1. Normal expression of this protein is limited to the brain, however alternatively spliced forms are found in malignant tissue (Buckanovich *et al*, 1993). Nova-1 is an RNA binding protein thought to play a role in alternative splicing of mRNA, the activity of which can be interrupted in-vivo by anti-Ri sera (Buckanovich *et al*, 1996).

1.4.5 Anti-CV2/ CRMP-5 Antibodies.

Honnorat *et al* (Honnorat *et al*, 1996) first described anti-CV2 antibodies in patients with cerebellar degeneration, uveitis and peripheral neuropathy in 1996. These antibodies stained a subpopulation of oligodendrocytes in adult rat brain and reacted with a 66 kDa protein belonging to the Ulip/CRMP family of proteins. The commonest underlying malignancy associated with anit-CV2 antibodies is SCLC (77 %) followed by thymoma (6 %). Comparison of patients with anti-Hu and anti-CV2 associated peripheral neuropathy showed that the former tended to suffer from a pure sensory

neuropathy as opposed to a mixed sensorimotor neuropathy associated with the presence of anti-CV2 antibodies (Antoine *et al*, 2001).

Tani *et al* (Tani *et al*, 2000) and Croteau *et al* (2001) have described cases of paraneoplastic chorea in-patients with an underlying SCLC whose sera contained antibodies directed against a 68 kDa and 79 kDa protein in each case respectively. The identity of these proteins was not documented. Later Yu and collegues (Yu *et al*, 2001) described antibodies against CRMP5 in patients with PND which is thought to correspond to the previously described autoantigens. It has more recently been suggested that anti-CV2 and anti-CRMP5 antibodies are directed against the same antigen (Posner and Dalmau, 2001; Honnorat *et al*, 2002).

In a study of 116 anti-CRMP-5 positive patients cerebellar ataxia, dementia, cranial neuropathy, chorea, loss of taste/smell and optic neuritis were among the most frequent manifestations of neurological dysfunction. Further reports described 16 anti-CRMP5-IgG positive patients with underlying malignancy and chorea, normally as part of a multifocal neurological disorder (Vernino *et al*, 2002). Chorea has rarely been described in anti-Hu positive patients with PEM (Heckmann *et al*, 1997), however, anti-Hu antibodies are often detected concurrently with the presence of anti-CRMP-5 IgG and chorea may thus represent a discrete syndrome that occurs in combination with PEM/SSN. The findings to date suggest that anti-CRMP-5 is the most frequently occurring marker of paraneoplastic chorea.

1.4.6 What Are We Missing?

Posner and Dalmau's editorial 'Yet another paraneoplastic antibody' (Posner and Dalmau, 2000) implies efforts to identify novel anti-neuronal antibodies are becoming exhausted. As has been described, even the more common anti-neuronal antibodies occur infrequently, so what are the chances of finding novel antibody associations? In their study of 50 patients with limbic encephalitis Gultekin *et al* (Gultekin *et al*, 2000) found that 40 % of the patients were antibody negative. Similarly, Antoine *et al*s' study of paraneoplastic peripheral neuropathies showed 73 % of patients had no evidence for anti-neuronal antibodies (1999). In both studies the association of neurological and neoplastic disease was considered enough to consider the disease to be paraneoplastic. Our own investigations have shown that 23 % of patients with paraneoplastic disease have no anti-neuronal antibodies, a figure likely to be lower than the actual figure as the method for data collection was biased for antibody positive cases of PND (See Appendix 3). It is clearly possible that in some patients anti-neuronal antibodies simply do not occur. Inappropriate secretion of neurotoxic compounds or cytokines could feasibly trigger distant neurological dysfunction. Similarly a T cell mediated response could result in inflammation within the nervous system in the absence of B cell activity. However investigations conducted in a routine laboratory frequently detect anti-neuronal activity in patients with neurological disease and cancer that do not conform to the classical antibodies described in the preceding text.

Since antibodies associations are rare a large library of samples are required to demonstrate novel antibody associations. Uncommon immune responses may be dismissed as unique simply due to the infrequency with which samples arrive. Gephyrin, one of the latest target antigen described in the literature was characterised on

the basis of results obtained from investigation with the serum from one patient (Butler *et al*, 2000).

It must also be remembered that the detection of a single well-characterised anti-neuronal antibody may not be the complete story. We have already described how a number of tumour antigens can be recognised by antibodies in the serum of a single cancer patient (Anti-Hu and anti-CRMP-5 antibodies. See section 1.4.5). It is probable that patients have antibodies against numerous neuronal antigens at the same time. Bataller *et al* (Bataller *et al*, 2004) have recently described an antibody response to Zic4, a zinc finger protein in 27 % of serum samples with anti-Hu and CRMP5 antibodies. In a different study coexisting anti-neuronal antibodies were found in 31 % of 553 samples contained multiple antibodies (Pittock *et al*, 2004). It is possible that the phenotype of a disease may be the result of multiple immune responses against various antigens, some of which may remain unidentified.

To date most proteins identified as targets for anti-neuronal antibodies have been characterised by the screening of bacteriophage expression libraries. Antigen identification using this technique may not be universally applicable. For example, carbohydrate antigens and conformational epitopes may not be detectable. It may be for this reason that efforts to screen expression libraries with anti-Tr positive sera have not yielded a candidate antigen. As a result alternative techniques may be required in order to characterise as yet undetected antigens associated with PND. The benefit of characterising novel target antigens lies not just in the potential to develop diagnostic tests, but in broadening the understanding of the pathological mechanisms involved in PND and so developing effective therapeutic strategies.

1.5 Pathogenesis of Paraneoplastic Neurological Disease.

The T and B cells which form the adaptive arm of the immune system are capable of orchestrating an immune response against tumour cells which, as discussed, is evidenced by the presence of inflammatory infiltrates in tumour tissue and the presence of anti-tumour antibodies.

Normally an immune response will occur without any immune attack against normal healthy tissue. Autoimmunity is prevented by the deletion of self-reactive cells during their development. T cells, which react with self-antigens, are deleted during development in the thymus following recognition of self-antigen. Since not all antigens can be presented to T cells by the thymus some potentially reactive cells may reach the periphery. In this instance these cells are rendered either anergic due to incorrect presentation of the self-antigen, deleted because the antigen is in too low a concentration or remain inactive as the antigen is sequestered in such a way as to prevent T cell interaction. B cells also undergo selection in the bone marrow during development. However, since B cells undergo receptor editing during maturation the potential for B cells to become self-reactive is greater than for T cells.

The pathogenesis of most PND is still unknown however immunological abnormalities such as the presence of neuronal autoantibodies and presence of inflammatory change points towards an autoimmune mechanism. It is believed that expression of normally sequestered neuronal antigens by tumour cells results in the production of a cross-reactive immune response between the tumour and neuronal tissue. It is probable that T and B cells capable of recognising the antigenic targets of anti-neuronal antibodies are

activated as part of the wider immune response to tumour tissue. The presence of the anti-neuronal antibodies alone is not sufficient evidence to say that these syndromes are antibody mediated. For a disease to be classified as antibody mediated it is generally accepted that four criteria must be satisfied:

1: The antibodies should react with the correct tissue type to produce the clinical phenotype.

2: The passive transfer of antibodies from a patient into experimental animals should replicate the disease.

3: Immunisation of an experimental animal with purified antigen should induce the clinical phenotype.

4: Immunotherapy that reduces antibody titres must result in clinical improvement.

The circulating antibodies found in LEMS meet these criteria. In contrast only the first of the four postulates has been satisfied for the antibodies directed against intracellular targets. Further evidence of pathogenicity in these cases is far more circumstantial. The deposition of antibodies in neurones of patients with PND has been demonstrated and anti-Yo antibodies are taken up by cultured rat neurones (Greenlee *et al*, 1995). The mechanism by which antibodies penetrate the cell is unclear but may involve endocytosis. Such a mechanism would not allow antibodies to reach their nuclear antigens to allow them to exert a pathogenic effect. Epitope mapping of Hu (Kumagai *et al*, 1999) and Yo (Fathallah-Shaykh *et al*, 1991) shows that the antibodies are directed against the functional domains of these proteins. However in only a single report have

anti-Hu antibodies been reported to be directly pathogenic (Schafer *et al*, 2000). Transfer of antibodies (Tanaka K *et al*, 1999) or immunisation of experimental animals (Tanaka *et al*, 1994) has failed to replicate the disease despite the finding of high titres of anti-neuronal antibodies. Finally, treatments that aim to reduce the titre of anti-neuronal antibodies are consistently unsuccessful (Blaes, 2002). Therefore most investigators now believe that anti-neuronal antibodies associated with PND are an epiphenomenon, acting as markers of a disease mediated by a different process.

The failure to provide evidence for an antibody-mediated mechanism has led to the examination of the role of cell mediated immunity in PND. Analysis of the parenchymal inflammatory infiltrate has shown that the lymphocytes in situ are recruited, and activated following interaction with a specific antigen. Sub-classification of the $CD4^+$ T cells demonstrates that they are of the Th1 subtype and as such could be involved in cell mediated injury. Studies regarding the association between Human Leukocyte Antigen (Involved in antigen presentation) and PND in anti-Hu positive patients have failed to identify any haplotype implicated in the development of anti-Hu associated syndromes (Voltz *et al*, 1998). Stronger evidence exists for the role of cytotoxic T-cell lymphocytes in anti-Yo associated PCD. One study has identified cdr2 specific cytotoxic lymphocytes in 3/3 patients who were HLA A2.1 positive (Albert *et al*, 1998), while investigation of a Japanese cohorts of anti-Yo positive patients has demonstrated an association with HLA A24 (Tanaka and Tanaka, 1996). Restricted peptide epitopes from cdr2 that contain the appropriate anchor residues for these HLA molecules can stimulate cytotoxic effects (Tanaka *et al*, 2001). One of the most recent and convincing experiments regarding the need for a T-cell mediated immune response has recently been described. By transferring Mal activated $CD4^+$ Th1 lymphocytes between mice

investigators have been able to induce pathological changes in nervous tissue synonymous to those seen in PND (Pellkofer *et al*, 2004).

1.6 Anti-tumour immunity in PND

The immune response associated with PND seems to have considerable anti-tumour activity which results in the restriction of tumour growth and metastatic spread. There was initially some doubt as to whether this was a result of the neurological symptoms prompting investigation for a tumour early in the natural history. However, 16 to 25.5 % of neurologically normal patients with SCLC harbour low titres of serum anti-Hu antibodies (See section 1.4.1). These patients are more likely to have limited stage disease, a better response to treatment and improved survival when compared with antibody negative SCLC patients (Graus, *et al*, 1997). It remains to be determined whether the anti-tumour effects are a direct result of anti-tumour activity directed specifically against Hu, or whether the antibodies are a marker a more aggressive generalised activation of the immune system (Dalmau *et al*, 1990).

Analysis of SCLC cells has demonstrated that Hu is expressed at the surface of all cells and is therefore an accessible target to an immune response, but it is unclear why only a proportion of patients develop an immune response to this protein. Analysis of tumours of SCLC patients with neurological disease reveals MHC I expression to be more prevalent than on tumours from neurologically normal patients suggesting that these tumour cells are more immunogenic and more likely to elicit cell mediated immunity (Manley *et al*, 1995). Additional evidence for an anti-tumour effect comes from animal models. Vaccination of murine models of SCLC with a plasmid encoding Hu elicits

anti-tumour activity (Ohwada *et al*, 1999) and studies examining the lymphocytic infiltrate into murine neuroblastoma found a higher CD8: CD4 ratio in the tumours of Hu vaccinated animals (Carpentier *et al*, 1998).

1.7 Post-Infectious Neurological Diseases.

1.7.1 Paraneoplastic and Post-infectious Disease.

PND and some post-infectious neurological disorders share similarities in that they are both associated with the production of anti-neuronal antibodies and an immune response capable of cross-reacting with components of normal nervous tissue (Figure 1-1). The main difference is that in the case of paraneoplastic disease auto-immunity is thought to be triggered by the aberrant expression of normal self-antigen while post-infectious auto-immunity is thought to be initiated by an immune response to an exogenous antigen carried by a pathogenic agent. Methods employed in the identification of antigenic targets in post-infectious neurological disease can be utilised and applied to the detection of antigens in paraneoplastic disease and vice versa. The following sections provide an introduction into the principles behind post-infectious autoimmunity with some examples of post-infectious neurological diseases.

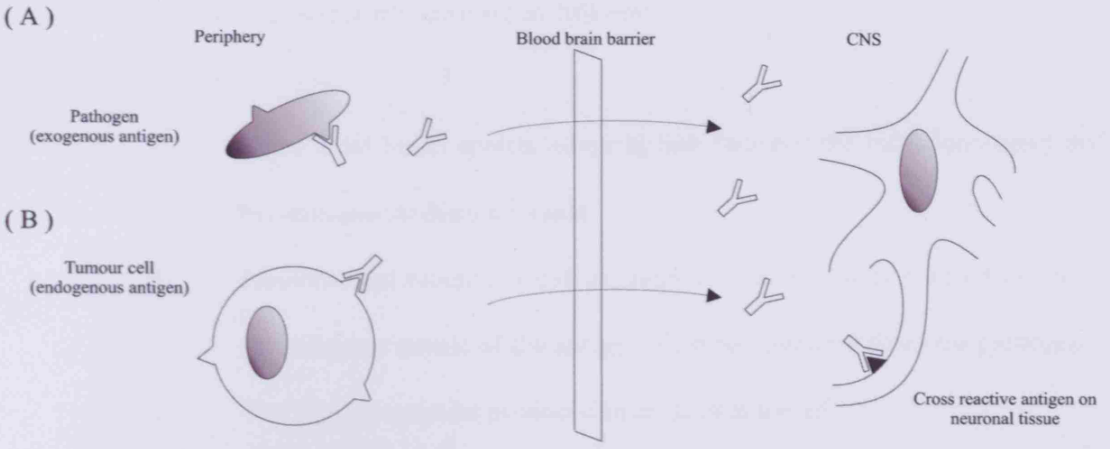


Figure 1-1: Production of cross-reactive anti-neuronal antibodies in post-infectious and paraneoplastic disease.

1.7.2 Breakdown of Tolerance and Molecular Mimicry.

As with paraneoplastic disease post-infectious autoimmune disease may be the consequence of an inappropriate immune response against self-antigens, which results in the damage and dysfunction of the target tissue. One explanation for the breakdown of tolerance and the initiation of a post-infectious autoimmune response is suggested by the theory of 'molecular mimicry'. This states that the infection of a susceptible host by a pathogen bearing antigens similar to those on host tissue but with sufficient variation provokes an immune response that then cross-reacts with normal tissue. In order for a disease to be the result of molecular mimicry it is generally accepted that four criteria must be satisfied (Rose and Bona, 1993; Benoist and Mathis, 2001). In the case of antibody mediated disease the criteria outlined in section 1.5 should also be kept in mind.

The criteria for molecular mimicry are as follows:

1: There must be an epidemiological link between the infectious agent and the immune-mediated disease.

2: Patients must mount a T cell or antibody response against self-antigen.

3: An antigenic mimic of the antigen must be identified from the pathogen.

4: The disease must be produced in an animal model.

1.7.3 Evidence for MolecularMimicry as a Cause of Neurological Disease.

Gullain-Barré syndrome (GBS) is a monophasic illness characterised by inflammation and degeneration of the peripheral nerves. Miller-Fisher syndrome (MFS) is considered a variant of GBS characterised by ataxia, areflexia, and ophthalamoplegia. The syndromes usually occur as a consequence of various bacterial or viral infections. Epidemiological studies have highlighted a clear association with Campylobacter jejuni (Rees *et al*, 1995). This bacterium carries lipopolysaccharides in the cell wall against which antibodies are produced with the capacity to cross-react with gangliosides present on the surface of peripheral nerves. Antibodies against the ganglioside GQ1b are detected in >90 % of patients with MFS while antibodies to GM1 and other gangliosides can be detected in 20 to 40 % of patients with in GBS. Passive transfer of antibodies from GBS patients to mice has the capacity to produce GBS symptoms (van den Berg *et al*, 1994). The findings of an epidemiological link with an infectious agent, the existence of a molecular mimic, the presence of cross-reactive antibodies and the capacity of the antibodies to transfer disease means GBS is considered by many to be the best example of a disease caused by molecular mimicry.

More recently, evidence for molecular mimicry causing HTLV-1 associated myelopathy/ tropical spastic paraparesis (HAM/TSP) following human T-lymphotropic virus type 1 (HTLV-1) infection has been presented. Levin *et al* (2002) have identified an antibody response against the neuronal protein hnRNP-A1 which cross-reacts with the tax protein of HTLV-1. These antibodies were able to completely abolish neuronal firing in patch clamp experiments. While all the criteria for molecular mimicry have yet to be satisfied for HTLV-1 induced HAM/TSP the evidence presented thus far suggests that HAM/TSP may be an excellent example of molecular mimicry resulting in neurological disease.

1.8 Post-Streptococcal Diseases of the CNS.

There is considerable interest in diseases of the CNS associated with recent infection with group A β haemolytic streptococcus (GABHS). The term post-streptococcal neurological disorders includes diseases such as Sydenham's chorea (SC) (Church *et al*, 2002), acute disseminated encephalomyelitis (ADEM) (Dale *et al*, 2001), paediatric autoimmune neuropsychiatric disorder associated with streptococcal infection (PANDAS) (Swedo *et al*, 1998), encephalitis lethargica (EL) (Dale *et al*, 2003) and Tourette's syndrome (TS) (Church *et al*, 2003). It has been postulated that these disorders may involve the process of 'molecular mimicry' described above. Indeed anti-neuronal antibodies have been described and the putative antigens characterised. An introduction to the clinical phenotype of a number of post-streptococcal neurological disorders is provided below. Table 1-4 summarises the main clinical, streptococcal and immunological findings for each disease. The evidence for the presence of anti-neuronal antibodies and the putative antigens are described (see section 1.9 and 1.10 respectively).

Table 1-4: Clinical findings, streptococcal association and anti-neuronal antibodies in proposed post-streptococcal neuropsychiatric diseases.

Syndrome	*Symptoms*	*Evidence for association with Streptococcus*	*Evidence for anti-neuronal antibodies*		*Outcome*
			Histochemistry	*Immunoblot*	
Sydenham's Chorea	Chorea, OCD, anxiety, ADHD ,depression . .Systemic signs associated with RF.	Yes. Raised in 63% (Ayoub and wannamaker 1966)	Cytoplasmic staining of caudate and subthalmis neurones (Husby *et al*, 1976). Anti-neural antibodies (Morshed *et al*, 2001).	Antibodies to neuronal tissue but no specific bands described (Morshed *et al*, 2001).. Antibodies against BG enriched proteins of 40, 45 and 60kDa (Church *et al*, 2002).	Complete remission in 50% by 2 years.
PANDAS	Motor Tics, OCD, ADHD	Yes. Required by definition (Swedo 1998).	Staining pattern similar to Sydenham's chorea. (Pavone 2004)	Antibodies against 40, 45 and 60kDa BG proteins (Church 2004)	Relapses associated with re-infection. Final outcome remains unclear.
Tourette's Syndrome	Motor and vocal tics OCD, ADHD	Evidence for elevated streptococcal serology in a subgroup of patients	An anti-neuronal response. Nuclear staining occasionally with sparing of nucleolus (Morshed *et al*, 2001).	Patients with Raised ASOT frequently have antibodies against 40, 45 and 60 kDa proteins (Church *et al*, 2003).	'waxing and waning'. Often resolves in adulthood.
Encephalitis Lethargica	Sleep disturbances, lethargy, movement disorders	Yes. 65% have elevated ASOT (Dale *et al*, 2003).	Cytoplasmic binding of antibodies to BG neurones (10/10 patients) (Dale *et al*, 2003).	Antibodies against neuronal 40, 45, 60 and 98kDa neuronal proteins (Dale *et al*, 2003).	Often relapsing course. Possibly fatal. 75% will have persistent neurological disease
ADEM	Various depending on site of lesion.	Yes: Patients with raised ASOT and preceeding infection have been reported.	Immunoflourescent staining of large striatal neurones (Dale *et al*, 2001)	Binding to basal ganglia enriched proteins (MW = 60, 62, 67, 80kDa) (Dale *et al*,1990).	Occasional relapse but frequently resolution of neurological symptoms.

ADEM: Acute disseminated encephalomyelitis. ADHD: Attention deficit hyperactivity disorder. BG: Basal ganglia. OCD: Obsessive compulsive disporder.

1.8.1 Sydenham's Chorea.

Rheumatic fever (RF) is a systemic disease precipitated by GABHS infection. The most common symptoms include fever, arthritis and carditis. SC, characterised by the rapid onset of bilateral chorea, often accompanied by behavioural changes is considered to be a late manifestation of RF. The disorder is more common in females with the peak incidence at 8.4 years (Cardoso *et al*, 1997). Medical intervention in western societies has resulted in the reduction of the number of cases of RF and thus SC. However RF remains endemic in developing countries.

In contrast to RF which typically occurs within 21 days of GABHS infection, SC often has a delayed onset ranging from 1 to 6 months (Murphy *et al*, 2000). This delayed period of onset presents problems for the diagnosis of SC as the presence of acute phase proteins and titers of anti-streptococcal antibodies have often subsided at the time of presentation, however the epidemiological association of GABHS and SC has been demonstrated (Ayoub and Wannamaker, 1966).

Since SC is rarely fatal only a limited number of pathological studies involving post-mortem tissue are available but consistently show inflammatory changes in the basal ganglia concentrated mainly to the caudate and putamen. Neuronal loss is an uncommon finding and may be misleading, being related to the increased severity of the disease process in patients with fatal SC. More commonly patients experience persistence of chorea which extends beyond two years in 50 % of cases. Patients may also experience later relapses associated with re-infection or around the time of pregnancy.

1.8.2 Paediatric Autoimmune Neurophsychiatric Disorders Associated With Streptococcal infection (PANDAS).

An apparent temporal association between an outbreak of GABHS and a 10 fold increase in the incidence of motor tics was recognised in the 1980's (Kiessling *et al*, 1993). In 1998 Swedo *et al* (1998) published an in-depth description of 50 patients with neuropsychiatric disease associated with recent infection with GABHS, and coined the term 'paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS)'.

To conform with a diagnosis of PANDAS, Swedo *et al* defined a number of criteria one of which stated that the neuropsychiatric disorder must occur in a pre-pubital patient, follow an episodic course and have evidence of GABHS infection. Since the phenomenon of PANDAS is fairly new there is limited information pertaining to the demographics and pathological basis of the illness. The Swedo paper describes a series of predominately male patients (2.6:1) with a mean age of onset of 6.3 and 7.4 years for tics and OCD respectively (Swedo *et al*, 1998). There are no published reports in which in-depth post-mortem analysis of brain tissue has been conducted. MRI scanning suggests a pathology localised to the basal ganglia since patients frequently have an enlarged caudate and putamen when compared to healthy controls (Giedd *et al*, 2000). The association with GABHS implies that PANDAS may be an immune-mediated post-infectious disorder. Indeed there is some evidence to suggest the existence of anti-neuronal antibodies in the serum and CSF of patients with PANDAS (see below) while the use of immunomodulatory therapies such as IVIG and plasma exchange have been shown to decrease symptoms (Perlmutter *et al*,. 1999). The disease by definition follows a 'saw-tooth' pattern with exacerbation's linked with recurrent GABHS

infection, however the natural history of the disease is unknown but is thought to be similar to the natural course of SC.

1.8.3 Acute Disseminated Encephalomyelitis (ADEM)

Acute disseminated encephalomyelitis is a monophasic inflammatory disease of the white matter which is commonly post-infectious but may also occur as a consequence of vaccination with killed or attenuated virus or bacteria. Cases of ADEM following infection with mycoplasma, influenza, enterovirus, Epstein-Barr virus and varicella have also been described. The clinical findings vary as a consequence of the anatomical localisation of the lesions. Among the more common findings are pyramidal signs, encephalopathy and bilateral optic neuritis. The disease is rare making the exact incidence difficult to determine. ADEM following mumps has an occurrence of 1:1000, while ADEM following rubella has an incidence of 1:20,000.

Pathological investigation reveals a perivenular inflammatory myelinopathy composed of lymphocytes and macrophages. Demyelination occurs in the absence of axonal damage. The pathological findings are consistent independent of the precipitating pathogen or vaccine. Investigation of the CSF and brain tissue for the presence of infection agents is consistently unsuccessful and has led to the suggestion that the disorder may be immune mediated. This is supported by the apparent benefits of immunomodulatory therapies in the treatment of the disease, some of which have been reported to have dramatic effects.

Although rare (6 % of ADEM patients), Streptococcal infection has been associated with ADEM. Dale *et al* presented a series of 10 patients with ADEM all of whom had

serological evidence for GABHS infection. This cohort were unusual in that 50 % of patients had a dystonic extrapyramidal movement disorders, an uncommon clinical finding in ADEM patients (Dale *et al*, 2001). The authors described an increased incidence of lesions in the basal ganglia (80 % compared to 18 % in non-streptococcal ADEM) and concluded that post-streptococcal ADEM represented a new post-streptococcal neurological disease.

1.8.4 Tourette's Syndrome

Tourettes syndrome is defined as a neuropsychiatric disease characterised by chronic motor tics with one or more vocal tics associated with psychiatric symptoms such as OCD, attention deficit hyperactivity disorder, anxiety and depression. The disorder affects 5:10,000, has a male predominance (M: F = 3:1) and is prepubital in onset (mean age = 7 years). The symptoms are typically described as 'waxing and waning' often with complete resolution by adulthood although some patients develop chronic disease. Excellent reviews can be found in (Jankovic, 2001; Leckman, 2002 and Robertson, 2000).

The underlying aeitology of TS is unclear. Undoubtedly, genetic and environmental factors play a role. Studies of the relatives of patients with TS suggest an autosomal dominant inheritance but no specific genetic markers have yet been described (Pauls, 2003). Imaging studies suggest the basal ganglia are often affected (Peterson *et al*, 1993).

Like SC and PANDAS some patients with TS have positive streptococcal serology compared to controls suggesting TS may in some cases be post-infectious in nature.

While the use of IVIG in TS has not been shown to have an effect some investigators have claimed the existence of anti-neuronal antibodies in patients with TS (see below).

1.8.5 Encephalitis Lethargica.

Encephalitis lethargica is a severe neuropsychiatric disease characterised by sleep disturbance (hypersomnolence, insomnia and sleep inversion), lethargy and extrapyramidal movement disorders (Parkinsonism and dyskinesia). The majority of survivors are left with significant neuropsychiatric deficits such as oculogyric crisis, opthalamoplegia, obsessive compulsive disorder (OCD), depression, apathy and mutism.

The first reported cases of EL formed an epidemic between 1916 and 1927 coinciding with the influenza pandemic of 1918. At that time EL was thought to be the result of direct infiltration of the brain parencyma by influenza virus (Ravenholt and Foege, 1982) but examination of acchived brain tissue from these EL cases for the presence of influenza virus failed to demonstrate its presence (McCall *et al*, 2001; Lo *et al* 2003).

Histochemical examination of brain tissue from the first epidemic and from sporadic cases demonstrates perivascular lymphocytic cuffing of vessels situated in the mid brain and basal ganglia which have more recently been identified as IgG producing plasma cells. These findings coupled with the presence of oligoclonal bands and the reportedly steroid responsive nature of the disease has lead to the suggestion that EL may be immune mediated.

In 2004 Dale *et al* reported a series of 20 patients with EL which were frequently preceded by an upper respiratory tract infection or tonsillitis (Dale *et al*, 2003). The association of basal ganglia and midbrain dysfunction and the post-infectious presentation of the disease led the investigators to question whether these cases of EL were post-streptococcal in nature. The groups were able to culture group A β-haemolytic streptococcus from the throats of 2 patients while 65 % of patients had a significantly elevated anti-streptolysin O titres (ASOT). In addition anti-neuronal antibodies were detected in 95% of patients.

1.9 Anti-Neuronal Antibodies in Post-Streptococcal Diseases of the CNS

The post-Streptococcal neurological diseases described above have all been postulated to be diseases in which the phenomenon of molecular mimicry may play a role in the pathogenesis. Epidemiological studies have demonstrated a relationship between GABHS in all the neurological diseases described above thus fulfilling the first criterion for molecular mimicry (see section 1.7.3). In addition circumstantial evidence for the involvement of an aberrant immune response in the pathogenesis of SC, PANDAS, TS, EL and ADEM can be inferred from the apparent therapeutic benefit of immunomodulatory therapies such as the use of IVIG, Plasmapheresis and immunosuppression. Finally, two independent studies have been able to produce stereotypical movements in rats after infusion of serum from patients with TS suggesting the presence of a serum soluble factor capable of inducing disease (Taylor *et al*,.2002; Hallet *et al*, 2000).

More compelling evidence for an autoimmune response has been provided by studies that have detected anti-neuronal antibodies in the serum and CSF of patients with post-

streptococcal neurological disorders. The first evidence for anti-neuronal antibodies was provided in 1976 by Husby *et al* (1976). Immunoflourescent staining of the cytoplasm of neurones in the caudate and thalamus was found in 46 % of SC patients compared to 14 % of patients with RF. In addition a correlation between antibody titre and clinical status was described. The existence of anti-neuronal antibodies associated with SC has been confirmed by a number of subsequent studies one of which (Morshed *et al*, 2001) described a similar cytoplasmic staining of neurones to that described by Husby *et al*. However, probing immunoblots of rat brain with patients' serum failed to demonstrate a consistent anti-neuronal response.

In 1998, Singer *et al* demonstrated the existence of anti-neuronal antibodies in the serum of patients with TS. Using both an enzyme linked immunosorbent assay (ELISA) and immunoblot of human caudate and putamen tissue a more frequent anti-neuronal response was demonstrated in TS patients compared to controls. The immunoblots demonstrated a frequent antibody response against 83, 67 and 60 kDa antigens. Attempts to refine the detection methods using a neuroblastoma cells line proved disappointing. The association of antibodies against proteins of a specific molecular weight was lost unless powerful statistical tests were applied to the immunoblots (Singer *et al*, 1998).

Church *et al* re-examined the existence of anti-neuronal antibodies in patients with SC using both an ELISA and immunoblot of human basal ganglia tissue. The results broadly concurred with previous findings. Anti-neuronal antibodies were detected in 95% (ELISA) and 100% (Immunoblotting) of patients with acute SC (Church *et al*, 2002). In addition, immunoflourscence detected staining of neuronal tracts in the

caudate, although a detailed description of the staining pattern was not provided. The most important finding of this paper was the description of anti-neuronal antibodies directed against a discrete set of proteins with molecular weights of 40, 45 and 60 kDa. Following the successful demonstration of anti-neuronal antibodies in SC the group looked at other post-streptococcal neurological disorders. Antibodies against a similar set of proteins to those described in SC were also detected in the serum of patients with PANDAS (40, 45 and 60 kDa) (Church *et al*, 2004) while immunoflourescent studies replicated the staining pattern of basal ganglia neurones (Church *et al*, 2003). In addition antibodies against the 40, 45 and 60 kDa antigens were detected in patients with post-streptococcal EL (Dale *et al*, 2003) and antibodies against a 60 kDa protein were also detected in the serum of patients with post-streptococcal ADEM (Dale *et al*, 2001).

1.10 Putative Antigens in Post-streptococcal Diseases of the CNS

Given the close association between streptococcus and the neurological and psychiatric symptoms in these disorders it is reasonable to suggest that the antibodies may recognise common antigenic targets. Thus, serum was used in combination with various protein purification techniques to characterise the target antigens. Thus far, four potential antigenic targets have been identified. These are α and γ enolase, aldolase C and pyruvate kinase (PYK).

First discovered in 1934, α-Enolase is a 47 kDa glycolytic enzyme that catalyses the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate in the forward direction of the second half of the Emden Myerhoff-Parnas glycolytic pathway. The reaction occurs in the reverse direction during gluconeogenesis. The enzyme is classed as a

metalloenzyme due to the requirement of divalent cations (naturally Mg^{2+}) as a cofactor for its function.

Enolase exists as three isoenzymes: α-Enolase is found in a variety of tissues, β-Enolase is restricted almost entirely to muscle tissue and γ-Enolase is restricted to neuronal and neuroectodermal tissue. Enolase is one of the most abundant cytosolic proteins but has also been demonstrated to exist on the surface of a variety of eukaryotic cells. In addition, α-Enolase has been reported to occur as a surface protein on GABHS making it an interesting candidate in the molecular mimicry hypothesis of immune mediated disorders. α-Enolase has been shown to bind other glycolytic enzymes with a high affinity, one of which, pyruvate kinase (PYK) has been implicated as a potential antigen in post-streptococcal immune mediated diseases of the central nervous system.

α-Enolase has been implicated in a number of diseases. Levels of α-Enolase are increased in association with neuroblastoma and small cell lung cancer while antibodies to the protein have been implicated in cancer-associated retinopathy (CAR) and have been detected in a wide variety of autoimmune diseases ranging from SLE, ulcerative colitis and autoimmune hepatitis. Interestingly, anti-α-Enolase antibodies have previously been hypothesised to have an important role in the development of post-streptococcal movement disorders (Fontan *et al*, 2000). It is unclear why an antibody to a ubiquitous enzyme should result in unassociated autoimmune disorders. One explanation may be that the epitope recognised by the antibodies is different between diseases. An epitope study into CAR sera revealed that it was only pathogenic when it recognised a certain epitope (Adamus *et al*, 1998). It is possible that a reaction to a distinct epitope could result in a given pathology.

Aldolase C is one of the three forms of the enzyme fructose-1, 6-bisphosphate aldolase found in mammals. Aldolase A and B have similar enzymatic activities as Aldolase C on the substrates fructose-1, 6-bisphosphate and fructose-1-phosphate but act with differing affinities. Aldolase A is essentially ubiquitously expressed in all tissues acting as a key enzyme in the glycolytic pathway. Aldolase B expression is restricted to the liver. Expression of Aldolase C is enriched within the cerebellum distributed in stripes in the Purkinje cell layer of the cerebellum as well as in the inferior olives and sensory neurones of the posterior horn of the spinal cord. This distribution has lead to the hypothesis that the protein has functions beyond that of glycolysis within the nervous system (Buono *et al*, 2001). As with α-Enolase the aldolases are a conserved group of molecules. Forms exist in GABHS thus making aldolase a potential molecular mimic. The occurrence of an antibody response against the C isoform of the protein is compatible with the production of a neurological disease.

1.11 Post-Infectious Opsoclonus-myoclonus.

As discussed in section 1.2.4 OM may have a paraneoplastic or post-infectious cause. The incidence of PIOM is difficult to estimate for a number of reasons:

1: OM is rare.

2: Many case reports document the absence of a tumour but do not thoroughly investigate the patient for an identifiable causative agent. Often tumour negative OM cases will be labelled as 'Idiopathic'.

3: Neuroblastoma, commonly associated with OM may undergo spontaneous remission.

Despite these limitations Digre's (Digre, 1986) review of 58 case reports of OM was able to attribute a paraneoplastic cause to only 19 % of cases. In the remaining 34 cases an infectious aetiology was implied in 26 and a pathogen positively identified in 8 cases. The study may have carried a selection bias for infectious cases or been conducted before the development of high resolution scanning techniques capable of detecting small tumours but it implies that infection is a common cause of adult OM. The finding that patients with post-infectious illness were often younger than 40 years old is broadly supported by the investigations of Bataller *et al.* Indeed there were a number of common findings between Batallers and Digre's findings (Table 1-5).

Table 1-5: Comparison of two cohorts of post-infectious OM.

Finding	*Digre et al*	*Batallers et al*
Total number of cases,	58	24
Number of 'Idiopathic' cases	38	10
Mean age onset, years (range)	35 (19-73)	40 (24-80)
Male: Female	0.8:1	1:1
Clinical features,		
Opsoclonus	100 %	100 %
Myoclonus	47 %	70 %
Ataxia	64 %	100%
Outcome,		
Complete remission	79%	60%
Mild neurological deficit	9%	40%
Severe neurological deficit	0%	0 %
Death	6%	0 %
Unknown	6%	0 %

Identified infectious agents in OM include Psittacosis, Rickettsia, Coxsakie virus (B3 and B2) and Salmonella. More recent reports have suggested that the onset of OM may follow infection with Epstein-Barr virus (Delreux, *et al*, 1989; Verma and Brozman, 2002) and Enterovirus (Imtiaz and Vora, 1999;Wiest *et al*, 1997).

The underlying pathological mechanism for PIOM is unclear. Post-mortem studies described similar changes to those seen in POM. There is limited evidence for a consistent anti-neuronal response, although there is evidence for the benefit of immunomodulatory therapies. Plasmapheresis (Yiu *et al*, 2001) and IVIG (Glatz, *et al*, 2003) have both been associated with improvement in the clinical syndrome. In

Batallers adult cohort eight patients had complete recovery which was hastened by steroid therapy. In two patients the OM followed a relapsing course which was effectively treated with IVIG.

Perhaps the most interesting point regarding PIOM is the frequency with which recovery occurs. Between 60 and 79 % of patients are reported to have complete resolution of neurological symptoms. This is in stark contrast to findings in adult paraneoplastic disease in which the average survival is nine months, and paediatric POM in which 50 % of patients will continue to experience significant neurological deficit.

1.12 Summary and Aims

With respect to this thesis the B cell response to antigens in the nervous system represents an interesting phenomenon for a number of reasons. These include the identification of novel insights into the biology of tumours and the nervous system while providing antigenic targets which may later be utilised in diagnostic test. To date a number of anti-neuronal antibodies have been described, however there is reason to believe from our own experience and recent research that as yet uncharacterised antibodies remain undetected. There is significant overlap between the pathology of paraneoplastic and post-infectious neurological disease. Methods that apply to antigen identification can be applied to antibodies associated with both paraneoplastic and post-infectious neurological disease.

I propose to use a range of approaches involving molecular biology and proteomic techniques to detect as yet unrecognised anti-neuronal antibodies and identify the autoantigens. The broad aims of the thesis are:

1: The cloning and production of recombinant forms of the putative antigens recognised by antibodies in the serum of patients with post-streptococcal neurological disease in order to substantiate previous observations and develop the techniques for application to as yet undefined antigens associated with paraneoplastic and post-infectious disease.

2: The identification of patient(s) with as yet undescribed anti-neuronal antibodies in paraneoplastic and post-infectious neurological disease.

3: The characterisation of putative antigens by the application of established protein purification techniques or bacteriophage library screening.

4: The development of recombinant forms of putative antigens for the substantiation of findings and screening of larger patient populations.

The methods used and an overview of the principles used in the following chapters are detailed therein. In addition, a methods section detaling common protocols is provided in chapter 8 and will be refered to at the relevant points throughout the chapters.

2 *Chapter 2: Production of putative antigens recognised by antibodies of patients with post-infectious neurological disease.*

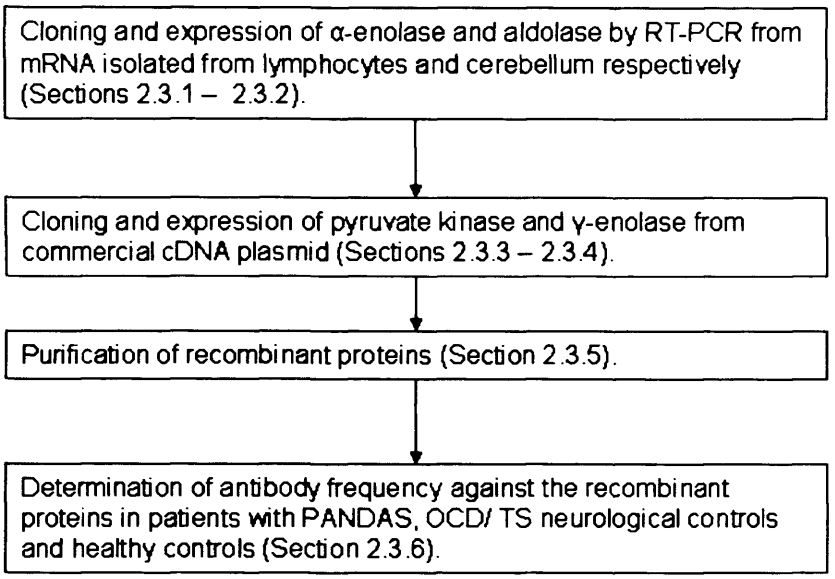
2.1 Introduction.

As described in the opening chapter an increasing number of neuropsychiatric disorders are thought to be immune mediated. Evidence to support this theory comes from the apparent success of immunomodulatory therapies and the detection of antibodies directed against neuronal tissue. Four potential auto-antigens recognised by antibodies in the sera of patients with post-streptococcal neuropsychiatric disease have been described. These are PYK, aldolase C and α and γ-enolase. Recombinant forms of these proteins were required in order to:

1: Confirm that these proteins were indeed the targets of an antibody response.

2: Determine the frequency with which antibodies against these proteins occur in patient and control groups.

An introduction to the principles of the techniques used in this chapter is provided below. A summary of the experiments is provided on the following flow chart.



2.1.1 Cloning cDNA.

The polymerase chain reaction (PCR) is the most convenient way to amplify a specific sequence of DNA. The reaction requires the use of two oligonucleotide primers complementary to sequences at either end of the DNA sequence to be amplified, orientated in such a way as to allow the polymerase to proceed through the region between the two primers. Following denaturation of the DNA template the primers bind their target sequence. This allows bases complementary to the rest of the DNA template to be added to the 3' end of the primer by the enzyme *taq* polymerase. This reaction produces a new template which can be denatured and hybridised by the primers. Repeated cycles of denaturation, hybridisation and extension result in the exponential accumulation of PCR product.

Reverse transcription followed by PCR (RT-PCR) is a method by which RNA isolated from a suitable source can be converted into cDNA. The major attraction of this method with respect to the production of recombinant proteins is that the cDNA produced

represents a 'mature' form of gene expression in which the introns have been removed to leave the exons in a continuous stretch of coding sequence. The downstream primer necessary for first strand synthesis can take the form of random hexamers or oligo-dT primers designed to hybridise the polyA sequences at the 3' end of mature mRNA. If the sequence of the desired product is known then primers can be designed to target that specific sequence of RNA in first strand synthesis resulting in the increased specificity of the amplification process. In the experiments conducted below RT-PCR reactions were conducted using the SuperScript one-step RT-PCR kit (Invitrogen).

2.1.2 Primer Design.

Knowledge of the sequence to be amplified in the RT-PCR procedure and the vector into which the resulting cDNA is to be ligated allows the investigator to design primers that amplify the desired sequence and introduce appropriate restriction sites into the cDNA. By incorporating a different restriction site at each end of the amplified sequence cloned products can be directionally inserted into an appropriate vector. This is achieved by extending the 5' sequence of the oligonucleotide primers used in the RT-PCR or PCR to introduce the appropriate restriction enzyme recognition sequence.

2.1.3 Bacterial Expression Vectors.

A number of bacterial expression vectors are available for recombinant protein production. Such vectors usually incorporate a multiple cloning site (MCS) into which DNA sequences coding the desired protein can be inserted. The vectors allow cloning of the insert into the correct reading frame either through the use of restriction sites or through the provision of 3 independent vectors covering all three reading frames. In many vectors an affinity tag will be situated at the 3'- or 5'- end of the MCS in order to

allow the purification of a fusion protein product. In designing any vector suitable for the transcription and translation of the inserted DNA a number of basic factors are required. These include strong, inducible promoters for transcription, a ribosome binding site, a start and stop codon for optimal translation efficiency and a transcription terminator sequence. The plasmid should also carry an origin of replication (Ori) as well as genes conferring antibiotic resistance as a means to facilitate selection of transformed bacteria.

2.1.4 pRSET

The bacterial expression vectors pRSETA, pRSETB and pRSETC (Figure 2-1) are derived from the pUC expression vectors and are designed for high-level expression and purification of recombinant proteins from cloned genes in *E. coli.* Cloned DNA sequences are positioned downstream of a T7 promoter which makes high-level expression possible. Located downstream from the T7 promoter is an ATG start codon, polyhistidine tag, a T7 gene 10 leader sequence to stabilise RNA transcripts, an Xpress epitope and an enterokinase cleavage site. A MCS containing 11 unique restriction sites follows these sequences. The polyhistidine tag provides a metal binding domain through which fusion peptides can be purified by affinity chromatography. The enterokinase site located between the 3 kDa His-tag and the recombinant protein allows the removal of the tag from the recombinant protein assuming that an enterokinase sequence is not encoded within the inserted DNA sequence.

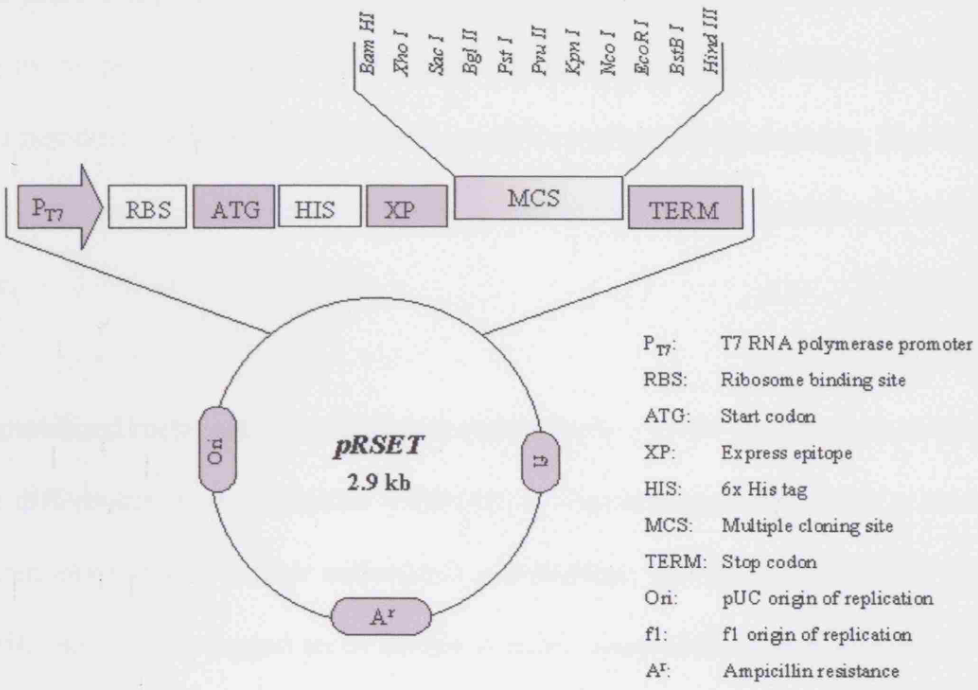


Figure 2-1: The pRSET bacterial expression vector.

2.1.5 Purification of Recombinant Proteins.

The detection and purification of recombinant proteins from crude bacterial homogenates has been simplified through the introduction of bacterial expression vectors capable of expressing proteins which have been suitably 'tagged'. The tag is created by the integration of a short stretch of DNA encoding a known amino acid sequence on to the 5'- or 3'- terminus of the target gene. The resulting hybrids are then expressed as fusion proteins with the affinity tag attached to the N- or C- terminal of the protein. Knowledge of the physical and chemical properties of the tag allows the selection of a suitable method for subsequent purification of the target protein.

The pRSET expression vector used in these experiments results in the fusion of a His-tag to the protein of interest. The purification of His-tagged proteins exploits the fact that histidine is not an abundant amino acid in most bacterial proteins. The introduction of stretches of histidine residues to the protein results in the creation of a metal binding site.

Immobilised metal chelate affinity chromatography (IMAC) is a technique that exploits the differences in the affinities exhibited by various amino acids for a metal ligand, commonly nickel, for their subsequent purification. The technique can be used for the purification of His-tagged recombinant proteins. Essentially, metal ions are immobilised onto a matrix backbone contained within a chromatography column which has been chemically modified by the addition of a polydentate ligand. The addition of the metal ions produces a metal ion chelate complex with the potential to form co-ordinant bonds with electron donor groups located on the surface of proteins. IMAC procedures are based on bond formation between the imidazoyl side chain of histidine with immobilised Nickel (II) ions. Bacterial homogenates can be passed through and washed from the column before specifically bound proteins are eluted by the introduction of imidazole. The imidazole acts as a competitive ligand for the metal ions thus disrupting the metal-protein interaction resulting in elution from the column.

Although the His-tag system has been widely employed the most important limitation with the system is the reduced selectivity of the IMAC column for the fusion protein when there is an abundance of host proteins. This is potentially problematic in situations where the recombinant proteins are required for immunoblot analysis or ELISA in which patients serum is employed since most patients will have anti-*E. coli* antibodies resulting in some background signal in the various tests.

Contamination of recombinant protein preparations with host proteins can be reduced by the inclusion of a low concentration of imidazole in the binding buffer. The low concentration of imidazole acts by competing with the aromatic and aliphatic amines of host proteins whilst remaining low enough to facilitate the binding of the fusion-protein to the metal ions. In addition, the use of an elution gradient in which the concentration of imidazole is gradually increased can aid purification by eluting weakly-bound bacterial proteins before the His-tagged proteins are eluted.

2.2 Methods.

2.2.1 Preparation of Lymphocytes for mRNA Isolation.

Lymphocytes were isolated from whole blood of a healthy volunteer using the Accuspin System-Histopaque-1077 (Sigma). Histopaque tubes were brought up to room temperature prior to use. Fresh, anti-coagulated blood was collected and lymphocytes isolated within 2 hours. 15 ml of blood were poured into the Accuspin tube which was then centrifuged at 800 g for 15 min. The plasma layer was aspirated and discarded and the mononuclear band transferred to a sterile centrifuge tube. The cells were suspended in 10 ml PBS and centrifuged at 250 g for 10 min. This wash step was repeated twice. The cells were resuspended for a final time in 15 ml PBS and the cell count determined by microscopy. The cells were aliquotted into 1 ml fractions containing approximately $1x10^6$ cells, snap-frozen in liquid nitrogen and stored at minus 80°C until required.

2.2.2 *Preparation of Brain Tissue for mRNA Extraction.*

Snap-frozen human cerebellar tissue from a neurologically normal individual was obtained from the UCL brain bank. 0.5 cm^2 sections of tissue weighing no more than 50 mg were prepared under liquid nitrogen and stored individually at -80°C until required.

2.2.3 *Total mRNA Isolation.*

Total mRNA was isolated using a Micro-FastTrack kit (Invitrogen). Micro-FastTrack lysis buffer was prepared by the addition of 20 µl of Protein/RNase degrader per ml of lysis buffer. 1 ml of isolated lymphocytes or one section of brain tissue was resuspended in 1 ml lysis buffer. The lysate was passed through a 1 cc syringe fitted with a 21 gauge needle until the solution was no longer viscous then incubated at 42°C for 20 min to promote protein degradation and inactivation of ribonucleases. The NaCl concentration of the lysate was standardised by the addition of 63 µl 5 M NaCl to each 1 ml of lysate which was then added to a vial of oligo-dT cellulose and left to stand for 2 min. The vial was rocked at room temperature for 20 min and centrifuged at 4000 g for 5 min to precipitate the oligo-dT cellulose. The supernatant was aspirated from the cellulose and discarded. 100 µl of elution buffer was stirred into the cellulose slurry, which was transferred to a spin-column and centrifuged prior to the addition of a further 100 µl of elution buffer. 10 µl of 2 mg/ml glycogen carrier, 30 µl 2 M sodium acetate and 600 µl of absolute alcohol were added to the flow-through from the column and the solution frozen on dry ice. The sample was thawed and centrifuged at 16000 g for 15 min at 4°C. The supernatant was aspirated and the mRNA resuspended in 2.5 µl of elution buffer. The isolated mRNA was used immediately.

2.2.4 *RT-PCR Amplification of α-Enolase and Aldolase C.*

The coding sequences for human α-Enolase (GenBank accession no: M14328) and aldolase C (GenBank accession no: NM_005165) were obtained and analysed using Webcutter v2.0 (www.rna.lundberg.gu.se/cutter2). Sense and anti-sense primers were designed to flank the complete coding sequence and introduce unique restriction sites suitable for ligation into the selected expression vector (Table 2-1). All primers were synthesised by Sigma Genosys. RT-PCR was used to amplify cDNA encoding α-Enolase and aldolase C using mRNA isolated from lymphocytes and brain respectively.

The RT-PCR reaction was performed using a Superscript one-step RT-PCR kit (Invitrogen, UK). The following components were combined in a 0.5 ml tube: 25 µl 2x reaction mix, 2.5 µl of isolated mRNA, 1 µl sense primer (10µM), 1 µl anti-sense primer (10 µM), 2 µl RT/Platinum *taq* HiFi mix. DNase/RNase free water was added to a final volume of 50 µl. A control reaction substituting Ampli*taq* gold for RT/Platinum *taq* HiFi mix was used to confirm the absence of genomic DNA in the mRNA preparation. In a second control RNA was substituted with 2.5 µl H_20. All RT-PCR reactions were conducted under the conditions shown in Table 2-2. RT-PCR reaction products were analysed on a 0.7 % agarose gel (Chapter 8).

Table 2-1: mRNA/cDNA source, primers and predicted length of cDNA encoding the target antigens following RT-PCR or PCR.

Protein	*mRNA/ cDNA source*	*Primers*	*Product Size (bp)*	*Required vector*
α-Enolase	Human lymphocytes	Sense (BamHI site underlined): 5'-CA GGA TCC ATG TCT TAT TCT CAA GAT CCA T-3' Anti-sense (Hind III site underlined): 5'- GCC CAA GCT TAC TTG GCC AAG GGG TTT C-3'	1321	pRSETA
Aldolase C	Human cerebellum	Sense (BamHI site underlined): 5'-TCC AGG ATC CAT GCC TCA CTC GTA CC -3' Anti-sense (Hind III site underlined): 5'- GCT AAA GCT TCA GTA GGC ATG GTT GGC AAT G -3'	1095	pRSETA
PYK	I.M.A.G.E clone (ID: 3859987)*	Sense (Bam HI site underlined): 5'- CGC GGA TCC ATG TCG AAG CCC C -3' Anti-sense (Hind III site underlined): 5' – AAA GAA TTC TCA CGG CAC AGG AAC AAC ACG –3'	1595	pRSETA
γ-Enolase	I.M.A.G.E clone (ID: 3629603)*	Sense (Xho I site underlined): 5'- GGT ACT CGA GAT GTC CAT AGA GAA GAT CTG GGC -3' Anti-sense (Hind III site underlined): 5' – TCC AAG CTT CAC AGC ACA CAC TGG GAT TAC G –3'	1304	pRSETA
γ-Enolase (Truncated)	I.M.A.G.E clone (ID: 3629603)*	Sense (Xho I site underlined): 5'- GGT CAG ACT CGA GAT CCT GGA CTC CCG C -3' Anti-sense (Hind III site underlined): 5' – TCC AAG CTT CAC AGC ACA CAC TGG GAT TAC G –3'	1274	pRSETA

* I.M.A.G.E clone contains cDNA which was amplified directly by PCR.

Table 2-2: Reaction conditions for the RT-PCR amplification of α-Enolase and aldolase C.

First strand synthesis and pre-denaturation	PCR amplification		Final extension
1 cycle	35 cycles		1 cycle
55°C for 30min	Denature:	94°C for 15s	72°C for 10min.
94°C for 2min	Anneal:	60°C for 30s	
	Extend:	68°C for 1.5min	

2.2.5 The Pyruvate Kinase and γ-Enolase I.M.A.G.E Clones.

Bacteria carrying plasmids containing cDNA for PYK M1 (I.M.A.G.E ID: 3859987) and γ-Enolase (I.M.A.G.E ID: 3629603) were obtained from the I.M.A.G.E consortium library (MRC geneservice, Cambridge). The bacteria were streaked onto SOB ampicillin plates and incubated at 37°C overnight. The following day individual colonies were grown at 37°C in 5 ml SOB ampicillin over night. 1 ml of bacteria was used to produce glycerol stocks which were stored at -80°C. Plasmids were extracted from the remaining culture (Chapter 8).

2.2.6 PCR Amplification of Pyruvate Kinase and γ-Enolase.

The coding sequences of PYK and γ-Enolase were analysed using Webcutter v2.0. Sense and anti-sense primers were designed to flank the complete coding sequence and introduce suitable restriction sites for ligation into the selected expression vector (see Table 2-1). A sense primer for the amplification of truncated γ-Enolase was designed 10 bp downstream of the start codon. PCR was conducted using Ampli*taq* gold polymerase. Plasmid DNA was omitted from the control reaction. cDNA was amplified

directly from the plasmid using the reaction conditions shown in Table 2-3. PCR products were analysed by electrophoresis in a 0.7% agarose gel (Chapter 8).

Table 2-3: PCR reaction conditions for the amplification of PYK, γ-Enolase and truncated γ-Enolase.

Denaturation	*Amplification*	*Final extension*
1 cycle	35 cycles	1 cycle
94°C for 10min.	Denature: 94°C for 30s.	72°C for 10min.
	Anneal: 55°C for 30s	
	Extend: 72°C for 2min	

2.2.7 Preparation for Expression of Recombinant Proteins.

The required pRSET expression vector (A, B or C) was selected following application of the translate programme (www.expasy.org/tools/dna) to determine which vector would allow expression of full length protein (see Table 2-1).

Following PCR or RT-PCR the amplified DNA fragments were purified from the agarose gel and sequentially digested (Chapter 8) using the restriction enzymes specified in Table 2-1. The required pRSET expression vector was digested with the corresponding enzymes and the DNA and vector ligated. Chemically competent TOP10F' cells were transformed and selected on SOB containing ampicillin and tetracycline. The following day colonies were selected, grown in 5 ml SOB and plasmids isolated. Single enzyme restriction analysis was used to determine if ligation

had been successful. DNA sequencing was performed (Chapter 8) and the sequence analysed using the Translate (www.expasy.org) and BLAST (www.ncbi.nih.gov/BLAST) tools to confirm ligation into the correct reading frame and the identity of the ligated DNA respectively. The pI/MW prediction programme (www.expasy.org) was used to predict the molecular weight of the protein. Expression vectors containing cDNA in the correct reading frame were used to transform competent *E. coli* BL21 (DE3) pLysS in preparation for expression of recombinant protein.

2.2.8 Expression of recombinant antigens

E. coli BL21 (DE3) pLysS (Invitrogen) containing the required plasmid were grown on SOB chloramphenicol, ampicillin plates. Seed cultures were prepared the night before expression by inoculation of SOB broth containing ampicillin and chloramphenicol with a single colony. The following day the cultures were diluted to an optical density (OD) of 0.1 in pre-warmed SOB (no antibiotics). These cultures were grown at 37°C to an OD of 0.4 to 0.6 and protein production induced by addition of 1 mM IPTG. After 3 hours the cells were harvested by centrifugation, resuspended in His-binding buffer (8M urea, 20mM Tris-HCL, 0.5M NaCl, 20mM Imidazole, 1mM 2-mercaptoethanol) containing bacterial protease inhibitors (Sigma) and subjected to four 10 second rounds of sonication on ice. The crude bacterial homogenates produced were used in immunoblots to confirm expression of recombinant protein prior to purification.

2.2.9 Purification of Recombinant Antigens.

Each of the recombinant antigens were purified using a HiTrap Chelating column (Amersham biosciences, UK) and elution gradient as described in Chapter 8. Purified proteins were subjected to polyacrylamide gel electrophoresis (PAGE) and stained to

confirm purification. The total protein concentration of each antigen was determined and diluted as required (see 2.2.11).

2.2.10 Patients and Controls.

Patient and control samples were investigated for the presence of antibodies against recombinant PYK, aldolase C and α and γ-Enolase. 131 blinded serum samples were kindly provided by Dr R Dale. All diagnoses had been confirmed by the supplying clinician although specific clinical details were not available. The number in each group is given in Table 2-4.

Table 2-4:Patient and control serum samples used for antibody detection.

Serum Source	*Number*
Healthy control	31
Neurological control	30
Encephalitis	10
Epilepsy	10
Developmental	10
PANDAS	30
OCD	20
TS	20

2.2.11 Antibody Detection Using Recombinant Protein Immunoblots.

Unblinded serum samples with and without antibodies against each antigen were determined using the method for anti-neuronal antibody detection described by Dale *et al* (2001). Serial immunoblots blots were then conducted using various dilutions of recombinant antigen in order to determine the concentration of antigen that gave the best discrimination between antibody positive and negative samples. Total protein was calculated as described (Chapter 8).

1.5 μg, 5 μg , 6.5 μg, and 13 μg grams of recombinant α-enolase, γ-enolase, aldolase C and PYK respectively were loaded into a single 4-12 % Bis-Tris gels and (Invitrogen) and electrophoresed in MES buffer for 40 minutes prior to transfer and blocking of nitrocellulose. Serum samples were diluted 1:300. A positive control antibody was included on each blot. Interpretation was conducted blindly by Drs Church, Chapman and Dale. Blots were assessed as positive, negative or equivocal. Two similar responses were required to label a sample as positive or negative. Equivocal blots (ie no consensus reached) were repeated once and counted as negative if no consensus was reached on the second blot.

2.3 Results.

2.3.1 Cloning and Expression of Human α-Enolase.

2.3.1.1 RT-PCR Amplification and Subcloning of Human α-Enolase.

The RT-PCR amplification of human α-Enolase was conducted using mRNA isolated from two aliquots of snap frozen lymphocytes. Electrophoretic analysis of the RT-PCR reaction mixture demonstrated the amplification of a 1300 bp fragment of DNA (Figure

2-2). The absence of DNA in the *taq* and water controls confirmed the absence of genomic or mRNA contamination in the lymphocyte mRNA preparation or reaction reagents.

Following ligation, transformation and antibiotic selection eight colonies of TOP10F' were isolated. There were no colonies on the negative control plate. Single restriction enzyme analysis of the recovered plasmids revealed six of the colonies contained plasmid DNA of approximately 4300 bp size indicating that ligation of the RT-PCR product had occurred (Figure 2-3). These plasmids were designated pRSETAαENO.

A BLAST search using the DNA sequence of the cDNA confirmed that the encoded protein was indeed that of α-Enolase. Application of the translate program confirmed that cDNA had been ligated into the correct reading frame. The predicated molecular weight of the protein was 50 kDa.

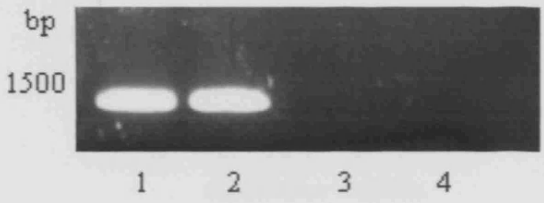


Figure 2-2: RT-PCR amplification of human α-Enolase

Lane 1 and 2: amplification of mRNA isolated from lymphocytes. Lane 3: *taq* control. Lane 4: no mRNA added. A fragment of DNA approximately 1300 base pairs in length was successfully amplified from the mRNA isolated from human lymphocytes. Both control reactions were negative signalling that the amplified DNA was not from contamination.

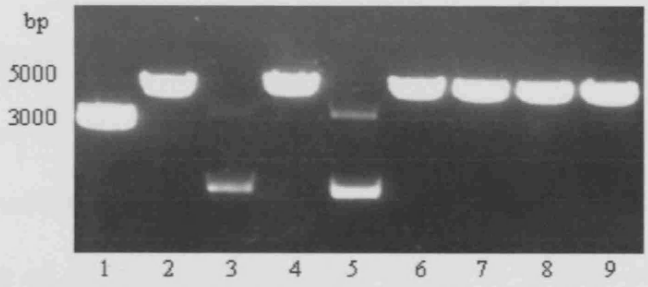


Figure 2-3: Selection of ligated pRSETαAENO.

Plasmids were extracted from transformed *E. coli* TOP10F' and subjected to restriction analysis. Lane 1: Un-ligated pRSETA (control). Lanes 2-9: plasmid extracted from transformed colonies. Colonies 2, 4, 6-9 contained a plasmid of approximately 4300 bases, the predicted length of successfully ligated pRSETAαENO. These plasmids were sequenced to confirm the identity and reading frame of the insert.

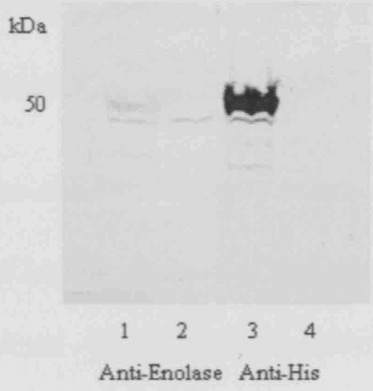


Figure 2-4: Detecion of recombinant α-Enolase in bacterial homogenate.

Bacterial homogenates of pRSETAαENO and pRSETA only were probed with antibodies against α-Enolase or His tag. Lane 1 and 3: pRSETAαENO. Lanes 2 and 4 pRSETA only. The dominant band recognised by the anti-α-Enolase and anti-His antibodies at 50 kDa was not present in the pRSETA control.

2.3.1.2 Confirmation of Recombinant α-Enolase Expression.

Immunoblot analysis of homogenates of induced BL21 (DE3) pLysS containing pRSETAαENO or pRSETA control were probed with anti-α-Enolase or anti-His antibodies to confirm protein expression. Bands corresponding to the predicted molecular weight of recombinant α-Enolase could be clearly seen in homogenates prepared from bacteria containing pRSETAαENO when probed with both the anti-α-Enolase and anti-His antibodies (Figure 2-4). In contrast, the anti-His antibody did not react with the pRSETA control homogenate. A protein in both the pRSETAαENO and pRSETA homogenates was detected by the anti-α-Enolase antibody. This protein is thought to represent an *E. coli* protein. The recombinant protein had undergone some degradation as evidenced by reactivity of the anti-His antibody with proteins smaller than 50 kDa but this was limited in comparison to the reactivity of the intact protein.

2.3.2 Cloning and Expression of Human Aldolase C.

2.3.2.1 RT-PCR Amplification and Subcloning of Aldolase C.

The RT-PCR amplification of human aldolase C was conducted using mRNA isolated from normal human cerebellum. Electrophoretic analysis of the RT-PCR reaction mixture demonstrated an amplified DNA molecule of approximately 1100 bp (Figure 2-5). The absence of DNA in the *taq* and water controls confirmed that the DNA was not amplified from genomic or mRNA contamination in the cerebellar mRNA preparation or in the reaction reagents.

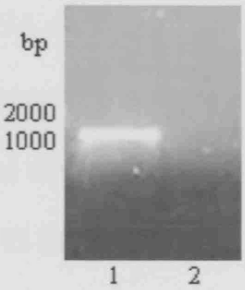


Figure 2-5: RT-PCR amplification of human aldolase C.

Lane 1: amplification of mRNA isolated from human cerebellar tissue. Lane 2: *taq* control. An RT-PCR product of approximately 1100 base pairs was successfully amplified from mRNA isolated from human cerebellum.

Ligation and transformation of TOP10F' yielded an excess of colonies in comparison to the control plate. Twenty one of these colonies were selected to determine if ligation of the cDNA had been successful. Single restriction enzyme analysis of seven plasmids isolated from transformed TOP10F' confirmed successful ligation in all seven colonies (Figure 2-6). These plasmids were designated pRSETAaldoC.

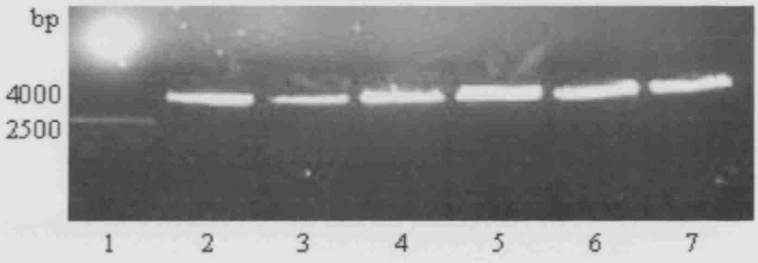


Figure 2-6: Restriction enzyme analysis of transfected colonies using HindIII.

Lane 1: Unligated, digested pRSET A. Lanes 2-7: Plasmids extracted from transformed colonies. All plasmids were approximately 4100 base pairs as predicted. Plasmids were sequenced to check the cDNA sequence and reading frame. Successfully ligated plasmids were designated pRSETAaldoC.

Sequencing and analysis of the DNA confirmed that the encoded protein was aldolase C. Application of the translate programme confirmed that the DNA had been ligated into the correct reading frame of the expression vector. The predicted molecular weight of the recombinant aldolase C was 42 kDa.

2.3.2.2 Confirmation of recombinant aldolase C expression.

Immunoblot analysis of homogenates of induced BL21 (DE3) pLysS containing pRSETAaldoC or pRSETA demonstrated the presence of His-tagged recombinant protein in homogenates containing pRSETAaldoC but not in the control preparation (Figure 2-7). A smaller protein was also recognised by both the anti-aldolase and anti-His antibodies in the pRSETAaldoC homogenate but not in the pRSETA homogenate. These smaller proteins are thought to represent breakdown products of the recombinant protein.

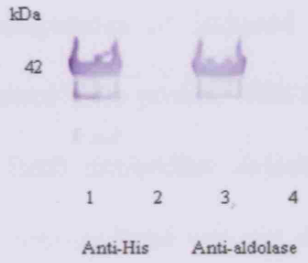


Figure 2-7: Detection of aldolase C in crude bacterial homogenates.

Lanes 1 and 3: Homogenate from *E. coli* containing pRSETAaldoC. Lanes 2 and 4: Homogenate from *E. coli* containing pRSETA control. The homogenates were probed with anti-His antibody or anti-Aldolase antibody. The anti-His and anti-aldolase antibody both detected an antigen at 42 kDa that was not present in the control preparation. This dominant band represents recombinant His-tagged aldolase C.

2.3.3 Cloning and Expression of Pyruvate Kinase.

2.3.3.1 PCR Amplification and Subcloning of Human PYK from I.M.A.G.E Clone 3859987.

PCR amplification of the cDNA contained within the plasmid I.M.A.G.E clone 3859987 resulted in the amplification of a DNA fragment of 1600 bp. No DNA amplification was detected in the control preparations. Ligation and transformation of TOP10F' resulted in the production of a number of colonies all of which contained ligated vector as determined by single enzyme restriction analysis. Ligated plasmids were designated pRSETAPYK. Sequencing analysis confirmed that the DNA encoded PYK. Application of the translate programme confirmed ligation into the correct reading frame of the expression vector.

2.3.3.2 Confirmation of Recombinant PYK Expression.

Immunoblot analysis of homogenates of induced BL21 (DE3) pLysS containing pRSETAPYK or pRSETA control were probed with anti-PYK or anti-His antibodies to confirm protein expression. Both antibodies detected a protein of 62 kDa in the pRSETAPYK homogenate. A similar band was not detected when homogenates of the control pRSETA *E. coli* strain were probed. Some proteins were detected by the anti-PYK antibody but these all corresponded to a protein recognised in the anti-His lane suggesting that these were a result of protein degradation. The concentration of the intact protein was greater than that of the smaller proteins.

2.3.4 Cloning and Expression of Recombinant γ-Enolase from I.M.A.G.E Clone 3629603

PCR amplification of cDNA encoding human γ-Enolase produced a DNA fragment of approximately 1300 base pairs. TOP10F' cells transformed with ligated vector were analysed by single enzyme digestion. Sequencing analysis confirmed the identity of the ligated cDNA and that it had been positioned in the correct reading frame. Crude homogenates of induced *E. coli* (pRSETAγENO and pRSETA control) were probed with anti-His and anti-γ-Enolase antibody and a protein of the correct molecular weight detected. Purification resulted in the isolation of a single protein band. During initial experiments to detect anti-γ-Enolase antibodies in serum samples it was noted that the HRP conjugated detector antibody reacted independently with the protein. Thus, a truncated version of recombinant γ-Enolase in which the first 10 amino acids were removed was produced. This was achieved by repositioning the sense primer downstream by 30 base pairs.

γ-enolase cDNA was amplified from I.M.A.G.E clone 3629603 using the new start primer. An amplified fragment of approximately 1300 base pairs was obtained and ligated into the expression vector. Single restriction enzyme analysis was used to confirm ligation. Ligated plasmids were designated pRSETAγENO. Analysis of the sequence data using the translate programme confirmed ligation in the correct reading frame.

2.3.4.1 *Confirmation of Recombinant γ-Enolase Expression.*

Homogenates of BL21 (DE3) pLysS containing pRSETAγENO or pRSETA probed with anti-γ-Enolase and anti-His antibody detected a 49 kDa protein in the pRSETAγENO preparation but not in the control. No proteins were detected when probed with anti-Human HRP conjugated detector antibody.

2.3.5 *Purification of Recombinant Proteins.*

Each antigen was successfully purified from the crude bacterial homogenate. An example of pre and post purification is shown in figure (Figure 2-8).

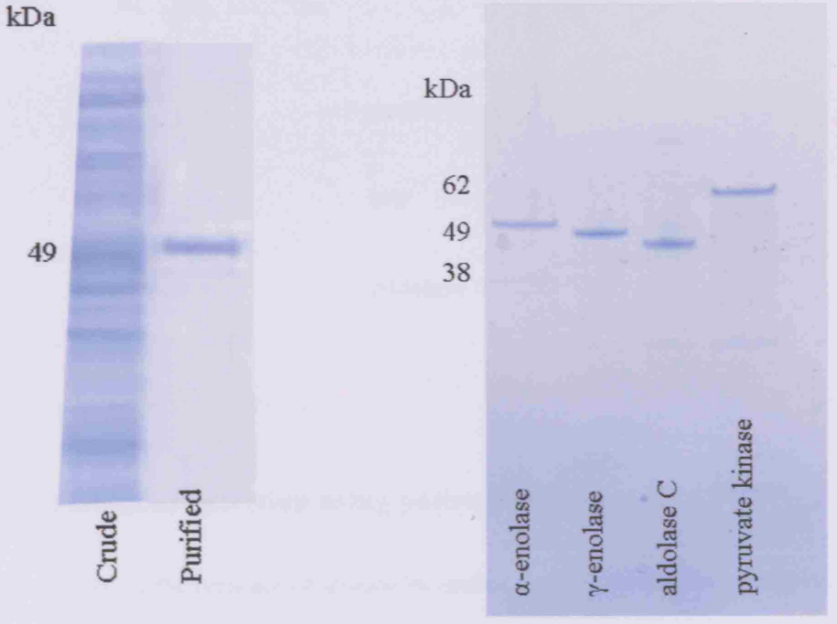


Figure 2-8: Purifcation of recombinant antigens.

Left hand figure: Pre and post purification of recombinant α-enolase. Post purification demonstrates clear enrichment of the recombinant antigen from the crude bacterial homogenate. Right hand figure: PAGE electrophoresis of all four purified antigens. Enrichment of each antigen is clearly shown. The protein concentrations used are the same as those used for antibody detection in patients serum.

2.3.6 Reactivity of Patients Serum with Recombinant Putative Antigens.

Each purified antigen was probed with commercial, patient and control antibodies. Representative Immunoblots are shown in Figure 2-9.

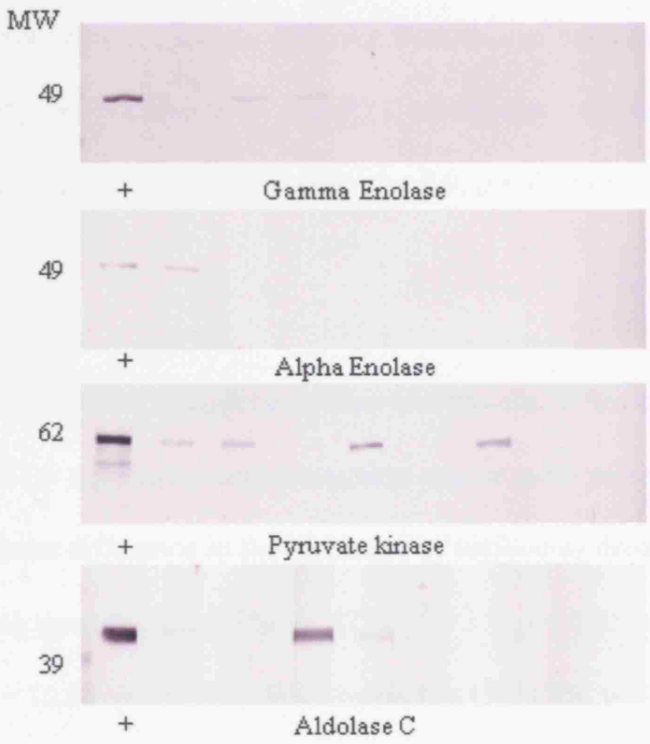


Figure 2-9: Recombinant detection using patients serum.

Patients serum was tested for the presence of antibodies against the four recombinant antigens. Representative immunoblots are shown. + = positive control antibody.

Sera from patients with PANDAS was positive against α-enolase, γ-enolase, aldolase C and PYK was positive in 23%, 32%, 10% and 35% of samples respectively (Figure 2-10). The incidence of antibodies against recombinant γ-enolase in PANDAS was significantly higher than the incidence in the healthy control population (32% vs. 6%, $\chi^2 = 6.6$, $p = 0.025$) but not significantly different from that of neurological controls (32% vs. 20%). Similarly, the incidence of anti-PYK antibodies was significantly different from the healthy control group (35% vs. 6%, $\chi^2 = 7.9$, $p < 0.01$) but not the neurological control group.

Some patients had antibodies against more than one protein. When reactivity against any of the antigens i.e. a positive response against one or more proteins was considered there was a significant difference in the incidence of antibodies detected in patient sera compared with both neurological (68% vs. 47%, $\chi^2 = 3.9$, $p < 0.05$) and healthy controls (68% vs. 26%, $\chi^2 = 10.09$, $p < 0.001$). With respect to OCD/TS, we were unable to detect a significant difference in the incidence of antibodies against any of the antigens. This was also true when a response to any i.e. one or more, antigen was considered.

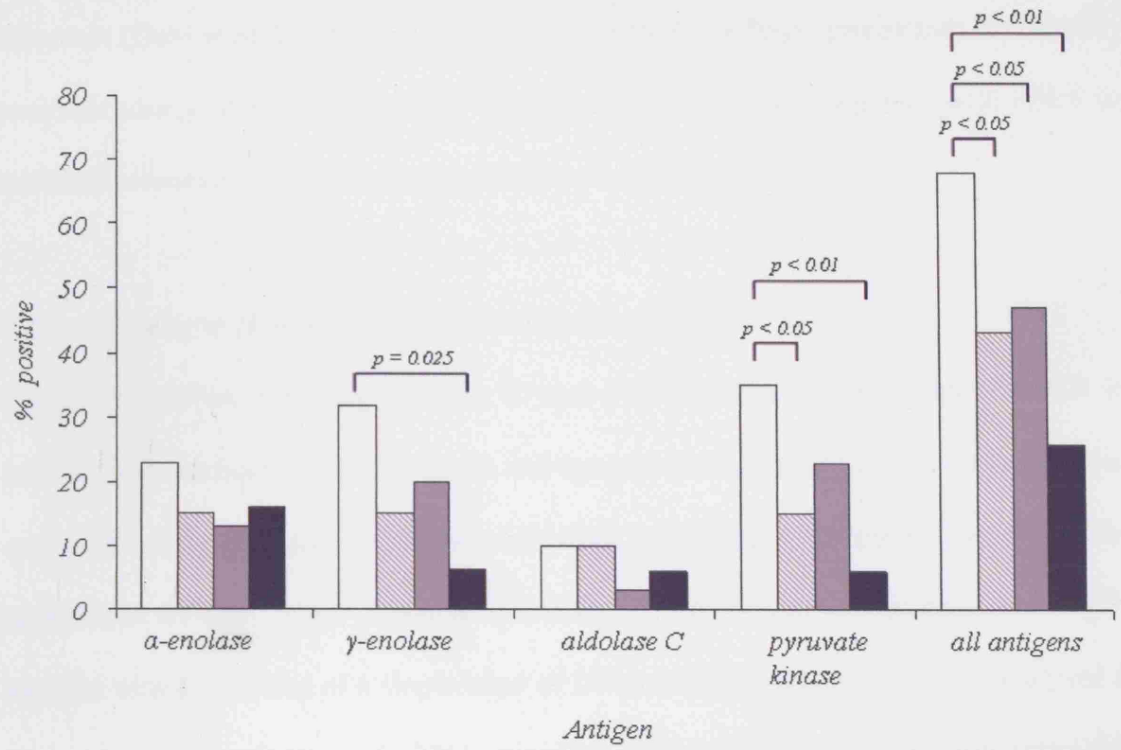


Figure 2-10: Reactivity of patient and control serum with recombinant antigens.

Serum from patients with PANDAS (□) or OCD/TS (▨), neurological (▩) or healthy (■) controls was tested against each of the putative recombinant antigens. There was no significant difference between the incidence of antibodies against α-enolase or aldolase C between any of the groups. There was a significantly increased incidence of antibodies against γ-enolase and pyruvate kinase between patients with PANDAS and healthy controls. In addition there was a significantly increased incidence of antibodies against pyruvate kinase between patients with PANDAS and OCD/TS. Antibodies against any of the antigens when considered as a whole were significantly increased in patients with PANDAS compared to patients with OCD/ TS, neurological and healthy controls.

2.4 Discussion.

Using a combination of techniques including ammonium sulphate precipitation, ion exchange chromatography and 2D gel electrophoresis the proteins α and γ-Enolase, aldolase C and PYK had been identified as candidate auto-antigens recognised by

antibodies in the serum of patients with a host of post-streptococcal neurological disorders (Dale *et al*, 2006). In order to confirm these findings, production of a readily available source of each antigen was required to determine the frequency with which an antibody response occurred in both patients and controls.

2.4.1 Cloning of Human Aldolase C, α-Enolase and PYK.

Cloning of cDNA encoding aldolase C and α-enolase was achieved by RT-PCR of mRNA isolated from cerebellar tissue and lymphocytes respectively. The reaction was conducted using gene specific primers allowing for the rapid, directional ligation of the cDNA into the expression vector. Amplification of cDNA for both proteins was highly specific with production of a single band of DNA at the correct length when analysed in an agarose gel. Production of cDNA encoding aldolase C and α-enolase was simplified by the absence of splice variants of these two proteins.

In contrast, PYK is known that two forms of the protein exist; M1 and M2. These two proteins differ only by the inclusion of Exon 9 or 10 in isoform M1 and M2 respectively (Takenaka, *et al*, 1991). The use of flanking primers would have been unsuitable for the production of cDNA from tissue mRNA, as amplification of both forms of the enzyme would have occurred. The size of the reaction products would have been similar, the M2 isoform being only fifteen bases longer than M1. This is too small to enable their separation via agarose gel electrophoresis. As a result an alternative method of cloning was required i.e. oligonucleotide screening of phage libraries. It was fortuitous that a clone containing the entire coding sequence for PYK M1 was available from the MRC geneservice. The acquisition of this clone ensured that cDNA encoding the correct PYK

isoform could be amplified while avoiding the cost, inconvenience and potential problems associated with mRNA purification and amplification and library screening.

2.4.2 Cloning and Expression of Recombinant γ-Enolase.

Unexpected problems were encountered during the detection of recombinant human γ-Enolase. Amplification of the cDNA from the I.M.A.G.E clone proved straightforward. However, problems were associated with the detection of the recombinant protein by immunoblot. Negative control lanes containing anti-Human IgG only (no primary antibody) showed evidence of binding. This had not occurred in the cases of aldolase C, α-Enolase or PYK and was not due to interaction with bacterial protein since the detector antibody did not react with crude homogenates of control *E. coli* in earlier experiments. The reasons for this phenomenon are unclear. Crude analysis (not shown) of the sequence encoded from the pRSETA start codon and the cDNA suggested an area of homology between IgG and the recombinant protein. Possible solutions to this problem involve the use of a different vector, the cleavage of the His-tag from the existing protein in an attempt to interrupt non-specific interaction between anti-Human IgG and the recombinant protein and the production of a truncated protein using downstream gene specific primers. We opted to produce a slightly truncated version of the protein by amplifying the cDNA from the IMAGE clone using a sense primer 30 base pairs downstream of the start codon in the cDNA. The resulting recombinant protein was still detected by the commercial anti-γ-enolase antibody and patients' sera whilst preventing interaction with anti-human IgG-HRP detector antibody. Thus it appears that the removal of 10 amino acids from the N terminal of the protein did not disrupt the epitope required for antibody recognition.

2.4.3 The Purification of Recombinant Proteins.

Production, purification and detection of all four recombinant proteins was successfully achieved. In some cases there was some proteolytic degradation of the recombinant proteins as evidenced by the number of proteins detected by the specific anti-His antibody. However in all cases the production of the mature protein was far in excess of these smaller fragments. In the case of α-enolase, the antigen-specific antibodies also reacted with the control bacterial homogenates. It is possible that the detected proteins represent the bacterial homologues of the human antigens.

The purification of the recombinant antigens using an elution gradient was successful. The recovery of the recombinant proteins was much greater than that of any contaminating proteins. Whilst the protein preparations used here proved suitable for antibody detection by immunoblot, the existence of homologous *E. coli* proteins and the presence of anti-*E. coli* antibodies in the serum samples may demand refinement of the purification protocol if other assays such as ELISA are to be employed. One simple method which may merit investigation would be to separate the bacterial homogenate into soluble and insoluble proteins prior to IMAC. This could remove some contaminating *E. coli* proteins in a simple step prior to column purification. Alternatively a yeast-based expression system could be employed. It may be that antibodies against yeast are found with less frequently than against *E. coli* which is a common human commensal organism.

2.4.4 Recognition of Recombinant Protein by Patients' Sera.

Since the identification of potential auto-antigens in post-streptococcal disease of the nervous system by Dale *et al* (2006a) one group has attempted to determine the

significance of the antibody response towards these proteins (Singer *et al* 2005). Their study utilised commercially available preparations of α-enolase, γ-enolase, aldolase C and PYK in immunoblots and concluded that antibodies against these proteins did not differentiate between clinical groups. However methodological concerns have been raised with regard to these findings specifically pertaining to the quantity and type of antigen used (Dale *et al*, 2006b).

In earlier publications the presence of antibodies against the putative antigens using basal ganglia immunoblots had a specificity and sensitivity of 100 % and 93 % respectively in acute SC and 69 % and 93 % in chronic SC (Church *et al*, 2002). A similar study of patients with TS revealed antibodies against the putative proteins in 24 % of patients compared with 4 % of controls whilst a study of patients with OCD detected antibodies in 42% of patients compared to 10% of controls. However, no study has yet examined the frequency with which antibodies against these putative proteins can be detected in cohorts of PANDAS patients or how recombinant forms of the antigens fare in a diagnostic test.

With respect to the data presented here it is encouraging to see that there is a significant increase in incidence of antibodies to γ-enolase, PYK and 'all antigens' in patients with PANDAS. These findings are less compelling than the figures presented for SC however, the less stringent diagnostic criteria for the diagnosis of PANDAS may by associated with a more heterogeneous cohort of patients. In addition since clinical details were not available it was not possible to correlate antibody positivity with the stage of the illness or the severity of the symptoms. Since PANDAS is said to follow a 'saw tooth' pattern of exacerbations, a serum sample taken during a 'trough' could

potentially give a negative result. Future study would require investigation of serial samples for the presence of antibodies together with clinical evaluation at each time point using a recognised disease severity score to allow the correlation of antibody positivity and clinical status.

In a recent study Kansy *et al* (2006) used quantitative immunoblotting to evaluate the antibody response against PYK in patients with TS with or without OCD. They detected an elevated antibody response in those patients with newly acquired GABHS infection and symptomatic TS. With regard to the data presented, while there was a greater number of OCD/ TS patients with an antibody response this was not significantly higher than that seen in the control populations. When OCD and TS were considered separately, 30 % and 60 % of patients were positive respectively. To further delineate the importance of anti-PYK antibodies in TS it will be important to better define the study population with regard to symptom severity and precipitating factors. In addition the use of quantitative techniques such as ELISA or quantitative immunoblotting may be required to demonstrate elevated antibody titres in study groups compared to controls.

One of the major problems with the study appears to be the frequency with which antibodies can be detected in the sera of controls. Antibodies against 'any antigen' could be detected in 47 % of neurological controls and 26 % of healthy controls. These figures are significantly higher than those quoted by other investigators and may reflect a problem with the recombinant immunoblot assay.

Although every effort was made to optimise the immunoblotting method used here a few points should be borne in mind. Firstly, no alteration was made to the dilution of serum which was maintained as that used in papers in which basal ganglia immunoblots were used i.e. 1 in 300. A systematic investigation of serum dilutions is required to determine if alternative dilutions provides enhanced differentiation between patient and control groups. In addition, alteration of the concentrations of the antigens in the immunoblots may have a similar effect. It may be of benefit for those samples deemed to be positive for antibodies to be re-screened at increasing dilutions to determine if it is possible to further differentiate between groups. These methods may reduce the number of positives to a level more in keeping with that of the literature.

Secondly, while negative control groups were included in the study (ie healthy and neurological controls) it may have been prudent to include an antigen control in the form of an irrelevant recombinant protein. Testing the samples from each group against this protein would have highlighted the degree to which false positivity occurred as a result of non-specific interaction with contaminating proteins.

Another consideration is that of epitope specificity. It is possible that a discreet antibody response against a specific epitope is required in order to produce the disease. An example of this phenomenon has been demonstrated for patients with CAR. Adamus *et al* (1996) detected antibodies against α-enolase antibodies in 11/16 (69 %) patients with CAR compared to 10/110 (11 %) of healthy controls. However, in a later study epitope mapping demonstrated an epitope recognised by patient but not control samples (Adamus *et al* 1998). To demonstrate this in post-streptococcal disorders would require epitope mapping of each antigen. In a similar manner, despite the fact that the nature of

the antigens was determined by probing denatured proteins, a subgroup of patients antibodies may require a conformational epitope in order to recognise the antigenic target.

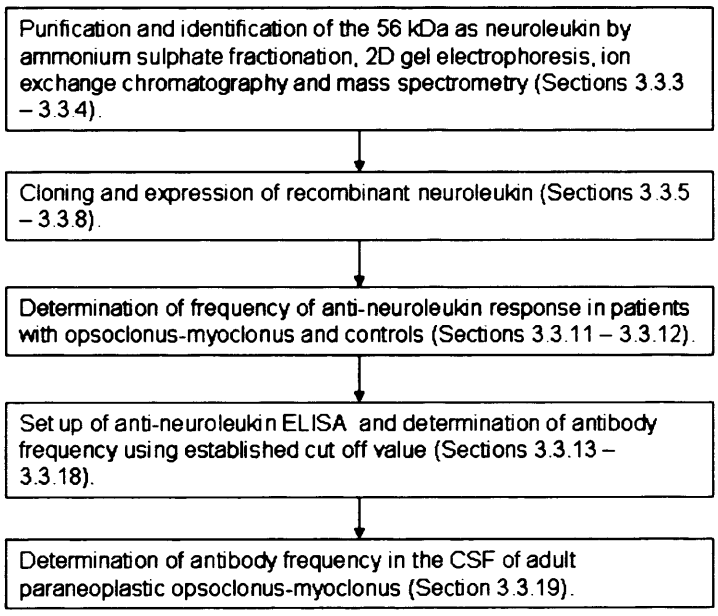
Finally, by their nature, immunoblots are subject to interpretation. Whilst the blots here were all interpreted by three investigators, there is likely to be variability in the interpretation of the immunoblots. A quantitative assessment of antibody incidence by ELISA would provide a quantitative cut off against which a positive test can be clearly defined.

In 2006, Dale *et al* showed increased rates of apoptosis in neuronal cell cultures incubated with commercial antibodies against each of the putative antigens. However, the data presented here suggest that the antibodies are not detected with increased frequency in PANDAS. Unless there is a role for antibodies against a specific epitope (see above) it is unlikely that pathogenic antibodies can be found in such a high proportion of healthy and neurological controls. Indeed, the high incidence of the antibodies in the neurological control groups cast doubt on any hypothesis that the antibodies represent anything other that an epiphenomenon of a separate disease process.

3 *Chapter 3: Identification of Neuroleukin, a 56 kDa antigen recognised by antibodies in the serum and CSF of patients with opsoclonus-myoclonus.*

3.1 Introduction.

Opsoclonus-myoclonus may have a post-infectious or paraneoplastic aetiology. We received serum samples from two patients with post-infectious OM who on routine testing for anti-neuronal antibodies were found to have antibodies against a 56 kDa antigen. We sought to determine the nature of the antigen using a proteomic approach consisting of ammonium sulphate fractionation (ASF), ion exchange (IEX) chromatography and two-dimensional (2D) gel electrophoresis. These techniques had already been used to characterise γ and α-Enolase, PYK and aldolase C as potential auto-antigens in post-streptococcal neurological disease (Dale *et al*, 2006). We then aimed to determine the frequency with which the antibody response occurred in a cohort of paediatric and adult OM. An introduction to the principles of some of the techniques used in this chapter is provided while a summary of the experiments can be found in the following flow sheet.



3.1.1 Ammonium Sulphate Fractionation.

Ammonium sulphate fractionation represents a useful technique as it provides a convenient method by which complex solutions of protein can be simplified. The distribution of charged molecules on the surface of a protein and the properties of the solvent into which it is dissolved (including ionic strength and pH) governs the solubility of proteins in a given solution. Thus, manipulation of the solvent can alter the solubility of the proteins dissolved within. This principle is exploited by ASF. Addition of ammonium sulphate to a solution results in the precipitation of proteins. These precipitated proteins can be separated from the solvent by centrifugation. Further addition of ammonium sulphate to the supernatant results in the precipitation of more soluble proteins. This process can be continued until the solvent is saturated. ASF is not suitable for the single step purification of proteins, especially when the starting protein solution is a homogenate of complex tissue, thus further purification steps would be required. However, by taking large fractions in the first instance (0, 25, 50, 75, 100 % ammonium sulphate saturation) the fraction containing the target protein can be selected and further dissected using smaller ammonium sulphate fractions spanning that saturation. The result of ASF is the production of a number of fractions, in one of which the protein of interest will be relatively enriched.

3.1.2 Ion Exchange Chromatography.

Similarly to ASF, IEX chromatography was previously utilised in the characterisation of potential auto-antigens (Dale *et al*, 2006). IEX chromatography is a method of protein purification that exploits the electrostatic attraction between the surface charge of a protein molecule and charged groups displayed on the matrix of a chromatography column. During protein separation, the IEX column and proteins are equilibrated in the

same buffer, thus the charged groups displayed on the surface of the column matrix and the proteins in solution will be associated with ions from the buffer. Introduction of the protein solution into the column displaces ions from the column and the resulting electrostatic attraction between matrix and protein results in bond formation, the strength of which is governed by the overall charge on the surface of a given protein molecule. This characteristic is exploited during the elution of proteins from the column which is achieved by increasing the ionic strength of the buffer by the addition of NaCl. The added ions compete with the proteins for the polar groups of the matrix. At a given ionic strength a protein will no longer be attached to the column and will be successfully eluted. The gradual increase of NaCl allows those proteins that are weakly bound to the column to be eluted earlier than strongly bound proteins. The result is the differential elution and thus separation of proteins. Important aspects of IEX chromatography include the selection of an appropriate column, the isoelectric point of the protein of interest and the pH of the buffer system. The use of a buffer with a pH one unit above or below the pI of the protein of interest will result in the production of a net negative or positive charge respectively. This influences the choice of the IEX column, which exist as anionic or cationic exchangers. Anionic exchanger's bind negatively charged proteins, while cationic exchangers bind positive proteins. Selection of the pH and column can ensure that the protein is retained on the column for later elution. In other instances a pH may be selected at which a protein has the same charge as the column. In this instance the protein will not bind and be collected in the flow-through while other proteins will remain attached to the column (Scopes, 1993).

3.2 Methods.

3.2.1 Patients with Opsoclonus-myoclonus.

Serum and CSF from the index paediatric cases of OM were supplied by Dr R Dale. These samples had been taken as part of the patient's diagnostic work up. Serum from further paediatric cases of OM were kindly supplied by Professor A Vincent. The collection of serum had the appropriate ethical approval however some demographic data was not available. Clinical information was withheld until requested. Samples from 7 adult patients with POM were kindly supplied by Professor J Dalmau. Specific clinical details were not available.

3.2.2 Control Patients.

Serum samples from children with and without neurological disease were supplied by Dr R Dale. The details are listed in Table 3-1. CSF samples from 14 adult patients with inflammatory neurological disease were provided by Dr G Giovannoni. These samples consisted of three encephalitis and 11 multiple sclerosis samples.

Table 3-1: Clinical details of serum and CSF samples (adult and paediatric)

Sample	*Number*
Serum	
Paediatric	
Opsoclonus-myoclonus	11
Inflammatory conditions	16
ADEM	8
Multiple sclerosis	4
Rasmussen's encephalitis	2
Cerebral lupus	2
Non-inflammatory neurological conditions	14
Post-streptococcal disorders.	8
Sydenham's Chorea	4
Glomerulonephritis	4
Healthy controls.	16
CSF	
Paediatric	
Opsoclonus-myoclonus	2
Inflammatory conditions.	16
ADEM	8
Multiple sclerosis	4
Rasmussen's encephalitis	2
Cerebral lupus	2
Non-inflammatory neurological conditions	14
Adult	
Opsoclonus-myoclonus	7
Encephalitis	3
Multiple sclerosis	11

ADEM = acute disseminated encephalomyelitis

3.2.3 Sample Preparation.

Human brain tissue provided by the UCL brain bank was snap-frozen within 12 hours of acquisition. Wistar rat brains (B&K) were isolated and snap-frozen immediately after death. Brain tissue was homogenised in a Teflon homogeniser with Tissue protein extraction reagent (1 ml/g brain tissue) (Tper, Pierce, UK) and mammalian protease inhibitors (50 µl/g brain tissue) (Sigma, UK). The homogenate was centrifuged at 10,000g at 4°C for 12 minutes, the supernatant was aliquotted and stored at –80°C until required.

3.2.4 Ammonium Sulphate Fractionation.

Ammonium sulphate fractionation was performed using 300 µl of rat brain supernatant dissolved in 10 ml distilled water containing 50 µl Tris-HCl (pH 9.0). Ammonium sulphate was added in stages to give the following saturations: 0-20 %, 20-40 %, 40-60 %, 60-80 % and 80-100 % as described (Scopes, 1993). At each stage the solution was stirred for 1 hour and proteins precipitated by centrifugation at 10,000g for 20 minutes. The precipitated proteins were stored at -80°C until required and the supernatant used for further ASF. Precipitated proteins were resuspended, subjected to PAGE, stained or immunoblotted and probed with the serum from the index patients to determine which fraction contained the antigen of interest.

3.2.5 2D Gel Electrophoresis

2D electrophoresis was conducted using the Powerease 500 system and preformed pH 3-10 isoelectric focussing (IEF) gels (Invitrogen, UK). The 40-60 % ammonium sulphate fraction of rat neuronal tissue was buffer exchanged into sodium phosphate buffer (pH 7.0) and concentrated using the Microcon system (Millipore, UK). Proteins

were initially separated by IEF according to pI. 100 µl of sample was suspended in 125 µl IEF sample buffer (Invitrogen, UK) and 25 µl 0.05 % SDS. Samples were left to stand at room temperature for at least 20 min before continuing with further steps. Anode and cathode buffer (Invitrogen, UK) were prepared by dilution in water and allowed to reach room temperature. A single pH 3 to 10 IEF gel was washed in cathode buffer before being locked into the gel tank. Cathode buffer was then poured into the inner chamber and anode buffer into the outer chamber. 10 µl of IEF marker was added to the first lane. 25 µl of sample was then loaded into each subsequent well. IEF was conducted as follows:

Step 1	Step 2:	Step 3:
1hr at 100v	1hr at 200v	30min at 500v
18mA	18mA	18mA.

Once the focusing was finished the gel was removed from its plastic casing and immersed in fixing solution (Appendix 2) for 30 min. The solution was then drained and the gel washed a minimum of three times in water. The gel was immersed in Coomassie blue stain for 5 minutes followed by destain solution. De-staining was continued for approximately 1.5 hr. The gel was then placed in deionised water until required.

Following separation by pI proteins were separated by PAGE. A lane of stained focused proteins were cut from the IEF gel and immersed in solubilisation buffer for a minimum of 5 min. In the meantime a 1 well PAGE gel was prepared. A section of filter paper was cut to match the dimensions of the well in the PAGE gel and labelled 10 and 3 to

correspond with the pH of the IEF gel. Once the proteins in the cut section of the IEF gel had been re-solubilised the gel was rinsed in the same MOPS buffer (Invitrogen, UK) as that used to prepare the PAGE gel. The running buffer was then decanted and the filter paper applied to the gel strip. The gel was then transferred using the filter paper and positioned into the large well of the PAGE gel so that it was flush with the well bottom. The proteins were then subject to electrophoresis as described for standard PAGE (See chapter 8). Gels were subjected to silver staining or immunoblotted as required. Proteins of interest were subjected to in-gel trypsinolysis and identified using a Q-Tof hybrid quadrupole / orthogonal acceleration time of flight spectrometer (See section 3.2.7).

3.2.6 Ion Exchange Chromatography.

IEX was performed using the ÄKTA fluid phase liquid chromatography (FPLC) system and the anionic exchange HiTrap Mono Q FF (5 ml) column (Amersham Biosciences). Proteins from the 40-60 % ammonium sulphate fraction were exchanged into IEX binding buffer (20 mM Tris-HCl pH 8.35) and injected onto the column. Proteins were eluted over a gradient of increasing ionic strength by injection of elution buffer (20 mM Tris-HCl 1.5 M NaCl pH 8.35) 0-100 % over 50 ml. Fractions were collected using a Frac-900 fraction collector and those corresponding to peaks on the chromatogram selected, subjected to PAGE and immunoblotted. The 56 kDa antigen was detected using serum from patient one. Further IEX was conducted using proteins from the initial IEX separation and a lower pH binding buffer (20 mM Tris-HCl pH 8.0). Proteins were subjected to gradient elution as described using 20mM Tris-HCl 1.5M NaCl pH 8.0.

Fractions were collected, subjected to PAGE and silver stained or immunoblotted as required. Silver stained proteins were subjected to in gel trypsinolysis and identified using a Q-Tof hybrid quadrupole / orthogonal acceleration time of flight spectrometer (see section 3.2.7).

3.2.7 Mass Spectrometry.

Proteins were digested from silver stained PAGE gels following 2D gel electrophoresis, IEX or immunoaffinty chromatography. The antigens were identified by MOLDI-TOF mass spectrometry by Dr R. Wait using the following method:

In gel trypsinolysis was performed using an Investigator Progest (Genomic Solutions, Huntingdon, UK) robotic digestion system, as previously described (Wait *et al*, 2001). Tandem electrospray mass spectra were recorded using a Q-Tof hybrid quadrupole / orthogonal acceleration time of flight spectrometer (Micromass, Manchester, UK) interfaced to a Micromass CapLC capillary chromatograph. Samples were dissolved in 0.1% aqueous formic acid, and 6 µl injected onto a Pepmap C18 column (300 µm x 0.5 cm; LC Packings, Amsterdam, NL), and washed for 3 min with 0.1 % aqueous formic acid. The flow rate was then reduced to 1 µl per min, the stream select valve was switched to the data acquisition position, and the peptides were eluted into the mass spectrometer with an acetonitrile / 0.1% formic acid gradient (5% to 70 % acetonitrile over 20 minutes).

The capillary voltage was set to 3,500 V, and data dependant MS/MS acquisitions were performed on precursors with charge states of 2, 3 or 4 over a survey mass range 540-

1200. Known trypsin autolysis products and keratin-derived precursor ions were automatically excluded. The collision voltage was varied between 18 and 45 V depending on the charge and mass of the precursor. Product ion spectra were charge-state de-encrypted and de-isotoped with a maximum entropy algorithm (Max Ent, Micromass). Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in SwissProt/TREMBL, using ProteinLynx Global Server (Versions 1 and 1.1, Micromass). One missed cleavage per peptide was allowed, and the fragment ion tolerance was set to 100 ppm. Carbamidomethylation of cysteine was assumed, but other potential modifications were not considered in the first pass search. All matching spectra were reviewed manually, and in cases where the score reported by ProteinLynx global server was less than 100, additional searches were performed against the NCBI database using MASCOT. Identifications were verified by manual sequencing using the MassLynx program Pepseq (Micromass).

3.2.8 Production of Neuroleukin Specific Antibodies.

Anti-neuroleukin (NLK) antibodies were raised in New Zealand rabbits (CovalAb, UK) as described (Niinaka *et al* 1998). The peptide YFQQGDMESNGKYITK was used as the immunogen as this sequence is common to both human and rat NLK.

3.2.9 NLK Detection Using Cultured Neurones.

Live neurones were kindly cultured by Dr S Griffin using the following method: Pregnant Sprague Dawley rats were sacrificed by cervical dislocation, foetuses were extracted and cortical tissue removed. Neurones were isolated by mechanical and enzymatic digestion of tissue. Cells were suspended at a final concentration of 1 x 10^6 cells/ml in neurobasal medium (NB) containing 2 mM glutamine, 25 μM Glu and 2 %

B27 serum supplement. Glass cover slips previously coated in 0.01 % poly-D-lysine were incubated in 100 µl of NB containing 2 mM glutamine, 62.5 µM Glu 2 % B27, 5 % horse serum and 5 % foetal bovine serum. 100 µl of neuronal suspension was suspended directly onto the coverslips and left for 1 hr prior to the addition of 2 ml supplemented NB. The cells were housed at 37°C in an atmosphere of 5 % CO_2/ 95 % air at < 90 % humidity. Cell medium was replaced on day 3 and 7 with 1 ml of astrocyte-conditioned NB containing cytosine arabinofuranoside at a final concentration of 10 µM.

On day 10 cells were rinsed in Tris- buffered saline (TBS) and fixed by addition of ice-cold methanol for 10min. The methanol was removed, the cells rinsed in blocking buffer (TBS, 1 % BSA, 10 % serum (corresponding to the species of the secondary antibody) for 2 hours at room temperature. Blocking solution was removed and rabbit anti-NLK (diluted 1:1000) or patient serum (diluted 1:70) applied and incubated overnight at 4°C. Cells were washed twice in TBS and incubated with the appropriate secondary antibody (Swine anti-rabbit TRITC, rabbit anti-human IgG/FITC) (Dako) for 1 hr at room temperature. The cells were washed and stained with 0.01 % 4, 6-diamidino-2-phenylindole HCL (DAPI) for 10 minutes. Cells were mounted on glass cover slip slides using 10 µl of citiflour (Dako). Omission of primary and secondary antibodies and omission of cells were used as negative controls. A patient with anti-Hu antibodies was used as a control to determine if the cell membrane had been left intact. Cells were visualised by confocal microscopy using a Zeiss 510 laser scanning confocal microscope.

3.2.10 Production of Recombinant Neuroleukin.

mRNA was isolated from human cerebellum using the Micro-FastTrack kit (Invitrogen) (See section 2.2.3). cDNA was synthesised using human cerebellar mRNA and gene specific primers designed to amplify the entire open reading frame of NLK (GenBank Accession No: K03515). The following primers were used. (Sense primer, 5'-CCGGAATTCATGGCCGCTCTCTCACCC-3' (*EcoRI* site underlined). Anti-sense primer 5'-GCCCAAGCTTATTGGACTCTGGCCTCG-3' (*HindIII* site underlined).

The RT-PCR reaction was performed using a Superscript one-step RT-PCR kit (Invitrogen, UK). The following components were combined in a 0.5 ml tube: 25 µl 2x reaction mix, 2.5 µl of isolated mRNA, 1 µl sense primer (10 µM), 1 µl anti-sense primer (10 µM), 2 µl RT/Platinum *taq* HiFi mix. DNase/RNase free water was added to a final volume of 50 µl. A control reaction substituting Ampli*taq* gold for RT/Platinum *taq* HiFi mix was used to confirm the absence of genomic DNA in the mRNA preparation. In a second control RNA was substituted with 2.5 µl H_20. The RT-PCR reaction was conducted as described in section 2.2.4.

Amplified cDNA was gel-purified and ligated into the pRSETB bacterial expression vector (Invitrogen, UK) prior to transformation of *E. coli* TOP10F'. Following antibiotic selection purified plasmids were analysed by single restriction enzyme analysis, sequenced (Chapter 8) and the Translate and BLAST programmes used to confirm the identity of the DNA and its ligation into the correct reading frame. Successfully ligated plasmids (designated pRSETBNLK) were used to transform *E. coli* BL21 (DE3) pLysS (Invitrogen, UK) in preparation for expression of the recombinant protein.

3.2.11 Expression and Purification of Recombinant NLK.

Expression of recombinant NLK (rNLK) was conducted using the method described (See sections 2.2.8). A crude bacterial homogenate was used in immunoblots to confirm recombinant protein expression prior to purification. Purification of rNLK was conducted using the method described for other recombinant antigens (see section 2.2.9).

3.2.12 Detection of Anti-NLK Antibodies.

Recombinant NLK was subjected to PAGE and immunoblotted. Serum samples were diluted 1:300 in 0.2 % milk 0.9 % saline solution. CSF samples were diluted 1:50 in 0.2 % milk 0.9 % saline solution. anti-NLK antibodies were used as a positive control. Secondary HRP-conjugated detector antibodies (swine anti-rabbit IgG and rabbit anti-human IgG) were diluted 1:5000 in 0.2 % milk 0.9 % saline solution and washed after 1 hour. Blots were developed using Enhanced chemiluminescence (ECL).

3.2.13 Determination of Sequence Homology.

A comparison of the amino acid sequences of human NLK and streptococcal proteins was conducted using the protein-protein BLAST application (www.ncbi.nih.gov/BLAST) set with the default parameters.

3.2.14 Anti-His, Antigen and Detection Antibody Concentration for rNLK ELISA.

Initial attempts to produce a NLK ELISA for the detection of anti-NLK antibodies in patient's serum were based upon the use of the anti-His antibody to selectively bind

rNLK from crude homogenates of *E. coli* pRSETBNLK. During the initial set up of the ELISA the rabbit anti-NLK antibody was used as a positive control.

Anti-His antibodies were diluted 1:500 in 0.05 M carbonate buffer and 100 µl used to coat 36 wells. The plate was incubated overnight at 4°C. The following day the plate was rinsed 3 times in 0.9 % saline and incubated with 300 µl of 2.5 % bovine serum albumin (BSA) for 1h at room temperature. 100 µl of crude homogenate of *E. coli* containing pRSETBNLK was diluted 1:10 to 1:5000 in 0.9 % saline containing 0.2 % (w/v) BSA. Omission of bacterial homogenate was used as a negative control. Each dilution was incubated with 2 wells containing anti-His antibodies. After incubation at room temperature for 1 hr the plate was washed 6 times in ELISA wash solution (0.2 % BSA, 0.9 % saline 0.05 % tween). Rabbit anti-NLK antibodies were diluted 1:1000 or 1:5000 in wash solution and 100 µl applied to each well. Omission of anti-NLK antibodies was used as a further control. The plate was incubated at room temp for 1 hr, washed 6 times and incubated with 100 µl of swine anti-rabbit HRP conjugated detector antibody (Dako) diluted 1:1000 in wash solution. The plate was left for 1 hr at room temp, washed as previously described and developed by the application of 100 µl of 3, 3', 5, 5'- tetramethylbenzidine (TMB) liquid substrate (Sigma). The reaction was continued as appropriate, stopped by the application of 50 µl 1 M HCl and read using the Wallac 1420 multilabel counter. Absorbencies were averaged and plots produced using Microsoft Excel.

Following the selection of appropriate dilutions of capture antibody and antigen preparation patients' sera was tested. Serum from 9 patients with OM and 9 neurologically normal controls were investigated. Serum from patient 1 was always

included as a positive control as were rabbit anti-NLK antibodies. The ELISA was conducted as described above using anti-His capture antibody at 1:500 dilution, a serum and antigen dilution of 1:100 and rabbit anti-Human IgG-HRP diluted 1:1000. The mean absorbency was calculated. Patients were considered to have a positive ELISA result for the presence of anti-NLK antibodies if the absorbencies were greater than the mean +2 times the standard deviation of the average absorbencies of the 9 normal control serum. As each plate was internally controlled using serum from patient 1 the development time of the assay was not standardised at this stage.

Following the initial investigations with patients' sera an appropriate dilution of the HRP-conjugated antibody was sought. Anti-His antibodies diluted 1:500 in 0.05 M carbonate buffer were used to coat the ELISA wells. Serum from patient 1 was applied in duplicate wells at dilutions of 1:100, 1:500 and 1:1000. A blank well containing no patient serum was included. HRP-conjugated antibody was incubated with the well at the following dilutions 1:1000, 1:2000, 1:4000 and 1:8000. The absorbencies were determined, the mean calculated and divided by the corresponding blank value to determine which dilution gave the greatest difference between signal and background. The appropriate dilution of antibody was selected and the ELISA using patients' sera repeated using serum diluted 1:100 from the same 18 patients and controls and a HRP-conjugated antibody diluted 1:8000. Sera were considered to contain anti-NLK antibodies if the mean absorbencies over two wells were greater than the mean +2SD of the absorbencies of the controls.

3.2.15 Determining Factors Responsible for Background Signal

To determine areas of possible improvement a number of blank wells were set up to systematically determine factors responsible for the production of background signal. Each well was set up with the appropriate solution added in the order described in the initial ELISA set up (see section 3.2.14). When a layer of the ELISA was omitted wash solution was substituted. Anti-His antibodies were diluted 1:500 in 0.05 M carbonate buffer. Serum from patient 1 was diluted 1:100 in 0.9 % saline, 0.2 % BSA and HRP conjugated antibody diluted 1:8000 in 0.9 % saline, 0.2 % BSA. When anti-His antibody was omitted purified rNLK was incubated in 0.05 M carbonate buffer at a dilution of 1:10 and the blocking and detection steps continued as normal. As a final experiment the anti-His capture antibody was substituted for the rabbit anti-NLK antibody diluted 1:500 in 0.9 % saline, 0.2 % BSA and the experiment conducted using patients' sera and the appropriate blank.

3.2.16 Comparison of Blocking Agents

It was noted that some patients and controls had a raised signal when serum was incubated with blocked blank wells. To determine whether rabbit serum or BSA provided a more suitable blocking agent the serum of patients and controls was tested against wells blocked with 2.5, 5 and 10% solutions of BSA or normal rabbit serum (Sigma) in saline. Serum from 7 patients and controls was diluted 1:100 in 0.9 % saline, 0.2 % BSA or 0.9% saline/ 0.2 % normal rabbit serum and detector antibody was diluted at 1:1000 0.9 % saline, 0.2 % BSA or normal rabbit serum. Results were expressed as the mean absorbance of the samples +/- the SD.

3.2.17 Comparison of Crude Bacterial Homogenates and His-Purified rNLK

To determine whether His-purified rNLK represented an improved antigen preparation over crude homogenates of pRSETBNLK an ELISA was conducted using anti-His antibodies (1:500), crude or purified rNLK (1:10), serum (from 7 anti-NLK negative samples) diluted 1:100 and anti-human HRP antibodies diluted 1:8000. All dilutions used 0.9 % saline, 0.2 % normal rabbit serum. The ELISA was conducted as described in section 3.2.14, the absorbencies averaged and compared for the crude and purified rNLK preparations. The results were analysed using a Mann Whitney U test to determine if there was a statistically significant difference in the absorbencies obtained through the use of the different rNLK preparations.

3.2.18 Use of Antigen Coated Wells.

As an alternative to the use of a capture antibody we investigated the principle of using purified rNLK applied directly to the wells. As an initial investigation antigen was applied to the wells at a dilution of 1:50 in 0.05 M carbonate buffer and incubated overnight at 4°C. Wells were blocked and washed as described above. The serum of the index patient was tested at a dilution of 1:100, 1:300, 1:500 and 1:1000 and the appropriate dilution selected. The rabbit anti-human HRP antibody was also tested at various dilutions. A dilution of 1:300 for the patient's serum and 1:1000 for a HRP conjugate was selected. All dilutions used 0.9 % saline, 0.2 % normal rabbit serum. Opsoclonus-myoclonus and normal control patients were compared. All sera were also run against blank wells (Rabbit serum coated, no antigen). In one experiment the blank value was subtracted from the test wells to allow for non-specific binding. When more than one ELISA plate was required serum from patient 1 was included and absorbencies

from other patients expressed as a percentage of the absorbance of patient 1 to allow for intra-assay variation. Patients were considered to have a positive result by ELISA if the absorbance value was greater than the mean + 2SD of the average absorbencies of the control group. The coefficient of variation (CV) for the assay was determined at the same time using the serum of 1 patient and 2 controls, each repeated 16 times each and the average of the CVs taken.

3.3 Results.

3.3.1 Case History One

One week after a febrile illness and pharyngitis, a previously well 10 year-old girl presented with chaotic, multi-directional eye movements. The opsoclonus progressed rapidly over the next few days, and was complicated by myoclonus and ataxia. In addition, she became profoundly insomniac and suffered a change in personality. Her speech became pressured, disinhibited and inappropriate, and she experienced auditory hallucinations. MRI brain, EEG and echocardiogram were normal. CSF revealed 85 lymphocytes/mm^3, CSF protein of 0.48 g/dl and normal CSF glucose and lactate. CSF gram stain was negative. The patient was started on acyclovir and ceftriaxone pending CSF PCR for herpes simplex, varicella and enterovirus all of which were negative. No organisms were cultured from the CSF. Extensive serology for mycoplasma, influenza, Chlamydia, adenovirus, Epstein-Barr virus and measles virus were all negative or normal. ASOT was elevated (400 IU/ml, normal < 200 IU/ml). Throat culture was negative. Biochemistry including copper metabolism, liver and thyroid function tests were normal. Urinary vanillylmandelic acid and homovanillic acid were negative. Ultrasound of abdomen and metaiodobenzylguanidine scanning were normal. She was treated with ACTH 40 U/day for 3 days, and then oral prednisolone 2 mg/kg for 2

weeks. In addition, she was given penicillin 500 mg bd for 2 weeks. Within 1 week, her sleep pattern and movement disorder significantly improved although her mood became labile. Convalescent ASOT performed 6 weeks after the first was < 200 IU/ml. The prednisolone dose was tapered over 6 weeks, during which time her opsoclonus and movement disorder steadily improved leaving only a residual intention tremor. One year after her illness, she had no neurological signs, although she remained hyperactive, a finding not reported prior to the onset of the neurological disease.

3.3.2 Case History Two

A 16 year-old girl presented with a neurological disorder one week after a febrile illness characterised by pharyngitis and rash. The neurological dysfunction was initially characterised by gait disturbance followed by generalised myoclonus. In addition, her eye movements demonstrated jerky pursuit and reduced pupillary response to accommodation. MRI brain, EEG, ECG and echocardiogram were normal. CSF was acellular with CSF protein 0.5 g/dl and normal CSF glucose and lactate. CSF PCR for herpes simplex, varicella and enterovirus were negative. Serology for mycoplasma, Chlamydia, Epstein-Barr virus, HIV, Lyme disease and measles virus were all negative or normal. ASOT was elevated (800 IU/ml, normal < 200 IU/ml) although throat culture was negative. Biochemistry including copper metabolism, urine toxicology, liver and thyroid function tests, autoimmune profile and immunoglobulins were all normal. Urinary vanillylmandelic acid and homovanillic acid were negative and an ultrasound of the abdomen was normal. She was treated with oral prednisolone and 2 g/kg intravenous immunoglobulin over 24 hours. Her illness was resistant to the initial treatment, and progressed over the next month with the development of frank opsoclonus. In addition, her illness became complicated by the development of psychiatric symptoms,

particularly anxiety and low mood. A repeat ASOT 6 weeks after the first had fallen to 235 IU/ml. The patient remained on 2 mg/kg of prednisolone for 2 months. The dose was tapered over a further 2 months. A repeat MRI at 6 months remained normal. Her OM had completely resolved by 9 months, although 2 years later she required rehabilitative care for her impaired motor function resulting from her prolonged admission.

3.3.3 Anti-neuronal Antibody Detection

Antibodies in the serum and CSF of both patients reacted with a 56 kDa protein present in both human and rat brain homogenate (Figure 3-1, A) which was also present in the 40 to 60% ammonium sulphate fraction of rat brain (Figure 3-1, B).

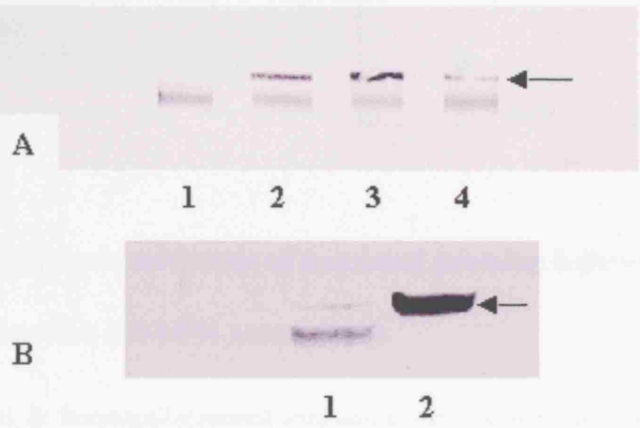


Figure 3-1: Antibodies against a 56 kDa protein in serum and CSF of OM patients.

A: Lane 1: Control serum. Lane 2: Serum from patient 1. Lane 3 and 4 serum and CSF from patient 2 respectively. Arrow: 56 kDa antigen. The common lower band represents human IgG within the tissue preparation. **B**: Comparison of human and rat brain. Serum from patient 1 detects the 56 kDa antigen in both human brain (lane 1) and the 40-60 % ammonium sulphate fraction of the rat brain homogenate (lane 2) (Arrow). The lower band (lane 1) represents human IgG in the preparation of the tissue homogenate.

3.3.4 Identification of the 56 kDa Auto-antigen.

Following ammonium sulphate fractionation the 40 to 60 % rat brain fraction was subjected to further purification. 2D gel electrophoresis achieved good separation of proteins. Immunoblotting showed that the 56 kDa protein had an approximate pI of 8.0. Figure 3-2 shows the immunoblot and silver stained gel, with the candidate 56 kDa protein arrowed. The protein was removed from the gel, digested and subjected to Q-TOF mass spectrometry.

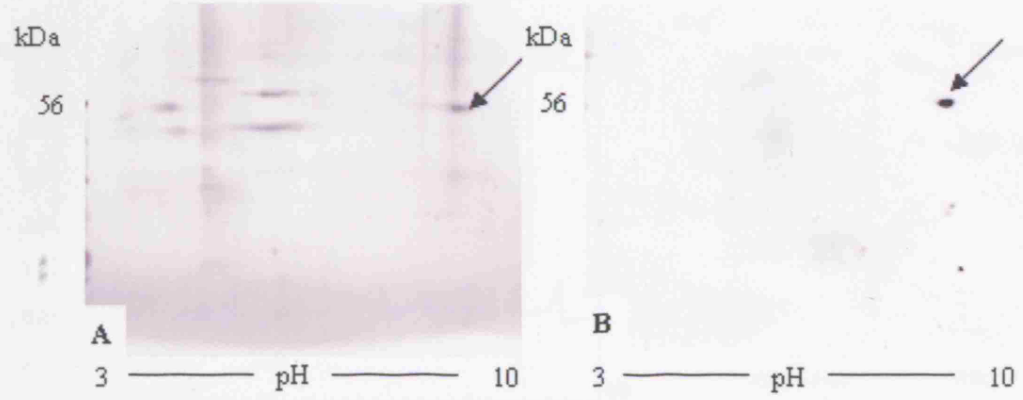


Figure 3-2: 2D gel electrophoresis of neuronal proteins following ammonium sulphate fractionation (40-60% saturation).

A: Silver stained gel. **B**: Immunoblot probed with serum from patient 1. A strong reactivity could easily be identified on the immunoblot with a corresponding protein clearly visible on the stained gel. The protein was digested from the gel and subjected to mass spectrometry.

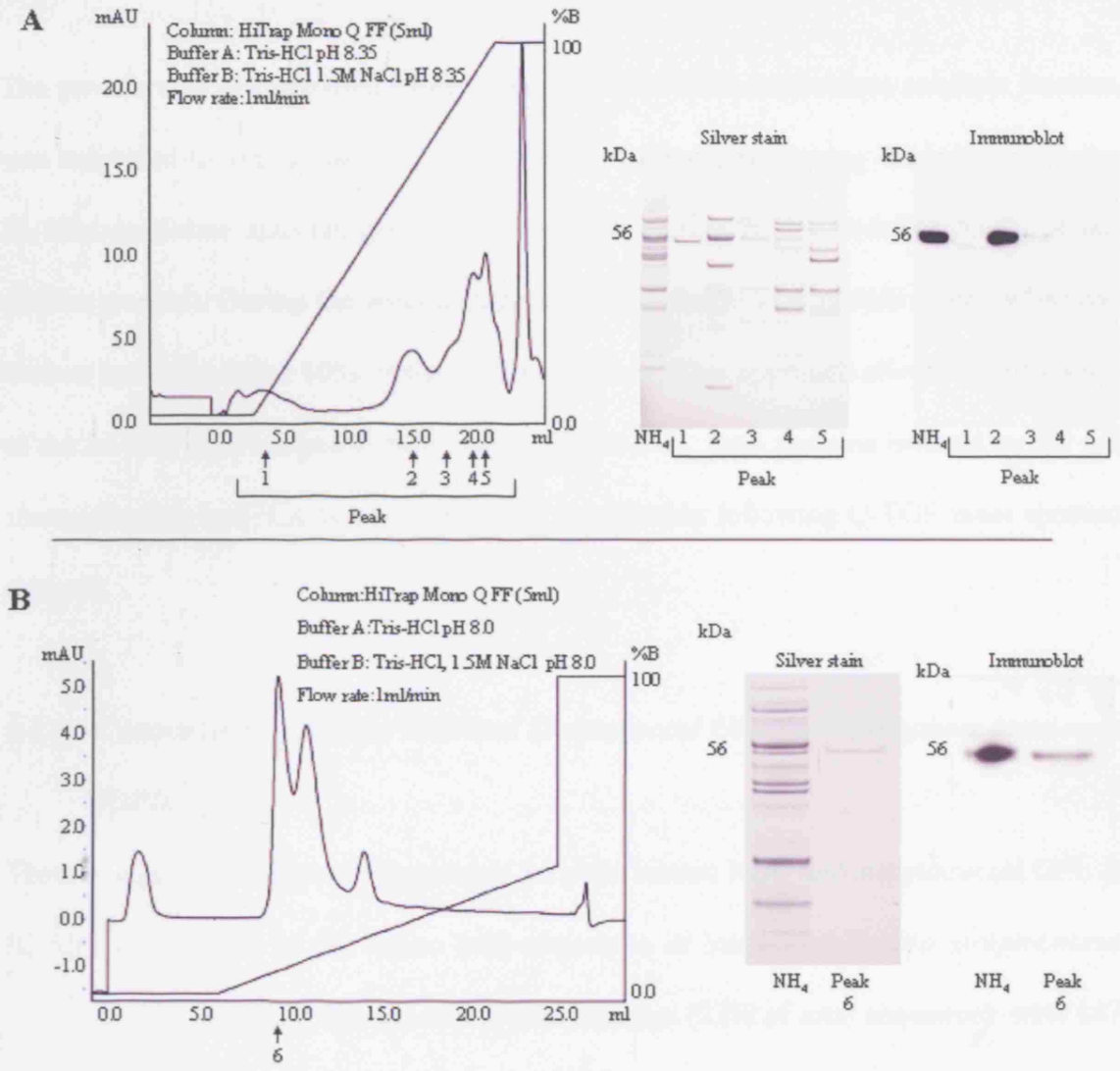


Figure 3-3: Two rounds of ion exchange chromatography were used to purify the 56 kDa antigen.

A: Partially purified proteins from the 40-60 % ammonium sulphate fraction were separated. Peaks 1 - 5 were selected, separated by PAGE and the gel stained or immunoblotted and probed with serum from patient 1. Immunoblotting showed that the antigen was contained within peak 2. **B**: The proteins from peak 2 were subjected to further IEX under different conditions. A number of peaks were selected and the antigen again detected by immunoblotting. The antigen was contained in peak 6. A silver stain showed a single protein at 56 kDa, the molecular weight of the antigen. No other proteins were observed in the gel. The band was digested and subjected to mass spectrometry. (NH_4 = pre-IEX 40-60 % ammonium sulphate fraction of rat brain homogenate).

The protein was also purified using IEX. The 40 to 60% ammonium sulphate fraction was subjected to ion exchange chromatography in 2 stages. During the initial step, the 56 kDa candidate auto-antigen eluted when the elution buffer equalled 55 % of the elution gradient. During the second stage of elution the 56 kDa protein eluted when the elution buffer equalled 10% of the elution gradient. This approach allowed purification of the 56 kDa auto-antigen to near purity (Figure 3-3). Both proteins isolated by 2D gel electrophoresis and IEX were identified as neuroleukin following Q-TOF mass spectral analysis.

3.3.5 Comparison of Human NLK and Streptococcal Glucose-6-phosphate Isomerase (GPI).

There is significant sequence homology between human NLK and streptococcal GPI. A BLAST comparison of the amino acid sequences of human NLK with *streptococcus pyogenes* GPI revealed 89 exact amino acid matches (23% of total sequence), with 147 close matches (38%) (expected chance homology was less than 1×10^{-6}).

3.3.6 RT-PCR Amplification of Human Neuroleukin.

Electrophoresis of the RT-PCR reaction mixture revealed a dominant band of approximately 1700 bp. In addition, a number of smaller reaction products could be visualised ranging from approximately 900 to 1200 bp. No reaction product could be seen in the *taq* or water control suggesting that the amplified fragments were not a result of contamination of the mRNA preparation with genomic DNA or due to contamination of the reagents.

3.3.7 Analysis of Ligated pRSETB

Thirteen colonies were isolated from the plates containing bacteria transformed with ligated pRSETBNLK. A large number of colonies were also found on the negative control plate (pRSETB only) suggesting that double digestion of pRSETB had not been particularly efficient. Single restriction enzyme analysis of the recovered plasmids demonstrated that 2 of the 13 colonies contained a plasmid into which the cDNA had been successfully ligated (Figure 3-4).

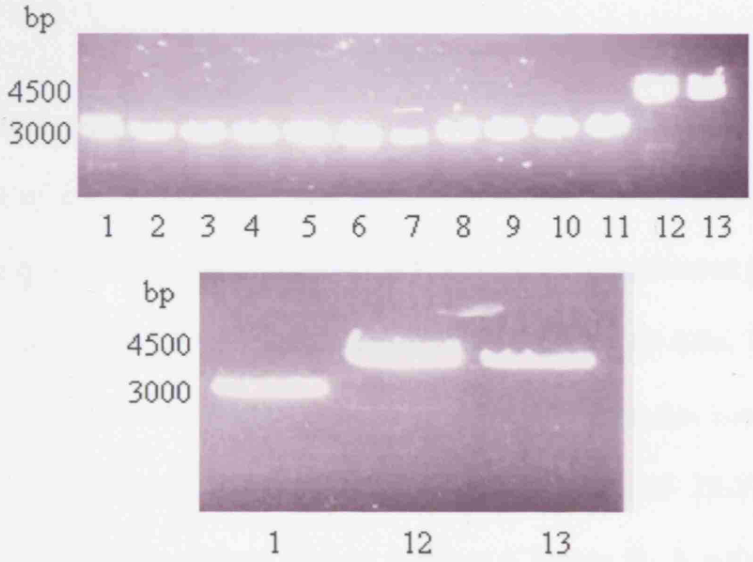


Figure 3-4: Selection of pRSETBNLK

Plasmids were extracted from transformed *E. coli* TOP10F' and subjected to restriction analysis. Top figure: 13 colonies were selected and digested with *EcoRI*. Lane 1: Un-ligated pRSETB (control). Lanes 2-13: plasmid extracted from transformed colonies. Colonies 12 and 13 appeared to contain ligated plasmid. Lower figure: Plasmids from colonies 12 and 13 were redigested and ligation confirmed. Lane 1 = Un-ligated pRSETB control.

3.3.8 Sequencing of Successfully Ligated pRSETB

Sequencing of the ligated pRSETB was conducted in order to check the cDNA represented the complete open reading frame of NLK (according to the published sequence: GenBank Accession No: K03515) and to determine whether the cDNA had been inserted into the correct reading frame. Sequence analysis of the two ligated plasmids revealed that they both contained cDNA encoding the entire reading frame of neuroleukin and that the sequence was in the same reading frame as the start codon on pRSETB.

3.3.9 Production and Purification of Recombinant Neuroleukin.

Immunoblots of crude bacterial homogenates in which protein expression had been induced were probed with anti-His antibody to detect the recombinant protein. The anti-His antibody detected a protein with a molecular weight of 60 kDa. The antibody did not recognise a protein when used to probe bacterial homogenates containing pRSETB only. rNLK was efficiently isolated from homogenates of BL21 (DE3) pLysS pRSETBNLK by IMAC using an elution gradient (Chapter 8). A stained PAGE gel of the homogenate, flow-through and elution fractions revealed that a protein of approximately 60 kDa was eluted from the column during the elution phase. The eluted protein was immunoblotted (see chapter 8) and probed with both anti-His and rabbit anti-NLK antibodies. Both antibodies reacted with the 60 kDa protein confirming that it was rNLK.

3.3.10 Probing Purified Neuroleukin with Patients serum

An immunoblot of purified rNLK probed with a rabbit anti-neuroleukin antibody and serum from the index patients used to identify the antigen revealed that both antibodies

reacted with a protein at approximately 60 kDa thus confirming that the patient had an immune response against NLK. In addition a number of bands were recognised by the patients' sera both above and below the 60 kDa antigen. These proteins had not reacted with the anti-His antibody in the previous experiment, nor did they react with the rabbit anti-neuroleukin antibody, therefore these proteins most likely represent contaminating *E. coli* proteins that had not been removed by the purification process.

3.3.11 Probing rNLK with Sera from a Cohort of OM Patients.

We tested the sera of a further nine patients with OM for the presence of anti-NLK antibodies, seven of which were positive (9/11 total (82 %)) (1 result was equivocal). Five of 54 (9%) controls showed reactivity with rNLK (cerebral lupus n = 1, normal n = 3, encephalitis n = 1). A representative blot is shown (Figure 3-5, A) There was a significant difference between patients and controls (Fischer's exact test. p = <0.0001). The sensitivity and specificity for the western blot was 82 and 91% respectively. CSF was only available for the index cases but both patients showed reactivity against rNLK. The blot using CSF from patient 2 is shown (Figure 3-5, B). Only 1 of 30 (3%) CSF controls was positive. This patient had cerebral lupus with
cognitive impairment and gait disturbance.

Table 3-2: Paediatric OM patient details and investigation results for anti-NLK antibodies.

			Immunoblot			
Patient	*Sex*	*Aetiology*	*Serum*	*CSF*	*ELISA*	*Cell Staining*
1	F	Post-infectious	+	+	+	+
2	F	Post-infectious	+	+	+	+
3	F	Neuroblastoma	-	NA	-	-
4	M	Idiopathic	+/-	NA	+	-
5	F	Post-infectious	+	NA	+	+
6	F	Neuroblastoma	+	NA	+	NA
7	F	Post-infectious	+	NA	+	+
8	F	Idiopathic	+	NA	+	NA
9	M	Post-infectious	+	NA	+	NA
10	F	Idiopathic	+	NA	-	NA
11	M	Idiopathic	+	NA	-	NA

NA = Not available.

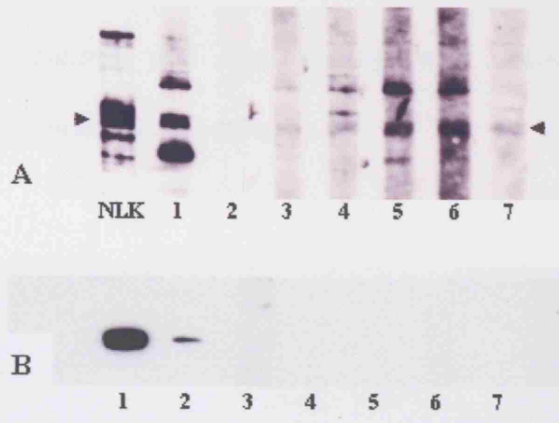


Figure 3-5: Immnuoblot using patients serum and CSF.

A: Immunoblot of rNLK probed with patients serum. NLK: Rabbit anti-NLK (positive control). Lane 1: Patient 1 (index case). Lanes 2 - 7: Patients 3 - 8. Anti-NLK antibodies were detected in all samples (arrow head) with the exception of patient 3 (who was anti-Hu positive). **B**: Antibodies against rNLK were detected in the CSF. Lane 1: Rabbit anti-NLK (positive control). Lane 2: CSF from patent 2. Lanes 3 - 7: Control CSF.

3.3.12 Immunocytochemistry

Permeabilisation steps were omitted during the preparation of freshly cultured rat neurones in an attempt to determine whether NLK is present on the plasma membrane. No cytoplasmic or nuclear staining was observed in neurones stained with the rabbit anti-NLK antibody. An area of bright staining continued around the perimeter of the cell (Figure 3-6). Serum from patients 1, 2, 5 and 7 produced a staining similar pattern to that of rabbit anti-NLK. Serum from patients 3 and 4 were negative. No nuclear staining was produced when the serum of patient 3 (Hu positive) was used suggesting that the integrity of the plasma membrane had been maintained during the preparation of the slides. The results largely correlated with those produced with immunoblots of rNLK with the exception of serum from patient 4 who was positive on immunoblot

(Figure 3-5, A. Lane 3) but negative on immunofluoresence. Secondary antibodies and serum from one healthy control and one patient with acute disseminated encephalomyelitis (ADEM) did not stain the cells.

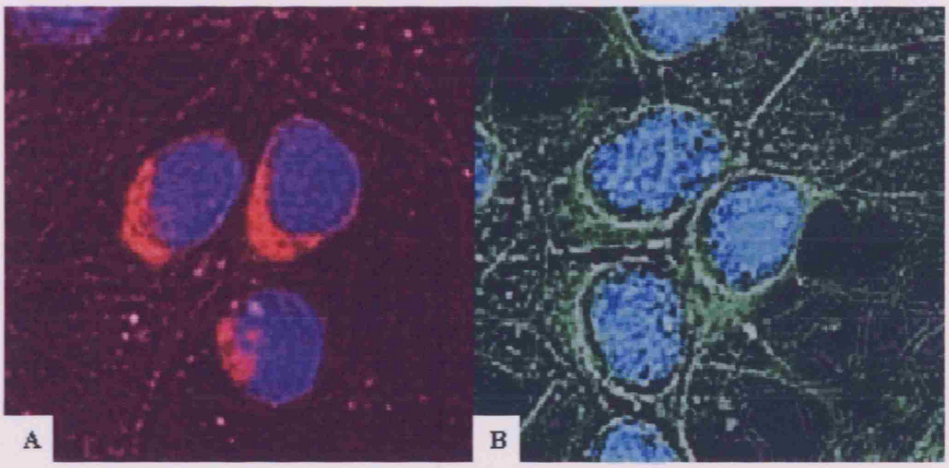


Figure 3-6: Immunocytochemistry.

Live rat neurones were incubated with **A**: rabbit anti-NLK antibody and **B**: serum from patient 1. Staining can clearly be seen localised to the plasma membrane. Staining of the cytoplasm did not occur as evidenced by the separation of the nucleus (blue) from the stained membrane.

3.3.13 The His Capture ELISA.

As proof of principle rabbit anti-NLK antibodies were used in the initial stages of ELISA development. Signal was raised above background when rNLK was diluted 1:1000 and 1:100 for 1:1000 and 1:5000 dilutions of anti-NLK antibodies respectively. Signal continued to increase with application of lower antigen dilutions when rabbit anti-NLK antibodies were applied at either 1:1000 or 1:5000 dilutions suggesting that the rabbit anti-NLK antibodies were in excess of the antigen. Omission of rabbit anti-

NLK established that there was no interaction of the detector antibody with the other components of the ELISA (See Figure 3-7).

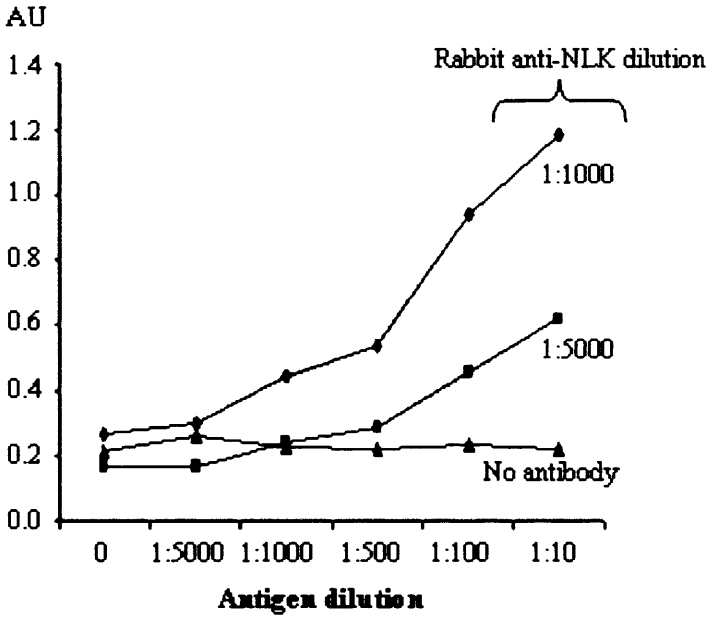


Figure 3-7: Investigating the use of a capture antibody (anti-His) ELISA for the detection of anti-NLK antibodies.

Anti-His capture antibody was diluted 1:500. Antigen (crude bacterial homogenate of *E. coli* pRSETBNLK) was incubated with capture antibodies at various dilutions. Rabbit anti-NLK antibodies were used at 1:1000, 1:5000 dilutions or omitted (control). Swine anti-rabbit HRP detector antibodies were diluted 1:1000. Background signal was similar for both dilutions of anti-NLK antibody when no antigen was incubated in the wells. Signal began to rise with an antigen dilution of 1:1000 and 1:100 when the anti-NLK antibodies were diluted 1:1000 and 1:5000 respectively. The continued rise in signal at lower dilutions of antigen suggests that there was an excess of anti-NLK antibodies over antigen. Signal produced when anti-NLK antibodies were omitted was minimal thus excluding cross-reactivity with other components of the ELISA.

The ELISA was repeated with the substitution of rabbit anti-NLK antibodies with patient and neurologically normal control serum diluted 1:100. Anti-His antibodies were

used at a dilution of 1:500 and antigen 1:100. Development of this ELISA produced disappointing results. High signal approaching the limits of the linear range of the detecting equipment were obtained within 5 min of development.

3.3.14 Determination of Detector Antibody Concentration.

The application of detector antibody was investigated at increasing dilutions and the signal to noise ratio calculated. A 1:8000 dilution of detector antibody was found to give the highest signal to noise ratio (Figure 3-8).

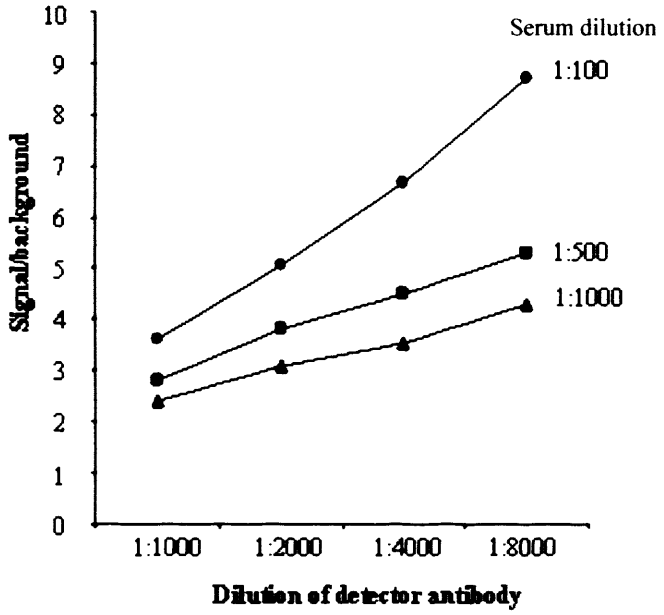


Figure 3-8: Determination of optimal detector antibody dilution.

To determine the appropriate dilution of detector antibody doubling dilutions were applied to wells containing anti-His antibody (1:500), rNLK (1:10) and patients serum diluted 1:100, 1:500 or 1:1000. Values were expressed as a ratio of signal compared to blank well (no serum). A dilution of 1:100 patients serum and 1:8000 HRP conjugated antibody provided the greatest discrimination between signal and background.

A repeat of the ELISA using patient and control serum demonstrated a difference between patient and control groups (Figure 3-9). However the results of this ELISA correlated poorly with those demonstrated by immunoblotting with rNLK and the colour reaction was complete in less than 5 minutes. In addition, absorbencies were raised in some blank wells (no NLK) when serum was applied. This problem had not been apparent during the development of the ELISA using the rabbit anti-NLK antibodies and suggested an underlying problem with the ELISA technique. This problem was not remedied by the application of patient serum at increased dilutions (1:500, 1:1000). In fact discrimination between the two groups grew weaker with increasing serum dilution.

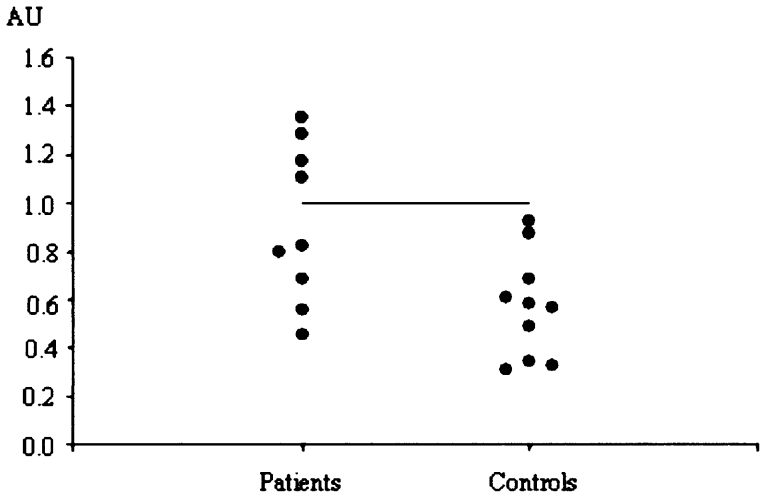


Figure 3-9: Initial NLK ELISA using patients serum.

An ELISA was conducted using anti-His antibody (1:500), rNLK (1:10), patient (n=9) or control serum (n=10) diluted 1:100 and rabbit anti-Human HRP (1:8000). A significant difference was observed between patients and controls (Fischer's exact - $p = 0.0345$), however the rapid development times and the poor correlation with the previous immunoblot resulted in attempts to improve the method.

3.3.15 Determining Factors Responsible for Background Signal

A series of controls were established to determine which, if any of the elements of the initial ELISA set-up were causing raised background. There was some cross-reaction of the rabbit anti-Human HRP detector antibody with the anti-His capture antibody (Figure 3-10, panel 3). This binding was not a result of reactivity with the blocking agent since wells coated with BSA only remained blank (Figure 3-10, panel 11). The well containing anti-His antibodies, patient serum and detector antibody (no antigen) produced absorbencies far in excess of other blank wells (Figure 3-10, panel 1). This effect may have been a result of the number of layers employed in the ELISA since substitution of the anti-His antibody for rabbit anti-NLK antibodies resulted in the production of similar background noise (data not shown). It is of interest that incubation of BSA coated wells with patients serum and HRP conjugated antibody produced raised background (Figure 3-10, panel 9). This observation led to the investigation of BSA as a suitable blocking agent (see section 3.3.16). Finally, antigen coated wells incubated with patient serum and HRP conjugated antibody also resulted in the production of signal (Figure 3-10, panel 5). This could have been a result of the non-specific reaction against the blocking agent as already described. However, the use of antigen coated wells was also investigated (see section 3.3.18).

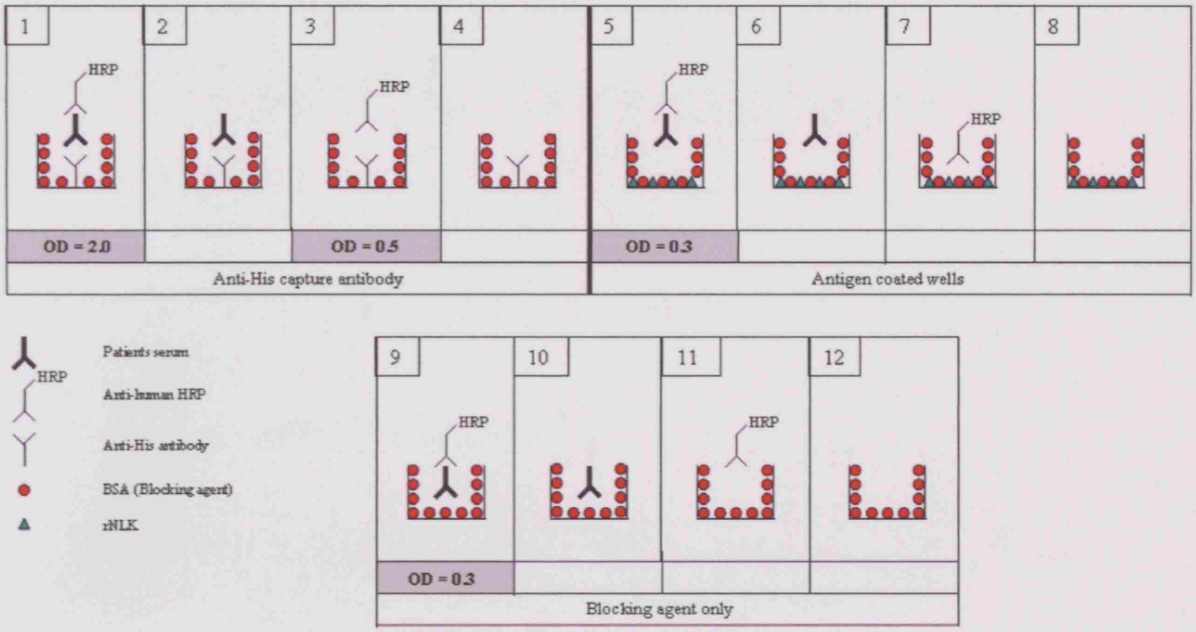


Figure 3-10: Determination of factors contributing to background signal in rNLK ELISA.

3.3.16 Comparison of Blocking Agents.

It was noted that some sera produced high absorbance values in blank wells (containing no antigen). Thus the suitability of BSA as a blocking agent was compared to that of normal rabbit serum. It was clear that rabbit serum was a much more suitable blocking agent. Background absorbance was lower than that produced in BSA-coated wells at all percentages (2.5, 5 and 10 % w/v). In addition use of rabbit serum produced more consistent absorbance (Figure 3-11). 2.5 % rabbit serum incubated for 1 hr at room temperature was sufficient to block the wells since any increase in the concentration of the blocking agent did not reduce the level of background.

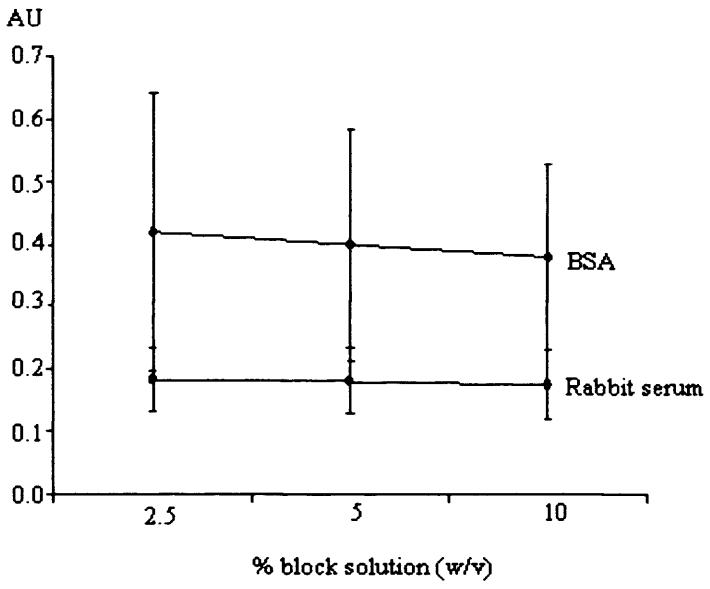


Figure 3-11: Comparison of BSA and rabbit serum as blocking agents.

Sera from 7 patients and controls incubated with wells blocked with increasing concentrations of blocking solution. The mean absorbance and the standard deviation were calculated for both blocking agents at each concentration. Rabbit serum was found to produce a consistently lower background signal than BSA.

3.3.17 Comparison of Crude Bacterial Homogenates and IMAC Purified rNLK

Comparison of the mean signal produced when crude and purified NLK were tested with control sera resulted in a non-statistically significant difference between preparations (Mann Whitney U = 21 p = 0.279) (Figure 3-12). The lack of significance may be attributed to the rise in signal produced by one sample upon use of purified NLK. However the mean absorbance produced by the purified preparation (0.683) was lower than that of the crude preparation (0.859) with a fall in absorbance in 6/7 (86 %) of the sera tested. Thus purified NLK was selected as a more suitable preparation for the ELISA.

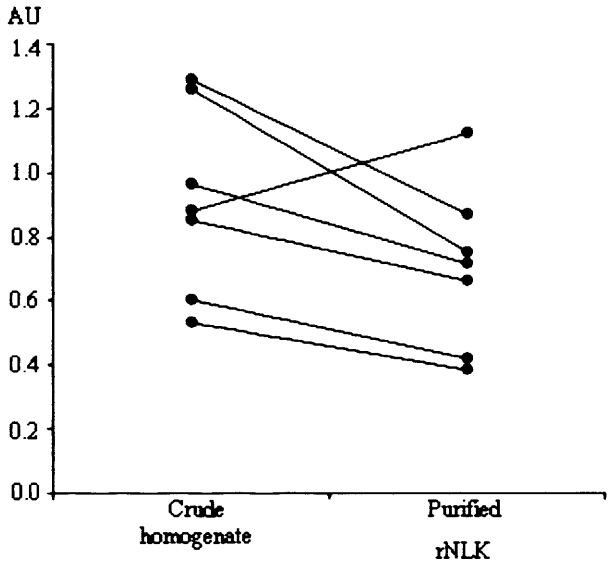


Figure 3-12: Comparison of crude homogenates of *E. coli* pRSETBNLK and purified rNLK in the NLK ELISA.

The mean absorbance for the ELISA using the crude antigen preparation was 0.859. The mean absorbance fell to 0.683 when the purified antigen preparation was used. The difference between the two preparations was not statistically different (Mann Whitney = 21 p = 0.279).

3.3.18 Use of Antigen Coated Wells.

The investigations into the cause of the background noise suggested that the use of antigen coated wells may provide an alternative form of ELISA. The initial investigations suggested that an antigen dilution of 1:50, a serum dilution of 1:300 and a detector dilution of 1:1000 would be suitable. The final ELISA utilised the serum of 11 patients and 13 controls (Figure 3-13). Anti-NLK activity was detected in 7/11 (63%) patients using the determined cut off (mean + 2SD). The difference between patients and controls was statistically significant (Fisher's exact test – p = 0.003). Although rabbit serum had been selected as a more suitable blocking agent some background signal was produced in blank wells (block only). To allow for this non-specific binding,

blank values were subtracted from the absorbencies produced in test wells (antigen coated). In this assay serum from 8/11 (72%) patients had a signal greater than the mean + 2SD of the anti-NLK negative controls. The difference between patients and controls was statistically significant (Fisher's exact test – $p = 0.0002$). The CV of the assay was calculated as 15%. The CV for the positive sample was 3%. The CV for the two negative samples was 33 and 26%. The results of the assay appeared to correspond to the intensity of the signal on the immunoblot of rNLK. Patients 3, 8 and 9 were negative for serum anti-NLK antibodies and a review of the immunoblot shows that the serum from these patients produced a faint band against rNLK on immunoblot (Patient 3 is shown, Figure 3-5 A, lane 3).

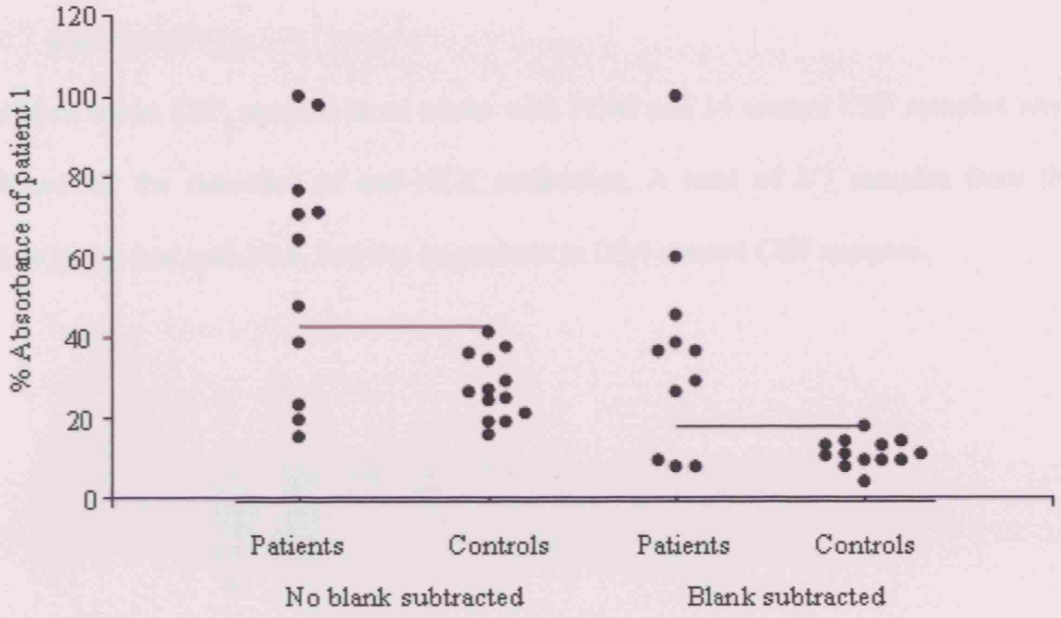


Figure 3-13: Final rNLK ELISA.

An ELISA was conducted using antigen-coated wells blocked with rabbit serum. Patient serum was diluted 1:300 and the detector antibody (rabbit anti-human HRP) diluted 1:1000. Results were expressed as a percentage of the signal produced by patient 1 to allow for interassay variation. In one experiment blank values were not subtracted. In this experiment 63 % of patients had a positive result (greater than the mean +2SD the absorbance of the controls). On subtraction of the blank 72 % of patients had a positive result. In both experiments there was a significant difference between patients and controls (Fisher's exact test; $p = 0.003$ (no blank subtracted) and Fisher's exact test; $p = 0.0002$ (blank subtracted)). The result correlated well with the immunoblotting. Patients 3, 8 and 9 were negative on the ELISA. On the immunoblot patient 3 was negative and patients 8 and 9 reacted weakly against rNLK.

3.3.19 Anti-NLK Antibodies in the CSF of Adults with Paraneoplastic Opsoclonus-myoclonus.

A total of seven CSF samples from adults with POM and 14 control CSF samples were analysed for the detection of anti-NLK antibodies. A total of 2/7 samples from the patient group had anti-NLK activity in contrast to 0/14 control CSF samples.

Figure 3-14: Detection of anti-NLK antibodies in the CSF of patients with OM.

Lane 1: Rabbit anti-NLK antibody (positive control). Lanes 2 – 5: CSF from adults with POM. Lanes 6 – 9: Representative CSF controls. Antibodies against NLK were detected in 2/7 patients (29 %) (Lanes 2 and 4) compared to 0/14 controls.

3.4 Discussion.

3.4.1 The Index cases.

We have described the first two documented reports of post-streptococcal OM. Both patients presented with a one-week history of febrile illness and upper respiratory tract infection prior to the onset of OM. Both patients had indirect evidence of streptococcal infection by way of a raised ASOT.

The cases were both unusual with regard to the histories. Based on the extreme differences between the mean age of onset of adult and paediatric OM it is unclear as to which of these the index cases should be classified. As discussed, paediatric OM occurs within the first 36 months of life while adult disease is most common over 50 years of age. The patients fit more easily into the 'idiopathic' definition applied to OM as used by Bataller *et al* (2001) Most importantly, the OM in both index patients all but resolved following the instigation of immunomodulatory therapies. Given these observations and the findings of published reports it may be more appropriate to regard OM as a rare post-infectious disease occurring at any age onto which the occurrence of adult and paediatric paraneoplastic OM can be superimposed. Given the post-streptococcal aetiology implied in the pathogenesis of OM in the index cases it will be of interest to determine the frequency with which streptococcal infection proceeds the onset of OM in other non-paraneoplastic cases.

3.4.2 Protein Purification and Identification.

As has been discussed, consistent anti-neuronal antibody markers of both paraneoplastic and post-infectious OM have not been described. Both patients reported in this thesis had antibodies against a 56 kDa protein found in homogenates of neuronal tissue. Given

the history of upper respiratory tract infection and the molecular weight of the recognised antigen these antibodies were initially thought to react with PYK (See chapter 2). However, testing of serum against recombinant PYK was negative, thus protein purification techniques were applied to purification of the target antigen.

Ammonium sulphate fractionation was used to provide a partially purified preparation of rat brain homogenate in which the 56 kDa antigen was relatively enriched. Simple 2D gel separation allowed the clear identification of a protein which was well separated from contaminating proteins and recognised by patients serum. The separation was sufficient to allow the protein to be digested from the 2D gel for characterisation by mass spectrometry without the need for further purification. As an additional check the 56 kDa protein was purified using IEX chromatography. The two proteins isolated by independent methods (2D and IEX chromatography) were analysed by mass spectrometry and in both cases the protein was identified as NLK. These findings were confirmed using rNLK. In addition by probing immunoblots of rNLK we were able to demonstrate the presence of anti-NLK antibodies in the CSF of both patients. This suggests that the antibodies have access to the antigen within the nervous system and thus the potential to induce disease.

3.4.3 Cohort Findings: Immunoblotting.

Serum samples from a group of 9 paediatric patients with OM were investigated for the presence of anti-NLK antibodies 7 of whom were found to be positive (9/11 total (82 %)) . Two patients in the cohort had an underlying neuroblastoma, five were post-infectious and two were 'idiopathic' i.e. no tumour detected. It would appear from these initial investigations that an anti-NLK response may be a marker of OM irrespective of

aetiology. ECL was used for the development of the immunoblots since the total volume of sera from the patients was limited and this method of development allows the use of smaller volumes of sample in the immunoblot. The majority of control immunoblots were easily interpretable. However, immunoblots using patient sera were a little harder to interpret. A consistent response against rNLK was observed, but multiple bands were detected. These additional bands may have been related to the presence of degraded recombinant proteins, contaminating bacterial proteins or the sensitive nature of ECL development. The subjective nature of immunoblotting leaves the technique open to some differences in interpretation.

3.4.4 Cohort Findings: rNLK ELISA.

The use of rNLK immunoblots demonstrated the existence of anti-NLK antibodies in a number of patients with OM. However, given the objective nature of the interpretation of the blots an ELISA was devised in an attempt to provide a quantitative assay for detection of anti-NLK activity.

Due to concerns regarding the existence of residual *E. coli* proteins in the rNLK preparation initial attempts at ELISA development utilised anti-His coated wells. Since the anti-His antibody is highly specific it was thought that this approach would prevent *E. coli* contamination. In fact the capture method was hindered by the non-specific interaction of some of the components, and was later adapted for the use of antigen coated wells.

To establish the cut-off value, serum samples from neurologically normal controls were selected. This may have artificially lowered the cut-off value as patients with neurological disease may have anti-NLK activity. Ideally, a further group of neurological controls should have been included to further test the cut-off value. Any positive samples could then be tested against rNLK by immunoblot to determine if a specific anti-NLK antibody response could be detected. With this caveat in mind, the ELISA demonstrated an anti-NLK response in 8/11 (73 %) patients. The results correlated roughly with the findings produced by immunoblot (see Figure 3-5 and Figure 3-13).

These initial investigations highlight a potentially frequent anti-NLK response in the serum of OM patients. Further investigations with a larger cohort are required to confirm the frequency with which anti-NLK antibodies occur. However, it is clear that the initial studies using sera from the index cases have led to the identification of antibody association in OM.

3.4.5 NLK.

NLK has both intracellular and extracellular function and is therefore an example of a moonlighting protein. Intracellularly NLK acts as the cytosolic enzyme, GPI, which catalyses the interconversion of glucose 6-phosphate and fructose 6-phosphate in the glycolytic pathway. Extracellularly the protein has various functions including the regulation of cell migration during tumour invasion and metastasis (autocrine motility factor, AMF) and cell maturation (maturation factor, MF).

The name NLK was ascribed as a result of the neurotrophic effects observed when the protein was incubated with cultures of spinal and sensory neurones (Gurney *et al*, 1986). Direct evidence for the role of NLK in the nervous system is limited. Most studies involved the detection of NLK expression in cell lysate, or the effects of the protein *in vitro*. It has been suggested that within the nervous system NLK is secreted and that its extracellular effects are mediated though interaction with the cell surface glycoprotein gp78 (Hage *et al*, 2000). It is also possible that a form of NLK is associated with the plasma membrane. This is supported by the fact that NLK has been detected within synaptosomal membrane preparations (Knull and Fillmore, 1985) and that other glycolytic enzymes have been shown to be associated with the plasma membrane (Nakajima *et al*, 1994). Evidence for the association of NLK with the plasma membrane is supported by the staining of neuronal cultures as described in this thesis.

A function for NLK in the nervous system can be inferred from its role in neurological disease. Antibodies against NLK have been implicated in motor-neurone disease (Gurney *et al*, 1984), interference with its function has been implicated in AIDS-associated neuropathology (Lee *et al*, 1987) and mutation in the NLK gene has been connected with neurological myopathy and mental retardation (Kugler *et al*, 1998). Thus, NLK is evidently required for the normal development and function of the nervous system and interference with that function has the capacity to cause disease. However the clinical phenotype of motor-neurone disease and OM are very different and it is unclear if anti-NLK antibodies could cause such distinct disorders.

3.4.6 NLK: A Reasonable Antigenic Target?

It may be of use to review the criteria antibody-mediated autoimmunity and for molecular mimicry provided in sections 1.5 and 1.7.2 respectively. The criteria satisfied by anti-NLK antibodies as agents in antibody-mediated disease and molecular mimicry are summarised in Table 3-3 and Table 3-4 respectivly.

Table 3-3: Criteria for pathogenicity of NLK and NLK antibodies.

Criterion	*Evidence*
Antibodies directed against the appropriate tissue type	NLK is found in association with the neuronal surface but its expression is not restricted to the nervous system.
Passive transfer of antibodies/ Immunisation	Neither passive transfer of antibodies nor immunisation of animals has been shown to cause OM
Immunotherapy	Limited evidence to suggest that symptoms improve following immunomodulatory therapies.

Table 3-4: Criteria satisfied for anti-NLK antibodies in molecular mimicry.

Criterion	*Evidence*
Epidemiological association with infectious agent.	Limited evidence. Many infectious agents have been associated with OM but evidence is limited to case reports. An extensive epidemiological investigation has not been conducted.
Presence of antibodies.	The data presented here suggest that anti-NLK antibodies may frequently be associated with OM.
An antigenic mimic must be identified.	NLK is an evolutionary conserved protein found on the surface of streptococcus.
Animal model.	An animal model of OM has not been produced.

As discussed in the introductory chapter a number of anti-neuronal antibodies have been described although only a few have been shown to be pathogenic. In the majority of reports, antibodies against intracellular targets such as Hu, NOVA1 and CDR62 have not been shown to cause disease, possibly because the target antigen is normally inaccessible. However, antibodies targeting cell surface proteins (Anti-Ach receptor, anti-VGCC) have been shown to cause disease. The extracellular location of NLK and its association with the plasma membrane make it an accessible antigenic target.

As has been discussed, patients with 'idiopathic' OM often have substantial if not complete resolution of their neurological symptoms which is consistent with the lack of pathological findings at autopsy (see sections 1.2.4 and 1.11). Thus, it is unlikely that the pathological mechanism of the disease is cell death. Alternatively, antibodies may subtly interfere with normal neuronal function following interaction with surface bound

NLK or from disruption of the interaction of NLK and gp78. Such a mechanism would not produce characteristic inflammatory features or neuronal cell loss.

Since NLK represents a component of the glycolytic pathway it is unsurprising that it is found in all cells. There are a number of explanations as to why antibodies against this globally expressed protein could cause neurological disease. One answer may lie in accessibility of the antigen as discussed previously. It may be that in most tissues NLK is only expressed within the cytoplasm of cells, in which case the antibodies may only interfere with the secreted or cell surface form of the protein and thus exert pathogenic effects specific to the nervous system. Since a rigorous study of NLK expression in systemic tissues has not been conducted we cannot say for certain that NLK does not appear on the cell surface of other cell lines.

There are two main reasons why anti-NLK antibodies may not have been previously detected in OM. Firstly, one of the commonest methods by which antibodies associated with disease are detected (prior to characterisation of the antigen) is to use photochemical or immunoblotting techniques. Since NLK is systemically expressed in the cytoplasm of all cells reactivity may have been disregarded due to non-neuronal specificity without realisation of the neuronal roles of NLK.

The second commonly-employed method for the identification of putative auto-antigens is that of bacteriophage expression library screening. The use of this technique with serum from OM patients has had little impact in defining commonly recognised antigens (Bataller *et al*, 2003). During the production of rNLK it was noticed that the anti-NLK antibodies reacted with a bacterial protein in the crude preparations of control

cultures (pRSET only). It is well-known that the development of the glycolytic pathway predates the split between eukaryotes and prokaryotes (Hattori *et al*, 1995) and thus it is not unreasonable to assume that *E. coli*, the bacteria used for library screening, carry a homologous protein to human NLK. If this was the case then library screening would be an unsuitable method by which NLK could be characterised as the antibodies would have been non-specifically bound to the bacterial homologue.

A BLAST search revealed 89 exact amino acid matches (23 % of the total sequence) with 147 close matches (38 %) (expected chance homology was less than 1×10^{6}) between streptococcal GPI and human proteins making this a potential molecular mimic and thus satisfying the third criteria for molecular mimicry. It is particularly interesting to find NLK associated with the membrane of streptococcus (Huges *et al*, 2002). However, it must be recognised that homology between bacterial and human proteins does not guarantee cross reactivity between species. It would be useful to check that the anti-NLK antibodies reacted with a purified streptococcal form of NLK and that reactivity with NLK could be removed by pre-incubation of patient's serum with homogenates of streptococcus. In addition, only two of the cases described here had evidence of streptococcal infection. However, since NLK represents a conserved protein it may be that a number of pathogens have the capability to induce autoantibody formation. Clearly, a rigorous study into the nature of associated pathogens is required in a larger cohort of patients.

3.4.7 NLK: A Unifying Antigenic Target?

The similarities between PIOM and paraneoplastic POM have been discussed. It is interesting that post-infectious and paraneoplastic processes could result in the same

clinical phenotype and may suggest that there is a common antigenic target in both paraneoplastic and post-infectious OM. We have presented data from a limited cohort of adult patients which suggests that anti-NLK antibodies can be detected in the CSF of POM cases although antibody detection was not a consistent finding. Clearly, a larger study is requied to expand on these findings. However, there is circumstantial evidence to suggest that this may be the case. Firstly,

NLK is thought to have a role in directing the metastatic spread of tumour following secretion from neoplastic tissue. In addition, patients who develop paraneoplastic OM frequently have tumours that are limited in size and metastatic spread when compared to neurologically intact controls. It is thus possible to theorise that anti-NLK antibodies could occur in a cancer patient as a result of the inappropriate secretion of normally sequestered NLK, and that these antibodies prevent NLK-mediated spread of neoplastic cells. The anti-NLK antibodies would also interfere with the normal neuronal functions of NLK. This would account for both the limited spread of the tumour and the neurological disease.

If antibodies are important in the pathogenesis of paraneoplastic and post-infectious OM why should one group do remarkably better? It has been clearly documented that patients with 'idiopathic' OM have a better neurological outcome than do the paraneoplastic patients. In one study the majority of the patients with idiopathic OM experienced complete or significant resolution of their neurological syndrome (Digre, 1986). In contrast the patients with an underlying malignancy fared worse. These findings are mirrored by those of Bataller *et al* (2001). The answer may lie in looking at the stimulus for antibody synthesis. In the case of post-infectious OM it is possible that

as the infectious agent is cleared by the host's immune system the stimulus for antibody production is removed resulting in a self-limiting illness. In the case of paraneoplastic OM, immunomodulation may lower the titre of pathogenic antibodies but does not remove the stimulus for antibody production and may in fact reduce anti-tumour activity thus allowing tumour growth. Indeed, surgical intervention is the only therapy found to have significant effect on paraneoplastic OM. However this theory does not explain why so many patients, especially in the paediatric population, are left with neurological deficit following successful tumour therapy.

3.4.8 Areas for Future Research.

The experimental findings presented here provide some evidence for an interesting antigenic target associated with the presence of serum antibodies in OM. It is clear that questions remain unanswered. In order to address these, a number of investigations can be conducted.

Firstly, a larger epidemiological study is required. The number of patients presented here is limited. This is a result of the rarity with which OM occurs. It would be necessary to acquire clinical data on a larger number of patients. In particular, it would be of interest to see what proportion of patients have evidence of an anti-NLK response and a clearly defined association with a pathogenic organism. Such a cohort could be acquired through the use of organisations such as the British National Neurological Surveillance (BNSU). In addition, further control data will be required to support the findings of this thesis. Increased numbers of neurological and healthy controls will be needed. More importantly, it would be of considerable value to determine the frequency of an anti-NLK response in post-streptococcal diseases and patients with neuroblastoma

but no neurological deficit. Similarly the testing of patients serum against unrelated recombinant proteins should be conducted as a matter of course to ensure any results are not the result of non specific reactivity with E. Coli proteins in recombinant protein preparations.'

Secondly, the experiments presented above have not addressed the issue of whether the antibodies are pathogenic. Incubation with cultured neurones and measurement of cell death or disruption of function would be of value.

It would be of interest to confirm that anti-NLK antibodies from the index patients cross-reacted with both human NLK and streptococci GPI. The use of streptococcal homogenates to absorb out anti-NLK antibodies would quickly resolve this issue. It would be of particular interest to determine if the immunisation of animals with streptococcal GPI could invoke an anti-NLK response and replicate OM.

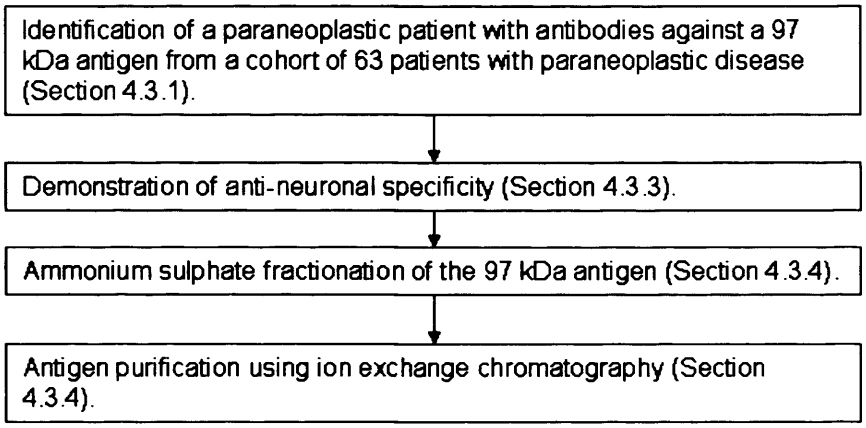
Finally, a cohort of patient with POM is required in whom malignant tissue is available. It would interesting to determine the level of NLK in patients serum or the arberrant expression of the antigen by malignant cells and determine if an antibody response is associated with limited metastatic spread.

Overview of Chapters Four, Five and Six.

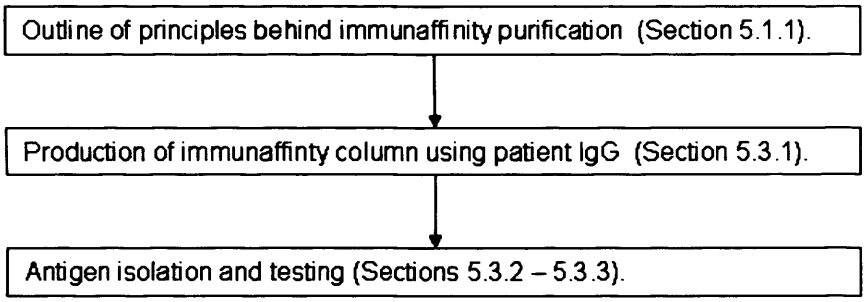
The following chapters describe a series of experiments in which a patient with a paraneoplastic cerebellar syndrome was selected on the basis of the presence of anti-neuronal antibodies against an unidentified 97 kDa antigen. The patient was selected from a series of patients with paraneoplastic disease who had been identified through a national surveillance programme. The clinical findings of each patient and associated antibodies are summarised along with the clinical characteristics of the selected patient. In the subsequent investigations 2D gel electrophoresis, ion exchange chromatography and immunoaffinty chromatography were applied in an attempt to purify the antigen in preparation for identification by mass spectrometry. The reasons for the failure of these methods to produce significant amounts of purified antigen are discussed.

Since the majority of anti-neuronal antibodies have been characterised by application of bacteriophage screening this method was used to determine the nature of the antigen recognised by the antibodies of our index patient. While the 97 kDa was not isolated the technique identified an antigen which was recognised by both the index patient and those found in the serum of a second patient with paraneoplastic cerebellar disease. The relevant experiments and the sections in which they can be found are summarised in the following flow chart.

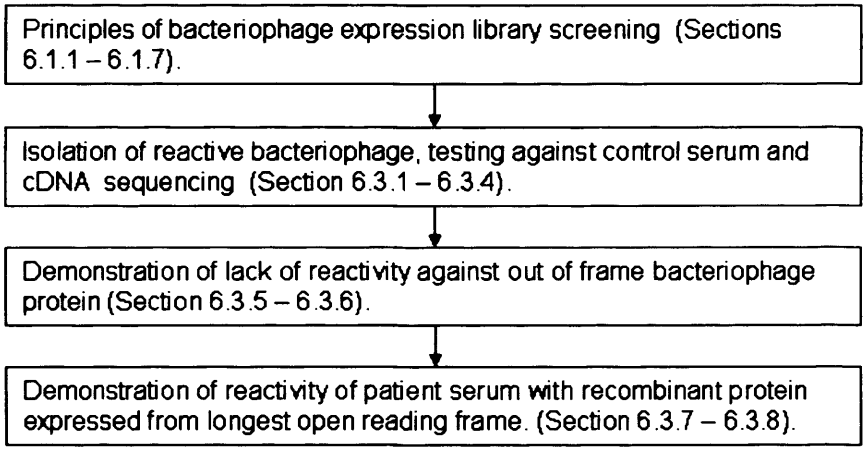
Chapter 4



Chapter 5



Chapter 6



4 *Chapter 4: Identification of a patient with a paraneoplastic syndrome and novel anti-neuronal antiboy directed against 97 kDa antigen.*

4.1 Introduction.

The majority of epidemiological studies concerning PND consist of series of selected patients with specific cancers (Elrington *et al*, 1991), specific anti-neuronal antibodies (Sillevis *et al*, 2002; Dalmau *et al,* 1992) or specific neurological syndromes (Gultekin *et al*, 2000; Peterson *et al*, 1992) but information of a more general nature is not readily available.

Using a nation-wide reporting scheme run by the BNSU data concerning patients with suspected PND was collected. We investigated the clinical spectrum of PND, the associated tumours and anti-neuronal antibodies, and the response of the PND to treatment of the underlying malignancy or immunomodulatory treatment. By reviewing anti-neuronal antibody reports we were able to identify patients with atypical anti-neuronal antibodies whose serum could be employed in antigen characterisation using previously successful methods.

4.2 Methods.

4.2.1 Paraneoplastic patients.

The British Neurological Surveillance Unit was used to consult members of the Association of British Neurologists (ABN) as to whether they had encountered cases of paraneoplastic disease affecting the CNS. Data collection was conducted between February 2000 and January 2001. Further information was sought by means of a questionnaire. Patients were included if they had a neurological disease in association

with a known malignancy or recognised anti-neuronal antibodies in the absence of a tumour. In some cases patients were referred directly to Dr J Rees. Patients were classified as either definite PND i.e. a neurological disorder occurring in association with an identified malignancy for which there was no other explanation, probable PND (anti-neuronal positive with no identified malignancy) or possible PND. Where possible, case notes were reviewed to obtain further clinical information. The study was approved by the Ethics Committee of the National Hospital for Neurology and Neurosurgery. In some instances anti-neuronal antibodies were identified as part of the routine diagnostic service offered at the National Hospital for Neurology and Neurosurgery. Serum was considered anti-neuronal antibody positive if the immunohistochemical staining of rat cerebellar tissue sections and immunoblotting of crude brain homogenate produced a pattern consistent with recognised anti-neuronal antibodies in PND (Table 1-3). On occasion the staining pattern produced or the molecular weight of the detected antigen did not conform to these patterns. Such samples were described as containing 'miscellaneous' anti-neuronal antibodies and were considered for use in antigen characterisation.

4.2.2 Sample Preparation, Ammonium Sulphate Fractionation and 2D Electrophoresis.

Pig cerebellum was selected as the tissue if choice as there was a ready supply available at the time of experimentation. Immunobloting was used to confirm the presence of the 97 kDa before the tissue was used in subsequent experiments. Cerebellum was isolated

and frozen within 10 minutes of sacrifice. Cerebellar homogenate was produced as described in section 3.2.3. ASF was conducted using the method described in 3.2.4. Initial wide fractions at 0 - 25, 25 - 50, 50 - 75, and 75 -100 % saturations were used. The 97 kDa protein was detected by immunoblot using sera from the index patient. The individual fractions were analysed using PAGE and Coomassie staining. The process was repeated using 10 % fractions from 0 – 60 %.

The 20 % fraction was selected as it contained relaivly enriched protein and buffer-exchanged into sodium phosphate buffer (pH 7.0) and concentrated using the Microcon system (Millipore, UK). Proteins were separated by pI and molecular weight as described in section 3.2.5. The target antigen was identified using patient serum and an immunoblot of the 2D gel. Reactive proteins were identified in the corresponding silver-stained gel and subjected to mass spectrometry if appropriate (See section 3.2.7).

4.2.3 Ion Exchange Chromatography.

IEX chromatography was performed using the ÄKTA FPLC system and the anionic exchange HiTrap Mono Q FF (5ml) column (Amersham Biosciences). Proteins from the 20 % ammonium sulphate fraction were exchanged into IEX binding buffer (20 mM Tris-HCl pH 8.00) and injected onto the column. Proteins were eluted over a gradient of increasing ionic strength by injection of elution buffer (20 mM Tris-HCl 1.5 M NaCl pH 8.35) 0-100% over 120 ml. Fractions were collected using a Frac-900 fraction collector and those fractions corresponding to peaks on the chromatogram selected, subjected to PAGE and immunoblotted. The 97 kDa antigen was detected using serum

from the index patient. Identified proteins were silver-stained and identified using mass spectrometry where appropriate.

4.3 Results.

4.3.1 Identified Patients, Underlying Tumours and Diagnosis of PND.

A total of 63 patients were identified (48 females, 15 males). Of these 52 patients were classified as having a definite PND. Detailed analysis of the cohort can be found in the publications at the back of the thesis. Of the 52 patients, 10 were known to have malignant disease before the onset of PND. The tumours in this group consisted of two SCLC, three breast (one with concurrent colonic cancer) 2 ovarian, 1 prostate, 1 mesothelioma and 1 endometrial carcinoma. In the remaining 42 patients the tumour was identified as a result of investigations for a suspected PND. Of these patients 22 were found to have lung cancer (17 SCLC, 5 non-SCLC (NSCLC)), 6 breast cancer, 4 adenocarcinoma of unknown primary, 4 Hodgkin's disease, 3 ovarian cancer, 1 melanoma, 1 malignant neuroendocrine tumour and 1 plasmacytoma.

A diagnosis of a probable PND was made in 10 patients because they had serum anti-neuronal antibodies (9 anti-Hu and 1 anti-Yo positive) but no tumour was found. One patient who presented with clinical features of limbic encephalitis and motor neuropathy was diagnosed as having a possible PND because the phenotype was compatible with a PND but no tumour or anti-neuronal antibody were identified.

4.3.2 Anti-neuronal Antibody Detection.

Results of anti-neuronal antibody screening were positive in 44 out of 58 patients (76 %). Of these, 39 had known antibodies (25 with anti-Hu antibodies, 11 with anti-Yo antibodies, 2 with anti-Tr antibodies and 1 with anti-amphyphysin antibodies). Five further patients had anti-neuronal antibodies demonstrated by immunohistochemistry or western blotting that did not recognise known antigens these were therefore classified as 'miscellaneous'. The tumour associations for each antibody are documented in Table 4-1. Of particular note is that anti-Yo antibodies were found in one patient with NSCLC and 2 patients with adenocarcinoma of unknown primary (AUP) and that one patient with anti-Tr antibodies had AUP.

Table 4-1: Tumour associations in patients with positive serum anti-neuronal antibodies.

	SCLC	*NSCLC*	*Breast*	*Prostate*	*Ovarian*	*AUP*	*Endometrial*	*NE*	*HD*	*NTF*	*Total*
Hu	9	3	3	1						9	25
Yo		1	3		3	2	1			1	11
Tr						1			1		2
Amphiphysin			1								1
Misc	1	1	3								5

SCLC = small cell lung cancer, NSCLC = non-small cell lung cancer, AUP = adenocarcinoma of unknown primary, NE= neuroendocrine, HD = Hodgkin's disease, NTF = no tumour found.

4.3.3 Details of Patient with Miscellaneous Anti-97 kDa Antibodies

A 61 year old right-handed female presented in 1999 following the acute onset of vertigo which settled after a few days leaving the patient with some difficulty in

walking. Two weeks later she experienced the abrupt onset of unsteadiness that caused her to fall. This episode was accompanied by shaking in all limbs. Since the second attack the patient had been unable to walk. Her condition remained stable until two months later when she developed diplopia. Her speech was unaffected but her mouth had become dry. There was no bladder or bowel disturbance. The patient had a history of laryngeal carcinoma in 1985 that was treated with radiotherapy. There was also a history of glaucoma and inflammatory bowel disease. There was no family history of note. The patient smoked 25 cigarettes a day.

The patient was able to stand with her feet together, but was rather unsteady. She was Romberg negative. She had a broad-based gait and truncal ataxia. There was no dysarthria. Eye movements were abnormal with reduced elevation in the left eye accompanied by diplopia on looking upwards, to the left and in the primary position. Upper limb co-ordination showed slight finger-nose ataxia, but definite dysdiadochkinesis in both hands. There was severe heel-shin ataxia. Power was normal throughout. Reflexes were diminished but post-pertantic potentiation could be elicited. Planters were flexor and sensation was normal in all modalities.

An MRI of the brain revealed a small right cerebellar cortical infarct which was not thought to be the cause of her ongoing problems. Investigation for anti-VGCC antibodies was positive (552, >100 = positive). Investigation for the presence of anti-neuronal antibodies reported the presence of an antibody against a 97 kDa protein in guinea pig brain homogenate on immunoblot. Histochemistry against rat cerebellar sections was reported as negative. The diplopia, dry mouth and potentiation of reflexes coupled with the positive anti-VGCC antibodies suggested a diagnosis of LEMS.

Electrophysiological testing confirmed this diagnosis. Due to the association of LEMS with underlying SCLC the patient was investigated for the presence of an underlying malignancy. A chest X-ray failed to demonstrate a focal lesion. However a full body FDG pet scan was conducted which showed increased uptake in the left upper lung field with a linear distribution extending towards the midline (Figure 4-1). A fine cut CT scan showed a 1 cm irregular nodule in the apical segment of the left lower lobe. A retrospective viewing of a CT scan conducted in 1999 showed that the mass had been present at that time, and that its appearance had not changed in the last 3 years. Bronchoscopy revealed no abnormality and broncho-alveolar lavage was negative for cytology. In view of the patients poor lung function an exploratory thoracotomy was not conducted. Instead the decision was taken to monitor the lesion using repeat CT scans. Treatment with 3, 4 - diaminopyradine was instigated for her LEMS.

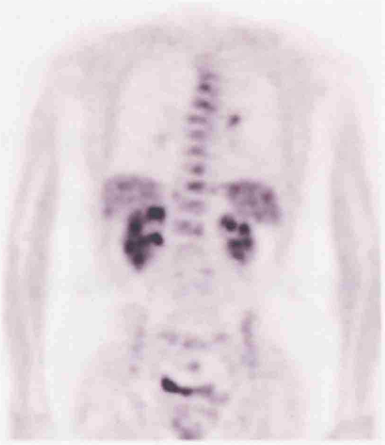


Figure 4-1: [18F]fluoro-2-deoxyglucose-PET scan.

The scan shows increased uptake in the left upper lung field with a linear distribution extending towards the midline.

Investigation for the presence of anti-neuronal antibodies was repeated. The immunoblot again demonstrated that the patient had antibodies against a 97 kDa protein

that was found in human and pig cerebellum. The reactivity could also be identified in pig cortex. The immunoblot of pig liver did not produce a similar band suggesting that the recognised antigen was neuron specific.

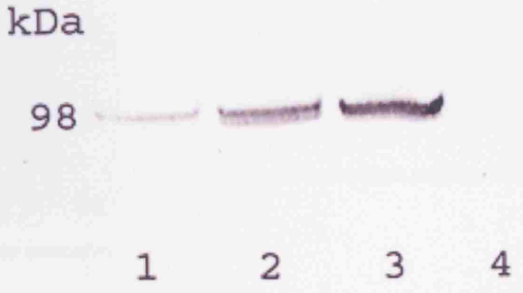


Figure 4-2: Evidence for an anti-neuronal response against a 97 kDa neuronal protein.

Immunoblot of 1: human cerebellum, 2: pig cerebellum, 3: pig cerebral cortex and 4: pig liver using serum from the index patient.

4.3.4 Ammonium Sulphate Fractionation.

An immunoblot of the 25, 50, 75 and 100 % ammonium sulphate fractions of pig cerebellar homogenate probed with patient serum demonstrated strongest reactivity with the 97 kDa antigen in the 50 % fraction. In addition a relatively small amount was also detected in the 25 % fraction (Figure 4-3, B). A stained gel of the fractions revealed the differential precipitation of proteins but no obvious individual protein at the 97 kDa level could be detected in either the 25 or 50 % fractions (Figure 4-3, A). In addition, fractionation uncovered antibody reactivity with three hitherto unrecognised proteins; one in the 75 % and two in the 50 % ammonium sulphate fraction (Figure 4-3, B). These proteins had not been detected when crude human or pig brain homogenates had been probed (see Figure 4-2).

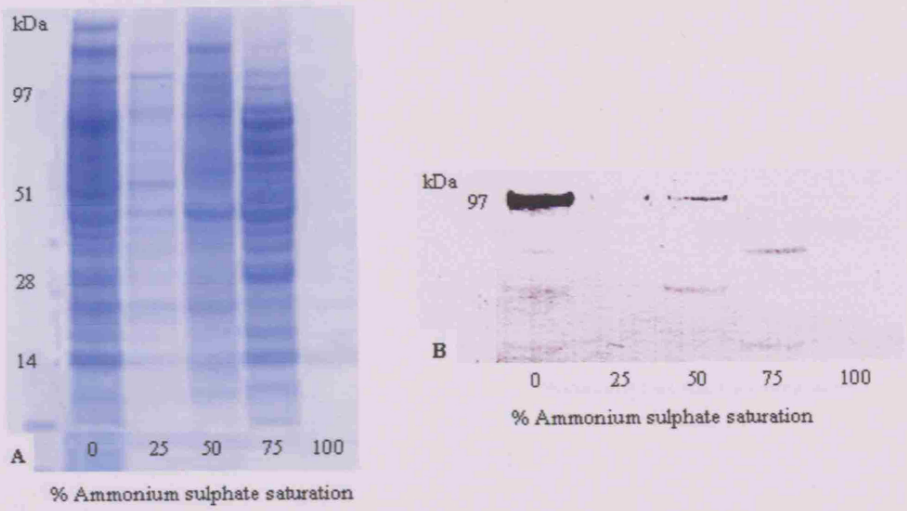


Figure 4-3: Ammonium sulphate fractionation of pig cerebellum (wide fractions).

Crude homogenates of pig cerebellum were subjected to ammonium sulphate fractionation using 25, 50, 75 and 100 % ammonium sulphate saturations. The precipitated proteins were subjected to PAGE and the gel **A**: stained or **B**: immunoblotted. The immunoblot detected some antigen in the 25 % fraction but the majority was precipitated at 50 % ammonium sulphate saturation. Other proteins also appeared to be reactive at a lower level at both 50 and 75 % ammonium sulphate saturation. The stained gel did not show discrete proteins at the 97 kDa level.

Repeat fractionation using 10 % fractions from 0-40 % demonstrated that the antigen precipitated in the 10, 20 and 30 % fractions, but was relatively enriched in the 20 % fraction (Figure 4-4, B). Staining revealed the differential precipitation of proteins but again no clear protein could be seen at the 97 kDa level (Figure 4-4, A). The additional bands observed at the higher ammonium sulphate saturations were not detected in any of the smaller fractions.

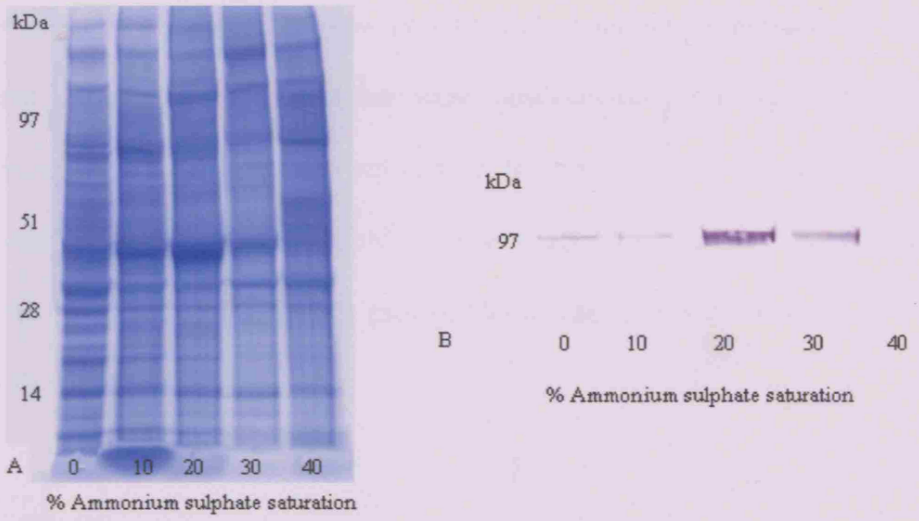


Figure 4-4: Ammonium sulphate fractionation of pig cerebellum (narrow fractions).

Ammonium sulphate fractionation was repeated using smaller fractions: 10, 20, 30 and 40 % ammonium sulphate saturation. Fractions were subjected to PAGE and A: stained or B: immunoblotted. The 97 kDa protein was precipitated at 10-30 % ammonium sulphate saturations with a relative increase in concentration in the 20 % fraction. The stained gel revealed a partial purification of the whole homogenate but no 97 kDa proteins were clearly discernible in any of the fractions. Further purification was therefore required.

4.3.5 2D Gel Electrophoresis.

2D gel electrophoresis was performed using the 20 % ammonium sulphate fraction of pig cerebellar homogenate. Silver staining revealed that the majority of the proteins migrated to a pI of 5 to10. A protein could be identified at the 97 kDa level with a pI of approximately 8. Immunoblotting and probing with patient's serum identified a protein with a higher molecular weight (between 97 and 198 kDa) (Figure 4-5, B). Both the silver stain and the immunoblot (developed using ECL) required extended developing

time possibly as a result of the low protein load. Attempts to increase the signal using increased concentration of proteins were unsuccessful resulting in poorly focused proteins or failure to detect the antigen on immunoblot. The initial results were not reproducible. In no instance could a clearly separated antigen be correlated with reactivity on an immunoblot, thus protein identification was deemed not to be possible using this approach.

4.3.6 IEX Chromatography.

Fractions corresponding to the peaks on the chromatogram clearly showed the separation of proteins in different fractions (Figure 4-6). However, the immunoblot (not shown) could not detect the 97 kDa antigen in any of the fractions. Repeated attempts using increased protein concentration did not facilitate purification or detection of the antigen.

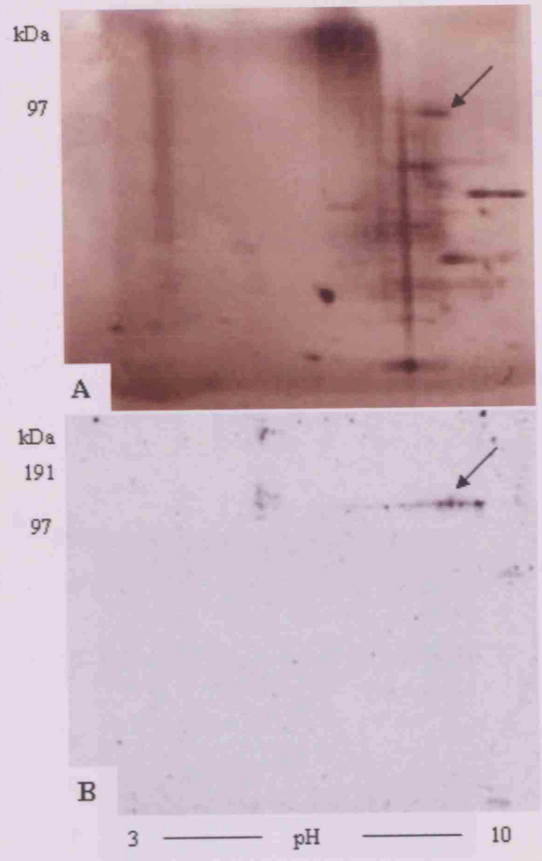


Figure 4-5: 2D gel electrophoresis of cerebellar proteins precipitated at 20 % ammonium sulphate saturation.

A: Silver stained gel. **B**: Immunoblot probed with patients serum. A band of protein with a molecular weight between 97 and 191 kDa extending from approximately pH10 was recognised by the patients serum. The stained gel revealed proteins at a lower molecular weight level. Both staining and ECL development required extended periods suggesting a low concentration of protein.

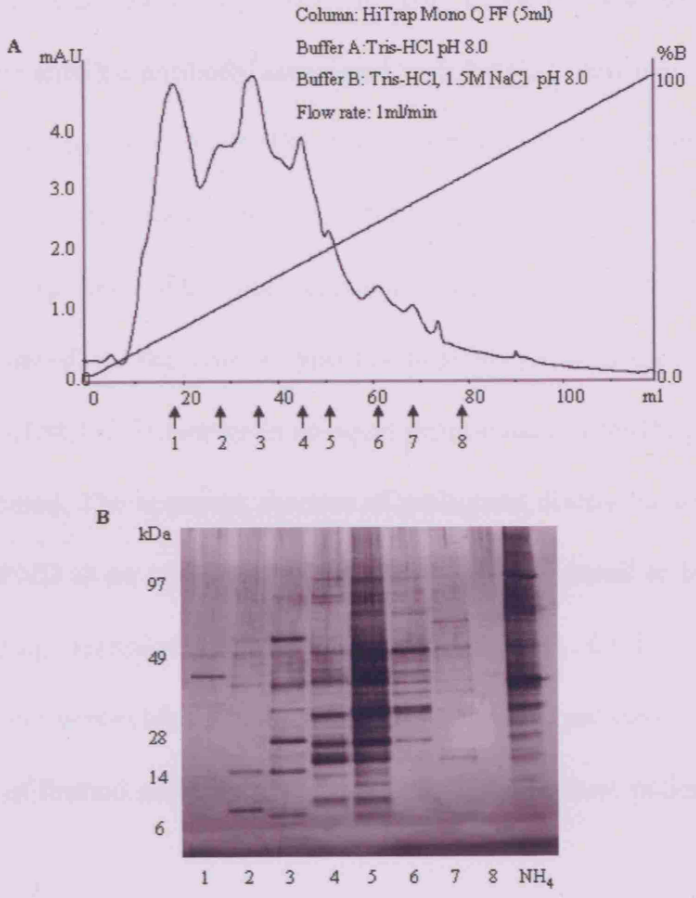


Figure 4-6: IEX purification of the 97 kDa antigen.

A: Partially purified proteins from 20 % ammonium sulphate fraction of pig cerebellum were separated. Peaks 1 - 8 were selected. **B** Proteins were separated by PAGE and the gel stained or immunoblotted (not shown) and probed with patients serum. IEX resulted in the separation of proteins, however patients serum did not detect an antigen in any of the lanes. (NH4 = pre-IEX 20 % ammonium sulphate fraction of pig cerebellar homogenate).

4.4 Discussion.

4.4.1 Anti-Neuronal Antibody Results.

The utility of anti-neuronal antibodies in both aiding diagnosis and directing the search for an underlying malignancy is well-documented (Dalmau *et al*, 1990; Anderson *et al*, 1998; Luque *et al,* 1991; Voltz *et al*, 1999). However, while most tumour associations

were consistent with those described in the literature we found four unusual associations, one anti-Yo antibody associated with NSCLC and two AUP and one anti-Tr antibody associated with AUP. The association of anti-Yo antibody with AUP has been previously reported (Peterson *et al*, 1992; Sutton *et al*, 2001) but is rare. However to the best of our knowledge the association of anti-Tr with AUP has not been previously described. In the case of anti-Hu positive patients the commonest tumour found was that of SCLC. However in an equal proportion of anti-Hu positive patients no tumour was located. The apparent absence of malignant disease is not inconsistent with a diagnosis of PND as on occasion the tumour may be too small to be detected even by sensitive imaging techniques (Anderson *et al*, 1998; Croft *et al*, 1965). The unexpectedly high percentage of tumour negative anti-Hu patients in this study may be a consequence of limited autopsy or PET investigations in these patients.

4.4.2 The Index Case

Five of the 63 patients (8 %) on whom data were collected were found to have antibodies against a neuronal protein which had not been previously described. Of these one was selected for further investigation on the basis of a compelling clinical picture, strong anti-neuronal response against crude brain preparation and availability of relatively large amounts of serum for use in investigations. Repeat investigation for anti-neuronal antibodies in the patient's serum confirmed a strong anti-neuronal antibody response against a 97 kDa protein expressed in the cerebellum and cerebrum but not in non-neuronal tissue. This patient had a sub-acute cerebellar syndrome and LEMS. It is important to note that an absolute diagnosis of PND can not be assigned since the patients poor lung function precluded tissue sampling of the suspected underlying lung mass. However abnormal uptake on FDG-PET scanning confirmed by

CT scanning in a smoker with LEMS (60% of cases are tumour associated) is strong evidence. The association of SCLC, cerebellar degeneration and LEMS has been previously described. In 1992, Clouston *et al* described a series of nine patients with LEMS and PCD usually associated with an underlying tumour and suggested that the association occurred more frequently than would be expected by chance. In a different study Mason *et al* (1997) detected anti-VGCC antibodies in up to 24% of patients with SCLC and PCD (in patients with clinical and subclinical LEMS). Whether the anti-VGCC antibodies are responsible for the PCD is unclear. In one study (Fukuda *et al*, 2003) a reduction of P/Q-type calcium channels in post-mortem specimens of PCD and LEMS. These findings are yet to be verified and significant levels of anti-VGCC antibodies in CSF samples have yet to be demonstrated.

It is likely that some patients have multiple antibody response to multiple onco-neuronal antigens. Indeed patients have been described with anti-Hu and anti-VGCC antibodies. In the case report described here, the patients had clearly demonstrable anti-VGCC antibodies and an antibody response against an unidentified 97 kDa neuronal protein. Reactivity with a 97 kDa protein by sera from PCD/ LEMS patients has not been reported. It is entirely possible that the antigen recognised by this patients antibodies are unique to this case of PCD/ LEMS, however, identification of the protein may provide insight into the nature of the disease in the index patient with a view to investigating a larger cohort of patients at a later date.

4.4.3 Antigen Purification by Ammonium Sulphate Fractionation, 2D Gel Electrophoresis and IEX Chromatography.

We attempted to characterise the 97 kDa antigen recognised by the antibodies in the serum of the index patient through the use of ASF, 2D gel electrophoresis and IEX chromatography. We were able to partially purify the protein using ASF. Interestingly the 50 and 100 % fractions of the pig brain homogenate revealed previously unrecognised antibody activity against proteins with a molecular weight lower than 97 kDa. The appearance of this antibody activity may be related to the relative enrichment of the target proteins within the fractions or a result of dissociation of peptides from the 97 kDa auto-antigen. Further characterisation of these proteins was not pursued although it provides evidence for the potential existence of other auto-antibodies, a factor which may have hampered attempts to immunopurify the 97 kDa antigen (see chapter 5). Attempts to separate the protein by 2D gel electrophoresis proved unsuccessful despite the application of increasing concentrations of protein. Similarly, the 97 kDa protein could not be detected following IEX chromatography. Previous experience in our laboratory has demonstrated that successful characterisation of antigens can be achieved using these well-established techniques of protein separation (see chapter 3).

Protein separation techniques have previously been applied by other groups to the characterisation of antigens recognised by the serum of neurologically normal patients with various forms of cancer. Brichory *et al* (2001) used 2D PAGE and immunoblotting to separate proteins isolated from both human tumour tissue or tumour cells lines and identified the protein 'protein gene product 9.5' as a target antigen recognised by antibodies from 9/64 (14 %) patients with lung cancer. In this example

the partial purification of proteins was not required as antigen characterisation could be achieved from the crude homogenates of whole tissue. Similarly, Prasannan *et al* (2000) used homogenates of neuroblastoma tumour tissue and neuroblastoma cell lines in 2D PAGE to identify β-tubulin as the antigen recognised by the antibodies of 11 patients with neuroblastoma. The use of cell lines in these experiments has obvious merit. A homogenous population of cells will contain similar proteins allowing reproducible result, while the absence of other cell types reduces the overall number of proteins that could hamper purification attempts. This approach is dependent on the availability of a suitable cell line. It should however be noted that antigen expression may vary from individual cell cultures from the same cell line depending on the conditions in which they are cultured thus repeated testing of antigen expression may be required prior to purification attempts. Future attempts at purification of the 97 kDa antigen could involve the selection of an appropriate cell line in order to simplify the composition of the initial homogenate and assist purification. Our experiments required the use of brain tissue for the identification of antigens. While we have had previous success using the proteomic approach it is well-documented that problems exist in the isolation and separation of proteins from brain tissue. Beranova-Giorgianna *et al* (2002) noted the lack of membrane bound proteins when mouse brain was used to produce 2D gels. Similarly, a two dimensional map of human brain proteins was recently found to be composed of only 2% membrane bound proteins (Langen *et al*, 1999) a figure much lower than the predicted 25-33% (Cascio *et al*, 2002). It is known that membrane proteins, with their low solubility (due to the existence of intramembrane hydrophobic regions) and tendency to aggregate during IEF, results in their under-representation on 2D gels using conventional conditions. One solution is to enrich a protein preparation for membrane-bound proteins by isolating membrane fractions from the brain

homogenate. Such a technique, coupled with various salt washes to detach peripheral proteins was employed by Friso and Wikström (1999) in their analysis of cerebellar membrane proteins. Their approach resulted in the production of a 2D map composed of a larger number of membrane proteins which was complementary to that produced from tissue treated in a standard manner (Beranova-Giorgianni *et al*, 2002). Problems relating to the solubility of the 97 kDa antigen could be a factor in our inability to characterise the antigen. In contrast to NLK which precipitated at 40-60 % ammonium sulphate, the 97 kDa protein precipitated at 20 % ammonium sulphate, suggesting that the protein is relatively hydrophobic and thus may be associated with a membrane. However, this does not explain the inability to separate the protein by IEX chromatography. Hydrophobic areas of a protein may reduce the affinity with which a protein binds to a column although the protein should still be present in the flow-through or elute upon a small increase in the ionic strength of the buffer. It is possible that the protein forms an aggregate that is unable to pass through the column. Future attempts to characterise the 97 kDa protein using proteomics should focus on ways to improve the preparation of the protein sample prior to analysis.

5 *Chapter 5: Characterisation of the 97 kDa neuronal antigen by immunoaffinity chromatography.*

5.1 Introduction.

Attempts to identify the 97 kDa antigen using established techniques of protein separation and purification was unsuccessful and the potential reasons for this have been discussed in the previous chapter. As a result a new technique was employed involving the use of the patients IgG to immunopurify the antigen.

5.1.1 Immunoaffinity Chromatography.

Affinity chromatography separates proteins or other molecules on the basis of a specific, reversible interaction with a ligand immobilised on a suitable matrix in a chromatography column. The technique can be applied whenever there is a suitable ligand for a target protein. With highly selective protein: ligand interactions a thousand-fold purification of specific molecules is possible. Many ligands have been described and applied in various situations. The general principles of affinity chromatography are shown in Figure 5-1.

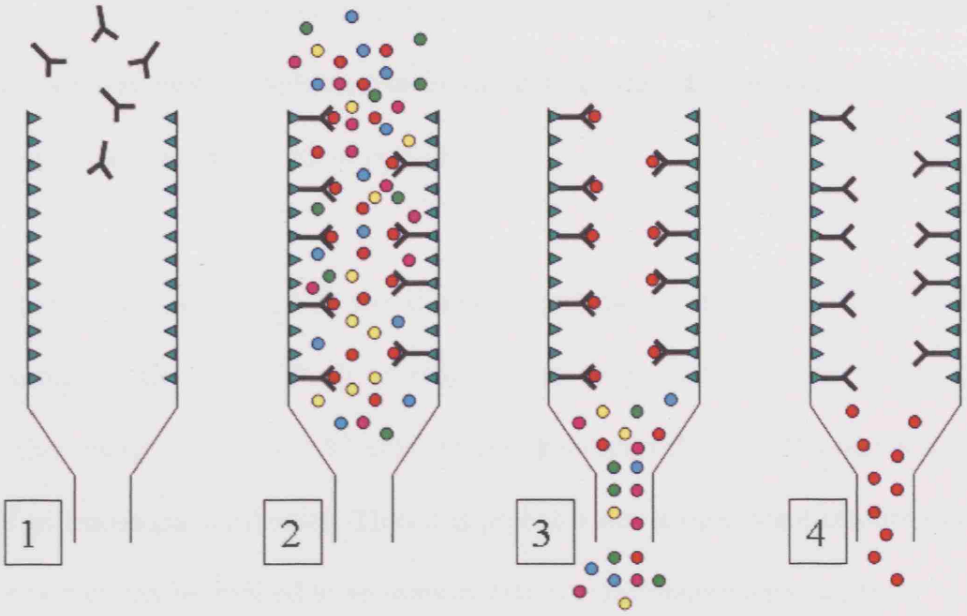


Figure 5-1: The principle of affinity chromatography.

1: A column containing a suitable matrix is coated with ligand (i.e. antibody) prior to the **2**: the introduction of the protein solution from which the target protein is to be isolated. **3**: The specific protein: ligand interaction allows retention of the protein while contaminants are washed away. **4**: The conditions within the column (pH, ionic strength) are altered to facilitate dissociation of the protein: ligand bonds. The purified protein can then be collected.

If a suitable ligand for a target protein is not available an antibody raised against the protein of interest can be used. Monoclonal antibodies provide a means of obtaining highly purified protein and are most useful when the identity of the target is established. When a suitable monoclonal antibody is not available polyclonal antibodies may be employed. Purification schemes using polyclonal sera commonly use antibodies raised in rabbits by repeated immunisation. Using this technique up to 10% of the total immunoglobulin may be specific for the target protein. The antibodies can then be coupled to a chromatography column. Theoretically, if 1 mg of immunoglobulin is attached to a column, only 0.1mg will be capable of antigen capture (Scopes, 1993).

Although only a small amount of protein would be purified from such a column, re-using the column over multiple runs or immobilising larger amounts of immunoglobulin to the matrix can circumvent these problems.

Patient serum has been used as the source of polyclonal antibodies in the following experiments. While it is difficult to estimate the proportion of antibodies in a given sample that are specific for the 97 kDa antigen, patients with PND frequently have high titres of anti-neuronal antibodies. Thus it is probable that a significant amount of anti-97 kDa antibodies can be applied to an immunoaffinity chromatography column.

5.1.2 Pre-Activated N-hydroxysucinnimide Columns Matrices

A pre-Activated N-hydroxysucinnimide (NHS) column was used for the production of an immunoaffinity column. These columns couple protein ligands through the amino groups in their structure. The NHS matrix consists of cross-linked agarose beads with a ten-atom spacer arm attached by epichlorodyrin and activated N-hydroxysucinnimide. Proteins couple to the matrix spontaneously by nucleophilic attack of the ester linkage to form a stable amide bond (Figure 5-2). 30 mg of IgG can be coupled per millilitre of NHS matrix. Once antibodies have been bound to the matrix, unoccupied binding sites can be blocked using an excess of a solution containing amino groups. Blocking the column in this way prevents non-specific binding of proteins to the column when protein solutions are introduced.

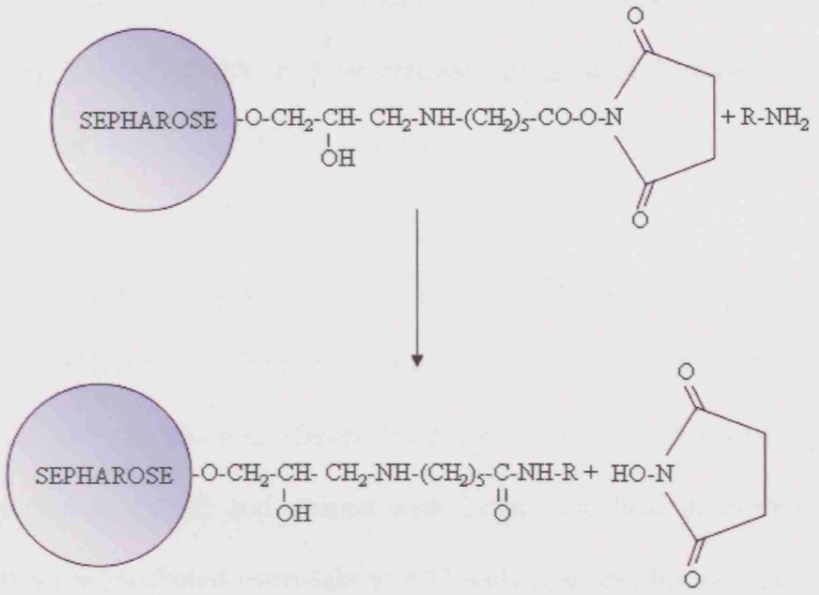


Figure 5-2: Protein binding by NHS activated chromatography column.

5.2 Methods.

5.2.1 Purification of IgG from Serum.

The High Trap rProtein A column is designed for the purification of IgG. A HiTrap rProtein A column (Amersham Bioscience) was installed onto the ÄKTA FPLC 920 and equilibrated with 10 mls of affinity elution buffer followed by 10 mls of affinity binding buffer. Serum was centrifuged (10,000g for 10min) to remove any insoluble proteins and the supernatent diluted in immunoaffinity binding buffer and loaded onto the 10 ml sample loop. IgG was purified from a total volume of 10 mls of serum in 2 ml purification steps. The sample was introduced onto the column at a flow rate of 0.5 ml/min. The flow of affinity binding buffer was continued until contaminating proteins had been removed and absorbance returned to baseline. The flow-through was collected for analysis. Purified IgG was removed from the column using a single step pH change

of 7 to 3 by introduction of 100% immunoaffinity elution buffer. 0.25 ml fractions were collected using the FRAC-900 and neutralised using neutralisation buffer. Fractions containing protein were selected with reference to the chromatogram and pooled.

The total protein concentration of the serum, flow-through and purified IgG fraction was calculated as described (Chapter 8). A commercial source of purified IgG was used as a control. Each sample was standardised to a total protein concentration of 0.05 mg/ml, subjected to PAGE and stained with Coomassie blue or immunoblotted. The immunoblots were incubated overnight at 4°C with goat anti-human IgG and goat anti-kappa/lambda antibodies both diluted 1:5000 in 0.2 % milk 0.9 % saline solution. Both antibodies were used in order to correlate which bands in the stained gel represented the kappa/lambda chains of the denatured antibody molecules. Immunoblots were washed and incubated with rabbit anti-goat HRP conjugated secondary antibody diluted 1:1000 in 0.2 % milk 0.9 % saline solution. After a second wash immunoblots were developed chromogenically. Isolated IgG was concentrated to a final volume of 1ml using the Microcon system prior to immobilisaiton on the chromatography column.

5.2.2 Coupling Purified IgG to the NHS Activated Column.

Purified IgG was buffer exchanged into IgG coupling buffer. The isopropanol preservative was removed from a 1 ml HiTrap NHS-activated HP column (Amersham bioscience) with 6 ml-ice cold HCl (1mM). 1 ml of the antibody solution was injected onto the column which was then left to stand for 30 min at room temperature to allow the coupling reaction to occur. The antibody solution was removed from the column by

injection of 3 ml of IgG coupling buffer prior to inactivation of the remaining binding sites in the column using alternate washes of NHS inactivation buffers A and B.

5.2.3 Isolation of Antigen from Cerebellar Homogenate.

The IgG-coupled NHS column was primed using 25 ml of affinity binding buffer followed by 25 ml affinity elution buffer and re-equilibrated in affinity binding buffer. The 20 % ammonium sulphate fraction of cerebellar homogenate (see chapter 4) was diluted 1:10 in affinity binding buffer and injected onto the column at a rate of 0.25 ml/min. Flow was continued until absorbance returned to baseline. At this point, antigen was eluted from the column into 0.25 ml fractions using affinity elution buffer. Fractions containing protein were pooled and neutralised. Seven cycles of purification were conducted using fractionated cerebellar homogenate each time.

5.2.4 Analysis of Eluted Proteins.

Proteins were concentrated using a YM-10 spin column and subjected to PAGE. Gels were then either stained using colloidal blue or transferred to nitro-cellulose. Immunoblots were incubated with the patient's serum at 4°C overnight; washed and incubated with rabbit anti-human HRP antibody diluted 1:15,000. Immunoblots were developed using ECL. Stained gels were sent for analysis by mass-spectrometry using the method described in section 3.2.7. Proteins around the 97 kDa molecular weight marker were analysed. Antigens were selected for further investigation if they were specific to the nervous system and had a molecular weight of between 95 and 110 kDa. To confirm the identity of the immunoaffinity purified antigen the 20% fraction of cerebellar homogenate was probed with antigen specific antibodies and patients serum.

5.3 Results.

5.3.1 Isolation of IgG from Patients Serum.

A representative chromatogram demonstrating IgG isolation from patient serum is shown (Figure 5-3, A). Fractions containing the flow-through and isolated IgG were pooled and subjected to analysis. Immunoblotting did not detect the presence of IgG within the flow-through fraction while it could be clearly identified in the IgG fraction as evidenced by the presence of a pattern identical to the commercial control (Figure 5-3, B). A Coomassie stained gel (not shown) of the same fractions showed that the purified IgG contained no other significant proteins not associated with the heavy or light chains of IgG.

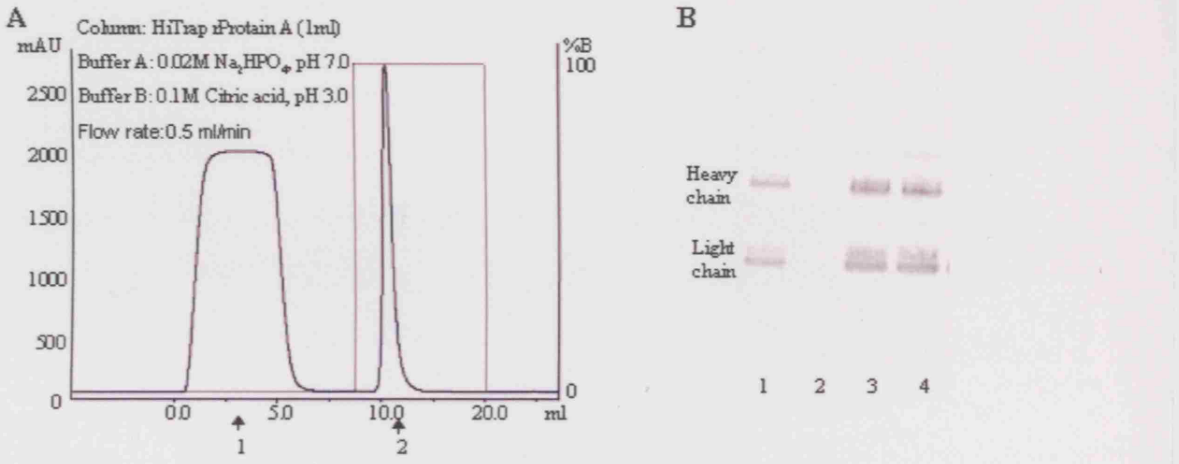


Figure 5-3: Purification of IgG from patients serum.

A: Spun serum was introduced to the column and the flow-through collected (peak 1). The column was washed until the absorbancy returned to baseline. IgG was eluted from the column by the introduction of 100 % elution buffer (peak 2). **B**: Whole serum (lane 1), flow-through (lane 2) and purified IgG (lane 3) were subjected to PAGE. Lane 4 = human IgG control. No IgG was detected in the flow-through fraction. IgG was efficiently purified.

5.3.2 Antigen Isolation from Cerebellar Homogenate.

Extractions were performed as described. A representative chromatogram is shown in Figure 5-4, A. The extracted and concentrated proteins were electrophoresed and stained (Figure 5-4, B). Some purification of protein had occurred however a larger number of contaminating proteins could be seen at a range of molecular weights. Distinct bands around 97 kDa could be visualised in the gel. Immunoblotting of the proteins showed that the 97 kDa antigen had been extracted following the immunoaffinity protocol, however two other reactive proteins were also identified (Figure 5-4, C) one of which was thought to be IgG heavy chain. The low yield of protein prevented further experimentation.

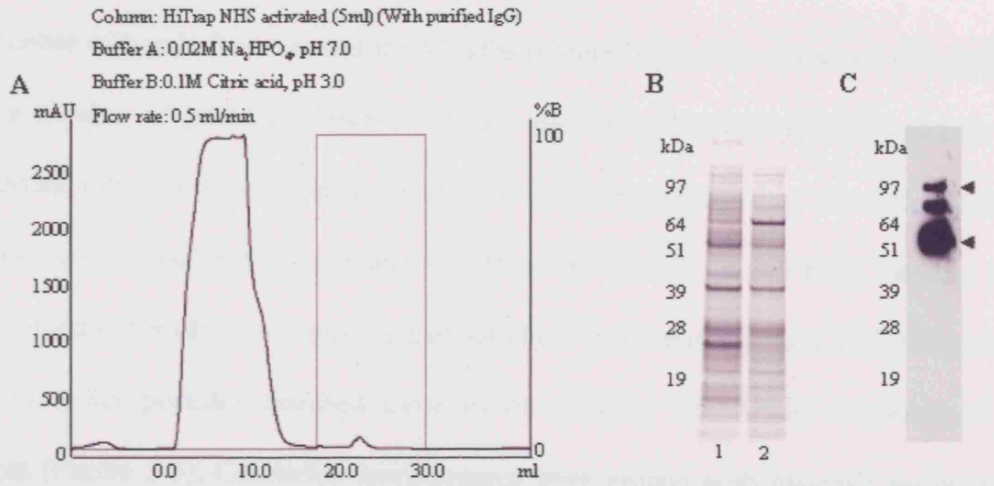
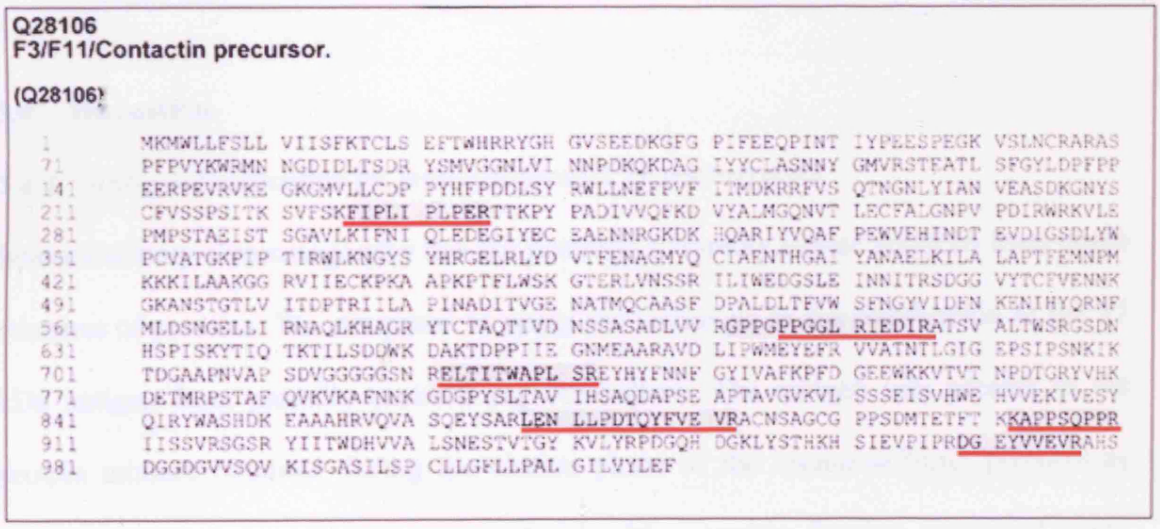


Figure 5-4: Immunoaffinty purification of the 97 kDa antigen.

Purified IgG was used to construct a tailored immunoaffinity column. The column was used to purify the 97 kDa antigen from the 20 % ammonium sulphate fraction of cerebellar homogenate. Purification was repeated 7 times using the protocol shown in **A.** The collected eluted proteins were subjected to PAGE and the gel stained. **B** shows the proteins collected from the first two runs. Protein from run 1 was immunoblotted (**C**) and probed with patients' serum. A 97 kDa protein was detected (top arrow head). Two other, previously undetected proteins were also present. It is thought the lowest band represents IgG contamination from the column (bottom arrow). Proteins around the 97 kDa molecular weight marker were subjected to analysis by mass spectrometry.

5.3.3 Mass Spectrometry.

A number of bands from around the 97 kDa protein level were extracted and analysed and a number of proteins identified. In an attempt to determine which protein was important subsequent analysis was restricted to those proteins that were related to the nervous system and had a molecular weight of between 95 to 105 kDa. Tandem mass spectrometry identified one protein that satisfied these requirements, F3/F11/contactin precursor. Six peptides matched those in the primary amino acid sequence of this protein (Figure 5-5). Cerebellar homogenates were probed with patient's serum and a commercial anti-contactin antibody (Figure 5-6). Both reacted with a protein in the cerebellar homogenate, however the anti-contactin antibody recognised a protein with a higher molecular weight than that recognised by the patients serum.



Q28106
F3/F11/Contactin precursor.

(Q28106)

```
   1    MKMWLLFSLL VIISFKTCLS EFTWHRRYGH GVSEEDKGFG PIFEEQPINT IYPEESPEGK VSLNCRARAS
  71    PFPVYKWRMN NGDIDLTSDR YSMVGGNLVI NNPDKQKDAG IYYCLASNNY GMVRSTEATL SFGYLDPFPP
 141    EERPEVRVKE GKGMVLLCDP PYHFPDDLSY RWLLNEFPVF ITMDKRRFVS QTNGNLYIAN VEASDKGNYS
 211    CFVSSPSITK SVFSKFIPLI PLPERTTKPY PADIVVQFKD VYALMGQNVT LECFALGNPV PDIRWRKVLE
 281    PMPSTAEIST SGAVLKIFNI QLEDEGIYEC EAENNRGKDK HQARIYVQAF PEWVEHINDT EVDIGSDLYW
 351    PCVATGKPIP TIRWLKNGYS YHRGELRLYD VTFENAGMYQ CIAENTHGAI YANAELKILA LAPTFEMNPM
 421    KKKILAAKGG RVIIECKPKA APKPTFLWSK GTERLVNSSR ILIWEDGSLE INNITRSDGG VYTCFVENNK
 491    GKANSTGTLV ITDPTRIILA PINADITVGE NATMQCAASF DPALDLTFVW SFNGYVIDFN KENIHYQRNF
 561    MLDSNGELLI RNAQLKHAGR YTCTAQTIVD NSSASADLVV RGPPGPPGGL RIEDIRATSV ALTWSRGSDN
 631    HSPISKYTIQ TKTILSDDWK DAKTDPPIIE GNMEAARAVD LIPWMEYEFR VVATNTLGIG EPSIPSNKIK
 701    TDGAAPNVAP SDVGGGGGSN RELTITWAPL SREYHYFNNF GYIVAFKPFD GEEWKKVTVT NPDTGRYVHK
 771    DETMRPSTAF QVKVKAFNNK GDGPYSLTAV IHSAQDAPSE APTAVGVKVL SSSEISVHWE HVVEKIVESY
 841    QIRYWASHDK EAAAHRVQVA SQEYSARLEN LLPDTQYFVE VRACNSAGCG PPSDMIETFT KKAPPSQPPR
 911    IISSVRSGSR YIITWDHVVA LSNESTVTGY KVLYRPDGQH DGKLYSTHKH SIEVPIPRDG EYVVEVRAHS
 981    DGGDGVVSQV KISGASILSP CLLGFLLPAL GILVYLEF
```

Figure 5-5: Peptide matches with Amino acid sequence of F3/F11/Contactin precursor.

Figure 5-6: Detection of contactin in cerebellar homogenate.

An immunoblot of pig cerebellar homogenate was probed with patients' serum (left) and a mouse anti-contactin monoclonal antibody (right). The detected proteins are not at the same level suggesting that the patients antibodies do not react with contactin.

5.4 Discussion.

5.4.1 Antigen Purification by Immunoaffinity Chromatography.

Immunoaffinity chromatography has the potential to rapidly isolate antigens from crude mixtures of proteins. We attempted to apply the technique to the purification of the 97 kDa antigen from partially purified pig cerebellum. The antigen was present in the protein mixture obtained during the elution phase of the immunoaffinity protocol as determined by immunoblot, however the resulting protein fraction contained many proteins.

It is possible that the immunoprecipitation of antigen was no more than an artefact of non-specific interaction of proteins with the column or IgG. Since total IgG and not antigen-specific antibody was coupled to the column, non-specific antibodies could

have isolated the contaminating proteins. It is clear that our patient has antibodies against VGCCs as well as antigens other than the 97 kDa antigen (see Figure 4-3), and we have already described the existence of auto-antibodies in cancer patients all of which may have bound proteins.

To circumvent this problem two methods could be employed to increase the proportion of antigen-specific IgG. Firstly, proteins could have been isolated from the 97 kDa level of PAGE gel and used to immunise animals in order to narrow the IgG repertoire immobilised on the column. Such a technique has proven successful in other studies, but is hampered by the expense of employing an external laboratory in which to raise the antibodies, and the production of specific antibodies to proteins that co-migrate in the PAGE gel. Alternatively, patient serum could be enriched for neuron-specific antibodies by the pre-incubation of patients serum with non-neuronal tissue to remove non-specific contaminating auto-antibodies. The resulting IgG preparation would have increased specificity for the antigen and would theoretically yield a protein fraction of increased purity following affinity purification of neuronal tissue. Such an approach, known as 'reverse immunoaffinity chromatography' has been successfully employed in the identification of auto-antigens in patients with autoimmune gastritis (Goldkorn *et al*, 1991). A drawback to this approach is the requirement of a large amount of starting material. The identification of auto-antigens from patients with autoimmune gastritis required IgG isolated from 40 ml of serum.

A second potential reason for the precipitation of a large number of molecules is the process of co-precipitation. The association of proteins in tight complexes can result in an increase in the number of proteins purified during affinity purification. The co-

precipitation of proteins during affinity purification is a recognised phenomenon and provides a means by which interacting proteins can be identified. This is eloquently demonstrated by Husi *et al* (2000) who employed immunoaffinity isolation of the N-methyl-D-aspartate (NMDA) receptor from mouse brain to study the associated proteins. Their approach resulted in the identification of 77 co-precipitated proteins. In this instance co-precipitation was desirable. With regard to the experiments presented here it is unlikely that all proteins other than the 97 kDa antigen were retained due to protein-protein interactions (see above), however adapting the method for the preparation of the brain homogenate may have reduced the level of contaminating proteins. Disruption of protein-protein interactions by denaturing proteins in SDS and reduction of di-sulphide bonds with DTT may be a suitable approach since the denatured 97 kDa antigen can be detected following denaturing PAGE electrophoresis and immunoblotting. Indeed, systematic investigation of various protein preparations could be employed to ascertain which preparation procedure yields the highest signal to noise ratio.

5.4.2 Investigation of Potential Auto-antigens.

Probing immunoblots of the purified proteins revealed that the target antigen was present in the proteins isolated from the column during the elution phase. However, a stronger signal was produced by a protein at approximately 55 kDa. Patients serum had not reacted previously with a protein of this molecular weight. It was thought that this protein corresponded to IgG although there was not a sufficient yield to determine whether this is true (by probing the proteins with anti-human IgG antibody only). It may have been possible to circumvent this by thorough washing of the column with elution buffer prior to introduction of brain homogenate, however, it is also possible that the

acid elution protocol resulted in the destruction of the IgG-column bond and thus the elution from the column. If this was the case the useful life of the column would be finite. Alternative elution strategies such as ionic elution may have proved more suitable and should have been investigated.

Despite the disappointing purification of the antigen a stained gel of the immunopurified proteins following PAGE showed a number of distinct bands around the level of 97 kDa. Analysis of these proteins was largely inconclusive. However one protein, F3/F11/Contactin precursor, was regarded as a potential antigen but was subsequently found to be incorrect following immunoblotting and comparison of the reactivity seen by patient's serum and an anti-contactin antibody.

6 *Chapter 6: Characterisation of the 97 kDa antigen by means of screening a bacteriophage expression library.*

6.1 Introduction.

Characterisation of the majority of well-recognised anti-neuronal antibodies associated with PND has been achieved through the screening of bacteriophage expression libraries. While protein purification techniques had been successfully applied to the characterisation of the 56 kDa protein associated with OM they did not facilitate purification of the 97 kDa protein recognised by antibodies in the serum of the index patient with paraneoplastic disease. Attempts to immunopurify the antigen were also unsuccessful. The potential reasons for this have been discussed. Due to the previously successful application of phage screening techniques to the identification of PND-associated antigens it was decided that further attempts to identify the 97 kDa antigen should be conducted using these methods. An introduction to the technique of library screening is provided below.

6.1.1 Bacteriophage Lambda.

Bacteriophage λ has become one of the most extensively studied viruses of *E. coli.* It has provided a wealth of information regarding the regulation of gene expression and has been manipulated to produce a useful tool in the creation of cDNA libraries.

Bacteriophage λ consists of a 150 nm tail attached to a 50 nm capsular head containing a linear 48.5 kbp double stranded DNA molecule. Infection of a cell follows the binding of a maltose receptor and injection of the entire strand of DNA into the host. The

overlapping 5' ends of the linear genome are then ligated by *E. coli* DNA ligase to form newly circularised DNA. Once a host cell has been infected the bacteriophage can enter one of two pathways in the continuation of its life cycle. These are the lytic and lysogenic lifecycles.

It is the lytic phase of the life cycle that is essential for the screening of a phage expression library. During this phase the circularised genome of bacteriophage is initially replicated producing progeny that are composed of circularised genomes before replication becomes asymmetric resulting in the production of linear DNA molecules. These molecules are packaged into the head of the bacteriophage and assemble with a tail. Accumulation of new phage results in the lysis of the host cell after which phage are free to infect new host bacteria. Lysis also allows proteins encoded by cDNA inserted into the bacteriophage genome to leave the host cell making them accessible for detection.

6.1.2 Early libraries.

Completion of the sequence of the Bacteriophage λ genome (Sanger *et al*, 1982) identified a non-essential DNA sequence and facilitated the development of early bacteriophage expression libraries. Young and Davis (1983) substituted this non-essential region with the complete *E. coli* LacZ gene, encoding beta-galactosidase and its promoter to produce bacteriophage λgt11. A λgt11 library is constructed through the fusion of foreign cDNA sequences into a unique *Eco RI* site at the beginning of the LacZ gene. cDNA sequences inserted in the correct orientation and reading frame can be expressed as fusion proteins by growing λgt11-infected *E. coli* on plates containing

IPTG. By screening these proteins with an appropriate antibody bacteriophage carrying cDNA for the target protein can be identified and analysed to determine the identity of the antigen. Modern vectors such as λZAP (see below) have been designed to facilitate this process through the inclusion of auto-excision protocols which allow automatic subcloning of inserted cDNA from the bacteriophage vector into a plasmid. These modern libraries draw on the understanding of the biology of the M13, f1 and fd coliphage viruses of *E. coli*.

6.1.3 Coliphage.

Following penetration of coliphage into the cytoplasm of the bacterial host the viral or (+) strand is released from its protein capsid, and rapidly converted into double stranded 'replicative form' (RF) DNA by the action of host RNA polymerases. The RF molecule is then replicated until approximately 100 RF molecules have been produced. At this point the accumulation of viral gene II protein directs asymmetrical replication by introducing a nick into the (+) strand of the RF DNA. Subsequent elongation of the 3' end of the nick displaces the old (+) strand via a rolling circle mechanism. The displaced strand is ligated to form a circle of single stranded DNA molecule which is then packaged into a viral particle and extruded non-lytically from the cell.

Investigation into coliphage replication has allowed the isolation of the 'functional origin of replication'. This sequence, when introduced into a plasmid allows it to enter the f1 replicative cycle upon superinfection of the host with wild type f1 helper phage. Importantly plasmids that enter the f1 cycle of replication are packaged as M13-like particles and extruded, along with wild type M13, into the culture medium. It was later discovered that the f1 origin of replication consists of two similar, overlapping

sequences, an initiator and a terminator (Dotto *et al*, 1984). Positioning these sequences on different areas of a plasmid, directs the formation of single stranded circular DNA molecules containing an intact f1 origin of replication and the interposed plasmid DNA.

6.1.4 λZAP

Short *et al* (1988) exploited the knowledge of the M13 life cycle to create the λZAP bacteriophage vector. Essentially, the λZAP vector is composed of three major regions:

1: The plasmid pBluescript (pBS) (see section 6.1.5) into which cDNA is inserted. This is flanked by:

2: The initiator and terminator regions of f1 all of which is inserted into:

3: The non-essential region of the bacteriophage.

The merit of this vector lies in the ease with which cDNA can be isolated from recombinant phage. Once a λZAP bacteriophage has been purified from a library, excision of pBS is achieved by co-infecting *E. coli* with recombinant phage and M13 'helper phage'. As M13 replicates, gene 2 protein recognises the initiator domain in the recombinant phage and initiates excision of the DNA between the initiator and terminator sequences (pBS and cDNA). The resulting single-stranded pBS DNA is packaged as M13 like virus particles and extruded from the cell into the culture medium. Removal of *E. coli* from culture medium by centrifugation leaves a solution containing wild type M13 viruses and the M13-like virus particle.

Re-infection of a second strain of *E. coli* which suppresses wild type M13 replication easily separates the virus and virus-like particles. Following infection of this second strain the single stranded pBS from the M13-like virus particles is converted to double stranded DNA. Selection of *E. coli* containing pBS is achieved by plating the bacteria on media containing ampicillin. The recovered plasmid contains the reactive cDNA suitable for production of recombinant protein, and can be isolated from the cells ready for sequencing.

6.1.5 pBluescript.

As described above, pBluescript (pBS) is central in the design of λZAP. The four major regions of the plasmid all have a role to play in the screening and recovery protocol applied to λZAP libraries. Ampicillin resistance provides a selectable marker while ColE1 serves as the initiation point for plasmid replication. The f1 origin of replication allows the plasmid to enter the f1 replicative cycle (see above) and plays a role in the auto-excision of pBS containing cDNA from selected recombinant bacteriophage. The final major area of pBS is the LacZ gene containing the multiple cloning site (MCS). The principles underlying the importance of this region are similar to those described for the use of the LacZ gene in λgt11 libraries. In contrast to LacZ in λgt11, which encodes the entire protein with only one restriction site (*EcoRI*), the LacZ gene in pBS is incomplete, encoding only the alpha chain of beta-galactosidase, but with the advantage of a multiple cloning site (MCS) containing 21 unique restriction sites. The 21 MCS is provided by the introduction of a synthetic polylinker into the LacZ gene. The MCS does not disturb the formation of a functional alpha peptide until introduction of cDNA causes insertional activation of the gene.

6.1.6 Construction of A λZAP Library.

Modern commercial libraries are prepared using a number of techniques which enhance their application. For example, libraries consist of cDNA developed using random primers in an attempt to develop a better representation of epitope expression from a given tissue (as opposed to the enrichment for 3' epitopes produced when poly-A primers are used during mRNA RT-PCR). In addition, directional insertion of cDNA into the vector ensures that cDNA is inserted in the correct orientation in preparation for expression. In doing so the total number of clones that need to be screened are reduced by maximising expression of inserted cDNA.

6.1.7 Screening a Bacteriophage Library.

All bacteriophage libraries are screened using similar protocols (Figure 6-1). Molten agar containing infected *E. coli* is poured over a solid agar base and incubated. This results in the production of a lawn of plaques that can be screened efficiently up to density of up to $5x10^4$ plaques per 90 mm plate. Traditional methods of screening involve the placement of IPTG soaked nitro-cellulose membranes over these plates at the beginning of plaque formation (approximately 3hrs after plating). This ensures a more complete representation of encoded epitopes by delaying the expression of potentially toxic foreign proteins. The membranes are then probed with the antibody of interest and the appropriate secondary antibody. By referring from the nitro-cellulose 'map' to the original agar plate, phage encoding a recognised protein can be rescued, eluted from the agar plug and processed through further rounds of screening to provide a purified phage. Due to the reported occurrence of false positives when screening it is common for duplicate nitrocellulose membranes to be created from each plate.

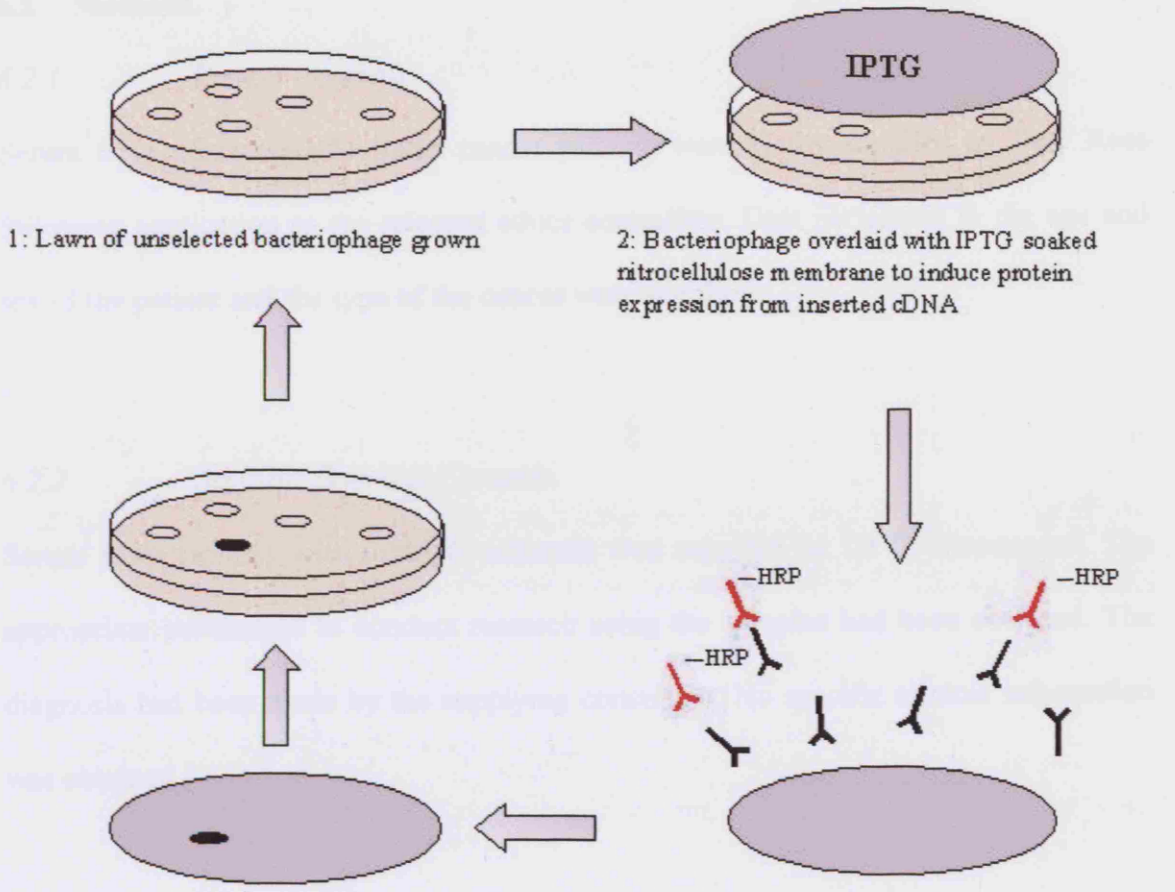


Figure 6-1: Screening a bacteriophage expression library.

6.2 Methods.

6.2.1 Cancer Controls.

Serum from neurologically intact cancer patients were kindly supplied by Dr J Rees following application to the relevant ethics committee. Data pertaining to the age and sex of the patient and the type of the cancer were supplied.

6.2.2 Multiple Sclerosis Controls.

Serum from patients with multiple sclerosis was supplied by Dr G Giovannoni. The appropriate permission to conduct research using the samples had been obtained. The diagnosis had been made by the supplying consultant. No specific clinical information was obtained

6.2.3 cDNA Library.

A commercial cerebellar cDNA library (Stratagene) was used. The library had been developed using cDNA from 11 cerebellar samples from males and females aged 22 to 70 years using random primers and directional recombination to create a library consisting of $2x10^6$ primary plaques with an average insert size of 1.9kb.

6.2.4 Preparation of XL1-blue MRF'.

E. coli XL1-blue MRF' were used for the propagation of bacteriophage. XL1-blue cells were streaked onto LB tetracycline plates and incubated overnight at 37°C. A single colony of XL1 blue cells was used to inoculate 50 ml LB broth supplemented with 10 mM $MgSO_4$ and 0.2 % maltose. The cells were grown overnight at 30°C to prevent overgrowth of bacterial cultures. The bacteria were harvested the following day by

centrifugation at 2000 rpm for 10 min and re-suspended in 10 mM $MgSO_4$ to an OD = 0.5. Cells were stored at 4°C and used within 5 days of preparation.

6.2.5 Titrating and Testing for cDNA Insertion.

2 ml of freshly prepared XL1-Blue MRF' were infected with dilutions of bacteriophage from the library ranging from 1:10 to 1:1000 in SM buffer. After incubation at 37°C for 20 min the *E. coli* were mixed in 25 ml of molten NZY top agar and applied to a large plate (243 mm x 243 mm x 18 mm) containing NZY base. The plates were incubated overnight at 37°C. The following morning an appropriate dilution for screening was selected. To ensure that cDNA had been ligated into the MCS of pBS 200 µl of prepared XL1-Blue MRF' were infected with 1 µl of bacteriophage from the library. The infected cells were mixed into 2 ml of molten NZY top agar which had been supplemented with 10 mM IPTG and Xgal (6.25 mg/ml). The agar was poured onto petri dishes containing NZY base, allowed to set and incubated at 37°C for 6 to 8 hours.

6.2.6 Infection and Plating of XL1 blue for screening.

Large (243 mm x 243 mm x 18 mm) NZY agar plates prepared 2 days prior to use were dried at 42°C for at least one hour. NZY top agar was melted and divided into 25 ml aliquots which were cooled to 50°C in a water bath. Stock bacteriophage library was diluted 1:66 in SM buffer and stored at 4°C until required. 1 µl of diluted bacteriophage was used to infect 1.2 ml of XL1 blue (prepared as above) by incubation at 37°C without shaking for 20 min. Infected bacteria were then combined with the molten NZY top agar, mixed gently and poured over the dried NZY base plates. The plates were left to set for 10 min before incubation at 42°C for 3.5 hours. Nitro-cellulose membranes

were labelled and soaked in 10 mM IPTG. The membranes were dried between paper towels and placed on the bacterial plates at the end of the 3.5 hr incubation. The temperature was reduced to 37°C and incubation continued overnight.

6.2.7 Probing Nitrocellulose Membranes.

Prior to the removal of the nitro-cellulose membranes the plate lids were removed for 30 min. The membranes and underlying agar were pierced using a pin to provide orientation marks for later reference and the membranes carefully removed to avoid disturbing the underlying top agar. The membranes were then placed directly into TNT buffer and any top agar removed using a gloved finger prior to blocking for 1hr in TNT blocking buffer. Blocking buffer was then substituted with patients serum diluted 1:1000 in TNT containing 0.2% dry milk powder. The membranes were incubated with this solution overnight at 4°C. Removal of anti-*E. coli* antibodies was not required at this stage as the density of *E .coli* antigen meant any background reactivity was sufficiently reduced to allow signal to be detected. Following incubation the antibody solution was decanted and saved for subsequent rounds of screening. The antibody solution was periodically used to probe immunoblots of pig cerebellum to ensure that reactivity with the 97 kDa antigen remained. The membranes were washed for 1 hr with 6 changes of TNT buffer containing 0.1% BSA and then incubated with HRP-conjugated rabbit anti-human IgG antibody solution (diluted 1:1000 in TNT buffer containing 0.2% dry milk powder) for 1 hr at room temperature prior to further rounds of washing. The membranes were then developed in chromogenic developing solution for one hour. Positive plaques were mapped onto acetate sheets which were then used to

refer back to the original plates. Plaques were cored using a pipette tip and placed in 1 ml SM buffer, vortexed and stored at 4°C for further investigation.

6.2.8 Subsequent Screens.

Positive bacteriophage were diluted 1:10, 1:100, 1:1000 and 1:5000 in SM buffer. 1 µl of each dilution of bacteriophage was used to infect 200 µl XL1 blue as described above and combined with 3 ml molten NZY top agar. The top agar was poured over 9 cm NZY plates and incubated overnight at 37°C. A suitable dilution of phage was selected to allow individual plaques to be selected and protein expression conducted using the IPTG-soaked membranes as detailed for the initial screening round. Successive rounds of screening were continued until greater than 90 % of the plaques yielded a positive signal. The final selected phage was designated phage 20A, B or C.

6.2.9 Testing Selected Phage Using Control Serum.

Step 1:

25 ml of NZY top containing 1200 µl of XL1 Blue infected with 1 µl of unselected phage from the raw library was poured over large NZY bases plates (243 mm x 243 mm x 18 mm). The plates were then incubated at 42°C for 3.5 hours before dry 9cm^2 squares of nitro-cellulose were placed over the top. The plates were then incubated overnight. The next day the nitro-cellulose membranes were prepared as described above with both washing and blocking steps before being placed into 10 well manifolds. Control sera diluted 1:1000 in 0.2 % milk, 0.9 % saline was then incubated in individual wells with the membranes overnight to reduce the level of *E. coli* antibodies. In the meantime, large (243 mm x 243 mm x 18 mm) plates of selected phage 20A were

prepared using a phage dilution that allowed the growth of well-defined plaques evenly distributed across the plate. After 3.5 hours incubation at 42°C 9 cm^2 sections of nitro-cellulose impregnated with 10 mM IPTG were placed over the phage and incubation continued at 37°C overnight. The next day the membranes were washed and blocked as described for the initial library screening, and placed into 10 well manifolds. The sera to be tested was then transferred from the manifold containing the non selected phage and used to probe for specific reactivity with the selected phage. Secondary rabbit anti human HRP antibody was diluted 1:1000.

Step 2:

Some serum required a second screening for anti-phage activity. Serum samples that were thought to have some anti-phage activity in stage 1 were tested using the second stage. Selected phage 20A and raw library were titered and the dilutions which gave approximately 50 plaques used. 200 µl of *E.coli* were infected with both phage 20A and non-selected phage using those dilutions and the *E. coli* spread on NZY plates. The resulting lawns consisted of 50 % selected phage. Membranes were produced as described and incubated with pre-absorbed serum (see step1). The serum from the index patient was always included as a positive control. Samples were defined as negative if there were no anti-phage antibodies or the percentage of positive plaques was greater than produced by the positive control as this would imply global background reactivity, not a phage specific response.

6.2.10 pBluescript Excision.

The SOLR strain of *E. coli* is designed for use in the pBluescript excision protocol. It allows only excised pBS phagmid to replicate and removes the possibility of co-infection with helper phage. SOLR were plated grown on LB kanamycin plates (75 ng/ml) and incubated at 37°C overnight. Single colonies of XL1 and SOLR were grown overnight at 30°C in LB broth supplemented with 10 mM $MgSO_4$ and 0.2 % maltose. The cells were harvested by centrifugation and re-suspended in $MgSO_4$ to OD = 1.0. 200 µl of XL1-Blue were combined with 250 µl of the selected bacteriophage and 1 µl of helper phage and incubated at 37°C for 15 min. 3 ml of LB broth was added to the excision reaction and incubated for a further 3 hrs at 37°C with shaking. The reaction mixture was then heated at 70°C for 20 min to kill the XL1-blue cells which were then removed by centrifugation. The supernatant containing the excised pBluescript packaged as filamentous particles was decanted and stored. 200 µl of SOLR cells were combined with 100 µl of the phage particles, incubated at 37°C for 15 min to allow infection to take place. SOLR containing the excised pBluescript were selected by plating 200 µl of the infected bacteria on the LB ampicillin plates and incubating overnight at 37°C. Cells were then grown in LB broth supplemented with the appropriate antibiotics and glycerol stocks were produced. In addition, the plasmid was extracted from 4 ml of culture using the method described (See Chapter 8) and used for characterisation of the encoded protein.

6.2.11 Restriction Analysis and Sequencing of pBluescript.

Restriction enzymes that cut as close as possible to the cloning site used in the creation of the cDNA library were selected. The restriction digest reaction consisted of 2 µl 10x

BSA, 2 µl buffer 2 (New England Biolabs), 1 µl DNA and 13 µl H_20. The reaction mixture was mixed gently by pipette and 1 µl of both *Bam HI* and *Xho I* added. The reaction was left under paraffin at 37°C for at least 4hrs before analysis by electrophoresis through a 0.7 % agarose gel. A 1 kb DNA ladder was also included to allow estimation of insert size. Digests were visualised under UV illumination and pictures recorded. The sequence of the inserted cDNA was determined using the method described in section 8.2.6.

6.2.12 Expression and Detection of Protein From pBluescript 20A.

Glycerol stocks of SOLR containing pBS recovered from bacteriophage 20A were plated on LB ampicillin plates and incubated overnight at 37°C. The following evening a single colony was selected and used to inoculate 5 ml of LB ampicillin broth. Protein expression was induced as described (section 8.2.10). The cells were pelleted by centrifugation at 4000 rpm for 10 min, the supernatant decanted and the cells snap frozen in liquid nitrogen. The cells were stored at -80°C until required. SOLR containing pBS1C1 isolated from a previous experiment with which the patients serum did not react were prepared as described above and used as a control.

Bacterial homogenates were prepared as described (see 8.2.11). The total protein concentration of the pBS20A and pBS1C1 homogenates was calculated and then standardised by the addition of water to the more concentrated sample. 65 µl of each sample were added separately to 25 µl of LDS and 10 µl DTT. The samples were subjected to PAGE using a 4-12 % Bis-Tris gel in the presence of MOPS buffer. Gels

were stained or immunoblotted. Immunoblots were probed with serum from the index patient diluted 1:1000 and developed using ECL.

6.2.13 Removal of Anti-E.coli Antibodies from Patients Serum.

The excessive background signal produced when probing the immunoblots of pBS20A and pBS1C1 highlighted the need to apply preparative steps to the patient's serum. A 5 cm^2 section of nitro-cellulose membrane was incubated with 2 ml of SOLR pBS1C1 bacterial homogenate at room temperature for 5 hours. The homogenate was decanted and the membrane blocked with 2 % milk 0.9 % saline solution. Patients serum was diluted 1:1000 and 3 mls of this solution incubated with the *E. coli*-coated membrane overnight. The serum was then used to probe immunoblots of the bacterial homogenates containing pBS20A or PBS 1C1.

6.2.14 Subcloning of 20A Into pRSETB.

Sequence data obtained from pBS20A, the published sequence of the pRSET vectors, and restriction analysis of the cDNA insert were used to select a vector into which the cDNA sequence could be inserted that would maintain the same reading frame as found in the pBS vector. The restriction enzymes *BamHI* and *XhoI* were selected to digest the cDNA from pBS20A. The resulting fragment was predicted to contain a small fragment of pBS at the 5' end of the molecule. pRSETB and pBS20A were both digested overnight at 37°C with both *BamHI* and *XhoI*, separated in a 0.7 % agarose gel and the appropriate fragment purified from the gel. 2 µl of CIP was included in the digestion reaction of pRSETB to reduce the level of background produced in the ligation reaction. The cDNA fragment from pBS20A and pRSETB was ligated and 2 µl of the ligation reaction was then used to transform competent TOP10F' cells.

Cells were grown in 5 ml SOB broth, harvested by centrifugation and the plasmids isolated. The plasmids were analysed by single digestion using *XhoI* and separated by electrophoresis through a 0.7 % agarose gel. Unligated pRSETB was used as a negative control. Colonies found to contain a successfully ligated vector were designated pRSETB20A and used to produce glycerol stocks.

6.2.15 Subcloning of 20A Into pRSETC.

To allow the production of full length protein sequence data from pBS20A, the pRSET vectors and genebank were used to determine which bacterial expression vector the cDNA could be inserted into to allow transcription of the longest open reading frame. pRSETC was selected for ligation with the cDNA. pRSETC and pBS were digested with *BamHI* and *XhoI*. The ligation reaction, transformation and analysis of colonies were conducted as described (Section 6.2.14).

6.2.16 Sequencing and Protein Prediction.

Ligated pRSET plasmids were sequenced using a primer complementary to the T7 promoter of the pRSET plasmid (5'-AATACGACTCGGG-3'). The known sequence of each pRSET plasmid and the inserted DNA from the bacteriophage was used to establish the reading frame of the inserted sequence. The predicted molecular weight of the recombinant protein was calculated using the translate and mw/pi tools on the Expasy website (www.expasy.org/tools). Once it had been established that the pBS fragment had been inserted into the correct reading frame the vectors were used to transform BL21 (DE3) pLysS in preparation for expression which was conducted

exactly as described for previous proteins expressed by the pRSET vectors (See chapters two and three).

6.2.17 Detection of Expression of His-Tagged Proteins From pRSETB20A.

E. coli containing pRSETB20A or pRSETB were grown, protein expression induced and homogenates produced. The total protein for both homogenates was calculated and standardised by the addition of water to the more concentrated sample. The same volume of both bacterial preparations was subjected to IMAC. Post-purification samples were prepared for PAGE, applied to a 4-12 % Bis-Tris gel and subjected to electrophoresis and immunoblotting as described (Chapter 8). Blots were probed using anti-His and patients serum, and developed using ECL. In addition, homogenates separated by PAGE were stained with colloidal blue to allow visualisation of bands corresponding to the recombinant proteins.

6.2.18 Comparison of Reactivity of Serum with Crude Bacterial Homogenates of pRSETB20A and pRSETC20A.

E. coli BL21 (DE3) pLysS containing pRSETC20A were grown overnight at 37°C on SOB agar containing chloramphenicol and ampicillin. Protein expression was conducted and homogenates of pRSETB, pRSETB20A and pRSETC20A were produced. 3 ml of lysate produced from cells containing pRSETB only was used to coat two 5 cm^2 sections of nitrocellulose membrane. Following incubation with the lysate for 5 hours at room temp the membrane was blocked for one hour using 2 % milk/ 0.9 % saline solution. Patients serum was diluted 1:1000 in 8 ml of 0.2 % milk/ 0.9 % saline solution applied to the membranes and incubated overnight at 4°C. The total protein of the three lysates was calculated, the concentration standardised and 25 µl of each

sample subjected to electrophoresis through a 4-12 % Bis-Tris PAGE gel. An immunoblot of the homogenates was then prepared as described (Chapter 8). Following blocking, the membranes were incubated overnight with the serum which had been pre-absorbed by incubation with the pRSETB membrane. Following washing rabbit anti-human HRP secondary antibody was applied to the membrane at a dilution of 1:5000. Blots were developed using ECL.

6.2.19 Avoiding Proteolysis of Recombinant Protein pRSETC20A:

6.2.19.1 Timed Expression of Recombinant Protein from pRSETC20A:

Cells containing pRSETC20A were grown to an OD of 0.4-0.6. A 1 ml aliquot was removed (designated time 0) and IPTG added to a final concentration of 1 mM. 1 ml aliquots were subsequently taken every 30 min for 3 hours. The aliquots were spun at 4000 rpm for 5 min, the supernatant removed and the pellet flash frozen in liquid nitrogen. Pellets were stored at -80°C until required. Homogenates were prepared from the pellets as described (Chapter 8). The protein concentration of each pellet was determined and adjusted to 1 mg/ml with H_2O. The samples were prepared for electrophoresis by the addition of 25 µl of LDS and 10 µl of DTT to 65 µl of each homogenate. The samples were heated at 65°C for 15 min and 25 µl of each electrophoresed through a 4-12 % Bis-Tris PAGE gel. Immunoblots were prepared as described (Chapter 8) and probed with anti-His antibody diluted 1:5000. Blots were developed using ECL.

6.2.19.2 Varying Bacterial Protease Inhibitors:

In an attempt to reduce the level of proteolytic degradation a variety of bacterial protease inhibitors were used. *E. coli* containing pRSETC20A were grown and induced as described (Chapter 8). Homogenates were prepared with the addition of

1: Bacterial protease inhibitor cocktail (Calbioscience).

2: Bacterial protease inhibitor cocktail (Sigma).

3: Bacterial protease inhibitor cocktail (Sigma) with the addition of PMSF.

The protein concentration of each homogenised sample was determined and standardised. Proteins were separated by PAGE and immunoblotted. Blots were probed with anti-His antibodies and developed using ECL.

6.2.19.3 Varying Homogenate Production:

To determine if expressed proteins were being degraded during preparation of the bacterial homogenates alternative methods of preparation were explored. Cells containing recombinant protein were produced and lysed as described. Alternatively cells were lysed with Bug Buster (Pierce, UK) containing protease inhibitors supplemented with PMSF with the omission of the sonication step. Samples were subjected to PAGE and immunoblotted. Blots were probed with anti-His antibodies and developed using ECL.

6.3 Results.

6.3.1 Antigen Identification Using Patient's Serum to Screen a Bacteriophage Expression Library.

The cerebellar library was selected as immunoblotting of cerebellar tissue had confirmed cerebellar expression of the antigen (See section 4.3.3). Serum from the patient of interest was used to screen a total of $1.75x10^6$ plaques. A single reactive plaque was identified and was purified through subsequent screens until greater than 90 % of the plated plaques reacted with the patient's serum (Figure 6-2).

6.3.2 Control Reactivity with Selected Phage.

During the initial testing of control serum phage 20A was used to create control membranes against which up to 10 patients sera could be tested for reactivity (Figure 6-3). The secondary antibody (rabbit anti-human HRP) did not react with the selected phage (Figure 6-3, lane 10). The results obtained using control sera are shown in Table 6-1 (centre column).

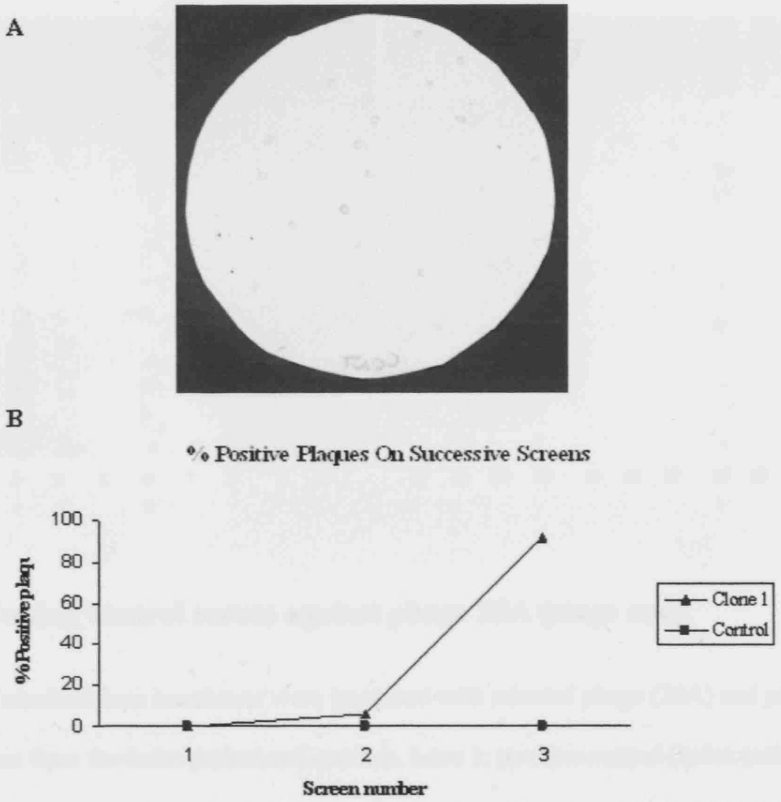


Figure 6-2: Screening for and selection of positive plaques.

A: Nitrocellulose membrane probed with patients serum after incubation with bacteriophage which had been passed thorough a number of rounds of screening to produce a population of which 90 % produced a protein recognised by patient's serum. **B**: Patients serum did not react with non-selected phage titred to give a similar density of plaques at each stage of screening. Anti-Human IgG did not react with the selected phage on any of the control blots.

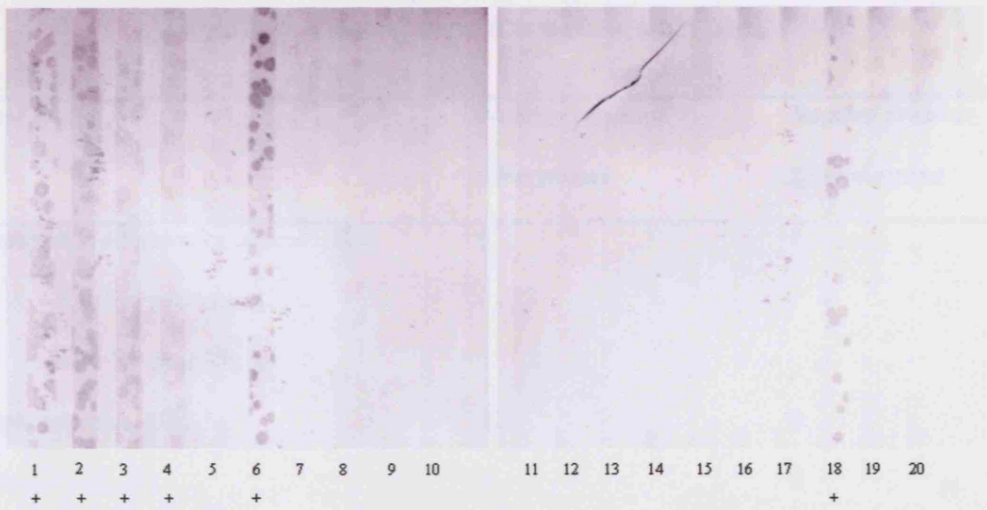


Figure 6-3: Testing control serum against phage 20A (stage one).

$9cm^2$ sections of nitrocellulose membrane were incubated with selected phage (20A) and probed with preabsorbed serum from the index patient and controls. Lane 1: positive control (index patient). Lanes 2-4 and 11-17: Anti-Hu positive. Lanes 5, 6 and 18, 19: Anti-Yo positive. Lanes 7-9 and 20: Neurologically normal adult control. Lane 10: Secondary antibody only. + = Positive for anti-phage antibodies.

Table 6-1: Control serum tested against phage 20A

Control group, n	*Number positive: First screen*	*Number positive: Second screen*
Paraneoplastic neurological disease, (19)	5	1
Anti Hu positive, (13)	3	0
Anti-Yo positive, (6)	2	1
Multiple sclerosis, (33)	12	0
Cancer controls, (4)	1	0
Encephalitis, (3)	0	NA
Movement disorders, (12)	0	NA
Dystonia, (9)	0	NA
Parkinson's disease, (3)	0	NA
Neurologically normal adult controls, (15)	1	0
Total, (86)	***19***	***1***

Unexpectedly, a total of 19 serum samples reacted with the selected phage despite pre-absorption of serum against *E. coli* and non-selected phage. In particular, serum from 12/ 33 (36%) patients with multiple sclerosis gave a positive result. However as a consequence of the method design a negative control lane containing unselected phage and *E. coli* was not included on the same blot. Thus the first screen only facilitated the detection of non-reactive serum controls. There was no way of determining whether a signal was due to specific phage reactivity or due to presence of residual anti-*E. coli* antibodies. Subsequently, sera that reacted during the first screen were subjected to testing against membranes prepared using a mixture of plaques containing 50 % phage 20A and 50 % unselected phage. This method provided an internal control. Samples

either did not react, reacted with all phage or reacted with selected phage. Samples that reacted with all phage could not contain phage specific antibodies. Representative membranes are shown in Figure 6-4.

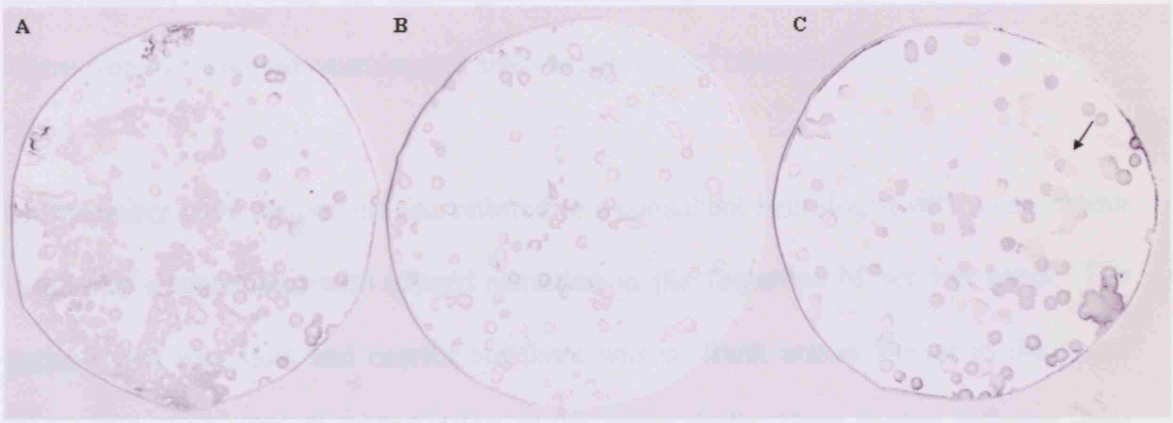


Figure 6-4:Testing control serum against phage 20A (stage two)

Membranes were produced from cultures infected with a 50:50 mix of selected phage 20A and unselected phage. Serum samples were considered negative for anti-phage antibodies if there was no reactivity or serum reacted with all phage. Membrane **A** shows a globally reactive serum sample. In contrast the serum from the index patient recognised only specific phage (membrane **B**). Only 1 of 86 control sera showed selective reactivity with the selected phage (membrane **C**). Clear delineation between background reactivity and true signal can be seen at over lapping plaques (arrow).

Control data from the second screen is given (Table 6-1), right hand column. Of a total of 86 control samples tested using the first and second screen protocols only one patient with PCD and anti-Yo antibodies had a specific anti-phage 20A antibodies. The serum from this patient clearly reacted more strongly with the selected phage (Figure 6-4, membrane C).

6.3.3 Case Details of Anti-Yo Positive Patient Reactive Against Phage 20A

A 46-year-old female underwent a left mastectomy with axillary node clearance for ductal carcinoma in July 1999. The patient subsequently received a course of chemotherapy following the histological identification of axillary node involvement. There were no reported neurological signs at the time of treatment.

In December 1999 the patient was referred to a consultant neurologist with a one month history of unsteadiness with altered sensation in the fingertips of her left hand. The patients gait was slow and careful but there was no frank ataxia. Power in the upper limbs was good with slight reduction in the lower limbs. Deep tendon reflexes were absent. Plantar responses were flexor. Position sense of the toes was absent and vibration sense was reduced. The consulting neurologist doubted a generalised neuropathy due to the asymmetrical nature of the signs and symptoms and suggested a metastatic or paraneoplastic cause relating to the original breast tumour.

Examination of the CSF revealed seven white cells and a protein of 1.8 g/l. Immunohistochemical investigation revealed weak staining of the Purkinje cell cytoplasm with stronger staining of the DRG. The serum also reacted with recombinant Yo. By March 2000 the patient's condition had deteriorated significantly however specific details were unavailable.

The patient died in July 2000 and an autopsy was performed. This revealed the presence of a carcinoma of the right lung with lymph node involvement and a broncho-pneumonia. Histology demonstrated a SCLC.

6.3.4 Sequencing pBluescript 20A, B, C.

Three plaques were selected from the final plate of bacteriophage. pBS was excised and designated pBS20A, pBS20B, pBS20C. Restriction analysis of each plasmid using double digestion yielded an identical band pattern suggesting that that the three selected clones contained an identical insert. The inserted cDNA was approximately 7400 bp in length.

The cDNA insert was sequenced as described. The sequence from each plasmid (pBS 20A, 20B and 20C) was identical. A BLAST search identified the insert as an incomplete fragment of transcription factor-like nuclear regulator (TFNR). TFNR cDNA has a 6762 open reading frame. Comparison of the published DNA sequence of TFNR (accession no: AJ238520) with the DNA sequence obtained from pBS 20A showed that the cDNA extended from base number 2017 and incorporated all the following 4745 bases listed in the published sequence to the stop codon. The remaining 2600 (approx) bases downstream of the stop codon could not be sequenced. Examination of the sequence data revealed that the inserted cDNA was not in the same reading frame as the start codon incorporated into the pBS plasmid (Figure 6-5).

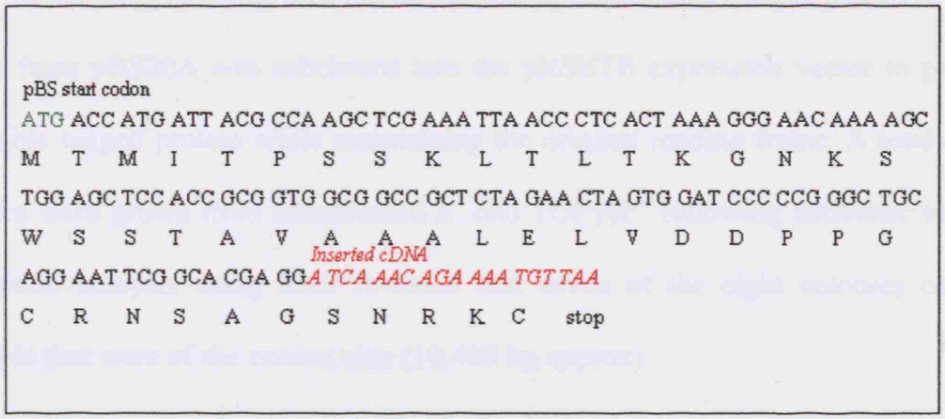


Figure 6-5: DNA and amino acid sequence of the TFNR cDNA inserted into pBS20A.

Only the final 5 amino acids are encoded by the inserted cDNA before a stop codon is encountered. This sequence, including the amino acids encoded by pBluescript encodes a peptide with a molecular weight of approximately 10 kDa.

6.3.5 Reactivity of Patients Serum with Proteins Produced from pBS20A

A colloidal stained gel of bacterial homogenates containing pBS20A or pBS1C1 (control pBS) were prepared as described. Interpretation of the gel was hampered since recombinant proteins were not purified from the crude homogenate resulting in a large number of bands corresponding to bacterial proteins. Colloidal blue staining failed to show an additional protein at the predicted molecular weight of 10 kDa in the pBS20A homogenate. Pre-absorbed serum using homogenates of pBS1C1 successfully reduced the level of background signal produced when probing immunoblots of bacterial homogenates with patient sera and ECL. However, no additional band could be observed when pBS20A bacterial homogenate was probed with the patient's serum.

6.3.6 Subcloning of cDNA 20A Into the Bacterial Expression Vectors pRSETB:

cDNA from pBS20A was subcloned into the pRSETB expression vector to provide a detectable tagged protein while maintaining the original reading frame. A total of eight colonies were grown from transformed *E. coli* TOP10F' following antibiotic selection. Restriction analysis using *XhoI* revealed that seven of the eight colonies contained plasmids that were of the correct size (10,400 bp approx).

Sequencing from the T7 promoter of the pRSET vector revealed that all seven plasmids contained the cDNA inserted into the expected reading frame and that the stop codon that terminated transcription in pBS20A had been preserved. The predicted molecular weight of the tagged recombinant protein was calculated as 8kDa, the final 5 amino acids encoded in the pBS20A vector were conserved (Figure 6-6).

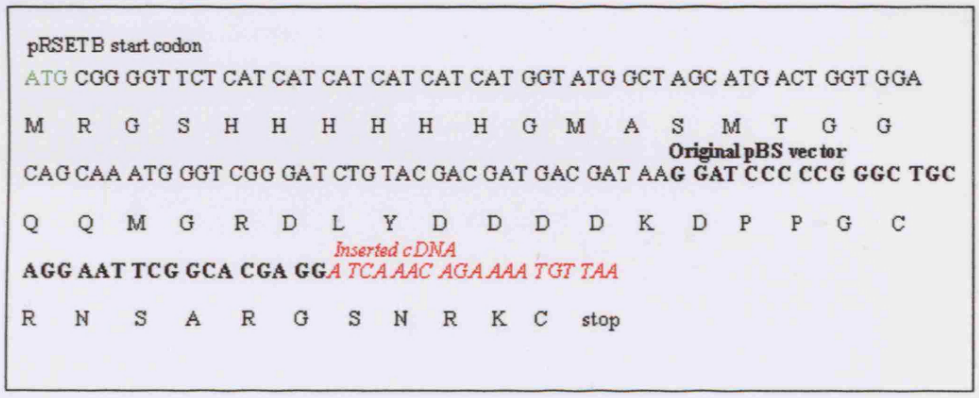


Figure 6-6:DNA and amino acid sequence following subcloning of TFNR from pBS20A into the pRSETB bacterial expression vector.

Subcloning of the cDNA from pBS20A into pRSETB allowed the addition of a His tag to the original peptide allowing easy purification for further analysis. The final 5 amino acids encoded by the cDNA were conserved.

Expression of recombinant protein was conducted from *E. coli* containing pRSETB or pRSETB20A and His-tagged proteins purified using IMAC. When proteins purified from the pRSETB20A vector were probed with anti-His antibody a protein with a molecular weight of approximately 8 kDa was detected (Figure 6-7, C). In contrast no protein was detected when the antibodies were used to probe post-IMAC proteins from pRSETB (not shown). The patient's serum reacted with numerous post-IMAC proteins from *E. coli* containing pRSETB20A (Figure 6-7, A) however these proteins were also recognised in post-IMAC preparations of pRSETB (Figure 6-7, B). Importantly, a specific reactivity at the molecular weight of the His-tagged proteins in pRSETB20A could not be detected (compare Figure 6-7 A and C). These results suggest that the patients serum did not react with the peptide encoded by the out of frame cDNA in pRSETB20A and thus did not recognise the protein encoded from the start codon of pBS in pBS20A.

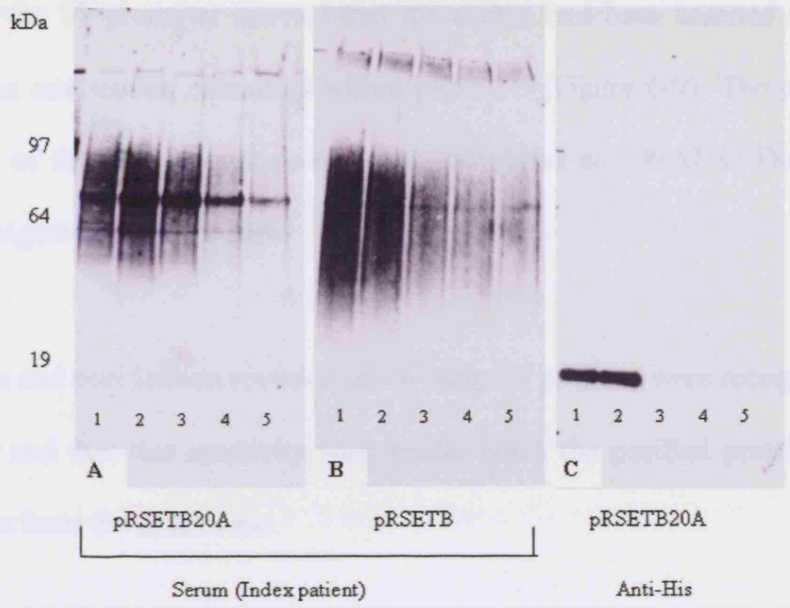


Figure 6-7: Reactivity of patients serum and anti-His antibodies against post-IMAC proteins from pRSETB20A and pRSETB.

As predicted, the anti-His antibody detects a protein with an approximate molecular weight of 8 kDa. Patient's serum also recognised a number of proteins however the reactivity was also present in the control preparation of *E. coli*. These results indicate that the antibodies in the patient's serum do not recognise the protein encoded by the out-of-frame TFNR cDNA as found in the original pBS20A plasmid.

6.3.7 Subcloning of cDNA 20A Into the Bacterial Expression Vectors pRSETC

cDNA from pBS20A was subcloned in to pRSETC in an attempt to express recombinant protein corresponding to the available open reading from of TFNR. Six colonies were grown from transformed TOP10F' following antibiotic selection. No colonies were grown from the plate inoculated with double digested pRSETC only. Single enzyme restriction analysis with *XhoI* revealed that all six colonies contained a successfully ligated plasmid of the correct size (10,400 bp).

Sequencing from the T7 promoter showed that the cDNA had been inserted in frame with respect to the start codon contained within pRSETC (Figure 6-9). The predicted molecular weight of the recombinant protein was calculated as 190 kDa. The ligated plasmids were designated pRSETC20A.

Protein expression and purification revealed that a range of proteins were recognised by anti-His antibody and that this reactivity was similar when the purified proteins were probed with serum from the index case.

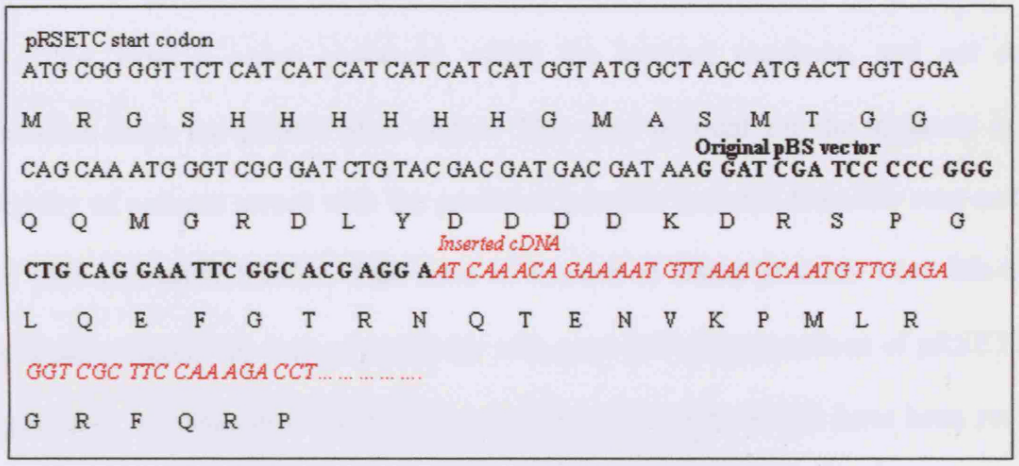


Figure 6-8: DNA and amino acid sequence following subcloning of TFNR cDNA frompBS20A into the pRSETC bacterial expression vector.

6.3.8 Reactivity of Patients Serum with Homogenates of pRSETB20A and pRSETC20A:

The patient's serum pre-absorbed with the crude homogenate produced from BL21 (DE3) pLysS still contained residual anti-*E. coli* as evidenced by the production of bands when the control homogenate was probed on immunoblot (Figure 6-9, lane 1). In confirmation of the previous experiment (Figure 6-9), the patient's serum did not

recognise a protein at the 8 kDa level when probing immunoblots of crude *E. coli* containing pRSETB20A. However, intense signal was produced over a range from 49 to 150 kDa which was not seen in the control lane. A similar pattern of intense signal was also seen when the pre-absorbed patient's serum was used to probe homogenates from pRSETC20A but with the addition of some bands at lower and higher molecular weights (28 - 188 kDa). The large number of reactive proteins in pRSETC20A suggests that proteolytic degradation had occurred which was later confirmed (see section 6.3.9). Since the cDNA inserted into pRSETB is out of frame the common bands seen in the pRSETB20A and pRSETC20A protein preparations must be a result of transcription of the cDNA from a codon contained within the inserted sequence, and not due to expression from the pRSET start codon. This may account for the apparent lack of reactivity of patients serum with the predicted proteins encoded from the start codon in both pBS and pRSETB20A. That none of the out of frame proteins were His-tagged would also explain the lack of reactivity with post-IMAC preparations of pRSETB20A (Figure 6-7, A) since any non-tagged recombinant proteins would have been removed from the column during the washing step.

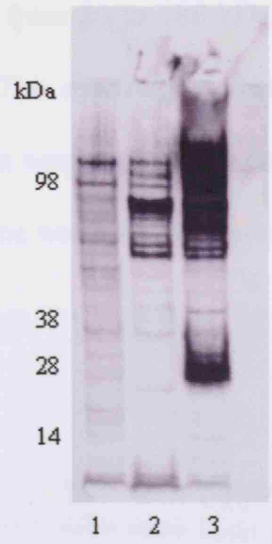


Figure 6-9: Comparison of reactivity of patients serum with pRSETB20A and pRSETC20A.

Immunoblot of *E. coli* homogenates containing 1: pRSETB 2: pRSETB20A 3: pRSETC20A were probed using patients serum following incubation with the pRSETB homogenate. Additional bands could clearly be identified in lanes 2 and 3.

6.3.9 Timed Expression of Recombinant Protein From pRSETC20A Confirms Degradation.

To determine whether the multiple reactive bands detected in the pRSETC20A homogenate were a result of proteolytic degradation and whether this breakdown could be prevented by reducing the induction time, expression was conducted and samples taken at 30 min intervals. Homogenates of each sample were prepared, immunoblotted and probed with the anti-His antibody.

Some His-tagged proteins could be detected at time zero indicating that there was some 'leak' of the expression vector. In addition a larger number of proteins were detected

with molecular weights ranging from 20 to 188 kDa. The number of bands increased with the length of induction. The reactivity corresponded roughly to the proteins detected when the patient's serum was employed to probe the pRSETC20A immunoblot (see Figure 6-9, lane 3). At no time was a single His-tagged protein detected.

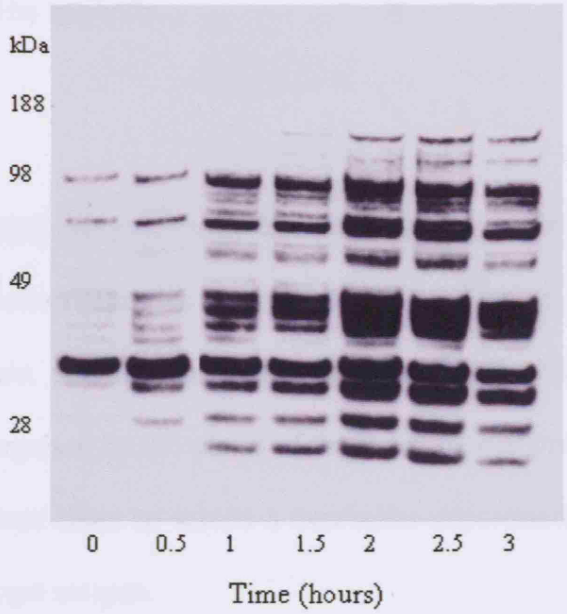


Figure 6-10: Timed protein expression from pRSETC20A.

Samples of induced BL21 pRSETC20A were taken at 30 minute intervals, homogenised, immunoblotted and probed with anti-His antibodies. Some tagged protein could be detected prior to induction with IPTG. Following induction the number of His tagged proteins visibly increased. These proteins had a MW ranging from 20 to 98 kDa (approx). The bands broadly corresponded to those seen when blots of pRSETC20A were probed with patient's serum.

6.3.10 Reducing proteolytic degradation of recombinant protein 20A

In an attempt to reduce the amount of proteolytic degradation of protein 20A the composition of the bacterial protease inhibitors was altered and their effect assessed by probing immunoblots of crude homogenates with the anti-His tagged protein. None of the different inhibitor cocktails or the inclusion of PMSF with the cocktail had any

noticeable effect on the prevention of degradation. To determine whether the process of sonication was increasing the breakdown of the recombinant protein a detergent lysis method was employed in the preparation of the bacterial homogenate. The use of this method had no noticeable effect in the reduction of the number of recombinant proteins detected by the anti-His antibody.

6.4 Discussion.

Many PND associated antigens have been characterised through the screening of bacteriophage expression libraries (see section 1.4). During our investigations the application of protein purification techniques to the identification of the 97 kDa neuronal protein recognised by the serum of the index patient proved unsuccessful. As a result the decision was taken to screen a cerebellar expression library as a means of characterising the target antigen.

6.4.1 Screening the Library: Technical Considerations.

A number of points regarding the methodology of screening a bacteriophage expression library should be noted. Firstly, the use of patient serum to screen a bacteriophage library carries with it potential problems. For example, given that a large number of plaques must be screened a large volume of serum may be required. In addition, using *E. coli* as a vehicle for the production of the recombinant protein may be problematic given the occurrence of anti-*E. coli* antibodies in serum samples. In the experiments described here both of these problems were avoided simply by re-using antibody solution over a number of nitro-cellulose membranes. The benefits of this are twofold. Firstly, the re-use of antibody solution dramatically decreases the total volume of serum required to efficiently screen a large number of phage while the quality of the antibody

solution can be easily assessed by its application to immunoblots of neuronal tissue. Secondly, reusing the antibody solution results in the removal of anti-*E.coli* antibodies with each membrane probed thus increasing the signal to noise ratio with each round of screening. In reality the use of dense lawns of plaques and large nitro-cellulose membranes in the initial screening process negates the need to remove contaminating anti-*E. coli* antibodies as the anti-*E. coli* response is spread so widely that no background signal can be detected even on the first use of the serum. However, the pre-absorption of *E. coli* antibodies becomes more important during subsequent rounds of screening when lower numbers of phage are plated.

As discussed in earlier chapters potential auto-antigens such as α-enolase and NLK may have been conserved during evolution. Probing homogenates of *E. coli* with commercial anti-NLK antibody resulted in the identification of a bacterial protein which could have conceivably been due to anti-NLK recognition of *E. coli* glucose-6-phosphate isomerase. With this in mind it may be a worthwhile precaution to determine if the antibody of interest can be absorbed out by preincubation with crude *E. coli* homogenates. Antibodies which do so will undoubtedly be unsuitable for screening bacteriophage libraries.

Finally, during the screening process it must be remembered that detection of a protein can only be achieved if a bacteriophage encoding the recognised antigen is plated. cDNA may be cloned into the expression vector in one of three reading frames thus increasing the total number of bacteriophage that must be screened to detect the protein. The process is even more difficult if the protein is relatively under-expressed or toxic to *E. coli* thus preventing its expression. While an attempt to avoid toxicity is made by

inducing protein expression after plaque formation, some proteins may simply not be detectable. These caveats should be remembered when attempting to screen a bacteriophage library and a decision made as to when screening should cease.

6.4.2 Screening with Patients Serum.

We used the serum of the index patient with a PND to screen a cerebellar expression library. Re-use of serum meant that only a small amount was required to screen the 1.75 x 10^6 plaques. The patients antibodies reacted with a single plaque which was purified until a homogenous population of bacteriophage were isolated.

The cDNA was rescued by application of the pBS recovery protocol in preparation for sequencing and protein expression. Initial attempts to express the protein from pBS proved problematic. Thus a novel strategy was employed to test control sera against the bacteriophage to determine if further investigation was worthwhile. This method used a two-stage process using a 50/50 mix of phage 20A and unselected phage. Control sera from 86 subjects were tested. A similar method for screening controls has previously been described by Bataller *et al* (2003). This group identified an association between anti-MAZ antibodies and Tr positive patients with PCD. Similarly to our experiments, attempts to immunoblot the MAZ recombinant protein from pBS were unsuccessful. The group suggested that this was a result of loss of a conformation epitope which was conserved during plaque formation but lost during sample preparation of proteins for immunoblotting, a suggestion which may not be true of the results presented here (see below 6.4.4). Of the 86 control sera tested only one sample contained antibodies that specifically reacted with phage 20A. The case history of this patient has been provided (see section 6.3.3).

6.4.3 The Reactive Patients.

There are similarities between the histories of the index and reactive control patient. Interestingly, both patients had undergone treatment for malignant disease prior to the onset of neurological symptoms, but in both cases neurological disease was thought to be related to the occurrence of a second malignancy. While malignant tissue was never isolated from the first case, the occurrence of LEMS with anti-VGCC antibodies and radiological changes was highly suggestive of a paraneoplastic disorder. In addition, it is most likely that the underlying malignancy was that of a SCLC. In the second case the occurrence of anti-Yo antibodies and the isolation of malignant tissue at autopsy provides compelling evidence for the occurrence of a PND. The findings presented here are further evidence that an anti-neuronal response may be composed of antibodies directed against multiple antigens. It is of note that the serum from the anti-Yo positive patient did not recognise a neuronal antigen with a molecular weight of 97 kDa.

The apparent lack of reactivity of serum from patients with inflammatory neurological disorders with phage 20A suggests that the antibodies are not an epiphenomenon. However, since it is likely that both reactive patients had SCLC the number of cancer controls screened may be insufficient. A larger number of samples from patients with a variety of cancers with an emphasis on SCLC are required to establish the specificity of the antibody in paraneoplastic disease. Similarly, the neurological symptoms experienced by both patients were different and calls into question the specificity of the antibody for a neurological syndrome. It is apparent that serum from a larger cohort of patients with paraneoplastic disease is required although this may me hampered by the rarity of the disorders.

6.4.4 Expression of Protein 20A.

As discussed, the 4745 bases of TFNR ligated into the pBS vector were out of frame with respect to the start codon in pBS. The protein encoded from the start codon had a predicted molecular weight of 10kDa with only 5 amino acids encoded by the cDNA before transcription was terminated. When the Hu antigen was originally identified using a similar technique one of the reactive clones contained the Hu cDNA in the incorrect reading frame with respect to the pBS vector but was still able to produce a reactive recombinant protein (Szabo *et al*, 1991). It can be concluded that transcription of the cDNA must have occurred from an in-frame start codon contained within the ligated cDNA for a reactive protein to be produced. In this situation two or more recombinant proteins could be produced from one cDNA sequence, one from the start codon of the expression vector and another from an internal start codon. The proteins produced from the internal start codons would not be tagged with (in the case of pBS) a portion of β-galactosidase. Thus these proteins would be harder to detect and purify using standard techniques. It is possible that the MAZ cDNA described by Bataller (2003) was also ligated in the incorrect reading frame. This may explain why their recombinant protein could not be detected in the bacterial homogenate as reactive protein may have not been expressed entirely as predicted.

In the present study serum from the index patient did not react with a protein of the predicted molecular weight when the recombinant protein was transcribed from the original pBS vector. The cDNA was ligated into the pRSETB vector to provide a simple means for purification and detection of recombinant antigen. Using this vector it was clear that recombinant protein was expressed from the cDNA but that this protein was

not recognised by the antibodies in the patient's serum. The reactivity with the bacteriophage plaques, reported full length expression from out of frame cDNA and the lack of reactivity with the recombinant proteins from pBS and pRSETB20A led to the ligation of the TFNR cDNA into the correct reading frame in order to produce full-length detectable protein which could be purified for subsequent investigation.

Production of recombinant proteins from pRESTC20A was conducted. An immunoblot of His-purified recombinant proteins probed with anti-His antibodies or patient's serum resulted in reactivity with a range of proteins by both antibodies with proteins ranging from 28 to 188 kDa. In addition, comparison of serum reactivity with preparations of pRSETB20A and pRSETC20A resulted in the identification of common proteins which were not expressed in the control preparation. This finding adds further support to the argument that reactive proteins were produced by the cDNA ligated into pBS20A which were expressed independently of the start codon contained within the pBS vector.

The number of reactive bands was surprising and warranted further investigation. Since bacteria had been transformed with only one vector all His-tagged proteins must have originated from this construct. It was thought that proteolytic cleavage may be occurring and a timed expression experiment confirmed this. Multiple His-tagged proteins could be detected even prior to induction by addition of IPTG. Although reactivity with the recombinant protein had been confirmed the applications for a degraded protein are limited. Since proteolysis was occurring quickly attempts to prevent 'downstream' proteolysis were thought likely to prove ineffectual and attempts to reduce proteolysis during protein extraction and purification were indeed unsuccessful. Given the previously documented findings that the only similar protein thus far described

undergoes extensive post-translational proteolysis (see below) a similar finding could have been anticipated. Despite this, the experiments confirmed that the patient's serum reacted with the protein encoded by pBS20A.

6.4.5 The Protein.

Subcloning and sequencing of the cDNA revealed that it encoded a large but incomplete portion of a protein known as TFNR. Relatively little is known about the function of this protein. A single paper describes the localisation of the gene, the mRNA and protein expression profile, the amino acid sequence and the identification of functional domains (Kelter *et al*, 2000).

The gene for TFNR has been mapped to 5q13. The gene produces a 9.5 kb transcript which, as demonstrated by Northern blotting, is expressed in all tissue but is relatively over expressed in the cerebellum. The coding sequence is 6762 bp in length and encodes a 2254 amino acid protein with a predicted molecular weight of 250 kDa. The sequence of the gene beyond the stop codon has not been mapped. This has been attributed to the poly-A tail in the 3' untranslated region. We encountered similar problems when sequencing beyond the stop codon of the cDNA.

The encoded protein is composed of 3 distinct regions. The first of these (aa 1-180) contains a bipartite nuclear localisation signal while the distal section (aa 1328-2254) has no significant homology to any known proteins.

The central portion of the molecule (aa 823 - 1327) encompasses a 55 aa motif repeated nine times. The only protein thus far described with a similar structure is the protein HCF of the herpes simplex virus. This protein has a complex post-translational pattern

as a result of proteolytic cleavage at the repeat sites. Histochemical staining of various cell lines (HeLa, fibroblasts and myogenic cells) using an anti-TFNR antibody confirmed the nuclear localisation of the protein within these cells. The same antibody reacted with a range of proteins (55 - 250 kDa) when used to probe homogenates of neuronal and muscular tissue. It must be noted however that the peptide (aa 215 - 229) used to produce the antibody in this case may react against a number of proteins. It may have been prudent to use a synthetic peptide from the central portion of the proteins in which the novel protein motifs are thought to lie.

6.4.6 TFNR as a Candidate Antigen in Paraneoplastic Neurological Disease.

The initial aim of employing the bacteriophage library for antigen identification was to characterise the 97 kDa antigen recognised by the serum of the index patient. Given the proposed molecular weight of the TFNR proteins reported it is probable that the antigen is different from the 97 kDa antigen recognised by antibodies in the serum of the index patient. To determine this, antibodies could be eluted from a membrane of reactive bacteriophage and used to probe an immunoblot of brain tissue. However, a review a cerebellar immunoblot probed with serum of the second patient (from the diagnostic service) showed that a 97 kDa protein had not been detected suggesting that the recognised protein is distinct from the 97 kDa antigen.

Despite uncertainty regarding the identity of the 97 kDa antigen the series of experiments conducted here have conclusively shown reactivity with the TFNR protein by antibodies in the serum of two patients with PND. As with NLK there are a number of matters relating to the suitability of this protein as an antigen in PND. The fact that

TFNR is relatively enriched in the cerebellum is interesting. However, from the limited data published it can be seen that TFNR is also expressed in a variety of systemic tissues. Thus unlike other anti-neuronal antibodies expression is not restricted to neuronal tissue. The basic cell staining techniques using anti-TFNR antibodies conducted by Kelter *et al* (2000) describe nuclear staining of cell from a variety of non-neuronal tissue. However, it is possible that, like NLK the pattern of expression is different in neuronal tissue. For example, TFNR protein may be secreted or associated with the plasma membrane of neurones, be confined to the nucleus or be expressed as a distinct isoform.

6.4.7 Areas for Further Investigation.

Despite the association of anti-VGCC antibodies, evidence of an abnormality in the thorax and a rapidly progressive cerebellar disease a tissue diagnosis of underlying malignancy was not available and thus while likely the diagnosis of PND cannot be made with absolute certainty. The identification of the second PND patient with anti-TNFR antibodies and confirmed malignant disease strengthens the argument that the occurrence of the antibody response and neurological disease is related to the occurrence of a tumour. Further investigation may identify similar cases in which tissue sections of underlying tumours are available for investigation. The cross reactivity of anti-TFNR antibodies and malignant tissue will ultimately need to be demonstrated. In addition to establishing the frequency of the anti-TFNR response in patients with PND, investigation into its occurrence in malignant tissue may be of value.

Although this thesis was principally concerned with the identification of antigens using proteomic and molecular biology techniques the matter of cross-reactivity with antigen and tumour tissue could be further investigated using immunohistochemistry. The elution of antibodies from membranes coated with protein from reactive phage could provided a relatively purified form of antibody which could then be utilised for the staining of neuronal and malignant tissues.

Finally given the paucity of publications pertaining to the role of TFNR in the nervous system it would be interesting to determine the role which the protein plays and the effects that disruption of its function(s) has. These future investigations are beyond the scope of this thesis, and given the currently limited number of patients with an anti-TFNR response are perhaps to be reserved pending investigation of the larger cohorts.

7 *Chapter 7: Summary of findings, areas for further investigation and conclusions.*

A summary of the findings of this thesis and areas for future research is provided below:

7.1 Antibodies Against Putative Targets in Post-infectious Neurological Disease.

All four putative auto-antigens proposed by Dale *et al* (2006a) were cloned and recombinant forms of the proteins recovered. The frequencies of antibody responses in patients with PANDAS, OCD, TS, neurological and healthy controls were determined. While there was no compelling evidence for a single antigen as a marker of the diseases studied this may be explained by the quality of the samples investigated and potential shortcomings in the method of detection. These reasons for this have been outlined.

Subsequent investigation would benefit greatly from a longitudinal study in which patients were monitored from their initial presentation and the occurrence of antibodies against each antigen correlated with the clinical phenotype. This would benefit from the development of a method for measuring antien specific IgG concentration rather than the simple positive or negative result obtained from an immunoblot.

7.2 Anti-neuroleukin Antibodies and Opsoclonus-myoclonus.

Two cases of post-streptococcal OM are described both of which had antibodies against a 56 kDa antigen. Protein purification techniques were employed and the antigen determined to be NLK. Investigation of a larger cohort of patients with OM revealed a frequent

response against this antigen regardless of the underlying aetiology of the disease. These results were consistent when both immunoblotting and ELISA were used to determine the presence of anti-NLK antibodies. Although NLK is widely expressed its specialised role in both the nervous system and tumour growth make it a potential unifying target in both POM and PIOM. In addition, we provided limited evidence for the presence of anti-NLK antibodies in the CSF of adults with POM.

Future work will require investigation of the frequency of an anti-NLK response in both adult and paediatric patients with OM in whom the underlying cause has been thoroughly investigated. It would also be of interest to determine any correlation between prognosis and antibody titre. This is especially true in those patients with POM in whom the antibodies may have a role in limiting malignant spread. Finally, while NLK represents an accessible antigenic target the criteria for it to be classed as an antibody mediated disease have yet to be fulfilled. Studies to determine the antibody effects on neuronal cells and the ability to establish disease in animal models will be required.

7.3 Antigen Identification in Paraneoplastic Disease.

The detection of anti-neuronal antibodies using crude brain homogenates sometimes demonstrates antibodies against neuronal proteins which have not yet been described.

63 patients with PND were identified by way of a surveillance programme and their anti-neuronal responses investigated. One of these, with antibodies against a 97 kDa antigen was selected for further investigation.

Protein purification techniques were employed following the successful characterisation of NLK however the application of ASF and 2D gel electrophoresis did not facilitate the isolation of the antigen. The potential reasons for this have been discussed. Immunoaffinty chromatography was employed and a partial purification of the antigen achieved. Analysis of the proteins around the 97 kDa level suggested one protein, contactin, may be of interest however further investigation excluded the possibility that contactin represented the 97 kDa protein.

My experiences suggest that future efforts to purify target antigens using these techniques could benefit from some simple steps which may increase the chances of success. For example, the number of proteins in the starting homogenate may be reduced by using a cell line found to express the protein. In addition, relative enrichment for a membrane or cytosolic fraction by differential centrifugation or salt washing, could further reduce the number of proteins in the starting homogenate.

With respect to immunoaffinty relative enrichment of neuronal specific IgG by incubating serum with non neuronal tissue may reduce the number of precipitated background proteins. In addition, treating the sample to destroy the bonds between proteins may reduce co-precipitated background resulting in the relative enrichment of the target antigen.

7.4 Identification of a Target Antigen by Screening a Bacteriophage Display Library.

A total of 1.75×10^6 plaques were screened and a single reactive plaque isolated. The reactive phage were passed through stages of enrichment until >90% of the plaques were

detected by antibodies in the patient serum. Phage were probed with patient and control serum using a two stage technique to account for any non-specific reactivity. Of the 86 samples tested one patient with a confirmed paraneoplastic disease was found to have antibodies against the expressed protein. Isolation and sequencing of the cDNA revealed that the protein was TFNR, a protein known to be relatively enriched in the cerebellum.

Efforts to express the protein from the original pBS plasmid were unsuccessful. As a result the cDNA was sub-cloned into the pRSET expression vector to provide a readily detectable tag and means of purification. By maintaining the original reading frame or the cDNA found in pBS an 8 kDa antigen was produced with was not detected by serum from the index patient.

It was hypothesised that protein expression from a start codon in the open reading frame was occurring. The cDNA was sub-cloned into the appropriate vector to allow the expression of the entire protein, which was shown to be recognised by the patient's serum. It is unlikely that this protein represented the 97 kDa antigen given the fact the second PND patient with antibodies against TFNR did not detect a similar 97 kDa protein on neuronal immunoblots.

It is of interest that this protein was detected by serum from two patients with paraneoplastic neurological disease and these experiments have highlighted an interesting area for future study. In the first instance, it would be of interest to confirm that the protein was not the 97 kDa recognised by antibodies from the index patient by eluting bound IgG

from blots of phage plaques and subsequently probing neuronal immunoblots. Secondly, for completeness, serum from the second patient should be used to confirm that the protein expressed from pRSETC was detected by the patient's antibodies. A larger cohort of patients with PND is required in order to determine the frequency and significance of an anti-TFNR response.

Unfortunately, malignant tissue was not available from either case of PND thus we were unable to confirm that the antigen was detectable in the underlying tumour. Systematic investigation into the expression of TFNR by various tissues and it cellular location would be of value. It is necessary to demonstrate aberrant expression of the antigen by tumour tissue.

7.5 Conclusion.

The role of anti-neuronal antibodies in neurological disease has long been a subject of research. While in some disease the role of antibodies in the pathogenesis i.e. myasthenia gravis or as diagnostic markers i.e. PEM/SSN and anti-Hu is undisputed there are other instances in which the role of antibodies is less well determined.

This studied aimed to determine the frequency and nature of the antigens recognised by anti-neuronal antibodies in paraneoplastic and post-infectious neurological disorders. A range of proteomic and molecular biology techniques were employed to accomplish these aims.

In the first instance we could find little supportive evidence for antibodies to enolase, aldolase or PYK in post-infectious neurological disease. However the experience gained in cloning and production of recombinant proteins means that these techniques to be readily adapted for later experiments.

We were able to isolate two proteins of potential interest namely NLK and TFNR. The role of these proteins in the disease requires further investigation in order to satisfy the criteria for an antibody mediated disease. However, in both instances the proteins have potential importance in the nervous system. Larger cohorts of both OM and PND should be investigated. Serial samples and clinical state should ideally be available. With respect to paraneoplastic disease tumour tissue is required to demonstrate aberrant expression of the target antigens.

8 Chapter 8: Methods.

The methods described below represent standard techniques in protein purification and molecular biology. They are subdivided into these two sections. Where adaptions have be made these are listed in the methods sections of the relevant chapters.

8.1 Protein Techniques.

8.1.1 Polyacrylamide Gel Electrophoresis and Immunoblotting.

Pre-cast gels (10 and 1 well 4-12% Bis-tris gels) sample and running buffers (MOPS, MES) were purchased for use in the Powerease 500 electrophoresis system (Invitrogen, UK). Gels were removed from the packaging, washed in running buffer and locked into the electrophoresis tank. The inner chamber was filled with running buffer and left to stand. Once it was clear that the inner chamber had no leaks the outer chamber was filled with the same buffer. The appropriate amount of sample was diluted in 4x LDS. DTT was added to a final concentration of 0.05 M. The samples were heated at 65°C for 15 min. The appropriate volume of sample was then loaded into the gel (25 µl per well in a 10 well gel, 200 µl per well in a single well gel) and electrophoresed. The majority of protein samples were electrophoresed at 200 v 120 mA for 35 min. In some cases the time was extended to allow the dye front to reach the bottom of the gel. Once finished, gels were stained or immunoblotted as required.

8.1.2 Colloidal Blue Staining.

Some gels were stained using colloidal blue kit (Invitrogen, UK). After electrophoresis the gel was placed in fixing solution on a rocker at room temperature for 10 minutes. The fixing solution was decanted and replaced with staining solution A and left for a further 10 minutes. 5 ml of stainer B was added to the solution and the gel left to stain at 4°C for a maximum of 16 hrs. Background stain was removed by incubation in distilled water for 1 day. Gels were scanned wet, or dried and mounted for future reference.

8.1.3 Silver Staining.

A silver staining kit was purchased from Amersham Biosciences, UK. Staining was conducted according to the protocol. Briefly, proteins were electrophoresed as required fixed in fixing solution for 30 min and then transferred to sensitising solution for 30 min. Following three 10 min washes in water, gels were incubated in silver solution. Silver solution was removed with 2, 1min washes in water. Developing solution was applied for a maximum of 5 min before the reaction was stopped by replacing developing solution with stop solution. Gels were given a final series of three, 5 min washes in water before being scanned and stored.

8.1.4 Immunoblotting.

When required proteins separated by PAGE were subjected to immunoblotting. Buffer reservoirs, filter paper and nitro-cellulose were soaked in transfer buffer (Invitrogen, UK). Two buffer reservoirs were then placed into the deep section of a blotting chamber. These were overlaid with 4 sheets of filter paper. The plastic case containing the gel was opened

and a further piece of the filter paper used to remove the gel from the plastic backing. The filter paper and gel were placed on top of the filter paper in the blotting chamber. A labelled section of nitro-cellulose was then carefully placed over the top of the gel. A gel knife was used to remove any trapped air bubbles and a further two pieces of filter paper positioned over the top followed by a sheet of cellophane. A further buffer reservoir was then added and the lid placed in position. The entire chamber was then placed in the electrophoresis tank and locked in position. Any spilled buffer was removed from the outer chamber and the inner chamber was filled with transfer buffer. Once it was clear that there were no leaks the outer chamber was filled with water. Proteins were transferred for 2 hr at 25v mA unless otherwise stated. Blots were blocked for at least one hour in 0.9 % saline containing 2% (w/v) non-fat dried milk powder prior to the addition of appropriate primary antibody. Immunoblots were incubated overnight at 4°C. The following day blots were washed 6 times with 10 min washes using 0.2 % (w/v) non-fat dried milk powder and 0.05 % tween in 0.9 % saline. The appropriate secondary antibody was diluted in wash solution and incubated with the blot for 1-2hrs. Washing was repeated and blots were developed using either ECL of chromogenic techniques (see section 2.2-6 and 2.2-7).

8.1.5 Enhanced Chemiluminescence.

After washing, immunoblots were rinsed in 0.9 % saline. ECL was performed using the Supersignal West Pico Chemiluminescent Kit (Pierce, UK). Equal volumes of luminol/ enhancer solution and stable peroxide solution were mixed and applied to the partially dried immunoblot. An acetate film was placed over the blot prior to exposure to the film (Hyperfilm, Amersham Biosciences, UK).

8.1.6 Chromogenic Development.

After washing, immunoblots were rinsed in 0.9 % saline. The saline was decanted and each blot incubated in 25 ml chromogenic developing solution. The blots were left to develop for a minimum of 30 min, rinsed to remove the developing solution, and dried. Blots were scanned for future reference.

8.1.7 Buffer Exchange Method.

Proteins were buffer exchanged using either a HiTrap desalting column (Amersham, Biosciences, UK) or a Slidealyzer dialysis cassette (Perce, UK). Samples to be buffer exchanged using the desalting column were injected into the sample loop of the ÄKTA FPLC. The pumps were prepared using the target buffer and the column equilibrated with that buffer. Samples were injected at 1 ml/min and proteins collected from the FRAC-900 using the chromatogram as a reference. When larger volumes were required to be buffer exchanged the slidealyzer system was used. Proteins were injected and the cassette incubated in the target buffer for at least 24 hours prior to use.

8.1.8 Total Protein Method.

Protein concentration was determined using the Bio-Rad D_c protein assay kit (Bio-Rad). A protein standard (2 mg/ml) was purchased (Sigma) and used to produce a range of standards ranging from 0 – 2 mg/ml. 10 µl of protein sample or standard was pipetted into two ELISA wells followed by 25 µl reagent A and 200 µl reagent B. The plate was incubated at room temperature for at least 20 mins to allow the colour reaction to proceed.

The plate was read using a Wallac 1420 multilabel counter. The standards were used for the production of the standard curve and the protein concentrations of the samples determined.

8.2 Molecular Biology Techniques.

8.2.1 Restriction Enzyme Digestions.

All restriction enzyme digestions were conduted according to the manufacturers guidelines for the given restrication enzyme. In instances where enzymes were not compatible DNA was digested with one enzyme, recorved from an agarose gel after electophoresis and digested with the second enzyme.

8.2.2 Agarose Gel Electrophoresis.

Agarose gels were prepared by the addition of the required amount of agarose to TBE running buffer. Unless otherwise stated, 0.7 % (w/v) agarose gels were used. The solution was heated until the agarose had dissolved. A minigel tray was assembled and the appropriate comb selected. Ethidium bromide was added to the agarose solution to a final concentration of 0.5 μg/ml and poured into the minigel tray. Samples were combined with loading dye, loaded into the gel and electrophoresed at 55 mv for approximately 45 min. 1-5μl of 1kb ladder (Promega) was loaded at each run to provide a reference. Gels were viewed using a gene genius bio imaging system (Synegene).

8.2.3 Gel Extraction.

DNA was extracted from agarose gels using a QIAquick gel extraction kit (Qiagen). Gels were viewed under UV illumination and DNA cut from the gel using a sterile scalpel. The gel slice containing the DNA was weighed; buffer QG added (1 ml/g) and heated at 50°C

for 10 min with occasional vortexing. The solution was applied to QIAquick spin column and centrifuged at 13,000rpm for 1 min. The flow-through was discarded. This was repeated until the entire solution had been passed through the column. 750 µl of buffer PE was centrifuged through the column, the flow-through was discarded and the column spun again to remove residual buffer. 30-50 µl of elution buffer or water was used to wash bound DNA from the column. DNA was collected from the column by centrifugation at 13,000rpm for one minute.

8.2.4 Digestion and Ligation of RT-PCR and PCR Products.

TOP10F' cells containing the required expression vector (pRSETA, B, C) were grown overnight at 37°C in 5 ml SOB broth containing ampicillin. The plasmids were isolated from the bacterial cultures using QIAprep miniprep kit as described (see section 8.2.5).

The RT-PCR and PCR products were extracted from a 0.7% agarose gel using a gel extraction kit and eluted in water. Expression vectors and the RT-PCR/PCR product to be inserted were digested with the same pair of restriction enzymes. Attempts at simultaneous double digestion of pRSETA and the RT-PCR with *Hind* III and *Bam* HI were unsuccessful. As a result a sequential digestion of the vector pRSETA and RT-PCR/PCR products was required. pRSETA vectors were concurrently treated with calf intestinal alkaline phosphatase (CIP) (NewEngland Biolabs) to prevent re-circularisation of singley digested vector. DNA was cleaned after each restriction reaction by extraction from a 0.7% agarose gel using a gel extraction kit and eluted in water. 5 µl of digested RT-PCR or PCR product, 3 µl of digested expression vector, 1 µl T4 DNA ligase and 1 µl T4 DNA ligase buffer (NewEngland Biolabs) were combined in micro-centrifuge tube and incubated in a

water bath at 16°C overnight. Substitution of the RT-PCR/PCR product with water formed a negative control to assess the level of background transformation.

8.2.5 Plasmid Extraction.

Plasmids were isolated from the bacterial cultures using QIAprep miniprep kit (Qiagen). Briefly, bacteria were isolated by centrifugation and re-suspended in 250 µl of buffer P1. The cells were transferred to a micro-centrifuge tube and lysed in 250 µl of buffer P2 for no more than 5 min. Lysis was then stopped using 350 µl of buffer N3 and insoluble material precipitated by centrifugation at 13,000 rpm for 10 min. The supernatant was decanted into a QIAprep spin column and centrifuged for 1 min. Flow-through was discarded. The plasmid DNA was washed in a total of 0.75 ml of buffer PE before elution from the membrane in 50 µl of elution buffer or water into a labelled micro-centrifuge tube.

8.2.6 DNA Sequencing.

DNA sequencing was conducted using plasmid DNA. 3 µl big dye terminator reaction kit v3.0, plasmid DNA and 1 µl of primer (10 µM) were combined in a PCR plate added H_20 added to a final volume of 15 µl. The sequencing reaction was conducted using a gene amp PCR system 9700 using 25 cycles of the following protocol:

Denaturation	Annealing	Extension
96°C for 15s	50°C for 15s	60°C for 4min

The reactions were then stored at 4°C overnight. The following day the reaction products were cleaned using the Montage SEQ_{96} sequencing reaction cleanup kit using the following

procedure. 20 µl of injection solution was added to each reaction and each sample transferred separately to a SEQ_{96} plate. The plate was positioned on a vacuum manifold and suction applied at 24" Hg until all wells were empty. Excess moisture was blotted from the bottom of the plate and a further 25 µl of injection solution added to each well. Wells were again dried by suction and a further 25 µl of injection solution applied. The reaction products were re-suspended by gently agitation and the plate loaded into a 3100 genetic analyser (ABI Prism) for electrophoresis and sequence determination. The obtained DNA sequence was analysed using BLAST and translate programs.

8.2.7 Production of Chemically Competent TOP10F'

E. coli TOP10F' (Invitrogen, UK) are suitable for the cloning, propagation and maintenance of plasmids. The cells were grown overnight at 37°C on SOB tetracycline plates. A single colony was selected and grown in 100 ml SOB broth at 37°C, 220 rpm to an OD = 0.3-0.5. Cells were then split into 50 ml falcon tubes and placed on ice for 10 min. The cells were harvested by centrifugation at 4000 rpm at 4°C for 10 min, re-suspended in 5 ml ice cold 0.1 M $CaCl_2$ (Sigma) and left on ice for at least 1 hr. The cells were spun at 4000 rpm at 4°C for 5 min before being re-suspended in 2 ml-ice cold 0.1 M $CaCl_2$. 150 µl DMSO was added to the cells, swirled to mix and left on ice for 10 min. A further 150 µl DMSO was added to the cells, which were then split into 100 µl aliquots in pre-chilled, labelled tubes. The cells were then snap frozen in liquid nitrogen and stored at -80°C until required.

8.2.8 Transformation of Chemically Competent TOP10F'

Ligated expression vector was used to transform TOP10F' cells. Competent cells were thawed for 30 min on ice. 50-100 ng DNA was added to the cells and left on ice for a further 30 min. Cells were then heat shocked at 42°C for 90 seconds before being returned to ice and left for a minimum of 2 min. 900 µl of pre-warmed SOC was added to the competent cells which were then incubated for 1 hr at 37°C, 220 rpm. 200 µL of this solution was then spread on tetracycline/ ampicillin SOB plates. Plates were incubated overnight at 37°C. The following day successfully transformed colonies were used to inoculate 5 ml SOB broth and cultured overnight. 1 ml of cells were snap frozen as described to provide a stock. Plasmids were extracted from TOP10F' thought to contain ligated vector using a miniprep kit as described and subjected to digestion using one of the proteins used for the initial double digestion of the cDNA. Control pRSET vector was also digested with the same enzyme as a control. Digested plasmids were analysed by electrophoresis through a 0.7 % gel and visualised under UV light.

8.2.9 Transformation of BL21 (DE3) pLysS.

The E.coli strain BL21 (DE3) pLysS is used for the expression of T7 regulated genes such as those cloned into the pRSET expression vectors. Purified plasmid was used to transform competent BL21 (DE3) pLysS as described (see section 8.2.8) and incubated overnight at 37°C on SOB plates containing chloramphenicol and ampicillin. Colonies were selected the next day, grown overnight in 5 ml SOB broth containing chloramphenicol and ampicillin and glycerol stocks prepared.

8.2.10 Induction of Recombinant Protein Production.

Strains of *E. coli* containing the appropriate plasmid were grown overnight in 5 ml SOB or LB broth containing the appropriate antibiotic at 37°C. The following day both cultures were diluted in 25 ml of pre-warmed SOB broth to OD 1.0 and grown to OD 4.0-6.0. Protein production was induced by the introduction of 1 mM IPTG (Sigma) and the cells grown for a further 3 hr. Cells were centrifuged at 4000 rpm for 10 min, the supernatant discarded and the cells were snap frozen in liquid nitrogen. When larger scale production of recombinant proteins was required volumes were scaled accordingly.

8.2.11 Preparation of Crude Bacterial Homogenates.

Homogenates were prepared on ice. 100 µl of bacterial protease inhibitors (Sigma, UK) were added to *E. coli* preparations and the cells resuspended in 100 µl of His-Protein binding buffer per ml of original culture volume. Cells were subjected to approximately 4 rounds of sonication and Triton X-100 added to a final concentration of 1 %. The cells were left rocking on ice for a further 30 min, spun at 16,000 for 20 min and the soluble fraction retained for further analysis.

8.2.12 IMAC Purification of His-tagged Recombinant Protein.

Unless otherwise stated His tagged recombinant proteins were purified using the following protocol. 1 ml HiTrap chelating columns (Amersham Biosciences, UK) installed on the ÄKTA fluid phase liquid chromatography (FPLC) system. The HiTrap chelating columns were flushed with 10ml distilled water and charged with Ni ions by the introduction 0.5 ml 0.1M $NiSO_4$. Un-bound ions were removed with a 10 ml wash with water. The column was

equilibrated with 10 ml His-binding buffer prior to the injection of bacterial homogenate at a rate of approximately 1 ml/min. Once the absorbance had returned to zero His tagged proteins were eluted using a gradient of His elution buffer (8 M urea, 20 mM Tris-HCL, 0.5 M NaCl, 0.5 M Imidazole, 1 mM 2-mercaptoethanol). Eluted proteins were collected using a Frac-900 fraction collector. Tubes containing protein were pooled and subjected to PAGE and immunoblotted to ascertain the purity and identity of the purified protein. The total protein of each recombinant protein was calculated using the method described. Proteins were alliquoted and stored at 20°C until required.

8.2.13 Confirmation of Recombinant Protein Expression.

Homogenates of bacteria containing ligated vector or control were prepared by combining crude homogenate with LDS and DTT and subjected to PAGE. Gels were either stained using colloidal blue or immunoblotted.

Immunoblots were probed with anti-HisG antibody or anti-antigen antibody (α-enolase, γ-enolase, PYK, aldolase C, NLK) as appropriate. The blots were washed and developed chromogenically. Development was stopped by replacement of developing solution with water. Immunoblots were scanned and retained for analysis.

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A:

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9 *Appendix 1: Materials, reagents and suppliers*

Solution	*Composition*	*Use*
SOB Agar (1 Litre)	Make up 1 Litre SOB broth as above. Once pH has been adjusted add 15g agar.	Growth and maintenance of recombinant bacterial strains.
SOC broth	Add 0.5ml of 50% glucose solution to 50ml SOB broth.	Preparation of transfected cell prior to plating
Solubalisation buffer	1ml 4xLDS sample buffer (Invitrogen), 1ml ddH_2O, 0.5ml Methanol	Treatment of IEF gel prior to PAGE electrophoresis.
10x TBE (1 litre)	121.1g Trisma Base, 61.8g Anhydrous boric acid, 7.4g EDTA, Check pH = 8.3.	Agarose gel electrophoresis of DNA.
DNA electrophoresis loading dye.	0.25% bromophenol blue, 40% (w/v) Sucrose, Dissolve in H_2O	Loading dye for DNA electrophoresis
TNT Buffer (5 Litres)	6.06g Tris, 34.5ml 1M HCl, 43.83g NaCl, 2.5ml Tween 20, pH 8.0	Preparation of membranes during bacteriophage screening.
TNT Blocking Buffer	Add 2% milk powder (w/v) to TNT buffer	Blocking of membranes during bacteriophage screen.
Chromagenic Developing Solution	20ml Methanol, 100 ml acetate buffer, 20mg 4-chloro-1-napthol, 120µl H_2O_2	Chromagenic development of immunoblots
Affinity elution buffer (0.5 litre)	8.61g Citric acid, 2.65 g Sodium Citrate, pH 3.0	Elution of IgG from HiTrap protein A column
Neutralisation buffer	50µl 1M Tric-HCl pH 9.0	Neutralise proteins after acid elution.
Collidal blue solutions	Fixing solution: 50% methanol, 10% acetic acid, 40% D.I water Stain A: 10ml methanol, 10ml stainer A, 55ml D.I water Stain B:	PAGE gel staining. Sterner A and B from Invitrogen.
IgG coupling buffer	0.2M $NaHCO_3$, 0.5M NaCl, pH 8.3	Coupling purified IgG to NHS column to produce immunoaffinity column.
ELISA wash solution	0.05% tween, 0.9% saline, 0.2% BSA or 0.05% tween, 0.9% saline, 0.2% normal	

	rabbit serum.	
IEX binding buffer	20mM Tris-HCl (pH 8.35 or pH 8.00)	
IEX elution buffer	20mM Tris-HCl 1.5M NaCl (pH 8.35 or pH 8.00)	
Silver stain fix	100ml ETOH, 25ml Acetic acid, 125 H_2O	
Silver stain sensitising solution	75ml ETOH, 10ml 5% Sodium Thiosulphate, 17g Sodium acetate, 163ml H_2O	
Silver solution:	25ml 2.5% silver nitrate solution, 0.1ml 37% formaldehyde, 225ml H_2O	
Carbonate buffer	0.69g sodium carbonate, 1.31g sodium hydrogen carbonate to 500 ml deionised water.	Protein binding to ELISA plates.
Silver stain stop solution	3.65g EDTA in 250ml ddH_2O	
Silver stain developing solution	6.25g Sodium carbonate, 0.05ml formaldehyde, 250ml H_2O	
NHS inactivation buffer A	0.5M ethanolamine, 0.5M NaCl, pH 8.3	Blocking active sites of NHS activated column after ligand has been introduced.
NHS inactivation buffer B	0.1M acetate, 0.5M NaCl, pH 4	Blocking active sites of NHS activated column after ligand has been introduced.
His-Protein Binding Buffer	8M urea, 20mM Tris-HCl, 0.5M NaCl, 20mM Imidazole, 1mM 2-mercaptoethanol, pH 8.0	Preparation of bacterial homogenate. Binding His-tagged recombinant proteins to IMAC column.
His-Protein Elution Buffer	20mM Tris-HCl, 0.5M NaCl, 0.5M Imidazole, 1mM 2-mercaptoethanol, pH 8.0	Elution of bound, His-tagged proteins from IMAC column.
Stock IPTG Solution	0.119g IPTG in 5ml IPTG. Final concentration 100mM. Store at 4°C.	Induction of recombinant protein production

Affinity binding buffer (0.5 litre)	0.87g Na_2HPO_4, 0.47g NaH_2PO_4, pH 7.0.	Binding IgG to HiTrap protein A column
Sodium phosphate buffer.	0.87g Na_2HPO_4, 0.47g NaH_2PO_4, pH 7.0.	
SM Buffer (1 Litre)	5.8g NaCl, 2.0g $MgSO_4.7H_2O$, 50ml of 1M Tris-HCl (pH 7.5), 5.0ml of 2% (w/v) gelatine. Autoclave before use.	Storage of bacteriophage
NZY Top (1 Litre)	As NZY base but substitute agar for 0.7% (w/v) agarose.	

Reagent	Catalogue Number	Company
1 well 4-12% Bis-Tris PAGE gel	NP0324 BOX	Invitrogen
10 well 4-12% Bis-Tris PAGE gel	NP0321 BOX	Invitrogen
ACCUSPIN System-HISTOPAQUE-1077	A7054	Sigma
Ampicillin	A 9393	Sigma
Anti-Contactin antibodies	610578	BD Biosciences
Anti-His antibodies	R940-25	Invitrogen
Bacterial protease inhibitor cocktail	P 8465	Sigma
BenchTop 5kb DNA ladder	G7541	Promega
BSA	A 2153	Sigma
BugBuster protein extraction reagent	70793	Novagen
Calcium Chloride ($CaCl_2$)	C 4901	Sigma
Calf intestinal phosphatase	M02908	New England Biolabs
Cerebellar cDNA library	937263	Stratagene
Chloramphenicol	C 0378	Sigma
Colloidal blue staining kit	LC6025	Invitrogen
Dimethyl sulfoxide (DMSO)	D8418	Sigma
Ethidium bromide	E7637	Sigma

HiTrap chelating HP column	17-0408-01	Amersham Biosciences
HiTrap desalting column	17-1408-01	Amersham Biosciences
HiTrap NHS-activated HP column	17-0717-01	Amersham Biosciences
HiTrap Q FF column	17-5156-01	Amersham Biosciences
HiTrap rProtein A FF	17-5079-01	Amersham Biosciences
Hyperfilm	28-9068-36	Amersham Biosciences
IEF Ph 3 –10 gels	EC6655A	Invitrogen
IEF andode buffer	LC5300	Invitrogen
IEF cathode buffer	LC5370	Invitrogen
IEF lane markers	39212-01	Invitrogen
IEF sample buffer	LC5311	Invitrogen
Imidazole	I5513	Sigma
IPTG	I 6758	Sigma
Kanamycin	K 4000	Sigma
LB Agar (1 Litre)	L 2897	Sigma
LB Broth (1 Litre)	L 3022	Sigma
Mammalian protease inhibitor cocktail	P 8340	Sigma
Mercaptoethanol	M6250	Sigma
Micro-fast track Mrna isolation kit	K1580-01	Invitrogen
MES running buffer	NP0002	Invitrogen
MOPS running buffer	NP0001	Invitrogen
Normal rabbit serum	R 4505	Sigma
NZY broth	N 3643	Sigma
Primers	As per order	Sigma genosys
Protein standard	P5619	Sigma
QIAprep spin miniprep kit	27104	Qiagen

QIAquick gel extraction kit	28704	Qiagen
Plusone silver staining kit	17-1150-01	Amersham Biosciences
PMSF	P7626	Sigma
Rabbit anti-human IgG/FITC	F0315	Dako
Rabbit anti-human IgG/HRP	P0406	Dako
Rabbit anti-human lambda/ kappa	P0212	Dako
RC DC protein assay kit I	500-0121	Bio-Rad
Restriction enzymes (various)	As per enzyme	New England Biolabs
Sodium dodecyl sulphate (SDS)	L3771	Sigma
SOB broth	H 8032	Sigma
Sodium Chloride (NaCl)	S 7653	Sigma
Supersignal west pico chemiluminescent kit	34080	Pierce
Swine anti-rabbit immunoglobulins-HRP	P 0217	Dako
Swine anti-rabbit TRITC		Dako
T4 DNA ligase	M0202T	New England Biolabs
Tetracycline	T 3258	Sigma
TMB	T4444	Sigma
Transfer buffer	NP0006-1	Invitrogen
Trizma base	T1503	Sigma
T-per	78510	Pierce
Tween 20	P 1379	Sigma
Triton X-100	T8787	Sigma
Urea	U5378	Sigma
Xgal	B 9146	Sigma

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10 Appendix 2: Publications

PAPER

A follow up study of patients with paraneoplastic neurological disease in the United Kingdom

P M Candler, P E Hart, M Barnett, R Weil, J H Rees

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Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease

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PAPER

Post-streptococcal opsoclonus-myoclonus syndrome associated with anti-neuroleukin antibodies

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