

**INVESTIGATING THE MOLECULAR TARGETS OF
ANTIPHOSPHOLIPID ANTIBODIES**

by

**Yiannakis Ioannou
Department of Medicine
University College London**

A Thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Science

2007

UMI Number: U592058

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U592058

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

The antiphospholipid syndrome (APS) is characterised by the presence of pathogenic antiphospholipid antibodies (aPL) that bind phospholipid via the N-terminal domain I (DI) of the protein cofactor beta2-glycoprotein I (β_2 GPI). This thesis aims to investigate and identify the nature of this interaction at the molecular level.

In order to study DI, a system for expressing and purifying sufficient quantities of this protein was developed using *Escherichia coli* (*E.coli*) as the expression host. This was achieved primarily by synthesising a gene with all codons optimised for *E.coli*, tight regulation of expression and recovery of DI from the periplasm to facilitate folding. Recombinant purified DI bound clinically relevant human monoclonal and polyclonal purified IgG aPL in both solid and fluid phase assays.

Hypotheses were generated that identified candidate epitopes for aPL binding as potentially involving residues D8, D9, E23, E26, E27, R39, G40, R43 and the DI-II interlinker region. In total, 15 mutants of DI targeting these areas were created and computer modelling employed to predict the likely structural effects of these mutations upon DI.

Expressed DI mutants were then tested in both solid and fluid phase assays for binding to polyclonal IgG derived from patients with APS and compared to wild-type DI. Some mutations, such as those targeting R39, caused loss of binding to aPL in the fluid phase whilst others caused enhanced binding over wild-type DI.

In conclusion, this thesis demonstrates that DI of β_2 GPI may be expressed using *E.coli* and binds clinically relevant IgG aPL. Detailed mutational studies support the concept that aPL bind discontinuous epitopes on DI involving regions D8 and D9, R39 to R43 and the DI-II interlinker which are in close proximity to each other in the tertiary structure. The ability to produce a mutant of DI with enhanced binding over wild-type holds therapeutic potential.

DEDICATION

I dedicate this thesis to one person, my wife Evi. It is through her love, patience and support that I derive my strength and motivation to achieve. My life is richer for having Evi by my side, now and for always.

ACKNOWLEDGEMENTS

I would like to thank my supervisors: - two people that I am particularly indebted to are David Isenberg for supporting me right from the start and making it all possible and Anisur Rahman for his insightful attention to detail and his unfailing ability to see light at the end of very long (and on occasion dark) tunnels. Both David and Anisur have been kind, wonderful supervisors and mentors. For me, they continue to represent inspirational role models. Thank you also to David Latchman for his most helpful meetings and advice on the meaning of acquired results and the merits of planned experiments.

I would like to thank all my colleagues within the Centre for Rheumatology at UCL and the Medical Molecular Biology Unit for their help, advice and friendship. In particular I am indebted to Ian Giles, for his initial help in basic molecular biology and ELISA techniques and constant, friendly availability as a useful sounding board for ideas. Thank you also to Charis Pericleous for enthusiastically carrying on the domain I baton, Nancy Lambrianides for her friendship and help with some of the monoclonal antibody binding work and Barry James and Miratul Muqat for patiently going through basic lab techniques.

Thank you to Dr Chris Richardson and Professor Pearl for their initial advice regarding recursive PCR and the use of the Juniper programme. Thank you also to Drs' M Iverson and M Linnik of La Jolla Pharmaceuticals for supplying monoclonal murine anti-DI antibodies.

I would also like to thank the Arthritis Research Campaign for funding this PhD through a Clinical Research Fellowship Award (4918).

Finally, thanks to my parents for their love and support over the years and to my two beautiful girls Georgia and Melissa, whom I adore more than I can put into words and who put everything else into perspective.

DECLARATION

The author carried out the work described in this thesis unless otherwise stated.

TABLE OF CONTENTS

Title page	1
Abstract	2
Dedication	3
Acknowledgments	4
Declaration	4
Table of contents	5
List of tables	12
List of figures	13
Abbreviations	17
CHAPTER 1 INTRODUCTION	21
OVERVIEW OF INTRODUCTION	22
1.1 THE IMMUNE SYSTEM: ANTIBODIES, ANTIGENS AND THE INTERPLAY OF PROTEINS	23
1.1.1 The immune response – innate and adaptive	23
1.1.1.1 <i>Components of the innate immune system</i>	23
1.1.1.2 <i>Cellular components of the adaptive immune response</i>	23
1.1.1.3 <i>Recognition of pathogens by the innate immune system: Toll-like receptors</i>	25
1.1.2 Antibodies in the immune system: structure and function	26
1.1.3 Autoimmunity and autoantibodies	28
1.1.4 Structural nature of proteins, antigens and antigenic determinants	29
1.1.5 Nature of the antigen : antibody interaction	31
1.2 THE ANTIPHOSPHOLIPID SYNDROME (APS)	32
1.2.1 Definition of the APS	32
1.2.2 Historical review of aPL and the APS	33
1.2.3 Classification of the APS	35
1.2.4 Standardisation of aPL assays	39
1.2.5 Epidemiology of APS and aPL as a risk for thrombosis	40
1.2.6 Thromboembolic and cardiovascular manifestations of the APS	43
1.2.7 Obstetric manifestations of the APS	44
1.2.8 Catastrophic antiphospholipid syndrome (CAPS)	47

1.2.9	'Primary' and 'secondary' APS: is there a true distinction?	48
1.2.10	Management and prognosis of the APS	50
1.2.10.1	<i>Secondary prophylaxis of venous and arterial thrombosis</i>	50
1.2.10.2	<i>Management of pregnant patients with the APS</i>	53
1.2.10.3	<i>Primary prophylaxis of aPL positive patients without APS</i>	55
1.2.10.4	<i>Management of non-thrombotic manifestations of the APS</i>	55
1.2.10.5	<i>Management of CAPS</i>	56
1.2.11	Prognosis in APS	57
1.3	SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)	57
1.3.1	Aetiology, epidemiology and natural course for SLE	58
1.3.2	Clinical features of SLE	59
1.3.3	Autoantibodies in SLE	62
1.3.4	Management of SLE	64
1.3.5	Can novel therapeutic candidates for SLE be applied to the APS	65
1.4	ANTIPHOSPHOLIPID ANTIBODIES (aPL)	68
1.4.1	Development of aPL – genetics versus environment	68
1.4.2	Distinguishing between pathogenic and non-pathogenic aPL	71
1.4.3	Evidence from animal studies for aPL promoting thrombosis	71
1.5	UNDERLYING MECHANISMS OF PATHOGENICITY IN THE APS	74
1.5.1	Effects of aPL on the coagulation pathways	75
1.5.1.1	<i>Direct effects of aPL on factors that promote coagulation</i>	75
1.5.1.2	<i>Inhibition of the protein C, thrombomodulin and fibrinolytic pathway</i>	77
1.5.1.3	<i>Effects of aPL on annexin V</i>	79
1.5.2	Cell mediated effects of aPL	79
1.5.2.1	<i>aPL induced activation of endothelial cells</i>	79
1.5.2.2	<i>Tissue factor in APS</i>	81
1.5.2.3	<i>Platelet pathology in APS</i>	82
1.5.2.4	<i>Activation of autoreactive CD4+ positive T cells</i>	84
1.5.2.5	<i>Intracellular signalling pathways in the APS</i>	85
1.5.3	Mechanisms for pregnancy morbidity, the complement pathway and cytokines	87
1.5.4	Atherosclerosis and aPL	90

1.5.5	Lessons from pathogenic mechanisms: current and future therapies	91
1.6	CHARACTERISATION OF β_2 GPI	93
1.6.1	Molecular structure and function of β_2 GPI	93
1.6.2	Effects of polymorphisms or deficiencies of β_2 GPI: clues to function and pathogenicity	96
1.6.3	Do aPL bind cryptic or non-cryptic epitopes on β_2 GPI?	97
1.6.4	DV of β_2 GPI: anchor to phospholipid	99
1.6.5	DIII and DIV of β_2 GPI: evidence for and against aPL binding	100
1.6.6	DI of β_2 GPI: evidence for aPL binding	102
1.6.7	Candidate epitopes within DI	103
1.6.7.1	<i>Clues from antigen mutational, binding and structural studies</i>	103
1.6.7.2	<i>Clues from aPL mutational studies</i>	107
1.6.7.3	<i>Conclusion on likely epitopes</i>	107
1.7	PRODUCTION OF RECOMBINANT PROTEINS	108
1.7.1	Production of eukaryotic proteins in prokaryotic cells	109
1.7.1.1	<i>Expression of target protein by E.coli</i>	109
1.7.1.2	<i>Consideration of codon usage</i>	111
1.7.1.3	<i>Choice of cellular compartment</i>	113
1.7.2	Production of proteins using mammalian cells	115
1.7.3	Advantages and disadvantages of other expression systems	116
1.8	PRODUCTION OF RECOMBINANT β_2 GPI	117
1.8.1	Established systems of recombinant β_2 GPI expression	117
1.8.2	Challenges in establishing expression of DI in <i>E.coli</i>	118
1.9	AIMS OF THESIS	120
CHAPTER 2	MATERIALS, METHODOLOGICAL PRINCIPLES AND	121
	DETAILS OF PROCEDURES	
	OVERVIEW OF CHAPTER 2	122
2.1	Chemicals, general materials and equipment	123
2.1.1	Chemicals	123
2.1.2	General materials and equipment	123
2.1.3	Enzymes	123

2.1.4	Reaction buffers	124
2.1.5	General buffers and solutions	124
2.1.6	Specific buffers and solutions	124
2.2	Bacterial strains	127
2.3	Antibiotic and growth media	127
2.4	Monoclonal antibodies (mAb)	128
2.4.1	Human monoclonal aPL – binding characteristics	128
2.4.2	Murine monoclonal antibodies	128
2.5	Human polyclonal IgG	129
2.6	Plasmids	129
2.7	Minipreparation of plasmid DNA from <i>E.coli</i>	129
2.8	Restriction digest of DNA	130
2.9	Separation of DNA fragments by agarose gel electrophoresis	134
2.10	Quantification of dsDNA concentration	134
2.11	Purification of DNA from agarose gels	135
2.12	Synthesis of synthetic DI gene using recursive PCR	135
2.12.1	Design of primers for recursive PCR	135
2.12.2	Recursive PCR	136
2.13	Ligation of amplified DNA from recursive PCR into pRSETb	136
2.14	Production of competent DH5 α and BL21(DE3) <i>E.coli</i> cells	137
2.15	Transformation of competent <i>E.coli</i> cells	137
2.16	Ethanol precipitation of plasmid DNA	137
2.17	Production of DI mutants	138
2.17.1	Computer modelling of DI and DI mutants	138
2.17.2	Site directed mutagenesis	139
2.18	Recombinant wild-type and mutant DI protein expression	142
2.18.1	Optimisation of protein expression protocol	142
2.18.2	Final optimised culture and induction experimental protocol	142
2.18.3	Preparation of periplasmic fraction	142
2.19	Recombinant wild-type and mutant DI purification	143
2.20	Protein quantification, detection and characterisation	144
2.20.1	Total protein quantification	144
2.20.2	SDS-polyacrylamide gel electrophoresis (PAGE)	144
2.20.3	Coomassie stain	145
2.20.4	Transfer of protein to nitrocellulose and western blotting	145
2.20.5	Immunodetection of proteins on western blots	146

2.20.6	Quantitative western blotting	146
2.21	Functional binding assays - enzyme-linked immunosorbent assay (ELISA)	146
2.21.1	Detection and quantification of total whole IgG molecules	147
2.21.2	Detection of aPL binding to cardiolipin in solid phase	148
2.21.3	Detection of aPL binding to phosphatidylserine in solid phase	148
2.21.4	Detection of aPL binding to β_2 GPI in solid phase	149
2.21.5	Detection of aPL binding of polyclonal IgG to pure human β_2 GPI coated on cardiolipin in solid phase	149
2.21.6	Detection of aPL binding to purified recombinant DI in solid phase	150
2.21.7	Detection of aPL binding in fluid phase	151
2.21.7.1	<i>Inhibition of aPL binding to DI</i>	151
2.21.7.2	<i>Inhibition of aPL binding to β_2GPI/cardiolipin</i>	151
2.22	Statistical analysis	151
 CHAPTER 3 THE DEVELOPMENT OF AN EXPRESSION SYSTEM FOR PRODUCING RECOMBINANT DOMAIN I OF HUMAN β_2GPI IN <i>E.COLI</i>		 153
OVERVIEW OF CHAPTER 3		154
3.1	Production of a synthetic gene encoding for DI of human β_2 GPI optimised for expression in <i>E.coli</i>	155
3.1.1	Introduction	155
3.1.2	Results from Juniper	156
3.1.3	Amplification of synthetic gene using recursive PCR	159
3.2	Cloning scheme for synthetic DI gene: expression is critically dependent on tight regulation of expression	160
3.2.1	Introduction	160
3.2.2	Ligation of synthetic Omp A-DI gene into pRSET-b expression plasmid	160
3.2.3	Ligation of synthetic Omp A-DI gene into pET-20b expression plasmid	163
3.2.4	Ligation of synthetic DI gene into pET-26b plasmid – tight regulation of expression using the high stringency T7/lac promoter	170
3.2.5	Ligation of the synthetic DI gene into pET-39b plasmid – incorporation of DsbA tag	174
3.3	Successful expression of DI in <i>E.coli</i>	177
3.3.1	Introduction	177
3.3.2	Expression and purification of periplasmic his ₆ -tagged DI	177
3.3.3	Efficient expression achieved with low levels of IPTG induction	184

3.3.4	Recombinant sequence optimised for <i>E.coli</i> is crucial to successful expression of DI	186
3.3.5	Expression of DI tagged with DsbA at the N-terminal	189
3.4	Discussion of results	192
CHAPTER 4 BINDING PROPERTIES OF RECOMBINANT DI OF β_2GPI		195
OVERVIEW OF CHAPTER 4		196
4.1	Recombinant DI binds to pathogenic monoclonal human aPL in both direct and inhibition assays	197
4.1.1	Introduction	197
4.1.2	Recombinant DI in solid phase binding to native IS4, IS4V _H /B3V _L , IS4V _H /UK-4V _L and variants containing point mutations in IS4V _H	199
4.1.3	Recombinant DI in the fluid phase binds to affinity purified IS4V _H /IS4V _L	202
4.2	Demographic data on polyclonal IgG patient samples	203
4.3	Binding profile of purified IgG to cardiolipin, phosphatidylserine and whole β_2 GPI	206
4.4	Recombinant DI binds polyclonal IgG derived from APS patients	210
4.5	Comparative sensitivities and specificities for detecting clinically relevant IgG	211
4.6	Correlation analyses of binding studies between DI, cardiolipin and whole β_2 GPI	212
4.6.1	Binding of aPL to DI correlates well with binding to cardiolipin	212
4.6.2	Identification of polyclonal aPL demonstrating species preference for β_2 GPI	213
4.6.3	Correlation of binding between DI and whole β_2 GPI	215
4.7	Identification and characterisation of aPL that bind whole β_2 GPI when bound to phospholipid	216
4.7.1	Identification of aPL that preferentially bind β_2 GPI bound to cardiolipin as opposed to an irradiated plate	219
4.8	Subset analyses of aPL samples	220
4.9	Binding of recombinant DI to polyclonal aPL samples in the fluid phase	223
4.10	Discussion of results	224
CHAPTER 5 DESIGN AND EXPRESSION OF MULTIPLE RECOMBINANT MUTANTS OF DI		230
OVERVIEW OF CHAPTER 5		231

5.1	Introduction: rationale for choice of regions to target	232
5.2	Design and production of DI mutant constructs	233
5.2.1	Computer modelling used to design nature of single / multiple point substitution mutations	233
5.2.2	Design of DI interlinker variants	237
5.2.3	Codon design of mutant amino acids	237
5.2.4	Results of constructs made by site directed mutagenesis	237
5.2.5	Production of synthetic mutant DI genes using recursive PCR	241
5.2.6	Successful synthesis of mutant DI gene is dependent on type of DNA polymerase employed in recursive PCR	245
5.2.7	Cloning of synthetic mutant DI genes into expression plasmid	247
5.3	Expression and purification of mutant DI proteins	252
5.3.1	Western blot analysis of expressed DI mutant proteins	253
5.4	The use of computer modelling to rationalise epitopes targeted for mutation	257
5.5	Discussion of results	259
CHAPTER 6 ELUCIDATING THE BINDING CHARACTERISTICS OF RECOMBINANT DI MUTANTS		261
OVERVIEW OF CHAPTER 6		262
6.1	Direct binding data of recombinant DI mutants to APS derived IgG	263
6.1.1	Direct binding of DI mutants to polyclonal purified IgG	263
6.1.2	Direct binding of DI mutants to monoclonal aPL	265
6.1.3	Assessing coating density of mutant antigens	266
6.2	DI mutants inhibit binding to APS derived IgG in the fluid phase	267
6.3	Computer models of mutants R39S and Mut 8+9	269
6.4	Discussion of results	273
CHAPTER 7 OVERVIEW AND IDEAS FOR FUTURE WORK		276
7.1	Summary of results	277
7.2	Inhibiting the biological properties of aPL	277
7.3	Work towards a smaller blocking peptide	279
APPENDICES		280
Appendix A	One and three letter codes for amino acids	280
Appendix B	Original articles arising from work described in this thesis	281

REFERENCES		282
LIST OF TABLES		
CHAPTER 1		
Table 1.1	Updated classification criteria for definite APS	38
Table 1.2	Clinical manifestations of APS	45
Table 1.3	Non-thrombotic manifestations of APS	46
Table 1.4	Preliminary criteria for the classification of CAPS	49
Table 1.5	Updated classification criteria for the diagnosis of SLE	60
Table 1.6	Key autoantibodies detected in patients with SLE and APS	63
Table 1.7	Amino acid sequences of domain interlinker regions within β_2 GPI	106
CHAPTER 2		
Table 2.1	DI mutations – designated names and positions of amino acids targeted	139
Table 2.2	Properties and sequences of primers used in site directed mutagenesis	141
Table 2.3	Composition of resolving and stacking gels for SDS-PAGE	145
CHAPTER 3		
Table 3.1	Transformation efficiencies significantly improved upon tightly regulating DI production	173
Table 3.2	Summary of key optimisation experiments for expressing recombinant DI	178
CHAPTER 4		
Table 4.1	Demographic details of subject samples	204
Table 4.2	Clinical details of APS patients	205
Table 4.3	Comparative sensitivities and specificities for each antigen test	211
CHAPTER 5		
Table 5.1	Results of computer modelling studies targeting negatively charged amino acids	235
Table 5.2	Results of computer modelling studies targeting Arg 39 to Arg 43	236
Table 5.3	Amino acid sequences of his ₆ -tag DI variants investigating the DI-II interlinker region	238

Table 5.4	Results of computer modelling studies targeting DI-II interlinker region	238
Table 5.5	Amino acid and corresponding codon changes of DI mutants	239
Table 5.6	Overlapping primers designed by Juniper for the synthesis of mutant DI genes in a recursive PCR	243

LIST OF FIGURES

CHAPTER 1

Figure 1.1	Summary of the coagulation and 'anti' coagulation pathways	76
Figure 1.2	Schematic representation of β_2 GPI binding PL	94
Figure 1.3	Schematic representation of expression vector and elements of regulation	112

CHAPTER 2

Figure 2.1	Plasmid map of expression vector pET-20b	131
Figure 2.2	Plasmid map of expression vector pET-26b	132
Figure 2.3	Plasmid map of expression vector pET-39b	133
Figure 2.4	Schematic diagram of DI gene synthesis using recursive PCR	136

CHAPTER 3

Figure 3.1	Results generated by the Juniper programme	157
Figure 3.2	Synthetic gene encoding for DI	158
Figure 3.3	Results of recursive PCR	159
Figure 3.4	<i>Bgl</i> II / <i>Nco</i> I digests of synthetic DI cloned into pRSET-b	161
Figure 3.5	Sequencing plot confirms synthetic sequence of Omp A-DI gene cloned into pRSET-b	162
Figure 3.6	PCR amplification of Omp A-DI-pRSET construct	164
Figure 3.7	<i>Nde</i> I / <i>Nco</i> I digests of synthetic Omp A-DI gene into pET-20b	164
Figure 3.8	Sequencing plot confirms synthetic sequence of Omp A-DI gene cloned into pET-20b	165
Figure 3.9	PCR amplification of DI gene from the Omp A-DI-pET-20b construct	166
Figure 3.10	<i>Nco</i> I / <i>Xho</i> I digests of synthetic DI gene cloned into pET-20b	167
Figure 3.11	Sequencing plot confirms synthetic sequence of DI gene cloned into pET-20b	168
Figure 3.12	Cloning scheme of DI into pET-20b plasmids	169

Figure 3.13	<i>Nco</i> I / <i>Xho</i> I digests of synthetic DI gene cloned into pET-26b	171
Figure 3.14	Sequencing plot confirms synthetic sequence of DI gene cloned into pET-26b	172
Figure 3.15	<i>Nco</i> I / <i>Xho</i> I digests of synthetic DI gene cloned into pET-39b	175
Figure 3.16	Sequencing plot of DI of human β_2 GPI cloned into pET-39b	176
Figure 3.17	Western blot of recombinant DI probed with an anti-his tag antibody	179
Figure 3.18	His ₆ -tagged DI elutes from nickel at low concentrations of imidazole	181
Figure 3.19	Coomassie stain of DI purification steps using nickel chromatography	182
Figure 3.20	Murine anti-DI antibodies bind recombinant DI	183
Figure 3.21	Growth curves of <i>E.coli</i> expression cultures following induction with varying concentrations of IPTG	184
Figure 3.22	Western blot of expression cultures induced with 1mM, 0.4mM and 0.1mM IPTG and an uninduced control	185
Figure 3.23	PCR amplification of human DI gene	187
Figure 3.24	Sequencing plot of native cDNA sequence of DI cloned into pET-26b	188
Figure 3.25	Western blot analysis of recombinant his ₆ -tagged DI probed with an anti-his ₅ -antibody	188
Figure 3.26	Expression of DI tagged with DsbA at the N-terminal	190
Figure 3.27	Western blot demonstrating inefficient cleavage of DI from DsbA using enterokinase	190
Figure 3.28	Coomassie stain of purified his-tagged DsbA protein for use as negative control	191

CHAPTER 4

Figure 4.1	Schematic of IS4V _H point mutations	198
Figure 4.2	Binding of IS4V _H /light chain variants to β_2 GPI coated on an irradiated plate	200
Figure 4.3	Binding of IS4V _H /light chain variants and IS4V _H mutants to DI coated on a nickel plate	201
Figure 4.4	Recombinant DI in the fluid phase inhibits native IS4 from binding to DI coated on a plate	202
Figure 4.5	Results of polyclonal IgG samples binding to cardiolipin	207

Figure 4.6	Results of polyclonal IgG samples binding to whole β_2 GPI coated on an irradiated plate	208
Figure 4.7	Results of polyclonal IgG binding to phosphatidylserine	209
Figure 4.8	Results of polyclonal IgG binding recombinant DI expressed from <i>E.coli</i>	210
Figure 4.9	Correlation scatter plot of purified IgG patient samples binding DI against binding to cardiolipin	212
Figure 4.10	Identification of an aPL with species specificity for human β_2 GPI	214
Figure 4.11	Correlation scatter plot of purified patient IgG samples binding DI against binding to whole β_2 GPI coated on an irradiated plate	215
Figure 4.12	Results of polyclonal IgG binding human β_2 GPI coated on cardiolipin	217
Figure 4.13	Correlation scatter plot of purified patient IgG samples binding DI against binding to human β_2 GPI when coated on cardiolipin	218
Figure 4.14	Identification of aPL that binds recombinant DI, β_2 GPI bound to cardiolipin but not β_2 GPI bound to a plate	219
Figure 4.15	Comparison of DI binding of aPL from patients with venous thrombosis only versus those with arterial thrombosis	221
Figure 4.16	Comparison of DI binding of aPL derived from patients with and without thrombocytopenia	222
Figure 4.17	Inhibition of IgG binding β_2 GPI/cardiolipin in the fluid phase by DI	223

CHAPTER 5

Figure 5.1	Ribbon structure of his ₆ -tagged DI of β_2 GPI	234
Figure 5.2	Sequencing plots of DI mutants produced by site-directed mutagenesis – D8S, D9G, Mut 8+9, E26S and E27S	240
Figure 5.3	Results from Juniper demonstrating design of gene for the E23S mutation	242
Figure 5.4	Recursive PCR amplification of DI mutants	244
Figure 5.5	Successful recursive PCR amplification of DI mutants is dependent on <i>Vent</i> DNA polymerase	246
Figure 5.6	<i>Xho</i> I / <i>Nco</i> I digested mutant constructs of E23S, Mut 23-26-27 and Mut 8-9-23-26-27	248
Figure 5.7	<i>Xho</i> I / <i>Nco</i> I digested mutants constructs of R39K, R39S, G40S and G40E	249
Figure 5.8	<i>Xho</i> I / <i>Nco</i> I digested mutants constructs of R43G, R43S, DII-III	250

	and DI ext	
Figure 5.9	Sequencing plots of DI mutants synthesised using recursive PCR	251
Figure 5.10	Protein gels of DI mutants	252
Figure 5.11	Quantitative western blot using microdensitometry	254
Figure 5.12	Western blots of DI mutants probing with anti-his tag antibody	255
Figure 5.13	Western blot of DI mutants probing with murine anti-DI antibody	256
Figure 5.14	Computer model of DI of β_2 GPI and the proposed aPL binding epitopes	258

CHAPTER 6

Figure 6.1	Direct binding of DI mutants to polyclonal APS derived IgG	264
Figure 6.2	Binding of native IS4 to mutant DI in the solid phase	265
Figure 6.3	Assessing density of antigen coated on the nickel plate	266
Figure 6.4	Binding of APS derived polyclonal IgG to wild-type DI and mutants in the fluid phase	268
Figure 6.5	Computer model of the DI mutant R39S	270
Figure 6.6	Binding of murine anti-DI antibodies to Mut 8+9 and R39S	271
Figure 6.7	Computer model of the DI mutant Mut 8+9	272

ABBREVIATIONS

Å	Angstrom
aCL	Anticardiolipin antibodies
ANA	Anti-nuclear antibodies
APASS	Antiphospholipid antibodies and stroke study
aPL	Antiphospholipid antibodies
apo A-I	Apolipoprotein A-I
apo ER2	Apolipoprotein E receptor 2
APS	Antiphospholipid Syndrome
arc	Arthritis Research Campaign
ATP	Adenosine triphosphate
β ₂ GP1	β ₂ -Glycoprotein I
BCA	Bicinchoninic acid
BIC	Bicarbonate buffer
BlyS	B lymphocyte stimulator
BSA	Bovine serum albumin
BV	Bed volume
cAMP	Cyclic-adenosine monophosphate
C-terminal	Carboxyl
CAPS	Catastrophic APS
CCP	Complement control protein
CDR	Complementarity-determining region
C _H	Constant region of heavy chain
CHO	Chinese, hamster, ovary
CL	Cardiolipin
C _L	Constant region of light chain
CMV	Cytomegalovirus
cPLA ₂	Phospholipase A ₂
Crry	Complement receptor 1-related protein y
DI – DV	Domain I to domain V of β ₂ GPI
DAF	Decay accelerating factor
ddH ₂ O	Distilled deionised water
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
dRVVT	Dilute Russell viper venom test

ds	Double stranded
DTT	Dithiothreitol
EC	Endothelial cell
<i>E. Coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
F(ab)	Fragment antigen binding
Fc	Fragment crystalline
FCS	Foetal calf serum
FDPs	Fibrinogen degradation products
FR	Framework region
GM-CSF	Granulocyte macrophage colony stimulating factor
Gp	Glycoprotein
HDL	High density lipoprotein
HELLP	Haemolysis, elevated liver enzymes, low platelets
His ₆	Hexahistidine
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen complex
HMG-COA	3-hydroxy-3-methylglutaryl-coenzyme A
HRP	Horse radish peroxidase
HUVEC s	Human umbilical vein endothelial cells
ICAM-1	Intracellular adhesion molecule-1
Ig	Immunoglobulin
IL	Interleukin
IRAK	IL-1 receptor associated kinase
iNOS	Inducible NOS
INR	International normalised ratio
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
LA	Lupus anticoagulant
LB	Lauria-Bertani
LDL	Low density lipoprotein
lpr	Lymphoproliferative
LPS	Lipopolysaccharide

mAb	Monoclonal antibody
MAPk	Mitogen activated protein kinase
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MW	Molecular weight
MWCO	Molecular weight cut off
MyD88	Myeloid differentiation protein
N-terminal	Amino terminal
NIH	National institute of health
NF-KB	Nuclear factor-KB
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PAPS	Primary APS
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-kinase
PL	Phospholipid
PS	Phosphatidylserine
PT	Prothrombin
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SEC	Sample, enzyme, conjugate dilution buffer
Sf9	<i>Spodoptera frugiperda</i>
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
ss	Single stranded
TAE	Tris acetate EDTA buffer

TB	'Terrific' broth
T _C	T cytotoxic cells
TGF-β	Transforming growth factor-beta
TIR	Toll/IL-1
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
T _H	T helper cells
TLR	Toll like receptor
T _M	Melting temperature
TNFα	Tumour necrosis factor α
tPA	Tissue plasminogen activator
TT	Transcription terminator
TTP	Thrombotic thrombocytopaenic purpura
UV	Ultraviolet
VCAM-1	Vascular adhesion molecule-1
VDRL	Venereal disease research laboratory
V _H	Variable region of heavy chain
V _L	Variable region of light chain

CHAPTER 1

INTRODUCTION

OVERVIEW OF INTRODUCTION

The aim of this chapter is to review the clinical and scientific literature relating to the antiphospholipid syndrome (APS) which is relevant to the content of this thesis. Given that this is an autoimmune condition an overview of the immune response is provided, with emphasis placed on mechanisms that undergo dysregulation in APS. Factors governing the structure of proteins and nature of antibody-antigen interactions are also discussed. The clinical syndrome of APS is then described in detail and, given the close association with systemic lupus erythematosus (SLE), this condition is also described briefly.

This chapter then shifts focus, describing the characterisation of antiphospholipid antibodies (aPL) and exploring in depth the likely pathogenic mechanisms of APS described to date in the literature. The key aPL antigenic target of beta 2-glycoprotein I (β_2 GPI) is then discussed in detail and current evidence citing candidate immunodominant domains of β_2 GPI is explored critically.

Finally, given that this thesis describes a novel method of expression of an important biological protein as outlined in Chapter 3, the production of recombinant proteins by various hosts is discussed, with particular emphasis placed on using prokaryotic expression platforms. This chapter concludes with a statement of aims that constitutes the scope of this thesis.

1.1 THE IMMUNE SYSTEM: ANTIBODIES, ANTIGENS AND THE INTERPLAY OF PROTEINS

1.1.1 The Immune response – innate and adaptive

The function of the immune response is to recognise a pathogen or other foreign material, neutralise the threat to host and eliminate it. This response may be broadly classified into two distinct types: innate and adaptive. The majority of micro organisms encountered daily in life are detected and destroyed within hours by mechanisms that are not antigen specific and do not require prolonged periods of induction. This early phase of the immune response is termed innate immunity and does not improve with each successive encounter. Only if this first line of defence is breached, does then an adaptive immune response ensue. Innate immunity also serves to keep the infection under some degree of control while the effector cells of the adaptive response are activated. The adaptive immune response is characterised by the two fundamental features of specificity and memory. In conjunction with this high degree of specificity, the adaptive immune response is also characterised by a high degree of diversity. These are key properties that are required in order to respond to many different foreign antigens whilst retaining the ability to recognise self from non-self. This highly specific response to a particular pathogen characteristically improves with each successive encounter and is triggered earlier. However, this response usually takes between four to seven days to mount.

1.1.1.1 Components of the innate immune system

The innate immune response is comprised of multiple barriers to infection. Surface epithelia may form a mechanical, chemical (e.g. fatty acids of skin, enzymes in tears and gut, low pH) and microbiological (e.g. normal flora of gut) barrier. Complement activation can proceed on the surface of some pathogens whilst sparing host cells even in the absence of specific antibodies. If epithelial barriers have been breached, tissue macrophages may recognise opsonised pathogens and trap, engulf and destroy them potentially preventing an infection from becoming established (Janeway et al., 2004, Roitt et al., 2001).

1.1.1.2 Cellular components of the adaptive immune response

The most important principle in adaptive immunity is clonal selection of lymphocytes, which are the key effector cells. Each 'naïve' lymphocyte bears a distinct receptor for a particular antigen. The lymphocytes that bear a receptor recognising self antigen

are eliminated early in development. When antigen interacts with a receptor on the surface of a mature lymphocyte, that cell is activated and becomes a blast cell. This lymphoblast then undergoes clonal expansion, dividing and giving rise to a clone of identical progeny, whose receptors all bind identical antigen. These cells then differentiate into effector cells such as those that secrete antibody. Effector cells eliminate the infection thereby removing the initial stimulus and the immune response ceases. However some lymphocytes differentiate into memory cells rather than effector cells, allowing host to respond more rapidly and intensely to a subsequent challenge by the same antigen (Janeway et al., 2004). There are two types of antigen specific lymphocytes that have been distinguished and these are termed B lymphocytes and T lymphocytes.

B lymphocytes comprise approximately 25% of the lymphocyte population. Each is genetically programmed to encode for a specific receptor, a membrane bound antibody specific for a particular antigen. When this antigen is encountered, B cells under the influence of cytokines and B cell growth factors undergo clonal expansion and differentiate into plasma cells and memory B cells. The function of plasma cells is to produce large amounts of circulating antibody that is identical to the original receptor molecule on the surface of the B cell from which the plasma cell was derived, thus maintaining specificity for the original antigenic stimulus. These cells generally have a life-span of only a few days and are the major effector cells of the humoral immune response, though it is recognised that a small number of plasma cells may be long-lived and survive for many months (Manz et al., 1998). Memory B cells have a longer life-span and continue to express surface receptor identical to the parent B cell. Mature B lymphocytes may be distinguished by the presence of CD19 and CD20 molecules on the surface, which are not present on the surface of plasma cells (Roitt et al., 2001, Morrow et al., 1999).

T lymphocytes may be divided into two major types, helper (T_H) cells and cytotoxic (T_C) cells. T_H cells express CD4 on the surface and T_C cells express CD8. T_H cells of the type-1 class (T_{H1}) promote immune responses that are primarily cell mediated or inflammatory. This process is mediated to a large extent via the production of specific cytokines: interleukin (IL)-2, IL-3 and interferon- γ . T_H cells of the type 2 class (T_{H2}) principally interact with B cells and help them initiate and reinforce clonal expansion, enhancing humoral immunity. The principal cytokines produced by T_{H2} cells are IL-4, IL-5, IL-6 and IL-10. However the polarisation of T_{H1} and T_{H2} cells

may be heterogeneous and these cells maintain their potential to be modified from one phenotype to the other (Mocci and Coffman, 1995). Recently, a subset of IL-17 producing CD4 positive T cells have been described that develop via a lineage distinct from the T_H1 and T_H2 cell lineages (Harrington et al., 2005). This functionally distinct T_H lineage of cells, that have been termed as T_H17 cells, develop from naïve T cells via cytokine signals such as IL-6 together with transforming growth factor-beta (TGF- β) and have been shown to have a crucial role in the induction of autoimmune tissue injury (Bettelli et al., 2006, Weaver et al., 2006).

T_C cells are responsible for the destruction of cells that have become infected by viruses or other intracellular pathogens. Like B cells, T cells also bear a receptor that recognises a specific antigen. However, the T cell receptor is unique in that it will only bind antigen when presented on the surface of another cell in association with a membrane protein termed the major histocompatibility complex (MHC). There are two different classes of MHC that differ subtly in structure and function: MHC class I and class II. MHC class I molecules are expressed by nearly all nucleated cells and recognise peptides processed from pathogens that replicate in the cytosol, principally viruses and some bacteria. These pathogens may only be destroyed by killing the cells containing them and the CD8 co-receptor helps T_C cells recognise peptide:MHC class I complexes on the surface of these infected cells. By contrast MHC class II is expressed primarily on cells of the immune system and binds peptides derived from intracellular vesicles. An antigen presented in association with MHC class II will bind to and activate T_H cells. This may in turn result in a number of possibilities all aimed at amplifying the immune response to the invading antigen. Typical antigen presenting cells are macrophages, dendritic cells and B cells. An activated T_H cell may in turn activate the infected macrophage to fuse its lysosomes with the vesicles containing the parasite, thus destroying it. External antigen internalised by a B cell via binding to its immunoglobulin receptor may trigger activation and clonal expansion of the cell (Janeway et al., 2004, Morrow et al., 1999, Roitt et al., 2001).

1.1.1.3 Recognition of pathogens by the innate immune system: Toll-like receptors

A significant advance over the past decade has been the realisation that the innate immune system, a universal and evolutionary ancient system, is more than just a simple mechanical or chemical barrier to infection (Janeway and Medzhitov, 2002). Once infection has breached these barriers the innate system has numerous

important functions such as opsonisation, activation of the complement and coagulation cascades, phagocytosis, activation of proinflammatory signalling cascades and apoptosis (Janssens and Beyaert, 2003). It is through these important functions that the adaptive immune response is activated and shaped. In order to achieve these functions the innate system must have a mechanism for distinguishing potentially harmful non-self from self. In contrast to the clonotypic, highly specific, receptors of the adaptive immune response present on T and B cells, the innate immune system uses a set of recognition molecules called pattern recognition receptors. These receptors bind conserved molecular structures found in large groups of pathogens (Janeway and Medzhitov, 2002). In *Drosophila*, which lack an adaptive immune system, a Toll receptor has a central role in the defensive response to infection by gram positive bacteria and fungi (Hoffmann, 2003). In mammals the identification of proteins similar to Toll, termed Toll-like receptors (TLRs) have been identified. TLRs appear to be the most important of the pattern recognition receptors in mammals and 10 types have been described in humans (Janssens and Beyaert, 2003, Kawai and Akira, 2005). To date the ligands to eight out of 10 TLRs have been described and enable these TLRs to discriminate an array of protozoan, fungal, bacterial and viral invaders (Yamamoto et al., 2004, Kirk and Bazan, 2005). The intracellular portion of a TLR has marked homology to the IL-1 receptor and is thus termed Toll/IL-1 (TIR) domain. All ten TLRs signal alarm to the cell via a core nuclear factor (NF)- κ B pathway utilising the adapter myeloid differentiation protein (MyD88) for most TLRs and / or the TRIF / TRAM adapter proteins for TLR 3 and 4 (Kawai and Akira, 2005). These peripatetic adapter proteins associate with the activated intracellular TIR domain, in turn recruiting members of the IL-1 receptor associated kinase (IRAK) family (IRAK-1 and 4) leading to the activation of the NF- κ B signalling cascade. Activated NF- κ B then enters the nucleus and activates proinflammatory genes coding for proinflammatory cytokines, interferons and adhesion molecules (Kawai and Akira, 2005, Yamamoto et al., 2004).

1.1.2 Antibodies in the immune system: structure and function

Antibodies are collectively termed immunoglobulins. These are a group of glycoprotein molecules present in the serum and tissues of all mammals. Some are carried on the surface of B cells, others are free in the blood or lymphoid circulation. They are constructed from four polypeptide chains. Two 50 kilodalton (kDa) chains are termed heavy chains each of which contains four domains, three constant (C_H) and one variable (V_H). The two smaller 25kDa chains are termed light chains and

contain two domains each, one constant C_L and one variable V_L region. Intra and interchain disulphide and covalent bonds serve to combine each unit to make up the immunoglobulin. There are only two types of light chains termed lambda (λ) and kappa (κ) and no functional differences have been found between them. By contrast, there are five distinct forms of C_H regions (μ , δ , γ , α , and ϵ) referred to as isotypes. These do determine functional activity of the antibody and are termed immunoglobulin M (IgM), IgD, IgG, IgA and IgE. IgG is the most abundant isotype in the plasma. Functional properties of each isotype are conferred by the carboxyl (C) terminal half of the heavy chain termed the fragment crystalline (Fc) portion. This is formed from the C_H portion of the heavy chain and regulates the effector functions of the antibody that include binding to cell surface Fc receptors and complement fixation leading to elimination of the bound antigen (Morrow et al., 1999, Janeway et al., 2004).

In contrast to the C_H and C_L regions that are highly conserved, the V_H and V_L regions contain great variation in amino acid sequence with short segments of hypervariable looping regions. This portion of the antibody is termed the fragment antigen binding (F(ab)) portion. It is responsible for conferring the antigen specificity of each antibody. Three hypervariable loops from the V_H region and three hypervariable loops from the V_L region come together to form the unique antigen binding site (Chothia et al., 1989). These hypervariable loops combine to form a surface complementary to the antigen and hence they are also termed complementarity determining regions (CDRs). The CDRs are separated within the V_H and V_L chains by more conserved regions termed framework regions (FRs) and these serve a more structural role (Wu and Kabat, 1970). The number of different antibody specificities available in an individual, otherwise known as the antibody repertoire, is in region of 10^{11} . This enormous diversity is generated by multiple mechanisms. The genes coding for different heavy and light chains come together in antibody producing cells and the antibodies produced by these cells derived from an inherited gene will bear the germ-line sequence. A large number of antibody genes use DNA rearrangement to create the antibody repertoire of an individual. However, this diversity is markedly enhanced by the presence of somatic mutation, whereby a limited number of inherited genes undergo multiple random mutations (Tonegawa, 1983).

1.1.3 Autoimmunity and autoantibodies

The huge potential diversity of reactivity means there is also the potential to create self-reactive lymphocytes. A variety of mechanisms exist both in the thymus and in the periphery to ensure that self-reactive T cells are deleted or removed. When there is a persistence of self-reactive lymphocytes then this indicates a breakdown in tolerance to self and forms the basis for the development of autoimmunity.

Autoimmune disease may be grouped into organ specific and non-organ specific / systemic autoimmune diseases. In organ specific autoimmune disease the condition tends to have a single or small number of autoreactive antibodies targeting a single or limited number of organ systems. Examples include anti-basement membrane antibodies targeted to the basement membrane of the kidneys and lungs causing Goodpasture's syndrome, or the anti-acetylcholine receptor antibodies associated with myasthenia gravis. The non-organ specific / systemic autoimmune diseases mostly comprise of the connective tissue diseases and autoimmune arthropathies. SLE and the APS are typical examples of systemic autoimmune diseases that target multiple organs or systems (Roitt et al., 2001).

However, the presence of auto reactive antibodies may be a physiological rather than a pathological phenomenon. Natural autoantibodies of low affinity to self-reactive antigens may be detected in normal healthy individuals (Baumgarth et al., 2005). These antibodies are produced by long-lived B-1 lymphocytes that are positively selected by interacting with self. This is in contrast to the more ubiquitous B-2 lymphocyte population, which produce high affinity antibodies, generate memory cells and are negatively selected when self is encountered (Baumgarth et al., 2005). The precise functions of natural autoantibodies are unknown but they are often of the IgM isotype and are sometimes able to bind more than one self or foreign antigen. The repertoire is remarkably stable between species, due in part to the fact that most natural antibodies use germ-line encoded V_H and V_L genes (Baumgarth et al., 2005).

Autoantibodies associated with SLE and APS however, tend to be of the IgG isotype and of high-affinity binding. Furthermore, most of these pathogenic autoantibodies use non-germ line encoded V_H and V_L genes, due to the presence of somatic mutations (Rahman et al., 2001, Giles et al., 2003b).

1.1.4 Structural nature of proteins, antigens and antigenic determinants

The term antigen was originally used for any molecule that was able to produce an antibody response from a B cell (antibody generator). However, the term antigen is now used more generically to determine any molecule recognised by key elements of the adaptive immune response, which includes both T and B cells (Roitt et al., 2001). Antigens that bind antibodies in humans and provoke an immune reaction tend to be proteins in the vast majority of cases, however polysaccharide coats of pathogens, nucleic acids and lipids may also occasionally be antigenic. The site on the antigenic protein that antibodies recognise is termed the antigenic determinant or epitope. Before considering the nature of antigenic determinants the general principles governing basic protein structure will be discussed.

Proteins consist of polypeptide chains, which are formed from individual amino acids linked by covalent peptide bonds (Stryer, 1988). Peptides are built from a repertoire of 20 amino acids and each protein has its own defined amino acid sequence. Frederick Sanger in 1953 demonstrated this for the first time when the amino acid sequence of the protein hormone insulin was first published (Sanger and Thompson, 1953a, Sanger and Thompson, 1953b). Amino acids are designated by either a three letter abbreviation or a one letter symbol as shown in appendix I. They consist of an amino group, a carboxyl group, a hydrogen atom and a distinctive R group all bonded to a central carbon atom. It is the nature of the R side chain that defines the type and ultimately the functional properties of each amino acid. The simplest one is glycine with just a single hydrogen atom as the side chain. Others have sulphur atoms such as cysteine, others an aromatic ring and others hydrocarbons up to three or four carbons long. At neutral pH amino acids may be either hydrophobic (non-polar) or hydrophilic (polar). All hydrophobic amino acids have a net neutral charge and these tend to be amino acids with long hydrocarbon side chains e.g. valine and leucine. Hydrophilic amino acids may have neutral, positive (basic) or negative (acidic) net electrical charges.

A striking feature of proteins is that they have a well-defined three-dimensional or tertiary structure and the conformation of a protein is the major determinant of its functional properties. There are a number of different factors that determine the final structure of a protein. Polypeptides often fold in to a regular α -helical repeating structure. These helical structures have 3.6 residues per turn such that amino acids spaced three and four apart in the linear sequence come into very close

approximation in an α -helix. Such α -helical coils form the main structural motif for many proteins such as haemoglobin. Alternatively peptides may be arranged into parallel flat β pleated sheets. The α -helix and β -sheet describe determinants of the secondary structure of proteins, but what elements determine the tertiary structure? These are primarily dictated by non-covalent molecular interactions and are mediated by hydrophobic properties of the non-polar amino acids and by three types of forces: electrostatic bonds, hydrogen bonds and van der Waal forces (Stryer, 1988). The strength of the non-covalent bond is critically dependent on distance. Electrostatic bonds may occur between positive and negative charges and the force is proportional to distance (d) between them ($1/d^2$). However, for van der Waal forces groups must come into closer approximation for these forces to be significant ($1/d^7$). The distance required for optimal hydrogen bond formation is longer than for electrostatic forces. These bonds are vitally important in the formation of α and β polypeptide units due to the formation of hydrogen bonds between the amino and carboxyl groups of adjacent units. However side chains of 11 amino acids may also participate in either receiving or donating one or more hydrogen bonds. The effect of hydrophobic side chains on the overall structure of amino acids is also very important. A polypeptide chain will fold spontaneously such that the hydrophobic side chains are buried deep in the protein whilst the polar, charged chains remain on the surface. The segments of main chains may be buried deep in the protein due to the formation of hydrogen bonds between the amino and carboxyl groups, which if left unpaired would markedly prefer water to a non-polar environment. This type of pairing may be neatly established in an α helical coil or β pleated sheet and the protein structure further stabilised by the formation of van der Waal forces between tightly approximating groups. Finally, the presence of a sulphur atom side-chain in cysteine allows for the formation of strong covalent disulphide bonds between cysteine residues located at different points in the primary structure, creating loops in the tertiary structure. Disulphide bonds can also link different polypeptide chains together and hence be integral to protein quaternary structures as seen with immunoglobulins (Stryer, 1988).

The tertiary structure of a given native protein antigen may allow multiple epitopes from different segments of the protein to come into close approximation and form the binding site for antibodies. These epitopes are termed discontinuous or conformational epitopes. Hence the effect of denaturing these antigenic proteins is to markedly reduce or abolish binding of antibody due to loss of conformation. In

contrast antibodies that bind linear or non-conformational epitopes will bind denatured antigen (Janeway et al., 2004, Roitt et al., 2001). Structural studies of the influenza derived protein neuraminidase complexed with monoclonal F(ab) provides a typical example of antibody binding non-conformational epitopes. The antibody binding site of this complex is comprised of 33 residues derived from eight chain segments of neuraminidase coming together to form the epitope (Tulip et al., 1992).

1.1.5 Nature of the antigen : antibody interaction

There exists a huge diversity of antibody repertoire as discussed. However, in order for this diversity to be of biological benefit, a high degree of specificity regarding the interaction between antibody and its corresponding antigen must exist.

Early investigations regarding the nature of the antigen : antibody interaction were limited in that only small molecules known as haptens were investigated as the binding antigen and not whole native protein. This is because potential sources of monoclonal antibody were limited and came from tumour cells producing antibody of unknown specificities. Hence only small compounds could practically be screened for binding to antibodies of unknown specificities. These initial structural studies of hapten ligands such as vitamin K or phosphorylcholine binding to antibody revealed that small antigens lie in a cleft made from the CDRs of both V_H and V_L . Small antigens such as these however may not come in to close contact with all CDRs as in the case of phosphorylcholine, which interacts with all three CDRs of the V_H but only CDR3 of the V_L (Davies et al., 1990, Janeway et al., 2004). Native protein antigens however are much larger than hapten molecules and often cannot be accommodated within the narrow cleft that haptens lie in. There have been few detailed X-ray crystallographic structural studies of antigenic proteins complexed with antibody. The two most common antigens studied have been the antigenic proteins hen egg white lysozyme (Amit et al., 1986, Sheriff et al., 1987) and influenza derived neuraminidase (Tulip et al., 1992) complexed with their corresponding monoclonal F(ab). These studies demonstrate that larger antigens typically make contact with all six CDRs. The surface area of the interaction is typically in the region of 700 angstroms (\AA) (Janeway et al., 2004).

Binding of an antibody to an antigen may be disrupted chemically by altering pH, adding detergents or inducing high salt concentrations. This implies that the nature of the interaction is non-covalent. Non-covalent forces between residues within a protein are essential for defining the tertiary structures of proteins. However, they

are also very important in defining the interaction between residues forming the epitopes of antigens and the paratopes of antibodies. However, in order for residues to come into close approximation and form non-covalent bonds there must be a good fit between the surface of the antigens and the surface of antibodies. As discussed the antibody has a surface complementary to the binding antigen. For example, the F(ab) of antibody to lysozyme shows marked complementarity, with 17 amino acid residues on the antibody making contact with 16 residues on the lysozyme molecule (Amit et al., 1986). A good fit between the antigenic epitope and the antibody creates the opportunity for the formation of hydrogen bonds and electrostatic attractive forces between residues. The formation of van der Waal forces requires close juxtaposition of residues and the hydrophobic non-polar residues may also attract by their mutual properties of repelling water. The strength of this bond between antibody and antigen is termed affinity (Roitt et al., 2001).

This thesis describes the molecular study and manipulation of a native peptide antigen which binds a group of autoantibodies termed aPL. These are the key pathogenic autoantibodies associated with the autoimmune disorder APS and are also seen frequently in patients with SLE. I will now discuss the clinical syndrome of the APS.

1.2 THE ANTIPHOSPHOLIPID SYNDROME

1.2.1 Definition of the APS

There is no standardised simple definition of the APS. The essential clinical features characterising APS are of arterial and venous thrombosis, thrombocytopenia, livedo reticularis or recurrent foetal loss. These clinical events typically occur in association with a group of circulating autoantibodies directed against phospholipid (PL) or PL binding cofactor proteins, the most important and best characterised being β_2 GPI (Harris et al., 1983, Hughes, 1983, Asherson et al., 1989). Though these are a heterogeneous group of antibodies, in routine, clinical practice they are detected by an enzyme linked immunosorbent assay (ELISA) directed against the anionic PL cardiolipin (CL). These aPL are also responsible for the paradoxical lupus anticoagulant (LA) effect, a test which detects an inhibitor of phospholipid dependent coagulation.

1.2.2 Historical review of aPL and the APS

The term APS was first coined relatively recently by Harris et al in an abstract submitted to the British Society of Rheumatology in 1987 (Harris et al., 1987a). Initial experiments leading up to the discovery of aPL were centred around the development of assays for syphilis. In 1906 a reagin test developed by Wasserman, and coined the Wasserman reaction, identified sera from patients infected with syphilis that reacted with syphilitic tissues (Wasserman et al., 1906). It was initially thought that infected serum was reacting with antigens derived from *Treponema pallidum* present in the syphilitic tissues. However, it was realised that infected sera must be reacting with a native antigen as the same effect was seen when non-infected normal human or mammalian tissue was used (Landsteiner et al., 1907). It was another 34 years before Pangborn et al in 1941 identified the antigenic component of the reagin test as being CL, sourced in these experiments from bovine hearts (Pangborn, 1941, Pangborn, 1942).

Subsequently the combination of CL, lecithin and cholesterol formed the basis of the test for syphilis known as the venereal disease research laboratory (VDRL) test. However, it was realised with the development of more specific tests, such as the *Treponema pallidum* immobilisation test, that the Wasserman reaction and VDRL test could produce false positive results for syphilis in non-infected individuals. In 1952 Moore and Mohr identified two circumstances in which false positive results for syphilis using the reagin test could be observed (Moore and Mohr, 1952). Transient false positives were seen in patients following an acute viral infection or post vaccination. Persistent false positive tests (more than six months) were observed in individuals with autoimmune disorders such as SLE, rheumatoid arthritis and Sjogren's syndrome (Moore and Mohr, 1952).

Also in 1952 Conley and Hartman were the first to describe a 'circulating anticoagulant' which caused prolongation of the prothrombin time in the serum of two patients with SLE who also had a false positive serological test for syphilis (Conley and Hartman, 1952). Subsequent studies in the mid to late 1950s demonstrated that this anticoagulant factor was in fact attributable to the biological false positive test result for syphilis in patients with SLE (Lee and Sanders, 1955, Laurell and Nilsson, 1957) though it was not until 1972 that the term 'lupus anticoagulant' was suggested by Feinstein and Rapaport (Feinstein and Rapaport, 1972). At the time it was puzzling to investigators in the field that this anticoagulant

entity, associated with SLE and the prolongation of all coagulation tests involving the use of lipid, was not accompanied by a tendency to bleed in the patient (Shapiro, 2005). Bowie and colleagues from the Mayo clinic in 1963 were the first to describe the paradoxical association of the presence of serum with this anticoagulant effect and the increased tendency to thrombose rather than bleed (Bowie et al., 1963). Interestingly however, it was Laurell and Nilsson in 1957 who first suggested the possible association of the anticoagulant phenomenon with a syndrome of recurrent miscarriages and also suggested that the prolonged tests might be associated with inhibitory effects against PL (Laurell and Nilsson, 1957). The mechanism of the LA was first published in 1980, when Thiagarajan et al described the properties of a purified IgM antibody from serum with marked LA activity. This purified IgM was found to inhibit PL dependent coagulation tests, but only in the presence of anionic PL, and not when neutral phosphatidylcholine or phosphatidylethanolamine were used as the lipid source (Thiagarajan et al., 1980). The first description of a method for measuring LA was published in 1986 and used a variation on the 'Stypven Time', a test making use of Russell Viper Venom and hence termed the dilute Russell Viper venom test (dRVVT) (Thiagarajan et al., 1986).

The possible association of LA activity with recurrent foetal loss was noted first in 1957, but it was not until the early 1980s' that multiple studies described the triple association of LA, recurrent foetal loss and prothrombotic tendencies (Soulier and Boffa, 1980, Carreras et al., 1981a, Carreras et al., 1981b). Hughes and colleagues at St Thomas's Hospital (London, UK) first described the association of cerebral thrombosis with recurrent miscarriage and LA in an editorial in the British Medical Journal in 1983 and suggested the presence of a distinct syndrome (Hughes, 1983). It was in Hughes's unit in the same year that Harris et al described a solid-phase radioimmunoassay for the detection of aPL reacting against CL as the target antigen (Harris et al., 1983). Subsequently, an assay for the detection of anticardiolipin antibodies (aCL) was described in 1985 (Loizou et al., 1985). A year later the term anticardiolipin syndrome was introduced by Hughes et al (Hughes et al., 1986) and was subsequently renamed the APS by the same group in 1987 (Harris et al., 1987a).

The aCL test required the presence of bovine or human serum to detect binding of aCL to CL coated on a plate. This led to a major discovery reported in 1990 by three independent laboratories that reactivity to CL was dependant upon the serum co-

factor β_2 GPI otherwise known as apolipoprotein H (Galli et al., 1990, Matsuura et al., 1990) (McNeil et al., 1990b). This co-factor of 50kDa in size and then of unknown function was found to bind anionic PL. β_2 GPI and its role in APS is described in detail in section 1.6. Although other co-factors have also been found to bind aPL such as protein C (Oosting et al., 1993), protein S (Oosting et al., 1993) and prothrombin (Bever et al., 1991), β_2 GPI remains the clinically most relevant and best studied.

1.2.3 Classification of the APS

The existence of APS without the presence of lupus or other autoimmune disease was first noted by Asherson in 1988, who coined the term "primary" APS (PAPS) and proposed classification criteria (Asherson, 1988). A year later three studies describing the clinical and serological features of series of patients with PAPS or APS and SLE were described (Asherson et al., 1989, Alarcon-Segovia et al., 1989, Alarcon-Segovia and Sanchez-Guerrero, 1989, Mackworth-Young et al., 1989). With subsequent larger studies of up to 1000 patients with APS it became apparent that in fact PAPS possibly constitutes the majority of patients with APS, accounting for just over 50% (Cervera et al., 2002).

Early attempts to establish classification criteria were made by many groups, with the consequent lack of absolute consensus (Hughes et al., 1986, Harris et al., 1987a, Asherson, 1988, Alarcon-Segovia and Sanchez-Guerrero, 1989, Alarcon-Segovia et al., 1989). The first attempt to form an international consensus for the classification of APS came in October 1998 during the Eighth International Symposium of the APS in Sapporo, Japan (Wilson et al., 1999). These preliminary criteria, also known as the Sapporo criteria, state that the diagnosis of APS should be made when one or more clinical outcome occurs in conjunction with specific laboratory criteria. The clinical outcomes include vascular thrombosis (arterial or venous) or pregnancy morbidity, either recurrent early (less than 10 weeks) foetal loss or pre-term birth (less than 34 weeks) due to placental insufficiency or miscarriage. Laboratory criteria constitute a positive LA and / or moderate to high IgG or IgM aCL titres on two or more occasions at least six weeks apart (Wilson et al., 1999). Validation studies by Lockshin et al showed that these criteria had high specificity of 0.98 but were relatively less sensitive at 0.71 (Lockshin et al., 2000). The criteria were an important tool in facilitating research studies, but there were some important manifestations of APS that were not covered and accounted for many of the false negative patients identified in the validation studies (Lockshin et al., 2000). These include non-thrombotic, non-pregnancy related clinical features such as livedo reticularis,

thrombocytopaenia or patients with anti- β_2 GPI antibodies, who constitute 3-10% of patients with APS but have consistently negative aCL and LA tests (Lee et al., 2003, Nash et al., 2004, Ebeling et al., 2003). Also, it was not entirely clear what constitutes a 'moderate' titre of aCL. These points have been addressed and the preliminary Sapporo criteria have been updated recently by a further international consensus statement agreed upon at a dedicated workshop convened before the Eleventh International Congress on APS in Sydney in 2004 (Miyakis et al., 2006). These classification criteria are summarised in table 1. The updated criteria differ from the Sapporo criteria, in that additional factors contributing to thrombosis should be assessed and that APS patients be stratified according to the presence or absence of risk factors contributing to thrombosis. These risk factors may be either inherited or acquired and include age (more than 55 years in men and 65 years in women), any of the established acquired risk factors for cardiovascular disease, inherited thrombophilia, oral contraceptive use, nephrotic syndrome, malignancy, immobilisation and surgery (Miyakis et al., 2006). The rationale behind this inclusion originates from concerns that the association of age and of commonly occurring risk factors for thrombosis and cardiovascular disease may cause classification bias. However, greater changes were made to the laboratory criteria required to make the diagnosis. A clear threshold for a positive result for aCL of more than 40 GPL and MPL units was given (see section 1.2.4 for standardisation of units). The committee further stated that APS should not be diagnosed if more than five years elapse between a positive clinical event and a positive serological result. The interval required for two positive successive results was increased from six to 12 weeks though this was due more to expert opinion rather than evidence based on previous validation studies (Miyakis et al., 2006). The most significant change was the inclusion of anti- β_2 GPI testing, either IgG or IgM isotype, which should be positive (more than 99th percentile) on two or more occasions at least 12 weeks apart. Finally, the statement was made that the traditional terms of 'primary' and 'secondary' APS should be abandoned, and that APS with or without the coexistence of SLE or other autoimmune disease be simply documented. Classification of the Catastrophic APS (CAPS) was outside the remit of these criteria. (CAPS is described in section 1.2.8 and preliminary classification criteria summarised in table 1.1).

It is recognised that patients may have clinical features of APS other than those detailed in the criteria. For example, a retrospective Greek study of 81 patients with SLE who had a renal biopsy found evidence of APS nephropathy i.e. thrombotic

microangiopathy in one third of aPL positive patients who did not meet the Sapporo classification criteria for APS (Tektonidou et al., 2004). The committee considered patients with thrombocytopaenia, livedo reticularis, cardiac valve disease, nephropathy and non-thrombotic neurological disease and proposed the terms aPL-associated thrombocytopaenia, aPL-associated livedo reticularis, aPL-associated cardiac valve disease and aPL-associated nephropathy. Pre-defined inclusion criteria were outlined for each group. (Miyakis et al., 2006). These distinct aPL-associated non-APS group of patients tend to present the greatest management dilemmas. The definition of an 'aPL-associated' condition should help in focussing prospective research studies designed to dissect the best treatment regimens for these groups of patients and also help stratify risk for the development of APS.

The updated criteria are likely to be an improvement over the Sapporo criteria of 1999 and future validation studies may confirm this. What was not addressed in the updated criteria was how to classify patients that have characteristic clinical features of the APS but without evidence of aCL, anti- β_2 GPI antibodies or LA using conventional assays tested repeatedly. The term 'seronegative APS' has been proposed by Hughes and Khamashta (St. Thomas' Hospital, London) to describe such groups of patients (Hughes and Khamashta, 2003). It is likely that the standard assays do not detect all aPL, given their marked heterogeneity. For example, the aCL assay utilises a bovine source for β_2 GPI, and the human β_2 GPI assay detects binding of aPL to β_2 GPI coated on a plastic surface, rather than PL as occurs physiologically. Furthermore, there may be many other co-factors involved in haemostasis that may be important antigenic targets for aPL. Given these potential factors perhaps it is unsurprising that not all patients with APS have detectable aPL as assessed using conventional assays.

CLINICAL CRITERIA	
1.	Vascular thrombosis: One or more episodes of arterial, venous or small vessel thrombosis in any tissue or organ
2.	Pregnancy morbidity: <ol style="list-style-type: none"> a) One or more unexplained death of a morphologically normal foetus at or beyond the 10th week gestation b) One or more premature births of a morphologically normal neonate before the 34th week of gestation because of i) eclampsia or severe pre-eclampsia or ii) recognised features of placental insufficiency c) Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal / maternal chromosomal abnormalities excluded
LABORATORY CRITERIA	
1.	LA present in plasma on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis (Brandt et al., 1995)
2.	aCL antibody IgG or IgM isotype, present in medium to high titre (i.e. more than 40 GPL or MPL units) on two or more occasions at least 12 weeks apart, as measure by standard ELISA
3.	Anit-β ₂ GPI antibody IgG or IgM isotype (more than 99 th percentile) on two or more occasions, at least 12 weeks apart, measured by a standardised ELISA, according to recommended procedures (Reber et al., 2004)

Table 1.1 Updated classification criteria for definite APS. Definite APS is considered to be present if at least one clinical and one of the laboratory defined criteria are met within five years of each other.

1.2.4 Standardisation of aPL assays

The association of aPL with thrombotic events or pregnancy morbidity underpins the diagnosis of APS. However, epidemiological studies designed to demonstrate these associations show seemingly heterogeneous results and consequently draw conclusions that differ from one another. These discrepancies may be due to a variety of factors specifically relating to patient inclusion / exclusion criteria, diagnostic criteria for a clinical event, clinical end-points, assay method employed to detect aPL and pre-defined cut-off points for determining a positive result. The final point is likely to be a major contributing factor towards the heterogeneity between studies observed.

IgG and IgM aCL are expressed in international standardised units as GPL and MPL units respectively. These standardised units are derived using standardised anti-IgG aCL and anti-IgM aCL calibrators distributed from the aPL standardisation laboratory in Galveston, (Texas, USA) (Harris and Pierangeli, 2002). The first International aCL workshop in 1986 defined GPL and MPL units as binding observed with 1 μ g of affinity purified polyclonal IgG and IgM that was distributed to participating laboratories (Harris et al., 1987b). Secondary standards were ultimately calibrated against the primary standards. Hence, different batches of calibrators will contain heterogeneous polyclonal aPL from different batches of patients. This has led to suggestions that these calibrators may not necessarily behave in a homogenous fashion when assayed at different dilutions or using different kits (Wong, 2004). This is underlined by the observation of considerable inter-laboratory variability (Harris and Pierangeli, 2002) even when using the same batch of calibrators. In one inter-laboratory study 12 serum samples were tested for both IgG and IgM aCL using the same assay in 56 laboratories. General consensus defined as an inter-laboratory agreement of more than 90% was achieved in 10 (42%) of the 24 cases only (Favaloro and Silvestrini, 2002). There is also a high degree of variability between different commercial kits for aCL detection when assessed within the same laboratory, with lower variability seen with different commercial anti- β_2 GPI kits (Audrain et al., 2004). For the reasons stated, the European Antiphospholipid forum have validated the efficacy of two other calibrators named HCAL (IgG) and EY2C9 (IgM) and claim less inter-laboratory variation than when using the standard aCL calibrators (Tincani et al., 2001). These calibrators are derived from a monoclonal anti- β_2 GPI antibody developed for use as a standard in assays to detect β_2 GPI dependent aCL and anti- β_2 GPI antibodies (Ichikawa et al., 1999, Ichikawa et al., 1994). It is unlikely that these standards will

displace the currently accepted international calibrators in use for detecting aCL, however the European Antiphospholipid Forum did suggest that they be intermittently used as external controls two to three times a year in laboratories that undertake routine aCL titre estimations (Tincani et al., 2004). Given the current absence of an internationally accepted anti- β_2 GPI calibrator, the same European group proposed the use of HCAL and EY2C9 humanised monoclonals as calibrators for the anti- β_2 GPI assay to validate comparisons between different assay systems (Reber et al., 2004). Internationally accepted criteria for testing LA were proposed in 1995 by the International Society for Thrombosis and Haemostasis (Brandt et al., 1995).

1.2.5 Epidemiology of APS and of aPL as a risk for thrombosis

As discussed in the previous section, there are some limitations in comparing different epidemiological studies due to lack of uniformity in assay methods and until relatively recently, lack of agreed classification criteria for APS. Furthermore, many studies investigating the prevalence of aPL in a given patient population presenting with thromboembolic events or pregnancy morbidity cannot be extended to form conclusions as to the prevalence of APS in these groups. This is because the majority of studies have estimated aCL or LA only once and not serially as required by the original Sapporo and updated classification criteria (Miyakis et al., 2006).

It is recognised that there is a relatively high prevalence of aPL and even LA in the general population though the majority do not have APS (Petri, 2000). Estimates for aCL vary from 1% (Pattison et al., 1993) to 5.6% (Shi et al., 1990) and for LA from 1.2% (Pattison et al., 1993) to 3.8% (Infante-Rivard et al., 1991). However these are estimates derived from the general adult population. For those above 48 years of age the frequency of aCL (both β_2 GPI dependent and independent) goes up and approximates up to 26% (Brey et al., 2001). The frequency of anti- β_2 GPI antibodies in the healthy population has been little studied. The largest case control study investigating the frequency of anti- β_2 GPI antibodies in patients presenting with stroke or myocardial infarction tested the serum of 1360 age matched (between ages 48 and 70 years of age) healthy controls. The frequency of β_2 GPI dependent IgG aCL was 12.1% and anti- β_2 GPI positivity 1.9% in this normal group (Brey et al., 2001).

Multiple prospective studies have demonstrated a statistically significant association between the presence of aCL and LA and the occurrence of venous thrombosis (Finazzi et al., 1996, Schulman et al., 1998, Galli, 2004). Furthermore, this risk is

enhanced if patients have additional risk factors for the development of venous thrombosis (Kassis et al., 2004, Hudson et al., 2003). Ginsburg et al performed a case-control sub-study as part of the Physicians Health Study and found a statistical association between aCL and venous thrombosis. LA unfortunately was not measured and methods for diagnosing thrombosis were not stated (Ginsburg et al., 1992). In another case-control study of 256 patients with venous thromboembolism there was a statistically significant association with the presence of LA but not of aCL as compared to the non-thrombotic arm (Ginsberg et al., 1995). However a weakness of this study was that the mean age of patients enrolled was 55 years of age, thus accounting for the high prevalence of aCL in the non-thrombotic arm. Given this the significance of aCL positivity in those over the age of 50 years is uncertain. The presence of anti- β_2 GPI antibody positivity and the risk of developing venous thrombosis was extensively reviewed in a meta-analysis by Galli et al (Galli et al., 2003). Of the 21 studies identified 12 showed a statistical association with venous thrombosis. The most likely reason accounting for this heterogeneity is that these studies employed 'home-made' anti- β_2 GPI detection assays with varying protocols and varying cut-off points defined. Most of the studies were performed between 1993 and 1998 prior to the publication of the 1999 Sapporo classification criteria.

If a patient is aCL or LA positive what is the risk of developing a second thrombotic episode after cessation of anticoagulation? A study by Schulman et al has addressed this question (Schulman et al., 1998). During four-year follow-up of 412 patients post six months anticoagulation after a first venous thrombotic episode, 29% with aCL developed a further episode as compared to 14% without aCL ($p=0.0012$). This positive association was seen despite a number of shortcomings in this study. Abnormal aCL was defined as any value above 5 GPL units and the number of patients who had significantly positive aCL (i.e. more than 40 GPL units) was only eight, of whom three developed a recurrence. The study did not estimate LA or attempt to define anti- β_2 GPI reactivity.

Differentiating between β_2 GPI dependent and independent aCL reactivity has been employed by Brey et al in identifying risk factors for developing arterial thrombosis (Brey et al., 2001). In a large case-control study of 374 men presenting with myocardial infarction and 259 with stroke, as compared to an age matched control group of 1360 men, there was a significantly greater incidence of β_2 GPI dependent IgG aCL in the group with arterial thromboses. However, a relatively low cut-off

value of 23 GPL units was used and LA activity was not estimated. Other studies have also demonstrated an association between aCL and stroke (1993, Tuhim et al., 1999) and myocardial infarction (Vaarala et al., 1995) whilst a few have failed to demonstrate an association with stroke (Ginsburg et al., 1992, Ahmed et al., 2000) or ischaemic heart disease (Phadke et al., 1993). Part of the reason may be due to small cohort numbers included in these studies and the fact that these conditions occur with high frequency in an elderly population who generally have a higher incidence of aCL. This may account for the stronger association of stroke with aCL and LA seen in patients under the age of 50 years (de Jong et al., 1993, Brey et al., 1990, Tietjen et al., 1993). Also others have demonstrated that aCL in excess of 100 GPL units constitutes an independent risk factor for stroke (Verro et al., 1998). Bas de Laat et al identified 58 blood samples from 198 patients with autoimmune disease that all had LA activity but only half the samples exhibited β_2 GPI dependency. The presence of LA activity associated with β_2 GPI dependency had a very strong association with thrombosis (odds ratio 42.3; 95% confidence interval, 194.3-9.9) whereas the β_2 GPI independent samples with LA activity had no association (de Laat et al., 2004).

The prevalence of APS in the general population is unknown. One editorial has suggested that as 2%-5% of the population have experienced a deep venous thrombosis (Coon et al., 1973) and that primary APS may account for 15-20% of all deep venous thrombotic (Ginsburg et al., 1992) episodes, then the prevalence of APS in the population is likely to be as high as 0.3% to 1% (Roubey, 2004). However, caution should be exercised when drawing conclusions from different studies investigating different population groups. Without formal epidemiological studies designed to address this question the true figure of APS within the population remains unknown.

In the largest survey of APS patients to date compiled by the Euro-Phospholipid Project Group the clinical features of 1000 patients with APS were analysed (Cervera et al., 2002). Of these APS patients, 53.1% had APS alone, 36% also had SLE, 5% a lupus like disease, 5.9% other autoimmune diseases (such as Sjogren's syndrome-2.2% or rheumatoid arthritis-1.8%) and 1% the catastrophic form of APS known as CAPS (see section 1.2.8). Ratio of females to males is high at 5:1 in all APS patients with 7:1 in those with APS and SLE as compared to 3.5:1 in those with APS alone. The presence of aPL as defined by the Sapporo criteria (Wilson et al., 1999) were:

aCL in 87.9%, LA in 53.6% and antinuclear antibodies (ANA) in 59.7% (Cervera et al., 2002). One study comparing 30 male versus 38 female patients with APS and no other autoimmune disease found stroke to be significantly more prevalent in females (Jara et al., 2005).

In patients with SLE, the frequency of LA has been estimated to be between 11% (Wong et al., 1991) and 30% (Cervera et al., 1990). The frequency of aCL has been estimated to be approximately 20-35% (Radway-Bright et al., 2000, Kutteh et al., 1993, Cervera et al., 1993) and the frequency of APS also around 10% (UCH cohort of 450 patients, via personal communication with Professor Isenberg). In a cohort of 380 patients (Hopkins Lupus Cohort) followed up for a mean of 12.3 years, dual positivity for LA and aCL was predictive of both venous and arterial thrombosis, whilst LA only was predictive of myocardial infarction and neither were predictive of atherosclerosis (Petri, 2004). Anti- β_2 GPI antibodies in patients with SLE or other autoimmune diseases have also been shown to predict thrombosis, especially if patients are also LA positive (de Laat et al., 2004) and may help stratify risk in SLE patients with LA and no history of a thrombotic event (Zoghiami-Rintelen et al., 2005). In women with SLE, the presence of aCL and LA is associated with increased risk of pregnancy loss (Kutteh et al., 1993).

1.2.6 Thromboembolic and cardiovascular manifestations of the APS

Thrombosis of one or more vessels is the most frequent clinical manifestation of the APS. Thrombosis may occur in a vessel of any size in either the arterial or venous circulation in the absence of vascular anomalies. Some authors have proposed that APS is unique in predisposing to both arterial and venous thrombosis (Kandiah et al., 1998). Even inherited thrombophilic disorders tend to be associated with either arterial thrombosis as seen in homocystinuria or venous thrombosis as seen in Factor V Leiden, Protein C or S deficiencies (Kandiah et al., 1998). The presenting clinical features of vascular thrombosis that may occur in the APS are listed in table 1.2 A) and 1.2 B). The most common clinical manifestation is venous thrombosis, notably deep venous thrombosis of the legs occurring in 29-55% of APS patients (Asherson et al., 1989, Vianna et al., 1994, Cervera et al., 2002). Pulmonary embolism occurs in approximately 20% (Cervera et al., 2002). Arterial thromboses are also commonly seen but less frequently. Stroke is the most frequently observed arterial manifestation accounting for up to 50% of arterial occlusions (Asherson et al., 1989) and affecting 20% of patients with APS (Cervera et al., 2002). APS is the commonest cause of thrombotic stroke presenting in patients under the age of 50 (Petri, 2000).

Myocardial infarction appears in approximately 5% of APS patients and constitutes the presenting clinical event for APS in half of these cases (Cervera et al., 2002).

The association of APS with atherosclerotic disease however remains contentious (Vlachoyiannopoulos and Samarkos, 2004) whereas it has been clearly established in the case of SLE (Manzi et al., 1997, Manzi et al., 1999). Studies reported from the Hopkins Lupus Cohort demonstrate that patients with SLE and LA, as compared to patients with SLE and no LA activity, have an increased probability of having a myocardial infarction but not of developing atherosclerotic plaques as assessed using carotid doppler studies (Petri, 2004). However, not all SLE patients with LA have or go on to develop APS. Furthermore, given the high propensity of atherosclerosis in patients with SLE, it may be difficult to definitively demonstrate an independent predisposition to atherosclerosis in a subset of SLE patients who also have APS. In contrast, other studies have demonstrated an increased prevalence of atherosclerosis in patients with primary APS using carotid doppler studies (Medina et al, 2003) and by measuring the ankle-brachial index in these patients as compared to an age and sex matched healthy control group in both sets of studies (Baron et al., 2005, Christodoulou et al., 2006). Hence current balance of evidence favours the increased predisposition of atherosclerosis in APS.

1.2.7 Obstetric manifestations of the APS

Early criteria proposed for the diagnosis of APS used terms such as recurrent 'foetal loss' (Harris, 1987) or recurrent 'spontaneous abortions' (Khamashta and Hughes, 1993) to describe the main obstetric condition. However, this nomenclature was confusing as at that time the classification of pregnancy loss was defined as an abortion if the event occurred prior to 20 weeks gestation and termed stillbirth or foetal loss after this period (Derksen et al., 2004). Subsequently, it was recognised that there exists the embryonic period prior to 10 weeks gestation and the foetal period that extends beyond 10 weeks (Derksen et al., 2004). There are multiple obstetric manifestations seen in the APS, with most outlined in the Sapporo classification criteria (Wilson et al., 1999) and remain unchanged in the updated criteria proposed in Sydney in 2004 (see table 1.1) (Miyakis et al., 2006). These are highlighted in tables 1.2 A) and 1.3.

ORGAN SYSTEM	CLINICAL MANIFESTATIONS OBSERVED IN THE APS DUE TO ARTERIAL OR SMALL VESSEL THROMBOSIS
CARDIAC	angina, acute coronary syndrome, myocardial infarction, coronary bypass rethrombosis, intramural thrombus, peripheral vascular disease
CEREBROVASCULAR	Stroke, transient ischaemic attack, multi-infarct dementia, amaurosis fugax
CUTANEOUS	Leg ulcers, pseudovasculitic lesions, digital gangrene
ENDOCRINE	Addison's syndrome due to adrenal infarction
GASTROINTESTINAL	Oesophageal or mesenteric ischaemia, bowel infarction, splenic infarction, pancreatic infarction
OBSTETRIC	Uteroplacental insufficiency causing miscarriage or pre-term birth (before 34 weeks gestation)
OPHTHALMOLOGICAL	Retinal artery thrombosis
PULMONARY	Pulmonary artery thrombosis
RENAL	Renal artery thrombosis, renal small vessel thrombosis causing: acute/sub-acute/chronic renal failure, hypertension, nephritic syndrome

Table 1.2 A)

ORGAN SYSTEM	CLINICAL MANIFESTATIONS OBSERVED IN THE APS DUE TO VENOUS THROMBOSIS
CEREBROVASCULAR	Dural sinus thrombosis
CUTANEOUS	Superficial thrombophlebitis
GASTROINTESTINAL	Budd-Chiari syndrome (hepatic vein thrombosis)
LIMB VESSELS	Deep venous thrombosis
OPHTHALMOLOGICAL	Retinal vein thrombosis
PULMONARY	Pulmonary embolism
RENAL	Renal vein thrombosis

Table 1.2 B)

Table 1.2 Clinical manifestations of the APS.

Table 1.2 A), arterial and small vessel thrombotic lesion; 1.2 B), venous thrombotic lesions.

ORGAN SYSTEM	CLINICAL MANIFESTATIONS OBSERVED IN THE APS DUE TO NON-THROMBOTIC DISEASE
CARDIAC	Cardiac valve vegetations (sterile), diffuse valve thickening, 'pseudoinfective' endocarditis
CUTANEOUS	Livedo reticularis, splinter haemorrhages
EAR, NOSE AND THROAT	Perforation of nasal septum
GASTROINTESTINAL	Pancreatitis, ascities
HAEMATOLOGICAL	Thrombocytopaenia, haemolytic anaemia, haemolytic uraemic syndrome
MUSCULOSKELETAL	Avascular osteonecrosis, atraumatic metatarsal stress fracture, bone marrow necrosis, algodystrophy
NEUROLOGICAL	Epilepsy, dementia, transverse myelitis, encephalopathy, migraines, mononeuritis multiplex, chorea
OBSTETRIC	Pre-eclampsia, eclampsia, HELLP syndrome (haemolysis, elevated liver enzymes, low platelet count), intrauterine growth retardation
PULMONARY	Pulmonary hypertension, alveolar haemorrhage, acute respiratory distress syndrome
RENAL	Hypertension, nephrotic syndrome, haematuria, renal artery stenosis, focal cortical atrophy

Table 1.3 Non-thrombotic manifestations of the APS.

In APS, pre-term labour (prior to 34 weeks gestation) is often due to hypertension associated disease such as pre-eclampsia or uteroplacental insufficiency. This feature has been included in the table 1.2 A) describing arterial thrombotic manifestations of the APS, however as will be discussed in section 1.3 relating to the pathogenesis of this condition, there is accumulating evidence supporting the concept that uteroplacental insufficiency in APS may be an inflammatory rather than thrombotic phenomenon predominantly.

1.2.8 Catastrophic antiphospholipid syndrome (CAPS)

This is a subset of the APS that is estimated to affect approximately 1% of patients suffering from this condition (Cervera et al., 2002). Its mode and pattern of presentation differs significantly from the classical picture of APS in that severe, acute multi-organ dysfunction occurs in the acute setting and is predominated by small vessel ischaemia and thrombosis (Erkan et al., 2003). The 'catastrophic' nature of presentation led Asherson in 1992 to coin the syndrome as such in his initial description (Asherson, 1992). Recently the clinical outcomes of 250 patients with CAPS have been described (Bucciarelli et al., 2006b). Based on this largest series to date, recovery occurred in 56% and death in 44%. The most common cause of death was cerebral pathology (thrombotic stroke, cerebral haemorrhage and encephalopathy) followed by cardiac disease. Typical clinical features include malignant hypertension, acute respiratory distress syndrome, disseminated intravascular coagulation, microangiopathic haemolytic anaemia, schistocytes on the blood film and thrombocytopenia (Erkan et al., 2003). Rather than pulmonary embolism being the dominant pulmonary lesion as seen in APS, acute respiratory distress syndrome is seen more frequently in CAPS affecting approximately 20% of patients diagnosed (Bucciarelli et al., 2006a). There are many similarities between CAPS and thrombotic thrombocytopenic purpura (TTP) though key differences do exist (Cervený and Sawitzke, 1999). TTP is more likely if there is a history of a viral prodrome, schistocytes on the peripheral blood smear, fever, profound rather than moderate thrombocytopenia, less severe renal impairment, purpura and histological evidence of platelet thrombi. Though none of these distinctions are absolute, it is important to make the distinction as anticoagulation in CAPS is an important part of treatment, whereas in TTP anticoagulation is inappropriate (Cervený and Sawitzke, 1999). Clearly physicians managing patients with autoimmune disease need to have a high degree of awareness for this potentially devastating but very rare condition, which can present acutely, mimic other conditions such as TTP and have a high mortality rate. In order to focus research on this small but important subset of

patients affected by the APS an international registry for CAPS patients has been established by the European Forum on aPL (Erkan et al., 2003). Furthermore, preliminary criteria for the diagnosis of definite and probable CAPS were proposed and accepted in Taormina, Sicily at the 10th International Congress on aPL (Asherson et al., 2003). These criteria are summarised in table 1.4.

1.2.9 'Primary' and 'secondary' APS: is there a true distinction?

This terminology is only relevant if there is a true clinical distinction between the two, either in terms of presentation, diagnosis, serology or management. A relatively large proportion of patients with APS also have SLE as discussed. It is this concordance that led to suggestions that the APS be classified as 'secondary' in the presence of SLE, and 'primary' in the absence of any other autoimmune disease (Mackworth-Young et al., 1989, Vianna et al., 1994). Precedents for such classifications in autoimmune rheumatic disease exist such as Sjogren's syndrome which may be classified as 'primary' or 'secondary' when associated with any other autoimmune disease (Baurmash, 2004).

The name 'secondary APS' implies that SLE is the primary disease and the development of APS occurs as a 'secondary' phenomenon to this. However, it is recognised that a small group of patients with APS alone go on to develop clinical features of SLE, estimated by one study to be approximately 8% (Gomez-Puerta et al., 2005) though found to be ~1% within the UCH cohort of 450 SLE patients (via personal communication with Professor Isenberg). Some studies suggest that the presence of anti-nucleosome antibodies (Abraham Simon et al., 2004) or hypocomplementaemia (Ramos-Casals et al., 2004) in patients with APS may predict the development of SLE in these patients. It is unknown if APS and SLE are two different diseases coinciding in the same individual, if underlying SLE offers a setting for the development of APS, or if APS and SLE represent two elements of the same process. Harris and Pierangeli have postulated that APS and SLE are not part of the same disorder but rather represent a super family of systemic autoimmune diseases (Harris and Pierangeli, 2004). One study of 130 patients with SLE has showed that aCL positivity may precede the onset of more severe SLE, as well as SLE complicated by features of the APS such as pregnancy loss or thrombosis (McClain et al., 2004).

1.	Evidence of involvement of ≥ 3 organs, systems, and/or tissues*
2.	Development of manifestations simultaneously or in < 1 week
3.	Confirmation by histopathology of small vessel occlusion in at least 1 organ/tissue†
4.	Laboratory confirmation of the presence of aPL (LA and/or aCL and/or anti- β_2 GPI antibodies)
DEFINITE CAPS: All 4 criteria	
PROBABLE CAPS:	
<ul style="list-style-type: none"> • All 4 criteria, except for the involvement of only 2 organs, systems, and/or tissues • All 4 criteria, except for the absence of laboratory confirmation of the presence of aPL at least 6 weeks after a first positive result (due to the early death of patients never tested for aPL before the onset of CAPS) • 1, 2, and 4 • 1, 3, and 4, plus the development of a third event in > 1 week but < 1 month, despite anticoagulation treatment 	

Table 1.4 Preliminary criteria for the classification of CAPS

*Usually clinical evidence of vessel occlusion, confirmed by imaging techniques when appropriate. Renal involvement is defined by a 50% rise in serum creatinine, severe hypertension ($> 180/100$ mm/Hg), and/or proteinuria (> 500 mg/24 hours).

†Significant histopathological evidence of thrombus must be present, though vasculitis may coexist occasionally.

Adapted from Asherson et al (2003) (Asherson et al., 2003).

There appears to be no difference in terms of clinical manifestations, complications, prognosis or frequency of aCL between those with APS alone and those with APS and SLE (Vianna et al., 1994, Harris and Pierangeli, 2004, Male et al., 2005). Genetic studies may be another means by which a distinction between the 'primary' and 'secondary' forms of APS may be drawn. One study has found a more frequent positive family history of autoimmune disease in patients with APS and SLE as compared to patients with APS alone. However, this was a relatively small study of 109 patients and HLA typing was not performed. Crucially, any potential genetic differences between 'primary' and 'secondary' may be due to genetic differences between APS and SLE. A difference between 'secondary' APS and SLE without APS would need to be demonstrated and at present no such evidence exists.

It would appear that there is no clear value in characterising APS into either a primary or secondary group. There is no evidence that such classification is helpful in aiding diagnosis, management or follow-up. Furthermore, the implication of the term 'secondary' APS is that this disease is a secondary phenomenon to the 'primary' disease of SLE. Moreover, we know that APS may co-exist with autoimmune conditions other than SLE. The recently updated classification criteria for the APS call for the terms primary and secondary APS to be abandoned (Miyakis et al., 2006). This consensus statement advised on simply using the term APS in the presence or absence of SLE. This will be the preferred terminology used for the purposes of this thesis.

1.2.10 Management and prognosis of the APS

The management of the APS will be discussed in terms of five main categories: secondary prophylaxis of thrombosis, secondary prophylaxis of pregnancy morbidity, primary prophylaxis, treatment of non-thrombotic manifestations of APS and management of CAPS.

1.2.10.1 Secondary prophylaxis of venous and arterial thrombosis

The only treatment to have shown any proven benefit for preventing recurrent venous or arterial thrombosis in patients diagnosed with the APS is oral anticoagulation. What is less certain is the intensity and duration of this anticoagulation. This is an important question as the cumulative incidence of a major haemorrhage occurring with oral anticoagulation within a 12-month period is estimated to be between 7% and 12% (McMahan et al., 1998, Landefeld and Goldman, 1989).

Regarding the duration of oral anticoagulation, early retrospective studies have shown high recurrence rates of thrombosis after discontinuation of oral anticoagulation. These range from 52-68% of patients developing another thrombotic event over a period of 5-6.4 years post discontinuation of warfarin (Khamashta et al., 1995, Krnic-Barrie et al., 1997, Rosove and Brewer, 1992). In prospective studies aPL positive patients had a higher risk of a recurrent venous thrombosis as compared to aPL-negative patients after discontinuation of oral anticoagulation originally lasting for only six months (Schulman et al., 1998). Regarding secondary prophylaxis of venous thrombosis, the weight of evidence seems to favour oral anticoagulation for longer than six months, but crucially there have been no prospective risk/benefit studies designed to study the consequences of continuing therapy for longer than six months in patients with APS presenting with one venous thrombosis and no other risk factors. Given this lack of evidence the British Society of Haematology guidelines state that oral anticoagulation is prescribed only for three to six months in this scenario (Greaves et al., 2000). However, a low threshold for instigating longer periods of anticoagulation is required, particularly if other risk factors for thrombosis exist (Meroni et al., 2003, Erkan and Lockshin, 2004).

Early retrospective studies of oral anticoagulation for the secondary prevention of venous thromboembolism recommended a target international normalised ratio (INR) of ≥ 3.0 . This high intensity regimen seemed to demonstrate reduced recurrent venous thromboembolism as compared to lower intensity regimens (Khamashta et al., 1995, Krnic-Barrie et al., 1997, Derksen et al., 1993). Nevertheless, some retrospective (Rosove and Brewer, 1992), and importantly prospective studies, have demonstrated that aiming for a relatively low target intensity INR of 2.0-3.0 is also effective (Ginsberg et al., 1995). Two randomised prospective studies each with approximately 100 APS patients enrolled have compared high intensity (target INR 3.1-4.0) to lower intensity (target INR 2.0-3.0) anticoagulation and found no difference in thrombotic recurrences (Crowther et al., 2003, Finazzi et al., 2005). However, it should be noted that the study by Crowther et al was flawed as the majority of patients in the high intensity arm who suffered events (four out of six) had an INR below 2.0 (Crowther et al., 2003). Furthermore, the study by Finazzi et al does not state the INR of the patients in the high intensity arm at the time of the thrombotic event (Finazzi et al., 2005). Given the evidence to date, it would seem

sensible to propose that for a single venous thromboembolic event (either deep venous thrombosis or pulmonary embolism) the initial target should be an INR of 2.5. Higher intensity oral anticoagulation (target INR of 3.5) should be the target when there are additional risk factors or if there has been further thromboembolic events despite oral anticoagulation at lower intensity regimens.

The case for high versus low intensity oral anticoagulation in preventing recurrent arterial thrombosis is even less well established (Gatenby, 2004, Ruiz-Irastorza and Khamashta, 2005). Two early retrospective studies of 70 patients (Rosove and Brewer, 1992) and 147 patients (Khamashta et al., 1995) with APS, of which approximately 45% had arterial thromboses, have suggested that prolonged treatment with high intensity warfarin (target $INR \geq 3.0$) is required to minimise the chance of re-thrombosis. Ruiz-Irastorza et al retrospectively studied 66 patients with APS who were managed to a target INR 3.5. The actual INR was frequently below 3.0 and episodes of life-threatening bleeds were estimated at six per 100 patient years with no fatalities. Approximately half had a history of thrombotic stroke and four had a recurrent arterial thrombosis, all of whom had an INR of between 2.1 and 2.6 (Ruiz-Irastorza et al., 2002). There have been two prospective comparison studies comparing high versus low intensity anticoagulation with warfarin, neither of which found a difference in the recurrence rate of arterial thrombosis. Both these studies included patients with mostly venous thrombosis (70% of patients enrolled in each study) and crucially excluded patients who had had a recent stroke or who had developed a thrombotic event whilst on anticoagulation, thus potentially biasing the results towards a relatively low-risk group of APS patients (Crowther et al., 2003, Finazzi et al., 2005). Derksen et al reported the results of prospective long-term follow-up of eight women with APS presenting with stroke who were only treated with anti-platelet therapy. After a median follow up of 8.9 years two developed a further stroke and it was concluded that the chances of a recurrence was as one would expect for aPL negative patients and that aspirin alone may be sufficient in treating this group of patients (Derksen et al., 2003). However, this study had an extremely low cohort number and did not have a warfarin treatment arm for comparison. The Antiphospholipid Antibodies and Stroke Study (APASS., 1993) took advantage of the randomised Warfarin versus Aspirin Recurrent Stroke Study and determined the aCL and LA activity of 1770 patients. It found similar end-points for the aPL positive versus aPL negative patients and concluded that aPL did not confer important knowledge for prognosis or treatment of patients with a recently

diagnosed ischaemic stroke and that warfarin was not associated with fewer thrombotic strokes as compared to aspirin in aPL positive patients (Levine et al., 2004). However, there are some potential flaws in this large study. Importantly this was a sub-group analysis of a study that was not designed to assess the outcomes outlined in the APASS study. Also the majority of patients were elderly (mean 62 years) and had aPL tested only once, with the titres in the majority falling in the low category (less than 40 GPL or MPL units). Thus, most of the patients did not have APS and furthermore could not be generalised to younger patients with APS. Hence upon critically reviewing this study, its relevance to the management of APS is questionable. It would appear that on balance evidence favours the use of long-term anticoagulation with warfarin. The current British Society of Haematology guidelines recommend long-term anticoagulation aiming for an INR 2.5 and if recurrence of arterial thrombosis / stroke occurs, to then increase the target to an INR of 3.5 (Greaves et al., 2000). Whether adding in aspirin to warfarin confers any additional benefit versus risk is unknown. The increased risk of haemorrhage if aspirin is co-administered with warfarin in non-APS groups of patients has been established (Meade and Miller, 1995). At present there exists no data for the long-term use of heparin or fondaparinux (Factor X inhibitor) for the secondary prevention of thrombosis.

1.2.10.2 Management of pregnant patients with the APS

Early, small, open-labelled studies recommended the use of prednisolone in combination with aspirin (Lubbe et al., 1983, Kwak et al., 1994) or other immunomodulatory treatment such as immunosuppressive agents (Gatenby et al., 1989) or intravenous immunoglobulins (Many et al., 1992). However larger, randomised controlled trials have shown no benefit in using corticosteroids, which were associated with significantly more maternal morbidity and more pre-term deliveries (Silver et al., 1993, Cowchock et al., 1992, Laskin et al., 1997). Hence the use of prednisolone in treating or preventing pregnancy morbidity in APS patients has been abandoned.

Three randomised trials have compared the efficacy of a combination of heparin and low dose aspirin to aspirin alone (Rai et al., 1997, Farquharson et al., 2002, Kutteh, 1996). One study also evaluated the outcome of pregnancies comparing aspirin combined with varying doses of heparin (Kutteh and Ermel, 1996). In these studies heparin was started either at the time of a positive pregnancy test (Kutteh, 1996, Kutteh and Ermel, 1996), when foetal heart activity was first confirmed (Rai et al.,

1997) or before 12 weeks gestation (Farquharson et al., 2002). Two of the three studies comparing the combination of aspirin and heparin to aspirin alone found that the combination arm had significantly higher live-birth rates. The rates were 71% and 80% in the combination arm as compared 42% and 44% in the aspirin-only arm for each respective study (Rai et al., 1997, Kutteh, 1996). The other prospective study by Farquharson et al revealed no additional benefit in adding heparin to aspirin (Farquharson et al., 2002). However, an aCL level of greater than 9 GPL units and 5 MPL units was deemed sufficient for entry. Hence, the majority of women included in this study did not fulfil the criteria for classification of APS published while the trial was being conducted between 1997 and 2000 (Wilson et al., 1999). In the study comparing varying doses of heparin in combination with aspirin, high and low doses of unfractionated heparin were associated with similar live-birth rates (Kutteh and Ermel, 1996). The long-term use of heparin raises potential concerns regarding bone loss over and above that seen in normal pregnancy. One study to address this question assessed 55 pregnant patients with APS on heparin and found no significant differences in their bone density within six months prior to conception and in the post partum period as compared to 20 non-APS pregnant control patients not on heparin (Carlin et al., 2004).

The balance of evidence favours the use of low molecular weight heparin and aspirin to be started once a patient with APS has a confirmed positive pregnancy test. Given that prothrombotic risk extends into the post-partum period, it would seem prudent to continue this regime up to six weeks post partum. This is the current management protocol recommended by the American College of Rheumatology (Derksen et al., 2004). However, guidelines for the management of pregnant patients fulfilling the classification criteria for APS have also been published under the auspices of the British Society of Haematology (Greaves et al., 2000) and do not concur with the those of the rheumatologists, stating that patients with APS who become pregnant should add heparin to aspirin once foetal heartbeat has been established and that heparin be discontinued at 34 weeks gestation. It would appear that some consensus between the haematological and rheumatological community is required. Until then it would seem prudent for rheumatologists, obstetricians and perhaps haematologists with an interest to agree local guidelines and co-manage this important group of patients, ideally in dedicated combined clinics.

1.2.10.3 Primary prophylaxis of aPL positive patients without APS

Ginsberg et al's study of aCL in the Physicians' Health Study cohort was the first to publish data regarding aspirin prophylaxis. This study demonstrated that moderate to high titres of aCL were associated with increased risk of venous thrombosis, which was unaffected by treatment with low dose aspirin (Ginsburg et al., 1992). However, it has since been recognised that the study was underpowered to detect the expected effect of aspirin prophylaxis (Alarcon-Segovia et al., 2003a). A retrospective cross-sectional study by Erkan et al compared the records of 77 patients with APS with those of 56 patients without APS but with aPL. When examining the six month period prior to thrombosis (for the thrombosis group) or prior to the last clinic visit for the asymptomatic group, the use of aspirin was significantly associated with asymptomatic status (Erkan et al., 2002). A consensus report has advocated the use of low dose aspirin prophylaxis in patients with moderate to high aPL but no clinical evidence of APS, though it conceded that prospective randomised trials were necessary to confirm that this was appropriate (Alarcon-Segovia et al., 2003a). Again advice by the haematologists differs in that prophylaxis in this group is not indicated, though a low-threshold for prophylaxis in the setting of additional risk factors should be maintained (Greaves et al., 2000). However, it is worth noting that these guidelines were issued two years prior to the publication of Erkan et al's study (Erkan et al., 2002). The use of aspirin in pregnancy as primary prophylaxis is more controversial. Currently accepted practice is that healthy women with no more than two embryonic losses and absence of foetal loss should not be advised to undergo aPL testing. The rationale being that if there is aPL positivity in this setting, there exists no evidence for improved outcomes with aspirin prophylaxis. However, close maternal-foetal monitoring is advised throughout pregnancy and the post-partum period (Derksen et al., 2004). Therefore, given the lack of evidence for or against, one cannot conclude with certainty that not treating pregnant patients with persistently positive aPL but no history thrombosis or miscarriage with aspirin is correct.

1.2.10.4 Management of non thrombotic manifestations of the APS

There are a number of manifestations for the APS which are not associated with thrombosis and for which oral anticoagulation is generally not helpful. However, data on how best to manage these manifestations is lacking and mostly limited to retrospective studies of generally small cohorts or case reports. Refractory thrombocytopaenia has been shown to respond transiently to intravenous

immunoglobulin therapy (Sturfelt et al., 1990, Sherer et al., 2000). Splenectomy in this group of patients may give good long-term responses (Hakim et al., 1998, Galindo et al., 1999). Intravenous immunoglobulins have also been successfully employed to treat autoimmune haemolytic anaemia associated with APS in one case report (Vandenberghe et al., 1996). For non-vascular manifestations such as non-ischaemic related epilepsy and chorea, very little evidence exists. Chorea has been associated with immune mediated excitatory effects of aPL causing striatal hypermetabolism as opposed to ischaemia as assessed by positron emission tomography imaging (Sunden-Cullberg et al., 1998, Furie et al., 1994). This may explain the reversibility that has been observed in a few cases treated with immunosuppressive therapy such as prednisolone in a patient with SLE and APS (Van Horn et al., 1996) and methotrexate in a patient with chorea and LA activity and high IgG-aCL titres with no other clinical features of either APS or SLE (Paus et al., 2001).

1.2.10.5 Management of CAPS

The management of CAPS is extremely challenging. The optimal treatment for this rare condition is unknown, it may be difficult to distinguish it from other acute conditions such as TTP or systemic vasculitis and multiple simultaneous organ failure is the typical presentation, necessitating prompt diagnosis and instigation of therapy. Patients may present with a mixture of haemorrhage (induced by profound thrombocytopenia and malignant hypertension for example) and multi-organ failure due to small vessel thrombosis, presenting difficult treatment dilemmas (Boura et al., 2005). Consensus guidelines for the management of CAPS have been produced though the authors acknowledge that these guidelines do not represent standardised treatment due to lack of prospective trial data (Asherson et al., 2003). Management of CAPS should have three broad aims: to treat any precipitating factors such as infection promptly, to prevent ongoing thrombotic events and to suppress the excessive 'cytokine' storm. Hence, treatment typically involves anticoagulation with intravenous heparin followed by oral anticoagulation, plasma exchange, intravenous immunoglobulins and if associated with a lupus flare cyclophosphamide (Asherson et al., 2003). Other options that have been used on an empirical basis include prostacyclin, defibrotide, danazol, cyclosporine, azathioprine, and splenectomy (Asherson et al., 1998, Asherson et al., 2001) though these treatments have been mostly used in single cases and are hence of doubtful benefit (Erkan et al., 2003). A recent study of 250 patients with CAPS suggests that a higher recovery rate is associated with combined treatment of anticoagulants, corticosteroids and plasma exchange and that concomitant treatment with cyclophosphamide yielded no added

benefit (Bucciarelli et al., 2006b). Monitoring and active management should occur in an intensive care setting once the diagnosis is made with facilities for mechanical ventilation and renal replacement therapy readily available (Erkan et al., 2003).

1.2.11 Prognosis in APS

In patients who develop a venous thrombosis and are then anticoagulated for six months only, re-thrombosis within a four-year follow-up period occurs in 29% of aPL positive patients as compared to 19% of aPL negative patients ($p=0.001$). This study by Schulman et al also demonstrated an increased mortality rate related to thromboembolic causes of 7% in the aPL positive group versus 2.2% in those that tested negative for aPL ($p=0.002$) (Schulman et al., 1998). A retrospective study of 52 patients with aCL, of whom 31 had APS, followed-up for 10 years revealed that nine (29%) of the patients with APS had a recurrent thrombosis and 10% of all patients died, despite all receiving either anticoagulant or antiplatelet therapy (Shah et al., 1998). A prospective five-year follow-up study of APS patients revealed similar re-thrombosis rates of 27%, with a mortality rate of 5.4% again despite anti-thrombotic therapy. This study by Turiel et al also confirmed that an IgG aCL of more than 40 GPL units was an independent risk factor, with 43.3% of patients with aCL titres in excess of 40 GPL units developing a recurrent thrombotic event as compared to 7.7% in patients who had titres below this level (Turiel et al., 2005).

In summary, upon critically reviewing the evidence there are limited treatment options with proven efficacy available to the clinician in managing patients with APS. Oral anticoagulation for the secondary prophylaxis of thrombosis is beneficial, but may be associated with hazards such as haemorrhage with some uncertainties surrounding the target intensities and duration of treatment. The treatment of pregnant APS patients is established, but the place for primary prophylaxis with aspirin in aPL positive patients remains unproven, with these uncertainties particularly underlined in pregnant aPL positive patients. What is clear is that there remains an unmet need to develop safer, targeted and arguably more effective therapies for APS.

1.3 SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Approximately 45% of patients with APS also have another autoimmune disease. This disease is SLE in approximately 80% of these cases (Cervera et al., 2002) and hence this syndrome will be described, albeit briefly.

SLE is a complex, multi-systemic autoimmune rheumatic disease characterised by widespread inflammation and mostly affecting young women. Classification criteria by Tan et al were published in 1982 (Tan et al., 1982) and subsequently revised in 1997 to include a 'positive finding of aPL' (Hochberg, 1997). Undoubtedly, these criteria have been an important research tool used in multiple studies designed to assess the efficacy of various treatment pathways for SLE. However, due to the fact that SLE can mimic so many other conditions they are also commonly used in routine practice when diagnosis is in doubt. The criteria are summarised in table 1.5 listing 11 criteria, of which at least four are required to make the diagnosis of SLE.

1.3.1 Aetiology, epidemiology and natural course for SLE

The precise aetiology of SLE remains uncertain other than there is clear evidence of dysregulation of the immune response in affected individuals. This includes the production of pathogenic autoantibodies, produced by hyperactive B cells, characteristically targeted to nuclear antigens such as nucleosomes. This occurs in the context of impaired cell mediated immunity with a predominance of circulating T_H2 cytokines. One currently popular theory proposed by Walport, amongst others and known as the 'waste disposal' hypothesis, suggests that tolerance to these ubiquitous autoantigens is broken due to the defective clearance of apoptotic cellular debris and immune complexes. Prolonged exposure to these autoantigens in genetically and perhaps hormonally susceptible individuals may trigger an autoimmune response to these autoantigens and lead to SLE (Walport, 2000). Surface blebs on apoptotic cells have been shown by Rosen and colleagues to cluster and expose autoantigens such as DNA, Ro, La, RNP as well as anionic PL (Rosen et al, 1994., Rosen et al., 1996). These cells are normally cleared by tingible body macrophages within the germinal centres of secondary lymphoid organs, thus avoiding contact of nuclear remnants with the immune system and preventing the production and affinity maturation of autoantibodies to nuclear material. Hermann and colleagues have shown that in patients with SLE, the number of these tingible body macrophages within the germinal centre of lymph nodes is reduced. This results in the accumulation of apoptotic cells exposing nuclear autoantigens which bind to follicular dendritic cells and may provide the survival signals for autoreactive B cells (Baumann et al., 2002). The most recent developments reviewing the likely pathogenesis of SLE have been summarised in a report covering the content of the 6th European Lupus meeting in March 2005, London, UK (Ioannou et al., 2005).

SLE is found worldwide with an increased preponderance in black females in the UK and USA. The female to male ratio approximates to 10:1 with the majority developing disease between the ages of 15 and 40 years of age. However, there is a subgroup of patients that develop SLE beyond the age of 50 years and the ratio drops in this group to 4:1, affecting approximately 10-15% of patients (Morrow et al., 1999). The prevalence rates per 100,000 in the UK for Caucasian, Asian and Black women with SLE are 36.2, 90.6 and 206 respectively (Johnson et al., 1995).

Four decades ago SLE was deemed to be a serious and frequently fatal disease with mortality rates estimated to be around 50% at five years. Since then there has been a marked improvement in survival with current estimates of standardised mortality ratio observed here at the Centre of Rheumatology, UCH to be approximately 4.0 (Moss et al., 2002). This reflects the easier identification of milder cases, the widespread availability of ANA and anti-double stranded (ds) DNA antibody estimations, the introduction of corticosteroids, immunosuppressive drugs, renal replacement therapy and renal transplantation. However 10% to 20% will succumb to either the disease, a side-effect of its treatment or both within 10 years of follow-up (Isenberg and Horsfall, 1998). The causes of death vary with disease duration showing a bimodal pattern of distribution. Early deaths within two years of diagnosis have a tendency to be related to SLE disease activity or infection, however deaths beyond two years of diagnosis tend to be due to atherosclerotic disease, cancer or infection (Abu-Shakra et al., 1995). The presence of APS has been shown to be an independent predictor of irreversible organ damage and mortality in SLE patients (Ruiz-Irastorza et al., 2004).

1.3.2 Clinical features of SLE

Non-specific symptoms are common in SLE. These include fatigue, fever, anorexia, weight loss and lymphadenopathy. Fatigue is often extremely severe and many patients find this the most disabling symptom of their SLE (Morrow et al., 1999). The most frequent symptom involving the musculoskeletal system is arthralgia, affecting approximately 90% of patients with SLE. However, overt arthritis with deformities is only present in approximately 5% (Morrow et al., 1999). The arthropathy is characteristically non-erosive and extreme deformities have a tendency to be correctable, otherwise known as Jaccoud's arthropathy (Spronk et al., 1992). Myalgia, muscle weakness and tenderness have been reported in up to 60% of patients, though only around 5% have a true myositis overlap condition (Isenberg and Snaith, 1981).

MANIFESTATION	DEFINITION/ CHARACTERISTICS
Malar rash	Erythematous rash over malar area, sparing nasolabial folds
Discoid rash	
Photosensitivity	
Oral ulcers	Usually painless and observed by physician
Arthritis	Non-erosive, involving two or more joints
Serositis	Pleuritis: pleural rub, history of pleural pain or pleural effusion AND / OR Pericarditis: evidence on echocardiogram or the presence of a pericardial rub
Renal disorder	Persistent proteinuria (>0.5g/24 hours or >3+ on dipstick if quantification unavailable) OR cellular casts on microscopy
Neurological disorder	Seizures OR psychosis (unprovoked)
Haemolytic disorder	Haemolytic anaemia OR leucopaenia (<4,000/mm ³ on ≥2 occasions) OR lymphopaenia (<1,500/mm ³ on ≥2 occasions) OR thrombocytopaenia (<100,000/ mm ³)
ANA	Raised titre at any point
Immunological disorder	Raised anti-native DNA antibody OR Presence of anti-Sm antibody OR Positive aPL (based on either an abnormal serum level of IgG or IgM aCL or positive LA or false positive test result for Syphilis sustained for at least six months)

Table 1.5 Updated classification criteria for the diagnosis of SLE

Adapted from Tan et al (Tan et al., 1982) and Hochberg et al (Hochberg, 1997). Out of the 11 criteria four are required, either serially or simultaneously, to make the diagnosis of SLE.

The classic butterfly rash is present in approximately one third of lupus patients though 85% will have skin involvement (Pistiner et al., 1991). Other dermatological lesions include maculopapular discoid lesions, alopecia (often diffuse and non-scarring), vasculitic lesions, mucosal ulcers, bullous lesions and nasal ulceration. Many of these lupus rashes are exacerbated by ultraviolet light (UV), with photosensitivity being more common among Caucasian patients (Morrow et al., 1999).

The most common pulmonary manifestation is pleuritic chest pain due to pleurisy, affecting approximately 40% of patients with SLE at some time. Lung parenchymal involvement has been reported in up to 18% of patients and includes interstitial fibrosis, pulmonary vasculitis and interstitial pneumonitis (Haupt et al., 1981). Pulmonary hypertension or pulmonary embolism occurs with greater frequency in SLE patients and aPL, in these circumstances, may be a contributing factor (Morrow et al., 1999).

Pericardial disease is the most frequent cardiac involvement and may be often clinically silent, with necropsy studies reporting that 66% of patients with SLE may be affected (Mandell, 1987). Clinical myocarditis is less common and affects 15% of patients. The classic Libman and Sacks endocarditis described in 1924 (Libman and Sacks, 1924), which is also seen in the APS (Hojnik et al., 1996), may be identified in up to 50% of autopsy cases but is often of no clinical significance (Morrow et al., 1999). There has been increasing recognition over the past two decades of the presence of accelerated atherosclerosis in patients with SLE. Women between the ages of 35 and 44 years exhibit rates of myocardial infarction that are up to 50-fold higher than that seen in age matched women without SLE (Manzi et al., 1997) with 40% of women with SLE having objective evidence of carotid atherosclerotic plaques as assessed by carotid doppler studies (Manzi et al., 1999).

Abdominal pain has been reported in up to 40% of patients. Common causes of an acute abdomen are vasculitis or thrombosis causing bowel perforation or bowel / organ infarction. Diagnosis of an acute abdomen may be difficult due to the masking of traditional signs of peritonitis by immunosuppressive therapy (Sultan et al., 1999). The nervous system, both peripheral and central, may be affected in a multitude of ways. Migraine affects approximately 30% of lupus patients (Isenberg et al., 1982) and seizures may occur in up to 20% (Isenberg and Horsfall, 1998). Peripheral

neuropathy, usually sensory, may affect 10% of patients. Cognitive dysfunction is observed in approximately 21% of patients as compared to 4% affected in an age matched disease (rheumatoid arthritis) and healthy control group (Hanly et al., 1993). Patients with lupus suffering from neuropsychiatric symptoms tend to have persistently elevated IgG aCL (Menon et al., 1999).

A high proportion of lupus patients also have haematological abnormalities. A normochromic normocytic anaemia is present in up to 70% of patients with lupus with a Coombs positive haemolytic anaemia affecting approximately 10%. Leucopaenia and lymphopaenia as defined in table 1.5 affects approximately 45-65% and 80% of lupus patients respectively. Chronic thrombocytopaenia as defined in table 1.5 may affect 20% of lupus patients (Morrow et al., 1999).

Renal disease until recently in many published series was the commonest cause of death (Isenberg and Horsfall, 1998). Given that symptoms only occur late on in the course of renal involvement once irreversible damage has occurred, a proactive approach for detecting early signs of renal involvement is required. Thus patients should have regular estimations of blood pressure, serum creatinine and urinary protein. The World Health Organisation has subdivided renal lupus in to five classes based on histological changes seen on renal biopsy and these have subsequently been revised under the auspices of the International Society of Nephrologists (Weening et al., 2004). Renal abnormalities may be further scored according to the degree for underlying activity and damage, which may be helpful in predicting prognosis and guiding treatment pathways (McLaughlin et al., 1991).

1.3.3 Autoantibodies and role of complement in SLE

The clinical diversity of SLE is matched by the diverse autoantibody profiles observed in patients with lupus and these autoantibodies with those also seen in APS are summarised in table 1.6. Antibodies to nuclear cellular components are the most frequently observed group and the common ones detected are ANA, anti-dsDNA, anti-nucleosome antibodies and antibodies to extractable nuclear antigens such as anti-Ro (SS-A), anti-La (SS-B), anti-ribonucleoprotein (RNP), anti-Sm antibodies and anti-histone antibodies. Antibodies to cell membrane components are also seen such as aPL, anti-erythrocyte and anti-platelet antibodies as well as antibodies to circulating proteins such as anti- β_2 GPI, anti-C1q and anti-IgG antibodies (rheumatoid factor) (Morrow et al., 1999).

The most sensitive test for SLE is the ANA, which is positive in 95% or more of patients. Anti-dsDNA antibodies are less sensitive, detected in approximately 70% of patients with SLE in a cohort of 450 SLE patients followed up at the Centre of Rheumatology, UCH (personal communication from Professor D Isenberg, see table 1.6). However, they are specific to SLE and rarely seen in patients with other disease or in healthy individuals (Isenberg et al., 1985). These antibodies generally correlate with renal damage and have been shown to deposit in the renal tissue of SLE patients (Hahn, 1998). However, as seen with aCL, these antibodies are heterogeneous in nature and not all are pathogenic. Patients may have high titres and not have renal disease for example and not all anti-dsDNA antibodies tested in animal models cause tissue damage (Ehrenstein et al., 1995).

Antibody Specificity	% Prevalence in SLE patients
ANA	96
dsDNA	69
‡Nucleosome	78
‡Histone	50-80
Sm	14
RNP	28
Ro	37
La	13
‡C1q	90
Fc IgG (Rheumatoid factor)	26
*Cardiolipin - IgG	24
*Cardiolipin - IgM	9
*Lupus anticoagulant	16
*‡β ₂ GPI	33

Table 1.6 Key autoantibodies detected in patients with SLE and APS. % prevalence figures are based on the first ~470 patients with SLE under long-term follow-up at the Centre of Rheumatology, SLE clinic, Univerisity College London Hospital (via personal communication with Professor Isenberg). *autoantibodies also associated with APS. ‡ anti-nucleosome (Ghillani-Dalbin et al., 2003); ‡ anti-histone (Egner, 2000); ‡ anti-C1q (Sjoholm et al., 1997); (‡ anti-β₂GPI (Loizou et al., 2000).

The complement system, primarily via the classical pathway, is involved in the sequence of inflammatory events seen in SLE. Early reacting components of the classical pathway, C1 (and its subcomponents C1q and C1s), C4, C2 and C3 are frequently reduced in active disease. Inherited deficiencies in these components predispose to the development of SLE and these patients do not tend to have autoantibodies to dsDNA (Morrow et al., 1999). C1q knockout mice develop glomerulonephritis with the presence of glomerular apoptotic bodies (Botto et al., 1998). *In vitro* experiments have shown that clearance of apoptotic cells is dependent upon the presence of C1q which binds these cells via IgM (Quartier et al., 2004). Anti-C1q antibodies are also strongly associated with the presence of lupus nephritis and induce disease in the context of immune complex deposition (Trouw et al., 2004).

1.3.4 Management of SLE

Successful therapy of SLE depends upon treating symptoms and the underlying inflammation, involving both pharmacological and non-pharmacological approaches (Ioannou and Isenberg, 2002). Patient education is essential, with emphasis on wearing UV protection and addressing traditional cardiovascular risk factors such as smoking and hypercholesterolemia, given the marked propensity for accelerated atherosclerosis and cardiovascular morbidity in lupus patients (Manzi et al., 1997). Adequate contraception is vital when patients are taking potent cytotoxic agents. Infection is one of the commonest causes of death and patients should have all appropriate vaccinations including influenza and pneumococcal vaccines. Live vaccines should be avoided in patients taking immunosuppression or prednisolone of more than 10 mg/day (Ioannou and Isenberg, 1999).

Patients should be stratified according to severity of disease and pharmacological treatment tailored accordingly. Patients with mild disease may be maintained on a combination of non-steroidal anti-inflammatory drugs (NSAIDs) and antimalarials such as hydroxychloroquine. Low dose oral corticosteroid (less than 7.5mg/day of prednisolone) or intermittent intramuscular corticosteroid injections may also be necessary. Addressing potential side-effects such as NSAID induced peptic ulceration or corticosteroid induced bone loss proactively with appropriate gastroprotection and bone protection is essential (Ioannou and Isenberg, 2002). More severe disease often associated with one or more major organ dysfunction that may occur over a relative short period of time requires prompt and more intense immunosuppression.

The ultimate aim is to prevent or minimise major organ damage. Higher doses of prednisolone (more than 7.5mg/day) in combination with cytotoxic agents are used. Drugs most frequently employed are oral azathioprine and cyclophosphamide. For the treatment of lupus nephritis a group from the National Institute of Health (NIH) in Bethesda, USA have shown in early studies that intravenous monthly pulses of cyclophosphamide for six months followed by three monthly pulses for two years has superior efficacy and toxicity profiles as compared to intravenous methylprednisolone (Boumpas et al., 1992). More recently mycophenolate mofetil has been compared to the NIH regimen of pulsed cyclophosphamide and appears to be equally effective with fewer side effects (Chan et al., 2000, Ginzler et al., 2005).

1.3.5 Can novel therapeutic candidates for SLE be applied to the APS?

The treatment for SLE up until now has relied on the use of broad-spectrum cytotoxic agents. Though this has improved mortality rates in SLE over the past four decades current conventional treatment regimens still rely on the use of toxic chemotherapeutic agents that favour the development of infection, which remains a major cause of morbidity and mortality (Moss et al., 2002). Similar dilemmas exist for the treatment of the APS with oral anticoagulation. This may need to be taken indefinitely, often at high intensity regimens and in combination with anti-platelet agents as reviewed in section 1.2.10. Though this has improved the treatment of the APS there exists the inherent problem of haemorrhage and the need to monitor therapy closely to ensure that the level of anticoagulation keeps within the desired therapeutic window. For both SLE and APS there remains an unmet need for therapy that is targeted and safer. This may only be developed through understanding the underlying pathogenesis. The past five years has seen the emergence of a number of novel therapeutic candidates for the treatment of SLE, many of which have demonstrated superior efficacy and safety profiles as compared to current treatments in the setting of clinical trials. SLE is characterised by dysregulation of the immune system with circulating pathogenic autoantibodies. The pathogenesis of APS will be discussed in the following section, however there is sufficient clinical evidence to imply that aPL are pathogenic and promote thrombosis (section 1.2.5). Given that SLE and the APS may be part of the same 'superfamily' of autoimmune disease, as has been proposed recently (Harris and Pierangeli, 2004), which of these novel candidates for the treatment of SLE may also be beneficial in treating APS? Rather than discuss an exhaustive list of numerous current therapies in development for the treatment of SLE, I will only focus on the key candidates for which published clinical trial data exist and which may conceivably be applied to APS based on our current

understanding of this condition. These trials were designed to study the effects on SLE and hence there exist no trial data on the effects of therapy on subgroups of patients who may also have had APS.

LJP394, also known as abetimus (Riquent[®]), is composed of four double-stranded oligodeoxynucleotides attached to a central branched platform. This drug irreversibly binds and cross-links anti-dsDNA B cell receptors on the cell surface, preventing them from receiving T cell help and thus causing them to selectively die (a process termed anergy), hence acting as a toleragen. This treatment was shown to be effective in treating lupus nephritis if patients had high affinity anti-dsDNA antibodies and had a good safety profile (Furie et al., 2001, Alarcon-Segovia et al., 2003b). Though abetimus is unlikely to be of benefit for the APS, the same principle of using peptide therapy composed of an aPL antigenic target for use as a toleragen may have therapeutic potential (Cockerill et al., 2003). The same company that developed LJP394 has pursued this concept and developed a compound named LJP1082 which tolerises B cells that produce antibodies to the N-terminal domain of β_2 GPI. This novel compound is in early clinical trial development (Horizon et al., 2003).

Another treatment that has proved effective for treating SLE also targets B cells by depleting them, but not in an antigen specific way. An anti-CD20 monoclonal antibody (mAb) named rituximab, originally used for the treatment of non-Hodgkin's lymphoma and then successfully extended for use in refractory rheumatoid arthritis (Edwards et al., 2004), has been used to treat patients with severe SLE refractory to all other standard therapies (Leandro et al., 2005). The majority of patients had a sustained beneficial response to just two infusions, with significant reduction in anti-dsDNA levels seen as well as a favourable safety profile. CD20 as well as CD19 is highly expressed on pre-B lymphocytes as well as on resting and activated B lymphocytes, hence rituximab depletes these cells. Not all antibodies are depleted and some antibodies such as protective anti-pneumococcal or anti-tetanus toxoid antibodies curiously remain unchanged (Cambridge et al., 2003). I have demonstrated in a small study that the levels of aCL are significantly depleted in patients with SLE when treated with rituximab (Ioannou et al., 2006a). The possibility of using rituximab for treating APS in cases refractory to oral anticoagulation in order to deplete pathogenic aPL may be an option for clinicians to consider. One recent report described the use of this rationale to treat three patients with refractory APS, one of whom had CAPS. A good response was observed in all

three patients treated with rituximab (Rubenstein et al., 2006). One other case report describes the successful use of rituximab to treat refractory thrombocytopaenia associated with APS in one patient with no other autoimmune disease. Levels of IgM aCL and anti- β_2 GPI antibodies that were positive pre-B cell depletion, became undetectable post-depletion (Trappe et al., 2006). Clearly further studies are required to assess the benefits and risks of such an approach. Depleting B cells may also be achieved by targeting a factor called B lymphocyte stimulator (BLyS) that enhances B cell proliferation and IgG production (Moore et al., 1999). BLyS levels have been shown to correlate with disease activity and anti-dsDNA production (Stohl et al., 2003). A human monoclonal anti-BLyS antibody named LymphoStat B has been developed that blocks the effects of BLyS in cynomolgus monkeys (Baker et al., 2003) and may be entering clinical-trial phase for SLE imminently. A factor such as this may conceivably deplete pathogenic aPL producing B cells.

Autologous stem cell transplantation for severe, refractory SLE has been employed with varying success. Studies in these groups of patients have demonstrated that this therapy induces long-term remission in the majority of patients receiving this aggressive therapy (Burt and Traynor, 2003, Traynor et al., 2000), though the treatment itself is associated with a mortality rate of approximately 7% (Tyndall et al., 1999). One recent study treated 46 patients with SLE, of whom 22 had APS refractory to oral anticoagulation. Post-transplantation, the majority of patients with a previously positive aPL (LA, aCL IgG or IgM - between 70-90%) became negative for a median follow-up of 15 months. 82% of patients had anticoagulation discontinued for a median of four months after transplantation, with 78% remaining thrombosis free for a median of 15 months post transplantation (Statkute et al., 2005).

There are other targets that have been investigated for the treatment of SLE with varying success. Examples include amelioration of T cell co-stimulation by using a CD-40 ligand mAb (IDEC-131 (Kalunian et al., 2002)) or by targeting interleukin-10 (IL-10) by using an anti-IL-10 mAb (Llorente et al., 2000). The rapid developments in a number of possible therapeutic options for SLE have arisen through understanding the underlying pathogenesis, and through rapid developments in biotechnology facilitating the development of this knowledge and the production of agents designed to target very specific components of the immune system. There

exists considerable epidemiological evidence implying that aPL may be pathogenic and enhance the risk of thrombosis (section 1.2.5). However, association does not necessarily define causation. The following section will discuss the aetiology of aPL and evidence from *in vivo* animal experiments supporting the thrombogenic potential of these antibodies.

1.4 ANTIPHOSPHOLIPID ANTIBODIES (aPL)

1.4.1 Development of aPL – genetics versus environment

The precise aetiology of aPL is unknown at present. What is likely, is that APS will arise in an individual who may be genetically predisposed to developing this autoimmune disease and who is exposed to certain antigenic stimulation. Family and population studies have attempted to address the degree to which genetic predisposition may be an aetiological factor, particularly by looking for associations with human leukocyte antigen (HLA) alleles.

A report in 1980 by Exner et al was the first to suggest a familial association, which described the presence of LA in two pairs of siblings (Exner et al., 1980). Subsequent family studies have demonstrated an increased frequency of aCL (Mackworth-Young et al., 1987) and APS (Radway-Bright et al., 2000) in first-degree family members of affected individuals, particularly if the affected patient has an additional autoimmune disease (Weber et al., 2000). LA associated with DR4 or DR7 haplotypes has been described by a number of investigators (Bussel et al., 1983, Rouget et al., 1982, Mackie et al., 1987). Dagenais et al found the haplotype A30; Cw3; B60; DR4; DRw53; DQw3 to be associated with aCL in an English Canadian family, some of whom had evidence of SLE and thrombotic disease and others who had aCL, but no evidence of APS. However, an HLA identical individual had no evidence of aCL (Dagenais et al., 1992). May et al also identified the haplotype DR4; DRw53; DQw3 in a set of twins and their mother who had SLE and APS, however two other HLA identical siblings who had lived all their lives in the same environment as their mother and twin siblings had no evidence of aPL or any other clinical / serological abnormality (May et al., 1993). These family studies indicate that HLA contribution may be an important but not sole determinant of aPL production or expression of the APS clinical phenotype.

With population studies it would be of interest to compare HLA associations between patients with APS and evidence of another autoimmune disease such as lupus, those

that have APS alone and those that have lupus, but no evidence of aPL or APS. This would help resolve the debate as to whether 'primary' or 'secondary' APS are distinct entities or not. Few studies have included cohort numbers of each group that are large enough to make such meaningful comparisons and none have demonstrated any significant differences between the two APS groups (Domenico Sebastiani et al., 2003). One study found that patients with APS and SLE had an increased frequency of HLA-DRB1*03 as compared to patients with APS alone. However, as the authors conceded, this haplotype is associated with SLE rather than specifically seen in patients with SLE and APS (Freitas et al., 2004).

Population studies on patients with APS as the only autoimmune disease have been relatively few. Asherson et al reported on 13 patients from the UK with APS and examined class II and class III genes of the MHC and found differences in the HLA class II region of DR4 and DRw53 in line with the family studies, whereas DR3 was absent (Asherson et al., 1992). Similar associations have been found in a small cohort of Spanish patients with APS and no other autoimmune disease (Camps et al., 1995). In a British study the haplotype DQB1*0604/5/6/7/9-DQA1*0102-DRB1*1302 was associated in 53 patients with APS and anti- β_2 GPI antibodies (Caliz et al., 2001). Studies on a small sample of Mexican patients with APS alone found an increase in the haplotypes HLA-DR5 (Vargas-Alarcon et al., 1995).

There have been many more studies investigating patients with SLE and aPL though in the majority of these studies it is unclear if patients with aPL studied had APS. Comparing different studies reveals a number of inconsistent associations, though it should be underlined many are from varying geographical areas with a mix of ethnic groups. As seen in studies in patients with APS, the presence of aPL in patients with SLE from Australia and the UK has been shown to be associated with an increased frequency of HLA-DR4 (McHugh and Maddison, 1989) and DRw53 (McNeil et al., 1990a). Also HLA-DR7 has been observed with increased frequency in patients with SLE and aPL from Northern Italy (Savi et al., 1988) and one large multicentre European study found all three of these haplotypes were observed with increased frequency (Hartung et al., 1992). In contrast no association with HLA-DR or DQ alleles in aCL positive patients with SLE was observed in a group of Caucasian and Black Americans (Gulko et al., 1993). Given that aPL are heterogeneous in nature, studies have investigated the possibility of genetic susceptibilities for the presence of different types of aPL. One very large multi-centre European study of 577 patients

with SLE found an association of aCL with the haplotype HLA-DRB1*04, -DRB1*07, -DQA1*0201, -DQA1*0301, -DQB1*0302, -DPB1*1501, -DPB1*2301, -DRB3*0301 whereas in the same population anti- β_2 GPI antibodies were associated with -DQB1*0302, -DPB1*0301, -DPB1*1901. No evidence of association with alleles at the DRB4 locus (DRw53) was found with DR4 associated in patients from Spain and Italy (Sebastiani et al., 2003). However, it was not stated in this study whether the aCL group of patients were differentiated into β_2 GPI dependent or independent groups, making interpretation of such results difficult. Arnett et al performed a study analysing the association of anti- β_2 GPI antibodies on three ethnic groups of patients with SLE in America. They found that HLA-DR4 haplotypes were strongly associated with anti- β_2 GPI antibodies with White and Mexican Americans and less so in Black Americans (Arnett et al., 1999).

It is difficult to draw definitive conclusions regarding the genetic susceptibility to develop APS. It is likely that some genetic predisposition to developing aPL exists, but at present it is not accurately defined. To add a further layer of complexity it is possible that genes outside the MHC region may contribute to the development of APS such as polymorphisms in the antigenic protein for example. Polymorphisms in the C-terminal domain V (DV) of β_2 GPI (valine instead of leucine at position 247) correlate with the production of anti- β_2 GPI antibodies in patients with APS (Hirose et al., 1999, Yasuda et al., 2005) (see section 1.6.2). Additional genetic factors in patients with APS may also confer thrombotic risk such as deficiencies in factor V Leiden (Letsas et al., 2005), protein C or protein S (Parke et al., 1992). Future studies aimed at genome wide linkage analyses may help assess genetic risk more precisely.

Regarding environmental triggers, infection with a variety of foreign antigens have been linked to the development of aPL and APS. Numerous infections have been linked to the development of aPL as reviewed by Cervera and Asherson (Cervera and Asherson, 2005). Examples include viruses (such as hepatitis C, human immunodeficiency virus (HIV), Epstein-Barr virus or cytomegalovirus (CMV)), bacterial infection (tuberculosis, leprosy, salmonella, *Coxiella burnetii*), spirochaetes (*Treponema pallidum*) and parasitic infections (malaria, toxoplasmosis). However, in the vast majority of cases the induction of aPL in response to infection is transient and does not result in the development of APS. Some have proposed that aPL may arise through the process of molecular mimicry. For example, peptides that share

peptide sequence homology with bacterial antigens (Pierangeli et al., 2004a) have been shown to bind and inhibit the thrombogenic effects of human aPL. Mice immunised with a CMV derived peptide (Gharavi et al., 2002) or whole bacteria such as *Haemophilus influenza* (Blank et al., 2002) produce aPL that demonstrate pathogenic properties *in vivo* in a mouse model of thrombosis. The development of CAPS has been linked with bacterial infection (Cervera et al., 2004). However, this association does not necessarily mean this is due to molecular mimicry. One possibility is that a systemic infection results in pathophysiological processes such as activation of ECs or exposure of anionic PL on the surface of activated platelets, for example that may lower the threshold at which circulating anti- β_2 GPI antibodies promote pathogenicity. Drugs such as phenytoin, propranolol, hydralazine and cocaine have also been linked to the development of aPL, but not APS (Robertson and Greaves, 2006).

1.4.2 Distinguishing between pathogenic and non-pathogenic aPL

As discussed in the previous section, the majority of aPL that arise from various infections do not give rise to the expression of APS in affected patients. The prevalence of aPL in the healthy population is estimated at between 1% to 5% (Pattison et al., 1993, Shi et al., 1990), yet there exists substantial epidemiological evidence that the presence of aPL promotes thrombotic risk in some people (see section 1.2.5). Hence, it is likely that aPL are very heterogeneous in nature, with some promoting pathogenesis and others not. The nature of aPL that promote thrombosis as opposed to those found in people with no history of thrombosis tend to be of the IgG isotype, have specificity for negatively charged PL and bind anionic PL only in the presence of β_2 GPI.

1.4.3 Evidence from animal studies for aPL promoting thrombosis

The association of aPL as a risk factor for thrombosis, as described in various clinical studies, does not necessarily mean that aPL themselves are thrombogenic. The most persuasive evidence supporting the thrombogenic potential of aPL comes from *in vivo* studies in mice. Two main methods have been employed to induce APS clinical manifestations in mice. One method has been by observing the pro-thrombotic effects of injecting purified aPL derived from humans with APS into mice, otherwise known as passive transfer. Another method is to take immune naïve mice, induce aPL production and observe the effects. This has been achieved by immunising mice with either aPL or an autoantigen (such a β_2 GPI or a peptide mimicking part of this protein) to provoke an antibody response.

Mouse models of thrombosis are few. Pierangeli et al demonstrated that when the femoral vein of a healthy mouse is subjected to a pinch stimulus, the size and longevity of the resultant thrombus is significantly enhanced if the mouse is pre-infused with polyclonal IgG derived from APS patients, as compared to control IgG (Pierangeli et al., 1994). Further studies using the femoral vein pinch model identified four out of seven monoclonal aPL derived from two patients with APS as having the potential to enhance thrombus formation (Pierangeli et al., 2000). In these studies, after the size of the resultant thrombus had been estimated, the cremaster muscle of the anaesthetised mouse was transilluminated under a microscope in order to observe *in vivo* leukocyte adhesion to blood vessel walls. This was confirmed for three out of the four monoclonal aPL that promoted thrombosis. Increased leukocyte adhesion *in vivo* was also observed when mice are given intraperitoneal injections of affinity purified aPL isolated from patients with APS (Pierangeli et al., 1999). Olee et al generated two monoclonal aPL derived from one patient with APS. Both bound CL in a β_2 GPI dependent manner, however only one had an *in vivo* thrombogenic effect using the femoral vein pinch model (Olee et al., 1996). The *in vivo* experiments described using this model highlight the heterogeneous nature of aPL in terms of promoting thrombosis. Blank et al demonstrated in 1991 that the passive transfer of either polyclonal or monoclonal aPL derived from a patient with APS into pregnant ICR mice was associated with lower fecundity rate, increased rates of foetal resorption and lower mean weights of embryos and placenta, mimicking the obstetric features of APS seen in humans (Blank et al., 1991). This effect was not observed on infusing pregnant mice with murine or human IgG lacking CL binding. Similarly pregnant BALB/c mice injected with monoclonal human β_2 GPI dependent aPL resulted in a significantly higher rate of foetal resorption and lower embryo and placental weight as compared to mice injected with a non-CL binding mAb (Ikematsu et al., 1998).

Given that around 30% of patients with SLE also have aPL with around half of them developing features of APS, it may be that some animal models of autoimmune prone disease mimicking lupus could also develop APS features. One such model was described in the NZWxBXSB F₁ strain. These mice develop a lupus-like syndrome with features of APS characterised by thrombocytopenia, coronary vascular disease, nephritis, vascular disease, foetal resorption and skin changes (Hang et al., 1981, Hashimoto et al., 1992). These mice also develop aCL, which recognise murine and

human β_2 GPI and increase in titre with age (Monestier et al., 1996). Another lupus-prone strain of mouse known as the MRL/lpr strain has a recessive lymphoproliferative gene (lpr) encoding for defective Fas, thus leading to defective Fas mediated apoptosis. These mice have been shown to have high levels of IgG aCL and clinical features characteristic of APS, such as poor pregnancy outcome, thrombocytopaenia (Gharavi et al., 1989) and histological evidence of cerebral thrombosis (Smith et al., 1990). Interestingly, a recent model described by Rauch and colleagues demonstrates the production of autoantibodies to SLE autoantigens with evidence of glomerulonephritis in mice in response to immunisation with human β_2 GPI in the presence of lipopolysaccharide (LPS) (Levine et al., 2006).

In 1992 Gharavi et al induced aPL production in mice following immunisation with purified human β_2 GPI (Gharavi et al., 1992). These mice developed thrombocytopaenia and when mated had a high foetal resorption rate (Blank et al., 1994). Pierangeli et al in the femoral vein pinch model observed larger and more persistent thrombus formation in mice immunised with β_2 GPI as compared to those immunised with human serum albumin as a negative control (Pierangeli et al., 1996). Thrombogenic aPL may also be generated by immunising mice with a peptide derived from the CMV virus (TIFI peptide) (Gharavi et al., 2002) or with a peptide bearing sequence homology to the C-terminal PL binding region of β_2 GPI (Gharavi et al., 1999). These experiments and their relevance to the pathogenesis of APS will be discussed in greater detail in section 1.5.

Active induction of APS has also been achieved through utilising the idiotypic network. Naïve BALB/c mice immunised with aPL leads to the generation of antibodies directed against the immunising antibody (anti-idiotypic). The animal subsequently develops autoantibodies against this anti-idiotypic antibody (anti-anti-idiotypic), which may have similar binding characteristics to the original immunising antibody. Bakimer et al immunised mice with a monoclonal human IgM aPL and observed the development of prolonged partial thromboplastin time and pregnancy morbidity. However, the monoclonal aPL was generated from the lymphocytes of a healthy subject who had been immunised with diphtheria and tetanus toxoid, raising potential questions as to the relevance of the findings to APS (Bakimer et al., 1992). A study by Cohen et al took purified IgG and IgM aPL derived from a patient with APS and used these antibodies as the immunising agents. The original IgG aPL was monospecific for binding CL (in the presence of β_2 GPI) binding at high titres, but the

IgM aPL bound CL with low affinity and also bound other antigens such as histones and dsDNA. Mice immunised with the CL specific IgG aPL developed prolonged partial thromboplastin time, thrombocytopenia and severe pregnancy morbidity. Mice immunised with polyspecific IgM aPL had much milder evidence of pregnancy morbidity and no other detectable clinical abnormalities (Cohen et al., 1993). However, it should be noted that other groups have failed to reproduce these experiments (Isenberg et al., 1991). In another mouse model of atherosclerosis (low density lipoprotein (LDL) receptor knockout mice) immunising these mice with human aCL accelerates atherosclerosis (George et al., 1997).

Experiments using mouse models of APS, reviewed by Radway-Bright and Isenberg (Radway-Bright et al., 1999), support the argument that aPL have thrombogenic potential. However, not one animal model encompasses all the multisystemic features of the clinical syndrome as seen in humans. Furthermore, it is apparent that different antibodies from the same or different patients have varying effects in these models. It is likely that this is a function of both the heterogeneous nature of the antibodies and also the models studied. The characterisation of chimpanzee β_2 GPI coupled with the observation of elevated anti- β_2 GPI antibodies in these animals holds the possibility of primate models for investigating the APS (Sanghera et al., 2001).

Having discussed the evidence implicating a causal pathogenic effect of aPL, I will now explore potential mechanisms by which aPL may promote thrombosis and pregnancy morbidity.

1.5 UNDERLYING MECHANISMS OF PATHOGENICITY IN THE APS

APS has a varied clinical phenotype and aPL, as discussed in the sections above, are a heterogeneous group of antibodies even within the same individual. Furthermore, when one considers the likely targets of aPL, PL and PL membranes are involved in numerous biological processes and the physiological function of β_2 GPI as yet, has not been fully elucidated (reviewed in section 1.6). For these reasons, multiple mechanisms through which aPL promote thrombosis have been proposed with no one satisfactory unifying hypothesis. As an overview, the key mechanisms proposed revolve around aPL effects on coagulation (particularly inhibition of fibrinolytic pathways), cell mediated effects (endothelial cells (ECs), platelets and placental trophoblasts) and mechanisms involved in the pathogenesis of atherosclerosis. Which

is the dominant one of these and whether thrombotic or inflammatory mechanisms predominate as yet has not been established.

1.5.1 Effects of aPL on the coagulation pathways

Pro-coagulant and 'anti-coagulant' / fibrinolytic pathways are finely balanced and complex biological processes. The coagulation cascade, ultimately resulting in the formation of fibrin as the key component of a thrombus, consists of an intrinsic and extrinsic pathway. However, this complex coagulation cascade with the potential to form large amounts of fibrin rapidly is regulated by the anticoagulant and fibrinolytic pathways with some factors such as thrombin being a key component of both. The two pathways of coagulation and fibrinolysis are schematically represented in figure 1.1 A) and 1.1 B). Pathology arises when there is a disturbance in the balance between these two pathways. There is evidence that aPL can upset this balance favouring a pro-thrombotic phenotype *in vivo*. This is achieved through aPL promoting components of the coagulation pathway and inhibiting key regulatory components of the fibrinolytic / 'anticoagulant' pathway. The ability of aPL to enhance the production of procoagulant factors may also occur indirectly through cell mediated processes that will be discussed in section 1.5.2.

1.5.1.1 Direct effects of aPL on factors that promote coagulation

Both the extrinsic and intrinsic pathways converge to activate factor X and result in the conversion of prothrombin to active thrombin (figure 1.1 A). The presence of anti-prothrombin (anti-PT) aPL *in vitro* would be likely to have an anticoagulant effect as observed in the LA test and first demonstrated by Bajaj et al in 1983 (Bajaj et al., 1983). However, the presence of these antibodies *in vivo* and the association with thrombosis has not been definitively established in the literature. Some studies report no association with anti-PT antibodies and the presence of thrombosis in patients with APS (Atsumi et al., 2000, Forastiero et al., 1997, Pengo et al., 1996) whilst other studies do report an association (Bertolaccini et al., 1998, Nojima et al., 2001). The most likely reason accounting for this discrepancy could lie in the assay methods employed. Anti-PT antibodies are heterogeneous in nature with some exhibiting different binding abilities depending on different detection methods employed (Galli et al., 1997). If instead of coating PT on a plate, this antigen is coated on an anionic lipid surface such as phosphatidylserine (PS), then serum levels of aPL that bind this PS-PT complex have been found to significantly correlate with LA activity and also correlate with the clinical features of APS (Atsumi et al., 2000).

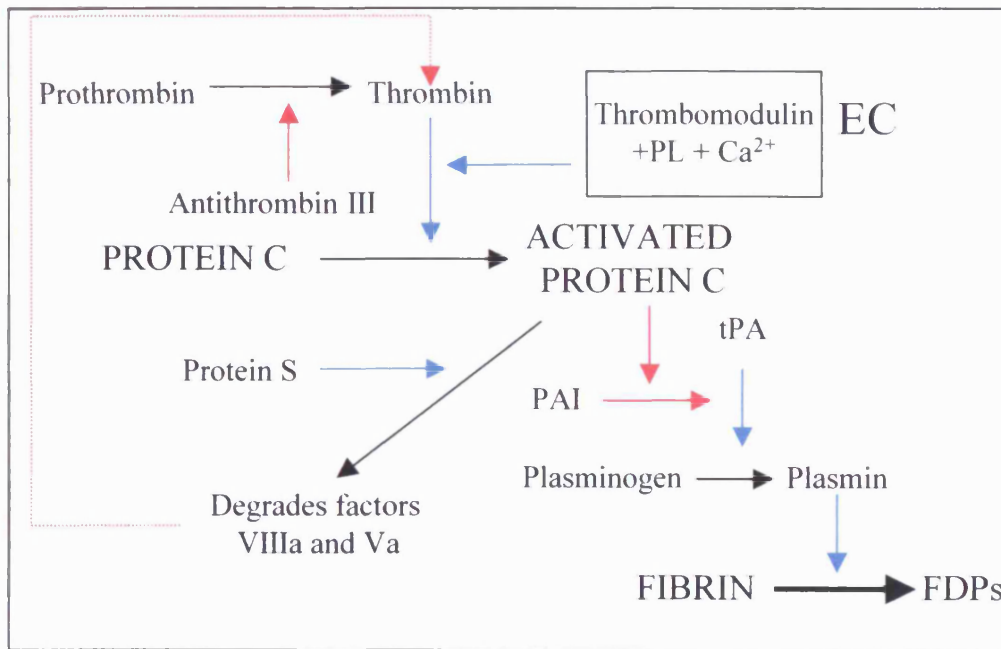
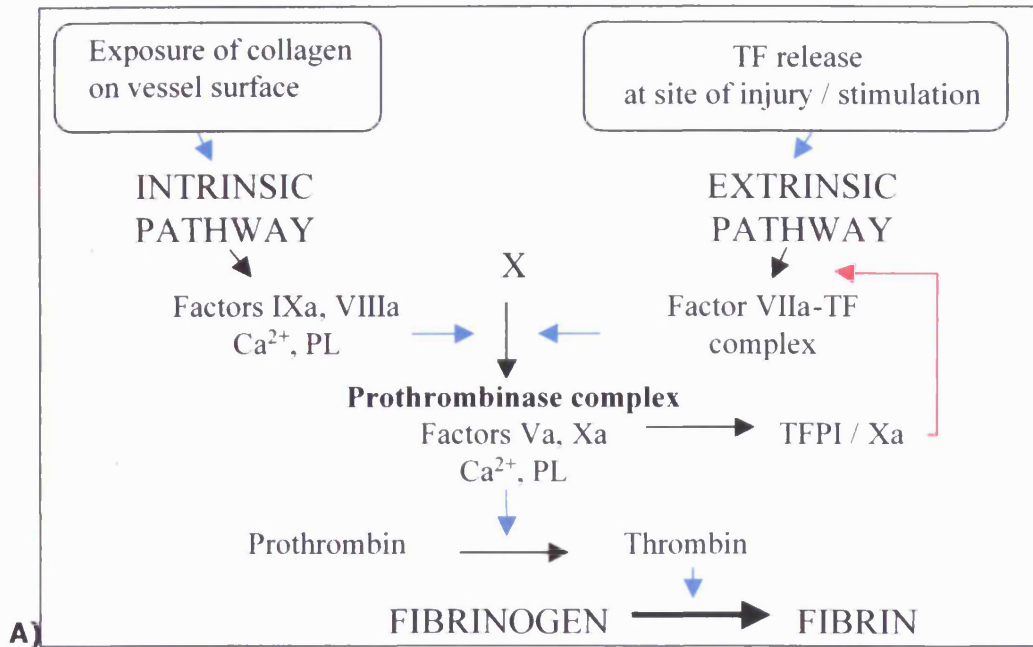


Figure 1.1 Summary of the coagulation and 'anti'coagulation pathways. A), extrinsic and intrinsic coagulation pathways; B) antithrombotic / fibrinolytic pathway. Abbreviations: PL, phospholipids; Ca^{2+} , calcium ions; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; EC, endothelial cell; FDP, fibrin degradation product; TF, tissue factor; TFPI, tissue factor pathway inhibitor. Blue arrows \rightarrow indicate promotion and red arrows \rightarrow inhibition of pathway.

Some advocate that the PS-PT assay be explored as a potential candidate for inclusion in the laboratory classification criteria for APS though further validation studies are required (Atsumi et al., 2004). However, it remains unclear how aPL binding to cryptic epitopes exposed on PT when bound to anionic PL enhance thrombosis rather than inhibit it. Inhibition is what one would intuitively expect and what is seen *in vitro*. One possible mechanism could be that pathogenic antibodies that bind cryptic epitopes on PT may also bind thrombin (given that it is derived from PT) and protect it from deactivation. Support for this hypothesis comes from the observation of a strong association between anti-PT and anti-thrombin antibodies seen in patients with APS, which have been shown *in vitro* to inhibit the deactivation of thrombin by antithrombin III (Hwang et al., 2001).

1.5.1.2 Inhibition of the protein C, thrombomodulin and fibrinolytic pathway.

Protein C is an endogenous vitamin K serine protease that has important antithrombotic mechanisms via an important feedback mechanism for controlling thrombin formation. As shown in figure 1.1 B), this protein is activated when thrombin binds thrombomodulin on the surface of ECs (Esmon, 2000). Protein C then catalyses the degradation of factors Va and VIIIa in the presence of protein S, hence leading to reduced thrombin production. This pathway has been identified as a target for aPL in the APS by numerous studies. The degradation of factor V by protein C has been shown *in vitro* to be inhibited by aPL derived from the serum of patients with APS (Oosting et al., 1993, Marciniak and Romond, 1989, Borrell et al., 1992). An inhibitory effect on the protein C/protein S complex by aPL has also been demonstrated (Malia et al., 1990) and the inhibitory effects of protein C mediated factor V degradation has been shown to be PL dependent, namely phosphatidylethanolamine (Smirnov et al., 1995). This particular PL in the oxidised form has been found to significantly enhance the function of activated protein C (Safa et al., 2001). There are elevated levels of oxidised PL in the serum of patients with APS (Pratico et al., 1999) and PL in the oxidised state has been shown to exhibit greater binding to aPL as compared to aPL binding PL in the reduced state (Horkko et al., 1996). Anti- β_2 GPI antibodies have been shown to bind β_2 GPI when anchored to oxidised PL (Horkko et al., 1997). A recent study by Safa et al demonstrated using *in vitro* studies that monoclonal anti- β_2 GPI, raised from mice immunised with this protein, had the effect of blocking PL oxidation dependent enhancement of activated protein C (Safa et al., 2005). If such a process occurs *in vivo*, then the protective effects of oxidised PL would be abrogated by aPL, leading to reduced activated protein C function and hence a hypercoagulable state.

Activated protein C can also inhibit the activity of plasminogen activator inhibitor (PAI) (van Hinsbergh et al., 1985). This ultimately leads to elevated levels of tissue plasminogen activator (tPA) resulting in the activation of plasmin and consequently fibrinolysis (figure 1.1 B). Hence, aPL inhibition of protein C activity may also promote thrombosis via inhibition of the fibrinolytic pathway. Data supporting the role of PAI and tPA in the APS remains uncertain with conflicting and confusing reports. One small study of 23 patients with SLE found elevated levels of PAI antigen and tPA in patients who had a history of thrombosis or pregnancy morbidity (Violi et al., 1990). Whether these patients had APS or not was not clarified. A study by Cugno et al demonstrated significantly elevated levels of antibodies to tPA in 14 out of 91 patients with APS as compared to the none out of 23 SLE or 3 out of 91 healthy control subjects studied. The two samples of IgG that were the best binders to tPA were shown to interact with the catalytic domain of tPA (Cugno et al., 2004). However, other studies have failed to demonstrate any significant differences between SLE disease control and aPL positive patients (Keeling et al., 1991) or APS patients (Mackworth-Young et al., 1995) with respect to levels of tPA and PAI. Again, as seen with the studies investigating binding of aPL to prothrombin, one reason for these discrepancies seen may be due to the assays employed. A recent study by Lu et al demonstrated significant binding of monoclonal and polyclonal IgG aPL to tPA only when associated with fibrin. The authors postulated that tPA when bound to fibrin exposes aPL binding epitopes (Lu et al., 2005).

The activation of protein C by thrombomodulin may also be another target for aPL though again there are conflicting reports in the literature. aPL derived from patients with positive LA activity have been shown to inhibit the activity of thrombomodulin (Freysinet et al., 1986, Cariou et al., 1988). However, other groups have failed to show evidence of aPL binding thrombomodulin (Keeling et al., 1993, Watson and Schorer, 1991) and it remains uncertain whether this is an important pathway in the pathogenesis of the APS.

An inherited deficiency of protein C results in recurrent thrombotic disease (Griffin et al., 1981). There have been a few case reports of patients with APS that have protein C or S deficiencies (Parke et al., 1992). However, one study of 74 patients with SLE found no association with these deficiencies and the occurrence of thrombosis (Hasselaar et al., 1989). These inherited deficiencies are typically

associated with venous rather than arterial thrombosis. The clinical pattern of APS is, however different, and is one of both arterial and venous thrombosis in addition to pregnancy morbidity. Despite the wealth of evidence described above, this incongruence makes aPL disruption of the protein C pathway difficult to accept as the dominant mechanism in inducing disease.

1.5.1.3 Effects of aPL on annexin V

This protein has potent anticoagulant activity by virtue of its high affinity for anionic PL surfaces. It is shaped like a concave disk and aggregates on the surface of PL membranes forming a crystal lattice that has the effect of displacing and blocking accessibility of PL by coagulation factors (Reutelingsperger et al., 1988). It is produced by ECs and is also likely to be an important natural anticoagulant in pregnancy as it is produced by placental precursor cells, trophoblasts, and is essential for the maintenance of placental integrity (Wang et al., 1999). Annexin V may be displaced from PL by aPL from patients with APS. This has been shown with both polyclonal (Rand et al., 1998) and monoclonal IgG aPL (Rand et al., 2003). This effect is not observed when using aPL derived from patients with syphilis (Wu et al., 2006) or patients with aPL and no history of thrombosis or pregnancy morbidity (Rand et al., 2004). Anti-annexin V antibodies are observed in 35-50% of patients with APS, but do not predict risk of thrombosis or pregnancy morbidity (de Laat et al., 2006a, Arnold et al., 2001, Ogawa et al., 2000). However, one polymorphism in annexin V (Cys to Thr at position 1) is an independent risk factor for pregnancy morbidity, which is an observation independent from APS (de Laat et al., 2006a). The levels of annexin V production by cells within the vascular and placental beds may be reduced by aPL induced cell mediated effects and this is discussed further in sections 1.5.2 and 1.5.3 respectively. However, uncertainties exist regarding the contribution of annexin V as an anticoagulant shield *in vivo* given the observation that annexin V deficient mice have no evidence of an increased propensity for thrombosis or pregnancy morbidity (Brachvogel et al., 2003).

1.5.2 Cell mediated effects of aPL

1.5.2.1 aPL induced activation of endothelial cells

The endothelium, previously thought to represent a 'passive' barrier between blood and tissues, is now known to be a dynamic and heterogeneous organ that plays an active role in the regulation of inflammatory responses, haemostasis and vessel tone (Cines et al., 1998). In 1995 two studies described the ability of polyclonal IgG aPL

to activate cultured human ECs by upregulating adhesion molecules (Simantov et al., 1995) and that this process was dependent upon the presence of serum containing the cofactor β_2 GPI (Del Papa et al., 1995). These findings have been confirmed and extended to monoclonal aPL by other groups and the most common adhesion molecules upregulated by aPL and detected as part of the proinflammatory phenotype include E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and P-selectin (George et al., 1998c, Pierangeli et al., 1999). Levels of soluble ICAM-1 and VCAM-1 in the plasma of patients with APS and thrombosis are elevated (Kaplanski et al., 2000, Stalc et al., 2005). Pierangeli et al demonstrated *in vivo* activation of ECs by aPL by direct visualisation of the adherence of leukocytes to the inner walls of venules within the cremaster muscle in anaesthetised mice (Pierangeli et al., 2000). This aPL induced leukocyte adherence was significantly reduced in ICAM-1 deficient mice and completely abrogated in mice deficient in both ICAM-1 and P-selectin. Infusion of an anti-VCAM-1 antibody also reversed the aPL induced leukocyte vessel wall adherence. The normal mice in these experiments developed enhanced thrombus formation when infused with aPL, which again was reversed in ICAM-1/P-selectin deficient mice and in mice infused with anti-VCAM-1 antibody. (Pierangeli et al., 2001). Similar inhibition of *in vivo* leukocyte vessel wall adherence accompanied by reduced thrombus formation was observed in C3 and C5 deficient mice, suggesting that an intact complement pathway is necessary for aPL induced EC activation and thrombosis (Pierangeli et al., 2005). IgG aPL have also been shown in one study to induce production of monocyte chemoattractant protein-1 when incubated with human ECs (Cho et al., 2002). These studies confirm that aPL bind to ECs and induce a proinflammatory and proadhesive phenotype.

ECs are integral to the function of normal vessel tone. These cells are capable of producing a number of factors that may either cause vasoconstriction, such as endothelin-1 or platelet activating factor, or vasodilatation such as prostacyclin or nitric oxide (NO) (Cines et al., 1998). In fact, the first report of aPL having a potential effect on ECs was made by Carreras et al on detecting reduced levels of prostacyclin in a woman with circulating LA (Carreras et al., 1981a). One study has demonstrated elevated levels of endothelin-1 in patients with APS and a history of arterial thrombosis (Atsumi et al., 1998). However, there are other conflicting studies that have not shown elevated levels of endothelin-1 or of other adhesion molecules in the serum of APS patients (Williams et al., 2000). One method of assessing EC

dysfunction is by measuring the degree of brachial artery vasodilatation that may be induced by increased blood flow-shear (known as flow-mediated vasodilatation) or glycerol trinitrate (Vogel, 2001). A study of 25 APS patients versus 25 age and sex matched control subjects found reduced flow-mediated and nitrate induced brachial artery vasodilatation in the APS group. In addition these APS patients had elevated levels of soluble VCAM-1 and ICAM-1 (Stalc et al., 2005). Delgado-Alves et al demonstrated increased production of inducible NO synthase (iNOS) in response to a monoclonal aPL (IS4) in an *ex vivo* and *in vivo* rat model of NO production. This was coupled with down-regulation of the physiological endothelial NOS (eNOS), resulting in a phenotype consistent with oxidative stress (Delgado Alves et al., 2006). Prolonged exposure to IS4 in mice resulted in overall reduced production of NO (Delgado Alves et al., 2005).

1.5.2.2 Tissue factor in the APS

Tissue factor (TF) is a glycoprotein responsible for being the major initiator of the extrinsic coagulation cascade, often at a site of vascular injury, and is an essential cofactor for factor VIIa to efficiently cleave factor X to its active form Xa (see figure 1.1 A). Functional TF is expressed on the surfaces of ECs, monocytes, leukocytes and other cells in contact with free-flowing blood in response to stimulation with a variety of agents including P-selectin, chemokines, LPS and inflammatory cytokines such as IL-1 and tumour necrosis factor- α (TNF- α) (Wolberg and Roubey, 2004b). Most studies investigating the role of TF in the pathogenesis of the APS have focussed on experiments using monocytes. Numerous groups have found that APS derived serum, plasma, monoclonal and purified polyclonal aPL had the effect of enhancing TF expression and procoagulant activity on normal monocytes (Kornberg et al., 1994, Schved et al., 1992, Amengual et al., 1998, Reverter et al., 1998). APL induction of TF expression may be achieved using the F(ab)₂ portion of monoclonal aPL suggesting that this process is not Fc mediated (Schved et al., 1992). Circulating levels of TF are significantly greater in patients with aPL and APS as compared to patients with aPL and leprosy without clinical features of APS (Forastiero et al., 2005).

Non-functional TF is produced by many cell types in an encrypted form, thought to be due to dimerisation of TF molecules (Bach and Moldow, 1997). Rapid dissociation of these dimers into the active or de-encrypted form of TF is a method of producing significant amounts of active TF very rapidly. There is no change in the number of TF molecules and very little delay that would otherwise be observed if increased TF

activity in response to stimulants was due solely to cellular up-regulation of TF. Stimulants that have been found to cause de-encryption of TF are calcium ionophore, hydrogen peroxide and freeze/thaw cycles (Wolberg and Roubey, 2004b). Anionic PL expression may also lead to changes in the TF molecule (Wolberg et al., 1999). Current evidence points towards aPL causing increased TF activity by the more gradual process via inducing increased cellular expression of TF, rather than by causing de-encryption of inactive TF. *Ex vivo* monocytes derived from patients with APS and thrombosis, grown in tissue culture, were found to express elevated levels of TF (Cuadrado et al., 1997) and have increased levels of TF messenger ribonucleic acid (mRNA) as compared to monocytes grown from patients with APS and no thrombosis, thrombosis and no APS and healthy control subjects (Dobado-Berrios et al., 1999). A later study by Wolberg and Roubey found no evidence of increased TF de-encryption when culturing non-stimulated monocytes with β_2 GPI and affinity purified anti- β_2 GPI antibodies, whereas significant activation of TF was observed upon adding calcium ionophore (Wolberg and Roubey, 2004a).

Tissue factor pathway inhibitor (TFPI) complexes with factor Xa and acts as a regulator of the TF/VIIa complex (see figure 1.1 A). Reduced levels or activity of the TFPI regulator would be expected to cause an enhanced thrombotic tendency. One study demonstrated an increase in TF stimulated factor Xa generation in plasma when incubated in the presence of anti- β_2 GPI antibodies. The authors postulated that this was indirect evidence of suppression of TFPI-dependent inhibition of TF (Salemink et al., 2000). Functional anti-TFPI antibodies have been identified in a small subgroup of patients with APS (Adams et al., 2001) and have been found to be associated with arterial thrombosis and stroke (Forastiero et al., 2003).

1.5.2.3 Platelet pathology in APS

These cells adhere and aggregate at a site of injury very rapidly, forming a platelet plug. Platelets may be activated by a number of platelet or EC derived activating factors such as P-selectin, which may also subsequently bind to and cause activation of the complement system (Del Conde et al., 2005). Activated platelets express anionic PL on their surface, providing a surface through which PL dependent coagulation reactions may occur (see figure 1A) (Heemskerk et al., 2002). Clearly platelets play a central role in coagulation yet their role in the pathogenesis in APS is not as well characterised as that of the ECs. Joseph et al analysed the degree of activation of platelets derived from 20 patients with APS and compared the results to platelets derived from patients with SLE, but no APS and healthy subjects. Only

platelets derived from the APS group demonstrated significant activation as assessed by detecting CD63 expression and conformational change in glycoprotein IIb-IIIa (GPIIb-IIIa) by binding to a specific monoclonal antibody named PAC-1 that recognises this protein (Joseph et al., 2001). Monoclonal anti- β_2 GPI antibodies have been shown to enhance adherence of platelets to vascular subendothelium as shown in an *in vitro* model of platelet activation (Font et al., 2002). Robbins et al demonstrated that aPL induced activation of platelets, as assessed by measuring thromboxane A2 production, is dependent upon the presence of β_2 GPI (Robbins et al., 1998). Pre-incubating platelets with agonists of cyclic-adenosine monophosphate (cAMP) has the effect of reducing aPL/ β_2 GPI induced thromboxane A2 production (Opara et al., 2003).

Convincing evidence for platelets having a direct role in the pathogenesis of APS comes from a study by Lutters et al which describes the use of an *in vitro* flow system in which whole blood from healthy donors is passed over collagen. Increased platelet deposition on the collagen surface was observed when anti- β_2 GPI antibodies were added. Also dimers of β_2 GPI, which have been shown in previous studies to exhibit LA activity (Lutters et al., 2001), had the same effect of enhancing platelet adherence to collagen. Co-immunoprecipitation revealed that dimeric β_2 GPI interacted with apolipoprotein E receptor 2 (apo ER2), a member of the LDL receptor family present on platelets (Lutters et al., 2003). Pierangeli et al demonstrated that mice infused with a monoclonal antibody against GPIIb-IIIa or mice deficient in this glycoprotein receptor do not develop enhanced thrombus formation on infusion of aPL (Pierangeli et al., 2004b). Similar results were observed in a hamster model of photochemical induced thrombosis. A human monoclonal anti- β_2 GPI antibody with cross reactivity to hamster β_2 GPI caused enhanced formation of platelet rich thrombus in the carotid artery of an anaesthetised hamster (Jankowski et al., 2003).

A recent study has shown that β_2 GPI dependent aPL crosslink β_2 GPI bound to the surface of human platelets via the cell surface receptor GP Ib α -IX-V complex (Shi et al., 2006). Activation of platelets by aPL in the presence of β_2 GPI is inhibited by anti-GPIb α monoclonal antibodies and β_2 GPI was shown to bind GPIb α *in vitro*. Given that ECs are also capable of expressing this surface receptor complex (Beacham et al., 1997, Beacham et al., 1999) this may provide a unifying mechanism through which β_2 GPI dependent aPL activate ECs and platelets and promote a thrombophilic

phenotype. Whether placental trophoblasts also express this cell surface glycoprotein receptor is currently unknown.

The mechanism of thrombocytopenia in patients with APS who do not have SLE is less clear. It tends to be mild and does not usually lead to problems of bleeding. It may be due to increased consumption though this has not been demonstrated. Antibodies to platelet glycoproteins have been demonstrated in patients with APS and thrombocytopenia and may be involved (Galli et al., 1994, Godeau et al., 1997). An interesting observation made recently in one study was that patients suffering from SLE and / or APS with LA activity also had a tendency to have prolonged bleeding times, even in the absence of thrombocytopenia or any drugs that could affect platelet function. The authors went on to assess platelet function using *in vitro* systems and also assess levels of von Willebrand factor, which were normal (Urbanus et al., 2004). This paradoxical finding of a prolonged bleeding time in patients with a probable tendency to develop thrombosis (much like LA activity) could not be explained and it is likely that aPL have complex effects on haemostasis and platelet function.

1.5.2.4 Activation of autoreactive CD4+ positive T cells

The production of aPL by activated B cells is dependent on T cell help. Autoreactive CD4+ T cells specific for β_2 GPI were found in eight out of 18 patients with APS, but not found in healthy controls (Visvanathan and McNeil, 1999). Hattori et al identified β_2 GPI specific T cell clones derived from all 12 APS patients with anti- β_2 GPI antibodies tested. However, β_2 GPI reactive CD4+ T cell clones were also identified in 12 out of 25 patients who did not have APS and were either patients with SLE or healthy controls (Hattori et al., 2000). All β_2 GPI reactive T cell clones would only react to reduced and not to native β_2 GPI. The authors postulated that this was due to β_2 GPI reactive T cell clones recognising cryptic epitopes exposed on reduced β_2 GPI and hidden when in the native state. This work was then extended in an attempt to identify the dominant peptide region of β_2 GPI responsible for activation of β_2 GPI autoreactive T cells. A total of 14 β_2 GPI reactive T cell clones were generated from three APS patients and were shown to react to a peptide encompassing residues 276 to 290 within DV (Arai et al., 2001). This is the PL binding region within DV (section 1.6.4). This led to experiments demonstrating that macrophages or dendritic cells pulsed with PL bound β_2 GPI induce a response of T cell clones specific for the β_2 GPI derived 276-290 peptide. This effect was not observed when macrophages were pulsed with native β_2 GPI not bound to PL or to PL alone, suggesting that processing

and antigen presentation of β_2 GPI bound to PL is essential for β_2 GPI autoreactive T cell activation (Kuwana et al., 2005).

1.5.2.5 Intracellular signalling pathways in the APS

There is a relatively large wealth of knowledge on the functional outcomes of cells when stimulated by aPL, but relatively little work up until recently has been done on dissecting the intracellular signalling events. The past five years has seen considerable advancement of our knowledge in this area in the APS. The three groups of cell types in which aPL induced intracellular signalling pathways have been described are ECs, platelets and monocytes.

Meroni et al stimulated human umbilical vein ECs (HUVECs) with polyclonal and monoclonal anti- β_2 GPI antibodies and demonstrated expression of adhesion molecules coupled with activation of NF- κ B, as observed when cells were stimulated with TNF α used as a positive control (Meroni et al., 2001). Shortly after this report, a study from Dunoyer-Geindre et al confirmed the same findings. This study correlated aPL induction of TF and adhesion molecules by ECs with NF- κ B translocation from the cytoplasm to the nucleus, but found that the observed response was not as rapid or pronounced as that seen when cells were stimulated with TNF α (Dunoyer-Geindre et al., 2002). This would suggest that the upstream pathways from aPL and TNF α cellular activation leading to NF- κ B activation are not the same. A further study by Meroni's group dissected this out further (Raschi et al., 2003). TRAF and MyD88 are adapter proteins that associate with the intracellular (TIR) domain of an activated TLR to phosphorylate IRAK leading to the activation of the NF- κ B signalling cascade (see section 1.1.1.3). TRAF-2 is employed in TNF α signalling pathways whereas TRAF-6 is critical to IL-1 and LPS signalling pathways along with MyD88 (Muzio et al., 1998). Raschi et al used ECs transfected with negative dominants of TRAF-2/-6 or MyD88 and found that incubation with monoclonal and polyclonal anti- β_2 GPI antibodies induced a signalling cascade comparable to that activated by LPS or IL-1. Assessment of the IRAK phosphorylation time kinetic suggested that aPL activate ECs through TLR-4 involved in the LPS pathway (Raschi et al., 2003). Another study demonstrated that aPL induction of TF production in ECs is dependent upon phosphorylation of the LPS induced kinase p38 mitogen activated protein kinase (MAPK), upstream of NF- κ B activation (Vega-Ostertag et al., 2005). One hypothesis is that there exists molecular mimicry between TLR-4 binding ligands and β_2 GPI. As briefly discussed in section

1.4.1, viral and bacterial derived peptides that constitute the natural ligands for TLRs bind anti- β_2 GPI antibodies. It is possible that aPL bind to TLR-4 and β_2 GPI on the cell surface, resulting in cross-linking of this complex and cellular activation. The co-receptor annexin II may also be involved as aPL have been shown to cross-link annexin II via bound β_2 GPI (Zhang and McCrae, 2005). As this protein does not display an intracellular domain, however, TLR-4 is required to effect cellular activation. A polymorphism in the *tlr-4* gene associated with a blunted LPS response is seen less frequently in patients with APS as compared to healthy subjects (Pierangeli et al., 2005b). However, direct binding of aPL to TLR has as yet not been demonstrated and only inferred (Raschi et al., 2003). Furthermore, contamination with LPS, the natural ligand for TLR-4, may occur at levels of LPS minute enough to escape detection by conventional LPS assay systems (Rifkin et al., 2005).

Less is known about the intracellular events in platelets and monocytes that occur following activation by aPL. One study treated platelets with low dose thrombin and IgG aPL, both whole IgG and their F(ab)₂ fragments. A significant increase in the phosphorylation of p38 MAPK (as seen in ECs), but not ERK-1/2 MAPKs was seen in the aPL treated platelets. Downstream to phosphorylation of p38 MAPK in platelets, there is calcium dependent phosphorylation of the enzyme phospholipase A₂ (cPLA₂) which catalyses the formation of thromboxane A₂ from acetylsalicylic acid. Phosphorylation of cPLA₂ induced by aPL was also demonstrated and a specific inhibitor of this prevented aPL induced thromboxane A₂ production (Vega-Ostertag et al., 2004). A recent study has shown that platelet activation of β_2 GPI dependent aPL via the GPIb α surface receptor induces activation of the PI3K / AKT pathway downstream of GPIb α activation (Shi et al., 2006). This is also the pathway activated by von Willebrand factor induced platelet activation (Jackson et al., 1994), which is an important pathway for the induction of platelet adhesion integrins crucial for mediating platelet to platelet / vessel interactions (Chen et al., 2004). Phosphorylation of p38 MAPK also occurs in monocytes when stimulated with aPL. Bohgaki et al demonstrated that monocytes incubated with a monoclonal β_2 GPI dependent aPL induced mRNA expression of TF, which was dependent upon p38 MAPK activation. NF- κ B nuclear translocation was shown to occur downstream to p38 MAPK phosphorylation (Bohgaki et al., 2004). A subsequent study by Lopez-Pedrerera et al confirmed these findings in monocytes with polyclonal IgG anti- β_2 GPI antibodies and also demonstrated the simultaneous activation of another MAPK cascade, namely the ERK/MAP pathway (Lopez-Pedrerera et al., 2006). This pathway

specifically phosphorylates and transactivates transcription factors such as Elk-1 which then regulates expression of genes such as TF (Chang et al., 2003).

1.5.3 Mechanism for pregnancy morbidity, the complement pathway and cytokines

The mechanisms for pregnancy morbidity in APS are complex. Direct evidence for aPL having a pathogenic effect comes from studies on pregnant mouse models (as reviewed in section 1.4.3) and are supported by the strong association of aPL and adverse foetal outcomes in patients with APS (section 1.2.5). β_2 GPI dependent aPL have also been shown to bind to human trophoblast cells *in vitro* (Di Simone et al., 2000). No evidence for thrombosis has been observed in the foetuses of pregnant mothers with APS, and aPL causing uteroplacental insufficiency is the most common cause. There are no pathognomonic histological changes seen in the placental beds of APS patients who have associated pregnancy morbidity. Characteristic histological features as reviewed by Gharavi et al include hyperplastic vessels, vasculopathy of uterine arteries (spiral arteries) with fibrinoid necrosis and infiltration of lipid-laden macrophages accompanied by areas of placental infarction (Gharavi et al., 2001). Worse outcomes tend to occur when multifocal areas of uteroplacental thrombosis are observed. One mechanism by which aPL may lead to a hypercoagulable state in the uteroplacental bed may be via annexin V (see section 1.5.1.3). Annexin V is expressed at high levels on the surface of trophoblastic cells. Decreased annexin V has been found on the villi of APS placenta (Rand et al., 1994) and aPL have been shown to reduce annexin V expression on cultured trophoblasts and placental villi (Rand et al., 1997, Vogt et al., 1997). However, the precise role of annexin V in the pathogenesis of the APS remains uncertain with the observation that annexin V deficient mice do not have an increased propensity for thrombosis or pregnancy morbidity (Brachvogel et al., 2003).

Hyperplasia of vessels with infiltration of inflammatory cells and low grade vasculopathy rather than profound vasculopathy and thrombosis seem to be the dominant lesions seen in affected placentas from patients with APS (Stone et al., 2005). The gross histological changes in the placental beds of patients with APS are almost indistinguishable from those changes seen in the placentas derived from patients with pre-eclampsia and no evidence of aPL / APS (Gharavi et al., 2001). Hence, it seems likely that aPL may mediate uteroplacental pathogenesis through other non-thrombotic mechanisms. Within the last several years, two lines of evidence exist for aPL having an autoimmune inflammatory effect on the

uteroplacental bed. There is emerging evidence of oxidative stress being important in contributing to placental insufficiency seen in conditions such as pre-eclampsia leading to foetal growth retardation (Roberts and Hubel, 2004). LDL may be oxidised by cells such as the trophoblast at the maternal-foetal interface (Bonet et al., 1998) and this can inhibit trophoblast invasion seen *in vitro*. aPL have been found to interact with oxidised LDL and may mediate placental injury through oxidative damage of the placental vascular endothelium (Hasunuma et al., 1997, Horkko et al., 1996). However, there is lack of direct evidence of aPL effecting oxidative damage at the maternal-foetal interface. LDL-receptor deficient mice have been studied to investigate pro-atherogenic effects of aPL (George et al., 1998a). It would be of interest to study the effects of aPL induced pregnancy morbidity in this strain of mice.

A second mechanism for which more convincing evidence exists is the role of complement. A fully active complement system at the foetal-maternal interface is essential to the progression of normal pregnancy as reviewed by Girardi et al (Girardi et al., 2006). However, this needs to be controlled by complement regulatory proteins such as decay-accelerating factor (DAF, also known as CD55), CD59 and complement receptor 1-related protein y (Crry). Deficiency of complement regulatory proteins in utero, such as Crry, leads to progressive embryonic lethality (Xu et al., 2000). Salmon and colleagues demonstrated that pregnancy morbidity observed in mice upon passive transfer of IgG aPL can be abrogated with C3 inhibitors such as Crry or inhibitors of C5 activation. Furthermore, mice deficient in either C3 or C5 did not have a tendency to develop aPL induced pregnancy morbidity (Holers et al., 2002, Girardi et al., 2003). The therapy for pregnant patients with APS is heparin (section 1.2.10.2) and the ability of heparin to inhibit complement has been known for many years (Weiler et al., 1978). Heparin given to mice can prevent aPL induced pregnancy morbidity and is associated with reduced complement activation. No protection with oral anticoagulation such as a thrombin inhibitor (hirudin) or a factor Xa inhibitor (fondaparinux) was observed in these mice (Girardi et al., 2004). Complement has also been implicated in the production of thrombosis in APS. Passive transfer of IgG with anti- β_2 GPI activity promoted the development of thrombosis in rats, which was not observed upon the passive transfer of IgG depleted of β_2 GPI binding antibodies. Histological examination of the mesenteric vasculature of aPL treated rats revealed co-localisation of C3 and C9. Rats deficient

in C6 or pre-treated with anti-C5 antibody were protected from anti- β_2 GPI induced thrombosis (Fischetti et al., 2005).

There exists convincing evidence as detailed above that complement plays an important role in the pathogenesis of APS. However, there are some inconsistencies that warrant discussion. A study by Jankowski et al demonstrated in a hamster model of carotid artery thrombosis that passive transfer of F(ab)₂ antibodies capable of binding β_2 GPI promoted thrombosis to a similar degree as when transferring whole IgG aPL (Jankowski et al., 2003). Other studies have also demonstrated the ability of F(ab)₂ aPL fragments to cause activation of platelets (Lutters et al., 2003, Robbins et al., 1998). F(ab)₂ fragments are unable to fix complement, which is dependent on the presence of the Fc portion of antibody. Hence, it seems likely that aPL induce thrombosis through mechanisms other than fixing complement. One possibility is that platelet activation is known to cause activation of the complement cascade, possibly via the production of P-selectin (Del Conde et al., 2005)

Cytokine abnormalities have also been implicated in the pathogenesis of foetal loss. Mice deficient in C3 or C5 and protected from aPL induced foetal loss also have reduced serum levels of circulating TNF α . TNF α release occurs downstream to complement activation and blockade of TNF α in pregnant mice prevents aPL induced morbidity, providing a potential target for therapeutic intervention (Berman et al., 2005). Normal placental development has been shown to be promoted by cytokines IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF). Levels of IL-3 in normal pregnant women peak at delivery and sharply fall upon delivery of the placenta (Fishman et al., 1992b). Pregnant BALB/c mice immunised with a human monoclonal aPL develop foetal loss and have reduced levels of IL-3 and GM-CSF (Fishman et al., 1992a). Passive transfer of aPL into ICR pregnant mice causes foetal resorption, which can be prevented if IL-3 is co-administered (Fishman et al., 1993). Levels of IL-3 are reduced in pregnant patients with APS (Shoenfeld et al., 1998, Fishman et al., 1996). Fishman et al also showed that monocytes cultured *in vitro* can be stimulated to produce IL-3 if they are incubated with low dose aspirin while higher doses of aspirin failed to induce IL-3 production. The authors suggested that low-dose aspirin protection in pregnant patients with APS could in part be related to effects on IL-3 production (Fishman et al., 1996).

1.5.4 Atherosclerosis and aPL

The risk of aPL promoting atherosclerosis is contentious. Current clinical and epidemiological studies support the theory that aPL enhance the risk of developing arterial thrombosis in the presence of atherosclerosis or other traditional risk factors, but do not cause progression of atherosclerosis per se (discussed section 1.2.6). The balance of evidence investigating the pathogenic role of aPL in atherosclerotic plaque development is equally contentious.

Evidence of pathogenic mechanisms by which aPL may promote atherogenesis have been explored by multiple groups. β_2 GPI is found in atherosclerotic plaque lesions (George et al., 1999). Immunisation of atherosclerosis-prone mice with β_2 GPI accelerates plaque formation, and transfer of β_2 GPI-reactive lymphocytes in LDL deficient mice promotes the development of precursor lesions to atherosclerosis such as fatty streaks (George et al., 2000). LDL-receptor deficient mice immunised with β_2 GPI also develop early atherosclerotic lesions (George et al., 1998a). There is some controversy as to whether patients with APS have anti-oxidised LDL antibodies that cross-react with β_2 GPI (Vaarala et al., 1993, Tinahones et al., 1998). A ligand that binds β_2 GPI derived from oxidised LDL has been isolated by one group. Liposomes that contain this ligand are taken up by macrophages *in vitro* and this process is enhanced in the presence of β_2 GPI (Kobayashi et al., 2001). Pathogenesis may also be mediated by aPL targeting protective elements of atherogenesis. High-density lipoprotein (HDL) inhibits the oxidation of LDL and apolipoprotein A-I (apo A-I) stabilises paraoxonase, an antioxidant enzyme within the HDL particle. Patients with APS have been shown to exhibit elevated levels of antibodies to HDL and apo A-I that can cross-react with CL (Delgado Alves et al., 2003). Levels of anti-HDL antibodies have an inverse correlation to levels of paraoxonase activity in patients with APS (Delgado Alves et al., 2002).

Whether aPL promotes atherogenesis remains unclear as there is evidence to suggest that antibodies to oxidised-LDL may have a protective role. Hypercholesterolaemic rabbits immunised with oxidised LDL exhibit reduced atherosclerotic development (Ameli et al., 1996). Mice deficient in apo ER2 or LDL receptors immunised with oxidised LDL produce anti-oxidised LDL antibodies that also seem to have a protective role in atherosclerotic plaque development (George et al., 1998b, Zhou et al., 2001). This protective immunity is reduced if mice are

depleted of B cells (Caligiuri et al., 2002). Passive infusion of aPL also reduces the development of atherosclerosis in LDL deficient mice (Nicolo et al., 2003).

It seems likely that given the heterogeneity of aPL, there exist populations of aPL which have either protective or harmful effects upon the development of atherosclerosis. Identifying epitopes recognised by the protective group of antibodies may aid the development of future therapies.

1.5.5 Lessons from pathogenic mechanisms: current and future therapies

A host of pathogenic mechanisms have been described accounting for the pathogenic effects of aPL. Given the ubiquitous and heterogeneous nature of PL matched by the heterogeneity of aPL, it is perhaps unsurprising that there exist many different complex mechanisms by which aPL can effect pathogenicity as described above. What has become apparent is that current therapies may work through mechanisms that were not anticipated when these therapies were first used in APS. Heparin used in pregnancy may primarily work by inhibiting complement rather than through a direct anti-thrombin III effect (Girardi et al., 2004). Aspirin may work through multiple mechanisms. As well as an antiplatelet effect, low-dose aspirin may stimulate the production of IL-3 that may have a protective role in pregnancy (Fishman et al., 1996). More recently aspirin has been shown to inhibit aPL activation of ECs *in vitro* (Dunoyer-Geindre et al., 2004), which could be mediated through aspirin inhibition of NF- κ B activation (Kopp and Ghosh, 1994, Weber et al., 1995). Other newer oral anticoagulants may have some benefit over warfarin. Ximelagatran is an oral thrombin inhibitor, as effective as warfarin given in a fixed dose with the advantage of not requiring monitoring (Gustafsson and Elg, 2003). However, there are some concerns regarding liver toxicity and the drug at present is not licensed in the UK or the USA.

A number of therapies for SLE may be applicable to APS as discussed in section 1.3.5. TNF α blockade, an established treatment for rheumatoid arthritis, has been shown to prevent aPL induced pregnancy morbidity in mice and may represent a future therapeutic agent (Berman et al., 2005). Though current licence guidance in the UK prohibits the use of TNF α blockade in pregnant women, emerging post-surveillance data suggests that continuing TNF α blockade therapy during pregnancy may not be associated with an adverse outcome (Salmon and Alpert, 2006).

Hydroxychloroquine, used frequently to treat patients with either SLE or rheumatoid arthritis, has anticoagulant properties that may make it useful in the treatment of APS. Patients with APS and SLE taking hydroxychloroquine have a reduced risk of developing thrombosis (Erkan et al., 2002). This drug when administered to mice reduced aPL induced thrombus size and may also inhibit activation of platelets *in vitro* (Edwards et al., 1997, Espinola et al., 2002). The anti-thrombotic and anti-inflammatory effects of hydroxychloroquine may occur through the interference with TLR-mediated cell activation (Rutz et al., 2004) and inhibition of LPS induced MAPK signalling (Weber et al., 2002).

Statins are potent inhibitors of cholesterol synthesis and act by inhibiting 3-hydroxy-3methylglutaryl-coenzyme A (HMG-CoA) reductase. Large clinical trials of statin therapy have shown beneficial effects in terms of primary and secondary prevention of cardiovascular related morbidity and mortality (4S., 1994, Furberg et al., 1994, Hebert et al., 1997). Experimental evidence suggests that the beneficial effects of statins may extend beyond lowering of cholesterol. Their effects include decreasing expression of adhesion molecules in monocytes and inhibiting leukocyte-endothelial interactions (Niwa et al., 1996, Weitz-Schmidt et al., 2001), down-regulating proinflammatory cytokines in ECs (Inoue et al., 2000), enhancing fibrinolysis (Essig et al., 1998, Bourcier and Libby, 2000), inhibition of TF expression by monocytes (Aikawa et al., 2001, Colli et al., 1997) and inhibition of platelet activation (Huhle et al., 1999). Statins have been shown to inhibit aPL induced activation of ECs *in vitro*, as assessed by adhesion molecule expression and activation by NF- κ B (Meroni et al., 2001). Upon passive transfer of aPL in Pierangeli's mouse model of thrombosis, fluvastatin reduces aPL induced thrombus size and leukocyte adherence to the cremaster muscle blood vessel walls *in vivo* (Ferrara et al., 2003).

The universal link in the majority of pathogenic mechanisms described is the interaction of aPL with antigen to effect the observed response. As described aPL from patients with APS bind PL via PL binding cofactors, of which β_2 GPI seems to be the most clinically relevant and consequently most studied (reviewed section 1.2.5). Understanding how aPL interact with β_2 GPI at the molecular level could facilitate the development of therapies designed to interfere with this interaction. Having discussed the clinical features of APS, the characterisation of aPL and the different pathogenic mechanisms that arise in APS, I will now discuss in more detail the β_2 GPI

protein and current knowledge base addressing the likely nature of the interaction between this protein and aPL at the molecular level.

1.6 CHARACTERISATION OF β_2 GPI IN APS

1.6.1 Molecular structure and function of β_2 GPI

β_2 GPI was first described in 1961 as a component of the beta-globulin fraction of human serum (Schultze et al., 1961). The gene encoding β_2 GPI maps to chromosome 17q23-24 (Steinkasserer et al., 1992) and the promoter has recently been characterised (Wang and Chiang, 2004). This protein has also been termed apolipoprotein H as approximately 40% is bound to lipoprotein, though it bears no structural similarity to other apolipoproteins (Lee et al., 1983). The major source of production of this glycoprotein is the liver, although mRNA coding for β_2 GPI has also been found in ECs, intestinal epithelial cells, trophoblasts, neurons and astrocytes (Caronti et al., 1999, Aversa et al., 1997, Chamley et al., 1997). It is synthesised as a single polypeptide chain and consists of 326 amino acids with a calculated molecular weight of 37.1 kDa. However, the protein has four N-linked carbohydrate side chains that account for approximately 20% (w/w) of the total molecular mass. Hence, the actual molecular weight of reduced β_2 GPI as assessed using protein gel electrophoresis is approximately 50kDa (Lozier et al., 1984). This non-complement protein belongs to the complement control protein (CCP) superfamily (Reid and Day, 1989) that also includes molecules such as complement receptor 1 and 2 (Fearon, 1979), IL-2 receptor or DAF (Nicholson-Weller et al., 1982). It consists of five short CCP repeating domains also termed 'sushi' domains. Of the five CCP domains (DI to DV) making up β_2 GPI, four consist of approximately 60 amino acids each. Within each of these four domains there are four cysteine residues forming two disulphide bonds that contribute to a 'loop-back' structure, with sequence homology between the domains ranging from 20% to 40%. DIII and DIV are the only domains to have three and one carbohydrate side chains respectively. The fifth C-terminal domain (DV) is aberrant, consisting of 82 amino acids due to a six-residue insertion and a 19-residue C-terminal extension cross-linked by an additional disulphide bond (described in more detail in section 1.6.4). Two independent groups have studied the crystal structure of β_2 GPI describing it as having a fishhook like appearance (Schwarzenbacher et al., 1999, Bouma et al., 1999). A schematic representation of β_2 GPI binding to PL is shown in figure 1.2.

β_2 GPI exhibits a high degree of conservation among mammals. Human, bovine, canine and mouse proteins all have low identity with 50% of 90% homology in the cytoplasmic domain, with the glycosylated form being the most conserved domain (Kawakami et al., 1995; Zhang et al., 1997; Cap et al., 1998). Chickens possess β_2 GPI that has a 59% identity with human β_2 GPI and even shares common polymorphisms (Satchere et al., 2001). β_2 GPI is one of the most abundant proteins

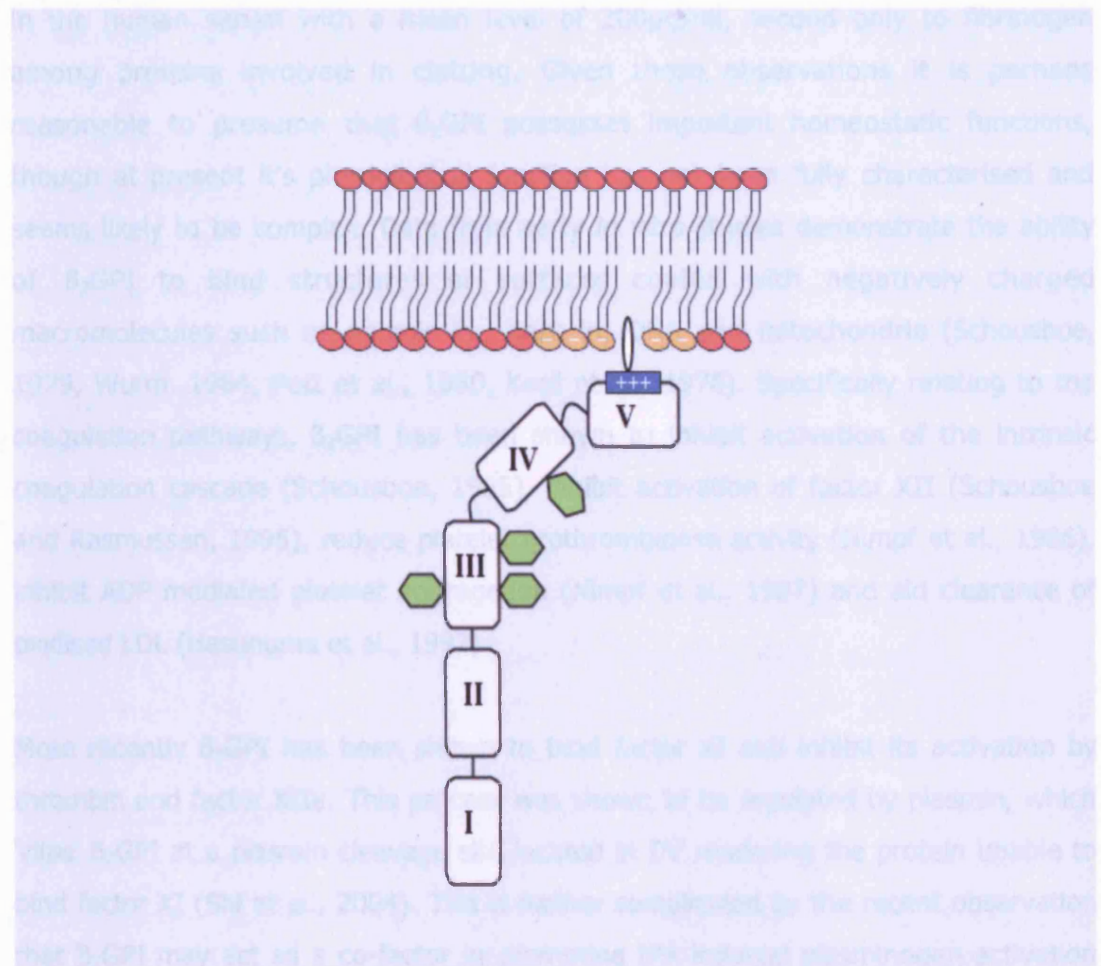


Figure 1.2 Schematic representation of β_2 GPI binding PL. Domains I-V are shown. N-linked carbohydrate chains are shown in green.

β_2 GPI exhibits a high degree of conservation among mammals. Human, bovine, canine and mouse proteins all have five domains with 60% to 80% homology in the amino acid sequence, with DV being the most conserved domain (Steinkasserer et al., 1991, Sheng et al., 1997, Gao et al., 1993). Chimpanzee β_2 GPI has a 99.4% amino acid sequence homology with human β_2 GPI and even shares common polymorphisms (Sanghera et al., 2001). β_2 GPI is one of the most abundant proteins in the human serum with a mean level of 200 μ g/ml, second only to fibrinogen among proteins involved in clotting. Given these observations it is perhaps reasonable to presume that β_2 GPI possesses important homeostatic functions, though at present it's physiological function has not been fully characterised and seems likely to be complex. Data from early *in vitro* studies demonstrate the ability of β_2 GPI to bind structures or surfaces coated with negatively charged macromolecules such as anionic PL, heparin, DNA and mitochondria (Schousboe, 1979, Wurm, 1984, Polz et al., 1980, Kroll et al., 1976). Specifically relating to the coagulation pathways, β_2 GPI has been shown to inhibit activation of the intrinsic coagulation cascade (Schousboe, 1985), inhibit activation of factor XII (Schousboe and Rasmussen, 1995), reduce platelet prothrombinase activity (Nimpf et al., 1986), inhibit ADP mediated platelet aggregation (Nimpf et al., 1987) and aid clearance of oxidised LDL (Hasunuma et al., 1997).

More recently β_2 GPI has been shown to bind factor XI and inhibit its activation by thrombin and factor XIIa. This process was shown to be regulated by plasmin, which 'clips' β_2 GPI at a plasmin cleavage site located in DV rendering the protein unable to bind factor XI (Shi et al., 2004). This is further complicated by the recent observation that β_2 GPI may act as a co-factor in promoting tPA induced plasminogen activation to plasmin, thus promoting the fibrinolytic pathway (see figure 1.1 B) (Lopez-Lira et al., 2006). β_2 GPI has also been shown to promote the clearance of apoptotic bodies (Pittoni et al., 2000) and liposomes (Chonn et al., 1995) *in vivo*. In the liver where the bulk of β_2 GPI is synthesised, it is emerging that it plays an important role in the survival of hepatocytes through as yet uncharacterised mechanisms (Averna et al., 2004).

Clearly the functions of β_2 GPI are broad, complex and not fully elucidated. The balance favours this protein functioning as a natural occurring anticoagulant in both health and disease.

1.6.2 Effects of polymorphisms or deficiencies of β_2 GPI: clues to function and pathogenicity

Given the ubiquitous nature of this protein and varied functions described, one would intuitively expect the absence of functional β_2 GPI to have considerable adverse physiological consequences. Curiously, epidemiological genetic studies on humans have found that a deficiency of β_2 GPI is not a major risk factor for either thrombosis or bleeding (Bancsi et al., 1992, Hoeg et al., 1985). β_2 GPI deficiency, defined arbitrarily as 77% less than the mean of the study population, was found to occur in approximately 7%, corresponding to the approximate frequency of heterozygous deficiency (Yasuda et al., 2000, Bancsi et al., 1992). In contrast a total lack of β_2 GPI is uncommon and occurs in approximately 0.3% of individuals (Hoeg et al., 1985). One study identified one man homozygous for β_2 GPI deficiency with a history of venous thrombosis. This was inherited in an autosomal dominant pattern and he had one brother who was also homozygous deficient for β_2 GPI who had no history of thrombosis aged 35 years (Bancsi et al., 1992). One Japanese study of two families with complete β_2 GPI deficiency (due to a single nucleotide deletion resulting in a frameshift in the β_2 GPI gene) found all affected members apparently healthy. Curiously, one family with β_2 GPI deficiency had elevated levels of LDL though the relevance of deficient β_2 GPI is questionable as the other β_2 GPI deficient family had normal levels (Yasuda et al., 2000). It may be that there are enough 'biological buffers' for reduced levels of β_2 GPI to be tolerated by individuals for normal physiological functioning. The effects of being homozygous for β_2 GPI deficiency as yet, have not been fully elucidated in humans as it occurs in the population at a very low frequency. Studies in β_2 GPI gene knockout mice show no clinical evidence of thromboses with this genotype. These mice, however do exhibit diminished rates of thrombin generation compared to β_2 GPI replete ($^{+/+}$) mice (Sheng et al., 2001b). Pregnant null mutant mice (β_2 GPI $^{-/-}$) had a modest reduction of 18% in number of viable implantation sites and reduced foetal / placental weights. Interestingly, the β_2 GPI $^{-/-}$ mice were protected from the more severe rates of foetal loss observed on passive transfer of β_2 GPI binding aPL (Robertson et al., 2004). It may be that individuals can tolerate diminished levels of β_2 GPI, however the presence of β_2 GPI in combination with aPL leads to disease.

Other single nucleotide polymorphisms (SNPs) have been identified in this protein, but few have functional effects. In total four SNPs have been identified at the DNA level that result in amino acid substitutions, one located in the region coding for DII

(Ser to Asn at position codon 88) and the other three are located in DV (Val to Leu at codon 247, Cys to Gly at codon 306, Trp to Ser at codon 316) (Sanghera et al., 1997a, Steinkasserer et al., 1993). The SNP at position 316 located in DV has been shown to disrupt the binding of β_2 GPI to PL with functional consequences (Mehdi et al., 2000). The presence of the β_2 GPI allele Val at 247 instead of Leu is seen more frequently than expected in patients with aPL and APS in the British, Mexican and Asian populations that have been studied (Atsumi et al., 1999, Prieto et al., 2003, Yasuda et al., 2005). One study of 140 patients presenting with cerebral haemorrhage in China detected the β_2 GPI allele Asn 88 to be associated with an increased frequency of cerebral haemorrhage as compared to the allele Ser 88 and attributed this finding to the generation of hypertension seen in patients with this SNP (Xia et al., 2004). However, dissecting genetic predisposition may be very complex. A recent paper has identified 142 SNPs located in the non-coding regions of the 20.3 kb β_2 GPI gene, which may occur in areas that affect gene regulation and ultimately function (Chen and Kamboh, 2006).

1.6.3 Do aPL bind cryptic or non-cryptic epitopes on β_2 GPI?

Several groups have found that β_2 GPI coated on an irradiated plate will bind monoclonal aPL (Matsuura et al., 1994) or polyclonal aPL (Chamley et al., 1999), but not when β_2 GPI is coated on polystyrene plates, even at high densities. The authors postulated that this effect was due to conformational change of β_2 GPI upon binding an irradiated plate facilitating aPL binding. Indirect methods to detect structural changes in β_2 GPI have been used such as infrared spectroscopy (Borchman et al., 1995), spectrophotometry to produce circular dichroism (Subang et al., 2000) and microcalorimetry (Hammel et al., 2001), which infers an alteration in structure of β_2 GPI upon binding PL. Hammel et al used X-ray scatter to infer an S-shaped structure of β_2 GPI in the fluid phase, as opposed to the fishhook structures of the published crystal. The authors postulated that DII and DIII were flexible and could be rotated. Modelling studies, in which these domains were rotated, revealed a change from the fishhook shape to that of the S-shape (Hammel et al., 2002).

There is also evidence to support that aPL binding to β_2 GPI may be dependent upon the density of β_2 GPI coated on a plate or PL, rather than the exposure of cryptic epitopes. Roubey et al demonstrated that aPL binds β_2 GPI coated on irradiated plates only at a density that exceeds that which may be coated on non-irradiated plates (Roubey et al., 1995). A variant of β_2 GPI that spontaneously dimerises has been produced using an insect cell expression system. This variant was shown to bind aPL

more effectively than wild-type β_2 GPI suggesting divalent binding (Sheng et al., 1998). Divalent binding has also been suggested by other work demonstrating that divalent and not univalent aPL $F(ab)_2$ bind β_2 GPI. Using surface plasmon resonance, molar ratios of monoclonal aPL binding to β_2 GPI immobilised on a chip approximated to 0.5, suggesting divalent binding (Regnault et al., 1999). In a more recent study, flow cytometry studies of β_2 GPI binding platelets expressing anionic PL found that β_2 GPI at physiological ionic strengths demonstrates poor binding to platelets, however at low ionic strengths or in the presence of bivalent $F(ab)_2$, this binding was greatly enhanced. The same effects were not observed with univalent $F(ab)$ fragments (Bever et al., 2005).

There is thus conflicting evidence for and against the presence of a cryptic epitope and this on going debate has been reviewed by Giles et al (Giles et al., 2003c). Given the heterogeneity of aPL, it is possible that some recognise cryptic epitopes and others bind β_2 GPI in a divalent fashion. Another possibility could be that both occur. Divalent binding of β_2 GPI by aPL is necessary for cell activation, and this binding may be facilitated by alteration in conformation of β_2 GPI upon binding PL. Two recent studies from de Groot's group support this theory. In one study the recombinant variant of β_2 GPI that dimerises was shown to bind apo ER2 (family member of LDL receptors) on the surface of platelets and cause spontaneous activation of these cells even in the absence of aPL, which were required to activate platelets in the same manner when native β_2 GPI was used (van Lummel et al., 2005). In another study two populations of anti- β_2 GPI antibodies were identified. One termed type A were derived from patients with thrombosis and were capable of binding β_2 GPI only if coated onto anionic PL and not in the fluid phase. The other population type B were derived from patients with no history of thrombosis / APS and were capable of binding β_2 GPI in both solid and fluid phase. Type A antibodies could, however, bind β_2 GPI in the fluid phase upon deglycosylation of this protein. These studies support the theory that β_2 GPI binds anionic PL and exposes epitopes due to displacement of carbohydrate side chains, which then allows aPL to bind clustered β_2 GPI facilitating dimerisation and effecting cell activation.

The location of immunodominant epitopes that are recognised by pathogenic aPL on the surface of β_2 GPI has been debated in the literature. Various groups have implicated a number of different domains as harbouring these epitopes. Each domain

will be discussed in turn and the evidence supporting each as containing aPL binding epitopes critically analysed.

1.6.4 DV of β_2 GPI: anchor to phospholipid

The β_2 GPI protein contains two highly conserved cationic regions rich in positively charged amino acids, one in DI and the largest in the C-terminal DV. A 2000 Å large patch of 14 positively charged amino acids between 274-Glu and 288-Cys within DV (12 Lys, one Arg and one His residue) provides the electrostatic interactions with the anionic head groups of PL. The area where cationic charge is particularly concentrated is between residues 282 and 287 (282-Lys-Asn-Lys-Glu-Lys-Lys-287 – KNKEKK). This is stabilised by a hydrophobic loop located between residues 311-Ser and 317-Lys that inserts into PL and is schematically represented in figure 1.2 (Schwarzenbacher et al., 1999, Bouma et al., 1999). Hunt et al demonstrated that a peptide sequence spanning the area 274-Glu to 288-Cys could inhibit binding of β_2 GPI to CL, and the inhibitory activity of this peptide was restricted to the particularly Lys rich region KNKEKK. Peptides that span other areas of DV had no such inhibitory effects (Hunt and Krilis, 1994). Further mutational studies by Sheng et al showed that altering Lys residues in the region 282 to 287 had the effect of reducing binding of β_2 GPI to CL. Reduction in binding was proportional to a number of Lys residues altered in this area (Sheng et al., 1996). The C-terminal end of the hydrophobic loop also constitutes a plasmin cleavage site and is a target *in vivo*. Plasmin cleavage of β_2 GPI between residues 317-Lys and 318-Thr has the effect of impairing the ability of this protein to bind PL. β_2 GPI cleaved in this manner has been termed in the literature as 'nicked' or 'clipped' (Hunt et al., 1993, Ohkura et al., 1998). Clipping β_2 GPI also impairs the ability of this protein to bind factor XI (Shi et al., 2004) and heparin, with heparin enhancing the ability of plasmin to clip β_2 GPI (Guerin et al., 2002). There are two polymorphisms within β_2 GPI at positions within or adjacent to the PL binding site. One at codon 316 is a substitution mutation of Trp to Ser located within the hydrophobic loop and another at codon 306 is a substitution of Cys for Gly, which disrupts the disulphide bond with 281-Cys, located within the cationic region. β_2 GPI purified from individuals homozygous for 316-Ser/316-Ser or compound heterozygote 316-Ser/306-Gly failed to bind PL (Sanghera et al., 1997b). Introducing these mutations into the native cDNA sequence of the β_2 GPI gene and expressing recombinant mutagenised β_2 GPI had the same effect (Mehdi et al., 2000).

Work by Gharavi et al has implicated DV as being important for aPL binding. Mice immunised with whole β_2 GPI develop aPL (Gharavi et al., 1992). Further work

demonstrated that a synthetic peptide named GDKV spanning 274-Gly to 288-Cys of DV could also induce aPL when used to immunise mice. The GDKV induced aPL enhanced thrombus formation *in vivo* and induced activation of ECs (Gharavi et al., 1999). A peptide derived from CMV named TIFI that is capable of binding PL has also been shown to induce thrombogenic aPL formation in mice (Gharavi et al., 2002).

Studying the crystal structure of β_2 GPI reveals the presence of the hydrophobic loop adjacent to the region homologous to the GDKV peptide used by Gharavi and colleagues. Given that this region is so closely applied to PL, it is unlikely that the majority of important aPL binding epitopes are contained within this domain. Also, given that the domains share up to 40% homology, it is possible that the GDKV peptide shares homology to other areas of β_2 GPI, which may be more amenable to aPL binding.

1.6.5 DIII and DIV of β_2 GPI: evidence for and against aPL binding

The production of mutants of β_2 GPI with one or more domains deleted has helped elucidate which domains harbour important antibody binding epitopes. These domain deletion mutants of β_2 GPI were produced using baculovirus to infect insect cells *Spodoptera frugiperda* (Sf9) as the expression host (Igarashi et al., 1996). Igarashi et al (Igarashi et al., 1996) and George et al (George et al., 1998d) demonstrated that a mutant β_2 GPI containing only the first four domains (DI-DIV), thus lacking DV, failed to bind CL. One would expect this, given the evidence described in the section above. This β_2 GPI mutant I-IV bound a murine anti- β_2 GPI antibody WB-CAL-1 better than whole β_2 GPI and mutant domain I-III (mutant β_2 GPI that lacked both DIV and DV) both in solid and fluid phase assays. Cof-21 is a mouse mAb directed against DIV. The authors demonstrated that human aPL could compete with binding of Cof-21 to β_2 GPI coated on a plate, but not with other mAbs that recognise epitopes present in DIV to DV (George et al., 1998d). The conclusions drawn were that DIV most likely contains the crucial aPL binding epitopes. However, these studies would have been more compelling if binding to DIV alone had been demonstrated.

Koike et al produced four mutants of whole β_2 GPI with incorporated single point mutations within DIV. These mutant forms of β_2 GPI exhibited reduced binding in the solid phase to 24 out of 30 polyclonal aPL samples derived from patients with APS (Koike et al., 2000). However, these mutant forms of β_2 GPI have subsequently been shown to bind aPL in the fluid phase (Iverson et al., 2002a). It therefore seems more likely that the mutations introduced within DIV by Koike et al produce a

conformational change resulting in either reduced binding of protein to the ELISA plate, or reduced exposure of the true epitopes to some aPL when this protein is bound to the plate. This theory is supported when one looks at the mutations incorporated. Three of the four mutants targeted negatively charged amino acids (193-Asp, 222-Asp, 228-Glu) mutating them to neutral Val. This would be likely to disrupt electrostatic bonds within the protein resulting in conformational change. Also, six out of 30 aPL samples tested, in fact, exhibited enhanced binding to the mutant forms of β_2 GPI, suggesting that the conformational change favoured the exposure of epitopes recognised by these aPL (Koike et al., 2000).

Blank et al identified two peptides that bind human anti- β_2 GPI mAbs ILA-3 and H-3 by screening hexapeptide phage display libraries. Both of these monoclonal aPL have been shown to exhibit thrombogenic pathogenicity in mice. The peptide that bound ILA-3 had sequence homology to a region located in DIII and that which bound H-3 had sequence homology to a region within DIV. The hexapeptides expressed, demonstrated low affinity binding to the mAb aPL tested and they were lengthened to 208-KDKATFGCHDGC-219 named peptide B (region in DIV) and 131-CATLRVYKGG-140 and named peptide C (region in DIII). Both these peptides inhibited their corresponding antibody from activating human ECs *in vitro* and also prevented the induction of foetal loss in pregnant BALB/c mice on infusion of ILA-3 and H-3 *in vivo* (Blank et al., 1999). These peptides were subsequently tested for binding to human polyclonal aPL derived from patients with APS. Binding at low to moderate levels was observed in only nine out of 43 samples tested, with high binding observed in only one patient sample. A subsequent multicentre study by Shoenfeld et al went on to characterise the binding of six peptides, two of which constituted peptide B (DIV region) and peptide C (DIII region), and another three (including the GDKV peptide) that have been shown to bind PL (DV cationic region). The ability of these peptides coated on a plate to bind sera derived from a total of 295 APS patients was tested. Binding levels three standard deviations (SDs) above a mean obtained by testing 100 healthy controls was considered positive. Approximately 85% of aPL samples bound at least one peptide. However, no individual peptide bound more than 50% of samples and no other irrelevant peptide was used as a negative control. In addition, it was unclear if standards were used for each individual plate to take account of interplate variability and binding to pure IgG aPL in the fluid phase was not tested (Shoenfeld et al., 2003).

On studying the crystal structure of β_2 GPI, bound to DIII and DIV are a total of four carbohydrate molecules (Schwarzenbacher et al., 1999) (figure 1.2). It is likely that these large sugar molecules would shield aPL from interacting with any epitopes that may be located within DIV. Recombinant whole β_2 GPI expressed from insect cells has a molecular weight of 43 kDa, as opposed to 50 kDa as observed when reduced β_2 GPI purified from humans is run on an electrophoretic protein gel (Igarashi et al., 1996). This discrepancy is likely to be due to inefficient glycosylation of proteins by insect cells as compared to mammalian cells. In theory less or altered glycosylation present in recombinant β_2 GPI expressed by insect cells could expose epitopes that would otherwise be hidden in native protein. On critically reviewing the evidence and from studying the structure of β_2 GPI, support favouring the presence of crucial aPL binding epitopes located in DIV or DIII is on the whole weak. It is more likely that biologically important epitopes bind other more amenable domains.

1.6.6 DI of β_2 GPI: evidence of aPL binding

Iverson et al studied the binding of β_2 GPI domain deleted mutants produced using the insect cell expression system developed by Igarashi et al (Igarashi et al., 1996). Mutants of β_2 GPI containing the N-terminal domain (DI) combined with other domains bound polyclonal IgG aPL derived from 11 patients with APS in both solid and fluid phase assays. However, domain deleted mutants of β_2 GPI that lacked DI exhibited very low or no evidence of binding to aPL. The β_2 GPI mutants that lacked DI were mutants with DII to DIV, DIII and DIV and DIV alone. If DIV was important in conferring binding to aPL as suggested by two other studies discussed in the section above (Igarashi et al., 1996, George et al., 1998d), then one would have expected these mutants to also bind aPL. The data by Iverson et al supports the theory that aPL binding epitopes are located within DI rather than DIV. This is underlined by the observation that binding of aPL to DI alone was demonstrated (Iverson et al., 1998b).

Further evidence exists from numerous other groups supporting DI as containing the probable immunodominant aPL binding epitopes. Reddel et al also tested binding of 27 serum samples from patients with APS against binding to a domain deletion mutant lacking DV (DI-IV) and one lacking DI (DII-V). All serum samples tested bound DI-IV mutants to a level comparable with wild-type β_2 GPI, whilst all showed significantly reduced binding to the DII-V mutants (Reddel et al., 2000). Surface plasmon resonance has been employed to study the binding of 106 APS serum samples to native β_2 GPI and various domain deletion mutants (II-IV) of β_2 GPI

immobilised on a chip. For those mutants lacking DI, a significant reduction in binding was observed, with almost half of the total APS patient serum samples showing almost complete loss of binding to the domain deletion mutants lacking DI (McNeeley et al., 2001). Incubation of soluble β_2 GPI with staphylococcal V₈ in another study resulted in a 50% reduction in binding to aPL serum samples derived from 15 APS patients. A cleavage site for this enzyme resides within DI (Guerin et al., 2000). Sheng et al characterised the LA activity of a panel of monoclonal aPL derived from either human patients with APS or mice immunised with β_2 GPI or NZW x BXSB mice with an APS / lupus like syndrome. Monoclonal aPL that bind DI cause considerable prolongation of the dRVVT and kaolin clotting time, thus exhibiting LA activity. Monoclonal aPL binding to DIII exhibited less LA activity, with no effect on kaolin clotting time whilst monoclonal aPL that bound DIV or DV had no LA activity (Sheng et al., 2001a). Another more recent study by de Laat et al has demonstrated that a sub-set of anti- β_2 GPI antibodies derived from patients with thrombosis bind DI and do not bind a variety of domain deletion mutants lacking DI. However, anti- β_2 GPI antibodies derived from patients with no history of thrombosis do bind other domain deletion mutants that lack DI (de Laat et al., 2005). This study suggests that aPL that bind DI are more likely to be pathogenic as compared to aPL that bind epitopes located within other domains of β_2 GPI.

1.6.7 Candidate epitopes within DI

The evidence for DI harbouring immunodominant binding epitopes for the majority of pathogenic aPL is compelling. This is underlined by the fact that DI is the most exposed of all the domains within β_2 GPI, as it is at the N-terminal and lacks glycosylation (figure 1.2), thus making it more amenable to aPL targeting. But which region of DI do the majority of aPL bind? Current evidence addressing this will be discussed and critiqued in some depth.

1.6.7.1 Clues from antigen mutational, binding and structural studies

One may argue that the differing effects of binding from one domain deletion mutant of β_2 GPI to the next may potentially be attributed to changes in conformation of whole protein introduced by virtue of the lack of one or more domains. In theory this could either expose neo-epitopes or result in the shielding of true epitopes present in the native protein. To account for this whole β_2 GPI has been produced by Iverson and colleagues with single point mutations incorporated within DI in an attempt to define further the precise immunodominant aPL binding epitopes. Phage-displayed peptide libraries were screened in which peptide fragments of DI were expressed as

a fusion with the protein pIII. The mutations were created by error-prone PCR such that an average of one mutation in DI was generated within each phage clone. A phage micropan assay was used to screen individual phage for the ability to bind affinity purified aPL from APS patients. Based on this system phages with substitution mutations covering half of the DI molecule were identified. Ten with variable effects on binding to aPL were chosen and these mutations were incorporated into whole β_2 GPI and whole protein expressed in insect cells (Iverson et al., 2002b). Reddel et al studied two of these β_2 GPI single point mutants, with one mutant substituting a Gly to Glu residue at position 40 (G40E) and the other Arg to Gly at position 43 (R43G). Binding was tested to 27 serum samples from patients with APS. A moderate decrease in binding was observed in 10 out of 27 serum samples to G40E and seven out of 27 to R43G. Also noted was significantly enhanced binding of one serum sample to R43G as compared to wild-type β_2 GPI. The mutant β_2 GPI with the Thr to Ala substitution at 50 (T50A) reduced binding to seven samples, but also significantly enhanced binding to seven other APS serum samples. These results suggested that G40 and R43 within DI could be important in conferring binding to aPL and also underlined the heterogeneous nature of these antibodies. Iverson et al went on to test G40E, R43G, T50A as well as another seven mutants of β_2 GPI with single point substitution mutations located within DI. All 10 mutants bound equally well in both solid and fluid phase assays to three murine mAb anti-DI antibodies that recognise conformationally restricted epitopes, suggesting that the incorporated mutations did not introduce significant conformational change within the protein structure. Binding to β_2 GPI affinity purified IgG aPL in the fluid phase was significantly reduced for mutants G40E and R43G and largely unchanged for the other mutants. Binding in the solid phase was less consistent and was reduced by more than 50% to only four out of eight aPL for G40E and five out of eight for R43G, with many other mutants such as D8A, K33E, M42K and M42V curiously resulting in enhanced binding to the majority of affinity purified aPL as compared to wild-type β_2 GPI (Iverson et al., 2002b). A recent study demonstrated that anti- β_2 GPI antibodies that do not bind β_2 GPI mutants G40E or R43G, are more likely to have LA activity and be derived from patients with a history of thrombosis as compared to anti- β_2 GPI antibodies that bind these mutants (de Laat et al., 2005).

These results prompted the authors Iverson et al to suggest that "the charged surface patch defined by residues 40-43 contributes to a dominant target epitope" for aPL binding DI (Iverson et al., 2002b). It seems likely that this region is indeed

important however, there remain a number of unanswered questions arising from these very important mutational binding studies. One criticism could be that the nature of the mutations incorporated were as a result of a random process through error prone PCR, and not through a hypothesis driven approach derived from structural or binding analyses of the protein. For example, the effects of the charge altering G40E mutation could be due to the fact that this residue is adjacent to Arg 39. On studying a computer model of β_2 GPI using Swiss-Model software (Guex and Peitsch, 1997, Peitsch, 1996) and the crystal structure of β_2 GPI (Schwarzenbacher et al., 1999) the residue Arg-39 has over 50% of its surface area exposed for potential interaction with other amino acids. This is more than any other residue within DI of β_2 GPI. Only half of the residues within DI were studied by Iverson's group and hence the effects of mutating R39 residue may not have been characterised. If aPL do recognise this linear epitope as suggested by the authors, then it is surprising that many do not bind to denatured β_2 GPI (Sheng et al., 2001b). Also, incubation of soluble β_2 GPI with staphylococcal V_8 results in reduced binding to aPL. This enzyme cleaves DI at position 20, hence one may expect to see binding largely unaffected if linear epitopes residing further along the protein from this region were dominant (Guerin et al., 2000). All these observations suggest that other regions of DI are also important and that aPL binding to DI may be more complex.

The binding abilities of a peptide (peptide A) that bears sequence homology to the N-terminal half of the DI-II interlinker region have been tested (table 1.6). This peptide prevented foetal loss induction by aPL in mice *in vivo* and activation of ECs *in vitro* by affinity purified aPL (Blank et al., 1999, Shoenfeld et al., 2003). Pierangeli et al demonstrated the ability of peptide A to inhibit aPL-mediated thrombosis in mice. A scrambled peptide used as a negative control had no such inhibitory effect (Pierangeli et al., 2004a). If the region 40-43 is dominant then one may reasonably expect peptide A not to have a significant aPL inhibitory effect *in vivo*. On studying the crystal structure of β_2 GPI, the region 40-43 comes into close approximation with the DI-II interlinker region (57-67) (Schwarzenbacher et al., 1999). It may be that aPL recognise discontinuous epitopes, or that mutations within the 40-43 region may disrupt the exposure of important epitopes elsewhere. The observation that a peptide homologous to the DI-II interlinker region binds aPL may in fact provide an explanation why some groups have published studies suggesting that DIV harbours aPL binding epitopes. (Igarashi et al., 1996, George et al., 1998d). Though possible, it is unlikely that aPL recognise completely different epitopes within the same

protein. It is more likely that aPL recognise homologous regions within β_2 GPI that may contain only slight differences in peptide sequences, and that DI has been identified as the domain containing the immunodominant epitopes given its accessibility. This theory of epitope spreading is supported by the fact that different domains within β_2 GPI have 20% to 40% homology. The DI-II interlinker region is homologous in amino acid sequence to the DIII-IV interlinker region, with no such homology existing between the other interlinker regions (Steinkasserer et al., 1991) (table 1.6). In theory epitopes within the DIII-IV interlinker region of a β_2 GPI mutant protein expressed by insect cells may have greater exposure than is present in native protein due to suboptimal glycosylation that occurs in the expression of heterologous protein by these cells. Thus epitopes present in DI homologous to epitopes exposed within other domains such as DIV may also bind aPL, perhaps accounting for the ongoing debate as to which domain binds the majority of aPL.

Another unanswered question from the Iverson et al study was the observation that the mutations D8A, K33E, M42K and M42V resulted in enhanced binding to seven out of eight β_2 GPI affinity purified aPL tested as compared to wild-type β_2 GPI (Iverson et al., 2002b). This finding was not discussed but is of importance as a peptide that can bind the majority of aPL better than native β_2 GPI may be of therapeutic potential. Hence, further studies investigating the possibility of producing such peptides are warranted and are feasible given these observations.

Protein region	Peptide sequence
Domain I-II interlinker (57-68)	<u>N</u> TLKCTPRV <u>C</u> PF
Domain II-II interlinker (113-124)	PELPVCAPIICP
Domain III-IV interlinker (174-188)	<u>N</u> WTKLPECREV <u>K</u> CPE
Domain IV-V interlinker (235-248)	WSAMPSCKASCKLP
Synthetic peptide A	<u>N</u> TLKTPRV <u>G</u> GC

Table 1.7 Amino acid sequences of domain interlinker regions within β_2 GPI

Homologous amino acid sequence between DI-II interlinker, DIII-IV interlinker and synthetic peptide A (Blank et al., 1999) is underlined.

1.6.7.2 Clues from aPL mutational binding studies

Investigations studying the consequences of alterations within aPL to binding CL or whole β_2 GPI may also be of some use in terms of inferring which aspects of the antigen may be important. Dr Ian Giles in our laboratory has performed such studies on IS4. This is a monoclonal human aPL which binds anionic PL and β_2 GPI and has proven thrombogenicity in mice *in vivo* (Pierangeli et al., 2000). Sequence analysis of IS4 and other human monoclonal aPL have revealed an accumulation of positively charged Arg and Lys residues in their antigen binding CDRs. These residues have arisen through somatic mutation and are present in numbers greater than one would expect by chance, inferring the presence of antigen driven preferential selection (Giles et al., 2003b). A mammalian expression system for aPL has been developed in this unit to produce whole antibodies with the V_H of one antibody paired up with the V_L of a different antibody. This development has been achieved through a transient expression system using COS-7 cells and more recently via a stable system using Chinese Hamster Ovary (CHO) cells (Lambrianides et al., 2004). These studies have demonstrated that IS4 V_H rather than IS4 V_L was dominant in conferring binding to CL (Giles et al., 2003a). Subsequently, mutational studies identified two adjacent Arg residues at positions 100 and 100g in IS4 V_H /CDR3 as having a major influence on binding to CL (Giles et al., 2005). Arg residues may interact with other amino acids either through the donation of H-bonds or given their positive charge through electrostatic interactions with negative charged residues (Stryer, 1988). On studying the amino acid sequence of DI of β_2 GPI, this domain is unique in that it contains two groups of clustered negatively charged residues - two Asp (D) residues at position 8 and 9 and three Glu (E) residues at position 23, 26 and 27. DIV also contains three E residues in a cluster and two D residues that are not adjacent to each other (Steinkasserer et al., 1991). Thus, one possibility is that positively charged Arg residues on aPL could form important electrostatic interactions with negatively charged epitopes exposed on DI.

1.6.7.3 Conclusion on likely epitopes

In conclusion, it is likely that the region identified by Iverson et al (40-43) is important. However, more detailed mutational studies need to be undertaken, particularly of charged residues lying adjacent to this region such as Arg 39 to define this area more thoroughly. Furthermore, one needs to rationalise how this fits in with the observations that the DI-II interlinker region is important, given the aPL inhibitory effects of peptide A homologous to this region. Crystal structure analysis

and computer modelling studies could help in identifying which regions lie adjacent to the region 39-43 and the interlinker region. Site-directed mutagenesis of DI could target these regions, and the negatively charged epitopes, to investigate what effects charge altering or charge conserving mutations would have on binding aPL. Also the observations that some mutations could enhance binding to affinity purified aPL over wild-type β_2 GPI merit further exploration given the therapeutic implications of such peptides. A study by de Laat et al suggests that carbohydrate molecules from other domains restrict access to DI when β_2 GPI is in the fluid phase, but on binding to anionic PL a conformational change in the position of carbohydrate residues opens up dominant epitopes within DI (de Laat et al., 2006b). A system of expressing and mutating non-glycosylated DI would aid in addressing the uncertainties surrounding the nature of these dominant epitopes. Such a platform of production could also be used to investigate the degree to which different mutations within DI could not only reduce but also enhance binding to aPL, which could aid in the eventual development of therapeutic blocking peptides or peptide mimetic agents. An expression system in *Escherichia coli* (*E.coli*) could offer such a platform of DI production, given the relative ease and efficiency of established prokaryotic expression systems. Other groups such as Iverson, Linnik and colleagues have attempted to establish such a system of DI production but have been unsuccessful (via personal communication). The following section will discuss recombinant protein biotechnology more broadly, with particular emphasis on prokaryotic platforms of protein production.

1.7 PRODUCTION OF RECOMBINANT PROTEINS

The rapid advancement in recombinant protein biotechnology over the last 10-20 years has seen a very diverse spectrum of proteins, peptides, monoclonal antibodies and antibody fragments produced for almost equally diverse purposes. In some applications a large array of proteins / antibodies are needed in relatively small quantities for scientific purposes such as verification of the identity of a protein or cloned gene, or studies on the function and structure of a protein. The high end of the spectrum involves the production of human therapeutic proteins that are required in quantities approaching the metric ton scale, whilst posing extreme demands in terms of product quality and safety. One report states that biotech drugs accounted for more than 35% of the 37 substances launched in 2001 (Tang et al., 2004). Most of these are antibodies and examples of successful drugs launched are remicade (Infliximab[®]), etanercept (Enbrel[®]), adalimumab (Humira[®]) and rituximab (Mabthera[®]) that have revolutionised the treatment of rheumatoid arthritis and other

inflammatory diseases (Maini et al., 1999, Moreland et al., 1999, Weinblatt et al., 2003, Edwards et al., 2004). The majority of therapeutic proteins have been produced in either mammalian cell-culture systems, with CHO cells representing the most common system, or in *E.coli* (Chu and Robinson, 2001, Swartz, 2001).

1.7.1 Production of eukaryotic proteins in prokaryotic cells

E.coli is the most frequently used prokaryotic expression host for the production of heterologous proteins. This popularity is by virtue of its efficiency, cost-effectiveness and potential for high-level production as an expression host (Hannig and Makrides, 1998). *E.coli* was the first host to produce a recombinant DNA pharmaceutical, enabling the approval of Eli Lilly's recombinant human insulin in 1982 (Kroeff et al., 1989). The success of this and subsequently bovine growth hormone, produced also in *E.coli*, was due to the cost-efficient, highly effective and scalable process of protein production that *E.coli* offers (Swartz, 2001). The disadvantages however, are that some eukaryotic proteins may be toxic to *E.coli*, there may be a tendency for proteins to not fold correctly and form inclusion bodies and proteins which require significant post-translational modifications, such as the addition of carbohydrate molecules cannot be achieved in *E.coli*.

Even with eukaryotic proteins that do not require glycosylation, there is no guarantee that a recombinant gene product will accumulate in *E.coli* at high levels in a full-length and biologically active form. Strategies for achieving these goals are focussed on optimising essential components of the expression vector, consideration of codon usage, choice of cellular compartment for protein expression, minimising proteolysis and the use of fusion proteins.

1.7.1.1 Expression of target protein by *E.coli*

Optimal design of the expression vector is crucial to the successful induction of target protein expression by *E.coli*. Factors to consider in the choice of expression vector include the type of promoter, incorporation of additional sequences (such as a leader sequence, fusion or tagged sequences), selective antibiotic resistance, plasmid copy number, restriction sites available within the multiple cloning site, nature of the inducing agent and the degree to which basal production of target protein can be regulated. Figure 1.3 schematically represents a typical example of target gene transcription and regulation, with transcription driven by the T7 promoter using a pET plasmid (Studier and Moffatt, 1986). The RNA polymerase specific for the T7 promoter is T7 RNA polymerase. Hence, the *E.coli* cell must have a gene encoding

for this polymerase otherwise expression of target gene cannot take place. If the cell has a chromosomal copy of the T7 RNA polymerase gene, then production of this polymerase specific for the T7 promoter can initiate transcription at this site on the plasmid. Transcription of the target gene by T7 polymerase will continue until a specific terminator sequence known as the transcription terminator (TT) is encountered downstream of the target gene. Translation of the mRNA sequence then requires a Shine-Dalgarno sequence followed by an initiation codon, which is most commonly AUG. Translation of the target gene mRNA occurs and is terminated upon encountering a stop codon such as UAA, UGA or UAG.

This process of protein expression must be tightly controlled, particularly if the target protein is toxic to *E.coli*. If a toxic protein is produced even at low levels prior to induction, the bacterial cells will not have the opportunity to replicate to a sufficient density to produce any significant amount of product. This process may be regulated by the production of factors either at the chromosomal level or plasmid level. The most common regulator employed is the production of a *lac* repressor by the *lacI* gene (Deuschle et al., 1986). A chromosomal copy of this gene can regulate the production of the transcribing RNA polymerase. For example, a chromosomal copy of the *lacI* gene results in the production of the *lac* repressor, which then binds at a *lacUV5* operator sequence present within the promoter upstream to the T7 RNA polymerase gene. On encountering lactulose, or more usually an analogue of lactulose with a longer cell half life such as isopropyl- β -D-thiogalactopyranoside (IPTG), this binds the *lac* repressor, freeing up the *lacUV5* promoter to enable host *E.coli* RNA polymerase to drive the production of T7 RNA polymerase and hence transcription of target gene. This process may be further regulated if a *lac* operator sequence is present immediately downstream of the promoter sequence within the plasmid. The plasmid must then also contain a copy of the *lacI* gene. Additional repression by *lac* at this T7/*lac* promoter would then also inhibit transcription of the target gene by any T7 RNA polymerase that may have 'leaked' out (Studier and Moffatt, 1986). The addition of IPTG binds the *lac* repressor preventing inhibition by the operator at both the chromosomal and plasmid levels, switching on or 'inducing' target protein production (see figure 1.3). The choice of promoter is governed by several criteria. It should be capable of induction in a simple manner, such as the addition of an inducing agent, like IPTG for example. However, for large-scale production of therapeutic proteins IPTG is not suitable given cost and toxicity (Hannig and Makrides, 1998). The introduction of a *lacI* thermosensitive repressor

provides a convenient alternative for the large-scale production of therapeutic proteins by *E.coli* (Adari et al., 1995, Hasan and Szybalski, 1995). The promoter should also exhibit minimal level of basal production and ideally be repressible. Importantly it should be a strong promoter and be capable of protein production, ideally in excess of 10% to 30% of total cellular protein (Hannig and Makrides, 1998). Contaminant *E.coli* or cells that lose plasmid will replicate rapidly and subsequently overgrow within the culture flask, inhibiting protein expression and growth of cells harbouring plasmid. The presence of an antibiotic resistance gene allows the supplementation of growth media with the relevant antibiotic killing cells lacking this gene. The addition of protein 'tags' such as hexahistidine (his₆) tags or glutathione S-transferase may aid subsequent purification of target protein using chromatography columns that bind the incorporated tag.

1.7.1.2 Consideration of codon usage

Different cell species derived from primates to yeast cells to prokaryotic cells each use a specific subset of the 61 available amino acid codons for the production of mRNA molecules (Wada et al., 1992, Lloyd and Sharp, 1992, Zhang et al., 1991). The appearance of rare codons within *E.coli* correlates with low levels of cognate tRNA species. There are approximately 20 codons that occur with a frequency of less than 10 per 1000 codons in *E.coli*, the least frequent being Arg coding codons AGG/AGA occurring 1.4 per 1000 codons (Kane, 1995) and the presence of many of these codons in a gene leads to reduced protein synthesis and cell growth (Zahn, 1996).

Hence target genes that contain many of these codons that are rarely used by *E.coli* will express at low levels. This is particularly evident if these codons occur in clusters of two to five codons in length (Kane, 1995). Broadly two strategies have been employed to minimise the effects of preferential codon usage by *E.coli*. One method is by modifying the codons within the target gene to alter them to 'major' ones that occur with greater frequency (Hu et al., 1996, Hua et al., 1994, Martin et al., 1995). Another method could be to expand the intracellular pool of tRNA by co-expressing genes that encode for such minor codons such as the *argU* (*dna Y*) gene that encodes the minor tRNA-Arg (AGG/AGA) (Brinkmann et al., 1989). This expansion may be achieved by using commercially available *E.coli* strains that contain an extra plasmid for the minor tRNAs. Although these strains may be efficient in producing large amounts of some proteins (Kleber-Janke and Becker, 2000), the commercially available plasmids at present only encode for up to six rare tRNAs. Hence one may

still expect translational problems if the eukaryotic sequence contains clusters of minor codons not supplied by these plasmids. This may explain why the former method of codon optimisation within the target gene is used more frequently (Kane, 1995).

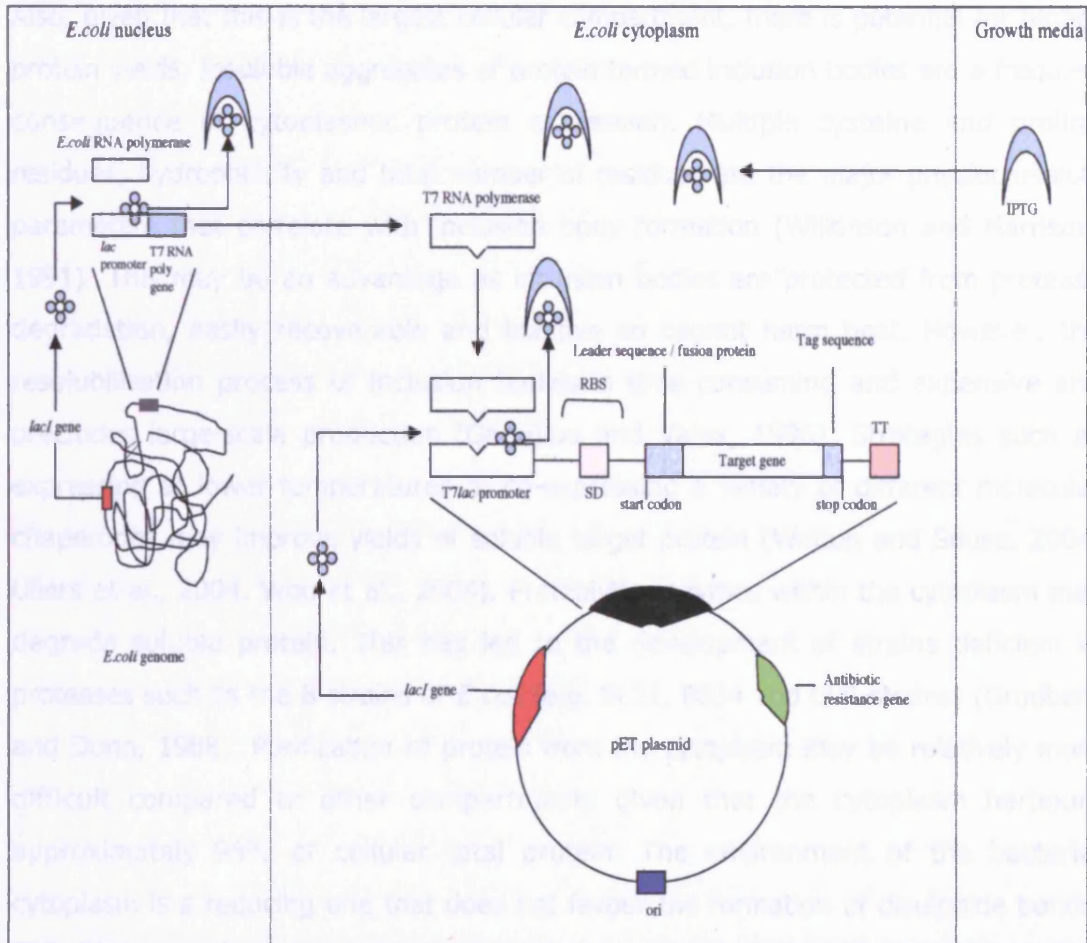


Figure 1.3 Schematic representation of expression vector and elements of regulation. Abbreviations: *E. coli*, *Escherichia coli*; T7 RNA poly gene, T7 RNA polymerase; SD, Shine-Delgarno; RBS, ribosomal binding site; TT, transcription terminator; *ori*, origin of replication; IPTG, isopropyl- β -D-thiogalactopyranoside.

1.7.1.3 Choice of cellular compartment

The three compartments where expressed proteins may be targeted are the cytoplasm, the periplasm and the culture medium. Each has its set of advantages and disadvantages.

Expressing protein within the cytoplasm has the advantage of requiring simpler plasmid constructs as additional leader sequences do not have to be incorporated. Also, given that this is the largest cellular compartment, there is potential for higher protein yields. Insoluble aggregates of protein termed inclusion bodies are a frequent consequence of cytoplasmic protein expression. Multiple cysteine and proline residues, hydrophilicity and total number of residues are the major physiochemical parameters that correlate with inclusion body formation (Wilkinson and Harrison, 1991). This may be an advantage as inclusion bodies are protected from protease degradation, easily recoverable and inactive so cannot harm host. However, the resolubilisation process of inclusion bodies is time consuming and expensive and precludes large-scale production (Georgiou and Valax, 1996). Strategies such as expressing at lower temperatures or co-expressing a variety of different molecular chaperones may improve yields of soluble target protein (Walton and Sousa, 2004, Ullers et al., 2004, Won et al., 2004). Proteolytic enzymes within the cytoplasm may degrade soluble protein. This has led to the development of strains deficient in proteases such as the B strains of *E.coli* (e.g. BL21, B834 and BLR strains) (Grodberg and Dunn, 1988). Purification of protein from the cytoplasm may be relatively more difficult compared to other compartments given that the cytoplasm harbours approximately 96% of cellular total protein. The environment of the bacterial cytoplasm is a reducing one that does not favour the formation of disulphide bonds. This is due to the presence of thioredoxin and glutaredoxin groups of reducing enzymes within the cytoplasm (Prinz et al., 1997). Mutant strains of *E.coli* are commercially available that lack these reducing enzymes and can enhance cytoplasmic disulphide bond formation and improve yields of soluble of target protein (Bessette et al., 1999).

Expressing in the smaller outer compartment of the bacterial cells, the periplasm holds a number of advantages over cytoplasmic expression. Periplasmic protein may be recovered whilst maintaining the integrity of the inner cell wall. Given the smaller pool of proteins within the periplasm this facilitates the isolation of purified target protein. The periplasm also lacks reducing enzymes and contains disulphide

isomerase enzymes, facilitating the formation of disulphide bonds. This has facilitated the expression of immunologically active anti-dsDNA F(ab)₂ fragments purified from the periplasm of *E.coli*, which requires the formation of five disulphide bonds within each fragment (Kumar et al., 2000, Kumar et al., 2001). Disulphide isomerase enzymes are exclusively periplasmic and are termed the Dsb group of enzymes (Bardwell et al., 1991). Further enhancement of periplasmic recombinant protein folding may be achieved by the overexpression of Dsb enzymes or by fusing target protein with DsbA (Hiniker and Bardwell, 2003). This strategy has facilitated the expression of recombinant tPA used for the treatment of acute myocardial infarction. This 527 amino acid protein contains 35 cysteine residues that participate in the formation of 17 disulphide bonds (Qiu et al., 1998). The disadvantage of periplasmic expression is that an N-terminal leader sequence needs to be included. This is necessary to transport protein from the cytoplasm to the periplasm. However, during the translocation process the leader sequence is cleaved by signal peptidase, and the cleaved leader sequence is then degraded by the enzyme signal peptide peptidase (Weihofen et al., 2002). Hence, target protein has a free N-terminus.

Finally, secretion of target protein into the extracellular culture media may be desirable. This compartment has the lowest level of proteolytic activity of all compartments and lowest number of contaminating proteins, thus facilitating purification. However, expressed protein needs to cross two membrane barriers. This translocation process is a highly specific one, particularly through the outer membrane where membrane translocation steps governing protein secretion are complex. Hence, this method has proved difficult generally producing low yields and is unsuitable for commercial production of recombinant protein (Swartz, 2001). To establish recombinant protein secretion three strategies have been employed. One is the utilisation of existing pathways for secreted proteins. For example, the *kil* gene mediates controlled export of periplasmic proteins and may be incorporated within an expression plasmid with some success, particularly if *Klebsiella planticola* is the expression host (Miksch et al., 1997). The second, is the use of signal sequences and translocator peptide sequences present at the carboxyl terminal of proteins secreted by *E.coli*, such as hemolysin. Hemolysin has a carboxyl HlyA signal sequence and HlyB and D translocator peptide sequences. These latter two peptide sequences may be employed to secrete other proteins if co expressed with the outer membrane protein TolC (Tzschaschel et al., 1996). Finally, mutant strains of *E.coli* have been engineered that lack the ability to synthesise an outer membrane and are termed L-

form strains. Thus, proteins targeted to the periplasm are released directly into the culture media (Gumpert and Hoischen, 1998, Rippmann et al., 1998).

1.7.2 Production of proteins using mammalian cells

Expressing eukaryotic proteins within mammalian host cells has the advantage of generating protein that has all the post-translational modifications included such as the addition of carbohydrate molecules. Mammalian cells also tend to recognise the same processing signals found in the original organism. In transient mammalian expression systems, only a fraction of the plasmid DNA delivered to the cell is incorporated into the host nucleus for transcription, with eventual export of mRNA to the host cytoplasm for protein production. In these systems gene expression from non-integrated plasmid DNA takes place. This non-integrated foreign DNA is degraded by nucleases or diluted by cell division and after approximately seven days its presence is no longer detected. Hence the disadvantages are of relatively low yields within a transient culture system, making it unsuitable for large-scale production of recombinant protein. However, transient expression systems are less time and labour intensive relative to stable mammalian systems, facilitating the production of high numbers of proteins for screening purposes. COS-7 cells derived from monkey kidney cell lines are often used as transient expression hosts and have been used to produce whole monoclonal native and mutant aPL in our unit (Giles et al., 2003a) as well as anti-ds DNA antibodies (Rahman et al., 1998). Some transient hosts such as the HEK293-EBNA cell line derived from human embryonic kidney cells have been cultured in bioreactor volumes of 1-3 litres to produce protein yields of a recombinant IgG1 antibody of 10-20mg/L of culture (Meissner et al., 2001).

In contrast stable expression occurs when the transgene spontaneously integrates with the host genome and replicates in synchrony with the cells. Cells containing integrated DNA are few and must be amplified by selection for drug resistance or identified as a result of phenotypic identification. This system requires several weeks to establish. However, the resulting cell lines form a stable source of protein production and hence larger quantities of recombinant protein yield may be obtained, making it the preferred method of protein production for biotech companies producing therapeutic recombinant proteins. The CHO and NS0 murine myeloma cell lines have established themselves as the predominant lines of choice for mammalian expression. The advantages that a stable mammalian expression system offers over a transient system were the reasons why a system for expressing monoclonal aPL within CHO cells was developed within our unit (Lambrianides et al.,

2004). Biotech companies employ CHO cell lines to produce therapeutics such as etanercept and rituximab and NS0 murine myeloma cell lines are used to produce infliximab (Chu and Robinson, 2001).

1.7.3 Advantages and disadvantages of other expression systems

Yeast cells are simple eukaryotic cells with a subcellular organisation that enables them to carry out some post-translational folding and modifications required for eukaryotic human proteins. Advantages over mammalian cells are that relatively simple transformation and fermentation conditions are required and they may be grown to high densities, facilitating high yields of protein. They may also be induced to secrete certain proteins into the growth medium for harvesting, such as the hepatitis B vaccine (McAleer et al., 1984). Disadvantages include less rapid growth as compared to prokaryotic cells and potentially less control over gene expression. Disadvantages over mammalian and insect cell systems are that there is incomplete glycosylation and occasionally refolding of protein may be required. The most frequently used strains of yeast are *Saccharomyces cerevisiae* and *Pichia pastoris* (Eckart and Bussineau, 1996, Buckholz and Gleeson, 1991).

Insect cells may be used as the expression host for eukaryotic proteins through the incorporation of genes into baculovirus vectors. Sf9 cell lines are the most frequently used lines (Altmann et al., 1999) and therapeutics such as the hepatitis C vaccine may be expressed using insect cells (Baumert et al., 1998). Advantages are that these cells are capable of more complex post-translational modification of protein, leading to correct folding of protein more frequently than both *E.coli* and Yeast systems. Cell growth is also marginally more rapid than mammalian cells. Disadvantages are that cell growth is significantly slower than both Yeast and *E.coli* systems. Expressed eukaryotic glycosylated proteins within insect cells are also often of lower molecular weight than native protein when run on an electrophoretic protein gel. This is most often due to proteolytic processing and incomplete glycosylation. Insect cells are only capable of simple glycosylation of proteins, lacking the ability to add sialic acid residues and form complex mammalian glycoproteins, which often consist of intricate scaffolds of N-linked acetylglucosamine, mannose, galactose, sialic acid and fucose residues (Altmann et al., 1999). These are likely to play a significant functional role as knock-out mice unable to synthesise complex N-glycans die in utero (Ioffe and Stanley, 1994, Metzler et al., 1994).

Spirin et al demonstrated that proteins may be synthesised using an *E.coli* based cell free system through an *in vitro* coupled transcription and translation process. *E.coli* cells are grown and lysed and the cell extract prepared. A feeding buffer composed of amino acids, ATP and GTP is added and protein synthesis is initiated upon adding template DNA. Protein may be synthesised for up to 40 hours in this continuous translation system (Spirin et al., 1988). The method was modified by Kigawa et al to produce up to 6mg/ml yields of recombinant chloramphenicol acetyl transferase (Kigawa et al., 1999). Recent advances in cell-free systems allow for the production of proteins directly from PCR products using a hollow-fibre membrane bioreactor (Nakano et al., 1999). This would enhance proteomic studies for high-throughput screening studies (Swartz, 2001). Wheat germ extract may also be used and can result in yields sufficient for nuclear magnetic resonance (NMR) spectroscopy structural studies (Vinarov et al., 2004). The disadvantages are that proteins are less likely to fold correctly and no post-translational modifications can take place, minimising the use of this system for creating bioactive proteins.

1.8 PRODUCTION OF RECOMBINANT β_2 GPI

1.8.1 Established systems of recombinant β_2 GPI expression

The vast majority of structural and functional studies of recombinant β_2 GPI over the past 10 years have been achieved by cloning cDNA sequence into a baculovirus which is then used to infect Sf9 insect cells as the expression host (Igarashi et al., 1996). Multiple groups have used this platform of production to perform structural and functional studies on β_2 GPI by expressing amounts sufficient to perform crystallographic studies (Schwarzenbacher et al., 1999, Bouma et al., 1999) and also produce mutants with one or more domain deleted to observe effects on binding to aPL (section 1.6). The yield from this production system is typically 2mg/L of supernatant (via personal communications with Dr M. Iverson, La Jolla Pharmaceuticals, San Diego, CA, USA). However, protein electrophoretic analysis of recombinant β_2 GPI expressed by insect cells reveals a band of 43 kDa as opposed to 50 kDa when native β_2 GPI is run on a protein gel (Igarashi et al., 1996). This size discrepancy between insect-driven recombinant proteins and authentic protein is a frequent occurrence and a major reason is due to a reduction in the amount and complexity of glycosylation residues by insect cells as compared to mammalian cells (as discussed in section 1.7.3.) (Altmann et al., 1999). If such a recombinant protein is used to study immunogenic determinants on epitopes that may otherwise be surrounded by carbohydrate molecules on the native protein, then it becomes

difficult to draw meaningful conclusions. This applies to DIII and DIV of β_2 GPI, which have N-linked glycosylated molecules and have been cited by some groups as containing the immunodominant binding epitopes (Igarashi et al., 1996, George et al., 1998d).

There is one early study from Kouts et al that describes the production of recombinant β_2 GPI using CHO mammalian cells (Kouts et al., 1993). It is unclear from reading the methods what yields were obtained from this system. Purified protein run on a protein gel and transferred on to a membrane for western blot analysis revealed two bands, one at 61 kDa and the other at 50-53 kDa level. In contrast to expression by insect cells, both bands are higher than the observed weight of 50 kDa for native β_2 GPI. Digestion by N-glycosidase which cleaves off all N-glycosylated molecules revealed a single diffuse band at 36-42 kDa as observed with native deglycosylated protein. This demonstrates that oligosaccharide processing amongst mammalian cells can be species and cell type dependent (Goochee et al., 1991) and may explain why this expression method does not seem to have been pursued by this or other groups.

1.8.2 Challenges in establishing expression of DI of β_2 GPI in *E.coli*

E.coli would be an inappropriate expression host for whole β_2 GPI due to the absence of glycosylation. Whole β_2 GPI has 38 cysteine residues forming 11 disulphide bonds that could potentially hamper protein folding in *E.coli*. However, this in itself would not preclude *E.coli* expression as bioactive tPA, which has 17 disulphide bonds may be expressed in the periplasm of *E.coli* when the periplasmic disulphide isomerase enzyme DsbA is co-overexpressed (Qiu et al., 1998). In fact the absence of glycosylation of protein by *E.coli* has been cited as an advantage given the consequent prolonged half-life of non-glycosylated rtPA *in vivo*, which is used as a thrombolytic drug in the treatment of acute myocardial infarction (Mattes, 2001). The bulk of evidence as discussed in section 1.6.6 supports DI as containing the immunodominant aPL binding epitopes and expressing this protein and multiple mutants could enhance our understanding of how this interacts with aPL and importantly, may facilitate the development of mutant peptides of DI that could have enhanced binding as compared to native protein. Given that this domain is not glycosylated in native protein and only has two disulphide bonds, expression may be possible in *E.coli*. This system is potentially the least labour intensive and time consuming of all the expression systems, genetic manipulation is relatively straightforward and there are multiple methods by which gene expression can be

controlled (Swartz, 2001). However, expressed protein would need to be transported to the periplasmic compartment by an appropriate leader sequence to facilitate correct folding. Fusion with DsbA may also enhance correct folding (Hiniker and Bardwell, 2003). The signal peptide is likely to be cleaved off by signal peptidase resulting in an authentic N-terminus (Hannig and Makrides, 1998). Osmotic shock breaks down the outer cell wall whilst retaining the integrity of the inner cell wall, resulting in the release of the relatively small pool of periplasmic proteins in to the media facilitating purification (Hannig and Makrides, 1998).

Another potential obstacle for expressing DI in *E.coli* is the observed set of codon preferences of *E.coli* that differs from the set of codons preferred by eukaryotic cells (section 1.7.1.2) (Kane, 1995). On studying the native cDNA sequence of DI, of the 61 codons 26 (43%) occur in *E.coli* at a frequency of less than 1% (Steinkasserer et al., 1991, Kane, 1995). These rare codons often occur in clusters of two to five codons in length. Hence one may expect translational problems in *E.coli* with an abundant mRNA species containing an excess of rare tRNA codons.

1.9 AIMS OF THESIS

1. To establish an expression system of DI of β_2 GPI using *E.coli* as the expression host. This system should be reliable, efficient and produce sufficient amounts of purified DI to use in direct and inhibition binding assays.
2. To characterise the binding of recombinant DI produced by *E.coli* to both monoclonal and polyclonal IgG aPL derived from patients with APS
3. To use this platform of production to create multiple mutants of DI. Analyses of binding and structural studies will be used to generate hypotheses as to the site and nature of the mutations that are likely to cause the greatest effects on binding.
4. To explore the possibility of creating mutant DI peptides which bind aPL better than native protein.
5. To determine effects of these mutations upon binding to monoclonal and polyclonal human aPL in both solid and fluid phase assays and compare binding to wild-type recombinant DI.

CHAPTER 2

MATERIALS, METHODOLOGICAL PRINCIPLES AND DETAILS OF PROCEDURES

OVERVIEW OF CHAPTER 2

This thesis represents the first reported successful attempt to express DI of human β_2 GPI in *E.coli*, express multiple variants of this protein and characterise the binding properties of the wild-type and mutant recombinant DI proteins to human antibodies relevant to the pathogenesis of APS.

The first part of this chapter, after listing the general materials used, describes in detail the cloning techniques necessary to produce a construct containing an insert encoding DI of human β_2 GPI. The rationale for producing a custom made gene encoding DI for use in a prokaryotic expression system is explained. A list of the mutant variants of DI that were made is also described and the techniques used to make these are outlined.

The second part then describes the methodological principles and design of the protocol for expressing wild-type and variant DI in *E.coli*. This is followed by a description of the target protein purification process, methods for target protein identification and assessment of quantity and purity.

The final part focuses on describing the principles and techniques of all the direct and inhibition assays that were performed in order to characterise the binding properties of the purified recombinant proteins.

2.1 Chemicals, general materials and equipment

2.1.1 Chemicals

BDH, Lutterworth, Leicestershire, UK supplied all chemicals used in this project with the following exceptions:

Absolute alcohol & propran-2-ol (Hayman Ltd, Witham, Essex, UK)
5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside in dimethylformamide (Xgal),
IPTG (Insight Biotechnology, Wembley, UK)

All solid chemicals were dissolved in ddH₂O, adjusted to correct pH with 0.1M HCl, glacial acetic acid, 5M KOH or 0.1M NaOH and autoclaved or filter sterilised with 0.22 μ m syringe driven filter units (Millipore, Bedford, MA, USA) unless otherwise stated. NaOH and KOH were not autoclaved. Ethidium bromide was stored in a light-tight container at room temperature (RT).

2.1.2 General materials and equipment

15ml and 50ml sterile tubes # 91015T, Helena Biosciences, Sunderland, UK
Bijou tubes # 39740, Bibby Sterilin Ltd, Staffordshire, UK
1.5ml and 0.5ml microfuge tubes Anachem, Luton, Bedfordshire, UK
1.5ml thin-walled microfuge tubes Eppendorf AC, Hamburg, Germany
Centrifuges Kendro Lab Products Ltd, Hertfordshire, UK
 Sorvall Biofuge Pico (and refrigerated version),
 Sorvall RT-7 Plus,
 RC26 Plus
PCR Cycler Machine Eppendorf Mastercycler, Hamburg, Germany
Spectrophotometer BioRad laboratories, CA, USA
Microdensitometer (GS-800) BioRad laboratories, CA, USA

2.1.3 Enzymes

Nco I # R6513, Promega, Southampton, UK
Xho I # R6161, Promega
Bgl II # R6081, Promega
T4 DNA Ligase # M1801, Promega
PfuTurbo® DNA Polymerase (Quikchange™ Site-directed mutagenesis kit,
Stratagene, CA, USA)
Vent DNA Polymerase (New England Biolabs (NEB), Herts, UK)

rEnterokinase

Novagen, Nottingham, UK

All enzymes and reaction buffers were stored at -20°C .

2.1.4 Reaction buffers

- a) 10x *PfuTurbo* DNA polymerase reaction buffer in Quikchange™ Site-directed mutagenesis kit (Stratagene, CA, USA) and 10x *Vent* DNA polymerase reaction buffer (NEB, Herts, UK)
 - 100mM KCl
 - 100mM $(\text{NH}_4)_2\text{SO}_4$
 - 200mM Tris-HCl, pH 8.8
 - 20mM MgSO_4
 - 1% Triton® X-100
 - 1mg/ml nuclease-free bovine serum albumin (BSA)
- b) 10x DNA ligase buffer (Promega, Southampton, UK)
 - 300mM Tris-HCl
 - 100mM MgCl_2
 - 100mM dithiothreitol (DTT)
 - 10mM adenosine triphosphate (ATP)
- c) 10x Promega Buffer D – for restriction enzymes *Nco* I and *Xho* I
 - 60mM Tris-HCl, pH 7.9
 - 1.5M NaCl
 - 60mM MgCl_2
 - 10mM DTT

All reaction buffers were stored at -20°C .

2.1.5 General buffers and solutions

- a) Phosphate buffered saline (PBS) pH 7.4. One PBS tablet (Invitrogen, Paisley, UK) was added per 500ml ddH₂O and autoclaved. To make PBS/0.1% Tween, 1ml “Tween 20” detergent was added to 1L PBS.
- b) Tris-EDTA (TE buffer), pH 7.5
 - 10mM Tris-HCl, pH 7.5
 - 1mM EDTA

2.1.6 Specific buffers and solutions

- a) Agarose gel electrophoresis

Agarose and low melting point agarose were supplied by Gibco BRL, Life Technologies, Paisley, Scotland, UK.

- Molecular weight markers:

- | | |
|------------------------------------|----------------------------|
| 100bp DNA MW marker | (Promega, Southampton, UK) |
| 1Kb DNA MW marker | (Invitrogen, Paisley, UK) |
| Lambda/ <i>Hind</i> III DNA marker | (Promega, Southampton, UK) |

- Tris Acetate EDTA (TAE buffer):

A stock of solution of 50 times working strength TAE buffer was made by dissolving the following components in 1L of ddH₂O:

- 242g/L Tris base
- 57.1ml glacial acetic acid
- 100ml 0.5M EDTA (pH 8.0)

Dilution to a working strength gave a final concentration of 40mM Tris acetate and 1mM EDTA.

- Loading Buffer:

The following components were added to 8.45ml autoclaved ddH₂O

- 25mg xylene cyanole
- 1.5g Ficoll 400 (Pharmacia Biotech, Uppsala, Sweden)

b) Plasmid DNA purification from bacterial cultures

The following buffers were supplied with Qiagen (Crawley, West Sussex, UK) plasmid purification kit:

- | | |
|-------------------------------------|--|
| ○ Buffer P1 (resuspension buffer) | 50mM Tris-Cl (pH 8.0)
10mM EDTA
100µg/ml RNase A |
| ○ Buffer P2 (lysis buffer) | 200mM NaOH
1% SDS |
| ○ Buffer N3 (neutralisation buffer) | 3.0 mM potassium acetate,
pH 5.5 |
| ○ Buffer EB (elution buffer) | 10 mM Tris-HCl (pH 8.5) |

c) His₆-tagged protein purification buffers

The following buffers were supplied with Novagen (Nottingham, UK) his₆-tagged protein purification kits:

- | | |
|---------------------|----------------|
| ○ 8x binding buffer | 40mM imidazole |
|---------------------|----------------|

- 8x charge buffer
 - 4x elute buffer
- 4M NaCl
160mM Tris-HCl (pH 7.9)
400mM NiSO₄
4M imidazole
2M NaCl
80mM Tris-HCl (pH 7.9)

d) Buffers for production of “super” competent DH5α and BL21(DE3) *E.coli* cells:

- Transformation Buffer 1
 - Transformation Buffer 2
- 10mM MES, pH 5.8
100mM RbCl₂
10mM CaCl₂
50mM MnCl₂
10mM PIPES, pH 6.5
75mM CaCl₂
10mM RbCl₂
15% v/v glycerol

e) ELISA reagents and buffers

- BSA # A7030, Sigma.
Goat anti-human IgG (Fc fragment specific) # I8885, Sigma.
CL # C1649, Sigma.
Phosphatidylserine (PS) # P7769, Sigma.
β₂GPI Scipac (Kent, UK)
Purified human IgG_λ # I5029, Sigma
Goat anti-human IgG_λ alkaline phosphatase conjugate # A3150, Sigma
p nitrophenyl phosphate tablets # 104-105, Sigma
- Sample, enzyme and conjugate (SEC) dilution buffer
 - 100mM Tris-HCl (pH 7.0)
 - 100mM Sodium Chloride
 - 0.02% Tween-20
 - 0.2% BSA
 - Bicarbonate buffer (BIC) (pH 9.6)
 - 0.5M Sodium bicarbonate
 - 0.05M Sodium dihydrogencarbonate

f) Western blot reagents

- Perfect Protein™ Western Markers Novagen, Nottingham, UK
HisTag® Monoclonal Antibody Novagen, Nottingham, UK

Horse radish peroxidase (HRP)–conjugated goat anti-mouse immunoglobulins (P 0447) Dako, Glostrup, Denmark

Anti-human IgG (γ -chain specific) peroxidase conjugate (A 8419) Sigma, Poole, UK

g) IgG purification buffers

IgG binding buffer	0.02M Sodium phosphate buffer pH 7 57.7% Na_2HPO_4 + 42.3 NaH_2PO_4
IgG elution buffer	0.1M glycine.HCl, pH 2.7

2.2 Bacterial strains

E.coli of strain DH5 α were supplied by Gibco, Paisley, UK

E.coli of strain BL21(DE3) and BL21 were supplied by Invitrogen, Paisley, UK

E.coli of strain W3110 were supplied by CellTech, Slough, UK

2.3 Antibiotic and growth media

Media were made in ddH₂O and sterilised by autoclaving.

a) Luria-Bertani (LB) medium:

- 1% (w/v) Bacto-tryptone (Duchefa, Haarlem, The Netherlands)
- 0.5% (w/v) Bacto-yeast extract (Duchefa)
- 1% (w/v) NaCl

Adjusted to pH 7.0 with NaOH.

For LB agar plates 1.5% (w/v) agar (Duchefa) was added to LB medium.

b) NZY+ Broth:

- 1% (w/v) NZ amine (casein hydrolysate), (Sigma)
- 5% (w/v) Bacto-yeast extract (Duchefa)
- 5% (w/v) NaCl

This was made up to 1L with ddH₂O, pH adjusted to 7.5 using NaOH and then autoclaved. Filter sterilised 1.5% 1M MgCl_2 , 1.25% 1M MgSO_4 and 1% 2M Glucose solution were added to the media prior to use.

c) "Terrific" Broth (TB):

- 1.2% (w/v) Bacto-tryptone (Duchefa)
- 2.4% (w/v) Bacto-yeast (Duchefa)
- 0.4% Glycerol

In a separate flask TB Salts were prepared, 1M KH_2PO_4 , monobasic and 1M K_2HPO_4 , dibasic.

TB and TB Salts were autoclaved separately and then combined in ratio of 1TB:10 TB Salts. To this filter sterilised 1% (w/v) glucose was added.

Where antibiotics were added to media and LB agar plates, final concentrations were of 50 μ g/ml ampicillin or 60 μ g/ml kanamycin. Filter sterilised stock solutions of ampicillin at 50mg/ml and kanamycin 60mg/ml in ddH₂O were kept in light-tight containers at -20°C.

2.4 Monoclonal antibodies (mAb)

2.4.1 Human monoclonal aPL – binding characteristics

IS4, B3 and UK-4 are all human IgG monoclonal antibodies produced from lymphocytes of three different patients. IS4 was derived from a patient with SLE/APS by Epstein-Barr virus transformation of peripheral blood mononuclear cells and fusion with human-mouse heterohybridoma K6H6/B5 cell line (Zhu et al., 1999). This was produced and kindly donated by Dr. P Chen, Department of Medicine, Division of Rheumatology, University of California at Los Angeles, LA, USA. B3 (Ehrenstein et al., 1993) and UK-4 (Menon et al., 1997) were isolated by fusion of peripheral blood lymphocytes from patients with cells of the mouse human heteromyeloma line CB-F7. B3 binds CL, single stranded (ss)DNA, dsDNA and histones (Ehrenstein et al., 1993, Kalsi et al., 1996). UK-4 binds negatively charged (but not neutral) PL in the absence of β_2 GPI (Menon et al., 1997). IS4 binds to CL only in the presence of bovine and human β_2 GPI and to human β_2 GPI alone (Zhu et al., 1999).

A system of production of monoclonal human IgG molecules has been established in our unit in which the heavy chain (V_H) is derived from the cloned cDNA of one human mAb and the light chain (V_L) from another (Rahman et al., 1998, Giles et al., 2003a). A stable expression system in CHO cells has been established to express three mAb variants - IS4 V_H /IS4 V_L , IS4 V_H /B3 V_L and IS4 V_H /UK-4 V_L (Giles et al., 2003a).

2.4.2 Murine monoclonal antibodies

6C4C10 and mAb-16 are anti-DI antibodies produced from mice immunised with recombinant DI of human β_2 GPI (Iverson et al., 2002b). These antibodies recognise conformational (but not linear) epitopes on DI and binding to whole β_2 GPI has been shown to be unaffected by 10 different single-point mutations within DI (Iverson et al., 2002b). Both these antibodies were kind gifts from Drs' Mike Iverson and Matt Linnik, La Jolla Pharmaceuticals (LJP), San Diego, USA.

2.5 Human polyclonal IgG

IgG was purified from serum collected from four groups of subjects: PAPS, APS and SLE, SLE with no clinical evidence of APS and finally healthy volunteers. 10-15mls of blood was collected in EDTA tubes, spun for 10 minutes at 1000g and serum collected and stored at -20°C pending IgG purification. Collection of samples was approved by the Joint UCL/UCLH Committees of Ethics of Human Research.

Purification of IgG from serum was performed using Protein G coated beads (Sigma, Poole, UK). Protein G 'slurry' was centrifuged at 200g for five minutes. Supernatant was then discarded and the beads washed with PBS and then centrifuged at 200g for five minutes. After three washes with PBS, serum was added to give 1:2 ratio of serum:beads. IgG binding buffer to give 2:1 ratio of IgG binding buffer:serum was also added and incubated at RT for two hours on a mixing table. The sample was then centrifuged at 200g and supernatant stored at -80°C as IgG free human serum. The IgG-coated Protein G beads were washed three times with IgG binding buffer and elution of IgG performed by adding 2ml of IgG elution buffer per 0.5ml serum. The sample was mixed for one minute and centrifuged. Supernatant was transferred as purified IgG and pH balanced with 1M Tris.HCl, pH 9.0. The elution step was repeated a second time.

2.6 Plasmids

The plasmid pRSET-b (Invitrogen, Paisley, UK) was used as the initial cloning vector. pET-20b (figure 2.1), pET-26b (figure 2.2) and pET-39b (figure 2.3) (Novagen, Nottingham, UK) were used as both cloning and expression vectors.

2.7 Minipreparation of plasmid DNA from *E.coli*

DH5 α -strain *E.coli* containing recombinant plasmid was stored in media containing 15% (v/v) glycerol at -80°C . Under sterile conditions a wire loop was used to streak the glycerol-stored cultures onto an LB agar plate supplemented with the appropriate antibiotic and incubated at 37°C overnight in a dry incubator. 5mls LB medium supplemented with the appropriate antibiotic in a 50ml falcon tube were inoculated with a colony and incubated at 37°C in a shaking incubator overnight. An uninoculated sample was also cultured overnight as a control to check that irrelevant bacteria had not contaminated the LB medium.

Plasmid DNA was extracted from overnight *E.coli* cultures using the QIAGEN QIAprep® Miniprep kit (Crawley, West Sussex, UK) according to the manufacturer's instructions. A bacterial pellet was obtained by centrifugation at 1864g for 10 minutes at 4°C and dried by inversion. The pellet was resuspended in 250µl of buffer P1 and transferred to a 0.5ml microfuge tube. Then 250µl of buffer P2 were added and the tube inverted six times. Following this 350µl of buffer N3 were added, mixed by inversion and then centrifuged for 10 minutes. All subsequent centrifugation steps were carried out at 13000 rpm in a microcentrifuge for one minute at RT. Supernatant from this step was decanted into a QIAprep spin column and centrifuged. DNA bound to the QIAprep spin column was washed by adding 0.75ml buffer PE and centrifuged. Flow through was discarded and the column centrifuged. After placing the QIAprep spin column in a clean 1.5ml microfuge tube recombinant DNA was eluted by adding 50µl of autoclaved water to the centre of each QIAprep column, which was left to stand for one minute before being centrifuged. Eluted DNA was then stored at -20°C.

2.8 Restriction digest of DNA

Plasmid DNA constructs or purified PCR products were digested with two different restriction enzymes, either *Bgl* II, *Nco* I or *Xho* I. Typically 1µg of plasmid DNA and 10 units of restriction enzyme were incubated in a total volume of 20µl for one hour with buffer D, at 37°C. The recipe for a typical small-scale digest would be as follows:

Plasmid DNA	5µl (1µg DNA)
Enzyme	1µl (10u/µl)
10x Buffer D	2µl
ddH ₂ O	to 20µl

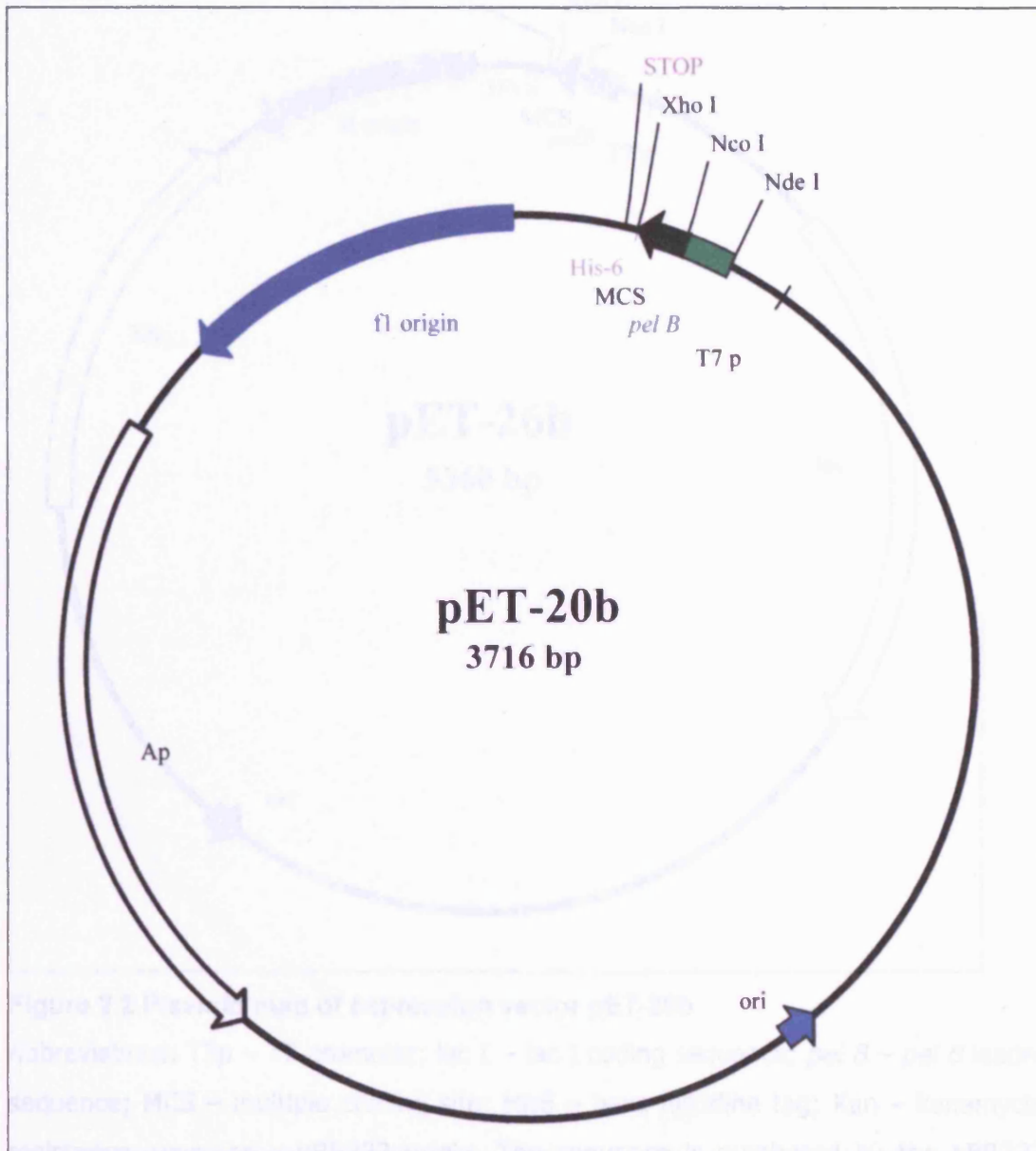


Figure 2.1 Plasmid map of expression vector pET-20b

Abbreviations: T7p – T7 promoter; *pel B* – *pel B* leader sequence; MCS – multiple cloning site; His6 – hexa-histidine tag; Ap – ampicillin resistance gene; ori - pBR322 origin. The sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map

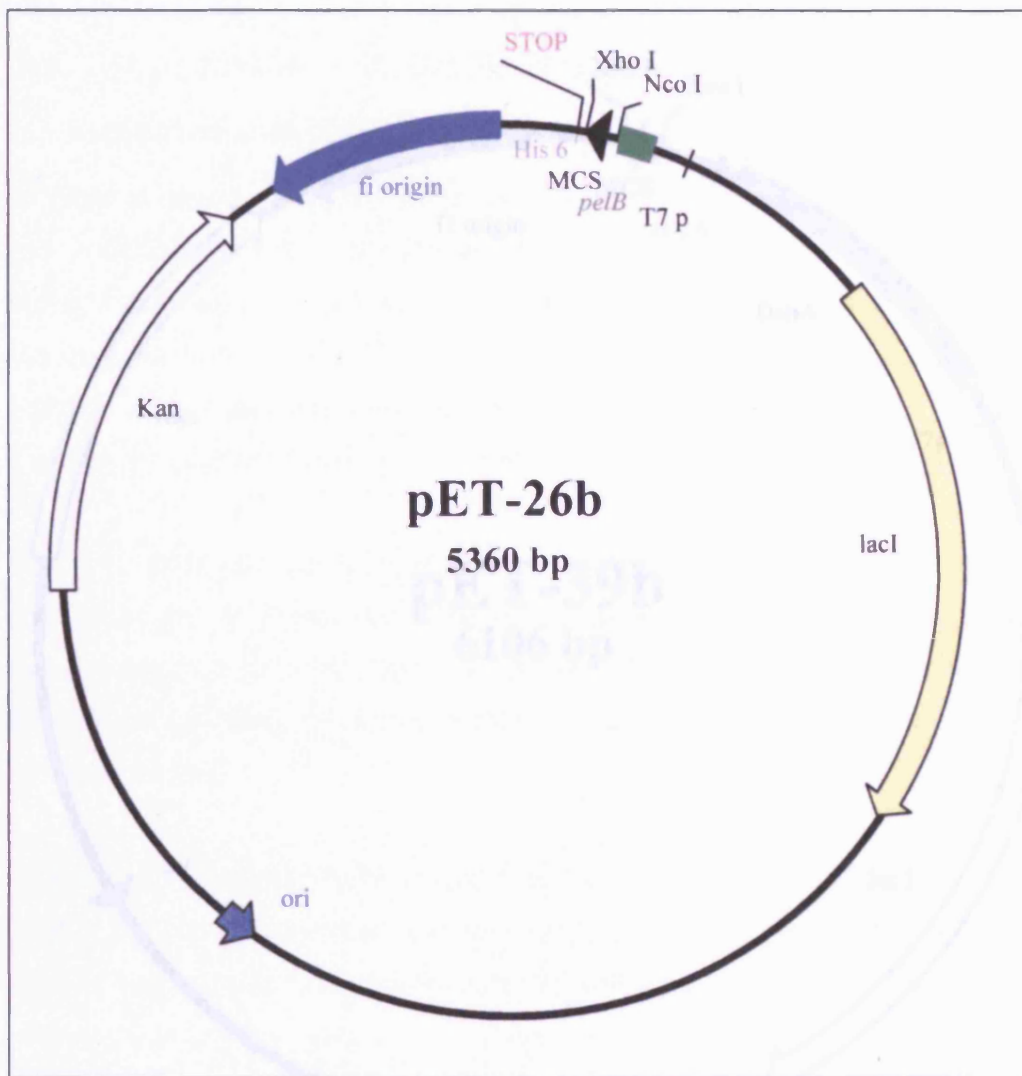


Figure 2.2 Plasmid map of expression vector pET-26b

Abbreviations: T7p – T7 promoter; lac I - lac I coding sequence; *pel B* – *pel B* leader sequence; MCS – multiple cloning site; His6 – hexa-histidine tag; Kan – kanamycin resistance gene; ori - pBR322 origin. The sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map.

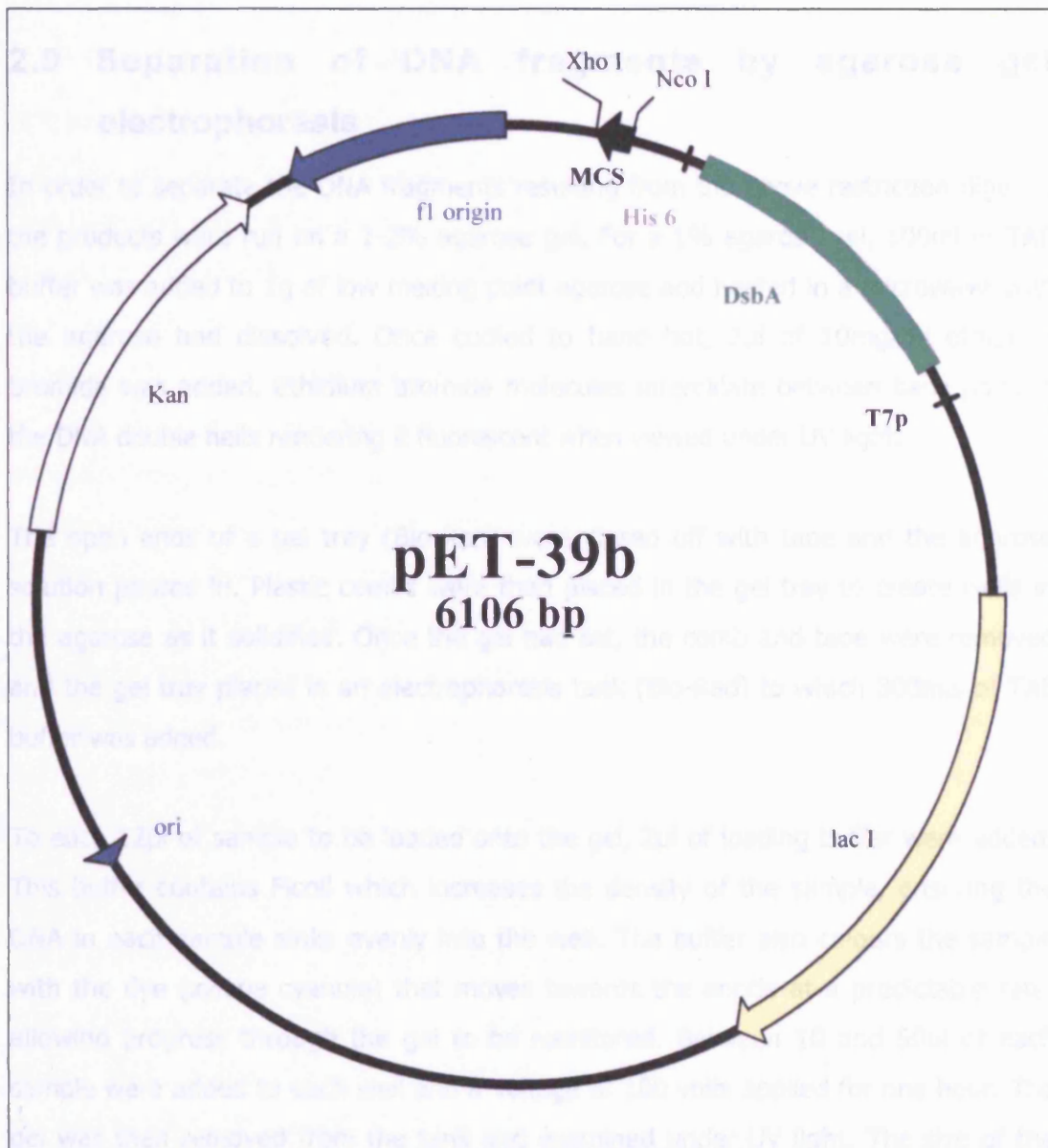


Figure 2.3 Plasmid map of expression vector pET-39b

Abbreviations: T7p – T7 promoter; lac I - lac I coding sequence; DsbA gene (DsbA leader sequence 408-465); MCS – multiple cloning site; His-6 – hexa-histidine tag; Kan – kanamycin resistance gene; ori - pBR322 origin. The sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map.

2.18 Quantification of dsDNA concentration

dsDNA concentrations were quantified by running standard DNA samples alongside unknown DNA samples on an agarose gel and comparing the intensity of the bands.

2.9 Separation of DNA fragments by agarose gel electrophoresis

In order to separate the DNA fragments resulting from the above restriction digests, the products were run on a 1-2% agarose gel. For a 1% agarose gel, 100ml of TAE buffer was added to 1g of low melting point agarose and heated in a microwave until the agarose had dissolved. Once cooled to hand hot, 2 μ l of 10mg/ml ethidium bromide was added. Ethidium bromide molecules intercalate between base pairs in the DNA double helix rendering it fluorescent when viewed under UV light.

The open ends of a gel tray (Bio-Rad) were closed off with tape and the agarose solution poured in. Plastic combs were then placed in the gel tray to create wells in the agarose as it solidified. Once the gel had set, the comb and tape were removed and the gel tray placed in an electrophoresis tank (Bio-Rad) to which 800mls of TAE buffer was added.

To each 12 μ l of sample to be loaded onto the gel, 2 μ l of loading buffer were added. This buffer contains Ficoll which increases the density of the sample, ensuring the DNA in each sample sinks evenly into the well. The buffer also colours the sample with the dye (xylene cyanole) that moves towards the anode at a predictable rate, allowing progress through the gel to be monitored. Between 10 and 50 μ l of each sample were added to each well and a voltage of 100 volts applied for one hour. The gel was then removed from the tank and examined under UV light. The size of the bands in the gel were estimated by comparison with a standard DNA molecular weight marker loaded into another well in the same gel. Photographs of the gel were taken using the Genesnap programme (Syngene, Cambridge, UK) and printed onto paper.

2.10 Quantification of dsDNA concentration

dsDNA concentrations were quantified by running linearised DNA samples alongside molecular weight markers of known DNA concentration on an agarose gel and comparing the intensity of the bands.

2.11 Purification of DNA from agarose gels

PCR samples and digested DNA fragments were purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Crawley, West Sussex, UK). An excised band of DNA weighing up to 400mg was placed in a 1.5ml microfuge tube and 1ml of buffer QG added. The mixture was then incubated at 55°C for 10 minutes until all agarose had dissolved. Then 1µl of isopropanol per 1mg of initial agarose gel dissolved was added and the tube inverted 10 times to mix. 800µl of this solution were then added to a spin column placed in a collection tube and centrifuged at 13000 rpm for 10 seconds. Flow through was discarded from the collection tube and the procedure repeated until all of the initial mixture had been passed through the spin column. A further 500µl of buffer QG was added to each spin column and centrifuged at 13000 rpm for 10 seconds. After discarding the flow through, 750µl of buffer, PE were added and centrifuged as above, flow through discarded and centrifuged again. Each column was then placed into a fresh 1.5ml microfuge tube and elution of DNA from each column performed by addition of 50µl of solution EB, allowing the column to stand for one minute followed by centrifugation as above. The product was then stored at -20°C.

2.12 Synthesis of synthetic DI gene using recursive PCR

The advantages of synthesising a gene encoding for DI of β_2 GPI rather than using cloned cDNA is that codons preferred by bacteria can be used, thus potentially increasing yield of expressed target protein by *E.coli*. Furthermore, convenient restriction sites can be incorporated to aid genetic manipulation. DI and 11 DI mutants (E23S, Mut 23-26-27, Mut 8-9-23-26-27, R39S, R39K, G40E, G40S, R43G, R43K, DI ext, II-III) were made using recursive PCR (Prodromou and Pearl, 1992).

2.12.1 Design of primers for recursive PCR

Design of the synthetic gene was performed using Juniper computer software, a kind gift from Dr. Chris Richardson and Professor Laurence Pearl, Institute of Cancer Research, London, UK. The amino acid sequence of DI of human β_2 GPI with an Omp A sequence at the N-terminal was entered into Juniper along with the desired flanking restriction sites. Juniper then designed a gene encoding this recombinant protein, but altered the codon usage, such that codons preferred by *E.coli* were used rather than those preferred by eukaryotic cells. Juniper then designed six 60mer oligonucleotide primers that together cover the length of this 270bp gene. The primers overlapped sequentially with each other by 17 nucleotides and were used in

a recursive PCR reaction to produce the desired synthetic gene optimised for *E.coli*. Thus, each primer effectively acted as a template (at the 3' end) and primer (at the 5' end). The principles of recursive PCR are schematically represented in figure 2.4.

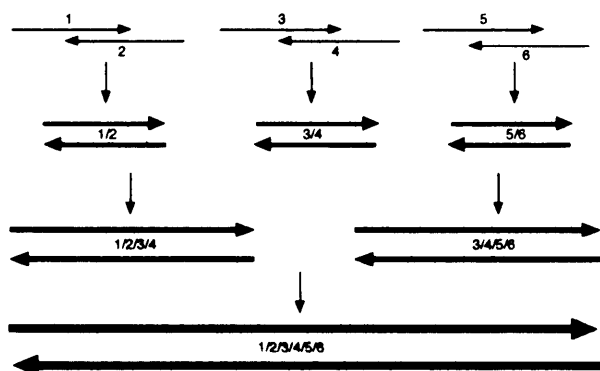


Figure 2.4. Schematic diagram of DI gene synthesis using recursive PCR. Oligonucleotides are numbered 1-6.

2.12.2 Recursive PCR

20pmol of each outer primer and two pmol of each internal primer were used in a reaction containing 2U of *Vent* DNA polymerase (NEB, Herts, UK) and 25mM of 2'-deoxynucleoside 5'- triphosphates (dNTPs') in 100µl of 10x supplied buffer and ddH₂O. Cycling parameters were as follows:

Initial denaturation of 94°C for eight minutes

Then 30 cycles as follows:	Denaturation	-	94°C for two minutes
	Annealing	-	57°C for one minute
	Extension	-	72°C for one minute
	Final extension	-	72°C for 10 minutes

The reaction was then kept at 4°C until the samples were removed. The PCR samples were run on a 2% agarose gel, the amplified gene excised and purified from the agarose gel. The purified recombinant gene was then digested with *Bgl* II and *Nco* I restriction enzymes and ligated (section 2.13) into the cloning vector pRSETb. Plasmid DNA of the pRSETb construct containing the recombinant DI insert was precipitated as described in section 2.16 and sent for automated sequencing.

2.13 Ligation of amplified DNA from recursive PCR into pRSETb

Gel purified, digested PCR products from step 2.12 were ligated into 50ng of pRSETb. Ranges from 3:1 to 1:3 insert:vector molar ratios were used. To 10µl of

insert:pRSETb mix, 2µl of 10x ligase buffer, 7.4µl autoclaved ddH₂O and 0.6µl (3U/µl) T4 DNA ligase were added. Samples were incubated for three hours at RT or at 4°C overnight. The ligation product was used to transform competent DH5α cells (section 2.14 and 2.15).

2.14 Production of competent DH5α and BL21(DE3) *E.coli* cells

DH5α or BL21(DE3) cells picked from a discrete colony upon an LB agar plate were grown overnight in 10mls of LB medium at 37°C in a shaking incubator (220rpm). The following morning 100µl of this culture was added to 100ml sterile LB in a sterile 2.5L flask and returned to the shaking incubator for three hours. When swirling clouds of bacteria were just visible the culture was removed from the incubator and divided into two 50ml falcon tubes, placed on ice for five minutes and then centrifuged at 1864g for 10 minutes at 4°C. Supernatant was discarded from each tube and the pellet resuspended in 20mls ice-cold transformation buffer 1 and incubated on ice for five minutes. Each tube was again centrifuged at 1864g for 10 minutes at 4°C and the supernatant discarded. Each pellet was then resuspended in 1ml of transformation buffer 2 and incubated on ice for 15 minutes. 100µl of this suspension was then aliquoted into pre-chilled 1.5ml microfuge tubes and stored at -80°C or used directly for transformations.

2.15 Transformations of competent *E.coli* cells

5µl of ligation mixture were added to 100µl of competent *E.coli* cells and incubated on ice for 30 minutes. A heat shock at 42°C for 60 seconds was then applied, following which the cells were incubated in 800µl of LB at 37°C for one hour. The cell suspension was then centrifuged at 700g for 10 minutes at RT, most of the supernatant poured off and the pellet resuspended in the remaining supernatant. This suspension was then spread on an LB agar plate containing the appropriate antibiotic. After drying on the bench for 15 minutes the plates were then incubated at 37°C overnight. The following day antibiotic resistant clones were picked, grown in 5ml LB with appropriate antibiotic and miniprep plasmid DNA produced as in section 2.7.

2.16 Ethanol precipitation of plasmid DNA

In the presence of salt (monovalent cations such as Na⁺) and at a temperature of -20°C or less, absolute ethanol efficiently precipitates nucleic acids. Therefore 0.1

volume of 3M Sodium Acetate, pH 5.2 and 2.5 volumes of 100% ethanol were added to the DNA preparation in a 1.5ml microfuge tube. This was placed in a -80°C freezer for one hour to precipitate the DNA.

To collect the precipitate, the precipitation was firstly spun in a microfuge for 10 minutes at 13000 rpm at 4°C . The supernatant was discarded and the pellet washed with 1ml 70% ethanol and centrifuged again as above. The 70% ethanol was then decanted and the sample left to dry under a lamp for one hour.

Dried precipitated plasmid DNA was sent for automated sequencing to MWG-Biotech, Ebensburg, Germany.

2.17 Production of DI mutants

DI mutants with single or multiple amino acid mutations were made using either site directed mutagenesis, or recursive PCR with the primers designed by Juniper (see section 2.12) and subsequently ligated into pET-26b as above. In total 16 constructs of DI mutants with single or multiple amino acid substitutions were made. Details of the amino acids targeted and the designated names given to each mutation are shown in Table 2.1.

2.17.1 Computer modelling of DI and DI mutants

Swiss-Model is a fully automated protein homology modelling server freely available on the internet via <http://swissmodel.expasy.org> website. Via the validated programme Swiss-Pdb Viewer (GlaxoSmithKline R&D and Swiss Institute of Bioinformatics), an accurate 3-dimensional template of a protein sequence can be made, and several proteins can be superimposed in order to deduce the effects of altering single amino acids on H-bond formation and overall conformation (Peitsch, 1996, Guex and Peitsch, 1997). This programme was used to minimise the probability of the desired mutations causing –

- i. significant conformational change
- ii. hydrogen bond or salt-bridge formation with neighbouring amino acids
- iii. large alterations in surface exposure of the epitopes of interest.

Mutation designated name	Amino acids substituted
D8S	Asp at 8 to Ser
D9G	Asp at 9 to Gly
Mut 8+9	Both Asp at 8+9 to Ser at 8 and Gly at 9
E23S	Glu at 23 to Ser
E26S	Glu at 26 to Ser
E27S	Glu at 27 to Ser
Mut 23-26-27	All three Glu at 23, 26 and 27 to Ser
Mut 8-9-23-26-27	Mutations of both Mut 8+9 and Mut 23-26-27
R39K	Arg at 39 to Lys
R39S	Arg at 39 to Ser
G40E	Gly at 40 to Glu
G40S	Gly at 40 to Ser
R43G	Arg at 43 to Gly
R43S	Arg at 43 to Ser
DI ext	Insertion of PRVCPF from position 61 to complete the DI-II interlinker
DII-III	Interlinker DII-III (RPIELPVCA at positions 53-61) substituted for wild-type interlinker (WPINTLKCT)

Table 2.1 DI mutations – designated names and positions of amino acids targeted

2.17.2 Site directed mutagenesis

The QuickChange site-directed mutagenesis kit (Stratagene) was found to be a convenient method to produce single amino acid substations. Five constructs encoding for mutant DI cloned into pET-26b were made - D8S, D9G, Mut 8+9, E26S and E27S. The basic procedure requires a dsDNA vector containing an insert of interest and the design of two specific oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complimentary to opposite strands of the vector, are extended during temperature cycling by *PfuTurbo* DNA polymerase. This enzyme has a 6-fold higher fidelity in DNA synthesis than *Taq* DNA polymerase and hence has a low potential for generating further mutations during PCR. Incorporation of the oligonucleotide primers generates a mutated plasmid. Following PCR, the product is treated with *Dpn I*, an endonuclease specific for methylated DNA

produced by DH5 α *E.coli*, which thus digests the parental DNA template but not the mutated plasmid. Vector DNA containing the desired mutation(s) is then transformed into XL-Blue supercompetent cells. Mutagenic oligonucleotide primers were designed according to manufacturers specifications. Primer length was between 25 and 45 bases with melting temperatures (T_m) \geq 78°C. The formula used to calculate T_m is shown in Table 2.2 (A) and the specific primers used in Table 2.2 (B).

Purified pET-26b was obtained as described in section 2.7. A series of PCR reactions were then set up containing 5ng, 10ng, 20ng and 50ng concentrations of plasmid DNA with the appropriate mutagenic primers (see Table 2.2 (B)), and the reaction solutions provided in the QuickChange site-directed mutagenesis kit (Stratagene) of plasmid DNA was mixed with 5 μ l 10x reaction buffer, 1 μ l of dNTP mix, 5 μ l forward primer (125ng), 5 μ l reverse primer (125ng), 1 μ l *PfuTurbo* DNA polymerase (2.5 units/ μ l) and autoclaved ddH₂O to a final volume of 50 μ l. As negative control no plasmid DNA was added to one reaction mixture. Each mixture was then taken, pulse spun, and placed into a PCR machine programmed according to the manufacturers instructions with the extension time of each reaction calculated at one minute per kb of plasmid length. Cycling parameters were as follows:

Initial denaturation at 95°C for 30 seconds

Then 12 cycles as follows -

Denaturation	- 95°C for 30 seconds
Annealing	- 55°C (for D8S, D9G) for one minute - 57°C (for Mut 8+9, E26S, E27S) for one minute
Extension	- 68°C for one minute per kb plasmid for sample reactions and six minutes for control reaction.

The reaction was then held at 4°C until the samples were removed.

Once the PCR step had been carried out the methylated template DNA containing the undesired sequence was digested by the addition of 1 μ l (10U) of *Dpn* I endonuclease restriction enzyme directly to each amplification reaction. The sample was mixed gently by pipetting up and down several times, after which it was pulse spun and incubated at 37°C for one hour. A separate 50 μ l aliquot of XL-Blue supercompetent cells was then transformed with 1 μ l of the *Dpn* I treated DNA from each control, sample reaction and transformational control plasmid (pUC-19) supplied by Stratagene.

A) $T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$

Where – N is the primer length in bases

Values for % GC and % mismatch are whole numbers

%GC = the percentage of GC bases in the primer sequence

% mismatch = the number of deletions / primer sequence x100

B)

PRIMER SEQUENCE		
1.	Forward	5'GGTCGTACCTGCCCGAAACCG <u>T</u> CCGACCTGCCGTTCTCC3'
2.	Back	5'GGAGAACGGCAGGTC <u>G</u> ACGGTTTCGGGCAGGTACGACC3'
3.	Forward	5'CGTACCTGCCCGAAACCGGAC <u>G</u> CTGCCGTTCTCCACC3'
4.	Back	5'GGTGGAGAACGGCAG <u>A</u> CCGTCCGGTTTCGGGCAGGTACG3'
5.	Forward	5'CGTACCTGCCCGAAACCG <u>T</u> CC <u>G</u> CTGCCGTTCTCCACC3'
6.	Back	5'GGTGGAGAACGGCAG <u>A</u> CC <u>G</u> ACGGTTTCGGGCAGGTACG3'
7.	Forward	5'ACCTTCTACGAACCGGG <u>TCC</u> GAAATCACCTACTCCTGC3'
8.	Back	5'GCAGGAGTAGGTGATTT <u>GGA</u> ACCCGGTTCGTAGAAGGT3'
9.	Forward	5'ACCTTCTACGAACCGGGTGA <u>TCC</u> ATCACCTACTCCTGC3'
10.	Back	5'GCAGGAGTAGGTGAT <u>GGA</u> ATCACCCGGTTCGTAGAAGGT5'

Table 2.2 Properties and sequences of primers used in site directed mutagenesis

A) Formula to calculate melting temperature of mutagenic primers

B) Mutagenic primers with sites for point mutations shown in bold and underlined. All primers were used to amplify DI cloned pET-26b and mutate:

Primers 1 and 2	Asp to Ser	Codon 8	-	D8S
Primers 3 and 4	Asp to Gly	Codon 9	-	D9G
Primers 5 and 6	Asp,Asp to Ser,Gly	Codons 8,9	-	Mut 8+9
Primers 7 and 8	Glu to Ser	Codon 26	-	E26S
Primers 9 and 10	Glu to Ser	Codon 27	-	E27S

This method is very similar to that described in section 2.15 with the following exceptions - a heat shock at 42°C of 45 seconds with recovery on ice for a further two minutes and 0.5ml of NZY medium preheated to 42°C was added to each transformation reaction. Colonies were picked from the sample transformation plates and inoculated into 5ml LB cultures containing 60µg/ml kanamycin and kept overnight in a 37°C shaking incubator. The following day glycerol stocks were made and plasmid DNA purified (see section 2.7). This was sequenced to confirm the presence of the desired mutation (see section 2.16).

2.18 Recombinant wild-type and mutant DI protein expression

2.18.1 Optimisation of protein expression protocol

An extensive range of experiments were carried out in an effort to establish and optimise the induction and expression protocol of recombinant DI detailed in this section below. Specifically expression experiments were performed using the pET-20b construct as well as the final expression construct pET-26b, altering media and culture volumes, varying culture and induction temperatures, using alternative *E.coli* strains, varying induction duration, inducing in the presence or absence of glucose in the culture media and varying levels of the inducing agent IPTG. These experiments are discussed in more detail in chapter 3.

2.18.2 Final optimised culture and induction experimental protocol

The final recombinant expression vector pET-26b, containing the DI gene and a kanamycin resistance gene, was transferred to the expression strain BL21(DE3) that contains a chromosomal copy of the gene for T7 RNA polymerase. Single colonies were picked from the transformants, and 5ml cultures of TB with 60µg/ml kanamycin in 50ml falcon tubes were inoculated and cultured at 30°C overnight in a shaking incubator. Fresh 250ml cultures of pre-warmed TB with 60µg/ml kanamycin were then set up using the overnight culture to inoculate the medium to an optical density (OD)₆₀₀ of 0.1 and grown at 30°C to an OD₆₀₀ of 0.6. Cultures were then induced with 0.1mM IPTG and allowed to grow for a further four hours at 30°C with shaking. OD₆₀₀ was recorded at periodic intervals before and after induction as a measure of bacterial growth in the culture.

2.18.3 Preparation of periplasmic fraction

The method for periplasmic extraction of protein from *E.coli* is based on previously published methods by our group for the expression of periplasmic F(ab)s of human

antibodies in W3110 *E.coli* (Kumar et al., 2000). Cultures were centrifuged four hours post induction at 4000g for 20 minutes at 4°C and the supernatant saved (at 4°C) for the detection of any leaked DI protein from cells. The cell pellet was then resuspended in ice-cold autoclaved ddH₂O (30ml ddH₂O per 1L culture) and stirred at 4°C for 30 minutes. This exposes the cells to osmotic shock, a process that degrades the outer-cell membrane releasing periplasmic protein into the medium whilst maintaining the integrity of the inner-cell membrane. The sample was then centrifuged at 8000g for 20 minutes at 4°C. The supernatant constitutes the periplasmic fraction containing recombinant DI, an aliquot of which was stored at -20°C for subsequent SDS-PAGE and western blot analysis.

2.19 Recombinant wild-type and mutant DI purification

DI was purified using nickel chromatography by virtue of the incorporated C-terminal his₆-tag, which binds divalent cations such as Ni²⁺ immobilised on resin. The HisBind® purification kit (Novagen, Nottingham, UK) was used. Conditions were optimised for DI purification as below.

All purification steps were done on ice. 0.6ml bed volume (BV) of resin was added per 1L expression culture to a chromatography column. This was then charged with nickel by applying five volumes of 1x charge buffer and equilibrated with three volumes of 1x binding buffer. The periplasmic extract was then applied to the column and subsequently washed successively with 5mM imidazole, then 45mM imidazole and DI eluted subsequently with 300mM of imidazole. The wash and elution buffers were made up by combining appropriate proportions of 1x binding buffer (5mM imidazole) and 1x elution buffer (1M imidazole) included in the kit. Imidazole binds to nickel cations in preference to a his₆-tag and thus displaces bound his₆-tagged DI. All washes and flow-through were collected and stored at -20°C for subsequent SDS-PAGE analyses.

DI elution sample was then dialysed against PBS-10% glycerol overnight at 4°C using dialysis visking tubing, MWCO-3500, Medicell (London, UK), aliquoted into 1.5 ml microfuge tubes, snap frozen in liquid nitrogen and stored at -80°C. The purity of eluted DI was assessed on SDS-PAGE 15% gels.

2.20 Protein quantification, detection and characterisation

2.20.1 Total protein quantification

Total protein was quantified by colorimetric detection using the bicinchoninic acid (BCA) protein assay kit (Pierce, IL, USA) according to manufacturers instructions. The method combines the reduction of Cu^{2+} to Cu^{1+} by protein within an alkaline medium with the sensitive and colorimetric detection of cuprous cation Cu^{1+} using a reagent containing BCA (Smith et al., 1985). The purple coloured reaction formed by the chelation of two molecules of BCA with one cuprous ion exhibits absorbance at 562nm that is linear with increasing protein concentrations over a range of 80-2,000 $\mu\text{g}/\text{ml}$. Varying known concentrations of 25 μl of a protein standard, BSA (in 0.9% saline and 0.05% sodium azide) supplied with the kit, is used to plot a line which is then used to quantify the total protein concentration in the 25 μl test sample.

2.20.2 SDS-polyacrylamide gel electrophoresis (PAGE)

Each gel consisted of 3mls 5% stacking gel layered on top of 5mls 15% resolving gel. The resolving gel was poured and immediately layered with isopropanol. After the gel had set, the isopropanol was removed and the stacking gel added to form the upper layer. A suitable comb was added prior to the polymerisation of the stacking gel. The composition of the resolving and stacking gels is described in table 2.3.

Total protein samples were separated into polypeptide units by SDS-PAGE according to the method of Laemmli, 1970 (Laemmli, 1970). Protein samples were added in a 1:1 ratio to sample buffer (2% (w/v) SDS, 50mM Tris-HCl, pH 6.8, 0.1% bromophenol blue, 10% (w/v) glycerol, 100mM DTT and denatured at 100°C for three minutes immediately before loading on to the gel. 20 μl of sample or 5 μl of molecular weight protein marker (Prestained Protein Marker, Broad Range (NEB, Herts, UK) or Rainbow™ Coloured Protein Molecular Weight Markers (Amersham Bio, Bucks, UK)) were loaded. SDS-PAGE gels were prepared in a vertical gel, one-dimensional, electrophoresis system (Gibco-BRL, Paisley, UK) and run in 1x running buffer (25mM Tris, 250mM glycine and 0.1% (w/v) SDS, pH 8.3) at 40 mA/gel at RT until the dye reached the bottom of the plate.

	10 mls 10% resolving gel	8 mls 5% stacking gel
ddH ₂ O	2.3 mls	5.5 mls
30% acrylamide mix ¹	5.0 mls	1.3 mls
1.5 M Tris, pH 8.8	2.5 ml	-
1.0 M Tris-HCl, pH 6.8	-	1.0 ml
10% SDS	0.1 ml	0.08 ml
10% APS ²	0.1 ml	0.08 ml
TEMED ³	0.004 ml	0.008 ml

Table 2.3. Composition of resolving and stacking gels for SDS-PAGE.

¹Acrylamide mix = N,N'-Methylene-bis-acrylamide. ²APS = ammonium persulfate.

³TEMED = NNNN-tetramethylethanediamine.

2.20.3 Coomassie stain

This stain was used to visualise the protein bands run on an SDS-PAGE gel. The gel was transferred from the electrophoresis apparatus to a plastic container and covered with 0.2% Coomassie gel stain (0.2% (w/v) Coomassie, 7.5% acetic acid, 40% methanol, dH₂O to a volume of 1L) for eight minutes. The gel was then destained with Coomassie destain (7.5% acetic acid and 5% methanol made up to 1L with dH₂O) for three hours. The gel was then dried on a gel dryer at 60-80°C for 30 minutes and kept as a permanent record.

2.20.4 Transfer of protein to nitrocellulose and western blotting

Protein separated on SDS-PAGE gels were transferred to Hybond C membranes (Amersham, Bucks, UK) using a wet-transfer method described by Towbin, 1979 (Towbin et al., 1979). The gel and the nitrocellulose membrane were pre-soaked in transfer buffer (50mM Tris, 180mM glycine, 0.1% (w/v) SDS) and 20% methanol) and blotted over one hour at 400mA at RT in a Trans-blot™ Cell (Bio Rad) according to manufacturers instructions. An ice-block was placed in the cell to avoid over-

heating. The membrane was subsequently removed, dried and the protein marker used highlighted.

2.20.5 Immunodetection of proteins on western blots

The membrane was blocked in PBS / 0.1% polyoxyethylene sorbitan monolaurate Tween 20 / 5% (w/v) skimmed milk for one hour at RT on a shaking platform. The primary antibody solution (in PBS / 0.1% Tween 20 / 5% milk) was then added and incubated for a further one hour, shaking at RT. The unbound primary antibody was then rinsed off by two 10 minute washes with PBS / 0.1% Tween 20, and the appropriate anti- IgG HRP conjugated secondary antibody (in PBS / 0.1% Tween 20 / 5% milk) added for one hour, RT on a shaker. Unbound secondary antibody was then rinsed off with two washes of 10 minutes with PBS / 0.5% Tween 20. The bound HRP was then detected using chemiluminescence (ECL™, Amersham, Bucks, UK) and the resultant light emissions exposed to X-ray film from one second to one hour, depending on the strength of the signal.

2.20.6 Quantitative western blotting

The concentration of purified target protein was further confirmed using quantitative western blotting. This technique was particularly useful for concentrations below 80µg/ml, which represents the lower sensitivity limit of the BCA protein assay (section 2.20.1). A known concentration of his₆-tagged DI (stock at 500µg/ml) was kindly donated by Dr M Iverson. Known serial dilutions were loaded onto a 15% SDS-PAGE mini gel (section 2.20.2) and a known dilution of the target protein also loaded. The proteins were then transferred onto a nitrocellulose membrane (section 2.20.4) and probed with an anti-his₅ antibody (Novagen). The intensity of signal displayed on the film is directly proportional to the amount of protein transferred onto the membrane. Digital microdensitometry analysis (GS-800, BioRad Lab, CA, USA) of the film produced arbitrary units representative of the intensity of signal. These results were used to plot a standard curve of his₆-tagged DI protein concentration versus arbitrary units, against which the concentration of expressed target protein could be estimated.

2.21 Functional binding assays – enzyme linked immunosorbent assay (ELISA)

In all the different ELISAs performed, 96 well plates used were supplied by either Nunc, VWR International, Leicester, UK (irradiated plates) or Xenopore, NJ, USA (nickel-chelate plates). OD was measured by the Genios plate reader (Tecan,

Reading, UK). Each plate was marked vertically to divide it into two halves; the test half and the control half. The 36 wells at the edge of the plate were not used and henceforth all descriptions of the plate refer to the inner 60 wells only.

2.21.1 Detection and quantification of total whole IgG molecules

A 'Sandwich' ELISA was used to detect the total whole IgG molecule concentration of each human monoclonal or affinity purified polyclonal IgG sample. Goat anti-human IgG_λ (Fc fragment specific) was dissolved in BIC buffer to give a working solution of 400ng/ml. 50μl of BIC buffer alone was added to the wells in the right (control) half of the plate. Each plate was then covered and incubated overnight at 4°C. After this the plates were washed three times with PBS / 0.1% Tween 20 and 100μl of PBS containing 2% BSA was added to each well to block non-specific binding of Ig to the plastic. The plates were incubated for one hour at 37°C and then washed three times with PBS / 0.1% Tween 20.

In order to dilute CHO cell supernatant or affinity purified antibody in SEC buffer serially, such that for each well in the test half of the plate there was a well in the control half containing the same supernatant at the same dilution, the following procedure was performed - 50μl of SEC was added to each well in both halves of the plate except the top wells. 100μl of neat CHO supernatant was added to the top well and diluted down the column such that each well contained supernatant at half the concentration of the well above. Purified human IgG of known concentration (100ng / ml) was loaded and diluted serially in the same way upon each plate to allow the construction of a standard curve relating OD to IgG concentration. The plates were incubated for one hour at 37°C and then washed three times with PBS / 0.1% Tween 20.

To detect bound antibody, 50μl goat anti-human λ light chain alkaline phosphatase conjugate (diluted 2500-fold in SEC dilution buffer) was added to the wells and incubated for one hour at 37°C. After one hour, the plates were washed three times with PBS / 0.1% Tween 20 and once with BIC buffer. One tablet of *p*-nitrophenyl phosphate substrate was dissolved in 5ml BIC buffer supplemented with 10μl of 2mM MgCl₂. 50μl of this solution was added to each well. The plates were then incubated at 37°C to allow a yellow colour to develop in each well. The OD of the reaction was read after 60 minutes at 405nm (reference 490nm). The final reading was calculated by subtracting the OD value of each control well from the OD value of the same sample in the corresponding test well. This ensured that only the conjugate bound

directly to IgG in the supernatant that had been captured by the anti-Fc IgG would contribute to the result.

The binding of whole IgG molecules in the supernatants to the plate depended on the presence of the Fc region whereas detection by the alkaline phosphatase conjugate depended on the presence of the λ light-chain, therefore this method only detected whole IgG $_{\lambda}$ antibodies.

2.21.2 Detection of aPL binding to cardiolipin in solid phase

A polysorb ELISA plate was coated with 50 μ l of CL (diluted to 2.5 μ g/ml in ethanol) on the test half and 50 μ l of ethanol on the control half. The plate was then incubated overnight at 4°C uncovered. Following that each plate was washed twice with PBS and 100 μ l of PBS containing 10% foetal calf serum (FCS) (Invitrogen) was added to each well to block non-specific binding of IgG to the plate surface. After incubation for one hour at RT the plate was washed twice with PBS.

Human polyclonal IgG was diluted in PBS containing 10% FCS to 20 μ g/ml and 50 μ l added to the test half and the corresponding well in the control half. All patient samples were tested in duplicate. IgG derived from a patient with APS whose sera was known to bind CL was used as a positive control in each plate tested. This positive control (P18) was serially diluted and used as the in-house standard against which to measure % binding activity to CL for each IgG sample. The plate was then incubated for 90 minutes at RT and washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in PBS containing 10% FCS and 50 μ l of this solution added to each well. After one hour incubation at RT the plate was washed three times with PBS and once with BIC buffer. Addition of substrate was carried out as in section 2.21.1, the plate incubated at RT and OD measurements taken at 30 minutes and one hour.

2.21.3 Detection of aPL binding to phosphatidylserine in solid phase

A polysorb ELISA plate was coated with 50 μ l of PS (diluted to 50 μ g/ml in methanol and chloroform 4:1) and 50 μ l of methanol and chloroform alone was added to the control half. The plate was then incubated overnight at 4°C uncovered. Following this all steps performed subsequently were as those described in the section 2.21.2 above.

2.21.4 Detection of aPL binding to β_2 GPI in solid phase

A maxisorp ELISA plate was coated with 50 μ l of β_2 GPI (diluted to 10 μ g/ml in PBS) on wells in the test half and 50 μ l of PBS alone in the control half. The plate was covered and incubated overnight at 4°C. Following that each plate was washed three times with PBS and then blocked by adding 100 μ l of 0.25% gelatin in PBS. After incubation at 37°C for one hour the plate was washed three times with PBS.

Monoclonal antibody samples were serially diluted in 0.1% BSA/PBS and then loaded upon each half of the plate, as well as a positive control of serum containing polyclonal anti- β_2 GPI antibodies leaving a final volume in each well of 50 μ l. Affinity purified polyclonal IgG samples were diluted to 40 μ g/ml and 50 μ l loaded on the test side only. IgG derived from a patient with APS whose sera was known to bind β_2 GPI was used as a positive control in each plate tested. This positive control (designated sample 'A96') was serially diluted and used as the in-house standard against which to measure % binding activity to β_2 GPI for each IgG sample. The plate was then incubated for two hours at 37°C after which it was washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in PBS containing 0.1% BSA/PBS and 50 μ l of this solution added to each well. A final incubation for one hour at 37°C was performed and the plate washed four times with PBS and once with BIC buffer. Addition of substrate and incubation were carried out as in section 2.21.1 with additional OD measurements taken at 30 minutes.

2.21.5 Detection of aPL binding of polyclonal IgG to pure human β_2 GPI coated on cardiolipin in solid phase

A polysorb ELISA plate was coated with 50 μ l of CL (diluted to 2.5 μ g/ml in ethanol) on both the test and control half. The plate was incubated overnight at 4°C uncovered. Following that each plate was washed twice with PBS and 100 μ l of 0.25% gelatin/PBS was added, incubated at RT for one hour and washed twice with PBS. Pure human β_2 GPI was diluted to 10 μ g/ml in PBS and 50 μ l coated on the sample side and 50 μ l per well of PBS alone was added on the control side. The plate was incubated at RT for one hour and washed twice with PBS.

Human polyclonal IgG was diluted in PBS containing 0.1% BSA to 40 μ g/ml and 50 μ l added to the test half and the corresponding well in the control half. All patient samples were tested in duplicate. IgG derived from a patient with APS whose sera was known to bind β_2 GPI and DI (designated sample 'A96') was used as a positive

control in each plate tested. This positive control was serially diluted and used as the in-house standard against which to measure % binding activity to β_2 GPI for each patient IgG sample. The plate was then incubated for 90 minutes at RT and washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in PBS containing 0.1% BSA and 50 μ l of this solution added to each well. After one hour incubation at RT the plate was washed three times with PBS and once with BIC buffer. Addition of substrate was carried out as in section 2.21.1, the plates incubated at RT and OD measurements taken at 30 minutes and one hour.

Given that the whole plate was coated with CL and subsequently only one half with whole β_2 GPI, the net OD represents the difference between binding to β_2 GPI in the presence of CL and to CL alone which is relevant clinically.

2.21.6 Detection of aPL binding to purified recombinant DI in solid phase

By virtue of the incorporated C-terminal his₆-tag, a direct ELISA could be performed by recombinant DI binding to a nickel-coated microwell plate. This would orientate DI correctly and allow binding to various aPL to be assayed (Iverson et al., 1998a).

Initially recombinant his₆-tagged DI was diluted to a concentration of 75 μ g/ml using PBS and 50 μ l added to the wells of a nickel chelate-coated microwell plate. For binding assays testing polyclonal IgG patient samples, it became apparent that coating the plate with 10 μ g/ml of DI was sufficient to detect binding and for all the polyclonal IgG assays this lower coating concentration was used. The test half was coated with DI and the control half with PBS. Plates were incubated at RT for two hours and then washed three times with PBS, blocked with 100 μ l 0.25% gelatin in PBS, incubated for a further one hour at RT and washed three times with PBS.

Affinity purified human polyclonal IgG samples were diluted in SEC to a concentration of 20 μ g/ml. 50 μ l was added in both the test half and the corresponding well in the control half. Each patient sample was tested in duplicate. IgG derived from a patient with APS whose sera was known to bind DI (P11 sample) was used as a positive control in each plate tested. This positive control was serially diluted and used as the in-house standard against which to measure % binding activity to DI for each IgG sample. Each monoclonal aPL sample to be tested, either affinity purified antibody or derived from CHO supernatant, was serially diluted in SEC. An identical concentration of primary antibody was loaded into a well in the test half and a corresponding well in the control half of the plate. The total volume in each well was 50 μ l. The plate

was then incubated for one hour at 37°C after it was washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate was diluted 1000 fold in SEC buffer and 50µl of this solution added to each well. After one hour at 37°C the plate was washed three times with PBS and one time with BIC buffer. Addition of substrate was carried out as in section 2.21.1, the plate incubated at RT and OD measurements taken at 30 minutes and one hour.

2.21.7 Detection of aPL binding in fluid-phase

2.21.7.1 Inhibition of aPL binding to DI

A direct binding ELISA carried out as above in section 2.21.6 and was used to determine the concentration of affinity purified IS4V_H/IS4V_L required to achieve ~50% maximum binding. DI or β₂GPI as test inhibitors were diluted in SEC at concentrations ranging from 0 (i.e. no inhibitor) to 30µM. Affinity purified aPL was then added to each sample of inhibitor to achieve final concentration of antibody required to achieve 50% maximum binding. The samples were incubated at RT for two hours and then tested for binding to DI on an ELISA plate as described in section 2.21.6.

The per cent inhibition for each concentration of inhibitor was determined from the following formula:

% inhibition = $(A_0 - A/A_0) \times 100$, where A_0 is the OD (A) from the well containing no inhibitor (corrected for background).

2.21.7.2 Inhibition of aPL binding to β₂GPI/cardiolipin

A direct ELISA was carried out as in section 2.21.5 and was used to determine approximately what concentration of each polyclonal IgG patient sample was necessary to achieve 50% maximum binding. This approximated to between 20-40µg/ml. DI and mutant DI samples were diluted in 0.1% BSA/PBS to a concentration of between 1.25 to 7µM and incubated with the appropriate concentration of human IgG aPL at RT for 90 minutes. The samples were then tested for binding to β₂GPI/CL as in section 2.21.5 and the per cent inhibition calculated as is in the section 2.21.7.1 above.

2.22 Statistical analysis

Comparisons between different clinical groups (APS, SLE disease control group and healthy controls) for each of the binding assays (DI, β₂GPI, PS, CL, β₂GPI/CL

complex) presented in Chapter 4 were made using a paired t-test. A paired t-test was also used to compare the binding of DI mutants with wild-type DI in the fluid phase (Chapter 6).

CHAPTER 3

THE DEVELOPMENT OF AN EXPRESSION SYSTEM FOR PRODUCING RECOMBINANT DOMAIN I OF HUMAN β_2 GPI IN *E.COLI*

OVERVIEW OF CHAPTER 3

This chapter focuses on the steps taken that led to the development of a successful and reliable platform of expression of DI of human β_2 GPI in *E.coli*. It is broadly divided into three main parts:

The first part elucidates the design and production of a synthetic gene encoding for DI, which crucially, is optimised for expression in *E.coli*.

The second part describes the cloning scheme that was undertaken. The first plasmid into which the DI synthetic gene was cloned was not the final expression construct used. This is because it became apparent that stability of the expression construct was dependent on the presence of tight regulation of expression. Thus multiple constructs were tested and this is discussed in detail.

The final part illustrates the successful expression and purification of his₆-tagged DI. Evidence is presented which supports the hypothesis that an optimised gene for *E.coli* improved yield of expression. Optimisation experiments using varying concentrations of the induction agent highlighted the fact that excessive levels of this eukaryotic protein are toxic to *E.coli*, underlying the need to regulate expression.

3.1 Production of a synthetic gene encoding for DI of human β_2 GPI optimised for expression in *E.coli*

3.1.1 Introduction

The expression of non-glycosylated eukaryotic proteins in bacteria has many advantages over other production systems, mostly relating to efficiency, reliability and convenience of production. However, the initial steps required in establishing a reliable prokaryotic expression protocol for a given eukaryotic protein can be difficult (Baneyx, 1999). Clearly an efficient production system for DI of β_2 GPI would facilitate the study of this potentially important antigenic target for aPL. A group in La Jolla (California, USA) who express DI using baculovirus to infect insect cells as the expression host (Iverson et al., 1998b, Igarashi et al., 1996) find this method laborious and time consuming. Consequently they attempted and failed to establish a system for expressing DI in *E.coli* (personal communication with Dr M Linnik, La Jolla Pharmaceuticals, CA, USA). I hypothesised that one reason accounting for the current absence of an *E.coli* expression system for DI could be due to the nature of the codons in the native gene coding for DI. The native DI gene has 61 codons and 26 (43%) are used by *E.coli* at a frequency of less than 1% (Kane, 1995). Optimising codon usage to facilitate expression by *E.coli* has been successfully employed to establish the production of many other eukaryotic proteins (Kane, 1995, Hua et al., 1994, Hu et al., 1996, Martin et al., 1995).

My aim was to produce a gene that coded for DI, but ensure that all codons within that gene are those most frequently used by *E.coli*. I intended to use the system of recursive PCR to synthesise this globally optimised gene (Prodromou and Pearl, 1992). This would involve designing primers that sequentially overlap by 15-20 nucleotides and cover the length of the DI gene, whilst consulting codon tables to ensure optimal ones for *E.coli* are incorporated. To do this manually is possible but likely to be considerably time consuming as the primers would also need to be designed to minimise the possibilities of non-specific annealing (Prodromou and Pearl, 1992). In order to avoid this delay I collaborated with the group that developed recursive PCR, who had also developed a computer programme named Juniper, which was written to perform this time-consuming task. Juniper consults codon usage tables to ensure good expression, inserts restriction sites where appropriate and checks for sequence overlap or complementarity to avoid non-

specific priming. It then designs overlapping regions with melting temperatures in a specific range to yield an even number of oligonucleotides of similar length suitable for recursive PCR. At the time of writing this thesis, Juniper was freely available on the internet at following website: <http://strubiol.icr.ac.uk/extra/juniper>

Hence the aim of these experiments was to synthesise an artificial gene encoding for an N-terminal Omp A leader sequence followed by DI, flanked by *Bgl* II and *Nco* I restriction sites whilst incorporating codons preferred by prokaryotic expression systems.

3.1.2 Results from Juniper

The amino acid sequence of the desired gene was entered into Juniper using FASTA format, and the desired flanking restriction sites, preferred oligonucleotide length, T_m and codon usage entered. Juniper then designed the gene, flagging all restriction sites and designed the overlapping oligonucleotides to the specifications as described above (see figure 3.1)

Juniper altered 41 out of the 61 codons (67%) present in the native sequence (Steinkasserer et al., 1991) to optimise expression in *E.coli* (figure 3.2).

Results from Juniper

```

      I      I      I      M      K      K      F      A      I      A
1:  G G G A G A T C T A T G A A A A A A A C C C C T A T C C C T
      N-Jg111

      I      A      V      A      L      A      G      I      A      I
31: A T C G C T C F T G C T C T C G G C F G G I T T C C C . A C C

      V      A      Q      A      G      K      F      C      F      K
61: G T T G C T C A G G C T G G T C G F A C C T G C C C G A A A

      P      D      D      L      P      I      S      F      V      V
91: C C G G A C G A C C T G C C G T T C T C C A C C C T T C T T

      P      D      K      T      I      Y      E      F      G      L
121: C C G C T G A A A A C C . T C T A C G A A C C C C G T C A A

      E      I      F      Y      S      C      K      F      G      Y
151: G A A A T C A C C . A C T C C T G C A A A C C C C G G . I A C

      V      S      R      G      G      M      K      K      I      I
181: G T T T C C C G T G G T G G T A T C C G I A A A T T C A T C
      N-Dex23K

      C      F      D      L      G      L      W      F      I      N      A      C
211: T G C C C G C T G A C C G C T C T G T G C C C C A T C A A C
      N-PinA1

      T      D      K      C      L      I      I      I      I      I
241: A C C C T G A A A T G C A C C T A G C C C A T G C T G .
      N-Nod1
  
```

Oligo overlaps:

```

1  GGGAGATCTATGAAAAAAACCGCTATCGGTATCGCGTGTGGCTCGGGCTGG
.....CCGACC

51  TTTGGCTACCG.....CCGAAACCGGACGACC
AAAGCGATGGCAACGAGTCCGACCAGCATGGACGGGGCTTGGCCGTGGTGG

101  TGGCGTCTCCACCGTGTGTCCGGTGA AAAACCTTCTACGAACCGG.....
.....TTTGGGAAGATGCTTGGCCCACTT

151  .....AAACCGGGTACCGTTCCTCCGTGGTGGTATGG
CTTTAGTGGATGAGGACCTTGGCCCAATGCAAAGG.....

201  TAAATTCATCTGCCCGCTGACCGGTCGT.....
.....GGCGACTGGCCAGACACCGGCTAGTGTGGGACTTAA

251  .....
CGTGGATCGGTACCACA
  
```

Forward oligos: 5' to 3'

1. GGGAGATCTATGAAAAAAACCGCTATCGGTATCGCGTGTGGCTCGGGCTGGTTCGGTACCC
2. CCGAAACCGGACGACCCTGGCGTTCCTCCACCGTGTGTCCCGTGA AAAACCTTCTACGAACCGG
3. AAACCGGGTACCGTTCCTCCGTGGTGGTAA.GCCTAAA.TCATCTGCCCGCTGACCGGTCGT

Reverse oligos: 5' to 3'

1. GC.CGTCCGGTTCCTCCGACGGTACGACCAGCC.TGAGCAACGGTACGGAAACCAGCC
2. GCAAACGTAACCCGGTTCGACGAGTACGTGAT.TCTTCACCCGGTTCGTAGAAGGT.TT
3. ACACCATGGCTAGGTGCAT.TCAGGGTC.TCATCGGCCACAGACCGGTCAGCCG

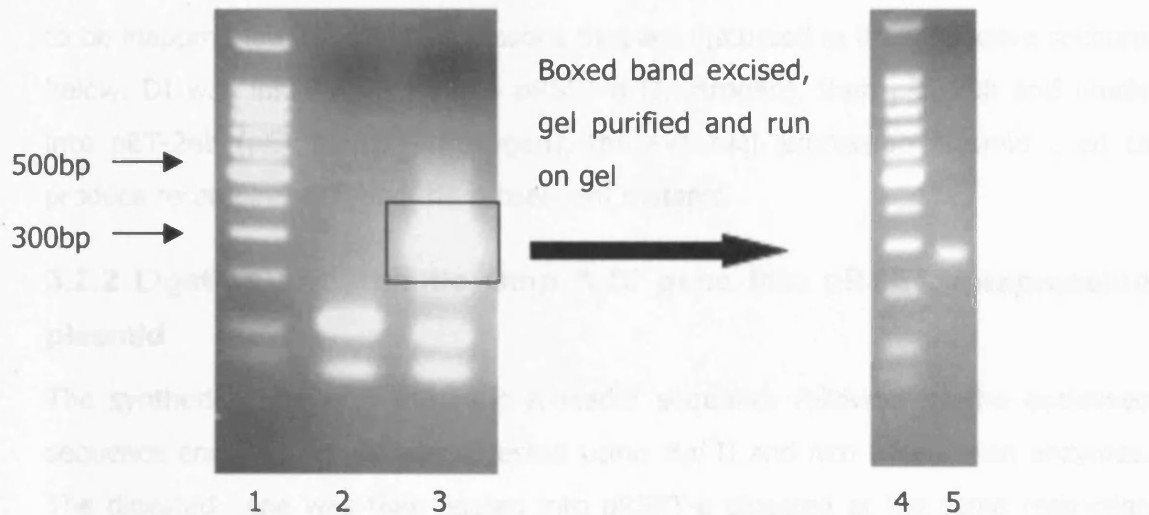
Figure 3.1 Results generated by the Juniper programme. Sequence of desired gene encoding for an N-terminal Omp A leader sequence followed by DI designed to incorporate *E.coli* preferred codon usage. Overlapping oligonucleotides designed for use in a recursive PCR to synthesise the desired gene product and primers required listed 5' to 3'.

M K K T A I A I A V A L A G F A T V A Q A G R T C P K P D D L P
 GGGAGATCTATGAAAAAACCGCTATCGCAATCGCTGTTGCTCTGGCTGGTTTCGCTACCGTTGCTCAGGCAEGTTCGTACC TGCCGAAACCGGACGACCTGCCG 105
 CCC TCTAGATACTTTTTTTGGCGATAGCGTTAGCGACAACGAGACCGACCAAAGCGATGGCAACGAGTCCGACCAGCATGGACGGGCTTTGGCCTGCTGGACGGC
*Bgl*III
 F S T V V P L K T F Y E P G E E I T Y S C K P G Y V S R G G M R K F I
 TTC TCC ACC GTT GTT CCG CTG AAA ACC TTT CAC GA ACC CGG GTG AAA GAA ATC ACC TAC TCCTG CAAA CCG EGT TAC GTT TCC CGT GGT GGT ATG CGT AAA TTC ATC 210
 AAG AGG TGG CAAC A GGC GAC TTT TGG AAG ATG CT TGG CCC C TTT TTT TAG TGG ATG AGG ACC TTT TGG CCA ATG CA AAG GC ACC ACC C TAC CCA TTT AAG TAG
 C P L T G L W P I N T L K C T
 TGC TCG CTG ACC GGT CTGTGG TCC ATCAAC ACC CTG AAA TGC ACC TAG CCA TGG CTGT
 ACG TCG GACTGGCC GAC ACC CGG TAG TTG TGG SACT TTT ACC TGG ATC GGT ACC ACA 268
*Nco*I

Figure 3.2 Synthetic gene encoding for DI. DI of human β_2 GPI underlined (amino acids) with Omp A leader sequence. Codons were chosen for optimal expression in *E.coli* by Juniper, altering 67% of the native sequence (boxed codons). Synthetic oligonucleotide primers are denoted by grey shading; oligonucleotides of the upper DNA sequence are read 5' to 3' (left to right) and the oligonucleotides of the lower DNA sequence are read 3' to 5' (left to right). Flanking restriction sites are denoted in the light grey font.

3.1.3 Amplification of synthetic DI gene using recursive PCR

Initial PCR reactions using the oligonucleotide primers at the recommended T_m of 55°C yielded primer-dimers and no target gene product. Optimisation of the PCR by raising the T_m to 57°C, performing a hot-start and altering the ratio of outer to internal primers to 10:1 yielded a band of approximately 270bp consistent with the size of the desired gene (figure 3.3)



Lanes:

- 1 and 4 100bp marker
- 2 10µl recursive PCR mixture using 100:1 outer:internal primer ratio
- 3 10µl recursive PCR mixture using 10:1 outer:internal primer ratio
- 5 gel purified band cut out from lane 3 (boxed area)

Figure 3.3 Results of recursive PCR. Mixtures were run on 2% agarose gel. Size of band in lane 5 consistent with desired 267bp gene. This was confirmed with subsequent sequencing of the gene cloned into pRSETb.

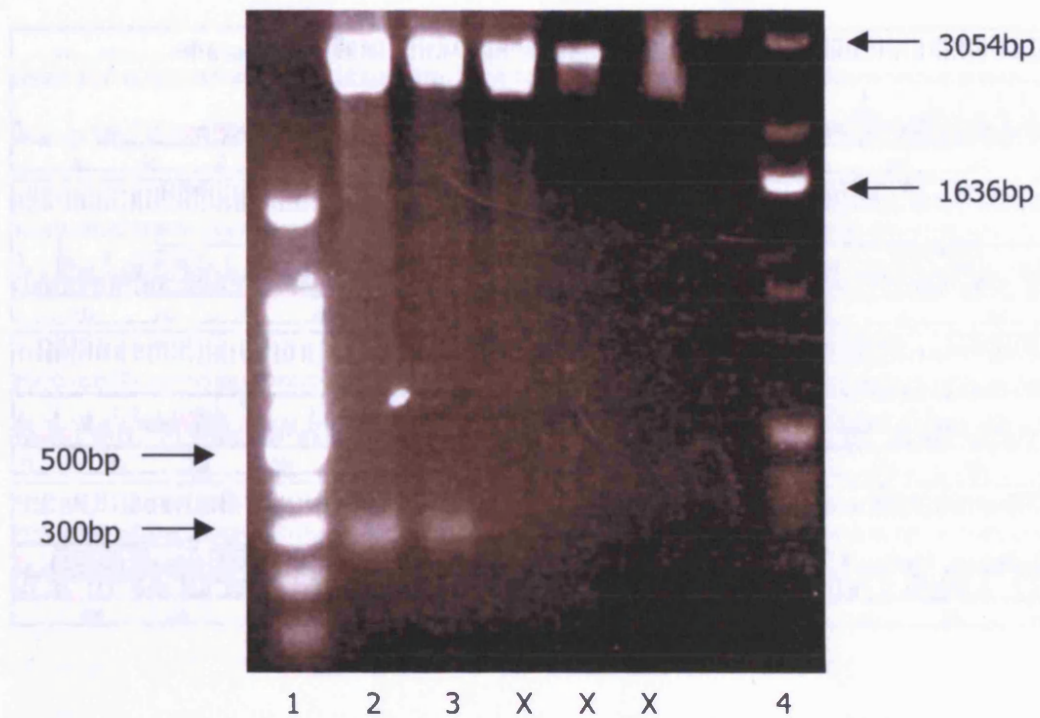
3.2 Cloning scheme for synthetic DI gene: expression is critically dependent on tight regulation of expression

3.2.1 Introduction

This section describes the rationale and results of the cloning scheme that led to the final expression construct being DI cloned into pET-26b (Novagen). The DI gene was cloned into several expression constructs before this one was made. Each was found to be inappropriate for different reasons that are discussed in the respective sections below. DI was initially cloned into pRSET-b (Invitrogen), then pET-20b and finally into pET-26b (pET series – Novagen), the eventual expression plasmid used to produce recombinant DI and the subsequent mutants.

3.2.2 Ligation of synthetic Omp A-DI gene into pRSET-b expression plasmid

The synthetic gene with the Omp A leader sequence followed by the optimised sequence encoding for DI was digested using *Bgl* II and *Nco* I restriction enzymes. The digested gene was then ligated into pRSET-b digested at the same restriction sites. A successful ligation was confirmed upon digesting the extracted plasmid DNA construct with *Bgl* II and *Nco* I enzymes and observing two distinct bands on the agarose gel. The larger band of 2673bp constitutes digested pRSET-b minus the insert and the smaller band of 267bp the synthetic DI gene with an Omp A leader sequence as shown in figure 3.4. Between five and 10 colonies were screened for the presence of the desired construct. This is because many colonies when grown were shown to harbour an empty plasmid indicating unsuccessful ligation or foreign plasmids from contaminant *E.coli* strains as shown in figure 3.4. Similar screening principles were observed for subsequent ligations using other expression plasmids. The plasmid was sequenced to ensure that PCR errors had not occurred and the sequencing plot is shown in figure 3.5.



Lanes

1 100bp marker

2 and 3 *Bgl* II / *Nco* I digested pRSET-b and Omp A-DI insert

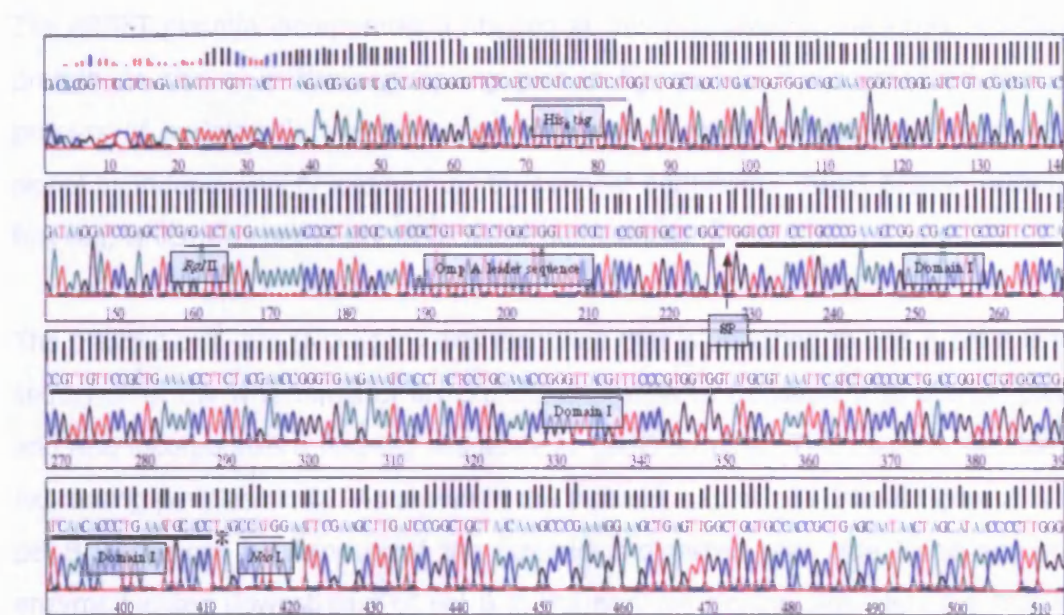
4 1kb marker

X lanes with either empty pRSET plasmids or foreign plasmid DNA

Figure 3.4 *Bgl* II / *Nco* I digests of synthetic DI cloned into pRSET-b. Band constituting synthetic gene insert is at 267bp and digested pRSET plasmid minus insert at 2673bp (lanes 2 and 3). 1.2% agarose gel.

3.2.3 Cloning of Synthetic Omp A-DI Gene into pRSET-b

3.2.3.1



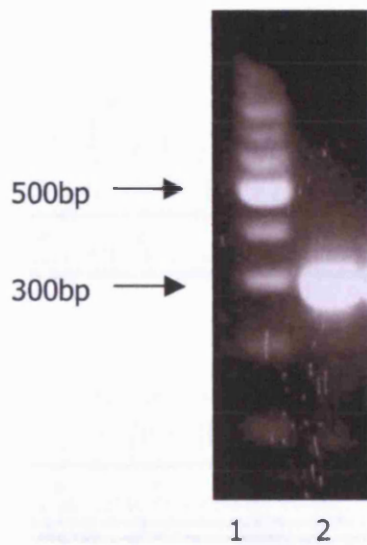
performed using the HindIII and XbaI restriction sites on the pRSET-b vector. Primers were designed to amplify the Omp A-DI synthetic gene which will insert into the HindIII and XbaI restriction sites to Nde I at the 5' end and Xba I at the 3' end (Figure 3.5). The PCR product was then digested with Nde I and Xba I restriction enzymes as per the manufacturer's protocol. The Omp A-DI gene was then ligated into the pRSET-b vector. **Figure 3.5 Sequencing plot confirms synthetic sequence of Omp A-DI gene cloned into pRSET-b.** Abbreviations and symbols: His₆, hexahistidine-tag; SP, signal peptidease cleavage site; *, stop codon.

When the BL21(DE3) cells with this construct were grown in the presence of IPTG, no cell growth was observed when they had reached mid-log phase. This was not observed when other host strains such as the cloning host DH5α were transformed. In case our cell growth was a function of the Omp A leader sequence, the BL21(DE3) strain, another PCR amplification of DI was performed using the Omp A-DI-pET-20b construct as the template. Primers were designed such that the DI gene was amplified without the Omp A leader sequence. Primers were located at the 5' end and Xba I at the 3' end (Figure 3.5). The Omp A-DI-pET-20b construct was then cloned into the pET-20b vector (Figure 3.5). This construct was expressed to include PCR error correction (PCR-ACC) and to include a stop codon at the 3' end. The resulting construct was then transformed into the BL21(DE3) strain. The resulting construct was then transformed into the BL21(DE3) strain. The resulting construct was then transformed into the BL21(DE3) strain.

3.2.3 Ligation of synthetic Omp A-DI gene into pET-20b expression plasmid

The pRSET plasmid incorporates a his₆-tag at the N-terminal of the expressed target protein as shown in the sequencing plot in figure 3.5. It was realised that the process of periplasmic transportation of protein cleaves off the signal peptide by signal peptidase. This process would thus render periplasmic target protein without a his₆-tag, which was necessary to facilitate purification.

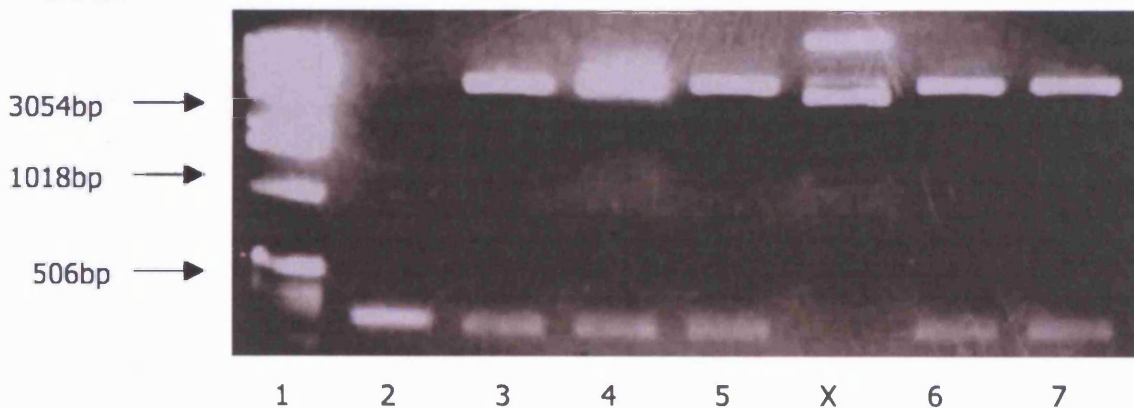
The plasmid pET-20b (Novagen) was identified that is designed to add a pel B leader sequence at the N-terminal of an expressed protein to transport it to the periplasm, and also incorporates a his₆-tag sequence at the C-terminal. This made it suitable for expressing periplasmic DI. As shown in the plasmid map of pET-20b in figure 2.1, the pel B leader can be removed if the plasmid is digested with *Nde* I and any other enzyme located downstream of pel B in the multiple cloning site. Hence a PCR was performed using the Omp A-DI-pRSET construct as the template with primers designed to amplify the Omp A-DI synthetic gene whilst simultaneously altering the restriction sites to *Nde* I at the 5' end and *Nco* I at the 3' end (figure 3.6). This PCR product was then digested with *Nde* I and *Nco* I restriction enzymes as was the native pET-20b plasmid, which in effect removed the pel B leader sequence. The Omp A-DI gene was then ligated into pET-20b (figure 3.7) and sequenced to exclude PCR errors (figure 3.8). However, on transforming the expression strain BL21(DE3) *E.coli* cells with this construct the cells grew poorly on the plate, which was not observed when other host strains such as the cloning host DH5 α were transformed. In case poor cell growth was a function of the Omp A leader sequence in the BL21(DE3) strain, another PCR amplification of DI was performed using the Omp A-DI—pET-20b construct as the template. Primers were designed such that only the DI gene was amplified without the Omp A sequence, flanked by *Nco* I restriction site at the 5' end and *Xho* I at the 3' end (figure 3.9). The *Nco* I / *Xho* I digested DI gene was then cloned into *Nco* I / *Xho* I digested pET-20b, in effect retaining the pel B leader sequence native to the pET-20b plasmid (figure 3.10). This was also sequenced to exclude PCR errors (figure 3.11). A schematic representation of the cloning steps described in this section are shown in figure 3.12.



Lanes:

- 1 100bp marker
- 2 Omp A-DI amplified gene

Figure 3.6 PCR amplification of Omp A-DI gene from the Omp A-DI-pRSET construct. 5µl of PCR mix run on a 2% agarose gel. 267bp band consistent with amplified gene.



Lanes:

- 1 kb DNA ladder
- 2 Omp A-DI gene from recursive PCR mix
- 3 to 7 *Nde* I / *Nco* I digested pET-20b and Omp A-DI insert
- X Lane with either empty pET-20b plasmid or foreign plasmid DNA

Figure 3.7 *Nde* I / *Nco* I digests of synthetic Omp A-DI gene cloned into pET-20b. Band constituting synthetic gene insert is at 267bp and digested pET-20b plasmid minus insert at 3600bp. 1.2% agarose gel.

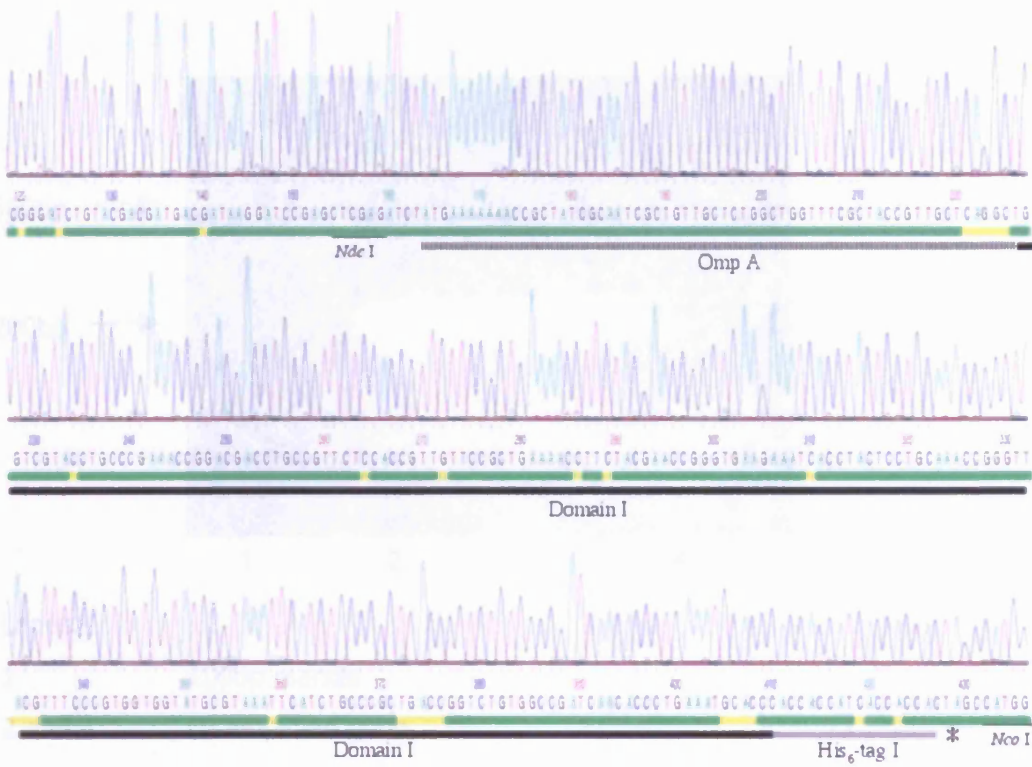
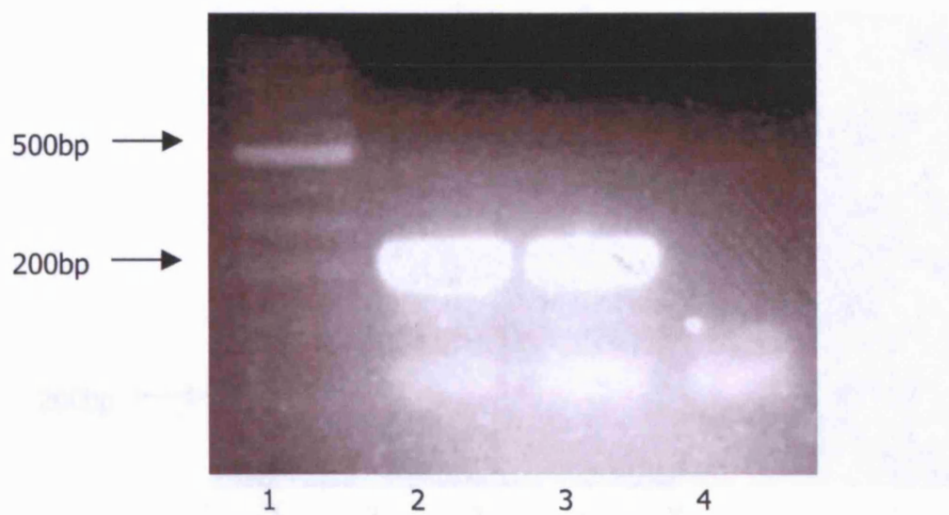


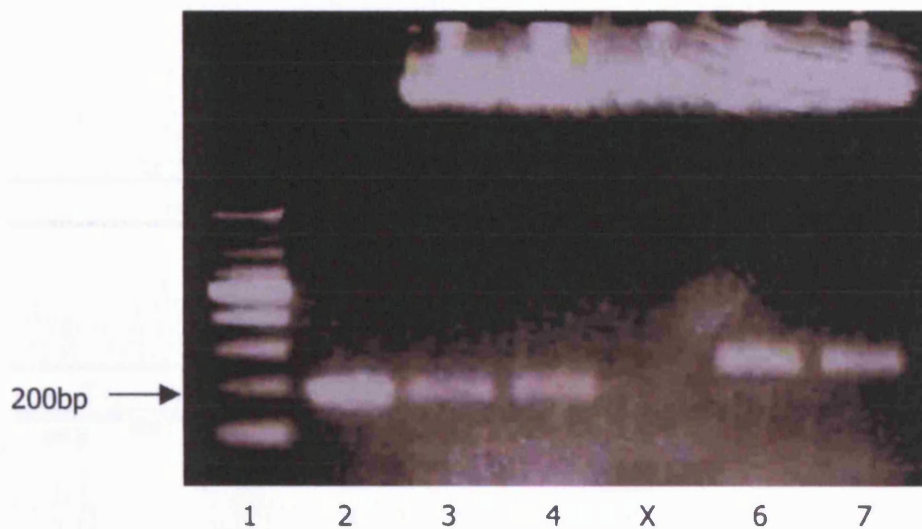
Figure 3.8 Sequencing plot confirms synthetic sequence of Omp A-DI gene cloned into pET-20b. Abbreviations and symbols: His₆, hexahistidine-tag; *, stop codon.



Lanes:

- 1 100bp marker
- 2 and 3 DI amplified gene
- 4 Negative control (PCR minus template DNA)

Figure 3.9 PCR amplification of DI gene from the Omp A-DI-pET-20b construct. 5µl of PCR mix run on a 2% agarose gel. 201bp band (lanes 2 and 3) consistent with amplified recombinant DI gene minus the Omp A leader sequence.



Lanes:

- 1 100bp marker
- 2 DI gene from PCR mix
- 3 and 4 *Nco* I / *Xho* I digested pET-20b and DI insert
- 6 and 7 *Nde* I / *Xho* I digested pET-20b and Omp A-DI insert
- X Lane with empty pET-20b plasmid

Figure 3.10 *Nco* I / *Xho* I digests of synthetic DI gene cloned into pET-20b. Band constituting synthetic gene insert is at 201bp and digested pET-20b plasmid minus insert at 3600 bp (lanes 3 and 4). DI gene from the PCR mix was run as a 201bp marker (lane 2). Omp A-DI cloned into pET-20b was also digested and run on the gel to act as a positive control for the *Xho* I enzyme and as a marker for the double cut pET-20b plasmid (lanes 6 and 7). 2% agarose gel.

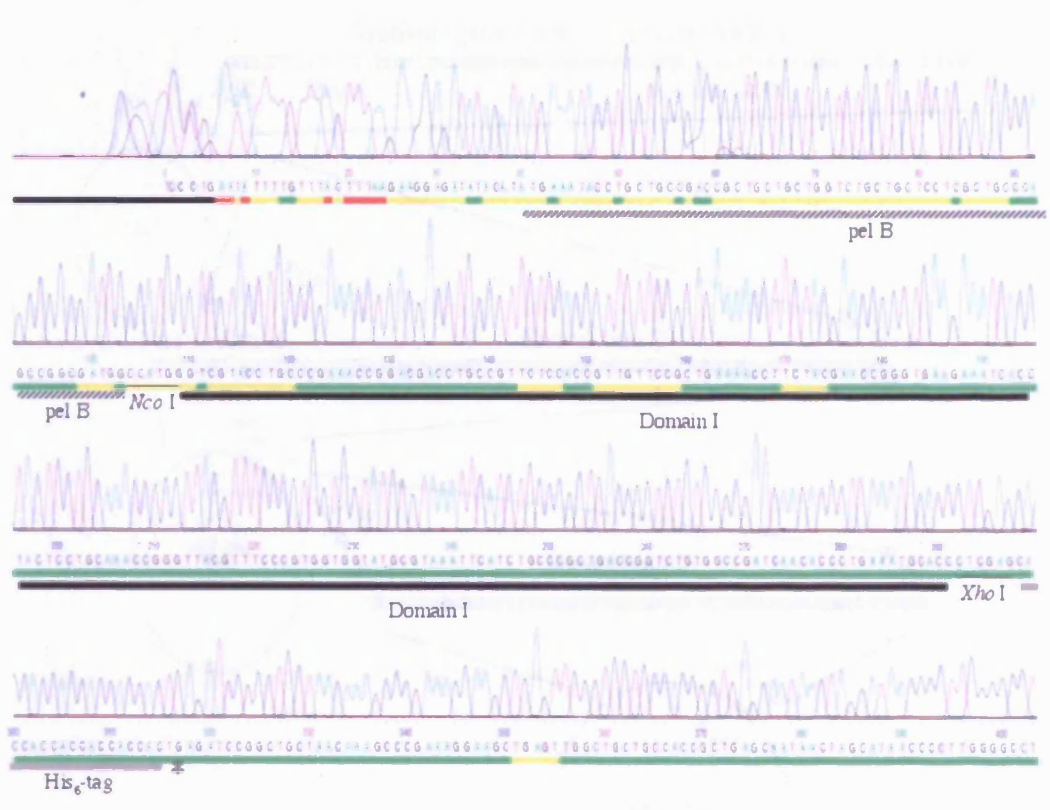


Figure 3.11 Sequencing plot confirms synthetic sequence of DI gene cloned into pET-20b. Site of gene cloned within plasmid results in retention of pel B leader sequence native to the pET-20b plasmid. Abbreviations and symbols: His₆, hexahistidine-tag; *, stop codon.

Figure 3.12 Cloning scheme of DI into pET-20b plasmids. The final construct using pET-20b includes the DI gene with an N-terminal pel B leader and a C-terminal His₆-tag. Abbreviations: pel B, hexahistidine tag.

3.2.4 Ligation of synthetic DI gene into pET-20b plasmid for the study of regulation of expression using the top string tag.

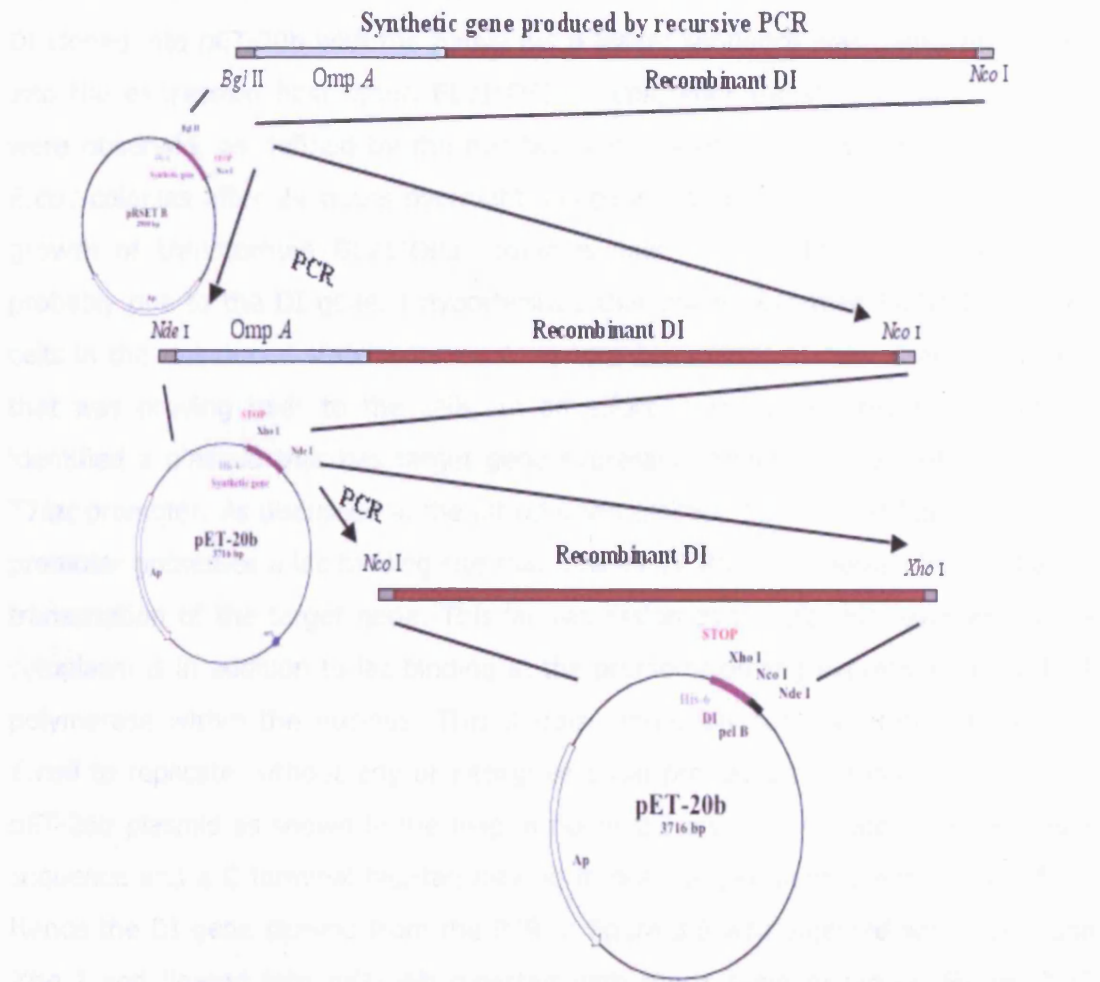
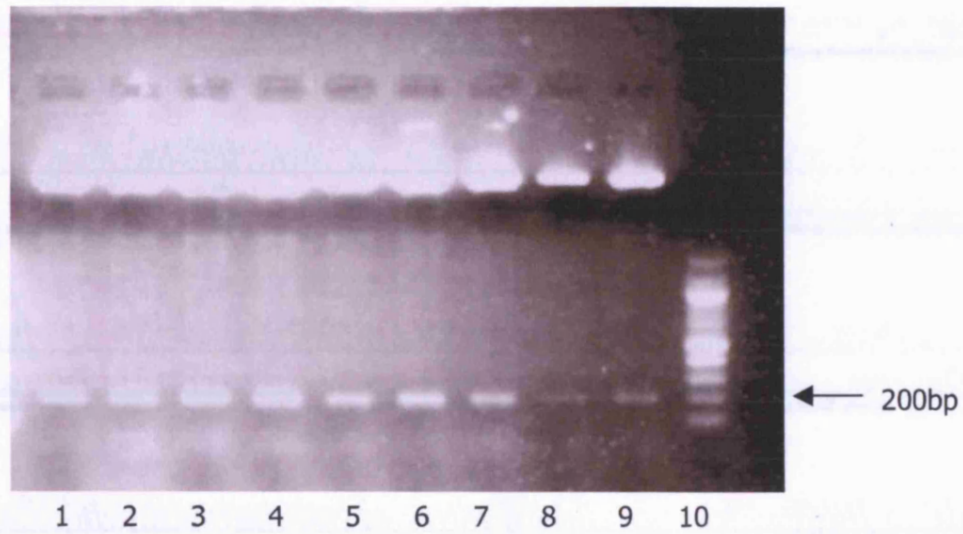


Figure 3.12 Cloning scheme of DI into pET-20b plasmids. The final construct using pET-20b included the DI insert with an N-terminal pel B leader and a C-terminal his₆-tag. Abbreviations: Ap, ampicillin, his-6, hexahistidine tag.

3.2.4 Ligation of synthetic DI gene into pET-26b plasmid - tight regulation of expression using the high stringency T7/lac promoter

DI cloned into pET-20b with the native pel B leader sequence was then transformed into the expression host strain BL21(DE3) *E.coli*. Poor transformation efficiencies were observed, as defined by the number and size of the transformed BL21(DE3) *E.coli* colonies after 24 hours overnight incubation at 37°C. This implied that poor growth of transformed BL21(DE3) colonies was not due to using Omp A and probably due to the DI gene. I hypothesised that the transformed BL21(DE3) *E.coli* cells in the uninduced state were exposed to a basal level of expressed DI protein that was proving toxic to the cells. In an effort to regulate expression further I identified a plasmid that has target gene expression driven by the high stringency T7/lac promoter. As discussed in the introduction (section 1.7.1.2 and figure 1.3), this promoter possesses a lac binding site that inhibits T7 RNA polymerase from initiating transcription of the target gene. This lac repression at the plasmid level within the cytoplasm is in addition to lac binding at the promoter driving expression of T7 RNA polymerase within the nucleus. This double repression has the effect of allowing *E.coli* to replicate without any or negligible basal production of target protein. The pET-26b plasmid as shown in the map in figure 2.3 also incorporates a pel B leader sequence and a C-terminal his₆-tag making it ideal for periplasmic expression of DI. Hence the DI gene derived from the PCR in figure 3.9 was digested with *Nco* I and *Xho* I and ligated into pET-26b digested with these same enzymes. Figure 3.13 shows the results of the ligation with the smaller band representing the 201bp DI gene and the larger band double digested pET-26b. The sequencing plot of figure 3.14 confirms the absence of PCR errors and that the DI gene was cloned in frame. Upon transforming the expression strain BL21(DE3) with pET-26b-DI construct, transformation efficiencies were considerably improved, supporting the hypothesis that tight regulation of expression is important. The transformation efficiencies for each plasmid-DI construct are summarised in table 1.1.



Lanes:

1 to 9 *Nco* I / *Xho* I digested pET-26b and DI insert

10 100bp marker

Figure 3.13 Sequencing data confirms correct synthetic sequence of DI insert

Figure 3.13 *Nco* I / *Xho* I digests of synthetic DI gene cloned into pET-26b. All nine colonies screened contained the desired construct. Band constituting synthetic gene insert is at 201bp and digested pET-26b plasmid minus insert at 5500bp. 1.2% agarose gel.

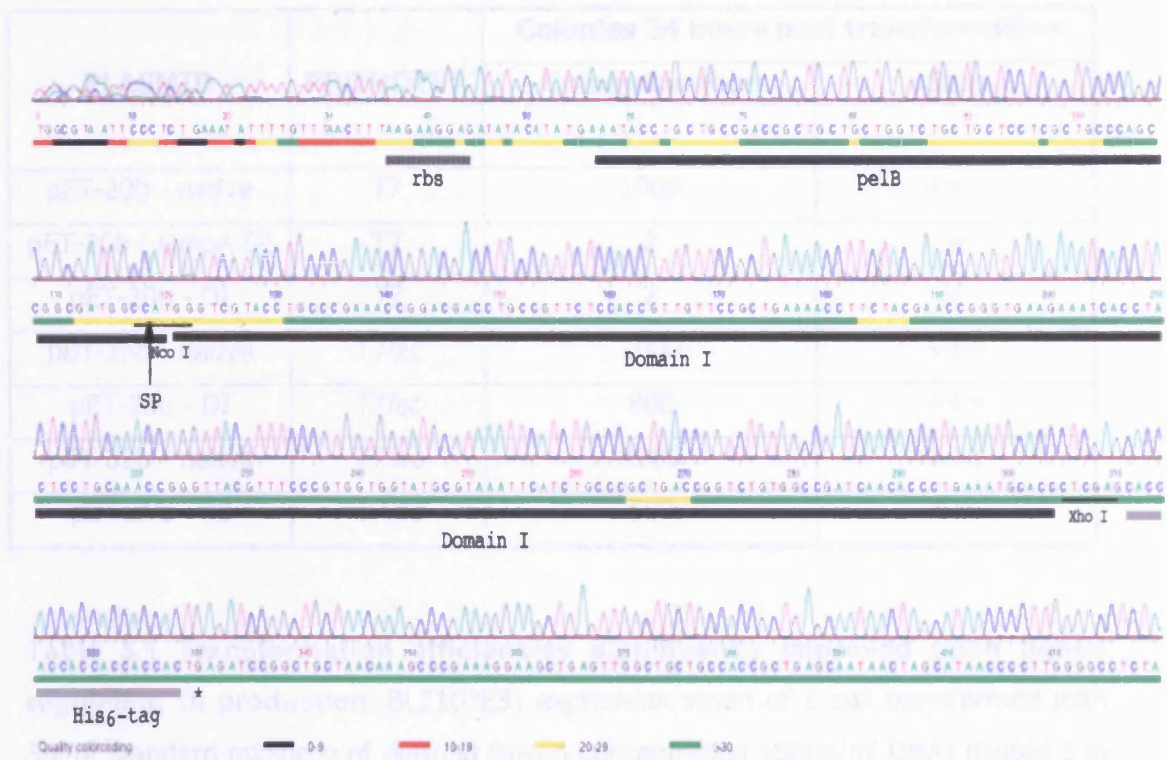


Figure 3.14 Sequencing plot confirms correct synthetic sequence of DI cloned into pET-26b. Abbreviations and symbols – rbs, ribosomal binding site; pelB, *pelB* leader sequence; SP, signal peptidase cleavage site; *, stop codon.

PLASMID	PROMOTER	Colonies 24 hours post transformation	
		Colony number	Size
pET-20b - native	T7	1000	+++
pET-20b - OmpA-DI	T7	2	+
pET-20b - DI	T7	2	+
pET-26b - native	T7/lac	1000	+++
pET-26b - DI	T7/lac	800	+++
pET-39b - native	T7/lac	1000	+++
pET-39b - DI	T7/lac	500	+++

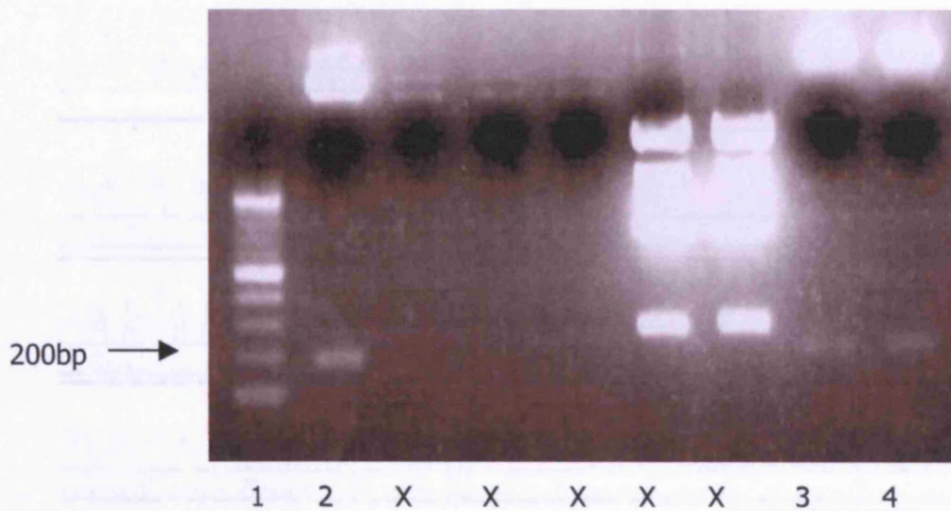
Table 3.1 Transformation efficiencies significantly improved upon tightly regulating DI production. BL21(DE3) expression strain of *E.coli* transformed with 5µl of standard miniprep of plasmid (mean concentration 150µg/ml DNA) diluted 1 in 100 in dH₂O. Colonies counted after 24 hour overnight incubation at 37°C. Size of colonies defined as: + ⇒ small, just visible; ++ ⇒ moderate size, easily visible, not merging; +++ ⇒ fat colonies with many merging on plate. Only pET-26b and pET-39b plasmids have expression driven by high stringency T7/lac promoter, hence transformation efficiency of native plasmid is maintained when plasmid harbours recombinant DI target gene.

3.2.5 Ligation of the synthetic DI gene into pET-39b plasmid – incorporation of DsbA tag.

There was a concern that despite the periplasm favouring the formation of disulphide bond formation through its oxidative environment, yields of soluble target protein would not be sufficient. The protein DsbA is a member of thioredoxin family. This 21kDa protein interacts with substrate proteins and oxidises them, thus catalysing the formation of disulphide bonds (Hiniker and Bardwell, 2003). To facilitate the formation of disulphide bond formation further and potentially improve yield of target protein, a construct was made which would encode for a DsbA-tagged DI expression product. The synthetic optimised gene encoding for DI was thus ligated into the multiple cloning site of the pET-39b plasmid (for plasmid map see figure 2.3). This construct encodes for a pel B leader sequence transporting target protein into the periplasm, an N-terminal DsbA-tag and a C-terminal his₆-tag. Expression remains tightly regulated through the T7/*lac* promoter as seen in the pET-26b construct. Figure 3.15 shows the results of the ligation with the smaller band representing the 201bp DI gene and the larger band double digested pET-39b. Given that expression is driven by the high stringency T7/*lac* promoter, like pET-26b, DI cloned into pET-39b results in good transformation efficiencies upon plasmid insertion into the expression strain BL21(DE3) as shown in table 1.1.

The main disadvantage with this construct however is that the expressed product is not DI with a free N-terminal. Rather it has the 21kDa DsbA protein attached at the N-terminal of DI. In order to attribute binding and biological properties of expressed protein to DI alone, DsbA would need to be cleaved off. Thrombin and enterokinase sites are incorporated at the C-terminus end of DsbA to facilitate this. These sites and the confirmed correct sequence of DI are shown in the sequencing plot figure 3.16.

Hence DI, which was originally cloned into pET-20b as shown in figure 3.12, was subsequently cloned into pET-26b and pET-39b to assess which construct would give greatest yield of soluble target protein.



Lanes:

- 1 100bp marker
- 2 *Nco* I / *Xho* I digested pET-20b and DI insert
- 3 and 4 *Nco* I / *Xho* I digested pET-39b and DI insert
- X Foreign / contaminant plasmid DNA

Figure 3.15 Sequencing plot of DI at various sites cloned into pET-39b.

Abbreviations: P, 50bp control; DI, DI gene; X, Xho I; N, Nco I; S, Sal I; K, Kpn I; B, Bam HI; H, Hind III; E, EcoRI; M, Msp I; X, Xho I; N, Nco I; S, Sal I; K, Kpn I; B, Bam HI; H, Hind III; E, EcoRI; M, Msp I.

Figure 3.15 *Nco* I / *Xho* I digests of synthetic DI gene cloned into pET-39b. Band constituting synthetic DI gene insert is at 201bp and digested pET-39b plasmid minus insert at 5995bp in lanes 3 and 4. Lane 2 constitutes digested DI and pET-20b minus insert at 3600bp which was used as positive control for the digest. 2% agarose gel.

3.3 Successful expression of DI in E.coli

3.3.1 Introduction

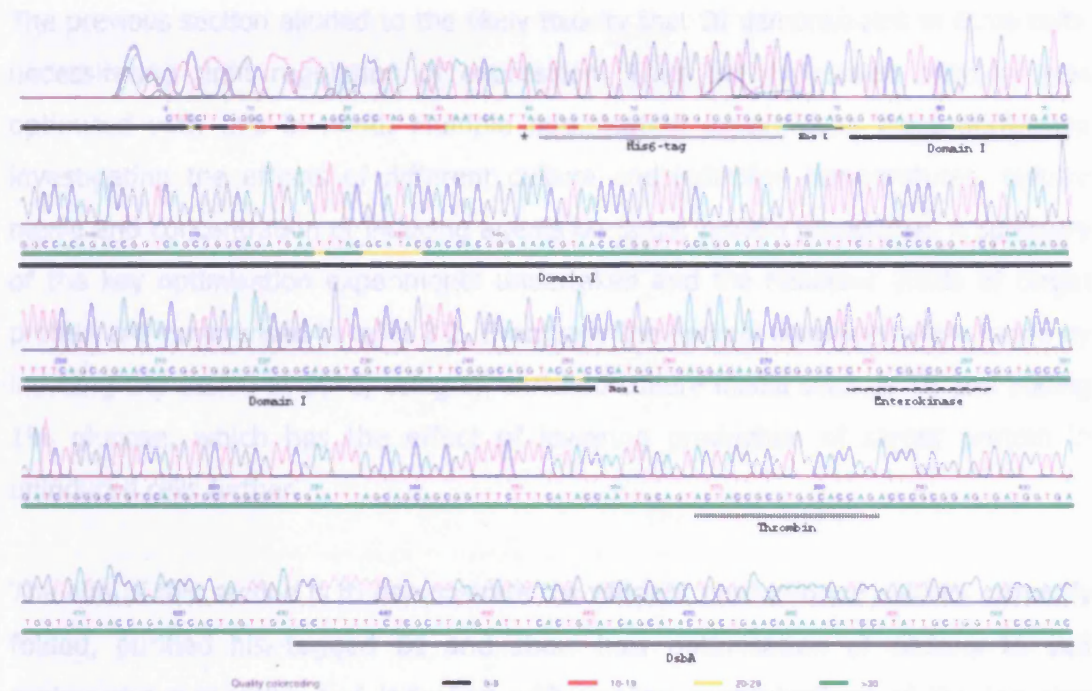


Figure 3.16 Sequencing plot of DI of human β_2 GPI cloned into pET-39b. Abbreviations: *, stop codon. Enterokinase cleavage site, thrombin cleavage site and the sequence encoding for the C-terminus end of DsbA is shown. The sequence reads 3' to 5' (left to right) as the T7 terminator was used as the sequencing primer. Given the size of DsbA, using the T7 forward primer for sequencing would have yielded a sequencing plot including most of DsbA, but not DI as only approximately 800 bases are included in any given plot.

3.3 Successful expression of DI in *E.coli*

3.3.1 Introduction

The previous section alluded to the likely toxicity that DI demonstrates in *E.coli* cells, necessitating tight regulation of expression. Thus the expression protocol was optimised with this in mind. Multiple optimisation experiments were performed investigating the effects of different culture and induction temperatures, culture media and concentration of inducing agents on target protein production. A summary of the key optimisation experiments undertaken and the resultant yields of target protein are summarised in table 3.2. Eventually the optimal conditions were found by inducing expression at 30°C, using an enriched culture media such as TB and adding 1% glucose, which has the effect of lowering production of target protein in uninduced cells further.

The aim of this section is to demonstrate the efficient production of soluble, correctly folded, purified his₆-tagged DI and show how optimisation of codons to suit prokaryotic expression and induction with varying concentrations of the inducing agent IPTG improved yield.

3.3.2 Expression and purification of periplasmic his₆-tagged DI

The host *E.coli* cells chosen for expression were of the strain BL21(DE3). These were chosen because this is a protease deficient strain, potentially reducing target protein degradation. Also this strain contains a chromosomal copy of the T7 RNA polymerase gene (λ DE3). The presence of a source of T7 RNA polymerase is crucial given that expression is driven by the T7 promoter.

BL21(DE3) cells were transformed with the expression construct DI cloned into pET-26b. Harvested induction cultures were exposed to ice-cold ddH₂O causing osmotic shock. This process lyses the outer membrane releasing periplasmic proteins whilst keeping the inner spherocyte intact. The periplasmic fluid then collected as supernatant was run on a 15% SDS-PAGE gel, electrophoretically transferred onto a nitrocellulose membrane and probed with a murine anti-his₅tag-antibody (Novagen). Figure 3.17 demonstrates the western blot confirming the presence of a his₆-tagged 7kDa protein consistent with expected size of the target protein.

Medium	Culture temp (°C)	Induction temp (°C)	IPTG concentration	Glucose	Target protein yield
LB	37	37	1mM	0	0
LB	RT	RT	1mM	0	0
TB	30	30	1mM	0	+
TB	30	30	1mM	1%	++
TB	RT	RT	1mM	1%	++
TB	30	30	0.4mM	1%	+++
TB	30	30	0.1mM	1%	++++

Table 3.2 Summary of key optimisation experiments for expressing recombinant DI. Abbreviations: temp, temperature. Target protein yield as assessed by western blot of periplasmic fraction: 0 ⇒ no detectable target protein; +++++ ⇒ saturated band on blot after exposure time of 3 seconds. Constructs used for expression were with DI cloned into pET-26b and pET-39b.

Periplasmic sample was then applied to a column containing resin charged with nickel. The washing steps were crucial to obtaining purest product. The purification kit recommended a second wash with 20mM imidazole, however this resulted in impure target protein on elution. Consistently, we found that 10mM imidazole partially eluted His₆-tagged DI as demonstrated by western blot in figure 3.18. Optimal washing with 45mM imidazole and elution at 100mM imidazole (higher than the recommended 1M elution) proved to be optimal for recovering purest sample of His₆-tagged DI, as shown in figure 3.19.

Once purified, the elution sample was then subjected to SDS-PAGE analysis overnight against 10% glycerol in PBS at 4°C to remove imidazole. Removal of imidazole is not only necessary for subsequent His₆-tag binding assays using nickel-coated microwell plates, it is also required for high target-protein quantification using the BCA protein assay (Pierce) as imidazole affects the sensitivity of this assay. Yields approximated 1 to 750µg of 2 per one litre of

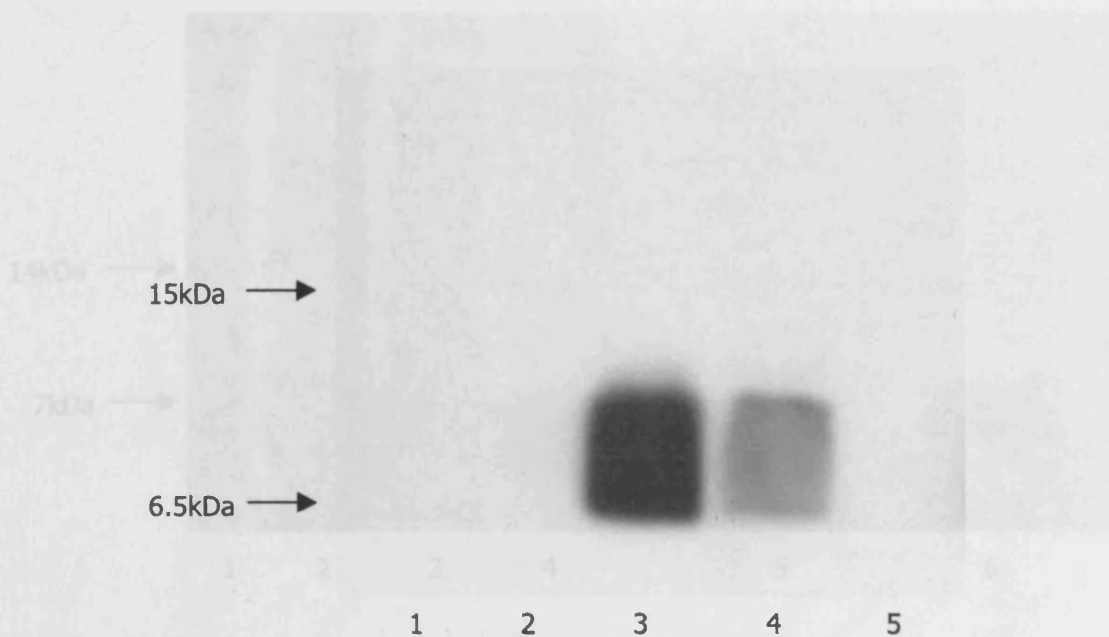
Lanes:
 1 His₆-tagged DI
 2 Periplasmic fraction

Figure 3.17 Western blot of recombinant DI probed with an anti-his tag antibody. Periplasmic fraction was transferred onto a nitrocellulose membrane from a 15% SDS-PAGE gel and probed with an anti-his₅-antibody. The ~7kDa band (lane 2) is consistent with DI.

Periplasmic sample was then applied to a column containing resin charged with nickel. The washing steps were crucial to obtaining purest product. The purification kit recommended a second wash with 30mM imidazole, however this resulted in impure target protein on elution. Conversely washing with 60mM imidazole partially eluted his₆-tagged DI as demonstrated by western blot in figure 3.18. Optimal washing with 45mM imidazole and elution at 300mM imidazole (rather than the recommended 1M elution) proved to be optimal for obtaining purest sample of his₆-tagged DI, as shown in figure 3.19.

Once purified, the elution sample was then subjected to extensive dialysis overnight against 10% glycerol in PBS at 4°C to remove traces of imidazole. Removal of imidazole is not only necessary for subsequent direct ELISA binding assays using nickel coated microwell plates, it is also required for total target protein quantification using the BCA protein assay (Pierce) as imidazole affects the sensitivity of this assay. Yields approximated to 750µg of target protein per one litre of expression culture, resulting in a working concentration of 150-200µg/ml of purified his₆-tagged DI.

Purified recombinant his₆-tagged DI was then coated on nickel-chelate coated microwell plates and binding confirmed to two murine monoclonal anti-DI antibodies, mAb-16 and 6C4C10 which recognise conformationally restricted epitopes (Iverson et al., 2002b), as shown in figure 3.20. Hence the correct size of target protein and observed binding of these antibodies on both the blot (figure 3.18) and the ELISA plate confirm the successful expression of DI, most likely in its correct conformational state.



Lanes:

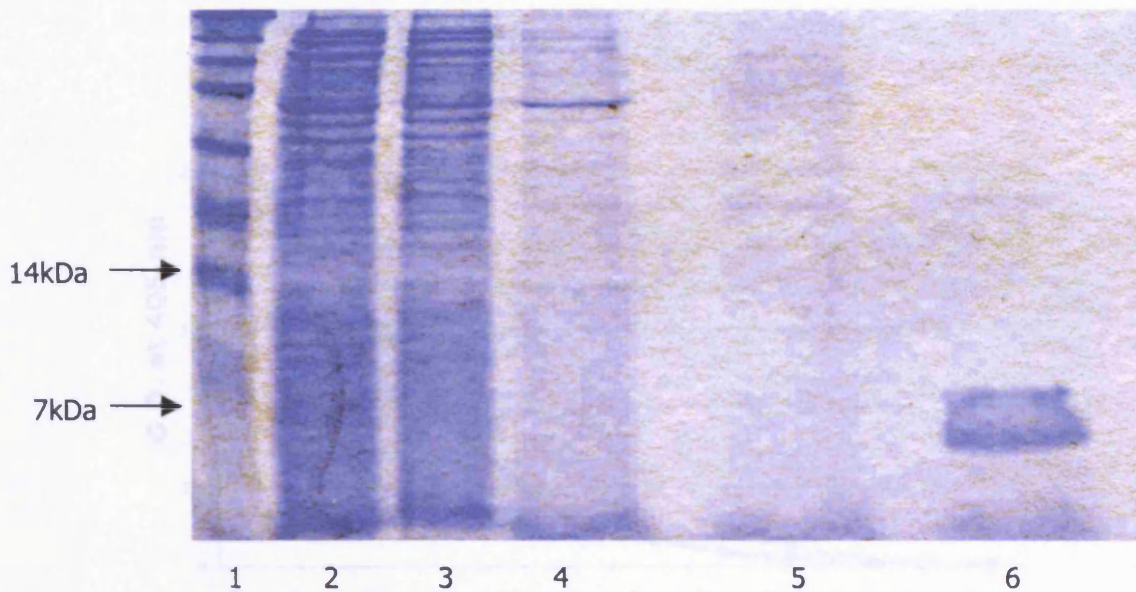
Lanes:

Protein molecular weight marker

- 1 Column flow through
- 2 Wash with 5mM imidazole
- 3 60mM imidazole - partial elution of his₆-DI
- 4 400mM imidazole - partial elution of his₆-DI
- 5 1M imidazole - no remaining target protein

Figure 3.18 Coomassie stain of DI purification steps using nickel

Figure 3.18 His₆-tagged DI elutes from nickel at low concentrations of imidazole. Samples were run on a 15% SDS-PAGE, transferred on to a nitrocellulose membrane and probed with the murine monoclonal anti-DI antibody 6C4C10. No detectable DI in the flow through suggests that all of the target protein binds nickel. DI could be eluted using between 60mM and 400mM of imidazole. Hence using 1M of imidazole as recommended by the manufacturers was not necessary as all target protein could be eluted at a lower concentration as demonstrated.



Lanes:

- | | |
|---|---|
| 1 | Protein molecular weight marker |
| 2 | Periplasmic extract |
| 3 | Column flow-through |
| 4 | Wash with 5mM imidazole |
| 5 | Wash with 45mM imidazole |
| 6 | Elution of pure his ₆ -DI with 300mM imidazole |

Figure 3.19 Coomassie stain of DI purification steps using nickel chromatography. Sample analysed by 15% SDS-PAGE. Washing with 5mM and then 45mM of imidazole and subsequent elution with 300mM imidazole yields an elute containing pure target protein.

3.1.3 Efficient expression achieved with low levels of IPTG induction

Inducing the expression culture with 1mM and 0.1mM of IPTG resulted in very similar reduction of culture growth rate as measured by OD_{600} . However, inducing at 0.1mM IPTG resulted in even slightly less inhibition of growth as compared to higher IPTG induction levels. This observation, that cells grow better under these gender induction conditions, may be due to the fact that higher IPTG concentrations result in higher production of target protein. It is possible that the amount of target protein produced is greater with 0.1mM IPTG and that the amount of target protein is sufficient to maintain the growth of the culture. The amount of target protein produced is dependent on the concentration of IPTG used to induce the culture.

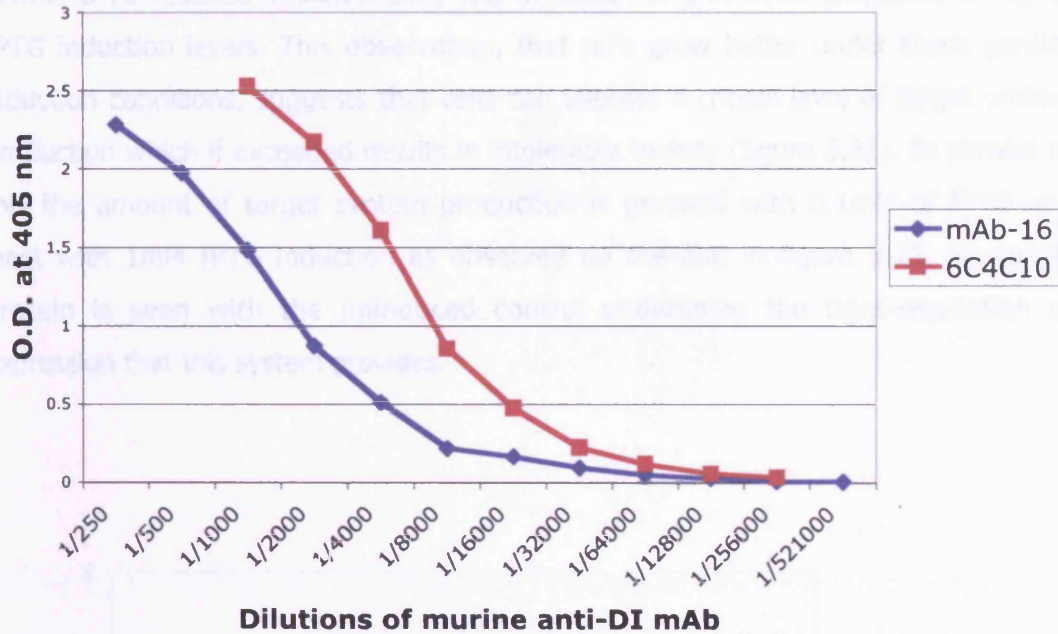


Figure 3.20 Murine anti-DI antibodies bind recombinant DI. Direct ELISA demonstrating strong binding to recombinant DI by two murine anti-DI monoclonal antibodies that bind conformationally restricted epitopes on DI, suggesting that expressed target protein is conformationally correct.

Figure 3.21 Growth curves of *E. coli* expression cultures following induction with varying concentrations of IPTG. OD_{600} represents the density of bacterial growth in the culture media. Samples were induced at OD_{600} of 0.6. Inducing with IPTG at 1mM and 0.1mM IPTG results in marked inhibition of cell growth. DI in the figure legend signifies that the proteins had been transformed with the DI-pET 20b construct.

3.3.3 Efficient expression achieved with low levels of IPTG induction

Inducing the expression culture with 1mM and 0.4mM of IPTG resulted in very similar reduction of culture growth rate as measured by OD₆₀₀. However inducing at 0.1mM IPTG resulted in significantly less inhibition of growth as compared to higher IPTG induction levels. This observation, that cells grow better under these gentler induction conditions, suggests that cells can tolerate a critical level of target protein production which if exceeded results in intolerable toxicity (figure 3.21). In parallel to this the amount of target protein production is greatest with 0.1mM of IPTG and least with 1mM IPTG induction as observed on the blot in figure 3.22. No target protein is seen with the uninduced control underlining the tight-regulation of expression that this system provides.

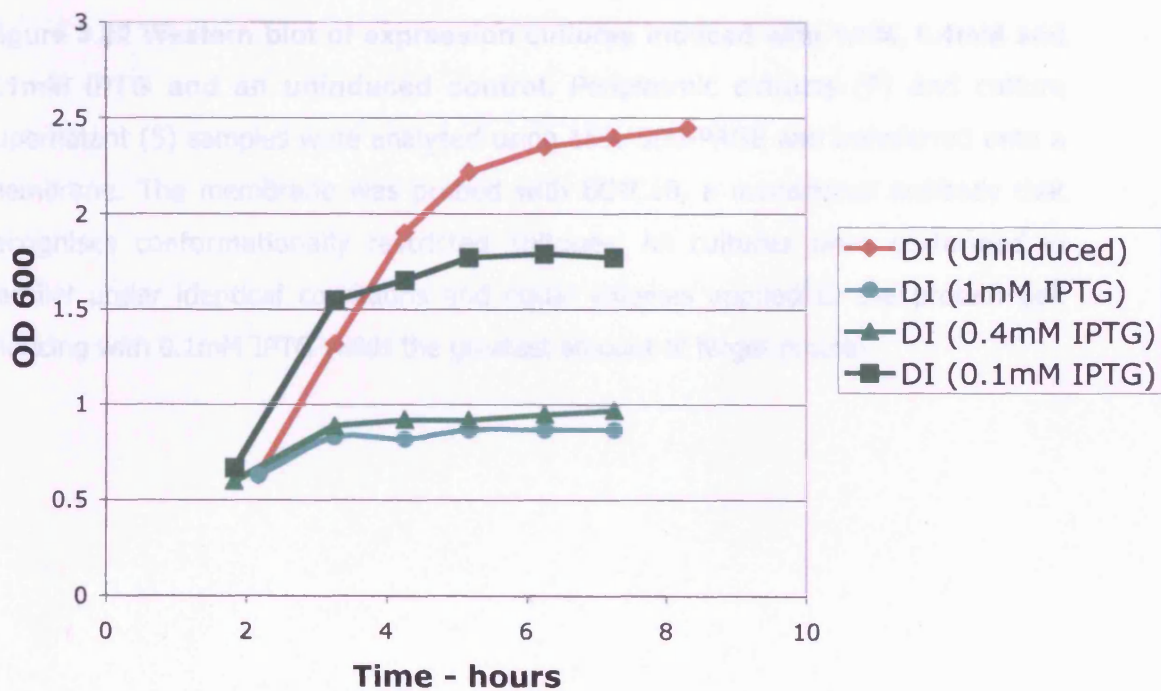


Figure 3.21 Growth curves of *E.coli* expression cultures following induction with varying concentrations of IPTG. OD₆₀₀ represents the density of bacterial growth in the culture media. Samples were induced at OD₆₀₀ of 0.6. Inducing with IPTG at 1mM and 0.4mM IPTG results in marked inhibition of cell growth. DI in the figure legend signifies that the bacteria had been transformed with the DI-pET 26b construct.

3.3.4 Recombinant sequence optimized for *E. coli* in order to maximize expression of Df

One possible explanation for this result may be the plasmid used and the specific expression and induction conditions. The hypothesis was that *E. coli* preferential device design was a better prokaryotic producer of Df in addition to the genetic code. To test this, a cDNA sequence of Df of human origin was obtained from GenBank (GenBank accession number U01451) and a pET-3a(+) plasmid (Novagen, Madison, WI, USA) was used for manufacturing. The pET-3a(+) plasmid contains a lacI^q gene, a lacO^q operator, and a lacP promoter. The lacI^q gene is a repressor of the lac operon. The lacO^q operator is a binding site for the lacI^q repressor. The lacP promoter is a strong promoter. The Df gene was cloned into the pET-3a(+) plasmid under the control of the lacP promoter. The resulting plasmid was transformed into *E. coli* BL21(DE3) cells. The cells were grown in LB medium containing 100 µg/ml ampicillin. After reaching an optical density of 0.6 at 600 nm, the cells were induced with IPTG. The culture supernatant (S) and periplasmic extracts (P) were analysed by Western blotting. The results are shown in Figure 3.22.

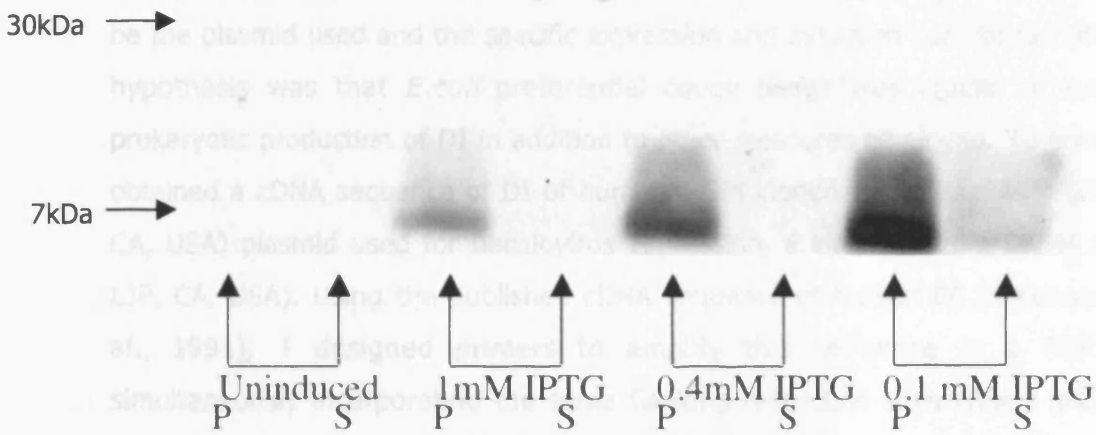


Figure 3.22 Western blot of expression cultures induced with 1mM, 0.4mM and 0.1mM IPTG and an uninduced control. Periplasmic extracts (P) and culture supernatant (S) samples were analysed using 15% SDS-PAGE and transferred onto a membrane. The membrane was probed with 6C4C10, a monoclonal antibody that recognises conformationally restricted epitopes. All cultures were performed in parallel under identical conditions and equal volumes applied to the protein gel. Inducing with 0.1mM IPTG yields the greatest amount of target protein.

3.3.4 Recombinant sequence optimised for *E.coli* is crucial to successful expression of DI

One possible explanation for successful expression of recombinant DI could simply be the plasmid used and the specific expression and induction conditions utilised. My hypothesis was that *E.coli* preferential codon usage was crucial to successful prokaryotic production of DI in addition to other measures employed. To prove this I obtained a cDNA sequence of DI of human β_2 GPI cloned into a BacPAK™ (Clontech, CA, USA) plasmid used for baculovirus expression, a kind gift from Dr. M Iverson, LJP, CA, USA). Using the published cDNA sequence of human DI (Steinkasserer et al., 1991), I designed primers to amplify this sequence in a PCR whilst simultaneously incorporating the same flanking restriction sites (*Nco* I and *Xho* I) present in the optimised synthetic gene. The PCR amplified human DI gene as shown in figure 3.23 was then cloned into pET-26b utilising the incorporated *Nco* I / *Xho* I flanking restriction sites and sequenced to exclude PCR errors (figure 3.24). Two parallel expression and induction cultures were then grown, one containing BL21(DE3) transformed with the construct containing the human cDNA sequence of DI and one containing the construct harbouring my synthetic gene which had been optimised for *E.coli*. Equal amounts of periplasmic fractions from each were then applied to the same 15% SDS-PAGE gel and transferred on to a membrane. Though some target protein was seen using the native cDNA sequence, much greater yield of target protein was seen when using the optimised sequence as seen figure 3.25.

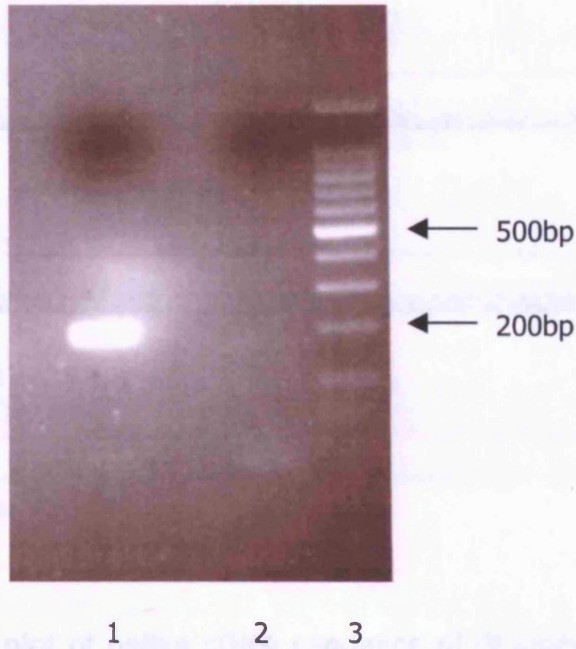


Figure 3.24 Sequencing gel of human DI gene. Lane 1, Human DI amplified gene; Lane 2, Negative control (PCR mix minus template DNA); Lane 3, 100bp marker.

3.23 PCR amplification of human DI gene. A PCR amplification of the human DI gene cloned into BacPAK™ plasmid was undertaken whilst simultaneously incorporating *Nco* I / *Xho* I flanking restriction sites. 5µl of the PCR mix was run on 2 % agarose gel and the 201bp band is consistent with the target gene.

Figure 3.25 Western blot analysis of recombinant His-tagged DI protein with anti-DI antibody. Lane 1, His-tagged recombinant DI protein; Lane 2, DI expressed using native DNA human antibodies; Lane 3, DI expressed using synthetic peptides. Equal amounts of protein extract were applied in lanes 1 and 2 on the protein gel.

3.3.5 Expression of DI tagged with DsA at the N-terminus

Though satisfactory yields were eventually obtained using the pET28a(+) construct, I

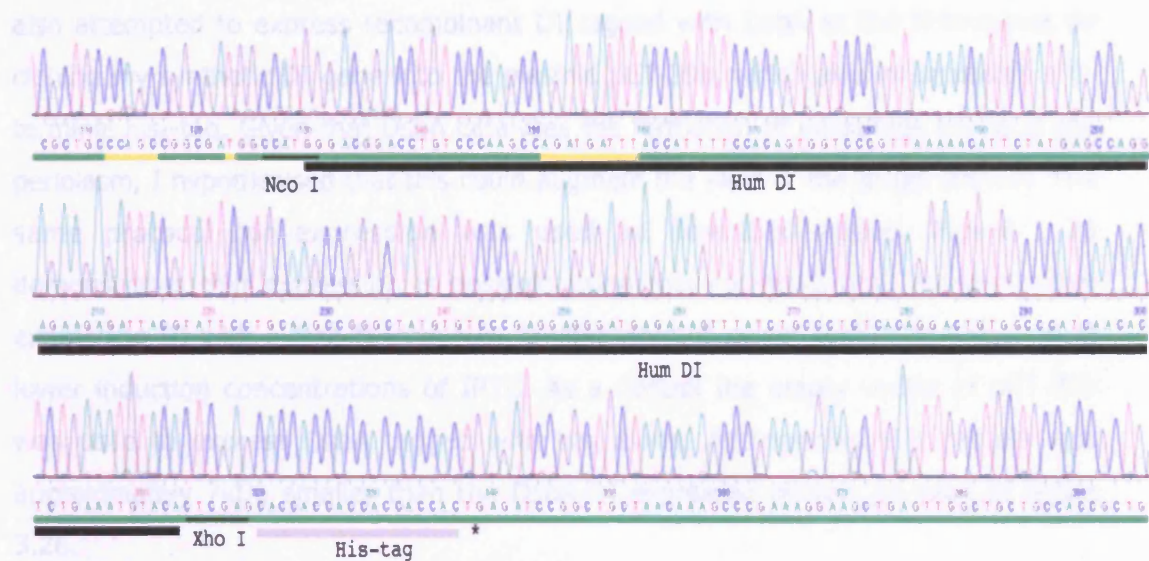


Figure 3.24 Sequencing plot of native cDNA sequence of DI cloned into pET-28b. Abbreviations – Hum DI, native human cDNA sequence of DI; *, stop codon.

of DI and the C-terminus of LysA (Figure 3.20), this site was specifically targeted.

The aim would be to cleave DI from LysA using recombinant antibodies which

according to the protocol in part of target protein (Figure 3.22) antibodies that

that including expression of DI with a low concentration of a protease inhibitor

or non-cleavage, which might cause major problems with protein degradation

and hence purified full of target protein with minimal amount of DI

DI detected. Varying the concentration of each enzyme, temperature and duration of

enzyme incubation neither improved yield nor efficiency of cleavage. Hence, this

method is not suitable for the cleavage of DI. For further experiments, only DI

and variants of this protein would be expressed using the pET-28b construct.

However, DNA was expressed in *E. coli* and purified and used as a marker of

positive control protein in subsequent binding assays (Figure 3.25).

Figure 3.25 Western blot analysis of recombinant his₆-tagged DI probed with an

anti-his₆-antibody. Lane 1, his₆-tagged molecular weight protein markers; lane 2,

DI expressed using native DNA human sequence; lane 3, DI expressed using

sequence optimised for *E. coli*. Equal amounts of periplasmic extract were applied to

lanes 1 and 2 on the protein gel.

15kDa →

1 2 3

3.3.5 Expression of DI tagged with DsbA at the N-terminal

Though satisfactory yields were eventually obtained using the DI-pET26b construct, I also attempted to express recombinant DI tagged with DsbA at the N-terminus by cloning my synthetic DI gene into the plasmid pET-39b, which also incorporates a C-terminal his₆-tag. Given that DsbA catalyses the formation of disulphide bonds in the periplasm, I hypothesised that this could augment the yield of the target protein. The same protocol for expression was used as described above. Figure 3.26 demonstrates that expression is possible using this construct and, as seen when expressing DI with a free N-terminus, greater yield of target protein is observed at lower induction concentrations of IPTG. As a control the empty vector of pET-39b was used to express DsbA tagged with his₆ alone. As expected this protein was approximately 7kDa smaller than the DsbA-DI expressed protein as seen in figure 3.26.

It was felt necessary to cleave DI off from DsbA before proceeding onto the planned experiments characterising the binding properties of DI. As there was an incorporated enterokinase cleavage site conveniently located between the N-terminus of DI and the C-terminus of DsbA (figure 3.16), this site was specifically targeted. The aim would be to cleave DI from DsbA using recombinant enterokinase whilst avoiding non-specific protein degradation of target protein. Figure 3.27 demonstrates that incubating expressed DsbA-DI with a low concentration of enterokinase resulted in non-cleavage, whilst higher concentrations resulted in non-specific protein degradation and hence marked loss of target protein with minimal amounts of free DI detected. Varying the concentration of enterokinase, temperature and duration of enzyme incubation neither improved yield nor efficiency of cleavage. Given that this method ultimately led to reduced yields of free DI, for further experiments native DI and variants of this protein would be expressed using the pET-26b construct. However, DsbA alone, expressed by using the empty pET-39b vector, was purified and used as a his-tagged negative control protein for subsequent binding assays (figure 3.28).



Figure 3.26 Expression of DI tagged with DsbA at the N-terminal. Periplasmic extract was transferred onto a membrane and probed with an anti-his₅ antibody. Lane 1, pET-39b empty vector expressing DsbA alone; lane 2, DsbA-DI induction with 1mM IPTG; lane 3, induction with 0.5mM IPTG; lane 4, induction with 0.1mM IPTG.

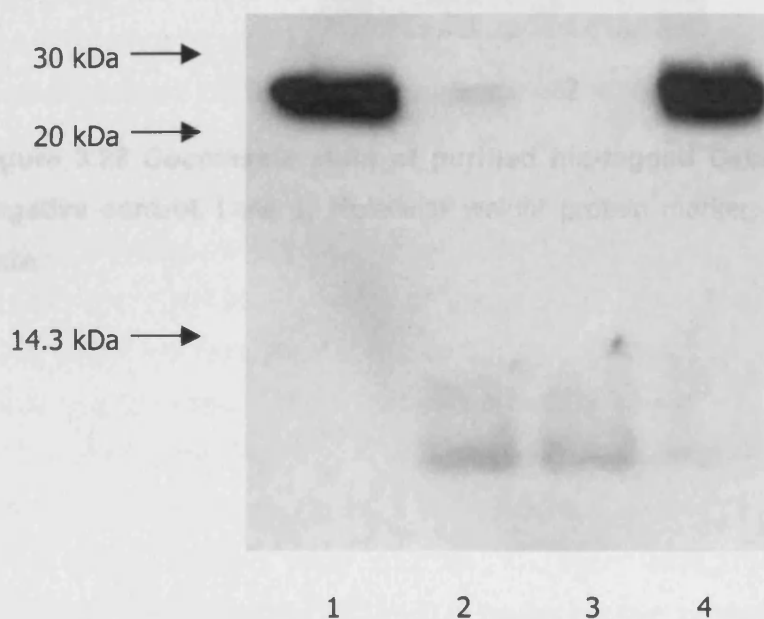


Figure 3.27 Western blot demonstrating inefficient cleavage of DI from DsbA using enterokinase. The membrane was probed with the anti-DI antibody 6C4C10. Lane 1, no enterokinase; lane 2, 2U/ μ l enterokinase; lane 3, 0.5U/ μ l enterokinase; lane 4, 0.1U/ μ l enterokinase. Low levels of cleaved 7kDa DI seen in lanes 2 and 3 due to non-specific degradation of protein by higher concentration of enterokinase.

3.4 Discussion of results

This chapter describes the results charting the development of an efficient system of recombinant DT expression in *E. coli*. It became apparent through sequential experiments that efficient expression of DT in *E. coli* was dependent upon identifying certain key factors. These factors were then used to develop a system for the efficient production of proteins, described in chapter 4.

to bacteria. These problems were addressed by the development of a system for the efficient production of proteins, described in chapter 4.

and control of the expression of the gene. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.

The cDNA for the protein was amplified by PCR using the primers described in chapter 4. The PCR product was then ligated into the expression vector, and the resulting construct was transformed into *E. coli*.

(Steinkasserer et al., 1993). The expression of the protein was induced by the addition of IPTG (43%) and the cells were harvested and the protein purified by ion exchange chromatography.

codons are known to cluster in the genome, suggesting that the genetic code may, in fact, be organized in a way that allows for the efficient production of proteins, described in chapter 4.

may, in fact, be organized in a way that allows for the efficient production of proteins, described in chapter 4. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.

containing an excess of residues with respect to the genetic code. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.

PCR has the advantage of being able to amplify a specific DNA sequence from a complex mixture of DNA. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.

of specifications in one single step. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.

Juniper ensured that all copies of the DNA were of the same length and that the DNA was of high quality. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.

however, involved specific steps of the genetic code, and did not involve any genetic code optimization as described in this chapter. The study has reported the successful expression of the individual genes from the genetic code, described in chapter 4.

elf2 gene by creating a gene with an optimal codon usage and a high GC content. However, this gene was created using a method using secondary PCR using a 5' primer and a 3' primer using reverse PCR. Other strategies that may be employed to address codon optimization involve increasing the GC content of the gene by co-expressing genes that encode rare tRNAs such as *argE* (*argE*) gene that encodes the minor codon Arg (AGG/AGA) (Andrews et al., 1996). This may be achieved by using commercially available *E. coli* strains that contain an extra plasmid for the minor tRNA change *E. coli* Argona (Novagen) or *E. coli* CodonPlus (Stratagene). Although this strategy may be effective in producing large amounts of some eukaryotic

proteins, it may not be suitable for the production of large amounts of some eukaryotic proteins. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.

proteins, it may not be suitable for the production of large amounts of some eukaryotic proteins. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.

proteins, it may not be suitable for the production of large amounts of some eukaryotic proteins. This system was developed by the development of a system for the efficient production of proteins, described in chapter 4.



Figure 3.28 Coomassie stain of purified his-tagged DsbA protein for use as negative control. Lane 1, Molecular weight protein marker; lane 2, purified DsbA elute.

3.4 Discussion of results

This chapter describes the results charting the development of an efficient system of recombinant DI expression in *E.coli*. It became apparent through successive experiments that efficient expression of DI in *E.coli* was dependent upon addressing certain key factors. These relate to codon usage for *E.coli*, control of basal production of protein, disulphide bond formation and the observation that DI is toxic to bacteria. These problems were addressed in turn as detailed in the sections above and consequently a novel platform of expression of DI of human β_2 GPI in *E.coli* developed.

The cDNA sequence of human DI contains clusters of codons used rarely by *E.coli* (Steinkasserer et al., 1991). Of the 61 codons present in the native DI gene, 26 (43%) are used by *E.coli* at a frequency of less than 1% (Kane, 1995). These rare codons are present in clusters of between two to five codons in length. Hence one may expect translational problems in *E.coli* with an abundant mRNA species containing an excess of rare low tRNA codons. Even a single rare codon can have a deleterious effect on heterologous protein expression (Hua et al., 1994). Recursive PCR has the advantage of being able to design and create a gene to meet a number of specifications in one simple PCR reaction (Prodromou and Pearl, 1992). Specifically Juniper ensured that all codons incorporated were those seen at relatively high frequencies in *E.coli*. The establishment of other eukaryotic protein expression systems in *E.coli* using the same codon optimisation principles has been described before (Hu et al., 1996, Hua et al., 1994, Martin et al., 1995). All these studies however, targeted specific areas of the genes of interest, and did not ensure pan-gene codon optimisation as described in this chapter. One study has reported the successful expression of the eukaryotic protein human translation initiation factor eIF2 alpha by creating a gene with all codons optimised (Ito and Wagner, 2004). However this gene was created in step-wise fashion using successive PCR steps, and not in a single reaction using recursive PCR. Other strategies that may be employed to address codon optimisation involve expanding the intracellular tRNA pool by co-expressing genes that encode rare tRNAs such as argU (*dnaY*) gene that encodes the minor tRNA-Arg (AGG/AGA) (Andrews et al., 1996). This may be achieved by using commercially available *E.coli* strains that contain an extra plasmid for the minor tRNAs such as *E.coli* Rosetta (Novagen) or *E.coli* CodonPlus (Stratagene). Although these strains may be efficient in producing large amounts of some eukaryotic

proteins, the commercially available plasmids only encode for up to six rare tRNAs. *E.coli* uses 18 codons at a frequency of less than 1% (Kane, 1995) so one may still expect translational problems if the eukaryotic sequence contains a cluster of minor codons not supplied by these plasmids. Synthesis of an artificial gene not only ensures the optimisation of all codons, but there is also the added advantage of being able to modify the sequence simultaneously to aid subsequent cloning steps, periplasmic localisation and to produce genes with mutations at multiple points of interest as I have done.

Periplasmic localisation of target protein facilitated the formation of disulphide bonds due to the oxidative environment of this compartment in bacteria (Hannig and Makrides, 1998). The target protein in the periplasm has a free N-terminal as the pel B leader sequence is cleaved by signal peptidase during the translocation process and subsequently degraded by cytoplasmic signal peptide peptidase (Weihofen et al., 2002). Furthermore, release of periplasmic proteins into the osmotic shock fluid potentially enhances efficiency of purification due to the significantly smaller pool of periplasmic bacterial proteins compared with the cytoplasm (Hannig and Makrides, 1998). Similar principles have been applied by this laboratory to express human anti-dsDNA antibody F(ab) in *E.coli* successfully (Kumar et al., 2000). Other approaches to improve solubility of protein with disulphide bonds could be to co-overexpress enzymes that catalyse the formation (DsbA) (Cervera et al., 2002) or isomerisation (DsbC) of disulphide bonds in the periplasm (Zapun et al., 1995), over-express molecular chaperones such as heat shock proteins, Gro-EL-GroES and trigger factor (Ito and Wagner, 2004), or express in *E.coli* cells with mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, potentially enhancing disulphide bond formation in the cytoplasm (Lobel et al., 2002). However, it transpired that these approaches were not necessary as sufficient quantities of soluble DI could be obtained from the periplasm.

Toxicity proved to be a major obstacle in terms of establishing efficient expression of DI. Tightly regulating expression by utilising the more stringent T7/*lac* promoter, as offered by pET-26b plasmid, partly overcame this problem. Other frequently used methods of tightly regulating T7 promoter systems are to express protein in BL21(DE3) cells containing the T7 lysozyme producing pLysS and pLysE plasmids (Dubendorff and Studier, 1991). This was not appropriate for my project as although T7 lysozyme regulates expression by inhibition of RNA polymerase (Huang et al.,

1999, Zhang and Studier, 1997), it has long been established that it can also cut specific bonds in the peptidoglycan layer of the *E.coli* inner cell wall, thus disrupting the periplasmic compartment (Inouye et al., 1973). Gentle induction of expression with low concentrations of IPTG was also crucial as inducing with the standard 1mM concentration resulted in reduced yields implying *E.coli* cells could tolerate only a certain level of DI production. The expression of periplasmic human anti-dsDNA F(ab) in *E.coli* by our laboratory found that greater yields of this protein were seen with the 1mM of IPTG induction rather than 0.1mM, implying that human anti-dsDNA F(ab) is less toxic to *E.coli* than DI of β_2 GPI (Kumar et al., 2001).

Finally, this expression system lends itself to detailed mutational studies of DI as the ease and relatively low cost of *E.coli* expression systems relative to other hosts facilitates the production of a number of DI mutants. However, before embarking on the task of producing numerous variants of DI and investigating the effects of introducing mutations at specific sites of interest, the binding characteristics of wild-type recombinant DI first need to be fully characterised.

CHAPTER 4

BINDING PROPERTIES OF RECOMBINANT DI OF β_2 GPI

OVERVIEW OF CHAPTER 4

Having developed an efficient system for expressing DI in *E.coli*, my next aim was to establish the binding properties of this purified recombinant protein. This Chapter's first focus is on presenting the results of recombinant DI binding assays to monoclonal aPL.

The focus then shifts to the assessment of DI binding polyclonal IgG purified from APS patient serum samples with two control groups included for comparison purposes (autoimmune disease and healthy controls). Patient selection details and criteria are presented. Purified IgG from all subjects is assessed for binding to a number of clinically relevant antigens such as CL, PS, whole β_2 GPI as well as recombinant DI. Statistical analyses of the binding results aims to detect differences between each of the patient groups studied within each of the antigen binding assays. Subset analyses are also performed for the APS group to detect if binding to DI can predict a specific clinical phenotype. Some IgG aPL exhibited interesting binding properties, such as displaying good binding to DI, whilst exhibiting relatively poor binding to whole β_2 GPI. These and other intriguing results are discussed, hypotheses for explaining them are put forward and results of experiments testing these hypotheses presented. Finally, results confirming the ability of recombinant DI to bind polyclonal aPL in the fluid phase are shown.

4.1 Recombinant DI binds to pathogenic monoclonal human aPL in both direct and inhibition assays

4.1.1 Introduction

IS4 is a human monoclonal antibody with proven pathogenicity derived from a patient with APS (Zhu et al., 1999). Our group have made two variants of this monoclonal aPL, swapping the light chain with that of an anti-DNA antibody B3 and another monoclonal aPL UK-4. The light chains are very similar to each other with more than 90% sequence identity. Thus the three monoclonal antibodies tested for their ability to bind recombinant DI were IS4V_H/B3V_L, IS4V_H/IS4V_L and IS4V_H/UK-4V_L. These have previously been shown to bind CL and β_2 GPI in the following order of descending magnitude: IS4V_H/B3V_L -- IS4V_H/IS4V_L -- IS4V_H/UK-4V_L (Lambrianides et al., 2004). Recently our group has demonstrated, using point mutational studies, that non-germ line encoded arginine residues in CDR-3 of the heavy chain of IS4 play a critical role in binding to CL (Giles et al., 2005). Three mutants named IS4V_H^{II&II}, IS4V_H^{III} and IS4V_H^{IV} contained mutations of arginine to serine at positions 96, 97 (for IS4V_H^{II&II}), 100 (for IS4V_H^{III}) and 100G (for IS4V_H^{IV}). These three mutants are schematically represented in figure 4.1 and have previously been shown to exhibit reduced binding to CL as a consequence of the incorporated mutations (Giles et al., 2005).

The aim was to demonstrate that DI bound to a plate exhibits binding to these monoclonal antibodies in a comparable way to that seen with whole β_2 GPI and CL. The ability of DI to bind affinity purified IS4V_H/IS4V_L in solution was also investigated and compared to that of whole β_2 GPI.

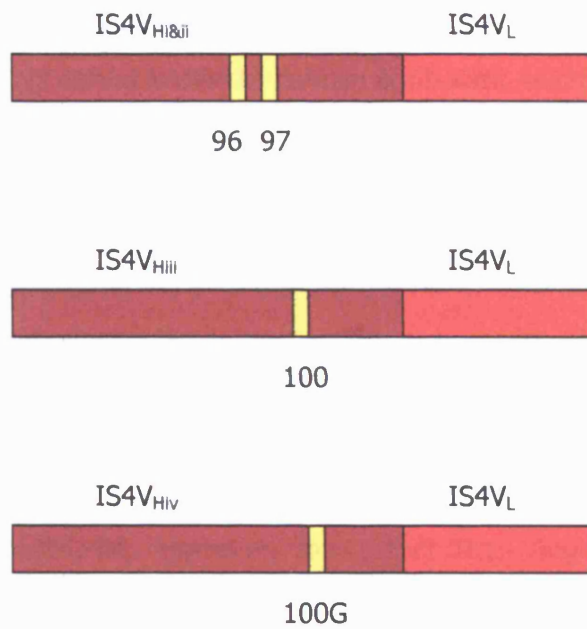


Figure 4.1 Schematic of IS4V_H point mutations. Yellow shading depicts the arginine to serine point mutations within each of the IS4V_H mutants. Each of the three IS4V_H mutants was paired with native IS4V_L.

4.1.2 Recombinant DI in solid phase binding to native IS4, IS4V_H/B3V_L, IS4V_H/UK-4V_L and variants containing point mutations in IS4V_H.

Binding of these monoclonal human aPL variants to whole β_2 GPI is shown in figures 4.2 and to DI in figure 4.3. Total IgG ELISAs were used to determine the precise stock concentrations of purified IgG aPL. The positive control in all ELISAs was a patient serum known to contain antibodies reactive to whole β_2 GPI. The same aliquot of patient serum was used for all direct binding assays.

As shown in figure 4.2, the strongest binding to whole β_2 GPI coated on an irradiated plate is observed with the monoclonal variant IS4V_H/B3V_L whilst almost no binding is observed with IS4V_H/UK-4V_L. The same order of binding with these three antibodies is observed to CL, except that low level (rather than negligible) binding occurs with the IS4V_H/UK-4V_L variant (Giles et al., 2005). Figure 4.3 demonstrates that DI also exhibits the strongest binding to native IS4 and IS4V_H/B3V_L and the least to IS4V_H/UK-4V_L. However, low rather than negligible binding, to IS4V_H/UK4-V_H is observed in line with the binding to CL. The variants of IS4 with point mutations in IS4V_H revealed no detectable binding to DI, suggesting that the arginine residues exposed on CDR-3 of IS4V_H are critical to binding DI of β_2 GPI.

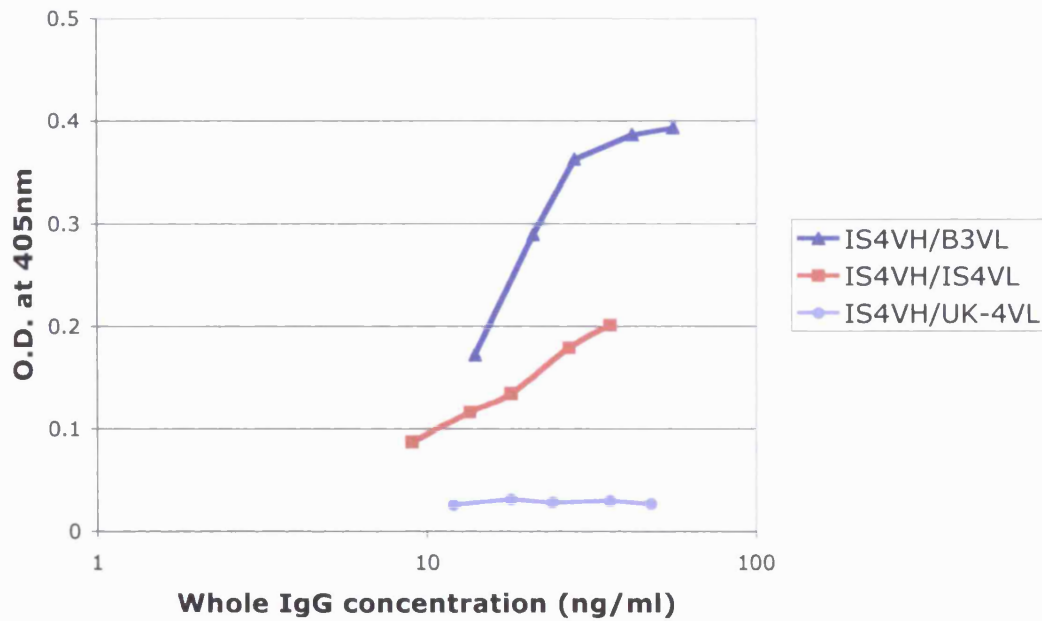


Figure 4.2 Binding of IS4V_H/light chain variants to β_2 GPI coated on an irradiated plate. Each heavy / light chain variant was expressed in CHO cells and secreted into the supernatant. Strongest binding was seen with IS4V_H/B3V_L and almost no binding was seen with IS4V_H/UK-4V_L variant.

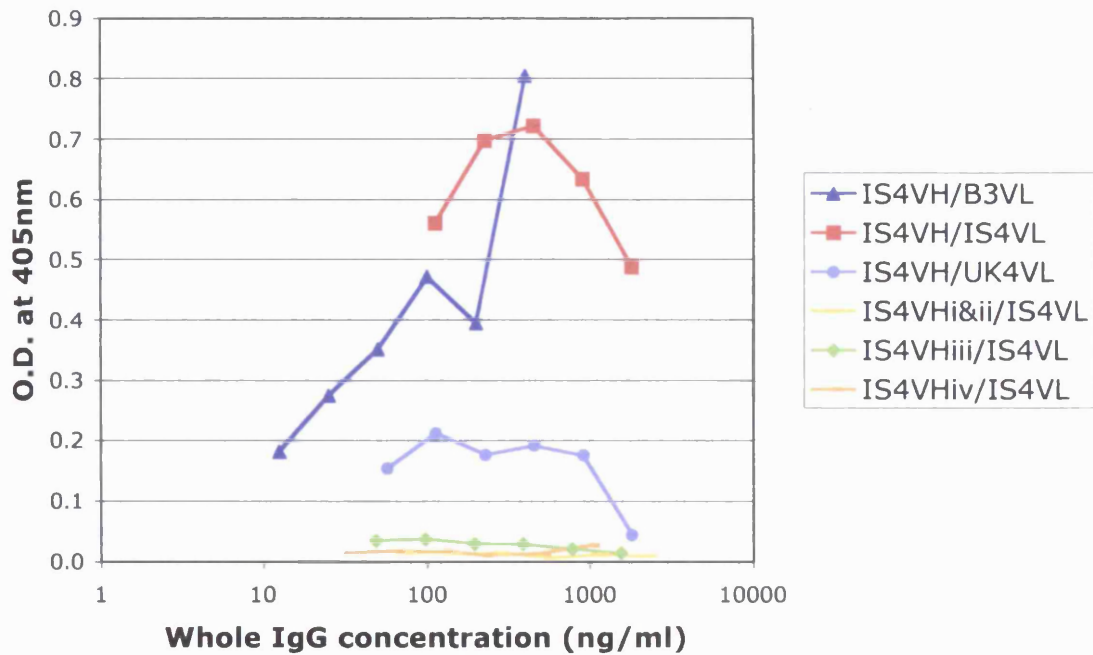


Figure 4.3 Binding of IS4V_H/light chain variants and IS4V_H mutants to DI coated on a nickel plate. Monoclonal antibodies were expressed in CHO cells and secreted in the supernatant. The strongest binding to DI was observed with native IS4V_H/IS4V_L and the variant IS4V_H/B3V_L and least with IS4V_H/UK-4V_L. The variants of native IS4 with the single point mutations in IS4V_H (IS4VHi&ii, IS4VHiii and IS4VHiv – see figure 4.1) revealed no detectable binding to DI, suggesting that arginine residues exposed on the CDRs of IS4V_H are critical to binding to DI.

4.1.3 Recombinant DI in the fluid phase binds to affinity purified IS4V_H/IS4V_L

Native IS4 was available in an affinity purified form. The ability of DI to bind IS4 in the fluid phase and inhibit IS4 binding to DI coated on a plate was ascertained and compared to whole β_2 GPI in the fluid phase. As β_2 GPI has a molecular weight approximately seven times greater than that of DI the concentration of inhibitors are expressed in μmol concentration rather than in $\mu\text{g/ml}$. As shown in figure 4.4, DI is capable of binding IS4 in the fluid phase. There is a clear threshold of concentration above which DI in solution markedly inhibits binding of IS4 to DI coated on a plate. The degree of inhibition of this antibody was greater than that of equivalent μM concentrations of whole β_2 GPI.

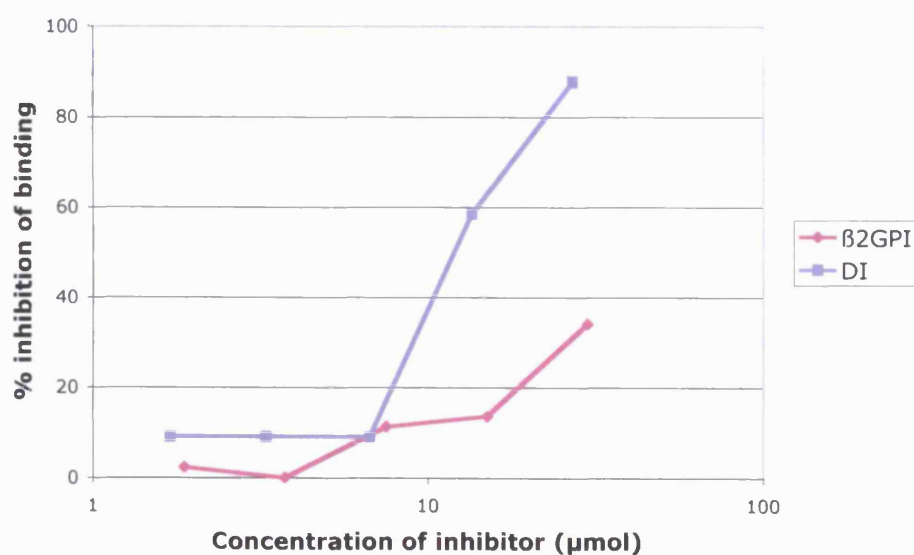


Figure 4.4 Recombinant DI in the fluid phase inhibits native IS4 from binding to DI coated on a plate.

4.2 Demographic data on polyclonal IgG patient samples

Polyclonal IgG was purified from the serum of three sample groups. Serum was obtained from the bank of SLE serum samples stored at -20°C or taken prospectively from patients / healthy volunteers after taking informed consent. Ethical approval for the sampling and use of serum within this project was granted by the UCL/UCLH Joint Research Committee on the 6th March 2003. The three sample groups studied were patients with APS, patients with either SLE or another autoimmune disease but no APS as disease controls and healthy volunteers. In total, IgG was purified from the serum of 54 individuals and the demographic information of these subjects are shown in table 4.1. Of the 24 APS patient samples, 18 (75%) had no other autoimmune disease whilst 6 (25%) also had SLE. The clinical details of each APS patient were recorded and shown in table 4.2 and all fulfilled the preliminary diagnostic classification criteria for APS (Wilson et al., 1999). The vast majority had a recorded positive IgG aCL (22/24, 91.7%) with a much smaller number also being positive for IgM aCL (7/24, 29.2%). None were positive for IgM aCL only. LA was positive in 17/24 (70.8%) and 9/24 (37.5%) at one point had a platelet count of less than $150 \times 10^3/\mu\text{l}$. There was a history of at least one miscarriage in 16/18 (88.9%) women with a history of pregnancy. 18/24 patients (75%) had a history of one or more thrombotic event. The most common venous thrombotic event was deep venous thrombosis (7/24, 29.2%) followed by pulmonary embolism (4/24, 16.7%). The most common arterial thrombotic event was a stroke (8/24, 33.3%) followed by myocardial infarction (2/24, 8.3%). The numbers of patients with a history of both arterial and venous thrombosis were 4/24 (16.7%). For the disease control group, of the 20 patients included 18 (90%) had SLE, one (5%) had autoimmune thyroid disease and one (5%) primary Sjogren's syndrome.

GROUP	n	Mean age (SD)	Gender	Ethnicity
APS	24	45.5 (9.1)	F=21 (87.5%) M=3 (12.5%)	C= 21 (87.5%) B= 3 (12.5%) O= 0 (0%)
*SLE / autoimmune disease (no APS)	20	36.4 (11.1)	F=19 (95%) M=1 (5%)	C= 19 (95%) B= 1 (5%) O= 0 (0%)
Healthy volunteers	10	31.8 (6.6)	F=7 (70%) M=3 (30%)	C=8 (80%) B=1 (10%) O=1 (10%)

Table 4.1 Demographic details of subject samples. Age is given as mean in years. Abbreviations: F, females; M, males; C, Caucasian; B, black; O, other which was Latin American. *Among the patients with SLE but no clinical features of APS, 6/20 (30%) were positive for either IgG aCL +/- or IgM aCL +/- or lupus anticoagulant.

Patient code	+/- SLE	ACL		LA	Low PI	Miscarriage number	Thrombotic event(s)
		IgG	IgM				
P1	-	+	-	+	-	Nulligravida	MI
P2	-	+	+	+	+	None	PE
P3	-	+	-	-	-	3	None
P4	-	+	-	-	-	6	PE, Stroke
P5	-	+	-	+	-	4	DVT
P6	-	+	-	+	+	2	DVT
P7	-	+	+	+	-	1	PE, Stroke (x2)
P8	-	+	-	+	-	4	None
P9	-	+	+	+	-	1	None
P10	-	+	-	+	-	2	Stroke (x3)
P13	-	+	-	-	+	1	None
P14	-	+	+	+	+	Nulligravida	TIA
P15	-	+	+	+	+	1	None
P16	-	+	+	+	+	N/A	Stroke (x2)
P18	-	+	-	+	+	N/A	Stroke
P19	-	+	-	+	-	Nulligravida	DVT (x2), PE, MI
P-H	-	+	-	+	+	N/A	DVT, CAPS
P-J	-	+	+	+	+	2	None
A46	+SLE	+	-	+	-	1	DVT
A73	+SLE	-	-	+	-	1	Stroke
A90	+SLE	+	-	-	-	1	Stroke
A96	+SLE	+	-	-	-	None	DVT, PE
A123	+SLE	+	-	-	+	5	DVT
A221	+SLE	+	-	-	-	2	Stroke

Table 4.2 Clinical details of APS patients. Abbreviations: Low PI, thrombocytopaenia; N/A, non-applicable for miscarriages as males; MI, myocardial infarction; PE, pulmonary embolism; DVT, deep venous thrombosis; CAPS, catastrophic antiphospholipid syndrome; TIA, transient ischemic attack. All patients fulfilled the preliminary consensus classification criteria for APS (Wilson et al., 1999). Thrombocytopaenia defined as platelet count less than $150 \times 10^3/\mu\text{l}$.

4.3 Binding profile of purified IgG to cardiolipin, phosphatidylserine and whole β_2 GPI.

Prior to performing binding assays to recombinant DI, I wanted to confirm that the binding characteristics of the purified IgG samples were as one would predict for the APS and control sample groups. Hence the purified IgG samples were assayed for binding to CL (figure 4.4), PS (figure 4.5) and whole β_2 GPI (figure 4.6). The results are expressed as a percentage of binding of an in-house polyclonal IgG standard derived from a patient with APS. All individual samples were tested in duplicate and the mean OD used to calculate percentage of binding activity. A binding level of 100% indicates the same level of binding compared to the standard, when used at the same IgG concentration on the same plate. A paired two-tailed t-test was used to calculate the probability of any difference observed between the groups as being statistically significant. For all three antigens, as one would expect, the majority of APS samples bound well and significantly better than either of the control groups. Figure 4.5 demonstrates a significant difference in binding to CL between the groups APS and autoimmune control ($p=0.007$) and APS and healthy controls ($p=0.03$). However, there was also a significant difference between the autoimmune control group and the healthy volunteers ($p=0.03$), though not as marked. Figure 4.6 demonstrates that when testing binding to whole β_2 GPI, there was also a statistically significant difference between APS and autoimmune controls ($p=0.02$), but again there was a significant difference between autoimmune controls and healthy controls ($p=0.0001$). The binding to PS was clearly not as sensitive at detecting APS as shown in figure 4.7. Though there was a significant difference between the APS group and the autoimmune disease control group ($p=0.03$), there was no difference between the APS and healthy control group ($p=0.1$), most likely due to the combination of a low number of healthy volunteer samples studied in contrast to the other groups and the relatively low number of APS samples which demonstrated binding to PS.

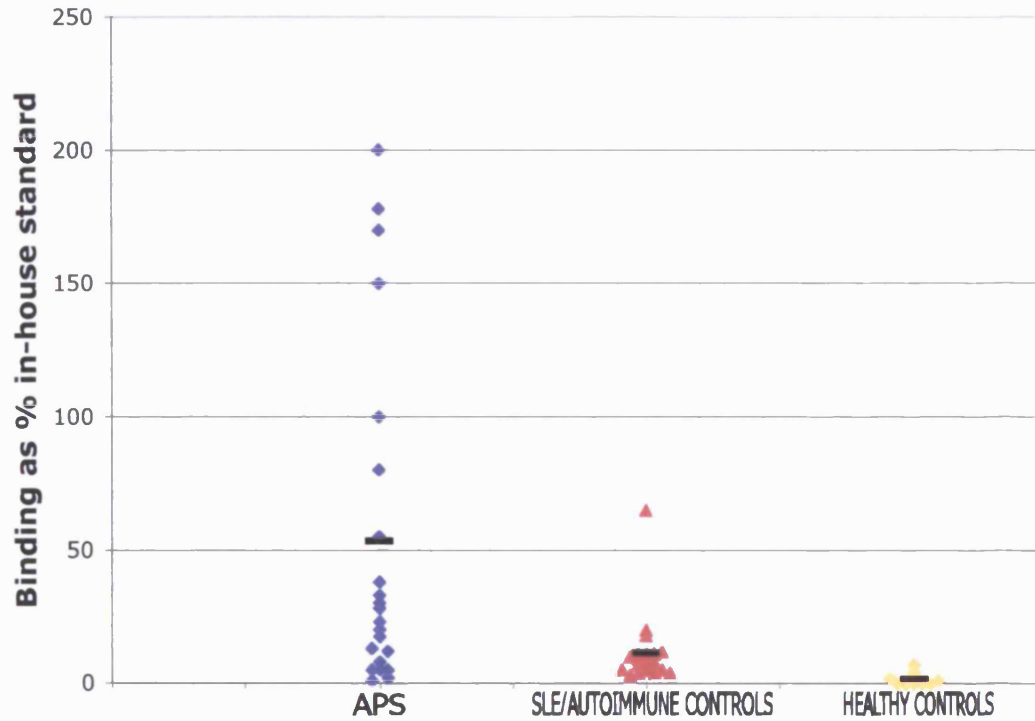


Figure 4.5 Results of polyclonal IgG samples binding to cardiolipin. APS, n=22; SLE/Autoimmune controls, n=20; healthy controls, n=10. **APS versus SLE/Autoimmune controls, $p=0.007$; *APS versus healthy controls, $p=0.02$; *SLE/Autoimmune controls versus healthy controls, $p=0.03$.

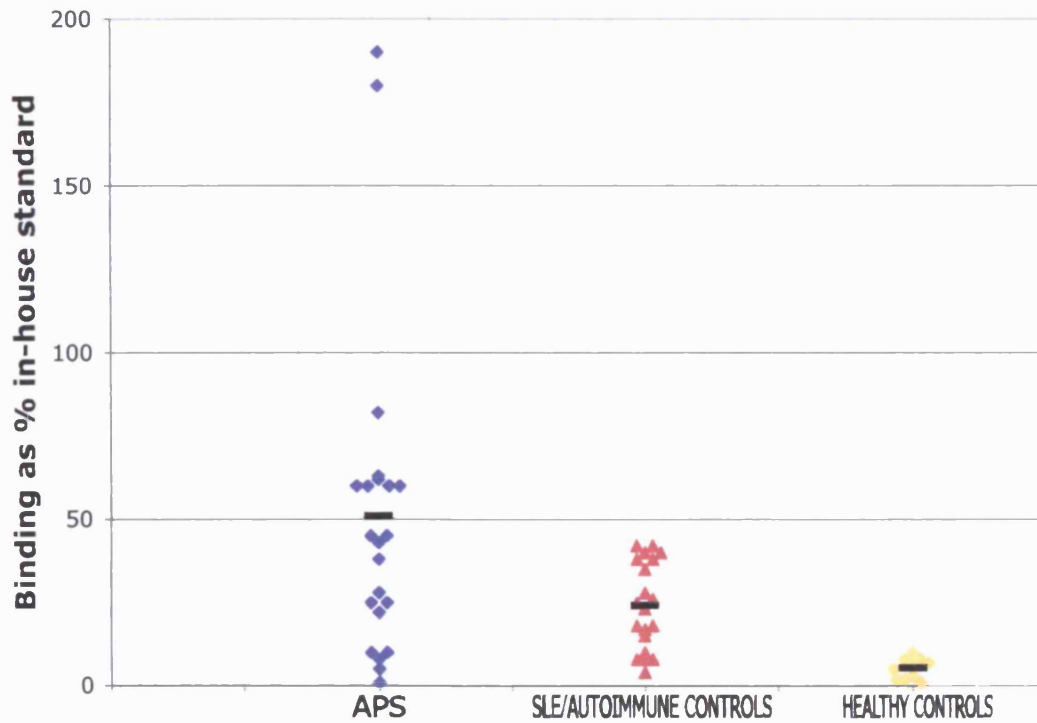


Figure 4.6 Results of polyclonal IgG samples binding to whole β_2 GPI coated on an irradiated plate. APS, n=22; SLE/Autoimmune controls, n=20; healthy controls, n=10. *APS versus SLE/Autoimmune controls, p=0.02; *APS versus healthy controls, p=0.007; **SLE/Autoimmune controls versus healthy controls, p=0.0001.

4.4 Recombinant DI binds polyclonal IgG derived from APS patients

Having established the binding characteristics of the polyclonal IgG samples to the standard antigens used in ELISAs in clinical practice, my aim was to ascertain whether these same antibody samples bind recombinant DI in a comparable pattern. Again an in-house standard from a patient with APS was identified which bound recombinant DI and this was used to calculate the binding activity of the other samples. Figure 4.8 demonstrates that the APS samples bound DI significantly better than the autoimmune disease control group ($p=0.002$) and the healthy control group ($p=0.007$). However, there was no significant difference between the two control groups studied ($p=0.24$). This is in contrast to the CL and whole β_2 GPI assays which did demonstrate a significantly greater difference between the autoimmune disease control and healthy control group (figures 4.5 and 4.6).

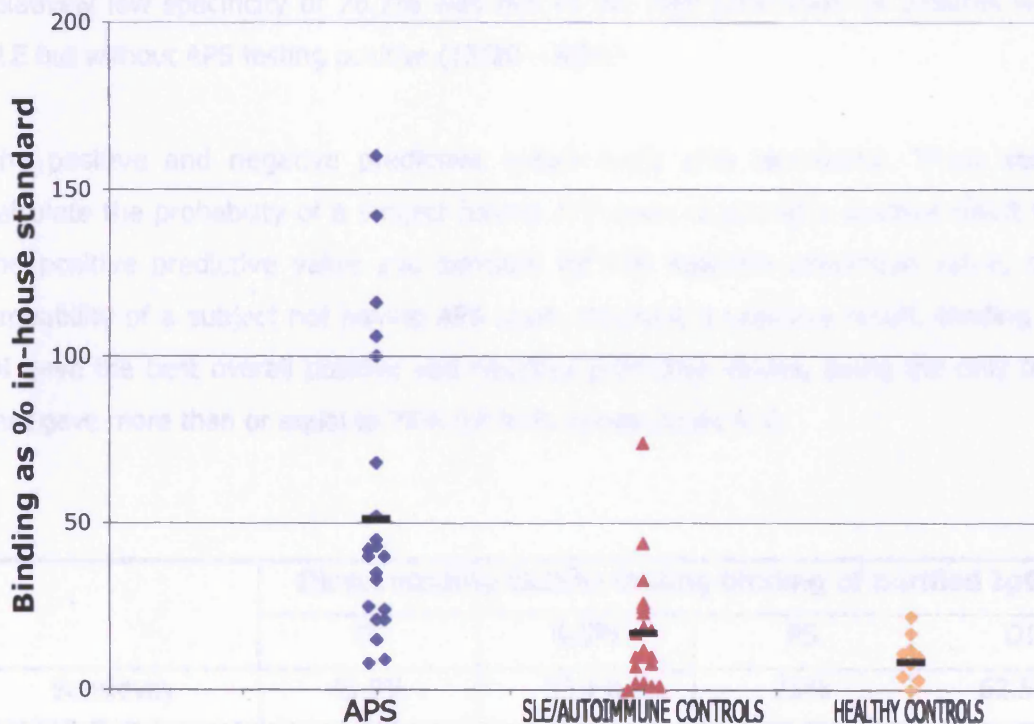


Figure 4.8 Results of polyclonal IgG binding recombinant DI expressed from *E.coli*. APS, $n=24$; SLE/Autoimmune controls, $n=20$; healthy controls, $n=9$. *APS versus SLE/Autoimmune controls, $p=0.002$; *APS versus healthy controls, $p=0.007$; SLE/Autoimmune controls versus healthy controls, $p=0.24$.

4.5 Comparative sensitivities and specificities for detecting clinically relevant IgG

If DI of β_2 GPI harbours the epitopes that the majority of pathogenic IgG aPL bind to, then one may expect a high degree of sensitivity and specificity in detecting clinically relevant IgG upon testing binding to this antigen. Inspecting the above data, it seems apparent that there is a large degree of variability between the sensitivity and specificity for each antigen test. Table 4.3 details the sensitivity and specificity of each antigen test in terms of detecting IgG derived from patients with APS. An arbitrary result of 30% binding of standard was used as a cut off above which a result would be defined as positive. The most sensitive test for detecting binding to clinically relevant purified IgG was binding to DI, with 62.5% sensitivity whilst also retaining high specificity of 90%. Binding to both CL and PS had marginally higher specificities of 96.7% but these tests were not as sensitive (45.5% and 25% respectively). Though binding to β_2 GPI had a moderate sensitivity of 59.1%, the relatively low specificity of 76.7% was due to the high proportion of patients with SLE but without APS testing positive (13/20 – 65%).

The positive and negative predictive values were also calculated. These tests calculate the probability of a subject having APS upon obtaining a positive result for the positive predictive value and similarly for the negative predictive value, the probability of a subject not having APS upon obtaining a negative result. Binding to DI gave the best overall positive and negative predictive values, being the only test that gave more than or equal to 75% for both values (table 4.3).

	Direct binding ELISAs testing binding of purified IgG to:			
	CL	β_2 GPI	PS	DI
Sensitivity	45.5%	59.1%	25%	62.5%
Specificity	96.7%	76.7%	96.7%	90%
+ve predictive value	91%	65%	86%	83.3%
-ve predictive value	70.7%	71.9%	61.7%	75%

Table 4.3 Comparative sensitivities and specificities for each antigen test.
Abbreviations: +ve, positive; -ve, negative; CL, cardiolipin; PS, phosphatidylserine.

4.6 Correlation analyses of binding studies between DI, cardiolipin and whole β_2 GPI

The aim of this section is to dissect the binding patterns of individual samples more thoroughly. It is of interest to ascertain whether the samples that bind well to recombinant DI are the same as those that also demonstrate good binding to CL or whole β_2 GPI. If there is good correlation between the different ELISA studies then one may hypothesise that the antibodies are binding the same or closely related epitopes within each antigen binding assay.

4.6.1 Binding of aPL to DI correlates well with binding to cardiolipin

Given the ubiquitous use of the CL assay in detecting aPL, correlation studies between DI and CL were felt to be of relevance as to ascertaining the significance of a positive DI binding test. As figure 4.9 demonstrates, there is a moderate to strong positive correlation between the aPL that bind CL with those that bind DI ($r=0.65$).

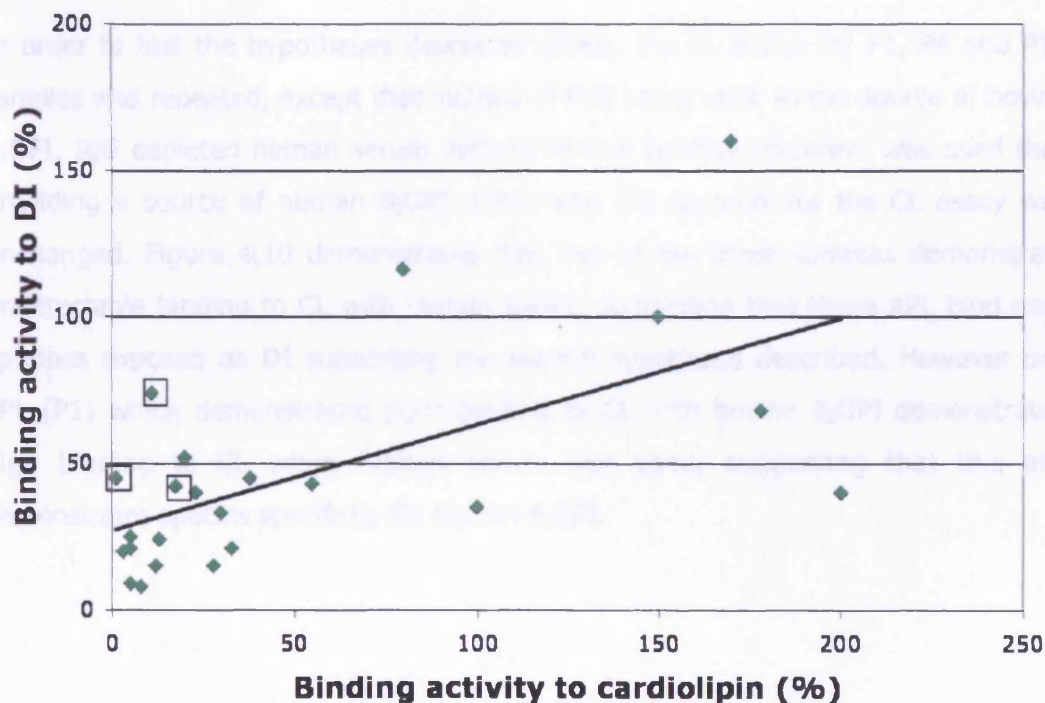


Figure 4.9 Correlation scatter plot of purified IgG patient samples binding DI against binding to cardiolipin. Linear regression line of best fit is shown in black. Boxed values are IgG samples that demonstrate >40% binding to DI but <20% binding to CL. Correlation coefficient $r=0.65$.

4.6.2 Identification of polyclonal aPL demonstrating species preference for β_2 GPI

Correlating binding of DI with binding to CL highlighted a small group of three aPL that bound DI well (more than 40%) but CL relatively poorly (less than 20%). These aPL samples have been highlighted in figure 4.9. Two of these samples were from PAPS patients (P1 and P4) and one from a patient that had SLE and no APS (P11). Given that the CL ELISA is performed using FCS as the source of bovine β_2 GPI, two different hypotheses were put forward that could explain the results described:

- The aPL P1, P4 and P11 may exhibit preferential recognition for human rather than bovine β_2 GPI and thus account for the poor binding to CL but relatively good binding to human recombinant DI.
- Recombinant DI alone may result in the exposure of different epitopes that are hidden when DI is attached to the rest of the β_2 GPI bound to CL.

In order to test the hypotheses described above, the CL ELISA for P1, P4 and P11 samples was repeated, except that instead of FCS being used as the source of bovine β_2 GPI, IgG depleted human serum derived from a healthy volunteer was used thus providing a source of human β_2 GPI. Otherwise the protocol for the CL assay was unchanged. Figure 4.10 demonstrates that two of the three samples demonstrate undetectable binding to CL with human β_2 GPI, suggesting that these aPL bind neo-epitopes exposed on DI supporting the second hypothesis described. However one aPL (P1) which demonstrated poor binding to CL with bovine β_2 GPI demonstrated high binding to CL when human serum was used, suggesting that this aPL demonstrates species specificity for human β_2 GPI.

4.5.3 Correlation of binding between CL and specific β_2 GPI

Intriguingly there was a weak, negative correlation between binding to CL and whole β_2 GPI as compared to CL (Figure 4.11). Two aPL were identified that demonstrated poor binding to whole β_2 GPI (less than 50%) but relatively good binding to CL (more than 40%) and these samples are highlighted in Figure 4.11.

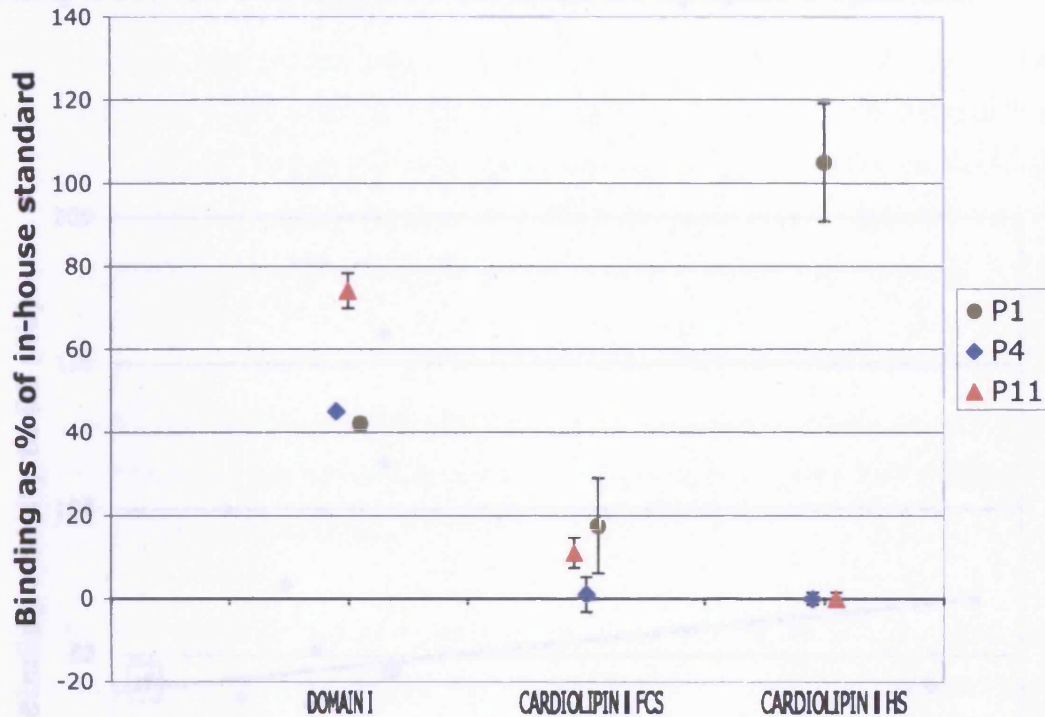


Figure 4.10 Identification of an aPL with species specificity for human β_2 GPI. Abbreviations: FCS, foetal calf serum; HS, IgG deplete human serum. P1 demonstrates preference for binding to CL in the presence of human serum. P4 and P11 exhibit poor binding to CL when both FCS and HS are used.

4.6.3 Correlation of binding between DI and whole β_2 GPI

Intriguingly there was a weaker positive correlation between binding to DI and whole β_2 GPI as compared to CL ($r=0.22$), figure 4.11. Two aPL were identified that demonstrated poor binding to whole β_2 GPI (less than 10%) but relatively good binding to DI (more than 40%) and these sample are highlighted in figure 4.11.

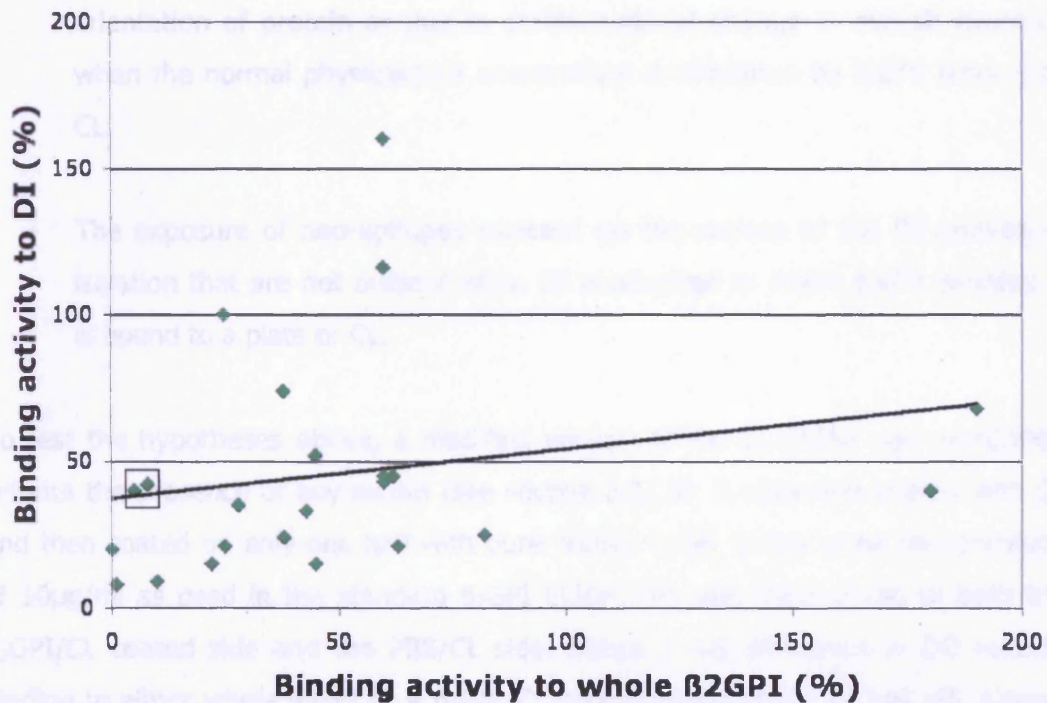


Figure 4.11 Correlation scatter plot of purified patient IgG samples binding DI against binding to whole β_2 GPI coated on an irradiated plate. Linear regression line of best fit is shown in black. Boxed values are IgG samples that demonstrate >40% binding to DI but <10% binding to whole β_2 GPI (P1 and P6). Correlation coefficient $r=0.22$.

4.7 Identification and characterisation of aPL that bind whole β_2 GPI when bound to phospholipid

One would expect all aPL that bound DI to also bind whole β_2 GPI. This apparent paradox may be explained by two possible hypotheses:

- That aPL may recognise epitopes exposed on recombinant DI that are hidden on whole β_2 GPI when stuck to an irradiated plate, due to the orientation of the protein. These epitopes then become exposed due to alteration in orientation of protein or due to conformational change in overall structure when the normal physiological environment is simulated by β_2 GPI binding to CL.
- The exposure of neo-epitopes exposed on the surface of the DI peptide in isolation that are not present when DI is attached to whole β_2 GPI whether it is bound to a plate or CL.

To test the hypotheses above, a modified version of the CL ELISA was performed without the presence of any serum (see section 2.21.5). A plate was coated with CL and then coated on only one half with pure human β_2 GPI at the same concentration of 10 μ g/ml as used in the standard β_2 GPI ELISA. aPL was then added to both the β_2 GPI/CL coated side and the PBS/CL side. Hence a net difference in OD reflects binding to either whole β_2 GPI or a β_2 GPI/CL complex only. Again an IgG aPL sample was identified which exhibited good binding to β_2 GPI within this modified β_2 GPI-CL ELISA and was used as an arbitrary in-house standard against which the binding activity of the other aPL samples was measured.

Figure 4.12 shows that this ELISA is a valid and relevant test for identifying pathogenic aPL. The majority of the aPL samples were tested and compared against the binding of IgG derived from the two control groups. In line with the standard CL, whole β_2 GPI and DI ELISAs, there was a significant difference between binding of IgG from APS patients and autoimmune disease controls ($p=0.003$) and APS and healthy controls ($p=0.018$). However, unlike the standard CL and whole β_2 GPI ELISA, there was no significant difference between the two control groups ($p=0.63$) in line with the DI ELISA. In fact there was undetectable binding in both control groups which underlines the low background of this assay. In line with previous antigen

tests determining the reliability of defining clinically relevant IgG (table 4.3), an arbitrary cut-off of 30% binding of standard was defined as a positive result. Using this the sensitivity of this assay was 50%, specificity 100%, positive predictive value 100% and negative predictive value 74.3%. Figure 4.13 shows that there is a much stronger correlation between this more physiological β_2 GPI/CL ELISA and DI ($r=0.47$) as compared to the standard β_2 GPI ELISA and DI (figure 4.11, $r=0.22$).

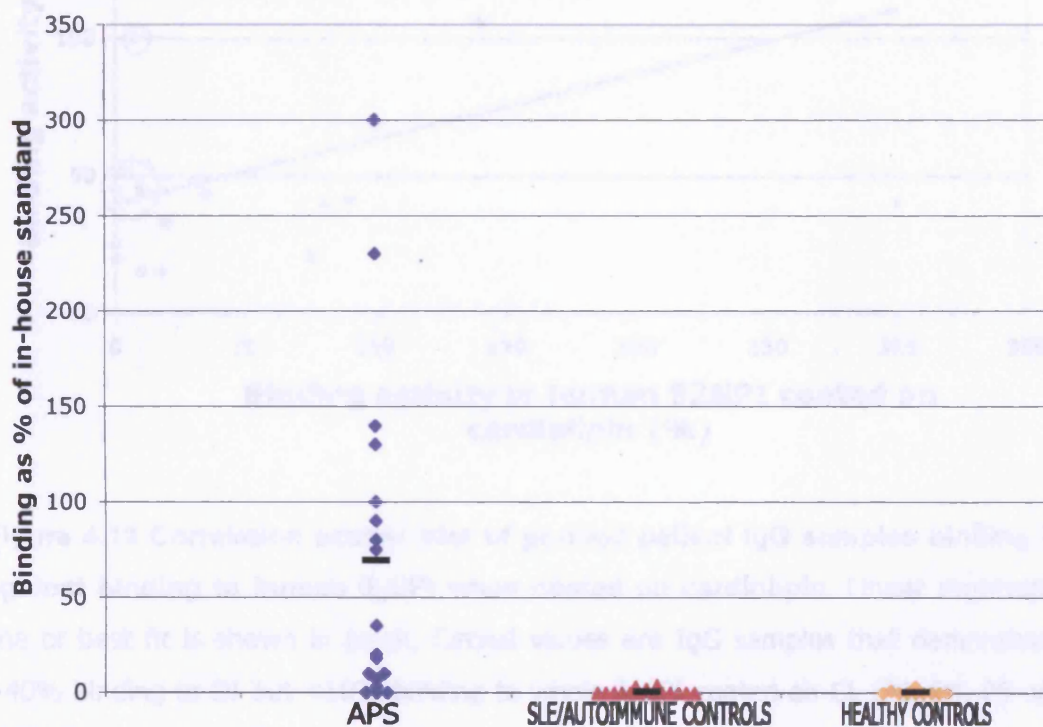


Figure 4.12 Results of polyclonal IgG binding human β_2 GPI coated on cardiolipin. APS, n=18; SLE/Autoimmune controls, n=16; healthy controls, n=10. **APS versus SLE/Autoimmune controls, $p=0.003$; *APS versus healthy controls, $p=0.018$; SLE/Autoimmune controls versus healthy controls, $p=0.63$.

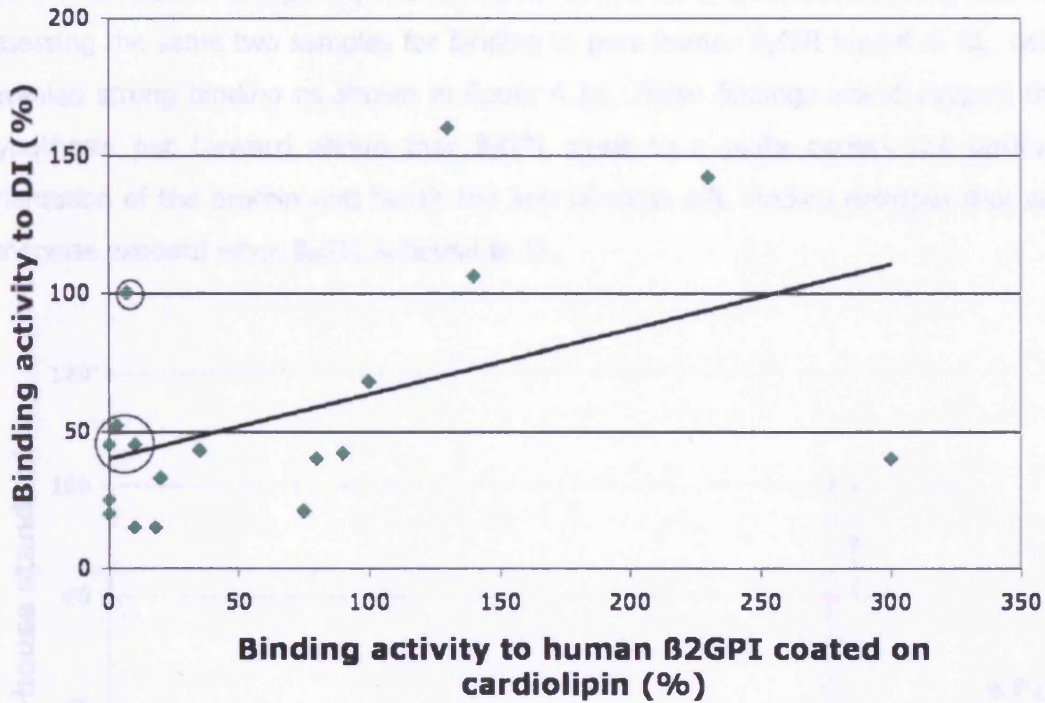


Figure 4.13 Correlation scatter plot of purified patient IgG samples binding DI against binding to human β_2 GPI when coated on cardiolipin. Linear regression line of best fit is shown in black. Circled values are IgG samples that demonstrate >40% binding to DI but <10% binding to whole β_2 GPI coated on CL (P2, P4, P5 and P10). Correlation coefficient $r=0.47$.

4.7.1 Identification of aPL that preferentially bind β_2 GPI bound to cardiolipin as opposed to an irradiated plate

As illustrated in figure 4.11, two IgG samples demonstrated poor binding to whole β_2 GPI when bound to a plate (P1 and P6) but bound DI in isolation relatively well. On assessing the same two samples for binding to pure human β_2 GPI bound to CL, both revealed strong binding as shown in figure 4.14. These findings would support the hypothesis put forward above that β_2 GPI stuck to a plate causes sub-optimal orientation of the protein and hence the loss of some aPL binding epitopes that are otherwise exposed when β_2 GPI is bound to CL.

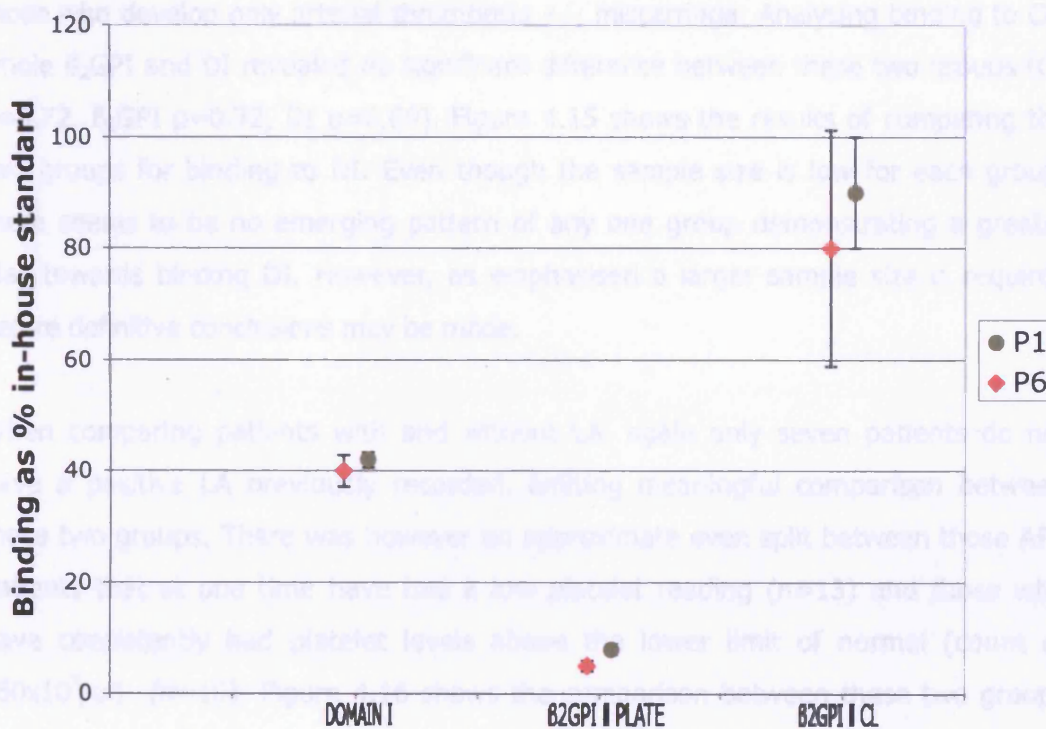


Figure 4.14 Identification of aPL that binds recombinant DI, β_2 GPI bound to cardiolipin but not β_2 GPI bound to a plate. Abbreviations: β_2 GPI-PLATE, β_2 GPI bound to an irradiated plate; β_2 GPI-CL, β_2 GPI coated on a CL surface.

4.8 Subset analyses of aPL samples

For meaningful subset analyses ideally large numbers of patients are required, unless the difference between the groups being analysed are marked. This limits the degree of subset analysis that may be possible in this study given the sample size of 24.

One possible method of stratifying different groups is to split them into those with a history of miscarriage and those who only present with thrombosis. However, once males and nulligravida women are excluded, there is only one female with a history of thrombosis and no miscarriage making this comparison unfeasible. Another option is to compare those presenting with only venous thrombosis +/- miscarriage against those who develop only arterial thrombosis +/- miscarriage. Analysing binding to CL, whole β_2 GPI and DI revealed no significant difference between these two groups (CL $p=0.72$, β_2 GPI $p=0.72$, DI $p=0.69$). Figure 4.15 shows the results of comparing the two groups for binding to DI. Even though the sample size is low for each group, there seems to be no emerging pattern of any one group demonstrating a greater bias towards binding DI. However, as emphasised a larger sample size is required before definitive conclusions may be made.

When comparing patients with and without LA, again only seven patients do not have a positive LA previously recorded, limiting meaningful comparison between these two groups. There was however an approximate even split between those APS patients that at one time have had a low platelet reading ($n=13$) and those who have consistently had platelet levels above the lower limit of normal (count of $150 \times 10^3/\mu\text{l}$) ($n=10$). Figure 4.16 shows the comparison between these two groups and binding to DI. There is no discernible difference between the two ($p=0.59$) with similar results attained when comparing the two groups for binding to CL ($p=0.38$) and whole β_2 GPI ($p=0.87$).

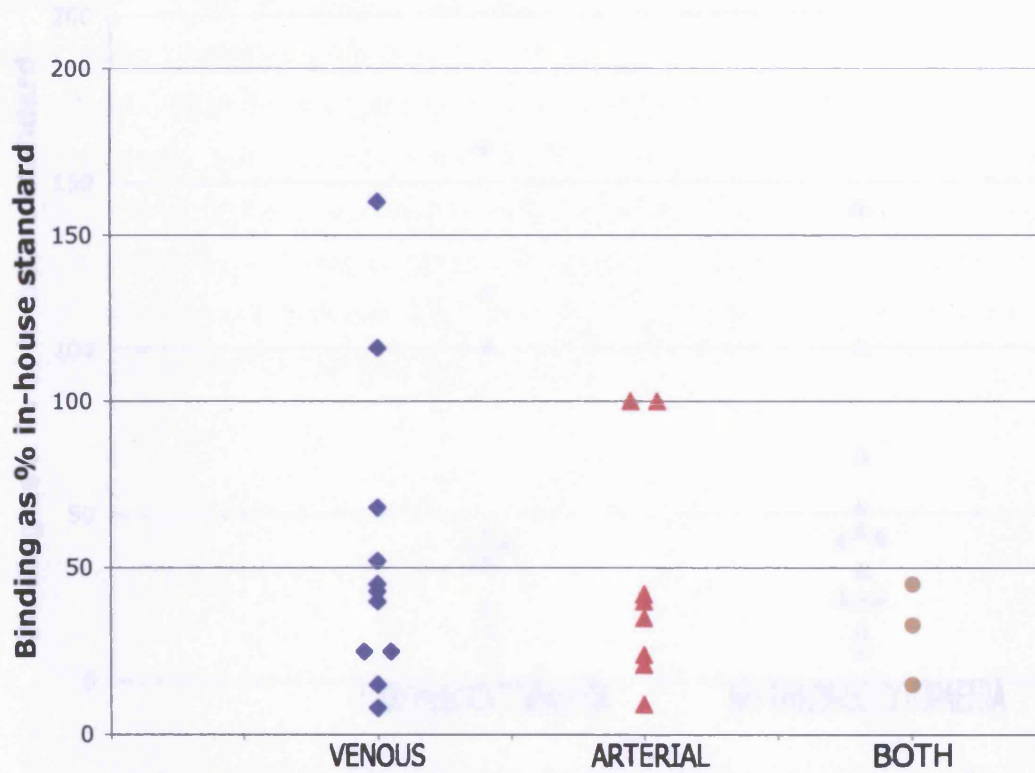


Figure 4.15 Comparison of DI binding of aPL derived from patients with venous thrombosis only versus those with arterial thrombosis. Each group included patients with miscarriage. No significant difference between the venous and arterial thrombosis groups was found ($p=0.69$).

Figure 4.15 Comparison of DI binding of aPL derived from patients with venous thrombosis only versus those with arterial thrombosis. Each group included patients with miscarriage. No significant difference between the venous and arterial thrombosis groups was found ($p=0.69$).

4.9 Binding of recombinant DI to polyclonal aPL samples in the fluid phase

The ability of DI to inhibit binding of polyclonal IgG derived from APS patients to human β_2 GPI was also tested. Eight samples were chosen that had been shown to exhibit strong binding to β_2 GPI coated DI as well as DI in the solid phase. As shown in Figure 4.17, DI in the fluid phase was able to inhibit binding of these IgG samples to human β_2 GPI coated on a CL surface. Data presented in the fluid phase chapter (Chapter 6) demonstrates that some of the single point assays of DI were found to exhibit an inhibition to these IgG samples in the fluid phase and hence a further experiment was set up to measure the effect of increasing the concentration of DI was 02.9% (+0.23.7%).

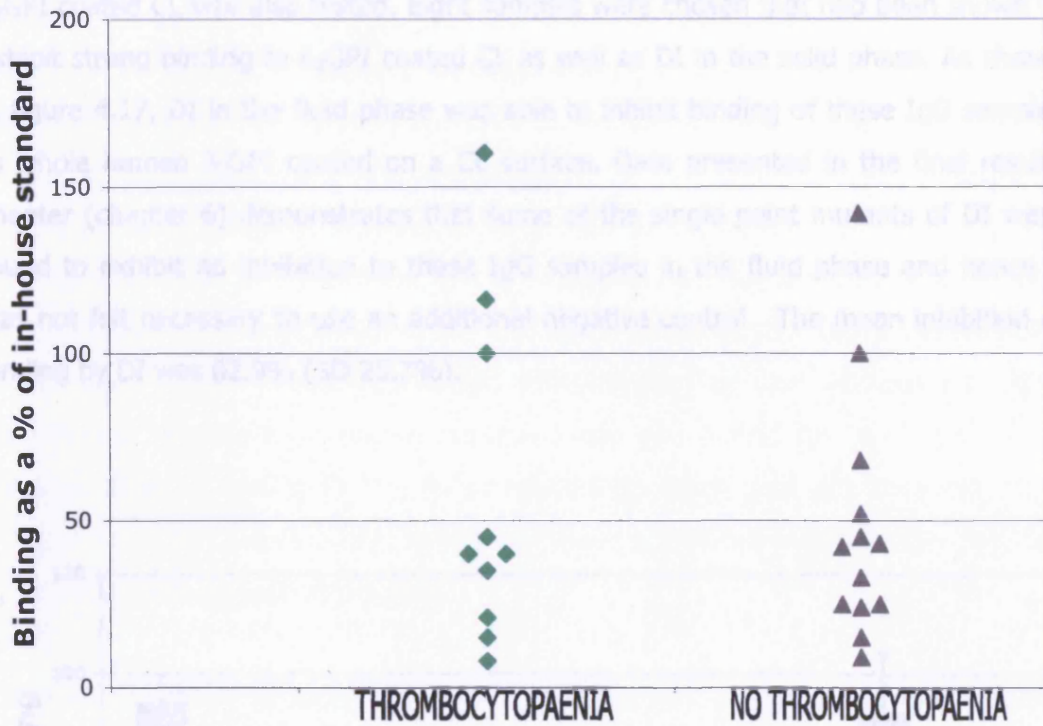


Figure 4.16 Comparison of DI binding of aPL derived from patients with and without thrombocytopenia. No discernable difference between the two groups was found with binding to DI ($p=0.59$).

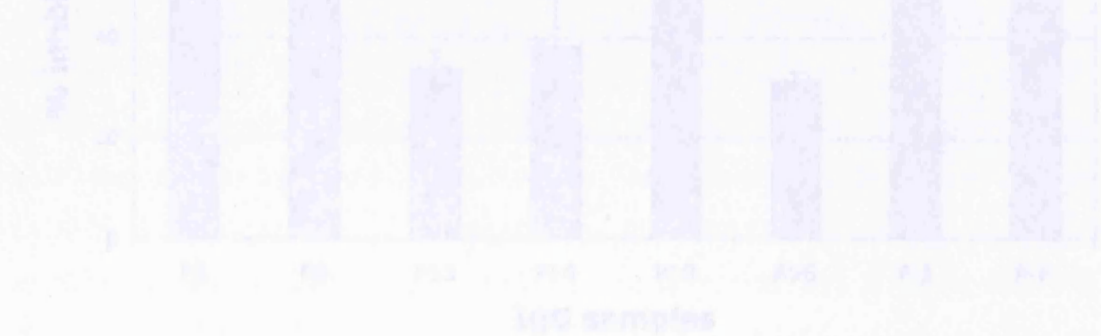


Figure 4.17 Inhibition of IgG binding to β_2 GPI-coated plates in the fluid phase by DI. DI used at 0.237% concentration was able to inhibit binding of 8 IgG samples to human β_2 GPI coated on a CL surface (mean \pm SD) 02.9% \pm 23.7%.

4.9 Binding of recombinant DI to polyclonal aPL samples in the fluid phase

The ability of DI to inhibit binding of polyclonal IgG derived from APS patients to β_2 GPI coated CL was also tested. Eight samples were chosen that had been shown to exhibit strong binding to β_2 GPI coated CL as well as DI in the solid phase. As shown in figure 4.17, DI in the fluid phase was able to inhibit binding of these IgG samples to whole human β_2 GPI coated on a CL surface. Data presented in the final results chapter (chapter 6) demonstrates that some of the single point mutants of DI were found to exhibit no inhibition to these IgG samples in the fluid phase and hence it was not felt necessary to use an additional negative control. The mean inhibition of binding by DI was 62.9% (SD 25.7%).

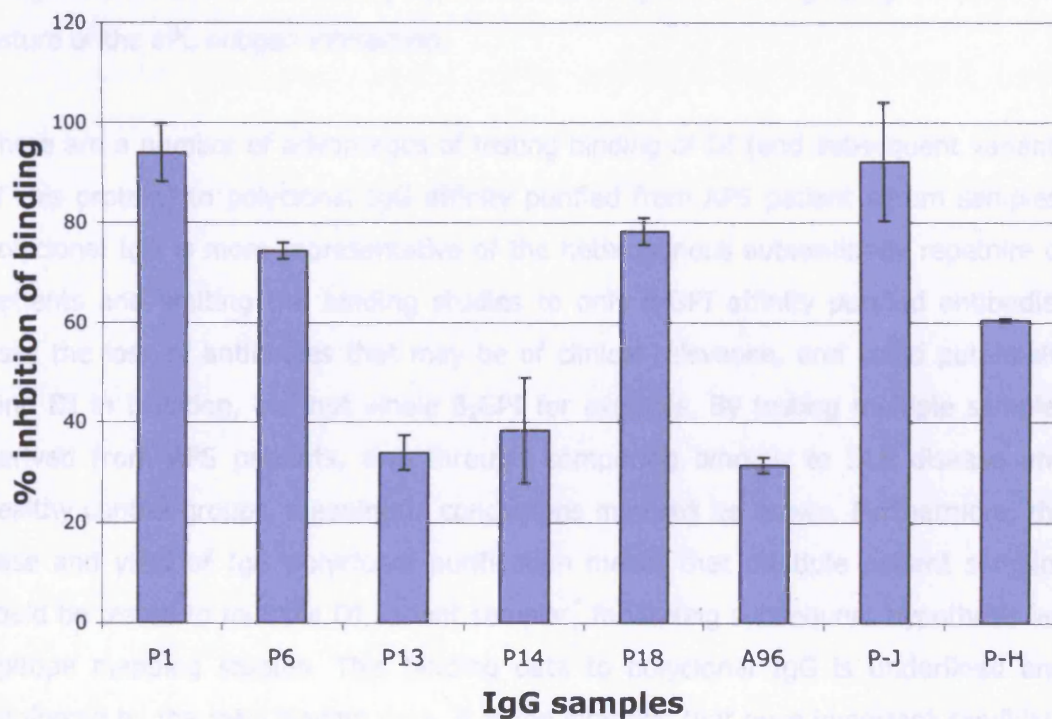


Figure 4.17 Inhibition of IgG binding β_2 GPI/cardiophilin in the fluid phase by DI. DI used at 3.25 μ M concentration was able to inhibit binding of 8 IgG samples to human β_2 GPI coated on a CL surface (mean +/- SD) 62.9% +/- 25.7%.

4.10 Discussion of results

The results presented in this section demonstrate that recombinant DI, produced by *E.coli* utilising the expression platform developed, binds clinically relevant monoclonal and polyclonal aPL derived from patients with APS. This supports the theory that DI of β_2 GPI harbours the epitopes that are bound by pathogenic aPL derived from patients with APS and furthermore validates analysis of IgG binding to DI in isolation. This was an essential pre-requisite prior to embarking upon multiple mutational studies of DI. Furthermore, the aPL tested were fully characterised in assays of binding to other antigens relevant to APS such as CL, whole β_2 GPI and PS. The binding properties of monoclonal aPL and the multiple monoclonal variants have already been fully characterised in our unit by Dr Ian Giles and Nancy Lambrianides (Giles et al., 2005). The polyclonal IgG samples, purified from patients with APS, autoimmune disease and healthy controls were also tested for binding to these antigens prior to testing to DI. By comparing DI binding of aPL to these other antigens such as CL and β_2 GPI, hypotheses were generated regarding the potential nature of the aPL-antigen interaction.

There are a number of advantages of testing binding of DI (and subsequent variants of this protein) to polyclonal IgG affinity purified from APS patient serum samples. Polyclonal IgG is more representative of the heterogenous autoantibody repertoire of patients and limiting the binding studies to only β_2 GPI affinity purified antibodies risks the loss of antibodies that may be of clinical relevance, and could potentially bind DI in isolation, but not whole β_2 GPI for example. By testing multiple samples derived from APS patients, and through comparing binding to SLE disease and healthy control groups, meaningful conclusions may still be drawn. Furthermore, the ease and yield of IgG polyclonal purification meant that multiple patient samples could be tested to multiple DI variant samples, facilitating subsequent hypothesis led epitope mapping studies. This binding data to polyclonal IgG is underlined and reinforced by the mAb binding data. It is the intention that once important candidate epitopes are identified, further binding studies to whole β_2 GPI and DI affinity purified antibodies will be undertaken.

Statistical comparisons between the different clinical groups were initially made using a paired t-test. This makes the assumption that the binding data is normally distributed. The data was also re-analysed using the non-parametric Mann-Whitney U test and this data is presented in the subsequent published paper presenting the

bulk of binding data presented in this thesis (Chapters 4 and 6; Ioannou et al., 2007 – appendix II for reference). For all group comparisons, statistical significance (or the lack of it) remained unchanged when both parametric and non-parametric statistical tests were used, thus not altering the ultimate conclusions drawn.

IS4, derived from a patient with APS, is a relevant monoclonal aPL to study given its proven thrombogenic pathogenicity in animal studies (Pierangeli et al., 2000). IS4 has Arg residues in the heavy chain CDR that have probably arisen through somatic mutation (Giles et al., 2003b). When these residues are mutated to neutral serine the result is loss of binding to whole β_2 GPI and CL (Giles et al., 2005). These Arg residues also seem to be crucial in conferring binding to DI, consistent with the hypothesis that these antibodies bind to similar epitopes on DI in isolation, DI when attached to the rest of β_2 GPI and within the standard CL assay.

Monoclonal aPL are derived from single B cell clones from a single patient. APS is characterised by the presence of heterogeneous aPL derived from patients with varying clinical phenotypes, a third of whom have SLE (Cervera et al., 2002). Would similar results be obtained by testing polyclonal aPL derived from multiple patients with APS? Also would DI in isolation open up neo-epitopes that could be recognised by non-pathogenic control IgG derived from patients with autoimmune disease other than APS or even by healthy volunteers? To answer these questions it was imperative that polyclonal IgG derived from multiple patients with APS and controls was tested. When testing the purified aPL for binding to CL and whole β_2 GPI using the standard ELISAs, as expected greatest binding was observed in the APS group, significantly greater than both the disease and healthy control group. However, there was also a significant difference (though not as marked) between the autoimmune disease control group and the healthy controls. This was not unexpected with the CL ELISA as it is known that around 30% of patients with SLE can develop aCL but only about half go on to develop APS (Petri, 2000). However, the observation that there may be a group of anti- β_2 GPI antibodies that may have a poor association with pathogenicity is a relatively new finding. Binding to DI predicted pathogenicity at least as well as CL and whole β_2 GPI. However, uniquely there was no significant difference between the SLE/autoimmune disease and healthy controls. This binding data, from a limited set of samples, would thus suggest that binding to DI of β_2 GPI in isolation is a better predictor of pathogenicity as compared to binding to whole β_2 GPI bound to a plate. It may be that β_2 GPI bound to a plate exposes domains

other than DI that are recognised by non-pathogenic aPL. This hypothesis is further supported by a recent report from Bas de Laat et al (de Laat et al., 2005). In this study 52 samples of anti- β_2 GPI antibodies have been characterised into two groups. One is termed Type A and bind DI only. The other group of aPL is type B and binds domains I to V as assessed by testing binding to a combination of domain deletion mutants. Types A antibodies have LA activity and are associated with thrombosis and type B antibodies have no LA activity and no association with thrombosis. However in this study all patients investigated had an autoimmune disease other than APS. The data reported in this section are more representative of the APS population with only 30% having SLE and the rest being patients with APS as their only autoimmune disease.

Correlation analyses between different antigens using different ELISA techniques need to be interpreted with caution. However I felt it important to perform these correlation studies as some insight into the potential nature of the interactions between DI, CL and human β_2 GPI bound to a plate with aPL may be gained. Binding of APS derived IgG to DI correlates well with binding to CL, as one would expect given that the CL ELISA is a measure not only of aPL binding to CL, but also potentially of bovine β_2 GPI binding. However, there was a small group of three aPL samples that bound DI well but CL relatively poorly. One aPL sample, previously demonstrating poor binding to CL with bovine serum, became strongly positive when human serum was used instead in the CL ELISA, suggesting that this aPL binds species specific epitopes on β_2 GPI present on the human protein but absent on bovine β_2 GPI. However, the other two aPL that bound DI well still bound CL poorly even when human serum was used. This highlights the heterogeneous nature of aPL and suggests that these aPL recognise epitopes on DI that are hidden on human β_2 GPI when bound to CL. Our unit has recently developed a monoclonal anti-dsDNA antibody with a single point mutation in the heavy chain resulting in loss of activity to dsDNA, but the emergence of binding to human β_2 GPI and to recombinant human DI but not to bovine whole β_2 GPI (Lambrianides et al – Appendix B). The sequence of bovine β_2 GPI has 89% homology to human β_2 GPI (Gao et al., 1993). In total nine amino acids in the sequence of human DI differ from bovine DI. The amino acids are {human \rightarrow Bovine}: {Asp 9 \rightarrow Gln 9}; {Thr 20 \rightarrow Arg 20}; {Glu 27 \rightarrow Gln 27}; {Thr 29 \rightarrow Val 29}; {Tyr 30 \rightarrow Phe 30}; {Lys 33 \rightarrow Gln 33}; {Ile 42 \rightarrow Met 42}; {Lys 44 \rightarrow Arg 44}; {Ile 46 \rightarrow Thr 46}. It is very likely that one or more of these epitopes are important in conferring binding to DI for the species specific aPL sample P1a.

The correlation between the binding of APS derived IgG to DI and binding to human β_2 GPI coated on a plate was not as strong as that between DI and CL. This poor correlation was primarily due to a small group of aPL that were poor binders to whole β_2 GPI immobilised on a plate but relatively good DI binders. This phenomenon may also be explained by the fact that DI not attached to the rest of β_2 GPI may present neo-epitopes. However, given that whole β_2 GPI is bound to a plate, one other possibility is that β_2 GPI may undergo a conformational change when bound to anionic PL as in the CL assay, and reveal aPL binding epitopes on DI which are also exposed when DI is present in isolation. When β_2 GPI is bound to CL, the previously poor β_2 GPI binders became good binders. Conversely, non-pathogenic samples from the autoimmune disease control group that bound β_2 GPI immobilised on a plate did not bind β_2 GPI immobilised on CL. Data presented in this section from the limited number of samples studied suggests that assessing binding of aPL to pure human β_2 GPI bound to CL is a far better predictor of pathogenicity than any of the other standard ELISAs tested and this also correlates well with binding to DI. However, this ELISA can only detect binding to IgG aPL purified from serum (thus free of native β_2 GPI) and so is not practical for use as a clinical assay. There has been considerable debate in the literature as to whether the epitopes on β_2 GPI that aPL recognise are cryptic or not and this has been reviewed by Giles et al (Giles et al., 2003c). The suggestion that pathogenic β_2 GPI antibodies recognise DI of β_2 GPI only after conformational change is supported by a further recent study by Bas de Laat et al (de Laat et al., 2006b). In this study pathogenic aPL as opposed to non-pathogenic aPL bound β_2 GPI only if coated on to a negatively charged hydrophilic plate and not when coated on a neutral plate or in the fluid phase. Furthermore on removing the carbohydrate chains of β_2 GPI, these pathogenic aPL were then able to bind the non-glycosylated β_2 GPI in the fluid phase. The suggestion is that large carbohydrate chains from other domains extend up and cover important aPL binding epitopes on DI. When β_2 GPI is then anchored to a negatively charged surface the sugar molecules flip down and expose these epitopes. If this is true it may explain the high sensitivity of the DI ELISA, given that *E.coli* expressed DI is not glycosylated. These important studies however did not investigate aPL from PAPS patients nor did they attempt to mimic the physiological *in vivo* environment more closely *in vitro* by assessing binding to pure β_2 GPI anchored to an anionic PL surface such as CL as I have done.

Finally, I have demonstrated that DI binds to aPL in the fluid phase, inhibiting the binding of aPL to β_2 GPI immobilised to CL. This was important to demonstrate as it shows that the aPL binding epitopes exposed when DI is anchored to a nickel plate are also exposed with DI in the fluid phase. Numerous other studies have also demonstrated the ability of DI to bind aPL in the fluid phase (Iverson et al., 1998b, Iverson et al., 2002b, de Laat et al., 2005). Generally these studies have demonstrated the ability of DI to inhibit aPL binding to whole β_2 GPI immobilised on plastic, but none have demonstrated the ability of DI to inhibit aPL binding to pure β_2 GPI immobilised on to CL.

To conclude, the results described in this section confirm that recombinant DI produced by this *E.coli* expression system binds clinically relevant aPL derived from APS patients. These data also reinforce the emerging evidence favouring DI as the dominant domain for harbouring important immunodominant aPL binding epitopes. Comparing the binding patterns of aPL to CL using either bovine or human serum as a source of β_2 GPI, β_2 GPI immobilised on to a plate and to β_2 GPI immobilised on CL has highlighted the heterogeneous nature of these antibodies. Broadly the conclusions are that it is likely that aPL binding epitopes on DI in isolation are relevant to pathogenicity, and that these epitopes on the whole β_2 GPI protein are best exposed when this protein is bound to CL. The importance of aPL binding epitopes exposed on DI is reinforced by the ability of DI in the fluid phase to inhibit aPL binding. The ability of a peptide to inhibit pathogenic aPL from binding native antigen may be of potential therapeutic value. However, in preference to a 7kDa DI or related peptide of a similar size being given therapeutically, a smaller peptide analogue modelled on the fine immunodominant epitopes exposed on the surface of DI or even a small molecule mimetic of this peptide analogue would be preferable for immunogenic and pharmacokinetic reasons (Tang et al., 2004, Gadek and McDowell, 2003). In order to ascertain the precise location of these epitopes mutational studies of DI need to be undertaken. Though there have previously been some reports of mutational studies within DI in the context of whole β_2 GPI (Iverson et al., 2002b, de Laat et al., 2005), there have been no published reports of the effects of mutations within DI in isolation.

The following section will describe the application of the *E.coli* expression system to produce multiple mutants of DI designed to test specific hypotheses. These hypotheses were generated from computer modelling studies of DI, previous

mutational studies within DI of whole β_2 GPI (Iverson et al., 2002b, de Laat et al., 2005) and work from this unit on mutational studies of monoclonal aPL expressed in mammalian expression systems (Giles et al., 2005).

CHAPTER 5

DESIGN AND EXPRESSION OF MULTIPLE RECOMBINANT MUTANTS OF DI

OVERVIEW OF CHAPTER 5

Work has been presented demonstrating both the development of an efficient expression system for DI in *E.coli* and the binding properties of this recombinant protein. This platform of DI production was then used to create multiple mutants of DI.

This chapter will describe the production of 15 of these mutants. The initial focus is on describing the rationale behind choosing which specific mutations to incorporate, a process facilitated by protein computer modelling studies. Evidence for the production of the mutant constructs is shown with sequencing plots confirming the correct mutation for each construct. Finally, results for the expression and purification of these mutants are demonstrated.

The majority of mutants expressed well, whilst a few had relatively low yields of purified target protein. Results of some experiments are shown which suggests potential explanations for the differences in yields between some mutants and these potential reasons are discussed.

5.1 Introduction: rationale for choice of regions to target

Each specific mutation chosen was designed to test a hypothesis. Results have been presented that demonstrate the importance of Arg residues, exposed on the surface of antigen binding regions of monoclonal aPL, in conferring binding not only to CL (Giles et al., 2005) and to whole β_2 GPI, but also to DI alone. It is possible that these positively charged residues on aPL are interacting with five negatively charged Asp and Glu residues clustered and exposed on the surface of DI. Hence these five residues were targeted. Computer modelling studies were used to predict which amino acid replacements at those positions would result in least overall conformational change of protein. Further evidence supporting the importance of two of these residues (Asp 8+9) comes from a study by Vlachoyiannopoulos et al (Vlachoyiannopoulos et al., 2004). An eight amino acid peptide derived from the region 239-245 of CD40 with 89% sequence homology to the 7-13 N-terminal region of DI has been shown to bind anti- β_2 GPI antibodies derived from patients with APS. This peptide harbours the neighbouring Asp 8 and 9 residues.

The second area to target was the region Arg at position 39 through to Arg at position 43. Iverson et al has reported that the G40E and R43S mutation in the context of whole β_2 GPI resulted in marked reduction in binding to the aPL tested (Iverson et al., 2002b). I aimed to introduce the same mutations into DI alone and also test the hypothesis that the effects on binding may in part be due to the fact that these residues lie adjacent to Arg 39, which has over 60% of its surface area exposed for potential antibody interaction. Finally I also chose to express DI with the whole DI-II interlinker region included. This was to test the hypothesis that this region may be important in conferring binding given its marked homology with the DIII-IV interlinker region (Steinkasserer et al., 1991) and the findings from some groups which suggest that important immunodominant aPL binding epitopes lie in DIV (George et al., 1998d, Igarashi et al., 1996). Another reason for targeting this region was that a peptide with homology to the DI-II interlinker region has been shown to bind aPL and inhibit aPL induced thrombosis in mice (Pierangeli et al., 2004a). The aim was to create mutations that would have the greatest effects on binding. Whilst some were expected to demonstrate reduced binding to aPL, it was anticipated that others might show enhanced binding compared to wild-type DI.

5.2 Design and production of DI mutant constructs

5.2.1 Computer modelling used to design nature of single / multiple point substitution mutations

A computer modelling programme, Swiss-Model (<http://swissmodel.expasy.org>), was used to build three-dimensional templates of his₆-tagged DI of human β_2 GPI. Swiss-Model has been validated as an accurate tool to predict protein structure (Peitsch, 1996, Guex and Peitsch, 1997) and furthermore the predicted structure of wild-type DI by this software is identical to the known crystal structure of this protein (Bouma et al., 1999, Schwarzenbacher et al., 1999). An example of a template demonstrating the location and surface exposure of negatively charged residues within a DI protein, tagged at the C-terminal with his₆, is shown in figure 5.1. Models of mutant DI were then made in order to deduce the effects of altering the targeted residues on H-bond / salt-bridge formation with neighbouring amino acids and the degree of surface exposure. Though it was anticipated that incorporating a mutation would inevitably cause some alteration in overall protein conformation as compared to wild-type, this software was used to help identify which mutation would cause least change. For example, as predicted by Swiss-Model, Glu 23 has 20% surface accessibility and forms H-bonds with Arg 2, Gly 25 and a double H-bond with Glu 26. The aim was design a mutation such that the negative charge at position 23 was lost while minimising the disruption in overall protein conformation. According to the model created by this software, mutating Glu 23 to Ser 23 would result in no change in surface accessibility of that residue but the H-bond with Arg 2 would be lost. This implies that the conformation is altered such that the residue at position 23 is no longer in close approximation with Arg 2 as seen in the wild-type protein. However, mutating Glu 23 to Gly 23 results in loss of all four H-bonds with no change in surface accessibility. Mutating to Ala 23 results in only loss of only one H-bond with Arg 2 as seen with Ser 23, but a reduction in surface accessibility from 20% to 10%. Hence the E23S mutation seemed the most appropriate. This systematic approach was made to all residues targeted for mutation and the results are shown in table 5.1. Regarding residues Gly 40 and Arg 43, the mutations were chosen on the basis that these were the mutations that Iverson et al (Iverson et al., 2002b) had incorporated in the whole β_2 GPI protein. The effects on conformation were nevertheless analysed and the results shown in table 5.2.

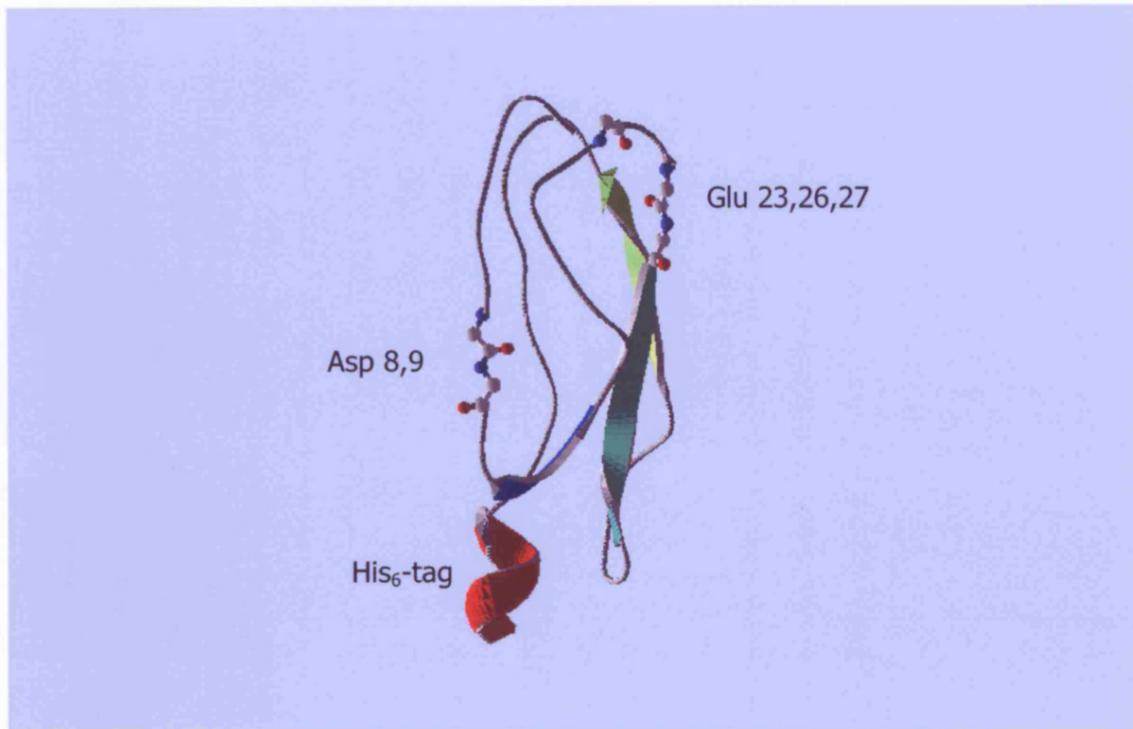


Figure 5.1 Ribbon structure of his₆-tagged DI of β_2 GPI. Molecular conformation of protein structure shows that Asp and Glu amino acid residues are located on surface exposed areas of the protein. Swiss-Model was used to create this model (Peitsch, 1996, Guex and Peitsch, 1997).

Mutation	Resultant loss of H-bond or salt-bridge with:	Resultant new H-bonds or salt-bridge with:	H-bond or salt-bridge remaining unchanged	Change in % amino acid surface area exposure from wild-type → to mutant
D8S	None	None	None	50% → 40%
D9G	Ser 13	None	None	30% → 25%
Mut 8+9	Asp 9 with Ser 13	None	None	D 8=50% → 45% D 9=30% → 30%
E23S	Arg 2	None	Gly 25, Glu 26(2x)	20% → 25%
E26S	Lys 19	None	Glu 23 (2x), Cys 47	15% → 15%
E27S	Thr 29, Lys 44 (2x)	None	None	20% → 25%
Mut 23-26-27	Ser 23 with Arg 2 Ser 26 with Lys 19 Ser 27 with: Thr 29, Lys 44 (x2)	None	Ser 23 with Ser 26 (x2) Ser 23 with Gly 25 Ser 26 with Cys 47	Glu 23= 20% → 25% Glu 26= 15% → 15% Glu 27= 20% → 25%
Mut 8-9-23-26-27	Asp 9 with Ser 13 Ser 23 with Arg 2 Ser 26 with Lys 19 Ser 27 with: Thr 29, Lys 44 (x2)	None	Ser 23 with Ser 26 (x2) Ser 23 with Gly 25 Ser 26 with Cys 47	Asp 8=50% → 40% Asp 9=30% → 25% Glu 23=20% → 25% Glu 26=15% → 15% Glu 27=20% → 25%

Table 5.1 Results of computer modelling studies targeting negatively charged amino acids. Abbreviations for multiple point mutations: Mut 8+9, D8-D9 mutated to S8-G9; Mut 23-26-27, E23-E26-E27 mutated to S23-S26-S27; Mut 8-9-23-26-27, D8-D9-E23-E26-E27 mutated to S8-G9-E23-S26-S27.

Mutation	Resultant loss of H-bond or salt-bridge with:	Resultant new H-bonds or salt-bridge with:	H-bond or salt-bridge remaining unchanged	Change in % amino acid surface area exposure from wild-type → to mutant
R39K	None	None	Arg 43	60% → 100%
R39S	None	None	Arg 43	60% → 40%
G40E	None	Gly 41	Ser 38	25% → 15%
G40S	None	None	Ser 38	25% → 20%
R43G	Arg 39, Thr 57	None	Tyr 30, Gly 41	15% → 10%
R43S	Arg 39, Gly 41, Thr 57	Gly 46	Tyr 30	15% → 20%

Table 5.2. Results of computer modelling studies targeting region Arg 39 to Arg 43.

5.2.2 Design of DI interlinker variants

The aim was to produce DI variants designed to test the hypothesis that the DI-II interlinker region was important in conferring binding to aPL. To test this hypothesis a variant of DI was designed with the remainder of the interlinker region attached on the C-terminal. This constitutes the addition of another five amino acids and the aim of this variant was to assess if the inclusion of the whole interlinker region enhances the binding properties to aPL as compared to DI expressed with only half the interlinker included. Another mutant of DI was designed substituting the DII-III interlinker region for the DI-II interlinker region hypothesising that this would lead to reduced binding. The amino acid sequences of the C-terminal portion of these recombinant DI variants are shown in table 5.3. Computer modelling studies of the interlinker region were undertaken to assess the degree of surface accessibility of each amino and to identify the H-bonds formed between the amino acids in the interlinker region and those located in other regions of DI (table 5.4).

5.2.3 Codon design of mutant amino acids

In total 16 mutant / variant DI constructs were thus planned for synthesis. In order to facilitate expression by prokaryotes, codons used frequently by *E.coli* were chosen to encode for the substitution mutations (Kane, 1995). The codons chosen for each mutant are shown in table 5.5.

5.2.4 Results of constructs made by site directed mutagenesis

Site-directed mutagenesis was used to make the mutants D8S, D9G, Mut 8+9, E23S and E27S. Automated sequencing of these constructs was undertaken to ensure absence of PCR errors and confirm correct sequence. The results of the sequencing plots are shown in figure 5.2.

	Amino acid sequence of C-terminal portion of protein
Wild-type	WPINTLKCTHHHHHH
DI ext	WPINTLKCT PRVP FHHHHHH
DII-III	<u>RPIELP</u> VCAHHHHHH

Table 5.3 Amino acid sequences of his₆-tag DI variants investigating the DI-II interlinker region. Abbreviations: DI ext, DI with remainder of interlinker region included (bold); DII-III, DI with DII-III interlinker substituted for wild-type interlinker (underlined). Protein sequences are shown in FASTA format.

Amino acid at position:	Forms H-bonds with:	surface accessibility
Trp 53	Gly 51	15%
Pro 54	Asn 56	20%
Ile 55	Thr 57	50%
Asn 56	Pro 54, Thr 57, Leu 58	35%
Thr 57	Arg 43, Ile 55, Asn 56,	35%
Leu 58	Asn 56, Leu 58, Cys 60	3%
Lys 59	Ser 38	30%
Cys 60	Leu 58	0.5%
Thr 61	Val 64, His 69, His 70	10%
Pro 62	Pro 65, Phe 66, His 70, His 71	15%
Arg 63	Phe 66, His 71, His 72	15%
Val 64	Thr 61, His 72	0%
Pro 65	Thr 61, Pro 62, His 67	0%
Phe 66	Pro 62, Arg 63, His 67, His 68	20%

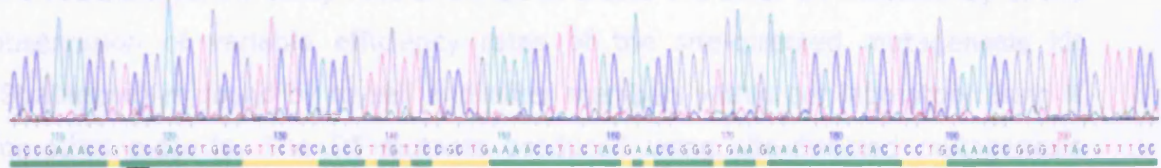
Table 5.4 Results of computer modelling studies targeting DI-II interlinker region.

DI mutant	Amino acid and codon changes: wild-type→mutant										
D8S	D	→	S								
	GAC		TCC								
D9G	D	→	G								
	GAC		GTT								
Mut 8+9	D	D	→	S	G						
	GAC	GAC		TCC	GTT						
E23S	E	→	S								
	GAA		TCC								
E26S	E	→	S								
	GAA		TCC								
E27S	E	→	S								
	GAA		TCC								
Mut 23+26+27	E	E	E	→	S	S	S				
	GAA	GAA	GAA		TCC	TCC	TCC				
Mut 8+9+23+26+27	D	D	E	E	E	→	S	G	S	S	S
	GAC	GAC	GAA	GAA	GAA		TCC	GTT	TCC	TCC	TCC
R39K	R	→	K								
	CGT		AAA								
R39S	R	→	S								
	CGT		TCC								
G40E	G	→	E								
	GGT		GAA								
G40S	G	→	S								
	GGT		TCC								
R43G	R	→	G								
	CGT		GGT								
R43S	R	→	S								
	CGT		TCC								
DI ext*	P	R	V	P	F						
	CCG	CGT	GTT	CCG	TTC						
DII-III	W	P	I	N	T	L	K	C	T	→	
	TGG	CCG	ATC	AAC	ACC	CTG	AAA	TGC	ACC		
	R	P	I	E	L	P	V	C	A		
	AGG	CCG	ATC	GAA	CTG	CCG	GTT	TGT	GCT		

Table 5.5 Amino acid and corresponding codon changes of DI mutants. The mutant codons substituted are shown with the altered bases highlighted in red. * DI ext – as this is not a substitution mutation only the additional amino acids inserted into the peptide at the C-terminal are shown with the relevant codons.

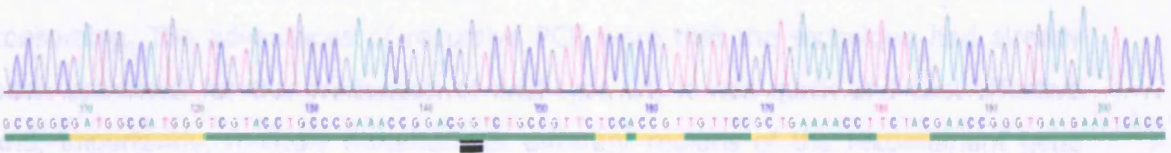
D8S

The recursive behind using recursive PCR to create the other DI mutants lay in the



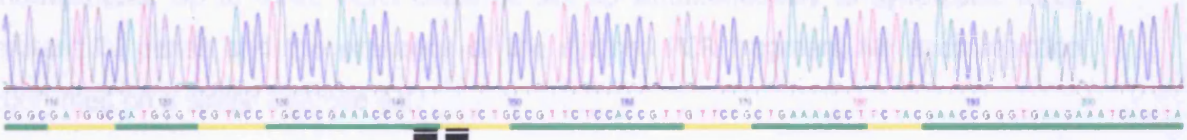
D9G

method was highly automated to save time, it proved to be both time and cost



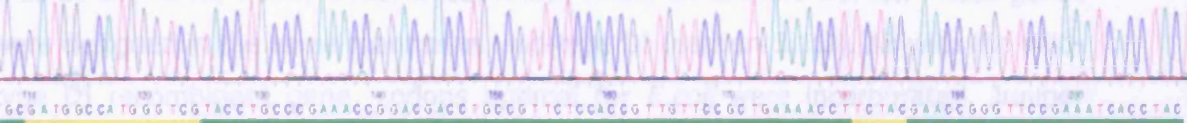
Mut 8+9

incorporated in a single PCR, which is not possible with site-directed



E26S

Juniper was used to design the recombinant mutant genes encoding for E26S. Mut 8-



E27S

used four primer overlapping primers spanning the length of the mutant genes

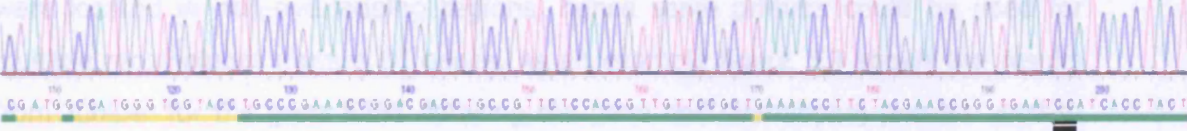


Figure 5.2 Sequencing plots of DI mutants produced by site-directed mutagenesis - D8S, D9G, Mut 8+9, E26S and E27S.

Indicates site of mutation.

5.2.5 Production of synthetic mutant DI genes using recursive PCR

The rationale behind using recursive PCR to create the other DI mutants lay in the observation of variable efficiency rates of the site-directed mutagenesis kit (Stratagene) as found by myself and other members within our laboratory using it for other projects. The DI mutants produced using site-directed mutagenesis required considerable optimisation of a number of parameters. Thus although this method was initially employed to save time, it proved to be both time and cost consuming. The advantages of recursive PCR were that the technique had already been optimised for the production of wild-type DI, it was quick and cost-effective and, importantly, multiple mutations at different regions of the recombinant gene could be incorporated in a single PCR, which is not possible with site-directed mutagenesis. Up to three PCRs could be set up simultaneously to synthesise three mutant DI genes and the presence of the desired PCR fragment for each reaction confirmed on a single agarose gel.

Juniper was used to design the recombinant mutant genes encoding for E23S, Mut 8-9-23-26-27, R39K, R39S, G40E, G40S, R43G, R43S, DI ext and DII-III. These genes were designed in the same manner as wild-type DI (section 3.1.2). As with the wild-type DI recombinant gene, codons optimal for *E.coli* were incorporated. Juniper designed four 60mer overlapping primers spanning the length of the mutant genes with flanking *Nco* I and *Xho* I restriction sites. The sites of the desired mutations were located in non-overlapping regions, hence some primers could be used for multiple recombinant mutant DI genes. As an example figure 5.3 shows the results from Juniper for the gene E23S detailing the gene and overlapping primers used to create this mutant recombinant gene.

The primers for each mutant as designed by Juniper were then used in a recursive PCR to create the 201bp recombinant mutant DI genes and these are shown in table 5.6. The PCR amplification products corresponding to the size of the desired synthetic mutant genes are shown in figures 5.4.

Results from Juniper

```

1      I      I      I      G      R      T      C      P      K      P
1 G G G G G C C A T G G G T C G T A C C T G C C C G A A A C C G
      \-HcoI

31     D      D      L      P      P      S      T      V      V      P
31 G A C G A C C T G C C G T T C T C C A C C G T T G T T C C G
      \-XmaI

61     L      K      T      F      Y      S      F      G      E      E
61 C T G A A A A C C T T C T A C T C C C C G G G T G A A G A A
      \-XmaI

91     I      T      Y      S      C      K      P      G      Y      V
91 A T C A C C T A C T C C T G C A A A C C G G G T T A C G T T
      \-Dex03R

121    S      R      G      G      M      R      K      F      I      C
121 T C C C G T G G T G G T A T G C G T A A A T T C A T C T G C
      \-PinAI

151    P      L      T      G      L      W      P      I      N      T
151 C C G C T G A C C G G T C T G T G G C C G A T C A A C A C C
      \-Sfr274I

181    L      K      C      T      I      I      I
181 C T G A A A T G C A C C C T C G A G T G T
      \-Sfr274I
  
```

Oligo overlaps:

```

1      GGGGCCATGGGGTCGTACCTGCCCGAAACCGGACGACTGCCCGTTCTCCAC
      .....GTG

51     CGTTGTTCCGCTGAAA.....CACCTACT
      GCAACAAGGCGACTTTTGGGAAGATCAGGGGCCCTTCTTTAGTGGATGA
      S      E E

101    CCTGCAAACCGGGTTACGTTTCCCGTGGTGGTATGCGTAAATTCATCTGC
      GGACGTTTGG.....TAAGTAGACG

151    CCGCTGAC.....
      GGCGACTGGCCAGACACCGGCTAGTTGTGGGACTTTACGTGGGAGCTCAC

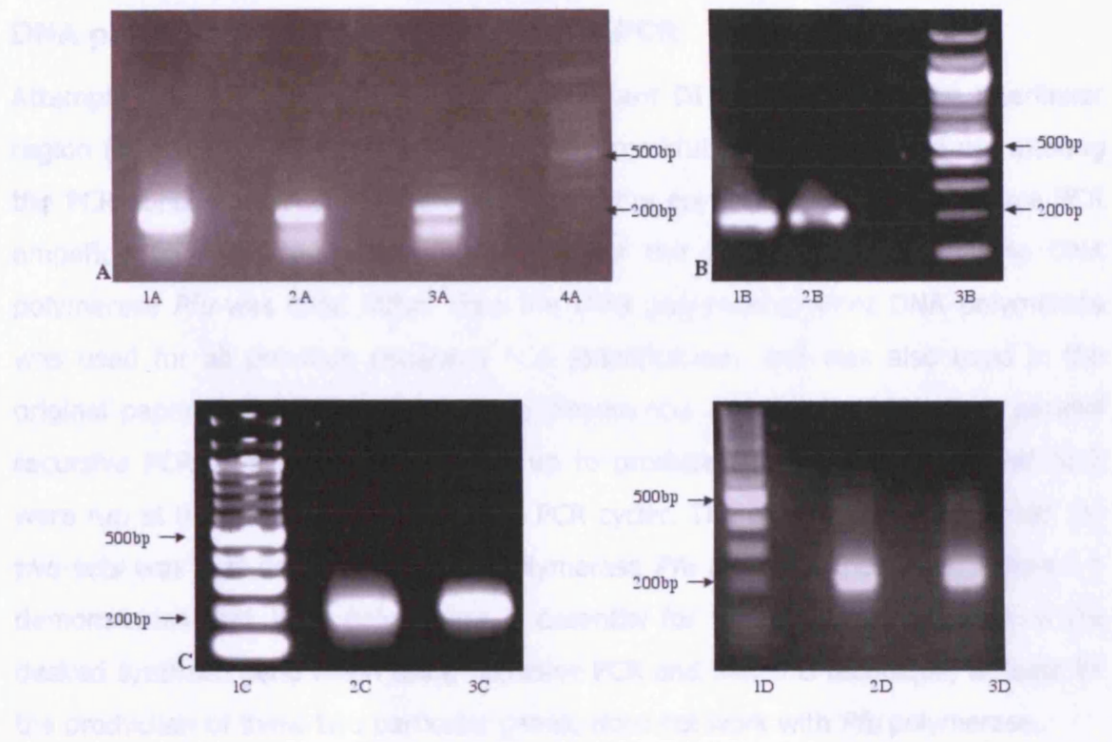
201 .
A
  
```

Figure 5.3 Results from Juniper demonstrating design of gene for the E23S mutation. Restriction sites are highlighted by Juniper. Overlapping primers to use in a recursive PCR designed and shown. Site of the E23S mutation is boxed and labelled in blue and other negatively charged amino acids boxed and labelled in black.

Primer	Sequence and mutants used for
DI-F1	5'-GGGGCCATGGGTCGTACCTGCCCGAAACCGGACGACCTGCCGTCTCCACCGTTGTTCCGCTGAAA-3' Primer A for: E23S, Mut 23-26-27, R39K, R39S, G40E, G40S, R43G, R43S, DI ext, DII-III
D8-9-F1	5'-GGGGCCATGGGTCGTACCTGCCCGAAACCG TCCGT CTGCCGTTCTCCACCGTTGTTCCGCTGAAA-3' Primer A for: Mut 8-9-23-26-27
E23-R1	5'-GGTITGCA GGG GTAGGTGATACCCGGGGAGTAGAAGGTTTTAGCGGAACAACGGTG-3' Primer B for: E23S
E23-27-R1	5'-GGTITGCA GGG GTAGGTGATTTCTTACCCGGGGAGTAGAAGGTTTTAGCGGAACAACGGTG-3' Primer B for: Mut 23-26-27 and Mut 8-9-23-26-27
DI-R1	5'-GGTITGCA GGG GTAGGTGATTTCTTACCCGGTTGGTAGAAGGTTTTAGCGGAACAACGGTG-3' Primer B for: R39K, R39S, G40E, G40S, R43G, R43S, DI ext, DII-III
DI-F2	5'-CACCTACTCCTGCAAACCGGGTTACGTTTCCCGTGGTGGTATGCGTAAATTCATCTGCCCGCTGAC-3' Primer C for: E23S, Mut 23-26-27, Mut 8-9-23-26-27, DI ext, DII-III
R39K-F2	5'-CACCTACTCCTGCAAACCGGGTTACGTTTCC AA GGTGGTATGCGTAAATTCATCTGCCCGCTGAC-3' Primer C for: R39K
R39S-F2	5'-CACCTACTCCTGCAAACCGGGTTACGTTTCC TC GGTGGTATGCGTAAATTCATCTGCCCGCTGAC-3' Primer C for: R39S
G40E-F2	5'-CACCTACTCCTGCAAACCGGGTTACGTTTCCCGT GAA GGTATGCGTAAATTCATCTGCCCGCTGAC-3' Primer C for: G40E
G40S-F2	5'-CACCTACTCCTGCAAACCGGGTTACGTTTCCCGT TCG GGTATGCGTAAATTCATCTGCCCGCTGAC-3' Primer C for: G40S
R43G	5'-CACCTACTCCTGCAAACCGGGTTACGTTTCCCGTGGTGGTATG GGT AAATTCATCTGCCCGCTGAC-3' Primer C for: R43G
R43S	5'-CACCTACTCCTGCAAACCGGGTTACGTTTCCCGTGGTGGTATG TCCA AATTCATCTGCCCGCTGAC-3' Primer C for: R43S
DI-R2	5'-ACACTCGAGGGTGCATTTAGGGTGTGATCGGCCACAGACCGGTCAGCGGGCAGATGAAT-3' Primer D for: E23S, Mut 23-26-27, Mut 8-9-23-26-27, R39K, R39S, G40E, G40S, R43G, R43S
DIext-R2*	5'-ACACTCGAG GAAC CGGAACACCGGGGGTGCATTTAGGGTGTGATCGGCCACAGACCGGTC-3' Primer D for: DI-ext
DII-III-R2	5'-ACACTCGAG AGCACAAACCGGGCAGTTCGATCGGCCT CAGACCGGTCAGCGGGCAGATGAAT-3' Primer D for: DII-III

Table 5.6 Overlapping primers designed by Juniper for the synthesis of mutant DI genes in a recursive PCR. All primers read 5' to 3' left to right. The site of the mutant codons are highlighted in red. *Boxed codons indicate that addition of five amino acids to complete the interlinker region.

6.2.5 Successful recursive amplification of mutant A genes with polymerase of type of



Lanes:

- | | |
|--|---|
| <p>A) 1A = E23S
 2A = Mut 23-26-27
 3A = Mut 8-9-23-26-27
 4A = 100bp marker</p> | <p>B) 1B = R39K
 2B = R39S
 3B = 100bp marker</p> |
| <p>C) 1C = 100bp marker
 2C = G40E
 3C = G40S</p> | <p>D) 1D = 100bp marker
 2D = R43G
 3D = R43S</p> |

Figure 5.4 Recursive PCR amplification of DI mutants.

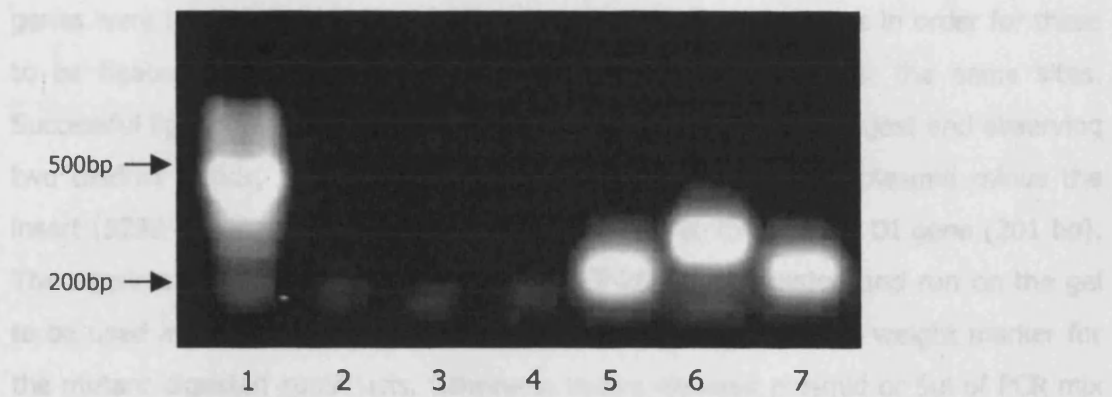
The technique of recursive PCR allowed for 2-3 mutants to be produced simultaneously. 10µl per lane of each PCR mixture was run on a 1.2% agarose gel. A-D show the individual agarose gels run confirming a band consistent with a 201bp mutant DI gene.

5.2.6 Successful synthesis of mutant DI genes is dependent on type of DNA polymerase employed in recursive PCR

Attempts made to produce the final two mutant DI genes altering the interlinker region (DI ext and DII-III) were initially unsuccessful. This was despite not altering the PCR conditions. The only difference from the previous successful recursive PCR amplifications was that different batches of the laboratory stock of the DNA polymerase *Pfu* was used rather than the *Vent* polymerase. *Vent* DNA polymerase was used for all previous recursive PCR amplifications and was also used in the original paper describing the technique (Prodromou and Pearl, 1992). Two parallel recursive PCR amplifications were set up to produce DI ext and DII-III and both were run at the same time in the same PCR cycler. The only difference between the two sets was that one had the DNA polymerase *Pfu* and the other *Vent*. Figure 5.5 demonstrates that *Vent* polymerase is essential for successful amplification of the desired synthetic gene when using recursive PCR and that this technique, at least for the production of these two particular genes, does not work with *Pfu* polymerase.

5.2.7 Cloning of synthetic oligonucleotide-templated engineering plasmid

The eleven synthetic oligonucleotide primers were used for recursive PCR as shown in figures 5.4 and 5.5. The purified mutant DI genes were

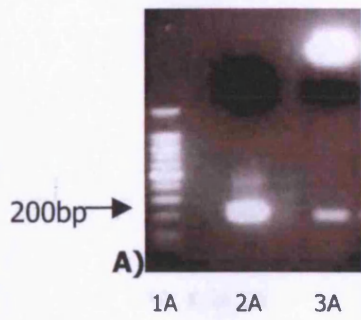


- Lanes: 1 = 100bp marker
2 = DI ext using *Pfu*
3 = DII-III using *Pfu*
4 = positive control (201bp) using *Pfu*
5 = DII-III (201bp) using *Vent*
6 = DI ext (213bp) using *Vent*
7 = Positive control (201bp) using *Vent*

Figure 5.5 Successful recursive PCR amplification of DI mutants is dependant on *Vent* DNA polymerase. 10 μ l of PCR mix from each amplification was added to the wells. As a positive control primers employed successfully in previous experiments (with *Vent* DNA polymerase) to produce R39K were used. 2% agarose gel.

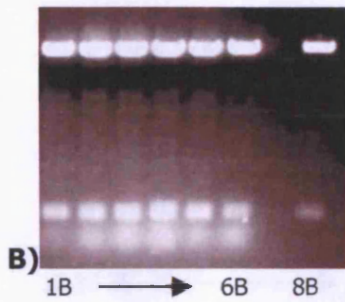
5.2.7 Cloning of synthetic mutant DI genes into expression plasmid

The eleven synthetic mutant DI genes produced by recursive PCR as shown in figures 5.4 and 5.5 were purified from the PCR mixture. The purified mutant DI genes were then digested with *Xho* I and *Nco* I restriction enzymes in order for these to be ligated into the pET-26b expression plasmid digested at the same sites. Successful ligation was confirmed by performing a *Xho* I / *Nco* I digest and observing two distinct bands, a large band corresponding to a digested plasmid minus the insert (5298 bp) and a smaller band corresponding to the mutant DI gene (201 bp). The construct of wild-type DI cloned into pET-26b was digested and run on the gel to be used as a positive control for the digest and as a molecular weight marker for the mutant digested constructs. Otherwise native digested plasmid or 5µl of PCR mix comprising a mutant DI PCR fragment was run to determine the respective levels of 5298bp and 201bp sizes on the agarose gel. Figure 5.6 shows the gels from the *Xho* I / *Nco* I digestion of the mutant constructs E23S, Mut 23-26-27 and Mut 8-9-23-26-27, figure 5.7 the digestion of the mutants R39S, R39K, G40E, G40S and figure 5.8 digests of the mutants R43S, R43G, DII-III and DI ext. The number of colonies screened for the presence of the desired ligated construct were between five and 10 as not all colonies when grown were shown to harbour the desired construct on extracting plasmid DNA, with many being either native plasmid or foreign plasmids derived from contaminant *E.coli* strains. This is illustrated in multiple gels. All mutant constructs were sequenced to confirm absence of PCR errors as shown in the sequencing plots of figure 5.9. The mutant construct Mut 8-9-23-26-27 contained a PCR error of a single base deletion resulting in frameshift. Hence the gene was re-synthesised using recursive PCR, cloned into pET-26b (figure 5.6B) and re-sequenced to confirm no errors in the second construct.



LANES

- 1A 100bp marker
- 2A E23S gene (PCR mix)
- 3A E23S *digested construct



- 1B to 6B Mut 23-26-27 digested*
construct
- 8B *Digested †positive control



- 1C to 5C Mut 8-9-23-26-27
*digested construct
- 6C *Digested †positive control

Figure 5.6 *Xho* I / *Nco* I digested mutant constructs of E23S, Mut 23-26-27 and Mut 8-9-23-26-27. Gels: A) = E23S digests, B) = Mut 23-26-27 digests and C) = Mut 8-9-23-26-27 digests. *All digests were performed using *Xho* I / *Nco* I restriction enzymes. †All positive controls were digested pET-26b with the wild-type DI insert. All agarose gels were 1.2%.

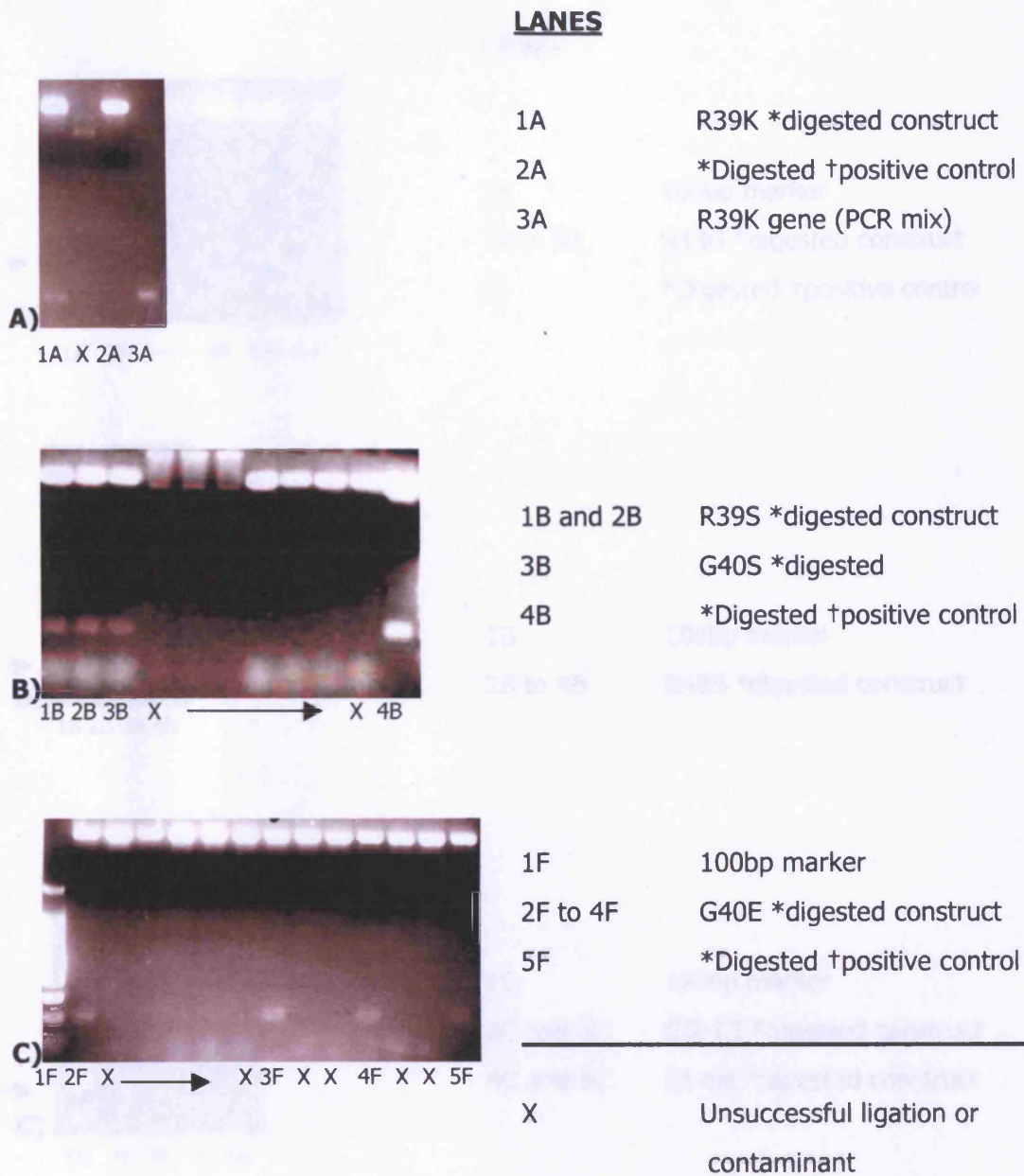


Figure 5.7 *Xho* I / *Nco* I digested mutant constructs of R39K, R39S, G40S and G40E. Gels: A) = R39K digests, B) = R39S and G40S digests and C) = G40E digests. *All digests were performed using *Xho* I / *Nco* I restriction enzymes. †All positive controls were digested pET-26b with the wild-type DI insert or native pET-26b. All agarose gels were 1.2%.

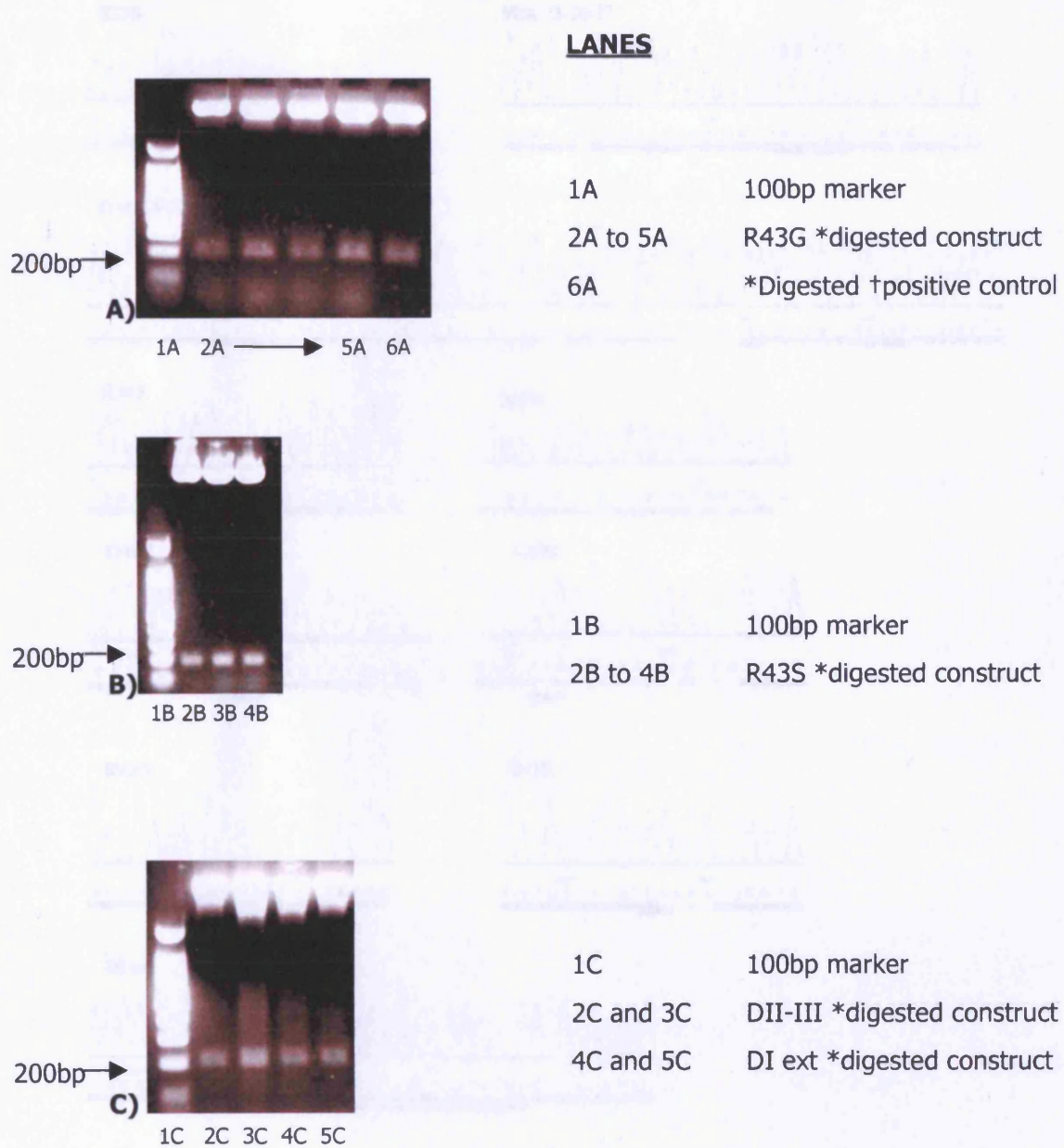
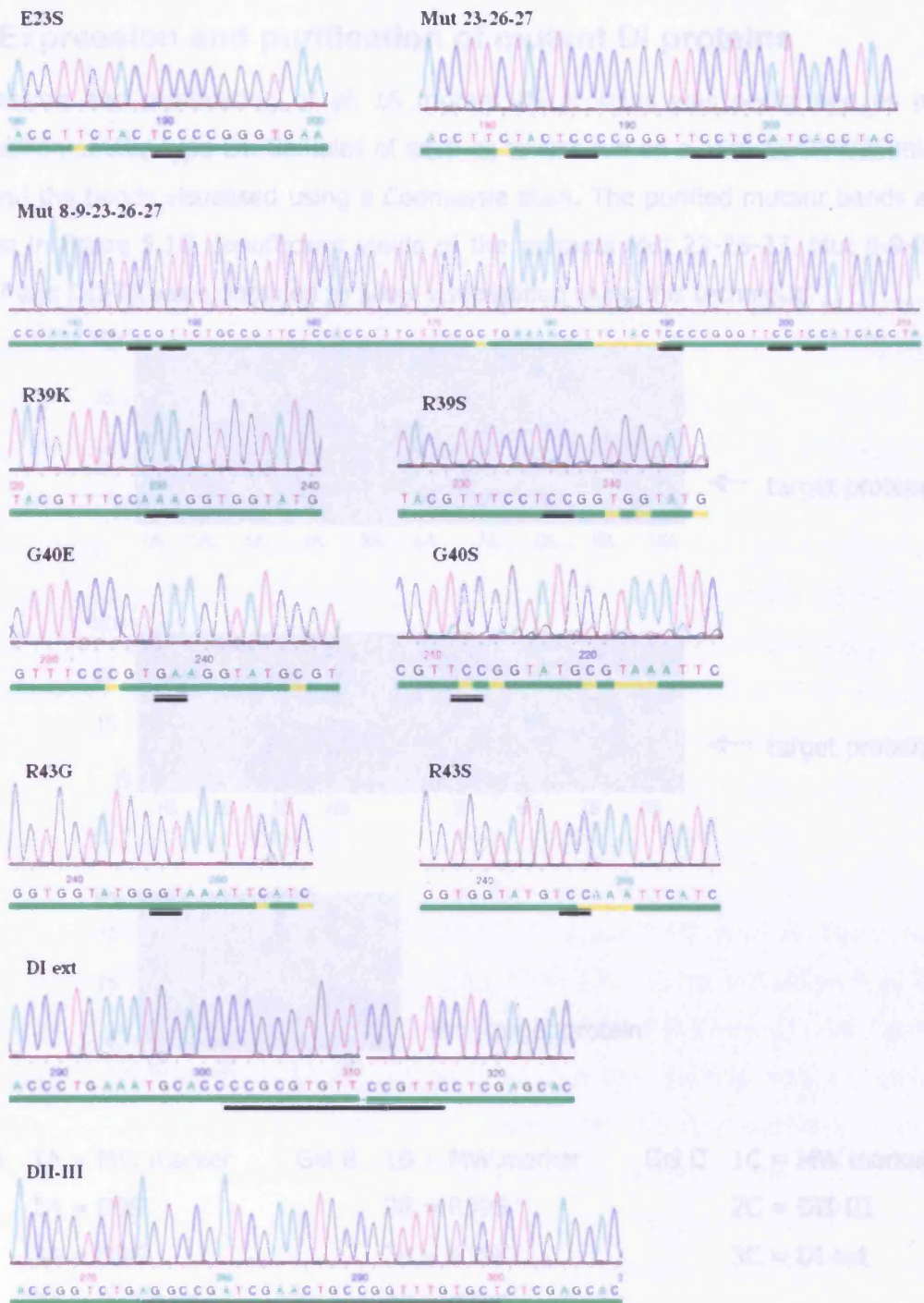


Figure 5.8 *Xho* I / *Nco* I digested mutant constructs of R43G, R43S, DII-III and DI ext. Gels: A) = R43G digests, B) = R43S digests and C) = DII-III and DI ext digests. *All digests were performed using *Xho* I / *Nco* I restriction enzymes. †All positive controls were digested pET-26b with the wild-type DI insert. All agarose gels were 1.2%.

Figure 5.9 Sequencing plots of DI mutants synthesized using recursive PCR. All gene sequences are underlined indicating the site of mutation substitution with introduction of DI ext, which corresponds to the start site of the DI protein.



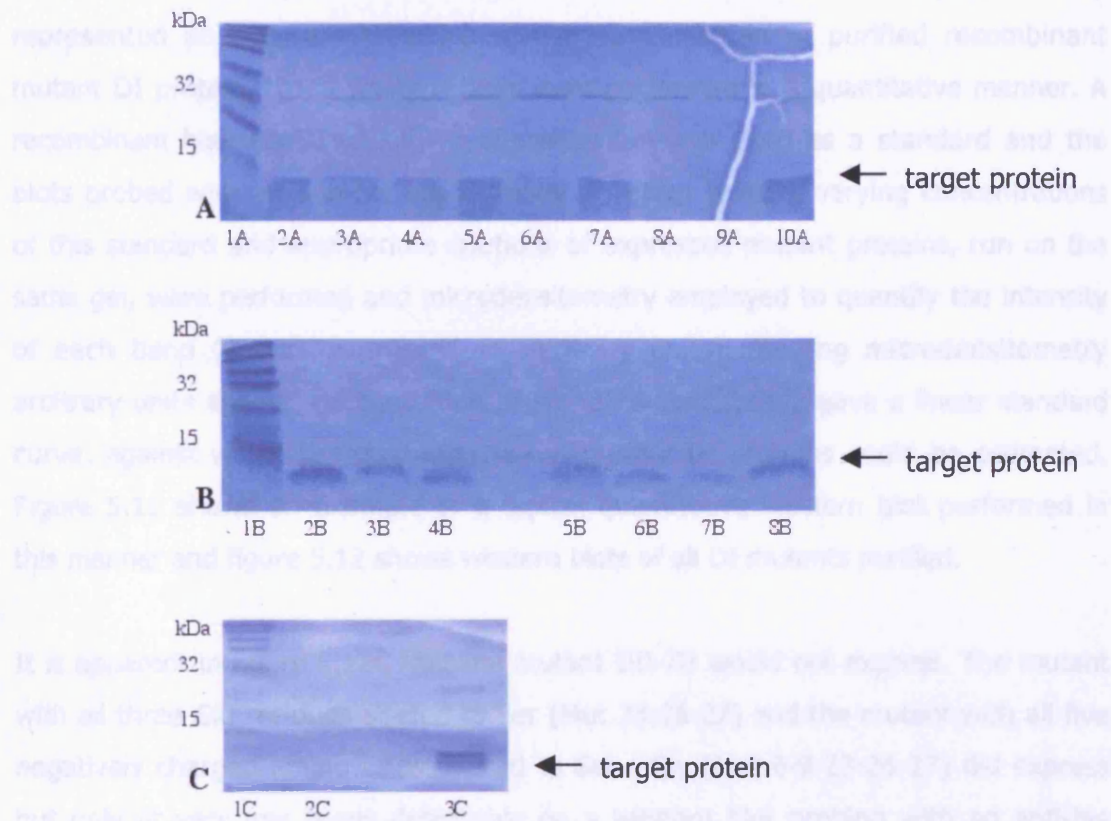
- 5A = E23S
- 5B = Mut 23-26-27
- 5C = Mut 8-9-23-26-27
- 5D = R39K
- 5E = R39S
- 5F = G40E
- 5G = G40S
- 5H = R43G
- 5I = R43S
- 5J = DI ext
- 5K = DII-III
- 5L = Wild-type DI

Figure 5.9 Sequencing plots of DI mutants synthesised using recursive PCR. All base sequences underlined represent site of mutation substitution with the exception of DI ext, which represents base sequence insertion.

5.3.1 Western blot analysis of expressed DI mutant proteins

5.3 Expression and purification of mutant DI proteins

Expression and purification of all 16 mutant DI proteins was performed as per expression of wild-type DI. Samples of each elute was run on a 15% SDS-PAGE mini-gel and the bands visualised using a Coomassie stain. The purified mutant bands are shown in figure 5.10. Insufficient yields of the mutants Mut 23-26-27, Mut 8-9-23-26-27 and DII-III were obtained to allow visualisation using this technique.



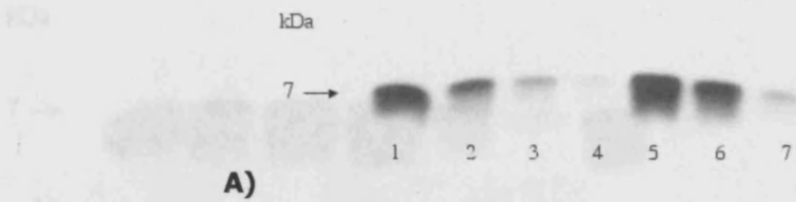
Gel A	1A = MW marker	Gel B	1B = MW marker	Gel C	1C = MW marker
	2A = D9S		2B = R39S		2C = DII-III
	3A = D9G		3B = R39K		3C = DI ext
	4A = Mut 8+9		4B = G40E		
	5A = E23S		5B = G40S		
	6A = E26S		6B = R43S		
	7A = E27S		7B = R43G		
	8A = Mut 23-26-27		8B = Wild-type DI		
	9A = Mut 8-9-23-26-27				
	10A = Wild-type DI				

Figure 5.10 Protein gels of DI mutants. Gels A-C, 15% SDS-PAGE.

5.3.1 Western blot analysis of expressed DI mutant proteins

Western blot analysis, probing with an anti-his tag antibody, was used to confirm that the purified 7kDa protein indeed contained a his-tag, making it highly likely to be the desired target protein. Furthermore, given the sensitivity of this technique, it was used to confirm whether any protein had been expressed for the three mutants not visualised on the less sensitive Coomassie stained gel (Mut 23-26-27, Mut 8-9-23-26-27 and DII-III). Simultaneously, in order to be certain that the protein assay represented an accurate measure of the concentration of purified recombinant mutant DI protein, these western blots were performed in a quantitative manner. A recombinant his₆-tag DI of known concentration was used as a standard and the blots probed with an anti-his tag antibody. Western blots of varying concentrations of this standard and appropriate dilutions of expressed mutant proteins, run on the same gel, were performed and microdensitometry employed to quantify the intensity of each band (results expressed as arbitrary units). Plotting microdensitometry arbitrary units against concentration of standard consistently gave a linear standard curve, against which the concentration of mutant DI proteins could be estimated. Figure 5.11 shows an example of a typical quantitative western blot performed in this manner and figure 5.12 shows western blots of all DI mutants purified.

It is apparent in figure 5.12C that the mutant DII-III would not express. The mutant with all three Glu residues altered to Ser (Mut 23-26-27) and the mutant with all five negatively charged amino-acids altered to Ser / Gly (Mut 8-9-23-26-27) did express but only at very low levels detectable on a western blot probing with an anti-his antibody (figure 5.12A). The most likely reason for the low expression levels observed for these mutants is due to the effects these mutations may have on the overall conformation of the protein as predicted in table 5.1. A marked conformational change would potentially lead to mis-folding and affect solubility resulting in lower yields. To test this theory, the same membrane as shown in figure 5.12A was stripped with 0.2M NaOH and re-probed with a monoclonal murine anti-DI antibody that recognises conformationally restricted epitopes (figure 5.13). This murine antibody bound wild-type and mutants of DI with single and double point mutations with bands almost identical to those seen when probing with the anti-his tag antibody (figure 5.11A). However, despite a long duration of film exposure, the bands for mutants Mut 23-26-27 and Mut 8-9-23-26-27 were not visible, suggesting an alteration in conformation to the extent that these mutants no longer bind the murine anti-DI antibody tested.



Lanes 1-4 represents his₆-tagged DI (500µg/ml neat) run at varying dilutions:
 1 = dilution six times - 83µg/ml
 2 = dilution eight times - 62.5µg/ml
 3 = dilution 12 times - 41.7µg/ml
 4 = dilution 16 times - 31.25µg/ml

Lanes 5-7 represent mutant DI samples:
 5 = Mut 8+9 dilution four times
 6 = E23S dilution two times
 7 = Mut 23-26-27 neat

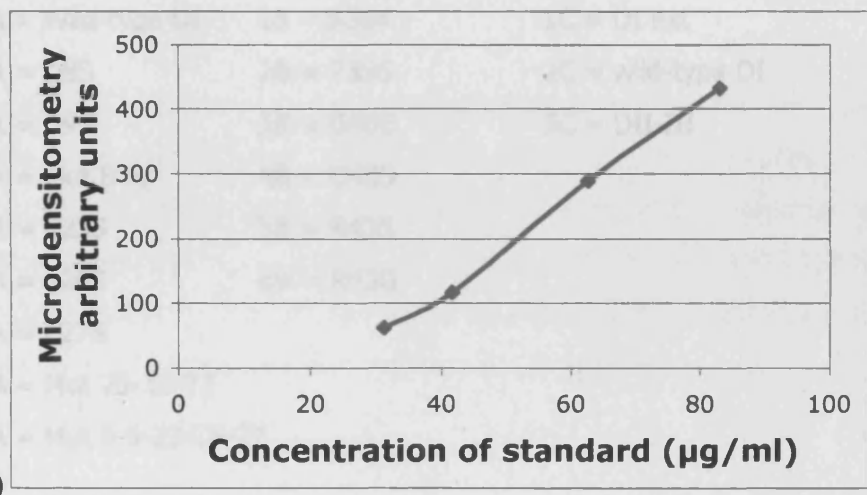


Figure 5.11 Quantitative western blot using microdensitometry. A) Western blot probed with an anti-his tag antibody. Microdensitometry arbitrary units are quantified for each band. A standard curve is drawn B), against which the concentration of the mutants may be calculated. Mut 8+9 (lane 5) microdensitometry reading was above the standard, necessitating the western to be repeated at a higher dilution.



Lanes:

1A = Wild-type DI	1B = R39K	1C = DI ext
2A = D8S	2B = R39S	2C = wild-type DI
3A = D9G	3B = G40E	3C = DII-III
4A = Mut 8+9	4B = G40S	
5A = E23S	5B = R43S	
6A = E26S	6B = R43G	
7A = E27S		
8A = Mut 23-26-27		
9A = Mut 8-9-23-26-27		

Figure 5.12 Western blots of DI mutants probing with anti-his tag antibody. Blots A) to C) were probed with an anti-his₅ antibody. Low signals are seen for the mutants E26S, Mut 23-26-27 and Mut 8-9-23-26-27 and there was insufficient quantities of these proteins for use in binding studies. Complete absence of a signal for the DII-III mutant implies lack of any soluble protein.

5.4 The use of computer modelling to rationalise epitopes targeted for mutation

Western blot analysis of mutants probed with murine anti-DI antibody. The membrane from figure 5.12A) was stripped and re-probed with mAb-16, a monoclonal murine anti-DI antibody that recognises conformationally restricted epitopes (Iverson et al., 2002b). Loss of signal for the mutants M23-26-27 and M8-9-23-26-27 suggest marked conformational change in the structure of these proteins relative to wild-type and other single point DI mutants.

Lanes: 1 = Wild-type DI

2 = D8S

3 = D9G

4 = Mut 8+9

5 = E23S

6 = E26S

7 = E27S

8 = Mut 23-26-27

9 = Mut 8-9-23-26-27

Figure 5.13 Western blot of mutants probed with murine anti-DI antibody. The membrane from figure 5.12A) was stripped and re-probed with mAb-16, a monoclonal murine anti-DI antibody that recognises conformationally restricted epitopes (Iverson et al., 2002b). Loss of signal for the mutants M23-26-27 and M8-9-23-26-27 suggest marked conformational change in the structure of these proteins relative to wild-type and other single point DI mutants.

5.4 The use of computer modelling to rationalise epitopes targeted for mutation

Broadly the three hypotheses formed to define the aPL binding epitopes targeted for mutation were: clustered negatively charged epitopes on DI, regions R39 to R43 with R39 being particularly important given its greater surface exposure and the DI-II interlinker (see section 5.1). However, on reflection it would seem that these hypotheses contradict each other. For example, if positively charged Arg residues in the V_H of aPL are important and bind negatively charged residues on DI then how does this hypothesis fit in with the positively charged patch in region 39-44 (RGGMRK) as also being important. Furthermore, if one or both these regions are important then how is it that the DI-II interlinker region may be important and that a short peptide, which bears some homology to this region, binds pathogenic aPL in the fluid phase (Blank et al., 1999)? It may be that polyclonal aPL being heterogeneous in nature bind varying linear regions of DI. Yet denaturing β_2 GPI results in loss of binding to aPL (Sheng et al., 2001b), suggesting that most aPL bind non-continuous epitopes. Studying the computer model of DI, which corresponds to the known crystal structure of DI within whole β_2 GPI, offers some rationale that may explain these apparent paradoxes. We know that H-bonds, which are stronger than Van der Waal forces, form between non-continuous amino-acids within a protein, bringing the interacting amino-acids in close proximity to each other, and play an important role in defining protein structure (Stryer, 1988). On studying the computer model of DI, Ser 13 is situated in very close proximity, just 4Å, from the interlinker region and Asp 9 forms a H-bond with Ser 13. This has the result of bringing Asp 8 and 9 residues to within 6Å of the interlinker region. Furthermore Arg 39 lies only 5 Å from the interlinker partly due to a H-bond between Arg 43 and Thr 57 within the interlinker region. Thus it is conceivable that these three discontinuous surface exposed areas, which lie in close proximity to each other within the tertiary structure of the protein as shown in figure 5.14, act together in either binding or stabilising the aPL-antigen interaction and that disrupting any of these areas could have an effect on binding.

5.5 Discussion of results

The results presented in this chapter demonstrate the successful application of the basic extraction and purification protocol, developed as shown in Chapter 3, to generate 1.5 μ g of DI. It was important that there existed a clear redox potential window for the installation. A redox potential window containing the residue chosen for the computer modeling analysis was identified to allow the site to be reduced and oxidized to the appropriate oxidation state.

Some residues were highlighted in the computer model. The residues R43, G40, R39, D8, and D9 were highlighted in the molecular model. The residues R39, D8, and D9 were highlighted in the molecular surface model. The residues R39, D8, and D9 were highlighted in the molecular surface model. The residues R39, D8, and D9 were highlighted in the molecular surface model.

Figure 5.14 Computer model of DI of β_2 GPI and the proposed aPL binding epitopes. a, molecular model of DI with the three regions within close proximity to each other highlighted. b, molecular surface of DI with electrostatic charge highlighted. The considerable surface area exposure of R39 is highlighted.

showing in the literature. The technique of attaching PCR to secondary probes to PCR ends, using a 5' or 3' DNA polymerase, which has the highest error rate is desirable and why the authors first described this technique used Vent (Embley and Peart, 1992). The DNA polymerase Vent and Pfu have proofreading capabilities and consequently have much lower PCR error rates. The polymerase Pfu has the greatest proofreading capability of the three and consequently the error rate is 1-10 fold lower than that of the non-proofreading Taq. The error rate for Vent has somewhere in between at approximately 4-5 fold over Taq (Chiu et al., 1996) shows that the primers do not tightly anneal, but rather have overlapping complementary regions. It is possible that the high fidelity Pfu control tolerate sub-optimal matching of oligonucleotide strands and hence will not amplify DNA under these circumstances.

5.5 Discussion of results

The results presented in this chapter demonstrate the successful application of the *E.coli* expression and purification platform, developed as shown in Chapter 3, to produce 15 mutants of DI. I felt it was important that there existed a clear rationale behind incorporating each specific mutation. A hypothesis existed for targeting the residues chosen and detailed computer modelling analyses were undertaken to choose the most appropriate mutants and analyse the effects on overall protein structure.

Some mutants did not express in sufficient quantities to use in further binding studies. These were E26S, Mut 23-26-27 and Mut 8-9-23-26-27. Glu 26 forms a H-bond with Cys 47. Cys 47 is integral to the structure of DI as it forms a disulphide bond with Cys 4. Hence mutating E26 may destabilise this bond and affect solubility of protein and hence yield. The multiple Glu mutants include a mutation at E26 and thus as expected these also had poor yields. Destabilising a disulphide bond is likely to produce significant structural changes within the protein (Stryer, 1988). This would have a detrimental effect on binding to antibodies that recognise conformational epitopes on DI as observed. The DII-III mutation would not express at all suggesting that this structure was very unstable.

The observation that recursive PCR only works when using *Vent* DNA polymerase and not *Pfu* is intriguing and I have not been able to find this phenomenon described elsewhere in the literature. The technique of recursive PCR is particularly prone to PCR errors, which is why *Taq* DNA polymerase, which has the highest error rate is unsuitable and why the authors that described this technique used *Vent* (Prodromou and Pearl, 1992). The thermostable polymerases *Vent* and *Pfu* have proofreading capabilities and consequently have much lower PCR error rates. The polymerase *Pfu* has the greatest proofreading properties of the three and consequently the error rate is 8-10 fold lower than that of the non-proofreading *Taq*. The error rate for *Vent* lies somewhere in between at approximately 4-5 fold lower than *Taq* (Cline et al., 1996). Given that the primers do not tightly anneal, but rather have overlapping complementary regions, it is possible that the high fidelity *Pfu* cannot tolerate sub-optimal matching of oligonucleotide strands and hence will not amplify DNA under these circumstances.

The areas targeted for mutation on native DI were analysed using the computer model of DI, which was identical to the published crystal structure. It was clear that these areas lay in close proximity to each other and raised the possibility that they could form a discontinuous epitope that the majority of aPL from patients with APS bind to. The fact that denatured β_2 GPI does not bind aPL (Sheng et al., 2001b) supports the hypothesis that aPL bind non-continuous rather than linear epitopes in this protein. It may be that some changes would reduce binding but it is also conceivable that others may have an enhanced binding effect. The assay studies presented in the final results chapter aim to test these possibilities

In conclusion, the *E.coli* platform of DI production was used to produce and purify sufficient quantities of 12 mutants of DI for use in binding assays. The site and nature of the mutations were chosen as a direct result of generated hypotheses and detailed computer modelling studies as used by the well validated Swiss-Model software (Peitsch, 1996, Guex and Peitsch, 1997). The data generated from using Swiss-Model led to the development of a unifying hypothesis potentially explaining how three different areas of DI could all be important in being responsible for conferring binding to aPL, given the close approximation these areas have to each other. Binding studies presented in the final chapter aim to delineate which region of DI has the greatest effect on aPL binding when altered.

CHAPTER 6

ELUCIDATING THE BINDING CHARACTERISTICS OF RECOMBINANT DI MUTANTS

OVERVIEW OF CHAPTER 6

This final results chapter aims to elucidate the binding characteristics of the purified DI mutants expressed by *E.coli*. The initial focus is on describing the binding data of these mutants to a panel of APS derived polyclonal IgG in the solid phase. Binding for each mutant is expressed as a percentage of binding to wild-type DI. Binding of mutants to a monoclonal aPL (IS4) is also assessed. The results show that some mutants bound the polyclonal and monoclonal IgG less well than wild-type DI, as predicted by the original hypotheses but others displayed unexpected binding characteristics such as enhanced binding. Experiments excluding variability in the coating densities of antigen on the plate as a potential cause for these observations are presented.

A panel of DI mutants with either reduced or enhanced binding to aPL over wild-type DI were then selected for assessment of binding to APS IgG in the fluid phase. Inhibition of IgG binding to β_2 GPI coated on a CL surface was tested. The results show that some mutations cause almost complete loss of binding to aPL in the fluid phase whilst others cause enhanced binding to the majority of IgG samples in line with the solid phase binding data.

Finally, computer modelling studies were employed to help form structural hypotheses explaining the observed binding data for some of the DI mutants with the greatest effects on binding. These results are then discussed and consequently hypotheses are generated that outline the precise nature of the aPL-DI interaction.

6.1 Direct binding data of recombinant DI mutants to APS derived IgG

In line with the DI ELISA outlined in section 2.21.6, plates were coated in duplicate with 10µg/ml of wild-type DI and the relevant DI-mutant being investigated on one half of the plate and PBS alone on the control half. Binding of either monoclonal IgG aPL or polyclonal purified IgG derived from APS patients was then assessed to both the wild-type and the mutant DI coated antigens. Net ODs were calculated and binding expressed as a percentage of binding to wild-type. Wild-type DI was coated on every plate to ensure that inter-plate variability would not be a factor contributing to the varying binding levels seen between the different DI mutants. As a negative control, a his-tagged irrelevant protein (DsbA) expressed and purified from native pET-39b was used and coated on the plate at the same concentration as the other DI mutant antigens.

6.1.1 Direct binding of DI mutants to polyclonal purified IgG

In total 12 mutants of DI could be expressed and purified in sufficient quantities for investigation in a direct binding assay. Binding was assessed to a panel of 10 IgG samples, all derived from patients with APS of whom two also had SLE. All samples bound well in direct assays of binding to wild-type DI, CL and β_2 GPI. As shown in figure 6.1, small changes in the DI protein can result in large alterations in binding to the majority of APS derived IgG tested as compared to wild-type DI. In the solid phase, the mutations with the greatest effect in reducing binding were D8S, D9G and the R39S mutation with a mean +/- SD binding as compared to wild-type of 48.7 +/- 11.5%, 46.6 +/- 8.3% and 53.8 +/- 6.4% respectively. Given that the D8S and D9G single point mutants caused reduced binding, surprisingly the double mutant Mut 8+9, with both these residues mutated, had the opposite effect of enhancing binding to the IgG samples tested as compared to wild-type DI (185.7 +/- 31.9%). Another unexpected finding was that the mutant E23S also showed enhanced binding compared to wild-type DI (182.5 +/- 30.5%). The DI variant with the remainder of the interlinker attached (DI ext) as predicted enhanced binding as compared to wild-type (188.3 +/- 24.1%). The his-tagged negative control protein revealed very low levels of binding.

6.1.2 Direct binding of DI mutants to monoclonal human aPL

Binding of the DI mutants in the solid phase was determined to CHO derived IgG, as shown in figure 6.1, to be very similar to the polyclonal IgG data. IgG could least well to the mutants R39S and D9G and had marginally enhanced binding to E27S compared with the polyclonal data (figure 6.1). However, it was clear that differences were noted, as no significant enhancement of binding over wild-type DI to CHO was observed with Mut 8+9 or DI ext.

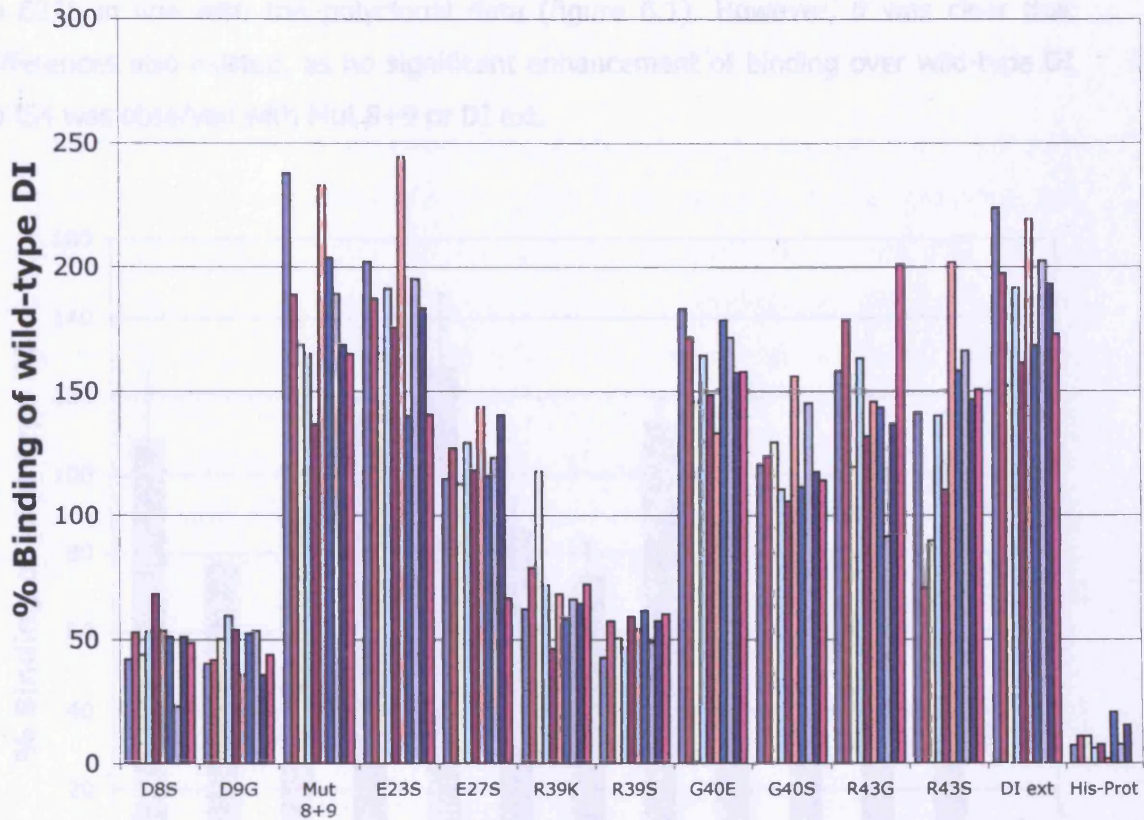


Figure 6.1 Direct binding of DI mutants to polyclonal APS derived IgG. The distribution of binding is shown for each mutant to each APS IgG derived sample (n=10) as compared to wild-type DI. The mutants with the least binding were D8S, D9G and R39S and those with marked enhanced binding over wild-type were Mut 8+9, E23S and DI ext. An irrelevant his₆-tagged protein (DsbA) coated at 10µg/ml was used as a negative control.

6.1.2 Direct binding of DI mutants to monoclonal human aPL

Binding of the DI mutants in the solid phase was determined to CHO derived IS4. As shown in figure 6.2, there were some similarities to the polyclonal IgG data. IS4 bound least well to the mutants R39S and D9G and had marginally enhanced binding to E27S in line with the polyclonal data (figure 6.1). However, it was clear that differences also existed, as no significant enhancement of binding over wild-type DI to IS4 was observed with Mut 8+9 or DI ext.

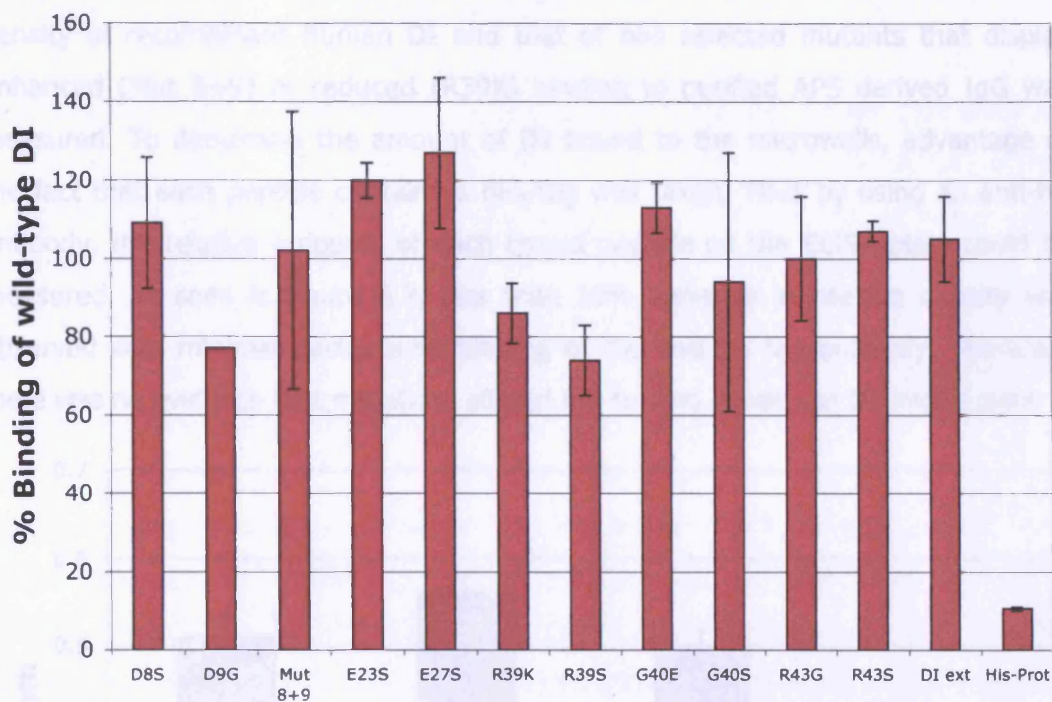


Figure 6.2 Binding of native IS4 to mutant DI in the solid phase. Least binding was observed to R39S and strongest to E27S. Error bars represent SD.

6.1.3 Assessing coating density of mutant antigens

The fact that polyclonal IgG bound some DI mutants better than others in direct ELISA could arise from two factors:

- a) The mutations could affect the interaction of aPL with DI or
- b) The mutations could alter binding of DI to the plate, affecting the coating density of the antigen.

The fact that the mutant DI proteins bind the plate through a his₆-tag that is not part of the domain itself make explanation b) less likely. To exclude this possibility, the density of recombinant human DI and that of two selected mutants that display enhanced (Mut 8+9) or reduced (R39K) binding to purified APS derived IgG was measured. To determine the amount of DI bound to the microwells, advantage of the fact that each peptide contains a his₆-tag was taken. Thus by using an anti-his antibody, the relative amounts of each bound peptide on the ELISA plate could be measured. As seen in figure 6.3, less than 10% variation in coating density was observed with minimal background binding of the anti-his tag antibody. Therefore there was no evidence that mutations altered the coating density on the nickel plate.

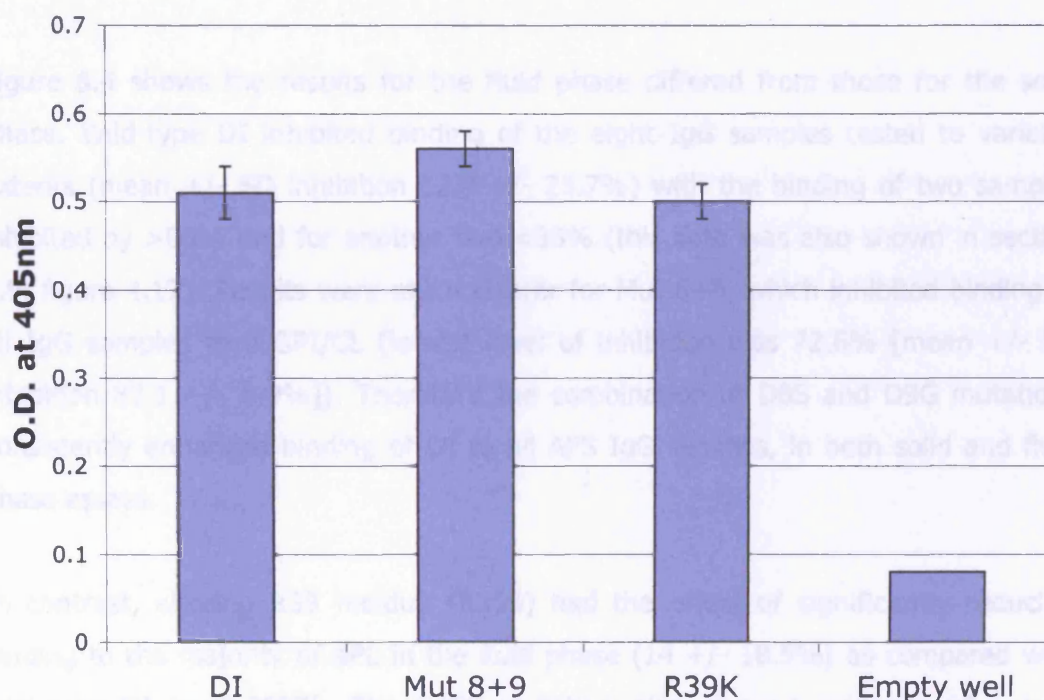


Figure 6.3 Assessing density of antigen coated on the nickel plate. By using an anti-his tag antibody, no significant differences in antigen coating densities on the plate were observed between the DI mutants. Mean OD +/- SD shown. (experiment planned by myself and performed by Charis Pericleous).

6.2 DI mutants inhibit binding to APS derived IgG in the fluid phase

Results in the solid phase assays suggested that binding to β_2 GPI coated on CL was a more specific assay for detecting pathogenic aPL (section 4.7) than either the standard CL ELISA or the standard β_2 GPI binding ELISA (section 4.5, table 4.3). I chose five recombinant DI proteins to inhibit a panel of eight purified IgG samples, derived from patients with APS, from binding to β_2 GPI/CL in this assay. All eight IgG samples were known to bind well in this ELISA.

The recombinant DI proteins chosen to test binding in the fluid phase were wild-type DI, two that displayed good binding in the direct ELISA (Mut 8+9 and DI ext), a poor binder (R39S), and one mutant that had been shown in the context of whole β_2 GPI to cause marked reduction in binding to clinically relevant aPL (G40E) (de Laat et al., 2005, Iverson et al., 2002b), although my solid phase ELISA had shown the opposite effect of this mutation in the context of DI in isolation (figure 6.1). After a series of experiments the optimum concentration of inhibitor was found to be 3.25 μ M.

Figure 6.4 shows the results for the fluid phase differed from those for the solid phase. Wild-type DI inhibited binding of the eight IgG samples tested to variable extents (mean +/- SD inhibition 62.9 +/- 25.7%) with the binding of two samples inhibited by >90% and for another two <35% (this data was also shown in section 4.9, figure 4.17). Results were much clearer for Mut 8+9, which inhibited binding of all IgG samples to β_2 GPI/CL (lowest level of inhibition was 72.6% [mean +/- SD inhibition 83.1 +/- 8.9%]). Therefore the combination of D8S and D9G mutations consistently enhanced binding of DI to all APS IgG samples, in both solid and fluid phase assays.

In contrast, altering R39 residue (R39S) had the effect of significantly reducing binding to the majority of aPL in the fluid phase (14 +/- 18.5%) as compared with wild-type DI (p=0.0007). The G40E mutation also showed evidence of overall reduced binding to the IgG samples as compared to wild-type DI (44.1 +/- 31.7%), but the effect was more variable than that observed with R39S. Although adding the remainder of the interlinker region onto the C-terminal of DI had the effect of enhancing binding in the solid phase, this effect was not observed in the fluid phase (43.3 +/- 26.7%, p=0.16).

6.3 Comparison of mutants R39S and Mut 8+9

In the previous chapter (section 5.4, figure 5.14) a computer model of DI shows that the region 234-251, the interdigit region and the D8 and D9 residues lie within the surface of DI. It is of interest to see how these residues are arranged in the tertiary structure of the protein. Swiss-Prot (P01012) contains the changes in the molecular structure of DI that have been reported. The changes are listed in table 6.1. The changes in the binding assays described in this chapter are also listed in table 6.1. The effect of the changes in the binding assays is shown in figure 6.4. The effect of the changes in the binding assays is shown in figure 6.4. The overall tertiary protein structure of DI is illustrated in figure 6.5.

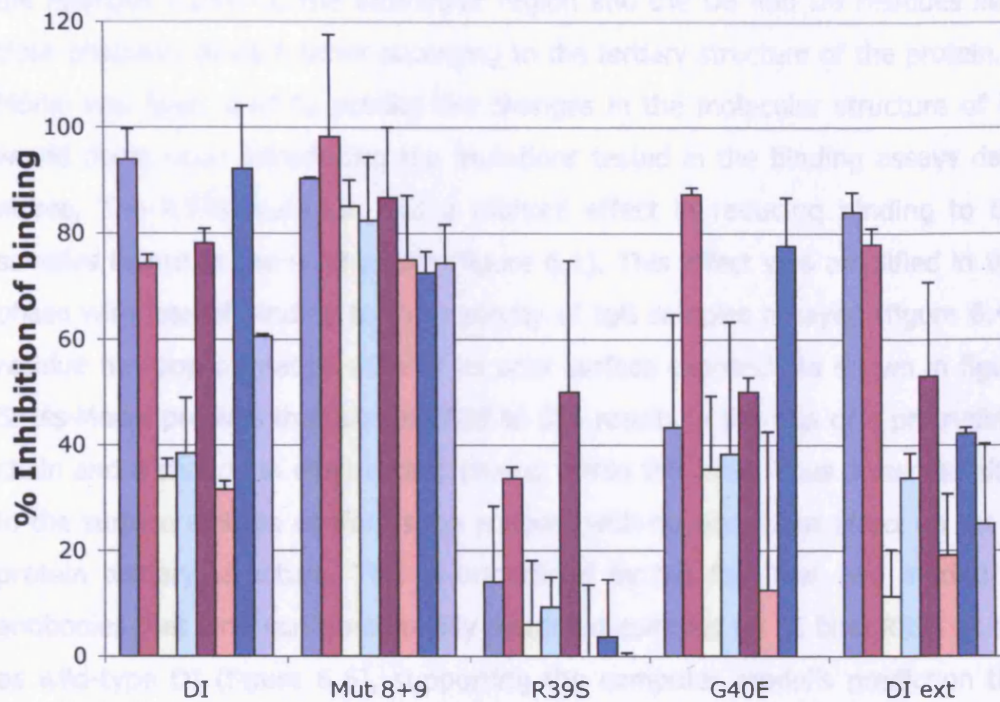


Figure 6.4 Binding of APS derived polyclonal IgG to wild-type DI and mutants in the fluid phase. Recombinant DI inhibitors were used at 3.25 μ M concentration. Variable inhibition was observed with wild-type DI. The R39S mutation resulted in loss of inhibition to the majority of IgG samples ($p=0.0007$) whilst the Mut 8+9 double point mutant caused enhanced binding to the majority of IgG samples in the fluid phase and thus a trend towards enhanced inhibition as compared to wild-type ($p=0.054$).

In the surface of DI at the site of the D6 and D9 residues that is more pronounced upon mutating these to E6 and G9. This is illustrated upon visualising the DI and Mut 8+9 models from multiple angles as shown in figure 6.7. Furthermore, Mut 8+9 causes a small reduction in binding to the two murine anti-DI antibodies (figure 5.5) suggesting some alteration in tertiary protein structure. What is clear however, is that the subtle change in protein structure, perhaps combined with the alteration in charge within this region of DI, has the effect of dramatically altering the binding pattern of DI, enhancing binding to aPL and hence making it likely to be an important region of DI that dictates binding to aPL.

6.3 Computer models of mutants R39S and Mut 8+9

In the previous chapter (section 5.4, figure 5.14) a computer model of DI shows that the epitopes R39-R43, the interlinker region and the D8 and D9 residues lie within close proximity to each other according to the tertiary structure of the protein. Swiss-Model was then used to predict the changes in the molecular structure of DI that would occur upon introducing the mutations tested in the binding assays described above. The R39S mutation had a marked effect in reducing binding to the IgG samples tested in the solid phase (figure 6.1). This effect was amplified in the fluid phase with loss of binding to the majority of IgG samples assayed (figure 6.4). This residue has approximately 60% of its area surface exposed. As shown in figure 6.5, Swiss-Model predicts that altering R39 to S39 results in the loss of a protruding side-chain and a change in electrostatic charge within this area. Thus a marked alteration in the surface epitope conformation is seen, with no significant effect on the overall protein tertiary structure. This is underlined by the fact that two murine anti-DI antibodies that bind conformationally restricted epitopes on DI bind R39S as strongly as wild-type DI (figure 6.6), supporting the computer model's prediction that the overall tertiary protein structure of DI is maintained.

The unexpected finding of a strong trend towards enhanced binding of Mut 8+9 to the majority of APS derived IgG samples tested in both solid and fluid phase assays is more difficult to explain. Figure 6.7 shows that the surface accessibility of mutated residues does not alter. The distance between D8 and D9 from the interlinker is 6Å, which does not alter upon mutating these residues to S8 and G9. However, a groove does exist in the surface of DI at the site of the D8 and D9 residues that is more pronounced upon mutating these to S8 and G9. This is illustrated upon visualising the DI and Mut 8+9 models from multiple angles as shown in figure 6.7. Furthermore, Mut 8+9 causes a small reduction in binding to the two murine anti-DI antibodies (figure 6.6) suggesting some alteration in tertiary protein structure. What is clear however, is that this subtle change in protein structure, perhaps combined with the alteration in charge within this region of DI, has the effect of dramatically altering the binding pattern of DI, enhancing binding to aPL and hence making it likely to be an important region of DI that dictates binding to aPL.

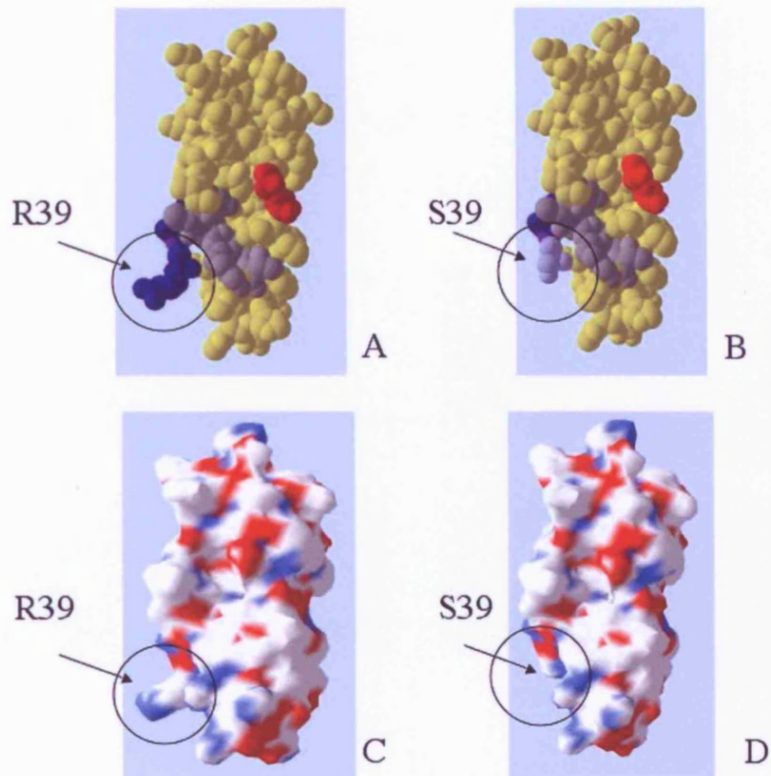


Figure 6.5 Computer model of the DI mutant R39S. Molecular structure for wild-type DI (A) and R39S (B). Figure 6.5-A also highlights the position of the interlinker region (grey) and the D8 and D9 residues (red). Surface structure with electrostatic charge for wild-type DI (C) and R39S (D). The above models show the marked change in the local epitope upon mutating R39 to S39 without any other marked change in overall protein conformation.

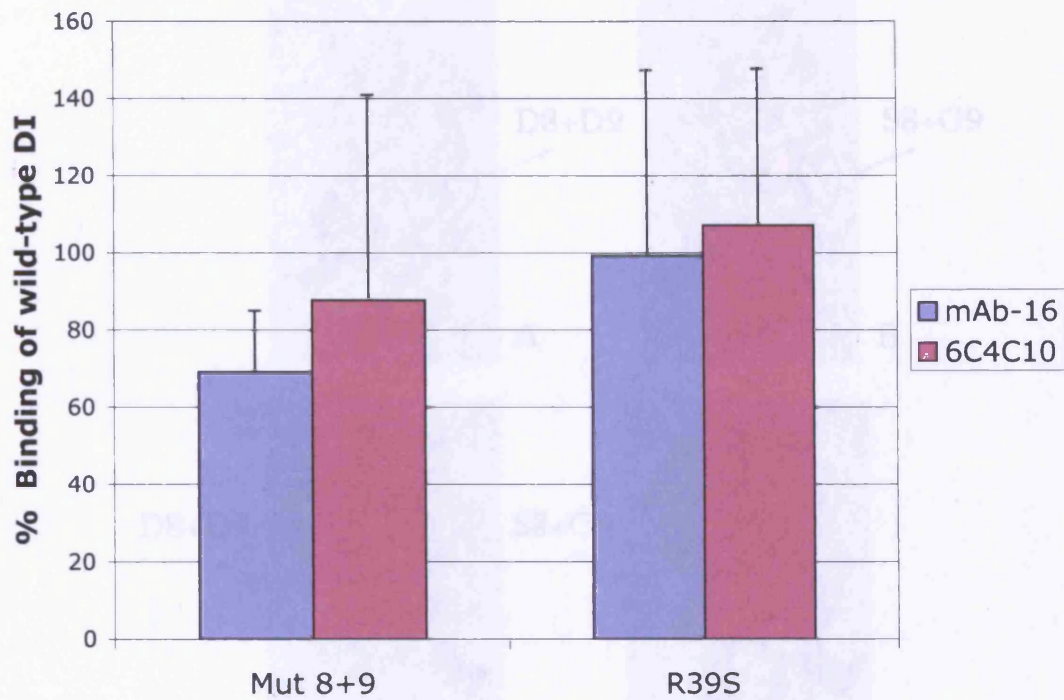


Figure 6.6 Binding of murine anti-DI antibodies to Mut 8+9 and R39S. As compared to wild-type DI, there was some reduction in binding to Mut 8+9 suggesting a small alteration in the tertiary structure as a result of this mutation. R39S revealed no obvious difference from wild-type DI in binding to these antibodies. Error bars represent SD calculated from triplicate wells.

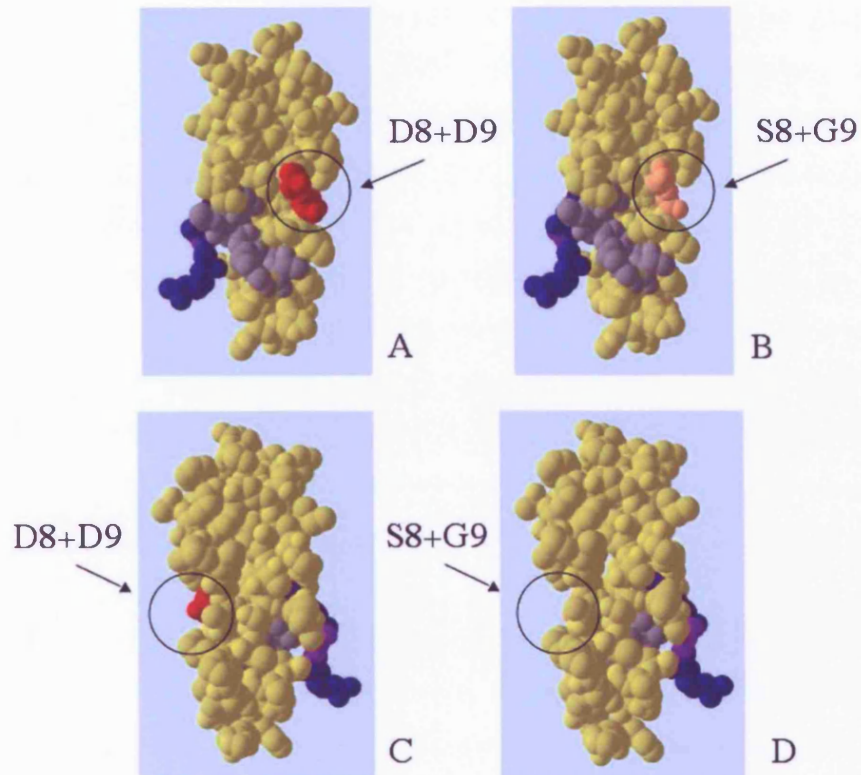


Figure 6.7 Computer model of the DI mutant Mut 8+9. Molecular structures for wild-type DI (A and C rotated 180° on its vertical axis) and Mut 8+9 (B and D rotated 180° on its vertical axis). All four figures also highlight the position of the interlinker region (grey) and the R39-R43 residues (blue). Mutating D8 and D9 to S8 and G9 creates a molecular groove that is better appreciated upon rotating the protein 180° on its vertical axis.

6.4 Discussion of results

The DI mutants created and tested were designed to test three hypotheses regarding immunodominant aPL binding epitopes outlined in the previous Chapters. The binding data of these mutants supported some of these hypotheses, but also yielded other unexpected, yet intriguing findings. In comparing my results with those of previous groups it is important to stress that I studied isolated recombinant DI, whereas investigators in most previous studies examined whole β_2 GPI containing mutations in DI. However, meaningful comparisons may be made, as I have demonstrated that DI in isolation binds clinically relevant IgG in a manner comparable to whole β_2 GPI and CL. Furthermore, it is recognised that as a general property of the CCP family of proteins, that β_2 GPI belongs to, isolated CCP domains are representative of their structure and dynamics as observed in the presence of neighbouring domains (O'Leary et al., 2004).

Despite this difference in methodology, my results confirmed the previous finding that region G40-R43 is likely to be important in binding to aPL, but these mutational studies lead to the new idea, and confirmed the hypothesis, that R39 plays a particularly important role. Altering R39 to serine had a greater effect on reducing binding to APS derived IgG in the fluid phase than the G40E mutation that had previously been studied in the context of whole β_2 GPI (Iverson et al., 2002b, de Laat et al., 2005). The R39S change reduced binding to IgG derived from APS patients in both the solid phase and fluid phase assays, whereas G40E reduced binding only in the fluid phase. Iverson et al studied 10 mutants of whole β_2 GPI with changes in DI, but none of these had changes at R39. Their selection of mutations was not hypothesis driven, but was based on an initial screen of a phage peptide display library of DI fragments in which mutations had been incorporated by a random process through error-prone PCR (Iverson et al., 2002b).

Some evidence for involvement of an epitope in the DI-II interlinker region was observed, as the extended DI (DI ext) molecule bound better than wild-type DI in the solid phase ELISA. However, these results were not confirmed in the fluid phase. It may be that mutations within this area are required in order to observe changes in the binding of aPL in the fluid phase. Unfortunately attempts to express a DI mutant with the DII-III interlinker substituted for the DI-II interlinker were unsuccessful, suggesting that point mutational studies of this region would be necessary. On

studying the structure of DI, the R43 residue forms a H-bond with both R39 and T57 located within the interlinker, which are both lost upon altering R43 to G43. Thus it would be of interest to target T57 for future mutational studies given the close proximity this residue has with the R39-R43 region.

Although the G40E mutation showed poor binding in the fluid phase as compared to wild-type DI, enhanced binding of this mutant and R43G was observed in the solid phase. The observation that recombinant human DI in isolation may behave differently in solid and fluid phase ELISAs is in keeping with previous observations by other investigators using whole β_2 GPI (de Laat et al., 2006b, Giles et al., 2003c, Iverson et al., 2002a). It is interesting to note in Iverson et al.'s point mutational study of DI within whole β_2 GPI, that the mutants M42K and M42V did show enhanced binding to affinity-purified aPL in the solid-phase as compared to wild-type β_2 GPI, but the increase was not as large, or as repeatable, in the fluid phase (Iverson et al., 2002b). The authors made no mention of these observations nor offered a potential explanation in their discussion. One possible explanation for this would be that some mutations in DI alter its ability to bind to the nickel plate, thus affecting the density of the antigen available for binding. Such alterations in coating density would affect binding to aPL in the solid phase not in the fluid phase ELISA. However, this is an unlikely explanation for the results observed in our tests, because binding to the plate occurred via a his-tag that was the same in all mutants, and because the direct measurements of binding of wild-type DI, Mut 8+9, and R39K using an anti-his-tag antibody showed these mutants coated the plate at similar densities despite giving widely divergent results in assays of binding to aPL. An alternative explanation is that some of the mutations alter the way in which the recombinant DI molecules pack together on the plate, thus affecting the way in which the epitopes are exposed to aPL. This underlines the importance of carrying out both solid and fluid phase ELISAs, because the latter are not affected by these plate-based considerations. The fact that the R39S mutant exhibited reduced binding to APS derived IgG, in both the solid and fluid phase assays, supports the hypothesis that this residue is particularly crucial for conferring binding to aPL due to its considerable degree of surface exposure, as shown in the computer models of DI.

Intriguingly, these mutational studies also demonstrate, for the first time, the production of a mutagenised variant of DI (Mut 8+9) in which mutations (D8S and D9G) are associated with markedly enhanced binding to the majority of APS derived

IgG tested, in both solid phase and fluid phase assays. This observation of enhanced binding upon altering D8 and D9 to S8 and G9, respectively, was unexpected and did not concur with my original hypothesis that interactions between the R residues in aPL and the D residues on DI contribute to the antigen-antibody interaction in a charge dependent manner. One may speculate that the N-terminal region of DI is likely to play a complex role in ensuring a stable aPL-DI interaction and altering this area seems to have a variable effect on binding, depending on the alteration. This complexity is underlined by the fact that the single mutations D8S and D9G both reduced binding to aPL even though the combination of D8S and D9G increased binding to the same panel of IgG samples. One possibility is that altering both residues creates a groove within DI that may favour a tighter fit with the antigen binding regions of the majority of aPL, shown in the computer model of figure 6.7. Although the cause of the binding properties of Mut 8+9 remains unclear, the fact that this product can potentially be expressed and purified from bacteria in large quantities and can inhibit binding of polyclonal aPL to whole β_2 GPI bound to anionic PL could have therapeutic implications.

CHAPTER 7

OVERVIEW AND IDEAS FOR FUTURE WORK

7.1 Summary of results

In this thesis work is described that expands our understanding about the precise nature of the molecular interaction of autoreactive aPL with their antigenic target DI of human β_2 GPI. These detailed mutational studies support the theory that the majority of aPL bind discontinuous epitopes neighbouring R39-R43 located within the tertiary structure of DI. Specifically the key achievements in this thesis were:

1. The development of an efficient system for expressing and purifying recombinant DI of β_2 GPI from *E.coli* cells.
2. The demonstration that recombinant DI expressed from *E.coli* binds clinically relevant monoclonal and polyclonal IgG in both the solid and fluid phase.
3. The use of this novel platform of DI production to create multiple mutants of DI with single / double point mutations targeting key areas of interest.
4. Confirmation that the region G40 to R43 is important but also propose the new idea that R39 is a particularly crucial residue participating in the aPL-DI interaction.
5. Definition of new areas within DI that are also likely to be involved in this interaction such as residues D8-D9 and the DI-II interlinker region.
6. The creation of a mutant of DI that binds the majority of clinically relevant polyclonal IgG better than wild type DI in both solid and fluid phase assays.
7. The use of computer modelling studies to demonstrate the close proximity of these discontinuous epitopes and visualise the changes in the molecular protein structure upon incorporating the mutations studied

7.2 Inhibiting the biological properties of aPL

Recombinant DI binds clinically relevant IgG in assays of binding *in vitro*, but does it necessarily follow that DI could bind to aPL and inhibit their functional effects in terms of promoting a thrombophilic phenotype? Currently one of the best animal models in which the thrombogenic effects of aPL have been demonstrated is the *in vivo* model of microcirculation developed by Pierangeli et al (Pierangeli et al., 1994). Both polyclonal and monoclonal antibodies including IS4 have been shown to be highly thrombogenic and to enhance endothelial activation in this *in vivo* model (Pierangeli et al., 2000, Pierangeli et al., 2001). I have developed a collaboration

with Professor Pierangeli who was very interested in investigating the ability of *E.coli* expressed DI to inhibit aPL enhancement of thrombosis in her femoral vein pinch mouse model. *E.coli* expressed recombinant wild-type DI was sent to Professor Pierangeli to perform these experiments. As a negative control an irrelevant his-tagged protein (DsbA) was also expressed and purified. Injecting the peritoneum of mice with 500µg of IgG purified from patients with APS enhanced thrombus formation and injecting mice with 40µg of the control his-tagged protein intravenously, prior to the femoral vein pinch, had no effect on the size of thrombus enhanced by aPL. However, if mice were injected with 40µg of recombinant DI, aPL enhancement of thrombus was inhibited down to the level of that seen with mice injected with control IgG. These data were presented at the American College of Rheumatology annual general meeting in 2006 (Ioannou et al., 2006b) and provides strong support for the clinical relevance of the binding studies presented in this thesis. It would also be of interest to investigate the ability of recombinant wild-type DI to inhibit the ability of aPL to activate human ECs and platelets *in vitro*.

It is intended that the above studies will be extended further to assess the relative ability of R39S and Mut 8+9 to inhibit aPL induced thrombus enhancement in mice as compared to wild-type DI. Furthermore, it would be of interest to test the abilities of these mutants to inhibit aPL induced activation of human ECs and platelets as compared to wild-type DI. I have set up a collaboration with Professor Krilis (Sydney, Australia) who has the necessary expertise in investigating the effects of aPL upon human platelets as well as ECs and I intend to perform such experiments through an approved Arthritis Research Campaign (**arc**) Clinician Scientist Award.

Finally La Jolla Pharmaceuticals have developed a drug called LJP 1082, which carries the wild-type DI molecule and can bind to DI-binding aPL on the surface of B cells (Cockerill et al., 2003, Horizon et al., 2003). Given that there is a lack of T cell epitopes, the B cell cannot recruit T cell help and dies. It is conceivable that the replacement of DI in this drug with a mutant of DI that binds a greater number of clinically relevant aPL (such as the mutant with the D8S/D9G mutations) would increase the ability of this drug to bind to and tolerate B cells producing anti-β₂GPI antibodies.

7.3 Work towards a smaller blocking peptide

This thesis demonstrates that whole DI and the mutant containing D8S/D9G are potent inhibitors of aPL. Particularly given that wild-type DI may inhibit the thrombotic effects of aPL *in vivo*, it raises the possibility as alluded to in the above section that such peptides may be used therapeutically to block the pathogenic effects of aPL. However, for reasons such as bioavailability, immunogenicity and ease of manufacture, smaller peptides containing parts of the DI structure may be better therapeutic agents. It is likely that such peptides will need to contain the epitopes described in this thesis and this raises issues as to how the peptides will fold and how stable they will be. To explore these issues I have set up a collaboration with Dr Alethea Tabor (Department of Organic / Chemical Biology, UCL) and plan to develop small peptides modelled on the epitopes described. These peptides could be produced through a variety of methods such as the development of mimetopes by screening peptide libraries based on the amino acid sequences of interest, or by coupling short peptides together again based upon the candidate discontinuous epitopes. Such peptides could then be tested in assays of binding and biological function and may serve as candidate therapeutic agents if shown to be stable and to inhibit the pathogenic effects of aPL. Again this is work I intend to explore in my **arc** funded post-doc studies.

Ultimately, it is envisaged that these ideas for future work, which have directly arisen from work described in this thesis, will lead to safer and more effective therapies for APS patients than that which is currently available.

APPENDICES

APPENDIX A

ONE AND THREE LETTER CODES FOR AMINO ACIDS

ONE LETTER CODE	THREE LETTER CODE	AMINO ACID
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

APPENDIX B

ORIGINAL ARTICLES ARISING FROM WORK DESCRIBED IN THIS THESIS

Ioannou, Y., Giles, I., Lambrianides, A., Richardson, C., Pearl, L., Latchman, D. S., Isenberg, D. A. and Rahman, A. (2006) A novel expression system of domain I of human beta2 glycoprotein I in *E.coli*. *BMC Biotechnology*, 6, 8-18.

Ioannou, Y., Pericleous, C., Giles, I., Latchman, D. S., Isenberg, D. A. and Rahman, A. (2007) Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of beta2-glycoprotein: mutation studies including R39 to R43. *Arthritis and Rheumatism*, 56, 280-90.

Lambrianides, A., Giles, I., Ioannou, Y., Mason, L., Latchman, D. S., Manson, J., Isenberg, D. A. and Rahman, A. (2007) Arginine mutation alters binding of a human monoclonal antibody to antigens to systemic lupus erythematosus and the antiphospholipid syndrome. *Arthritis and Rheumatism*, 56, in press.

REFERENCES

- APASS. (1993) Anticardiolipin antibodies are an independent risk factor for first ischemic stroke. The Antiphospholipid Antibodies in Stroke Study (APASS) Group. *Neurology*, 43, 2069-73.
- 4S. (1994) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet*, 344, 1383-9.
- Abraham Simon, J., Rojas-Serrano, J., Cabiedes, J. & Alcocer-Varela, J. (2004) Antinucleosome antibodies may help predict development of systemic lupus erythematosus in patients with primary antiphospholipid syndrome. *Lupus*, 13, 177-81.
- Abu-Shakra, M., Urowitz, M. B., Gladman, D. D. & Gough, J. (1995) Mortality studies in systemic lupus erythematosus. Results from a single center. II. Predictor variables for mortality. *J Rheumatol*, 22, 1265-70.
- Adams, M. J., Donohoe, S., Mackie, I. J. & Machin, S. J. (2001) Anti-tissue factor pathway inhibitor activity in patients with primary antiphospholipid syndrome. *Br J Haematol*, 114, 375-9.
- Adari, H., Andrews, B., Ford, P. J., Hannig, G., Brosius, J. & Makrides, S. C. (1995) Expression of the human T-cell receptor V beta 5.3 in *Escherichia coli* by thermal induction of the *trc* promoter: nucleotide sequence of the *lacI* gene. *DNA Cell Biol*, 14, 945-50.
- Ahmed, E., Stegmayr, B., Trifunovic, J., Weinehall, L., Hallmans, G. & Lefvert, A. K. (2000) Anticardiolipin antibodies are not an independent risk factor for stroke: an incident case-referent study nested within the MONICA and Vasterbotten cohort project. *Stroke*, 31, 1289-93.
- Aikawa, M., Rabkin, E., Sugiyama, S., Voglic, S. J., Fukumoto, Y., Furukawa, Y., Shiomi, M., Schoen, F. J. & Libby, P. (2001) An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. *Circulation*, 103, 276-83.
- Alarcon-Segovia, D., Boffa, M. C., Branch, W., Cervera, R., Gharavi, A., Khamashta, M., Shoenfeld, Y., Wilson, W. & Roubey, R. (2003a) Prophylaxis of the antiphospholipid syndrome: a consensus report. *Lupus*, 12, 499-503.
- Alarcon-Segovia, D., Deleze, M., Oria, C. V., Sanchez-Guerrero, J., Gomez-Pacheco, L., Cabiedes, J., Fernandez, L. & Ponce De Leon, S. (1989) Antiphospholipid antibodies and the antiphospholipid syndrome in systemic lupus erythematosus. A prospective analysis of 500 consecutive patients. *Medicine (Baltimore)*, 68, 353-65.
- Alarcon-Segovia, D. & Sanchez-Guerrero, J. (1989) Primary antiphospholipid syndrome. *J Rheumatol*, 16, 482-8.
- Alarcon-Segovia, D., Tumlin, J. A., Furie, R. A., McKay, J. D., Cardiel, M. H., Strand, V., Bagin, R. G., Linnik, M. D. & Hepburn, B. (2003b) LJP 394 for the prevention of renal flare in patients with systemic lupus erythematosus: results from a randomized, double-blind, placebo-controlled study. *Arthritis Rheum*, 48, 442-54.
- Altmann, F., Staudacher, E., Wilson, I. B. & Marz, L. (1999) Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj J*, 16, 109-23.
- Ameli, S., Hultgardh-Nilsson, A., Regnstrom, J., Calara, F., Yano, J., Cercek, B., Shah, P. K. & Nilsson, J. (1996) Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol*, 16, 1074-9.

- Amengual, O., Atsumi, T., Khamashta, M. A. & Hughes, G. R. (1998) The role of the tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. *Thromb Haemost*, 79, 276-81.
- Amit, A. G., Mariuzza, R. A., Phillips, S. E. & Poljak, R. J. (1986) Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. *Science*, 233, 747-53.
- Andrews, B., Adari, H., Hannig, G., Lahue, E., Gosselin, M., Martin, S., Ahmed, A., Ford, P. J., Hayman, E. G. & Makrides, S. C. (1996) A tightly regulated high level expression vector that utilizes a thermosensitive lac repressor: production of the human T cell receptor V beta 5.3 in *Escherichia coli*. *Gene*, 182, 101-9.
- Arai, T., Yoshida, K., Kaburaki, J., Inoko, H., Ikeda, Y., Kawakami, Y. & Kuwana, M. (2001) Autoreactive CD4(+) T-cell clones to beta2-glycoprotein I in patients with antiphospholipid syndrome: preferential recognition of the major phospholipid-binding site. *Blood*, 98, 1889-96.
- Arnett, F. C., Thiagarajan, P., Ahn, C. & Reveille, J. D. (1999) Associations of anti-beta2-glycoprotein I autoantibodies with HLA class II alleles in three ethnic groups. *Arthritis Rheum*, 42, 268-74.
- Arnold, J., Holmes, Z., Pickering, W., Farmer, C., Regan, L. & Cohen, H. (2001) Anti-beta 2 glycoprotein 1 and anti-annexin V antibodies in women with recurrent miscarriage. *Br J Haematol*, 113, 911-4.
- Asherson, R. A. (1988) A "primary" antiphospholipid syndrome? *J Rheumatol*, 15, 1742-6.
- Asherson, R. A. (1992) The catastrophic antiphospholipid syndrome. *J Rheumatol*, 19, 508-12.
- Asherson, R. A., Cervera, R., De Groot, P. G., Erkan, D., Boffa, M. C., Piette, J. C., Khamashta, M. A. & Shoenfeld, Y. (2003) Catastrophic antiphospholipid syndrome: international consensus statement on classification criteria and treatment guidelines. *Lupus*, 12, 530-4.
- Asherson, R. A., Cervera, R., Piette, J. C., Font, J., Lie, J. T., Burcoglu, A., Lim, K., Munoz-Rodriguez, F. J., Levy, R. A., Boue, F., Rossert, J. & Ingelmo, M. (1998) Catastrophic antiphospholipid syndrome. Clinical and laboratory features of 50 patients. *Medicine (Baltimore)*, 77, 195-207.
- Asherson, R. A., Cervera, R., Piette, J. C., Shoenfeld, Y., Espinosa, G., Petri, M. A., Lim, E., Lau, T. C., Gurjal, A., Jedryka-Goral, A., Chwalinska-Sadowska, H., Dibner, R. J., Rojas-Rodriguez, J., Garcia-Carrasco, M., Grandone, J. T., Parke, A. L., Barbosa, P., Vasconcelos, C., Ramos-Casals, M., Font, J. & Ingelmo, M. (2001) Catastrophic antiphospholipid syndrome: clues to the pathogenesis from a series of 80 patients. *Medicine (Baltimore)*, 80, 355-77.
- Asherson, R. A., Doherty, D. G., Vergani, D., Khamashta, M. A. & Hughes, G. R. (1992) Major histocompatibility complex associations with primary antiphospholipid syndrome. *Arthritis Rheum*, 35, 124-5.
- Asherson, R. A., Khamashta, M. A., Ordi-Ros, J., Derksen, R. H., Machin, S. J., Barquinero, J., Outt, H. H., Harris, E. N., Vilardell-Torres, M. & Hughes, G. R. (1989) The "primary" antiphospholipid syndrome: major clinical and serological features. *Medicine (Baltimore)*, 68, 366-74.
- Atsumi, T., Amengual, O., Yasuda, S. & Koike, T. (2004) Antiprothrombin antibodies--are they worth assaying? *Thromb Res*, 114, 533-8.
- Atsumi, T., Ieko, M., Bertolaccini, M. L., Ichikawa, K., Tsutsumi, A., Matsuura, E. & Koike, T. (2000) Association of autoantibodies against the phosphatidylserine-prothrombin complex with manifestations of the antiphospholipid syndrome and with the presence of lupus anticoagulant. *Arthritis Rheum*, 43, 1982-93.
- Atsumi, T., Khamashta, M. A., Haworth, R. S., Brooks, G., Amengual, O., Ichikawa, K., Koike, T. & Hughes, G. R. (1998) Arterial disease and thrombosis in the

- antiphospholipid syndrome: a pathogenic role for endothelin 1. *Arthritis Rheum*, 41, 800-7.
- Atsumi, T., Tsutsumi, A., Amengual, O., Khamashta, M. A., Hughes, G. R., Miyoshi, Y., Ichikawa, K. & Koike, T. (1999) Correlation between beta2-glycoprotein I valine/leucine247 polymorphism and anti-beta2-glycoprotein I antibodies in patients with primary antiphospholipid syndrome. *Rheumatology (Oxford)*, 38, 721-3.
- Audrain, M. A., Colonna, F., Morio, F., Hamidou, M. A. & Muller, J. Y. (2004) Comparison of different kits in the detection of autoantibodies to cardiolipin and beta2glycoprotein 1. *Rheumatology (Oxford)*, 43, 181-5.
- Averna, M., Paravizzini, G., Marino, G., Emmanuele, G., Cefalu, A. B., Magro, G., Bartoloni, G., Ragusa, M., Noto, D., Barbagallo, C. M., Callari, D., Mazzarino, M. C., Notarbartolo, A. & Travali, S. (2004) Beta-2-glycoprotein I is growth regulated and plays a role as survival factor for hepatocytes. *Int J Biochem Cell Biol*, 36, 1297-305.
- Averna, M., Paravizzini, G., Marino, G., Lanteri, E., Cavera, G., Barbagallo, C. M., Petralia, S., Cavallaro, S., Magro, G., Grasso, S., Notarbartolo, A. & Travali, S. (1997) Liver is not the unique site of synthesis of beta 2-glycoprotein I (apolipoprotein H): evidence for an intestinal localization. *Int J Clin Lab Res*, 27, 207-12.
- Bach, R. R. & Moldow, C. F. (1997) Mechanism of tissue factor activation on HL-60 cells. *Blood*, 89, 3270-6.
- Bajaj, S. P., Rapaport, S. I., Fierer, D. S., Herbst, K. D. & Schwartz, D. B. (1983) A mechanism for the hypoprothrombinemia of the acquired hypoprothrombinemia-lupus anticoagulant syndrome. *Blood*, 61, 684-92.
- Baker, K. P., Edwards, B. M., Main, S. H., Choi, G. H., Wager, R. E., Halpern, W. G., Lappin, P. B., Riccobene, T., Abramian, D., Sekut, L., Sturm, B., Poortman, C., Minter, R. R., Dobson, C. L., Williams, E., Carmen, S., Smith, R., Roschke, V., Hilbert, D. M., Vaughan, T. J. & Albert, V. R. (2003) Generation and characterization of LymphoStat-B, a human monoclonal antibody that antagonizes the bioactivities of B lymphocyte stimulator. *Arthritis Rheum*, 48, 3253-65.
- Bakimer, R., Fishman, P., Blank, M., Sredni, B., Djaldetti, M. & Shoenfeld, Y. (1992) Induction of primary antiphospholipid syndrome in mice by immunization with a human monoclonal anticardiolipin antibody (H-3). *J Clin Invest*, 89, 1558-63.
- Bancsi, L. F., Van Der Linden, I. K. & Bertina, R. M. (1992) Beta 2-glycoprotein I deficiency and the risk of thrombosis. *Thromb Haemost*, 67, 649-53.
- Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotechnol*, 10, 411-21.
- Bardwell, J. C., MCGovern, K. & Beckwith, J. (1991) Identification of a protein required for disulfide bond formation in vivo. *Cell*, 67, 581-9.
- Baron, M. A., Khamashta, M. A., Hughes, G. R. V. & D'Cruz, D. P. D. (2005) Prevalence of an abnormal ankle-brachial index in patients with primary antiphospholipid syndrome: preliminary data. *Annals Rheum Dis*, 64, 144-6.
- Baumann, I., Kolowos, W., Voll, R. E., Manger, B., Gaipl, U., Neuhuber, W. L., Kirchner, T., Kalden, J. R. & Hermann, M. (2002) Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum*, 46, 191-201.
- Baumert, T. F., Ito, S., Wong, D. T. & Liang, T. J. (1998) Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J Virol*, 72, 3827-36.
- Baumgarth, N., Tung, J. W. & Herzenberg, L. A. (2005) Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Semin Immunopathol*, 26, 347-62.

- Baurmash, H. D. (2004) Diagnosing primary and secondary Sjogren's syndrome. *J Oral Maxillofac Surg*, 62, 764-5; author reply 765-6.
- Beacham, D. A., Lian, J., Wu, G., Konkle, B. A., Ludlow, L. B. & Shapiro, S. S. (1999) Arterial shear stress stimulates surface expression of the endothelial glycoprotein Ib complex. *J Cell Biochem*, 73, 508-21.
- Beacham, D. A., Tran, L. P. & Shapiro, S. S. (1997) Cytokine treatment of endothelial cells increases glycoprotein Ib alpha-dependent adhesion to von Willebrand factor. *Blood*, 89, 4071-7.
- Berman, J., Girardi, G. & Salmon, J. E. (2005) TNF-alpha is a critical effector and a target for therapy in antiphospholipid antibody-induced pregnancy loss. *J Immunol*, 174, 485-90.
- Bertolaccini, M. L., Atsumi, T., Khamashta, M. A., Amengual, O. & Hughes, G. R. (1998) Autoantibodies to human prothrombin and clinical manifestations in 207 patients with systemic lupus erythematosus. *J Rheumatol*, 25, 1104-8.
- Bessette, P. H., Aslund, F., Beckwith, J. & Georgiou, G. (1999) Efficient folding of proteins with multiple disulfide bonds in the Escherichia coli cytoplasm. *Proc Natl Acad Sci U S A*, 96, 13703-8.
- Betteli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. & Kuchroo, V. K. (2006) Reciprocal development pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*, 441, 235-8.
- Bervers, E. M., Galli, M., Barbui, T., Comfurius, P. & Zwaal, R. F. (1991) Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. *Thromb Haemost*, 66, 629-32.
- Bervers, E. M., Janssen, M. P., Comfurius, P., Balasubramanian, K., Schroit, A. J., Zwaal, R. F. & Willems, G. M. (2005) Quantitative determination of the binding of beta2-glycoprotein I and prothrombin to phosphatidylserine-exposing blood platelets. *Biochem J*, 386, 271-9.
- Blank, M., Cohen, J., Toder, V. & Shoenfeld, Y. (1991) Induction of anti-phospholipid syndrome in naive mice with mouse lupus monoclonal and human polyclonal anti-cardiolipin antibodies. *Proc Natl Acad Sci U S A*, 88, 3069-73.
- Blank, M., Faden, D., Tincani, A., Kopolovic, J., Goldberg, I., Gilburd, B., Allegri, F., Balestrieri, G., Valesini, G. & Shoenfeld, Y. (1994) Immunization with anticardiolipin cofactor (beta-2-glycoprotein I) induces experimental antiphospholipid syndrome in naive mice. *J Autoimmun*, 7, 441-55.
- Blank, M., Krause, I., Fridkin, M., Keller, N., Kopolovic, J., Goldberg, I., Tobar, A. & Shoenfeld, Y. (2002) Bacterial induction of autoantibodies to beta2-glycoprotein-I accounts for the infectious etiology of antiphospholipid syndrome. *J Clin Invest*, 109, 797-804.
- Blank, M., Shoenfeld, Y., Cabilly, S., Heldman, Y., Fridkin, M. & Katchalski-Katzir, E. (1999) Prevention of experimental antiphospholipid syndrome and endothelial cell activation by synthetic peptides. *Proc Natl Acad Sci U S A*, 96, 5164-8.
- Bohgaki, M., Atsumi, T., Yamashita, Y., Yasuda, S., Sakai, Y., Furusaki, A., Bohgaki, T., Amengual, O., Amasaki, Y. & Koike, T. (2004) The p38 mitogen-activated protein kinase (MAPK) pathway mediates induction of the tissue factor gene in monocytes stimulated with human monoclonal anti-beta2Glycoprotein I antibodies. *Int Immunol*, 16, 1633-41.
- Bonet, B., Hauge-Gillenwater, H., Zhu, X. D. & Knopp, R. H. (1998) LDL oxidation and human placental trophoblast and macrophage cytotoxicity. *Proc Soc Exp Biol Med*, 217, 203-11.
- Borchman, D., Harris, E. N., Pierangeli, S. S. & Lamba, O. P. (1995) Interactions and molecular structure of cardiolipin and beta 2-glycoprotein 1 (beta 2-GP1). *Clin Exp Immunol*, 102, 373-8.
- Borrell, M., Sala, N., De Castellarnau, C., Lopez, S., Gari, M. & Fontcuberta, J. (1992) Immunoglobulin fractions isolated from patients with antiphospholipid

- antibodies prevent the inactivation of factor Va by activated protein C on human endothelial cells. *Thromb Haemost*, 68, 268-72.
- Botto, M., Agnola, C. D., Bygrave, A. E., Thompson, M. E., Cook, T. H., Petry, F., Loos, M., Pandolfi, P. P. & Walport, M. (1998) Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nature Genetics*, 19, 56-9.
- Bouma, B., De Groot, P. G., Van Den Elsen, J. M., Ravelli, R. B., Schouten, A., Simmelink, M. J., Derksen, R. H., Kroon, J. & Gros, P. (1999) Adhesion mechanism of human beta(2)-glycoprotein I to phospholipids based on its crystal structure. *Embo J*, 18, 5166-74.
- Boumpas, D. T., Austin, H. A., 3rd, Vaughn, E. M., Klippel, J. H., Steinberg, A. D., Yarboro, C. H. & Balow, J. E. (1992) Controlled trial of pulse methylprednisolone versus two regimens of pulse cyclophosphamide in severe lupus nephritis. *Lancet*, 340, 741-5.
- Boura, P., Papadopoulos, S., Tselios, K., Skendros, P., Dioritou, O., Malamis, G., Makris, P. & Lefkos, N. (2005) Intracerebral hemorrhage in a patient with SLE and catastrophic antiphospholipid syndrome (CAPS): report of a case. *Clin Rheumatol*, 24, 420-4.
- Bourcier, T. & Libby, P. (2000) HMG CoA reductase inhibitors reduce plasminogen activator inhibitor-1 expression by human vascular smooth muscle and endothelial cells. *Arterioscler Thromb Vasc Biol*, 20, 556-62.
- Bowie, E. J., Thompson, J. H., Jr., Pascuzzi, C. A. & Owen, C. A., Jr. (1963) Thrombosis In Systemic Lupus Erythematosus Despite Circulating Anticoagulants. *J Lab Clin Med*, 62, 416-30.
- Brachvogel, B., Dikschas, J., Moch, H., Welzel, H., Von Der Mark, K., Hofmann, C. & Poschl, E. (2003) Annexin A5 is not essential for skeletal development. *Mol Cell Biol*, 23, 2907-13.
- Brandt, J. T., Triplett, D. A., Alving, B. & Scharrer, I. (1995) Criteria for the diagnosis of lupus anticoagulants: an update. On behalf of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. *Thromb Haemost*, 74, 1185-90.
- Brey, R. L., Abbott, R. D., Curb, J. D., Sharp, D. S., Ross, G. W., Stallworth, C. L. & Kittner, S. J. (2001) beta(2)-Glycoprotein 1-dependent anticardiolipin antibodies and risk of ischemic stroke and myocardial infarction: the honolulu heart program. *Stroke*, 32, 1701-6.
- Brey, R. L., Hart, R. G., Sherman, D. G. & Tegeler, C. H. (1990) Antiphospholipid antibodies and cerebral ischemia in young people. *Neurology*, 40, 1190-6.
- Brinkmann, U., Mattes, R. E. & Buckel, P. (1989) High-level expression of recombinant genes in Escherichia coli is dependent on the availability of the dnaY gene product. *Gene*, 85, 109-14.
- Bucciarelli, S., Espinosa, G., Asherson, R. A., Cervera, R., Claver, G., Gomez-Puerta, J. A., Ramos-Casals, M. & Ingelmo, M. (2006a) The acute respiratory distress syndrome in catastrophic antiphospholipid syndrome: analysis of a series of 47 patients. *Ann Rheum Dis*, 65, 81-6.
- Bucciarelli, S., Espinosa, G., Cervera, R., Erkan, D., Gomez-Puerta, J. A., Ramos-Casals, M., Font, J. & Asherson, R. A. (2006b) Mortality in the catastrophic antiphospholipid syndrome: causes of death and prognostic factors in a series of 250 patients. *Arthritis Rheum*, 54, 2568-76.
- Buckholz, R. G. & Gleeson, M. A. (1991) Yeast systems for the commercial production of heterologous proteins. *Biotechnology (N Y)*, 9, 1067-72.
- Burt, R. K. & Traynor, A. E. (2003) SLE - hematopoietic stem cell transplantation for systemic lupus erythematosus. *Arthritis Res Ther*, 5, 207-9.
- Bussel, J., Miller, S., Hilgartner, M., O'reilly, R., Kempin, S., Pollack, M. & Miller, D. (1983) Transient appearance of the lupus anticoagulant in three family

- members sharing the A11B35DR4 haplotype. *Am J Pediatr Hematol Oncol*, 5, 275-8.
- Caligiuri, G., Nicoletti, A., Poirier, B. & Hansson, G. K. (2002) Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest*, 109, 745-53.
- Caliz, R., Atsumi, T., Kondeatis, E., Amengual, O., Khamashta, M. A., Vaughan, R. W., Lanchbury, J. S. & Hughes, G. R. (2001) HLA class II gene polymorphisms in antiphospholipid syndrome: haplotype analysis in 83 Caucasoid patients. *Rheumatology (Oxford)*, 40, 31-6.
- Cambridge, G., Leandro, M. J., Edwards, J. C., Ehrenstein, M. R., Salden, M., Bodman-Smith, M. & Webster, A. D. (2003) Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis. *Arthritis Rheum*, 48, 2146-54.
- Camps, M. T., Cuadrado, M. J., Ocon, P., Alonso, A., Gutierrez, A., Guil, M., Grana, M. I. & De Ramon, E. (1995) Association between HLA class II antigens and primary antiphospholipid syndrome from the south of Spain. *Lupus*, 4, 51-5.
- Cariou, R., Tobelem, G., Bellucci, S., Soria, J., Soria, C., Maclouf, J. & Caen, J. (1988) Effect of lupus anticoagulant on antithrombotic properties of endothelial cells--inhibition of thrombomodulin-dependent protein C activation. *Thromb Haemost*, 60, 54-8.
- Carlin, A. J., Farquharson, R. G., Quenby, S. M., Topping, J. & Fraser, W. D. (2004) Prospective observational study of bone mineral density during pregnancy: low molecular weight heparin versus control. *Hum Reprod*, 19, 1211-4.
- Caronti, B., Calderaro, C., Alessandri, C., Conti, F., Tinghino, R., Palladini, G. & Valesini, G. (1999) Beta2-glycoprotein I (beta2-GPI) mRNA is expressed by several cell types involved in anti-phospholipid syndrome-related tissue damage. *Clin Exp Immunol*, 115, 214-9.
- Carreras, L. O., Defreyn, G., Machin, S. J., Vermylen, J., Deman, R., Spitz, B. & Van Assche, A. (1981a) Arterial thrombosis, intrauterine death and "lupus" anticoagulant: detection of immunoglobulin interfering with prostacyclin formation. *Lancet*, 1, 244-6.
- Carreras, L. O., Vermylen, J., Spitz, B. & Van Assche, A. (1981b) "Lupus" anticoagulant and inhibition of prostacyclin formation in patients with repeated abortion, intrauterine growth retardation and intrauterine death. *Br J Obstet Gynaecol*, 88, 890-4.
- Cervený, K. C. & Sawitzke, A. D. (1999) Relapsing catastrophic antiphospholipid antibody syndrome: a mimic for thrombotic thrombocytopenic purpura? *Lupus*, 8, 477-81.
- Cervera, R. & Asherson, R. A. (2005) Antiphospholipid syndrome associated with infections: clinical and microbiological characteristics. *Immunobiology*, 210, 735-41.
- Cervera, R., Asherson, R. A., Acevedo, M. L., Gomez-Puerta, J. A., Espinosa, G., De La Red, G., Gil, V., Ramos-Casals, M., Garcia-Carrasco, M., Ingelmo, M. & Font, J. (2004) Antiphospholipid syndrome associated with infections: clinical and microbiological characteristics of 100 patients. *Ann Rheum Dis*, 63, 1312-7.
- Cervera, R., Font, J., Lopez-Soto, A., Casals, F., Pallares, L., Bove, A., Ingelmo, M. & Urbano-Marquez, A. (1990) Isotype distribution of anticardiolipin antibodies in systemic lupus erythematosus: prospective analysis of a series of 100 patients. *Ann Rheum Dis*, 49, 109-13.
- Cervera, R., Khamashta, M. A., Font, J., Sebastiani, G. D., Gil, A., Lavilla, P., Domenech, I., Aydintug, A. O., Jedryka-Goral, A., De Ramon, E. & Et Al. (1993) Systemic lupus erythematosus: clinical and immunologic patterns of

- disease expression in a cohort of 1,000 patients. The European Working Party on Systemic Lupus Erythematosus. *Medicine (Baltimore)*, 72, 113-24.
- Cervera, R., Piette, J. C., Font, J., Khamashta, M. A., Shoenfeld, Y., Camps, M. T., Jacobsen, S., Lakos, G., Tincani, A., Kontopoulou-Griva, I., Galeazzi, M., Meroni, P. L., Derksen, R. H., De Groot, P. G., Gromnica-Ihle, E., Baleva, M., Mosca, M., Bombardieri, S., Houssiau, F., Gris, J. C., Quere, I., Hachulla, E., Vasconcelos, C., Roch, B., Fernandez-Nebro, A., Boffa, M. C., Hughes, G. R. & Ingelmo, M. (2002) Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. *Arthritis Rheum*, 46, 1019-27.
- Chamley, L. W., Allen, J. L. & Johnson, P. M. (1997) Synthesis of beta2 glycoprotein 1 by the human placenta. *Placenta*, 18, 403-10.
- Chamley, L. W., Duncalf, A. M., Konarkowska, B., Mitchell, M. D. & Johnson, P. M. (1999) Conformationally altered beta 2-glycoprotein I is the antigen for anti-cardiolipin autoantibodies. *Clin Exp Immunol*, 115, 571-6.
- Chan, T. M., Li, F. K., Tang, C. S., Wong, R. W., Fang, G. X., Ji, Y. L., Lau, C. S., Wong, A. K., Tong, M. K., Chan, K. W. & Lai, K. N. (2000) Efficacy of mycophenolate mofetil in patients with diffuse proliferative lupus nephritis. Hong Kong-Guangzhou Nephrology Study Group. *N Engl J Med*, 343, 1156-62.
- Chang, F., Steelman, L. S., Lee, J. T., Shelton, J. G., Navolanic, P. M., Blalock, W. L., Franklin, R. A. & Mccubrey, J. A. (2003) Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia*, 17, 1263-93.
- Chen, J., De, S., Damron, D. S., Chen, W. S., Hay, N. & Byzova, T. V. (2004) Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. *Blood*, 104, 1703-10.
- Chen, Q. & Kamboh, M. I. (2006) Complete DNA sequence variation in the apolipoprotein H (beta-glycoprotein I) gene and identification of informative SNPs. *Ann Hum Genet*, 70, 1-11.
- Cho, C. S., Cho, M. L., Chen, P. P., Min, S. Y., Hwang, S. Y., Park, K. S., Kim, W. U., Min, D. J., Min, J. K., Park, S. H. & Kim, H. Y. (2002) Antiphospholipid antibodies induce monocyte chemoattractant protein-1 in endothelial cells. *J Immunol*, 168, 4209-15.
- Chonn, A., Semple, S. C. & Cullis, P. R. (1995) Beta 2 glycoprotein I is a major protein associated with very rapidly cleared liposomes in vivo, suggesting a significant role in the immune clearance of "non-self" particles. *J Biol Chem*, 270, 25845-9.
- Chothia, C., Lesk, A. M., Tramontano, A., Levitt, M., Smith-Gill, S. J., Air, G., Sheriff, S., Padlan, E. A., Davies, D., Tulip, W. R. & Et Al. (1989) Conformations of immunoglobulin hypervariable regions. *Nature*, 342, 877-83.
- Christodoulou, C., Zain, M., Bertolaccini, M. L., Sangle, S., Khamashta, M. A., Hughes, G. R. V. & D'Cruz, D. P. D. (2006) Prevalence of an abnormal ankle-brachial index in patients with antiphospholipid syndrome with pregnancy loss but without thrombosis: a controlled study. *Annals of Rheum Dis*, 65, 683-4.
- Chu, L. & Robinson, D. K. (2001) Industrial choices for protein production by large-scale cell culture. *Curr Opin Biotechnol*, 12, 180-7.
- Cines, D. B., Pollak, E. S., Buck, C. A., Loscalzo, J., Zimmerman, G. A., Mcever, R. P., Pober, J. S., Wick, T. M., Konkle, B. A., Schwartz, B. S., Barnathan, E. S., Mccrae, K. R., Hug, B. A., Schmidt, A. M. & Stern, D. M. (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood*, 91, 3527-61.
- Cline, J., Braman, J. C. & Hogrefe, H. H. (1996) PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res*, 24, 3546-51.

- Cockerill, K. A., Smith, E., Jones, D. S., Branks, M. J., Hayag, M., Victoria, E. J., Linnik, M. D. & Campbell, M. A. (2003) In vivo characterization of bioconjugate B cell toleragens with specificity for autoantibodies in antiphospholipid syndrome. *Int Immunopharmacol*, 3, 1667-75.
- Cohen, J., Bakimer, R., Blank, M., Valesini, G. & Shoenfeld, Y. (1993) Pathogenic serum IgG anticardiolipin antibodies and the idiotypic network. *Ann Rheum Dis*, 52, 742-8.
- Colli, S., Eligini, S., Lalli, M., Camera, M., Paoletti, R. & Tremoli, E. (1997) Vastatins inhibit tissue factor in cultured human macrophages. A novel mechanism of protection against atherothrombosis. *Arterioscler Thromb Vasc Biol*, 17, 265-72.
- Conley, C. L. & Hartman, R. C. (1952) A hemorrhagic disorder caused by circulating anticoagulant in patients with disseminated lupus erythematosus. *J Clin Invest*, 31, 621.
- Coon, W. W., Willis, P. W., 3rd & Keller, J. B. (1973) Venous thromboembolism and other venous disease in the Tecumseh community health study. *Circulation*, 48, 839-46.
- Cowchock, F. S., Reece, E. A., Balaban, D., Branch, D. W. & Plouffe, L. (1992) Repeated fetal losses associated with antiphospholipid antibodies: a collaborative randomized trial comparing prednisone with low-dose heparin treatment. *Am J Obstet Gynecol*, 166, 1318-23.
- Crowther, M. A., Ginsberg, J. S., Julian, J., Denburg, J., Hirsh, J., Douketis, J., Laskin, C., Fortin, P., Anderson, D., Kearon, C., Clarke, A., Geerts, W., Forgie, M., Green, D., Costantini, L., Yacura, W., Wilson, S., Gent, M. & Kovacs, M. J. (2003) A comparison of two intensities of warfarin for the prevention of recurrent thrombosis in patients with the antiphospholipid antibody syndrome. *N Engl J Med*, 349, 1133-8.
- Cuadrado, M. J., Lopez-Pedreira, C., Khamashta, M. A., Camps, M. T., Tinahones, F., Torres, A., Hughes, G. R. & Velasco, F. (1997) Thrombosis in primary antiphospholipid syndrome: a pivotal role for monocyte tissue factor expression. *Arthritis Rheum*, 40, 834-41.
- Cugno, M., Cabibbe, M., Galli, M., Meroni, P. L., Caccia, S., Russo, R., Bottasso, B. & Mannucci, P. M. (2004) Antibodies to tissue-type plasminogen activator (tPA) in patients with antiphospholipid syndrome: evidence of interaction between the antibodies and the catalytic domain of tPA in 2 patients. *Blood*, 103, 2121-6.
- Dagenais, P., Urowitz, M. B., Gladman, D. D. & Norman, C. S. (1992) A family study of the antiphospholipid syndrome associated with other autoimmune diseases. *J Rheumatol*, 19, 1393-6.
- Davies, D. R., Padlan, E. A. & Sheriff, S. (1990) Antibody-antigen complexes. *Annu Rev Biochem*, 59, 439-73.
- De Jong, A. W., Hart, W., Terburg, M., Molenaar, J. L., Herbrink, P. & Hop, W. C. (1993) Cardiolipin antibodies and lupus anticoagulant in young patients with a cerebrovascular accident in the past. *Neth J Med*, 42, 93-8.
- De Laat, B., Derksen, R. H., Mackie, I. J., Roest, M., Schoormans, S., Woodhams, B. J., De Groot, P. G. & Van Heerde, W. L. (2006a) Annexin A5 polymorphism (-1C>T) and the presence of anti-annexin A5 antibodies in the antiphospholipid syndrome. *Ann Rheum Dis*, 65, 1468-72
- De Laat, B., Derksen, R. H., Urbanus, R. T. & De Groot, P. G. (2005) IgG antibodies that recognize epitope Gly40-Arg43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. *Blood*, 105, 1540-5.
- De Laat, B., Derksen, R. H., Van Lummel, M., Pennings, M. T. & De Groot, P. G. (2006b) Pathogenic anti- β 2-glycoprotein I antibodies recognize domain I

- of β -glycoprotein I only after a conformational change. *Blood*, 107, 1916-24.
- De Laat, H. B., Derksen, R. H., Urbanus, R. T., Roest, M. & De Groot, P. G. (2004) β -glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. *Blood*, 104, 3598-602.
- Del Conde, I., Cruz, M. A., Zhang, H., Lopez, J. A. & Afshar-Kharghan, V. (2005) Platelet activation leads to activation and propagation of the complement system. *J Exp Med*, 201, 871-9.
- Del Papa, N., Guidali, L., Spatola, L., Bonara, P., Borghi, M. O., Tincani, A., Balestrieri, G. & Meroni, P. L. (1995) Relationship between anti-phospholipid and anti-endothelial cell antibodies III: β 2 glycoprotein I mediates the antibody binding to endothelial membranes and induces the expression of adhesion molecules. *Clin Exp Rheumatol*, 13, 179-85.
- Delgado Alves, J., Ames, P. R., Donohue, S., Stanyer, L., Nourooz-Zadeh, J., Ravirajan, C. & Isenberg, D. A. (2002) Antibodies to high-density lipoprotein and β -glycoprotein I are inversely correlated with paraoxonase activity in systemic lupus erythematosus and primary antiphospholipid syndrome. *Arthritis Rheum*, 46, 2686-94.
- Delgado Alves, J., Clapp, B. R., Stidwill, R., Chen, P. P., Hingorani, A. D., Singer, M. & Isenberg, D. A. (2006) Human monoclonal IgG anticardiolipin antibodies induce nitric oxide synthase expression. *Atherosclerosis*, 185, 246-53.
- Delgado Alves, J., Kumar, S. & Isenberg, D. A. (2003) Cross-reactivity between anti-cardiolipin, anti-high-density lipoprotein and anti-apolipoprotein A-I IgG antibodies in patients with systemic lupus erythematosus and primary antiphospholipid syndrome. *Rheumatology (Oxford)*, 42, 893-9.
- Delgado Alves, J., Mason, L. J., Ames, P. R., Chen, P. P., Rauch, J., Levine, J. S., Subang, R. & Isenberg, D. A. (2005) Antiphospholipid antibodies are associated with enhanced oxidative stress, decreased plasma nitric oxide and paraoxonase activity in an experimental mouse model. *Rheumatology (Oxford)*, 44, 1238-44.
- Derksen, R. H., De Groot, P. G. & Kappelle, L. J. (2003) Low dose aspirin after ischemic stroke associated with antiphospholipid syndrome. *Neurology*, 61, 111-4.
- Derksen, R. H., De Groot, P. G., Kater, L. & Nieuwenhuis, H. K. (1993) Patients with antiphospholipid antibodies and venous thrombosis should receive long term anticoagulant treatment. *Ann Rheum Dis*, 52, 689-92.
- Derksen, R. H., Khamashta, M. A. & Branch, D. W. (2004) Management of the obstetric antiphospholipid syndrome. *Arthritis Rheum*, 50, 1028-39.
- Deuschle, U., Gentz, R. & Bujard, H. (1986) lac Repressor blocks transcribing RNA polymerase and terminates transcription. *Proc Natl Acad Sci U S A*, 83, 4134-7.
- Di Simone, N., Meroni, P. L., De Papa, N., Raschi, E., Caliandro, D., De Carolis, C. S., Khamashta, M. A., Atsumi, T., Hughes, G. R., Balestrieri, G., Tincani, A., Casali, P. & Caruso, A. (2000) Antiphospholipid antibodies affect trophoblast gonadotropin secretion and invasiveness by binding directly and through adhered β -glycoprotein I. *Arthritis Rheum*, 43, 140-50.
- Dobado-Berrios, P. M., Lopez-Pedraza, C., Velasco, F., Aguirre, M. A., Torres, A. & Cuadrado, M. J. (1999) Increased levels of tissue factor mRNA in mononuclear blood cells of patients with primary antiphospholipid syndrome. *Thromb Haemost*, 82, 1578-82.
- Domenico Sebastiani, G., Minisola, G. & Galeazzi, M. (2003) HLA class II alleles and genetic predisposition to the antiphospholipid syndrome. *Autoimmun Rev*, 2, 387-94.

- Dubendorff, J. W. & Studier, F. W. (1991) Creation of a T7 autogene. Cloning and expression of the gene for bacteriophage T7 RNA polymerase under control of its cognate promoter. *J Mol Biol*, 219, 61-8.
- Dunoyer-Geindre, S., De Moerloose, P., Galve-De Rochemonteix, B., Reber, G. & Kruithof, E. K. (2002) NFkappaB is an essential intermediate in the activation of endothelial cells by anti-beta(2)-glycoprotein 1 antibodies. *Thromb Haemost*, 88, 851-7.
- Dunoyer-Geindre, S., Kruithof, E. K., Boehlen, F., Satta-Poschung, N., Reber, G. & De Moerloose, P. (2004) Aspirin inhibits endothelial cell activation induced by antiphospholipid antibodies. *J Thromb Haemost*, 2, 1176-81.
- Ebeling, F., Pettersson, T., Muukkonen, L., Vahtera, E. & Rasi, V. (2003) Beta-2-glycoprotein I antibodies in patients with thrombosis. *Scand J Clin Lab Invest*, 63, 111-8.
- Eckart, M. R. & Bussineau, C. M. (1996) Quality and authenticity of heterologous proteins synthesized in yeast. *Curr Opin Biotechnol*, 7, 525-30.
- Edwards, J. C., Szczepanski, L., Szechinski, J., Filipowicz-Sosnowska, A., Emery, P., Close, D. R., Stevens, R. M. & Shaw, T. (2004) Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med*, 350, 2572-81.
- Edwards, M. H., Pierangeli, S., Liu, X., Barker, J. H., Anderson, G. & Harris, E. N. (1997) Hydroxychloroquine reverses thrombogenic properties of antiphospholipid antibodies in mice. *Circulation*, 96, 4380-4.
- Egner, W. (2000) The use of laboratory tests in the diagnosis of SLE. *J Clin Pathol*, 53, 424-32.
- Ehrenstein, M., Longhurst, C. & Isenberg, D. A. (1993) Production and analysis of IgG monoclonal anti-DNA antibodies from systemic lupus erythematosus (SLE) patients. *Clin Exp Immunol*, 92, 39-45.
- Ehrenstein, M. R., Katz, D. R., Griffiths, M. H., Papadaki, L., Winkler, T. H., Kalden, J. R. & Isenberg, D. A. (1995) Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice. *Kidney Int*, 48, 705-11.
- Erkan, D., Cervera, R. & Asherson, R. A. (2003) Catastrophic antiphospholipid syndrome: where do we stand? *Arthritis Rheum*, 48, 3320-7.
- Erkan, D. & Lockshin, M. D. (2004) How much warfarin is enough in APS related thrombosis? *Thromb Res*, 114, 435-42.
- Erkan, D., Yazici, Y., Peterson, M. G., Sammaritano, L. & Lockshin, M. D. (2002) A cross-sectional study of clinical thrombotic risk factors and preventive treatments in antiphospholipid syndrome. *Rheumatology (Oxford)*, 41, 924-9.
- Esmon, C. T. (2000) The anticoagulant and anti-inflammatory roles of the protein C anticoagulant pathway. *J Autoimmun*, 15, 113-6.
- Espinola, R. G., Pierangeli, S. S., Gharavi, A. E. & Harris, E. N. (2002) Hydroxychloroquine reverses platelet activation induced by human IgG antiphospholipid antibodies. *Thromb Haemost*, 87, 518-22.
- Essig, M., Nguyen, G., Prie, D., Escoubet, B., Sraer, J. D. & Friedlander, G. (1998) 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors increase fibrinolytic activity in rat aortic endothelial cells. Role of geranylgeranylation and Rho proteins. *Circ Res*, 83, 683-90.
- Exner, T., Barber, S., Kronenberg, H. & Rickard, K. A. (1980) Familial association of the lupus anticoagulant. *Br J Haematol*, 45, 89-96.
- Farquharson, R. G., Quenby, S. & Greaves, M. (2002) Antiphospholipid syndrome in pregnancy: a randomized, controlled trial of treatment. *Obstet Gynecol*, 100, 408-13.
- Favaloro, E. J. & Silvestrini, R. (2002) Assessing the usefulness of anticardiolipin antibody assays: a cautious approach is suggested by high variation and limited consensus in multilaboratory testing. *Am J Clin Pathol*, 118, 548-57.

- Fearon, D. T. (1979) Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc Natl Acad Sci U S A*, 76, 5867-71.
- Feinstein, D. I. & Rapaport, S. I. (1972) Acquired inhibitors of blood coagulation. *Prog Hemost Thromb*, 1, 75-95.
- Ferrara, D. E., Liu, X., Espinola, R. G., Meroni, P. L., Abukhalaf, I., Harris, E. N. & Pierangeli, S. S. (2003) Inhibition of the thrombogenic and inflammatory properties of antiphospholipid antibodies by fluvastatin in an in vivo animal model. *Arthritis Rheum*, 48, 3272-9.
- Finazzi, G., Brancaccio, V., Moia, M., Ciaverella, N., Mazzucconi, M. G., Schinco, P. C., Ruggeri, M., Pogliani, E. M., Gamba, G., Rossi, E., Baudo, F., Manotti, C., D'angelo, A., Palareti, G., De Stefano, V., Berrettini, M. & Barbui, T. (1996) Natural history and risk factors for thrombosis in 360 patients with antiphospholipid antibodies: a four-year prospective study from the Italian Registry. *Am J Med*, 100, 530-6.
- Finazzi, G., Marchioli, R., Brancaccio, V., Schinco, P., Wisloff, F., Musial, J., Baudo, F., Berrettini, M., Testa, S., D'angelo, A., Tognoni, G. & Barbui, T. (2005) A randomized clinical trial of high-intensity warfarin vs. conventional antithrombotic therapy for the prevention of recurrent thrombosis in patients with the antiphospholipid syndrome (WAPS). *J Thromb Haemost*, 3, 848-53.
- Fischetti, F., Durigutto, P., Pellis, V., Debeus, A., Macor, P., Bulla, R., Bossi, F., Ziller, F., Sblattero, D., Meroni, P. & Tedesco, F. (2005) Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor. *Blood*, 106, 2340-6.
- Fishman, P., Bakimer, R., Blank, M., Sredni, D., Djaldetti, M. & Shoenfeld, Y. (1992a) The putative role of cytokines in the induction of primary anti-phospholipid syndrome in mice. *Clin Exp Immunol*, 90, 266-70.
- Fishman, P., Falach-Vaknin, E., Sredni, B., Meroni, P. L., Tincani, A., Dicker, D. & Shoenfeld, Y. (1996) Aspirin-interleukin-3 interrelationships in patients with anti-phospholipid syndrome. *Am J Reprod Immunol*, 35, 80-4.
- Fishman, P., Falach-Vaknine, E., Zigelman, R., Bakimer, R., Sredni, B., Djaldetti, M. & Shoenfeld, Y. (1993) Prevention of fetal loss in experimental antiphospholipid syndrome by in vivo administration of recombinant interleukin-3. *J Clin Invest*, 91, 1834-7.
- Fishman, P., Shoenfeld, Y., Hart, J., Zigelman, R., Shamir, Y., Feldberg, D., Inbar, O., Sredni, D. & Djaldetti, M. (1992b) Interleukin-3-like activity levels in pregnant women: possible modulation by progesterone. *J Reprod Immunol*, 22, 211-6.
- Font, J., Espinosa, G., Tassies, D., Pino, M., Khamashta, M. A., Gallart, T., Cervera, R., Escolar, G., Hughes, G. R., Ingelmo, M., Ordinas, A. & Reverter, J. C. (2002) Effects of beta2-glycoprotein I and monoclonal anticardiolipin antibodies in platelet interaction with subendothelium under flow conditions. *Arthritis Rheum*, 46, 3283-9.
- Forastiero, R. R., Martinuzzo, M. E. & Broze, G. J. (2003) High titers of autoantibodies to tissue factor pathway inhibitor are associated with the antiphospholipid syndrome. *J Thromb Haemost*, 1, 718-24.
- Forastiero, R. R., Martinuzzo, M. E., Cerrato, G. S., Kordich, L. C. & Carreras, L. O. (1997) Relationship of anti beta2-glycoprotein I and anti prothrombin antibodies to thrombosis and pregnancy loss in patients with antiphospholipid antibodies. *Thromb Haemost*, 78, 1008-14.
- Forastiero, R. R., Martinuzzo, M. E. & De Larranaga, G. F. (2005) Circulating levels of tissue factor and proinflammatory cytokines in patients with primary antiphospholipid syndrome or leprosy related antiphospholipid antibodies. *Lupus*, 14, 129-36.

- Freitas, M. V., Da Silva, L. M., Deghaide, N. H., Donadi, E. A. & Louzada-Junior, P. (2004) Is HLA class II susceptibility to primary antiphospholipid syndrome different from susceptibility to secondary antiphospholipid syndrome? *Lupus*, 13, 125-31.
- Freyssinet, J. M., Wiesel, M. L., Gauchy, J., Boneu, B. & Cazenave, J. P. (1986) An IgM lupus anticoagulant that neutralizes the enhancing effect of phospholipid on purified endothelial thrombomodulin activity--a mechanism for thrombosis. *Thromb Haemost*, 55, 309-13.
- Furberg, C. D., Adams, H. P., Jr., Applegate, W. B., Byington, R. P., Espeland, M. A., Hartwell, T., Hunninghake, D. B., Lefkowitz, D. S., Probstfield, J., Riley, W. A. & Et Al. (1994) Effect of lovastatin on early carotid atherosclerosis and cardiovascular events. Asymptomatic Carotid Artery Progression Study (ACAPS) Research Group. *Circulation*, 90, 1679-87.
- Furie, R., Ishikawa, T., Dhawan, V. & Eidelberg, D. (1994) Alternating hemichorea in primary antiphospholipid syndrome: evidence for contralateral striatal hypermetabolism. *Neurology*, 44, 2197-9.
- Furie, R. A., Cash, J. M., Cronin, M. E., Katz, R. S., Weisman, M. H., Aranow, C., Liebling, M. R., Hudson, N. P., Berner, C. M., Coutts, S. & De Haan, H. A. (2001) Treatment of systemic lupus erythematosus with LJP 394. *J Rheumatol*, 28, 257-65.
- Gadek, T. R. & Mcdowell, R. S. (2003) Discovery of small molecule leads in a biotechnology datastream. *Drug Discov Today*, 8, 545-50.
- Galindo, M., Khamashta, M. A. & Hughes, G. R. (1999) Splenectomy for refractory thrombocytopenia in the antiphospholipid syndrome. *Rheumatology (Oxford)*, 38, 848-53.
- Galli, M. (2004) Antiphospholipid antibodies and thrombosis: do test patterns identify the patients' risk? *Thromb Res*, 114, 597-601.
- Galli, M., Beretta, G., Daldossi, M., Bevers, E. M. & Barbui, T. (1997) Different anticoagulant and immunological properties of anti-prothrombin antibodies in patients with antiphospholipid antibodies. *Thromb Haemost*, 77, 486-91.
- Galli, M., Comfurius, P., Maassen, C., Hemker, H. C., De Baets, M. H., Van Breda-Vriesman, P. J., Barbui, T., Zwaal, R. F. & Bevers, E. M. (1990) Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *Lancet*, 335, 1544-7.
- Galli, M., Daldossi, M. & Barbui, T. (1994) Anti-glycoprotein Ib/IX and IIb/IIIa antibodies in patients with antiphospholipid antibodies. *Thromb Haemost*, 71, 571-5.
- Galli, M., Luciani, D., Bertolini, G. & Barbui, T. (2003) Anti-beta 2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. *Blood*, 102, 2717-23.
- Gao, B., Virmani, M., Romm, E., Lazar-Wesley, E., Sakaguchi, K., Appella, E., Kunos, G. & Takacs, L. (1993) Sequence of a cDNA encoding bovine apolipoprotein H. *Gene*, 126, 287-8.
- Gatenby, P. A. (2004) Controversies in the antiphospholipid syndrome and stroke. *Thromb Res*, 114, 483-8.
- Gatenby, P. A., Cameron, K. & Shearman, R. P. (1989) Pregnancy loss with phospholipid antibodies: improved outcome with aspirin containing treatment. *Aust N Z J Obstet Gynaecol*, 29, 294-8.
- George, J., Afek, A., Gilburd, B., Blank, M., Levy, Y., Aron-Maor, A., Levkovitz, H., Shaish, A., Goldberg, I., Kopolovic, J., Harats, D. & Shoenfeld, Y. (1998a) Induction of early atherosclerosis in LDL-receptor-deficient mice immunized with beta2-glycoprotein I. *Circulation*, 98, 1108-15.
- George, J., Afek, A., Gilburd, B., Levkovitz, H., Shaish, A., Goldberg, I., Kopolovic, Y., Wick, G., Shoenfeld, Y. & Harats, D. (1998b) Hyperimmunization of apo-E-

- deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. *Atherosclerosis*, 138, 147-52.
- George, J., Afek, A., Gilburd, B., Levy, Y., Blank, M., Kopolovic, J., Harats, D. & Shoenfeld, Y. (1997) Atherosclerosis in LDL-receptor knockout mice is accelerated by immunization with anticardiolipin antibodies. *Lupus*, 6, 723-9.
- George, J., Blank, M., Levy, Y., Meroni, P., Damianovich, M., Tincani, A. & Shoenfeld, Y. (1998c) Differential effects of anti-beta2-glycoprotein I antibodies on endothelial cells and on the manifestations of experimental antiphospholipid syndrome. *Circulation*, 97, 900-6.
- George, J., Gilburd, B., Hojnik, M., Levy, Y., Langevitz, P., Matsuura, E., Koike, T. & Shoenfeld, Y. (1998d) Target recognition of beta2-glycoprotein I (beta2GPI)-dependent anticardiolipin antibodies: evidence for involvement of the fourth domain of beta2GPI in antibody binding. *J Immunol*, 160, 3917-23.
- George, J., Harats, D., Gilburd, B., Afek, A., Levy, Y., Schneiderman, J., Barshack, I., Kopolovic, J. & Shoenfeld, Y. (1999) Immunolocalization of beta2-glycoprotein I (apolipoprotein H) to human atherosclerotic plaques: potential implications for lesion progression. *Circulation*, 99, 2227-30.
- George, J., Harats, D., Gilburd, B., Afek, A., Shaish, A., Kopolovic, J. & Shoenfeld, Y. (2000) Adoptive transfer of beta(2)-glycoprotein I-reactive lymphocytes enhances early atherosclerosis in LDL receptor-deficient mice. *Circulation*, 102, 1822-7.
- Georgiou, G. & Valax, P. (1996) Expression of correctly folded proteins in Escherichia coli. *Curr Opin Biotechnol*, 7, 190-7.
- Gharavi, A. E., Mellors, R. C. & Elkon, K. B. (1989) IgG anti-cardiolipin antibodies in murine lupus. *Clin Exp Immunol*, 78, 233-8.
- Gharavi, A. E., Pierangeli, S. S., Colden-Stanfield, M., Liu, X. W., Espinola, R. G. & Harris, E. N. (1999) GDKV-induced antiphospholipid antibodies enhance thrombosis and activate endothelial cells in vivo and in vitro. *J Immunol*, 163, 2922-7.
- Gharavi, A. E., Pierangeli, S. S., Espinola, R. G., Liu, X., Colden-Stanfield, M. & Harris, E. N. (2002) Antiphospholipid antibodies induced in mice by immunization with a cytomegalovirus-derived peptide cause thrombosis and activation of endothelial cells in vivo. *Arthritis Rheum*, 46, 545-52.
- Gharavi, A. E., Pierangeli, S. S., Levy, R. A. & Harris, E. N. (2001) Mechanisms of pregnancy loss in antiphospholipid syndrome. *Clin Obstet Gynecol*, 44, 11-9.
- Gharavi, A. E., Sammaritano, L. R., Wen, J. & Elkon, K. B. (1992) Induction of antiphospholipid autoantibodies by immunization with beta 2 glycoprotein I (apolipoprotein H). *J Clin Invest*, 90, 1105-9.
- Ghillani-Dalbin, P., Amoura, Z., Cacoub, P., Charuel, J. L., Diemert, M. C., Piette, J. C. & Musset, L. (2003) Testing for anti-nucleosome antibodies in daily practice: a monocentric evaluation in 1696 patients. *Lupus*, 12, 833-7..
- Giles, I., Lambrianides, N., Latchman, D., Chen, P., Chukwuocha, R., Isenberg, D. & Rahman, A. (2005) The critical role of arginine residues in the binding of human monoclonal antibodies to cardiolipin. *Arthritis Res Ther*, 7, R47-56.
- Giles, I. P., Haley, J., Nagl, S., Latchman, D. S., Chen, P. P., Chukwuocha, R. U., Isenberg, D. A. & Rahman, A. (2003a) Relative importance of different human aPL derived heavy and light chains in the binding of aPL to cardiolipin. *Mol Immunol*, 40, 49-60.
- Giles, I. P., Haley, J. D., Nagl, S., Isenberg, D. A., Latchman, D. S. & Rahman, A. (2003b) A systematic analysis of sequences of human antiphospholipid and anti-beta2-glycoprotein I antibodies: the importance of somatic mutations and certain sequence motifs. *Semin Arthritis Rheum*, 32, 246-65.

- Giles, I. P., Isenberg, D. A., Latchman, D. S. & Rahman, A. (2003c) How do antiphospholipid antibodies bind beta2-glycoprotein I? *Arthritis Rheum*, 48, 2111-21.
- Ginsberg, J. S., Wells, P. S., Brill-Edwards, P., Donovan, D., Moffatt, K., Johnston, M., Stevens, P. & Hirsh, J. (1995) Antiphospholipid antibodies and venous thromboembolism. *Blood*, 86, 3685-91.
- Ginsburg, K. S., Liang, M. H., Newcomer, L., Goldhaber, S. Z., Schur, P. H., Hennekens, C. H. & Stampfer, M. J. (1992) Anticardiolipin antibodies and the risk for ischemic stroke and venous thrombosis. *Ann Intern Med*, 117, 997-1002.
- Ginzler, E. M., Dooley, M. A., Aranow, C., Kim, M. Y., Buyon, J., Merrill, J. T., Petri, M., Gilkeson, G. S., Wallace, D. J., Weisman, M. H. & Appel, G. B. (2005) Mycophenolate mofetil or intravenous cyclophosphamide for lupus nephritis. *N Engl J Med*, 353, 2219-28.
- Girardi, G., Berman, J., Redecha, P., Spruce, L., Thurman, J. M., Kraus, D., Hollmann, T. J., Casali, P., Carroll, M. C., Wetsel, R. A., Lambris, J. D., Holers, V. M. & Salmon, J. E. (2003) Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest*, 112, 1644-54.
- Girardi, G., Bulla, R., Salmon, J. E. & Tedesco, F. (2006) The complement system in the pathophysiology of pregnancy. *Mol Immunol*, 43, 68-77.
- Girardi, G., Redecha, P. & Salmon, J. E. (2004) Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med*, 10, 1222-6.
- Godeau, B., Piette, J. C., Fromont, P., Intrator, L., Schaeffer, A. & Bierling, P. (1997) Specific antiplatelet glycoprotein autoantibodies are associated with the thrombocytopenia of primary antiphospholipid syndrome. *Br J Haematol*, 98, 873-9.
- Gomez-Puerta, J. A., Martin, H., Amigo, M. C., Aguirre, M. A., Camps, M. T., Cuadrado, M. J., Hughes, G. R. & Khamashta, M. A. (2005) Long-term follow-up in 128 patients with primary antiphospholipid syndrome: do they develop lupus? *Medicine (Baltimore)*, 84, 225-30.
- Goochee, C. F., Gramer, M. J., Andersen, D. C., Bahr, J. B. & Rasmussen, J. R. (1991) The oligosaccharides of glycoproteins: bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. *Biotechnology (N Y)*, 9, 1347-55.
- Greaves, M., Cohen, H., Machin, S. J. & Mackie, I. (2000) Guidelines on the investigation and management of the antiphospholipid syndrome. *Br J Haematol*, 109, 704-15.
- Griffin, J. H., Evatt, B., Zimmerman, T. S., Kleiss, A. J. & Wideman, C. (1981) Deficiency of protein C in congenital thrombotic disease. *J Clin Invest*, 68, 1370-3.
- Grodberg, J. & Dunn, J. J. (1988) ompT encodes the Escherichia coli outer membrane protease that cleaves T7 RNA polymerase during purification. *J Bacteriol*, 170, 1245-53.
- Guerin, J., Sheng, Y., Reddel, S., Iverson, G. M., Chapman, M. G. & Krilis, S. A. (2002) Heparin inhibits the binding of beta 2-glycoprotein I to phospholipids and promotes the plasmin-mediated inactivation of this blood protein. Elucidation of the consequences of the two biological events in patients with the anti-phospholipid syndrome. *J Biol Chem*, 277, 2644-9.
- Guerin, J., Sim, R., Yu, B. B., Ferluga, J., Feighery, C. & Jackson, J. (2000) Heterogeneous recognition of beta 2-glycoprotein I by antibodies from antiphospholipid syndrome patients. *Thromb Haemost*, 84, 374-80.

- Guex, N. & Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-Pdb Viewer: an environment for comparative protein modelling. *Electrophoresis*, 18, 2714-23.
- Gulko, P. S., Reveille, J. D., Koopman, W. J., Burgard, S. L., Bartolucci, A. A. & Alarcon, G. S. (1993) Anticardiolipin antibodies in systemic lupus erythematosus: clinical correlates, HLA associations, and impact on survival. *J Rheumatol*, 20, 1684-93.
- Gumpert, J. & Hoischen, C. (1998) Use of cell wall-less bacteria (L-forms) for efficient expression and secretion of heterologous gene products. *Curr Opin Biotechnol*, 9, 506-9.
- Gustafsson, D. & Elg, M. (2003) The pharmacodynamics and pharmacokinetics of the oral direct thrombin inhibitor ximelagatran and its active metabolite melagatran: a mini-review. *Thromb Res*, 109 Suppl 1, S9-15.
- Hahn, B. H. (1998) Antibodies to DNA. *N Engl J Med*, 338, 1359-68.
- Hakim, A. J., Machin, S. J. & Isenberg, D. A. (1998) Autoimmune thrombocytopenia in primary antiphospholipid syndrome and systemic lupus erythematosus: the response to splenectomy. *Semin Arthritis Rheum*, 28, 20-5.
- Hammel, M., Kriechbaum, M., Gries, A., Kostner, G. M., Laggner, P. & Prassl, R. (2002) Solution structure of human and bovine beta(2)-glycoprotein I revealed by small-angle X-ray scattering. *J Mol Biol*, 321, 85-97.
- Hammel, M., Schwarzenbacher, R., Gries, A., Kostner, G. M., Laggner, P. & Prassl, R. (2001) Mechanism of the interaction of beta(2)-glycoprotein I with negatively charged phospholipid membranes. *Biochemistry*, 40, 14173-81.
- Hang, L. M., Izui, S. & Dixon, F. J. (1981) (NZW x BXSb)F1 hybrid. A model of acute lupus and coronary vascular disease with myocardial infarction. *J Exp Med*, 154, 216-21.
- Hanly, J. G., Walsh, N. M., Fisk, J. D., Eastwood, B., Hong, C., Sherwood, G., Jones, J. V., Jones, E. & Elkon, K. (1993) Cognitive impairment and autoantibodies in systemic lupus erythematosus. *Br J Rheumatol*, 32, 291-6.
- Hannig, G. & Makrides, S. C. (1998) Strategies for optimizing heterologous protein expression in Escherichia coli. *Trends Biotechnol*, 16, 54-60.
- Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. & Weaver, C. T. (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*, 6, 1123-32.
- Harris, E. N. (1987) Syndrome of the black swan. *Br J Rheumatol*, 26, 324-6.
- Harris, E. N., Baguley, E., Asherson, R. A. & Hughes, G. R. V. (1987a) Clinical and serological features of the 'antiphospholipid syndrome' (APS). *Br J Rheumatol*, 26, (abstract) 19.
- Harris, E. N., Gharavi, A. E., Boey, M. L., Patel, B. M., Mackworth-Young, C. G., Loizou, S. & Hughes, G. R. (1983) Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet*, 2, 1211-4.
- Harris, E. N., Gharavi, A. E., Patel, S. P. & Hughes, G. R. (1987b) Evaluation of the anti-cardiolipin antibody test: report of an international workshop held 4 April 1986. *Clin Exp Immunol*, 68, 215-22.
- Harris, E. N. & Pierangeli, S. S. (2002) Revisiting the anticardiolipin test and its standardization. *Lupus*, 11, 269-75.
- Harris, E. N. & Pierangeli, S. S. (2004) Primary, Secondary, Catastrophic Antiphospholipid Syndrome: is there a difference? *Thromb Res*, 114, 357-61.
- Hartung, K., Coldewey, R., Corvetta, A., Deicher, H., Kalden, J. R., Krapf, F., Lang, B., Lakomek, H. J., Liedvogel, B., Peter, H. H. & Et Al. (1992) MHC gene products and anticardiolipin antibodies in systemic lupus erythematosus results of a multicenter study. SLE Study Group. *Autoimmunity*, 13, 95-9.

- Hasan, N. & Szybalski, W. (1995) Construction of lacI_{ts} and lacI_{qs} expression plasmids and evaluation of the thermosensitive lac repressor. *Gene*, 163, 35-40.
- Hashimoto, Y., Kawamura, M., Ichikawa, K., Suzuki, T., Sumida, T., Yoshida, S., Matsuura, E., Ikehara, S. & Koike, T. (1992) Anticardiolipin antibodies in NZW x BXS_B F1 mice. A model of antiphospholipid syndrome. *J Immunol*, 149, 1063-8.
- Hasselaar, P., Derksen, R. H., Blokzijl, L., Hessing, M., Nieuwenhuis, H. K., Bouma, B. N. & De Groot, P. G. (1989) Risk factors for thrombosis in lupus patients. *Ann Rheum Dis*, 48, 933-40.
- Hasunuma, Y., Matsuura, E., Makita, Z., Katahira, T., Nishi, S. & Koike, T. (1997) Involvement of beta 2-glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages. *Clin Exp Immunol*, 107, 569-73.
- Hattori, N., Kuwana, M., Kaburaki, J., Mimori, T., Ikeda, Y. & Kawakami, Y. (2000) T cells that are autoreactive to beta2-glycoprotein I in patients with antiphospholipid syndrome and healthy individuals. *Arthritis Rheum*, 43, 65-75.
- Haupt, H. M., Moore, G. W. & Hutchins, G. M. (1981) The lung in systemic lupus erythematosus. Analysis of the pathologic changes in 120 patients. *Am J Med*, 71, 791-8.
- Hebert, P. R., Gaziano, J. M., Chan, K. S. & Hennekens, C. H. (1997) Cholesterol lowering with statin drugs, risk of stroke, and total mortality. An overview of randomized trials. *Jama*, 278, 313-21.
- Heemskerk, J. W., Bevers, E. M. & Lindhout, T. (2002) Platelet activation and blood coagulation. *Thromb Haemost*, 88, 186-93.
- Hiniker, A. & Bardwell, J. C. (2003) Disulfide bond isomerization in prokaryotes. *Biochemistry*, 42, 1179-85.
- Hirose, N., Williams, R., Alberts, A. R., Furie, R. A., Chartash, E. K., Jain, R. I., Sison, C., Lahita, R. G., Merrill, J. T., Cucurull, E., Gharavi, A. E., Sammaritano, L. R., Salmon, J. E., Hashimoto, S., Sawada, T., Chu, C. C., Gregersen, P. K. & Chiorazzi, N. (1999) A role for the polymorphism at position 247 of the beta2-glycoprotein I gene in the generation of anti-beta2-glycoprotein I antibodies in the antiphospholipid syndrome. *Arthritis Rheum*, 42, 1655-61.
- Hochberg, M. C. (1997) Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum*, 40, 1725.
- Hoeg, J. M., Segal, P., Gregg, R. E., Chang, Y. S., Lindgren, F. T., Adamson, G. L., Frank, M., Brickman, C. & Brewer, H. B., Jr. (1985) Characterization of plasma lipids and lipoproteins in patients with beta 2-glycoprotein I (apolipoprotein H) deficiency. *Atherosclerosis*, 55, 25-34.
- Hoffmann, J. A. (2003) The immune response of *Drosophila*. *Nature*, 426, 33-8.
- Hojnik, M., George, J., Ziporen, L. & Shoenfeld, Y. (1996) Heart valve involvement (Libman-Sacks endocarditis) in the antiphospholipid syndrome. *Circulation*, 93, 1579-87.
- Holers, V. M., Girardi, G., Mo, L., Guthridge, J. M., Molina, H., Pierangeli, S. S., Espinola, R., Xiaowei, L. E., Mao, D., Vialpando, C. G. & Salmon, J. E. (2002) Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med*, 195, 211-20.
- Horizon, A., Weisman, M. H., Wallace, D. J., Merrill, J. T., Linnik, M. D., Cockerill, K. A. & Furie, R. A. (2003) Results of a Randomised, Placebo Controlled, Double-blind Phase 1/2 Clinical Trial (RCT) to Assess the Safety and Tolerability of LJP 1082 in Patients with Antiphospholipid Syndrome. *Arthritis Rheum*, 48, abstract S364.

- Horkko, S., Miller, E., Branch, D. W., Palinski, W. & Witztum, J. L. (1997) The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and beta2 glycoprotein 1 (and other proteins). *Proc Natl Acad Sci U S A*, 94, 10356-61.
- Horkko, S., Miller, E., Dudl, E., Reaven, P., Curtiss, L. K., Zvaifler, N. J., Terkeltaub, R., Pierangeli, S. S., Branch, D. W., Palinski, W. & Witztum, J. L. (1996) Antiphospholipid antibodies are directed against epitopes of oxidized phospholipids. Recognition of cardiolipin by monoclonal antibodies to epitopes of oxidized low density lipoprotein. *J Clin Invest*, 98, 815-25.
- Hu, X., Shi, Q., Yang, T. & Jackowski, G. (1996) Specific replacement of consecutive AGG codons results in high-level expression of human cardiac troponin T in *Escherichia coli*. *Protein Expr Purif*, 7, 289-93.
- Hua, Z., Wang, H., Chen, D., Chen, Y. & Zhu, D. (1994) Enhancement of expression of human granulocyte-macrophage colony stimulating factor by argU gene product in *Escherichia coli*. *Biochem Mol Biol Int*, 32, 537-43.
- Huang, J., Villemain, J., Padilla, R. & Sousa, R. (1999) Mechanisms by which T7 lysozyme specifically regulates T7 RNA polymerase during different phases of transcription. *J Mol Biol*, 293, 457-75.
- Hudson, M., Herr, A. L., Rauch, J., Neville, C., Chang, E., Ibrahim, R., Seguin, C., Kassis, J., Busque, L. & Fortin, P. R. (2003) The presence of multiple prothrombotic risk factors is associated with a higher risk of thrombosis in individuals with anticardiolipin antibodies. *J Rheumatol*, 30, 2385-91.
- Hughes, G. R. (1983) Thrombosis, abortion, cerebral disease, and the lupus anticoagulant. *Br Med J (Clin Res Ed)*, 287, 1088-9.
- Hughes, G. R., Harris, N. N. & Gharavi, A. E. (1986) The anticardiolipin syndrome. *J Rheumatol*, 13, 486-9.
- Hughes, G. R. & Khamashta, M. A. (2003) Seronegative antiphospholipid syndrome. *Ann Rheum Dis*, 62, 1127.
- Huhle, G., Abletshauer, C., Mayer, N., Weidinger, G., Harenberg, J. & Heene, D. L. (1999) Reduction of platelet activity markers in type II hypercholesterolemic patients by a HMG-CoA-reductase inhibitor. *Thromb Res*, 95, 229-34.
- Hunt, J. & Krilis, S. (1994) The fifth domain of beta 2-glycoprotein I contains a phospholipid binding site (Cys281-Cys288) and a region recognized by anticardiolipin antibodies. *J Immunol*, 152, 653-9.
- Hunt, J. E., Simpson, R. J. & Krilis, S. A. (1993) Identification of a region of beta 2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity. *Proc Natl Acad Sci U S A*, 90, 2141-5.
- Hwang, K. K., Grossman, J. M., Visvanathan, S., Chukwuocha, R. U., Woods, V. L., Jr., Le, D. T., Hahn, B. H. & Chen, P. P. (2001) Identification of anti-thrombin antibodies in the antiphospholipid syndrome that interfere with the inactivation of thrombin by antithrombin. *J Immunol*, 167, 7192-8.
- Ichikawa, K., Khamashta, M. A., Koike, T., Matsuura, E. & Hughes, G. R. (1994) beta 2-Glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with the antiphospholipid syndrome. *Arthritis Rheum*, 37, 1453-61.
- Ichikawa, K., Tsutsumi, A., Atsumi, T., Matsuura, E., Kobayashi, S., Hughes, G. R., Khamashta, M. A. & Koike, T. (1999) A chimeric antibody with the human gamma1 constant region as a putative standard for assays to detect IgG beta2-glycoprotein I-dependent anticardiolipin and anti-beta2-glycoprotein I antibodies. *Arthritis Rheum*, 42, 2461-70.
- Igarashi, M., Matsuura, E., Igarashi, Y., Nagae, H., Ichikawa, K., Triplett, D. A. & Koike, T. (1996) Human beta2-glycoprotein I as an anticardiolipin cofactor determined using mutants expressed by a baculovirus system. *Blood*, 87, 3262-70.

- Ikematsu, W., Luan, F. L., La Rosa, L., Beltrami, B., Nicoletti, F., Buyon, J. P., Meroni, P. L., Balestrieri, G. & Casali, P. (1998) Human anticardiolipin monoclonal autoantibodies cause placental necrosis and fetal loss in BALB/c mice. *Arthritis Rheum*, 41, 1026-39.
- Infante-Rivard, C., David, M., Gauthier, R. & Rivard, G. E. (1991) Lupus anticoagulants, anticardiolipin antibodies, and fetal loss. A case-control study. *N Engl J Med*, 325, 1063-6.
- Inoue, I., Goto, S., Mizotani, K., Awata, T., Mastunaga, T., Kawai, S., Nakajima, T., Hokari, S., Komoda, T. & Katayama, S. (2000) Lipophilic HMG-CoA reductase inhibitor has an anti-inflammatory effect: reduction of mRNA levels for interleukin-1beta, interleukin-6, cyclooxygenase-2, and p22phox by regulation of peroxisome proliferator-activated receptor alpha (PPARalpha) in primary endothelial cells. *Life Sci*, 67, 863-76.
- Inouye, M., Arnheim, N. & Sternglanz, R. (1973) Bacteriophage T7 lysozyme is an N-acetylmuramyl-L-alanine amidase. *J Biol Chem*, 248, 7247-52.
- Ioannou, Y., Bowman, S. J., Rahman, A. & Giles, I. P. (2005) Review of presentations at the 6th European Lupus Meeting 3-5 March 2005. *Lupus*, 14, 467-78.
- Ioannou, Y., Cambridge, G., Rahman, A., Leandro, M. J., Edwards, J. C. & Isenberg, D. A. (2006a) Effect of B cell depletion therapy on anti-cardiolipin antibody profiles in patients with systemic lupus erythematosus. *Rheumatology*, 45, (Suppl 1) Abstract 437.
- Ioannou, Y., Harper, T., Romay-Penabad, Z., Giles, I., Pericleous, C., Rahman, A. & Pierangeli, S. (2006b) Domain I of B2 glycoprotein I ameliorates thrombosis induced by antiphospholipid antibodies in mice. *Arthritis Rheum*, 54, Abstract 1350.
- Ioannou, Y. & Isenberg, D. A. (1999) Immunisation of patients with systemic lupus erythematosus: the current state of play. *Lupus*, 8, 497-501.
- Ioannou, Y. & Isenberg, D. A. (2002) Current concepts for the management of systemic lupus erythematosus in adults: a therapeutic challenge. *Postgrad Med J*, 78, 599-606.
- Ioffe, E. & Stanley, P. (1994) Mice lacking N-acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential role for complex or hybrid N-linked carbohydrates. *Proc Natl Acad Sci U S A*, 91, 728-32.
- Isenberg, D. A. & Horsfall, A. C. (1998) Systemic lupus erythematosus. In *Oxford textbook of Rheumatology* (Edited by Maddison P. J., Isenberg D. A., Woo P. and Glass D. N), Vol 2, p. 1147-80. Oxford, Oxford University Press.
- Isenberg, D. A., Meyrick-Thomas, D., Snaith, M. L., Mckeran, R. O. & Royston, J. P. (1982) A study of migraine in systemic lupus erythematosus. *Ann Rheum Dis*, 41, 30-2.
- Isenberg, D. A., Shoenfeld, Y., Walport, M., Mackworth-Young, C., Dudeney, C., Todd-Pokropek, A., Brill, S., Weinberger, A. & Pinkas, J. (1985) Detection of cross-reactive anti-DNA antibody idiotypes in the serum of systemic lupus erythematosus patients and of their relatives. *Arthritis Rheum*, 28, 999-1007.
- Isenberg, D. A. & Snaith, M. L. (1981) Muscle Disease in systemic lupus erythematosus: a study of its nature, frequency and cause. *J Rheumatol*, 8, 917-24.
- Isenberg, D. A., Katz, D., Le Page S., Knight, B., Tucker, L., Maddison, P., Hutchings, P., Watts, R., Andre-Schwartz, J. & Schwartz, R. S. (1991) Independent analysis of the 16/6 idotype lupus model. A role for an environmental factor? *J Immunol*, 147, 4172-7.
- Ito, T. & Wagner, G. (2004) Using codon optimization, chaperone co-expression, and rational mutagenesis for production and NMR assignments of human eIF2 alpha. *J Biomol NMR*, 28, 357-67.

- Iverson, G. M., Jones, D. S., Marquis, D., Linnik, M. D. & Victoria, E. J. (1998a) A chemically defined, toleragen-based approach for targeting anti-beta2-glycoprotein I antibodies. *Lupus*, 7, S166-9.
- Iverson, G. M., Matsuura, E., Victoria, E. J., Cockerill, K. A. & Linnik, M. D. (2002a) The orientation of beta2GPI on the plate is important for the binding of anti-beta2GPI autoantibodies by ELISA. *J Autoimmun*, 18, 289-97.
- Iverson, G. M., Reddel, S. W., Victoria, E. J., Cockerill, K. A., Wang, Y. X., Marti-Renom, M. A., Sali, A., Marquis, D. M., Krilis, S. A. & Linnik, M. D. (2002b) Use of single point mutations in domain I of β 2-glycoprotein I to determine fine antigenic specificity of antiphospholipid autoantibodies. *J Immunol*, 169, 7097-103.
- Iverson, G. M., Victoria, E. J. & Marquis, D. M. (1998b) Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. *Proc Natl Acad Sci U S A*, 95, 15542-6.
- Jackson, S. P., Schoenwaelder, S. M., Yuan, Y., Rabinowitz, I., Salem, H. H. & Mitchell, C. A. (1994) Adhesion receptor activation of phosphatidylinositol 3-kinase. von Willebrand factor stimulates the cytoskeletal association and activation of phosphatidylinositol 3-kinase and pp60c-src in human platelets. *J Biol Chem*, 269, 27093-9.
- Janeway, C. A., Jr. & Medzhitov, R. (2002) Innate immune recognition. *Annu Rev Immunol*, 20, 197-216.
- Janeway, C. A., Travers, P., Walport, M. & Shlomchik, M. J. (2004) *Immunobiology: the immune system in health and disease*, New York, Churchill Livingstone.
- Jankowski, M., Vreys, I., Wittevrongel, C., Boon, D., Vermylen, J., Hoylaerts, M. F. & Arnout, J. (2003) Thrombogenicity of beta 2-glycoprotein I-dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. *Blood*, 101, 157-62.
- Janssens, S. & Beyaert, R. (2003) Role of Toll-like receptors in pathogen recognition. *Clin Microbiol Rev*, 16, 637-46.
- Jara, L. J., Medina, G., Vera-Lastra, O. & Barile, L. (2005) The impact of gender on clinical manifestations of primary antiphospholipid syndrome. *Lupus*, 14, 607-12.
- Johnson, A. E., Gordon, C., Palmer, R. G. & Bacon, P. A. (1995) The prevalence and incidence of systemic lupus erythematosus in Birmingham, England. Relationship to ethnicity and country of birth. *Arthritis Rheum*, 38, 551-8.
- Joseph, J. E., Harrison, P., Mackie, I. J., Isenberg, D. A. & Machin, S. J. (2001) Increased circulating platelet-leucocyte complexes and platelet activation in patients with antiphospholipid syndrome, systemic lupus erythematosus and rheumatoid arthritis. *Br J Haematol*, 115, 451-9.
- Kalsi, J., Martin, A. C., Hirabayashi, Y., Ehrenstein, M., Longhurst, C. M., Ravirajan, C. T., Zvelebil, M., Stollar, B. D., Thornton, J. M. & Isenberg, D. A. (1996) Functional and modelling studies of the binding of human monoclonal anti-DNA antibodies to DNA. *Molecular Immunology*, 33, 471-83.
- Kalunian, K. C., Davis, J. C., Jr., Merrill, J. T., Totoritis, M. C. & Wofsy, D. (2002) Treatment of systemic lupus erythematosus by inhibition of T cell costimulation with anti-CD154: a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum*, 46, 3251-8.
- Kandiah, D. A., Sali, A., Sheng, Y., Victoria, E. J., Marquis, D. M., Coutts, S. M. & Krilis, S. A. (1998) Current insights into the "antiphospholipid" syndrome: clinical, immunological, and molecular aspects. *Adv Immunol*, 70, 507-63.
- Kane, J. F. (1995) Effects of rare codon clusters on high-level expression of heterologous proteins in Escherichia coli. *Curr Opin Biotechnol*, 6, 494-500.
- Kaplanski, G., Cacoub, P., Farnarier, C., Marin, V., Gregoire, R., Gatel, A., Durand, J. M., Harle, J. R., Bongrand, P. & Piette, J. C. (2000) Increased soluble vascular

- cell adhesion molecule 1 concentrations in patients with primary or systemic lupus erythematosus-related antiphospholipid syndrome: correlations with the severity of thrombosis. *Arthritis Rheum*, 43, 55-64.
- Kassis, J., Neville, C., Rauch, J., Busque, L., Chang, E. R., Joseph, L., Le Comte, M., Subang, R. & Fortin, P. R. (2004) Antiphospholipid antibodies and thrombosis: association with acquired activated protein C resistance in venous thrombosis and with hyperhomocysteinemia in arterial thrombosis. *Thromb Haemost*, 92, 1312-9.
- Kawai, T. & Akira, S. (2005) Pathogen recognition with Toll-like receptors. *Curr Opin Immunol*, 17, 338-44.
- Keeling, D. M., Campbell, S. J., Mackie, I. J., Machin, S. J. & Isenberg, D. A. (1991) The fibrinolytic response to venous occlusion and the natural anticoagulants in patients with antiphospholipid antibodies both with and without systemic lupus erythematosus. *Br J Haematol*, 77, 354-9.
- Keeling, D. M., Wilson, A. J., Mackie, I. J., Isenberg, D. A. & Machin, S. J. (1993) Role of beta 2-glycoprotein I and anti-phospholipid antibodies in activation of protein C in vitro. *J Clin Pathol*, 46, 908-11.
- Khamashta, M. A., Cuadrado, M. J., Mujic, F., Taub, N. A., Hunt, B. J. & Hughes, G. R. (1995) The management of thrombosis in the antiphospholipid-antibody syndrome. *N Engl J Med*, 332, 993-7.
- Khamashta, M. A. & Hughes, G. R. (1993) ACP Broadsheet no 136: February 1993. Detection and importance of anticardiolipin antibodies. *J Clin Pathol*, 46, 104-7.
- Kigawa, T., Yabuki, T., Yoshida, Y., Tsutsui, M., Ito, Y., Shibata, T. & Yokoyama, S. (1999) Cell-free production and stable-isotope labeling of milligram quantities of proteins. *FEBS Lett*, 442, 15-9.
- Kirk, P. & Bazan, J. F. (2005) Pathogen recognition: TLRs throw us a curve. *Immunity*, 23, 347-50.
- Kleber-Janke, T. & Becker, W. M. (2000) Use of modified BL21(DE3) Escherichia coli cells for high-level expression of recombinant peanut allergens affected by poor codon usage. *Protein Expr Purif*, 19, 419-24.
- Kobayashi, K., Matsuura, E., Liu, Q., Furukawa, J., Kaihara, K., Inagaki, J., Atsumi, T., Sakairi, N., Yasuda, T., Voelker, D. R. & Koike, T. (2001) A specific ligand for beta(2)-glycoprotein I mediates autoantibody-dependent uptake of oxidized low density lipoprotein by macrophages. *J Lipid Res*, 42, 697-709.
- Koike, T., Ichikawa, K., Atsumi, T., Kasahara, H. & Matsuura, E. (2000) Beta 2-glycoprotein I-anti-beta 2-glycoprotein I interaction. *J Autoimmun*, 15, 97-100.
- Kopp, E. & Ghosh, S. (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science*, 265, 956-9.
- Kornberg, A., Blank, M., Kaufman, S. & Shoenfeld, Y. (1994) Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. *J Immunol*, 153, 1328-32.
- Kouts, S., Bunn, C. L., Steinkasserer, A. & Krilis, S. (1993) Expression of human recombinant beta 2-glycoprotein I with anticardiolipin antibody cofactor activity. *FEBS Lett*, 326, 105-8.
- Krnic-Barrie, S., O'connor, C. R., Looney, S. W., Pierangeli, S. S. & Harris, E. N. (1997) A retrospective review of 61 patients with antiphospholipid syndrome. Analysis of factors influencing recurrent thrombosis. *Arch Intern Med*, 157, 2101-8.
- Kroeff, E. P., Owens, R. A., Campbell, E. L., Johnson, R. D. & Marks, H. I. (1989) Production scale purification of biosynthetic human insulin by reversed-phase high-performance liquid chromatography. *J Chromatogr*, 461, 45-61.

- Kroll, J., Larsen, J. K., Loft, H., Ezban, M., Wallevik, K. & Faber, M. (1976) DNA-binding proteins in Yoshida ascites tumor fluid. *Biochim Biophys Acta*, 434, 490-501.
- Kumar, S., Kalsi, J., Latchman, D. S., Pearl, L. H. & Isenberg, D. A. (2001) Expression of the Fabs of human auto-antibodies in *Escherichia coli*: optimization and determination of their fine binding characteristics and cross-reactivity. *J Mol Biol*, 308, 527-39.
- Kumar, S., Kalsi, J., Ravirajan, C. T., Rahman, A., Athwal, D., Latchman, D. S., Isenberg, D. A. & Pearl, L. H. (2000) Molecular cloning and expression of the Fabs of human autoantibodies in *Escherichia coli*. Determination of the heavy or light chain contribution to the anti-DNA/-cardiolipin activity of the Fab. *J Biol Chem*, 275, 35129-36.
- Kutteh, W. H. (1996) Antiphospholipid antibody-associated recurrent pregnancy loss: treatment with heparin and low-dose aspirin is superior to low-dose aspirin alone. *Am J Obstet Gynecol*, 174, 1584-9.
- Kutteh, W. H. & Ermel, L. D. (1996) A clinical trial for the treatment of antiphospholipid antibody-associated recurrent pregnancy loss with lower dose heparin and aspirin. *Am J Reprod Immunol*, 35, 402-7.
- Kutteh, W. H., Lyda, E. C., Abraham, S. M. & Wacholtz, M. C. (1993) Association of anticardiolipin antibodies and pregnancy loss in women with systemic lupus erythematosus. *Fertil Steril*, 60, 449-55.
- Kuwana, M., Matsuura, E., Kobayashi, K., Okazaki, Y., Kaburaki, J., Ikeda, Y. & Kawakami, Y. (2005) Binding of β 2-glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells. *Blood*, 105, 1552-7.
- Kwak, J. Y., Barini, R., Gilman-Sachs, A., Beaman, K. D. & Beer, A. E. (1994) Down-regulation of maternal antiphospholipid antibodies during early pregnancy and pregnancy outcome. *Am J Obstet Gynecol*, 171, 239-46.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-5.
- Lambrianides, N., Giles, I. P., Latchman, D. S., Isenberg, D. A. & Rahman, A. (2004) Stable transfection of CHO cells to express whole human IgG antiphospholipid antibodies. *Rheumatology*, 43 (Suppl 2), Abstract 263.
- Landefeld, C. S. & Goldman, L. (1989) Major bleeding in outpatients treated with warfarin: incidence and prediction by factors known at the start of outpatient therapy. *Am J Med*, 87, 144-52.
- Landsteiner, K., Muller, R. & Potzl, D. (1907) Zur Frage der Komplementbindungreaktion bei Syphilis. *Wein Klin Wschr*, 20, 1565-7.
- Laskin, C. A., Bombardier, C., Hannah, M. E., Mandel, F. P., Ritchie, J. W., Farewell, V., Farine, D., Spitzer, K., Fielding, L., Soloninka, C. A. & Yeung, M. (1997) Prednisone and aspirin in women with autoantibodies and unexplained recurrent fetal loss. *N Engl J Med*, 337, 148-53.
- Laurell, A. B. & Nilsson, I. M. (1957) Hypergammaglobulinemia, circulating anticoagulant, and biologic false positive Wassermann reaction; a study in two cases. *J Lab Clin Med*, 49, 694-707.
- Leandro, M. J., Cambridge, G., Edwards, J. C., Ehrenstein, M. R. & Isenberg, D. A. (2005) B-cell depletion in the treatment of patients with systemic lupus erythematosus: a longitudinal analysis of 24 patients. *Rheumatology (Oxford)*, 44, 1542-5.
- Lee, E. Y., Lee, C. K., Lee, T. H., Chung, S. M., Kim, S. H., Cho, Y. S., Yoo, B. & Moon, H. B. (2003) Does the anti-beta2-glycoprotein I antibody provide additional information in patients with thrombosis? *Thromb Res*, 111, 29-32.

- Lee, N. S., Brewer, H. B., Jr. & Osborne, J. C., Jr. (1983) beta 2-Glycoprotein I. Molecular properties of an unusual apolipoprotein, apolipoprotein H. *J Biol Chem*, 258, 4765-70.
- Lee, S. L. & Sanders, M. (1955) A disorder of blood coagulation in systemic lupus erythematosus. *J Clin Invest*, 34, 1814-22.
- Letsas, K. P., Filippatos, G. S., Kounas, S. P., Pappas, L. K., Gavrielatos, G. & Kardaras, F. (2005) Primary antiphospholipid syndrome and factor V Leiden mutation in a young patient with non-bacterial thrombotic endocarditis and transient ischemic stroke. *Thromb Haemost*, 94, 1331-2.
- Levine, J. S., Subang, R., Nasr, S. H., Fournier, S., Lajoie, G., Wither, J. & Rauch, J. (2006) Immunization with an apoptotic cell-binding protein recapitulates the nephritis and sequential autoantibody emergence of systemic lupus erythematosus. *J Immunol*, 177, 6504-16.
- Levine, S. R., Brey, R. L., Tilley, B. C., Thompson, J. L., Sacco, R. L., Sciacca, R. R., Murphy, A., Lu, Y., Costigan, T. M., Rhine, C., Levin, B., Triplett, D. A. & Mohr, J. P. (2004) Antiphospholipid antibodies and subsequent thrombo-occlusive events in patients with ischemic stroke. *Jama*, 291, 576-84.
- Libman, E. & Sacks, B. (1924) A hitherto undescribed form of valvular and mural endocarditis. *Arch Intern Med*, 33, 701-38.
- Llorente, L., Richaud-Patin, Y., Garcia-Padilla, C., Claret, E., Jakez-Ocampo, J., Cardiel, M. H., Alcocer-Varela, J., Grangeot-Keros, L., Alarcon-Segovia, D., Wijdenes, J., Galanaud, P. & Emilie, D. (2000) Clinical and biologic effects of anti-interleukin-10 monoclonal antibody administration in systemic lupus erythematosus. *Arthritis Rheum*, 43, 1790-800.
- Lloyd, A. T. & Sharp, P. M. (1992) Evolution of codon usage patterns: the extent and nature of divergence between *Candida albicans* and *Saccharomyces cerevisiae*. *Nucleic Acids Res*, 20, 5289-95.
- Lobel, L., Pollak, S., Lustbader, B., Klein, J. & Lustbader, J. W. (2002) Bacterial expression of a natively folded extracellular domain fusion protein of the hFSH receptor in the cytoplasm of *Escherichia coli*. *Protein Expr Purif*, 25, 124-33.
- Lockshin, M. D., Sammaritano, L. R. & Schwartzman, S. (2000) Validation of the Sapporo criteria for antiphospholipid syndrome. *Arthritis Rheum*, 43, 440-3.
- Loizou, S., Mccrea, J. D., Rudge, A. C., Reynolds, R., Boyle, C. C. & Harris, E. N. (1985) Measurement of anti-cardiolipin antibodies by an enzyme-linked immunosorbent assay (ELISA): standardization and quantitation of results. *Clin Exp Immunol*, 62, 738-45.
- Loizou, S., Samarkos, M., Norworthy, P. J., Cazabon, J. K., Walport, M. J. & Davies, K. A. (2000) Significance of anticardiolipin and anti- β_2 -glycoprotein I antibodies in lupus nephritis. *Rheumatology*, 39, 962-8.
- Lopez-Lira, F., Rosales-Leon, L., Martinez, V. M. & Ruiz Ordaz, B. H. (2006) The role of beta2-glycoprotein I (beta(2)GPI) in the activation of plasminogen. *Biochim Biophys Acta*.
- Lopez-Pedreria, C., Buendia, P., Cuadrado, M. J., Siendones, E., Aguirre, M. A., Barbarroja, N., Montiel-Duarte, C., Torres, A., Khamashta, M. & Velasco, F. (2006) Antiphospholipid antibodies from patients with the antiphospholipid syndrome induce monocyte tissue factor expression through the simultaneous activation of NF-kappaB/Rel proteins via the p38 mitogen-activated protein kinase pathway, and of the MEK-1/ERK pathway. *Arthritis Rheum*, 54, 301-11.
- Lozier, J., Takahashi, N. & Putnam, F. W. (1984) Complete amino acid sequence of human plasma beta 2-glycoprotein I. *Proc Natl Acad Sci U S A*, 81, 3640-4.
- Lu, C. S., Horizon, A. A., Hwang, K. K., Fitzgerald, J., Lin, W. S., Hahn, B. H., Wallace, D. J., Metzger, A. L., Weisman, M. H. & Chen, P. P. (2005)

- Identification of polyclonal and monoclonal antibodies against tissue plasminogen activator in the antiphospholipid syndrome. *Arthritis Rheum*, 52, 4018-27.
- Lubbe, W. F., Butler, W. S., Palmer, S. J. & Liggins, G. C. (1983) Fetal survival after prednisone suppression of maternal lupus-anticoagulant. *Lancet*, 1, 1361-3.
- Lutters, B. C., Derksen, R. H., Tekelenburg, W. L., Lenting, P. J., Arnout, J. & De Groot, P. G. (2003) Dimers of beta 2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2'. *J Biol Chem*, 278, 33831-8.
- Lutters, B. C., Meijers, J. C., Derksen, R. H., Arnout, J. & De Groot, P. G. (2001) Dimers of beta 2-glycoprotein I mimic the in vitro effects of beta 2-glycoprotein I-anti-beta 2-glycoprotein I antibody complexes. *J Biol Chem*, 276, 3060-7.
- Mackie, I. J., Colaco, C. B. & Machin, S. J. (1987) Familial lupus anticoagulants. *Br J Haematol*, 67, 359-63.
- Mackworth-Young, C., Chan, J., Harris, N., Walport, M., Bernstein, R., Batchelor, R., Hughes, G. & Gharavi, A. (1987) High incidence of anticardiolipin antibodies in relatives of patients with systemic lupus erythematosus. *J Rheumatol*, 14, 723-6.
- Mackworth-Young, C. G., Andreotti, F., Harmer, I., Loizou, S., Pottinger, B. E., Pearson, J. D., Davies, G. J., Maseri, A. & Walport, M. J. (1995) Endothelium-derived haemostatic factors and the antiphospholipid syndrome. *Br J Rheumatol*, 34, 201-6.
- Mackworth-Young, C. G., Loizou, S. & Walport, M. J. (1989) Primary antiphospholipid syndrome: features of patients with raised anticardiolipin antibodies and no other disorder. *Ann Rheum Dis*, 48, 362-7.
- Maini, R., St Clair, E. W., Breedveld, F., Furst, D., Kalden, J., Weisman, M., Smolen, J., Emery, P., Harriman, G., Feldmann, M. & Lipsky, P. (1999) Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT Study Group. *Lancet*, 354, 1932-9.
- Male, C., Foulon, D., Hoogendoorn, H., Vegh, P., Silverman, E., David, M. & Mitchell, L. (2005) Predictive value of persistent versus transient antiphospholipid antibody subtypes for the risk of thrombotic events in pediatric patients with systemic lupus erythematosus. *Blood*, 106, 4152-8.
- Malia, R. G., Kitchen, S., Greaves, M. & Preston, F. E. (1990) Inhibition of activated protein C and its cofactor protein S by antiphospholipid antibodies. *Br J Haematol*, 76, 101-7.
- Mandell, B. F. (1987) Cardiovascular involvement in systemic lupus erythematosus. *Semin Arthritis Rheum*, 17, 126-41.
- Many, A., Pauzner, R., Carp, H., Langevitz, P. & Martinowitz, U. (1992) Treatment of patients with antiphospholipid antibodies during pregnancy. *Am J Reprod Immunol*, 28, 216-8.
- Manz, R. A., Lohning, M., Cassese, G., Thiel, A. & Radbruch, A. (1998) Survival of long-lived plasma cells is independent of antigen. *Int Immunol*, 10, 1703-11.
- Manzi, S., Meilahn, E. N., Rairie, J. E., Conte, C. G., Medsger, T. A., Jr., Jansen-McWilliams, L., D'agostino, R. B. & Kuller, L. H. (1997) Age-specific incidence rates of myocardial infarction and angina in women with systemic lupus erythematosus: comparison with the Framingham Study. *Am J Epidemiol*, 145, 408-15.
- Manzi, S., Selzer, F., Sutton-Tyrrell, K., Fitzgerald, S. G., Rairie, J. E., Tracy, R. P. & Kuller, L. H. (1999) Prevalence and risk factors of carotid plaque in women with systemic lupus erythematosus. *Arthritis Rheum*, 42, 51-60.

- Marciniak, E. & Romond, E. H. (1989) Impaired catalytic function of activated protein C: a new in vitro manifestation of lupus anticoagulant. *Blood*, 74, 2426-32.
- Martin, S. L., Vrhovski, B. & Weiss, A. S. (1995) Total synthesis and expression in *Escherichia coli* of a gene encoding human tropoelastin. *Gene*, 154, 159-66.
- Matsuura, E., Igarashi, Y., Fujimoto, M., Ichikawa, K. & Koike, T. (1990) Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. *Lancet*, 336, 177-8.
- Matsuura, E., Igarashi, Y., Yasuda, T., Triplett, D. A. & Koike, T. (1994) Anticardiolipin antibodies recognize beta 2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. *J Exp Med*, 179, 457-62.
- Mattes, R. (2001) The production of improved tissue-type plasminogen activator in *Escherichia coli*. *Semin Thromb Hemost*, 27, 325-36.
- May, K. P., West, S. G., Moulds, J. & Kotzin, B. L. (1993) Different manifestations of the antiphospholipid antibody syndrome in a family with systemic lupus erythematosus. *Arthritis Rheum*, 36, 528-33.
- Mcaleer, W. J., Buynak, E. B., Maigetter, R. Z., Wampler, D. E., Miller, W. J. & Hilleman, M. R. (1984) Human hepatitis B vaccine from recombinant yeast. *Nature*, 307, 178-80.
- Mcclain, M. T., Arbuckle, M. R., Heinlen, L. D., Dennis, G. J., Roebuck, J., Rubertone, M. V., Harley, J. B. & James, J. A. (2004) The prevalence, onset, and clinical significance of antiphospholipid antibodies prior to diagnosis of systemic lupus erythematosus. *Arthritis Rheum*, 50, 1226-32.
- Mchugh, N. J. & Maddison, P. J. (1989) HLA-DR antigens and anticardiolipin antibodies in patients with systemic lupus erythematosus. *Arthritis Rheum*, 32, 1623-4.
- Mclaughlin, J., Gladman, D. D., Urowitz, M. B., Bombardier, C., Farewell, V. T. & Cole, E. (1991) Kidney biopsy in systemic lupus erythematosus. II. Survival analyses according to biopsy results. *Arthritis Rheum*, 34, 1268-73.
- Mcmahan, D. A., Smith, D. M., Carey, M. A. & Zhou, X. H. (1998) Risk of major hemorrhage for outpatients treated with warfarin. *J Gen Intern Med*, 13, 311-6.
- Mcneeley, P. A., Dlott, J. S., Furie, R. A., Jack, R. M., Ortel, T. L., Triplett, D. A., Victoria, E. J. & Linnik, M. D. (2001) Beta2-glycoprotein I-dependent anticardiolipin antibodies preferentially bind the amino terminal domain of beta2-glycoprotein I. *Thromb Haemost*, 86, 590-5.
- Mcneil, H. P., Gavaghan, T. P., Krilis, S. A., Geczy, A. F. & Chesterman, C. N. (1990a) HLA-DR antigens and anticardiolipin antibodies. *Clin Exp Rheumatol*, 8, 425-6.
- Mcneil, H. P., Simpson, R. J., Chesterman, C. N. & Krilis, S. A. (1990b) Antiphospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2- glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci U S A*, 87, 4120-4.
- Meade, T. W. & Miller, G. J. (1995) Combined use of aspirin and warfarin in primary prevention of ischemic heart disease in men at high risk. *Am J Cardiol*, 75, 23B-26B.
- Medina, G., Casaos, D., Jara, L. J., Vera-Lastra, O., Fuentes, M., Barile, L. & Salas, M. (2003) Increased carotid artery intima-media thickness may be associated with stroke in primary antiphospholipid syndrome. *Annals Rheum Dis*, 62, 607-10.
- Mehdi, H., Naqvi, A. & Kamboh, M. I. (2000) A hydrophobic sequence at position 313-316 (Leu-Ala-Phe-Trp) in the fifth domain of apolipoprotein H (beta2-glycoprotein I) is crucial for cardiolipin binding. *Eur J Biochem*, 267, 1770-6.

- Meissner, P., Pick, H., Kulangara, A., Chatellard, P., Friedrich, K. & Wurm, F. M. (2001) Transient gene expression: recombinant protein production with suspension-adapted HEK293-EBNA cells. *Biotechnol Bioeng*, 75, 197-203.
- Menon, S., Jameson-Shortall, E., Newman, S. P., Hall-Craggs, M. R., Chinn, R. & Isenberg, D. A. (1999) A longitudinal study of anticardiolipin antibody levels and cognitive functioning in systemic lupus erythematosus. *Arthritis Rheum*, 42, 735-41.
- Menon, S., Rahman, M. A., Ravirajan, C. T., Kandiah, D., Longhurst, C. M., McNally, T., Williams, W. M., Latchman, D. S. & Isenberg, D. A. (1997) The production, binding characteristics and sequence analysis of four human IgG monoclonal antiphospholipid antibodies. *J Autoimmun*, 10, 43-57.
- Meroni, P. L., Moia, M., Derksen, R. H., Tincani, A., McIntyre, J. A., Arnout, J. M., Koike, T., Piette, J. C., Khamashta, M. A. & Shoenfeld, Y. (2003) Venous thromboembolism in the antiphospholipid syndrome: management guidelines for secondary prophylaxis. *Lupus*, 12, 504-7.
- Meroni, P. L., Raschi, E., Testoni, C., Tincani, A., Balestrieri, G., Molteni, R., Khamashta, M. A., Tremoli, E. & Camera, M. (2001) Statins prevent endothelial cell activation induced by antiphospholipid (anti-beta2-glycoprotein I) antibodies: effect on the proadhesive and proinflammatory phenotype. *Arthritis Rheum*, 44, 2870-8.
- Metzler, M., Gertz, A., Sarkar, M., Schachter, H., Schrader, J. W. & Marth, J. D. (1994) Complex asparagine-linked oligosaccharides are required for morphogenic events during post-implantation development. *Embo J*, 13, 2056-65.
- Miksch, G., Neitzel, R., Fiedler, E., Friehs, K. & Flaschel, E. (1997) Extracellular production of a hybrid beta-glucanase from Bacillus by Escherichia coli under different cultivation conditions in shaking cultures and bioreactors. *Appl Microbiol Biotechnol*, 47, 120-6.
- Miyakis, S., Lockshin, M. D., Atsumi, T., Branch, D. W., Brey, R. L., Cervera, R., Derksen, R. H., Pg, D. E. G., Koike, T., Meroni, P. L., Reber, G., Shoenfeld, Y., Tincani, A., Vlachoyiannopoulos, P. G. & Krilis, S. A. (2006) International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost*, 4, 295-306.
- Mocci, S. & Coffman, R. L. (1995) Induction of a Th2 population from a polarized Leishmania-specific Th1 population by in vitro culture with IL-4. *J Immunol*, 154, 3779-87.
- Monestier, M., Kandiah, D. A., Kouts, S., Novick, K. E., Ong, G. L., Radic, M. Z. & Krilis, S. A. (1996) Monoclonal antibodies from NZW x BXSb F1 mice to beta2 glycoprotein I and cardiolipin. Species specificity and charge-dependent binding. *J Immunol*, 156, 2631-41.
- Moore, J. E. & Mohr, C. F. (1952) Biologically false positive serologic tests for syphilis. *JAMA*, 150, 467-73.
- Moore, P. A., Belvedere, O., Orr, A., Pieri, K., Lafleur, D. W., Feng, P., Soppet, D., Charters, M., Gentz, R., Parmelee, D., Li, Y., Galperina, O., Giri, J., Roschke, V., Nardelli, B., Carrell, J., Sosnovtseva, S., Greenfield, W., Ruben, S. M., Olsen, H. S., Fikes, J. & Hilbert, D. M. (1999) BLYS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science*, 285, 260-3.
- Moreland, L. W., Schiff, M. H., Baumgartner, S. W., Tindall, E. A., Fleischmann, R. M., Bulpitt, K. J., Weaver, A. L., Keystone, E. C., Furst, D. E., Mease, P. J., Ruderman, E. M., Horwitz, D. A., Arkfeld, D. G., Garrison, L., Burge, D. J., Blosch, C. M., Lange, M. L., McDonnell, N. D. & Weinblatt, M. E. (1999) Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial. *Ann Intern Med*, 130, 478-86.

- Morrow, W. J. W., Nelson, J. L., Watts, R. A. & Isenberg, D. A. (1999) *Autoimmune rheumatic disease*, Oxford, Oxford University Press.
- Moss, K. E., Ioannou, Y., Sultan, S. M., Haq, I. & Isenberg, D. A. (2002) Outcome of a cohort of 300 patients with systemic lupus erythematosus attending a dedicated clinic for over two decades. *Ann Rheum Dis*, 61, 409-13.
- Muzio, M., Natoli, G., Sacconi, S., Levrero, M. & Mantovani, A. (1998) The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J Exp Med*, 187, 2097-101.
- Nakano, H., Shinbata, T., Okumura, R., Sekiguchi, S., Fujishiro, M. & Yamane, T. (1999) Efficient coupled transcription/translation from PCR template by a hollow-fiber membrane bioreactor. *Biotechnol Bioeng*, 64, 194-9.
- Napoleone, E., Di Santo, A., Camera, M., Tremoli, E. & Lorenzet, R. (2000) Angiotensin-converting enzyme inhibitors downregulate tissue factor synthesis in monocytes. *Circ Res*, 86, 139-43.
- Nash, M. J., Camilleri, R. S., Kunka, S., Mackie, I. J., Machin, S. J. & Cohen, H. (2004) The anticardiolipin assay is required for sensitive screening for antiphospholipid antibodies. *J Thromb Haemost*, 2, 1077-81.
- Nicholson-Weller, A., Burge, J., Fearon, D. T., Weller, P. F. & Austen, K. F. (1982) Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J Immunol*, 129, 184-9.
- Nicolo, D., Goldman, B. I. & Monestier, M. (2003) Reduction of atherosclerosis in low-density lipoprotein receptor-deficient mice by passive administration of antiphospholipid antibody. *Arthritis Rheum*, 48, 2974-8.
- Nimpf, J., Bevers, E. M., Bomans, P. H., Till, U., Wurm, H., Kostner, G. M. & Zwaal, R. F. (1986) Prothrombinase activity of human platelets is inhibited by beta 2-glycoprotein-I. *Biochim Biophys Acta*, 884, 142-9.
- Nimpf, J., Wurm, H. & Kostner, G. M. (1987) Beta 2-glycoprotein-I (apo-H) inhibits the release reaction of human platelets during ADP-induced aggregation. *Atherosclerosis*, 63, 109-14.
- Niwa, S., Totsuka, T. & Hayashi, S. (1996) Inhibitory effect of fluvastatin, an HMG-CoA reductase inhibitor, on the expression of adhesion molecules on human monocyte cell line. *Int J Immunopharmacol*, 18, 669-75.
- Nojima, J., Kuratsune, H., Suehisa, E., Futsukaichi, Y., Yamanishi, H., Machii, T., Kitani, T., Iwatani, Y. & Kanakura, Y. (2001) Anti-prothrombin antibodies combined with lupus anti-coagulant activity is an essential risk factor for venous thromboembolism in patients with systemic lupus erythematosus. *Br J Haematol*, 114, 647-54.
- O'leary, J. M., Bromek, K., Black, G. M., Uhrinova, S., Schmitz, C., Wang, X., Krych, M., Atkinson, J. P., Uhrin, D. & Barlow, P. N. (2004) Backbone dynamics of complement control protein (CCP) modules reveals mobility in binding surfaces. *Protein Sci*, 13, 1238-50.
- Ogawa, H., Zhao, D., Dlott, J. S., Cameron, G. S., Yamazaki, M., Hata, T. & Triplett, D. A. (2000) Elevated anti-annexin V antibody levels in antiphospholipid syndrome and their involvement in antiphospholipid antibody specificities. *Am J Clin Pathol*, 114, 619-28.
- Ohkura, N., Hagihara, Y., Yoshimura, T., Goto, Y. & Kato, H. (1998) Plasmin can reduce the function of human beta2 glycoprotein I by cleaving domain V into a nicked form. *Blood*, 91, 4173-9.
- Olee, T., Pierangeli, S. S., Handley, H. H., Le, D. T., Wei, X., Lai, C. J., En, J., Novotny, W., Harris, E. N., Woods, V. L., Jr. & Chen, P. P. (1996) A monoclonal IgG anticardiolipin antibody from a patient with the

- antiphospholipid syndrome is thrombogenic in mice. *Proc Natl Acad Sci U S A*, 93, 8606-11.
- Oosting, J. D., Derksen, R. H., Bobbink, I. W., Hackeng, T. M., Bouma, B. N. & De Groot, P. G. (1993) Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? *Blood*, 81, 2618-25.
- Opara, R., Robbins, D. L. & Ziboh, V. A. (2003) Cyclic-AMP agonists inhibit antiphospholipid/beta2-glycoprotein I induced synthesis of human platelet thromboxane A2 in vitro. *J Rheumatol*, 30, 55-9.
- Pangborn, M. (1941) A new serologically active phospholipid from beef heart. *Proc Soc Exp Biol Med*, 48, 484-6.
- Pangborn, M. (1942) Isolation and purification of a serologically active phospholipid from beef heart. *J Biol Chem*, 143, 247-56.
- Parke, A. L., Weinstein, R. E., Bona, R. D., Maier, D. B. & Walker, F. J. (1992) The thrombotic diathesis associated with the presence of phospholipid antibodies may be due to low levels of free protein S. *Am J Med*, 93, 49-56.
- Pattison, N. S., Chamley, L. W., McKay, E. J., Liggins, G. C. & Butler, W. S. (1993) Antiphospholipid antibodies in pregnancy: prevalence and clinical associations. *Br J Obstet Gynaecol*, 100, 909-13.
- Paus, S., Potzsch, B., Risse, J. H., Klockgether, T. & Wullner, U. (2001) Chorea and antiphospholipid antibodies: treatment with methotrexate. *Neurology*, 56, 137-8.
- Peitsch, M. C. (1996) ProMod and Swiss-Model: Internet-based tools for automated comparative protein modelling. *Biochem Soc Trans*, 24, 274-9.
- Pengo, V., Biasiolo, A., Brocco, T., Tonetto, S. & Ruffatti, A. (1996) Autoantibodies to phospholipid-binding plasma proteins in patients with thrombosis and phospholipid-reactive antibodies. *Thromb Haemost*, 75, 721-4.
- Petri, M. (2000) Epidemiology of the antiphospholipid antibody syndrome. *J Autoimmun*, 15, 145-51.
- Petri, M. (2004) The lupus anticoagulant is a risk factor for myocardial infarction (but not atherosclerosis): Hopkins Lupus Cohort. *Thromb Res*, 114, 593-5.
- Phadke, K. V., Phillips, R. A., Clarke, D. T., Jones, M., Naish, P. & Carson, P. (1993) Anticardiolipin antibodies in ischaemic heart disease: marker or myth? *Br Heart J*, 69, 391-4.
- Pierangeli, S. S., Vega-Ostertag, M. E., Raschi, E., Liu, X., Borghi, M. O. and Meroni, P. L. (2005b) Toll like receptor 4 is involved in *in vivo* antiphospholipid-mediated thrombosis and endothelial cell activation. *Blood*, 106(11), Abstract 133.
- Pierangeli, S. S., Barker, J. H., Stikovac, D., Ackerman, D., Anderson, G., Barquinero, J., Acland, R. & Harris, E. N. (1994) Effect of human IgG antiphospholipid antibodies on an *in vivo* thrombosis model in mice. *Thromb Haemost*, 71, 670-4.
- Pierangeli, S. S., Blank, M., Liu, X., Espinola, R., Fridkin, M., Ostertag, M. V., Roye-Green, K., Harris, E. N. & Shoenfeld, Y. (2004a) A peptide that shares similarity with bacterial antigens reverses thrombogenic properties of antiphospholipid antibodies *in vivo*. *J Autoimmun*, 22, 217-25.
- Pierangeli, S. S., Colden-Stanfield, M., Liu, X., Barker, J. H., Anderson, G. L. & Harris, E. N. (1999) Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells *in vitro* and *in vivo*. *Circulation*, 99, 1997-2002.
- Pierangeli, S. S., Espinola, R. G., Liu, X. & Harris, E. N. (2001) Thrombogenic effects of antiphospholipid antibodies are mediated by intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and P-selectin. *Circ Res*, 88, 245-50.

- Pierangeli, S. S., Girardi, G., Vega-Ostertag, M., Liu, X., Espinola, R. G. & Salmon, J. (2005) Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. *Arthritis Rheum*, 52, 2120-4.
- Pierangeli, S. S., Liu, S. W., Anderson, G., Barker, J. H. & Harris, E. N. (1996) Thrombogenic properties of murine anti-cardiolipin antibodies induced by beta 2 glycoprotein 1 and human immunoglobulin G antiphospholipid antibodies. *Circulation*, 94, 1746-51.
- Pierangeli, S. S., Liu, X., Espinola, R., Olee, T., Zhu, M., Harris, N. E. & Chen, P. P. (2000) Functional analyses of patient-derived IgG monoclonal anticardiolipin antibodies using in vivo thrombosis and in vivo microcirculation models. *Thromb Haemost*, 84, 388-95.
- Pierangeli, S. S., Vega-Ostertag, M. & Harris, E. N. (2004b) Intracellular signaling triggered by antiphospholipid antibodies in platelets and endothelial cells: a pathway to targeted therapies. *Thromb Res*, 114, 467-76.
- Pistiner, M., Wallace, D. J., Nessim, S., Metzger, A. L. & Klinenberg, J. R. (1991) Lupus erythematosus in the 1980s: a survey of 570 patients. *Semin Arthritis Rheum*, 21, 55-64.
- Pittoni, V., Ravirajan, C. T., Donohoe, S., Machin, S. J., Lydyard, P. M. & Isenberg, D. A. (2000) Human monoclonal anti-phospholipid antibodies selectively bind to membrane phospholipid and beta2-glycoprotein I (beta2-GPI) on apoptotic cells. *Clin Exp Immunol*, 119, 533-43.
- Polz, E., Wurm, H. & Kostner, G. M. (1980) Investigations on beta 2-glycoprotein-I in the rat: isolation from serum and demonstration in lipoprotein density fractions. *Int J Biochem*, 11, 265-70.
- Pratico, D., Ferro, D., Iuliano, L., Rokach, J., Conti, F., Valesini, G., Fitzgerald, G. A. & Violi, F. (1999) Ongoing prothrombotic state in patients with antiphospholipid antibodies: a role for increased lipid peroxidation. *Blood*, 93, 3401-7.
- Prieto, G. A., Cabral, A. R., Zapata-Zuniga, M., Simon, A. J., Villa, A. R., Alarcon-Segovia, D. & Cabiedes, J. (2003) Valine/valine genotype at position 247 of the beta2-glycoprotein I gene in Mexican patients with primary antiphospholipid syndrome: association with anti-beta2-glycoprotein I antibodies. *Arthritis Rheum*, 48, 471-4.
- Prinz, W. A., Aslund, F., Holmgren, A. & Beckwith, J. (1997) The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the Escherichia coli cytoplasm. *J Biol Chem*, 272, 15661-7.
- Prodromou, C. & Pearl, L. H. (1992) Recursive PCR: a novel technique for total gene synthesis. *Protein Eng*, 5, 827-9.
- Qiu, J., Swartz, J. R. & Georgiou, G. (1998) Expression of active human tissue-type plasminogen activator in Escherichia coli. *Appl Environ Microbiol*, 64, 4891-6.
- Quartier, P., Potter, P. K., Ehrenstein, M. R., Walport, M. J. & Botto, M. (2004) Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages *in vitro*. *Euro J Immunol*, 35, 252-60.
- Radway-Bright, E. L., Inanc, M. & Isenberg, D. A. (1999) Animal models of the antiphospholipid syndrome. *Rheumatology (Oxford)*, 38, 591-601.
- Radway-Bright, E. L., Ravirajan, C. T. & Isenberg, D. A. (2000) The prevalence of antibodies to anionic phospholipids in patients with the primary antiphospholipid syndrome, systemic lupus erythematosus and their relatives and spouses. *Rheumatology (Oxford)*, 39, 427-31.
- Rahman, A., Haley, J., Radway-Bright, E., Nagl, S., Low, D. G., Latchman, D. S. & Isenberg, D. A. (2001) The importance of somatic mutations in the V(lambda) gene 2a2 in human monoclonal anti-DNA antibodies. *J Mol Biol*, 307, 149-60.

- Rahman, M. A., Kettleborough, C. A., Latchman, D. S. & Isenberg, D. A. (1998) Properties of whole human IgG molecules produced by the expression of cloned anti-DNA antibody cDNA in mammalian cells. *J Autoimmun*, 11, 661-9.
- Rai, R., Cohen, H., Dave, M. & Regan, L. (1997) Randomised controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies (or antiphospholipid antibodies). *Br Med J*, 314, 253-7.
- Ramos-Casals, M., Campoamor, M. T., Chamorro, A., Salvador, G., Segura, S., Botero, J. C., Yague, J., Cervera, R., Ingelmo, M. & Font, J. (2004) Hypocomplementemia in systemic lupus erythematosus and primary antiphospholipid syndrome: prevalence and clinical significance in 667 patients. *Lupus*, 13, 777-83.
- Rand, J. H., Wu, X. X., Andree, H. A., Ross, J. B., Rusinova, E., Gascon-Lema, M. G., Calandri, C. & Harpel, P. C. (1998) Antiphospholipid antibodies accelerate plasma coagulation by inhibiting annexin-V binding to phospholipids: a "lupus procoagulant" phenomenon. *Blood*, 92, 1652-60.
- Rand, J. H., Wu, X. X., Guller, S., Gil, J., Guha, A., Scher, J. & Lockwood, C. J. (1994) Reduction of annexin-V (placental anticoagulant protein-I) on placental villi of women with antiphospholipid antibodies and recurrent spontaneous abortion. *Am J Obstet Gynecol*, 171, 1566-72.
- Rand, J. H., Wu, X. X., Guller, S., Scher, J., Andree, H. A. & Lockwood, C. J. (1997) Antiphospholipid immunoglobulin G antibodies reduce annexin-V levels on syncytiotrophoblast apical membranes and in culture media of placental villi. *Am J Obstet Gynecol*, 177, 918-23.
- Rand, J. H., Wu, X. X., Lapinski, R., Van Heerde, W. L., Reutelingsperger, C. P., Chen, P. P. & Ortel, T. L. (2004) Detection of antibody-mediated reduction of annexin A5 anticoagulant activity in plasmas of patients with the antiphospholipid syndrome. *Blood*, 104, 2783-90.
- Rand, J. H., Wu, X. X., Quinn, A. S., Chen, P. P., Mccrae, K. R., Bovill, E. G. & Taatjes, D. J. (2003) Human monoclonal antiphospholipid antibodies disrupt the annexin A5 anticoagulant crystal shield on phospholipid bilayers: evidence from atomic force microscopy and functional assay. *Am J Pathol*, 163, 1193-200.
- Raschi, E., Testoni, C., Bosisio, D., Borghi, M. O., Koike, T., Mantovan, A. & Meroni, P. L. (2003) Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. *Blood*, 101, 3495-500.
- Reber, G., Tincani, A., Sanmarco, M., De Moerloose, P. & Boffa, M. C. (2004) Proposals for the measurement of anti-beta2-glycoprotein I antibodies. Standardization group of the European Forum on Antiphospholipid Antibodies. *J Thromb Haemost*, 2, 1860-2.
- Reddel, S. W., Wang, Y. X., Sheng, Y. H. & Krilis, S. A. (2000) Epitope studies with anti-beta 2-glycoprotein I antibodies from autoantibody and immunized sources. *J Autoimmun*, 15, 91-6.
- Regnault, V., Arvieux, J., Vallar, L. & T., L. (1999) Both kintec data and epitope mapping provide clues for understanding the anti-coagulant effect of five murine monoclonal antibodies to β 2-glycoprotein I. *Immunol*, 97, 400-7.
- Reid, K. B. & Day, A. J. (1989) Structure-function relationships of the complement components. *Immunol Today*, 10, 177-80.
- Reutelingsperger, C. P., Kop, J. M., Hornstra, G. & Hemker, H. C. (1988) Purification and characterization of a novel protein from bovine aorta that inhibits coagulation. Inhibition of the phospholipid-dependent factor-Xa-catalyzed prothrombin activation, through a high-affinity binding of the anticoagulant to the phospholipids. *Eur J Biochem*, 173, 171-8.

- Reverter, J. C., Tassies, D., Font, J., Khamashta, M. A., Ichikawa, K., Cervera, R., Escolar, G., Hughes, G. R., Ingelmo, M. & Ordinas, A. (1998) Effects of human monoclonal anticardiolipin antibodies on platelet function and on tissue factor expression on monocytes. *Arthritis Rheum*, 41, 1420-7.
- Rifkin, I. R., Leadbetter, E. A., Busconi, L., Viglianti, G. & Marshak-Rothstein, A. (2005) Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunol Rev*, 204, 27-42.
- Rippmann, J. F., Klein, M., Hoischen, C., Brocks, B., Rettig, W. J., Gumpert, J., Pfizenmaier, K., Mattes, R. & Moosmayer, D. (1998) Procaryotic expression of single-chain variable-fragment (scFv) antibodies: secretion in L-form cells of *Proteus mirabilis* leads to active product and overcomes the limitations of periplasmic expression in *Escherichia coli*. *Appl Environ Microbiol*, 64, 4862-9.
- Robbins, D. L., Leung, S., Miller-Blair, D. J. & Ziboh, V. (1998) Effect of anticardiolipin/beta2-glycoprotein I complexes on production of thromboxane A2 by platelets from patients with the antiphospholipid syndrome. *J Rheumatol*, 25, 51-6.
- Roberts, J. M. & Hubel, C. A. (2004) Oxidative stress in preeclampsia. *Am J Obstet Gynecol*, 190, 1177-8.
- Robertson, B. & Greaves, M. (2006) Antiphospholipid syndrome: An evolving story. *Blood Rev*, 20, 201-12.
- Robertson, S. A., Roberts, C. T., Van Beijering, E., Pensa, K., Sheng, Y., Shi, T. & Krilis, S. A. (2004) Effect of beta2-glycoprotein I null mutation on reproductive outcome and antiphospholipid antibody-mediated pregnancy pathology in mice. *Mol Hum Reprod*, 10, 409-16.
- Roitt, I., Brostoff, J. & Male, D. (2001) *Immunology*, London, Mosby.
- Rosen, L. A., Anhalt, G. & Rosen, A. (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med*, 179, 1317-30.
- Rosen, L. A., Rosen, A., Petri, M. & Schliessel, M. (1996) Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. *Proc Natl Acad Sci USA*, 93, 1624-9.
- Rosove, M. H. & Brewer, P. M. (1992) Antiphospholipid thrombosis: clinical course after the first thrombotic event in 70 patients. *Ann Intern Med*, 117, 303-8.
- Roubey, R. A. (2004) Antiphospholipid antibodies: immunological aspects. *Clin Immunol*, 112, 127-8.
- Roubey, R. A., Eisenberg, R. A., Harper, M. F. & Winfield, J. B. (1995) "Anticardiolipin" autoantibodies recognize beta 2-glycoprotein I in the absence of phospholipid. Importance of Ag density and bivalent binding. *J Immunol*, 154, 954-60.
- Rouget, J. P., Goudemand, J., Montreuil, G., Cosson, A. & Jaillard, J. (1982) Lupus anticoagulant: a familial observation. *Lancet*, 2, 105.
- Rubenstein, E., Arkfeld, D. G., Metyas, S., Shinada, S., Ehresmann, S. & Liebman, H. A. (2006) Rituximab treatment for resistant antiphospholipid syndrome. *J Rheumatol*, 33, 355-7.
- Ruiz-Irastorza, G. & Khamashta, M. A. (2005) Stroke and antiphospholipid syndrome: the treatment debate. *Rheumatology (Oxford)*, 44, 971-4.
- Ruiz-Irastorza, G., Khamashta, M. A., Hunt, B. J., Escudero, A., Cuadrado, M. J. & Hughes, G. R. (2002) Bleeding and recurrent thrombosis in definite antiphospholipid syndrome: analysis of a series of 66 patients treated with oral anticoagulation to a target international normalized ratio of 3.5. *Arch Intern Med*, 162, 1164-9.

- Rutz, M., Metzger, J., Gellert, T., Luppa, P., Lipford, G. B., Wagner, H. & Bauer, S. (2004) Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur J Immunol*, 34, 2541-50.
- Safa, O., Esmon, C. T. & Esmon, N. L. (2005) Inhibition of APC anticoagulant activity on oxidized phospholipid by anti- β 2-glycoprotein I monoclonal antibodies. *Blood*, 106, 1629-35.
- Safa, O., Hensley, K., Smirnov, M. D., Esmon, C. T. & Esmon, N. L. (2001) Lipid oxidation enhances the function of activated protein C. *J Biol Chem*, 276, 1829-36.
- Salemink, I., Blezer, R., Willems, G. M., Galli, M., Bevers, E. & Lindhout, T. (2000) Antibodies to beta2-glycoprotein I associated with antiphospholipid syndrome suppress the inhibitory activity of tissue factor pathway inhibitor. *Thromb Haemost*, 84, 653-6.
- Salmon, J. E. & Alpert, D. (2006) Are we coming to terms with tumor necrosis factor inhibition in pregnancy? *Arthritis Rheum*, 54, 2353-5.
- Sanger, F. & Thompson, E. O. (1953a) The amino-acid sequence in the glycol chain of insulin. I. The identification of lower peptides from partial hydrolysates. *Biochem J*, 53, 353-66.
- Sanger, F. & Thompson, E. O. (1953b) The amino-acid sequence in the glycol chain of insulin. II. The investigation of peptides from enzymic hydrolysates. *Biochem J*, 53, 366-74.
- Sanghera, D. K., Kristensen, T., Hamman, R. F. & Kamboh, M. I. (1997a) Molecular basis of the apolipoprotein H (beta 2-glycoprotein I) protein polymorphism. *Hum Genet*, 100, 57-62.
- Sanghera, D. K., Nestlerode, C. S., Ferrell, R. E. & Kamboh, M. I. (2001) Chimpanzee apolipoprotein H (beta2-glycoprotein I): report on the gene structure, a common polymorphism, and a high prevalence of antiphospholipid antibodies. *Hum Genet*, 109, 63-72.
- Sanghera, D. K., Wagenknecht, D. R., McIntyre, J. A. & Kamboh, M. I. (1997b) Identification of structural mutations in the fifth domain of apolipoprotein H (beta 2-glycoprotein I) which affect phospholipid binding. *Hum Mol Genet*, 6, 311-6.
- Savi, M., Ferraccioli, G. F., Neri, T. M., Zanelli, P., Dall'aglio, P. P., Tincani, A., Balestrieri, G., Carella, G. & Cattaneo, R. (1988) HLA-DR antigens and anticardiolipin antibodies in northern Italian systemic lupus erythematosus patients. *Arthritis Rheum*, 31, 1568-70.
- Schousboe, I. (1979) Purification, characterization and identification of an agglutinin in human serum. *Biochim Biophys Acta*, 579, 396-408.
- Schousboe, I. (1985) beta 2-Glycoprotein I: a plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. *Blood*, 66, 1086-91.
- Schousboe, I. & Rasmussen, M. S. (1995) Synchronized inhibition of the phospholipid mediated autoactivation of factor XII in plasma by beta 2-glycoprotein I and anti-beta 2-glycoprotein I. *Thromb Haemost*, 73, 798-804.
- Schulman, S., Svenungsson, E. & Granqvist, S. (1998) Anticardiolipin antibodies predict early recurrence of thromboembolism and death among patients with venous thromboembolism following anticoagulant therapy. Duration of Anticoagulation Study Group. *Am J Med*, 104, 332-8.
- Schultze, S., Heide, K. & Haupt, H. (1961) Über ein bisher unbekanntes niedermolekulares Beta2-Globulin des Humanserums. *Naturwissenschaften*, 48, 719.
- Schved, J. F., Gris, J. C., Ollivier, V., Wautier, J. L., Tobelem, G. & Caen, J. (1992) Procoagulant activity of endotoxin or tumor necrosis factor activated monocytes is enhanced by IgG from patients with lupus anticoagulant. *Am J Hematol*, 41, 92-6.

- Schwarzenbacher, R., Zeth, K., Diederichs, K., Gries, A., Kostner, G. M., Laggner, P. & Prassl, R. (1999) Crystal structure of human beta2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *Embo J*, 18, 6228-39.
- Sebastiani, G. D., Galeazzi, M., Tincani, A., Scorza, R., Mathieu, A., Passiu, G., Morozzi, G., Piette, J. C., Cervera, R., Houssiau, F., Smolen, J., Fernandez Nebro, A., De Ramon, E., Goral, A. J., Papasteriades, C., Ferrara, G. B., Carcassi, C., Bellisai, F. & Marcolongo, R. (2003) HLA-DPB1 alleles association of anticardiolipin and anti-beta2GPI antibodies in a large series of European patients with systemic lupus erythematosus. *Lupus*, 12, 560-3.
- Shah, N. M., Khamashta, M. A., Atsumi, T. & Hughes, G. R. (1998) Outcome of patients with anticardiolipin antibodies: a 10 year follow- up of 52 patients. *Lupus*, 7, 3-6.
- Shapiro, S. S. (2005) Lupus anticoagulants and anticardiolipin antibodies: personal reminiscences, a little history, and some random thoughts. *J Thromb Haemost*, 3, 831-3.
- Sheng, Y., Hanly, J. G., Reddel, S. W., Kouts, S., Guerin, J., Koike, T., Ichikawa, K., Sturgess, A. & Krilis, S. A. (2001a) Detection of 'antiphospholipid' antibodies: a single chromogenic assay of thrombin generation sensitively detects lupus anticoagulants, anticardiolipin antibodies, plus antibodies binding beta(2)-glycoprotein I and prothrombin. *Clin Exp Immunol*, 124, 502-8.
- Sheng, Y., Herzog, H. & Krilis, S. A. (1997) Cloning and characterization of the gene encoding the mouse beta 2-glycoprotein I. *Genomics*, 41, 128-30.
- Sheng, Y., Kandiah, D. A. & Krilis, S. A. (1998) Anti-beta 2-glycoprotein I autoantibodies from patients with the "antiphospholipid" syndrome bind to beta 2-glycoprotein I with low affinity: dimerization of beta 2-glycoprotein I induces a significant increase in anti-beta 2-glycoprotein I antibody affinity. *J Immunol*, 161, 2038-43.
- Sheng, Y., Reddel, S. W., Herzog, H., Wang, Y. X., Brighton, T., France, M. P., Robertson, S. A. & Krilis, S. A. (2001b) Impaired thrombin generation in beta 2-glycoprotein I null mice. *J Biol Chem*, 276, 13817-21.
- Sheng, Y., Sali, A., Herzog, H., Lahnstein, J. & Krilis, S. A. (1996) Site-directed mutagenesis of recombinant human beta 2-glycoprotein I identifies a cluster of lysine residues that are critical for phospholipid binding and anti-cardiolipin antibody activity. *J Immunol*, 157, 3744-51.
- Sherer, Y., Levy, Y. & Shoenfeld, Y. (2000) Intravenous immunoglobulin therapy of antiphospholipid syndrome. *Rheumatology (Oxford)*, 39, 421-6.
- Sheriff, S., Silverton, E. W., Padlan, E. A., Cohen, G. H., Smith-Gill, S. J., Finzel, B. C. & Davies, D. R. (1987) Three-dimensional structure of an antibody-antigen complex. *Proc Natl Acad Sci U S A*, 84, 8075-9.
- Shi, T., Giannakopoulos, B., Yan, X., Yu, P., Berndt, M. C., Andrews, R., Rivera, J., Iverson, G. M., Cockerill, K. A., Linnik, M. D. & Krilis, S. (2006) Anti-beta2-glycoprotein I antibodies in complex with beta2-glycoprotein I can activate platelets in a dysregulated manner via glycoprotein Ib-IX-V. *Arthritis Rheum*, 54, 2558-67.
- Shi, T., Iverson, G. M., Qi, J. C., Cockerill, K. A., Linnik, M. D., Konecny, P. & Krilis, S. A. (2004) Beta 2-Glycoprotein I binds factor XI and inhibits its activation by thrombin and factor XIIa: loss of inhibition by clipped beta 2-glycoprotein I. *Proc Natl Acad Sci U S A*, 101, 3939-44.
- Shi, W., Krilis, S. A., Chong, B. H., Gordon, S. & Chesterman, C. N. (1990) Prevalence of lupus anticoagulant and anticardiolipin antibodies in a healthy population. *Aust N Z J Med*, 20, 231-6.
- Shoenfeld, Y., Krause, I., Kvapil, F., Sulkes, J., Lev, S., Von Landenberg, P., Font, J., Zaech, J., Cervera, R., Piette, J. C., Boffa, M. C., Khamashta, M. A.,

- Bertolaccini, M. L., Hughes, G. R., Youinou, P., Meroni, P. L., Pengo, V., Alves, J. D., Tincani, A., Szegedi, G., Lakos, G., Sturfelt, G., Jonsen, A., Koike, T., Sanmarco, M., Ruffatti, A., Ulcova-Gallova, Z., Praprotnik, S., Rozman, B., Lorber, M., Vriezman, V. B. & Blank, M. (2003) Prevalence and clinical correlations of antibodies against six beta2-glycoprotein-I-related peptides in the antiphospholipid syndrome. *J Clin Immunol*, 23, 377-83.
- Shoenfeld, Y., Sherer, Y. & Fishman, P. (1998) Interleukin-3 and pregnancy loss in antiphospholipid syndrome. *Scand J Rheumatol Suppl*, 107, 19-22.
- Silver, R. K., Macgregor, S. N., Sholl, J. S., Hobart, J. M., Neerhof, M. G. & Ragin, A. (1993) Comparative trial of prednisone plus aspirin versus aspirin alone in the treatment of anticardiolipin antibody-positive obstetric patients. *Am J Obstet Gynecol*, 169, 1411-7.
- Simantov, R., Lasala, J. M., Lo, S. K., Gharavi, A. E., Sammaritano, L. R., Salmon, J. E. & Silverstein, R. L. (1995) Activation of cultured vascular endothelial cells by antiphospholipid antibodies. *J Clin Invest*, 96, 2211-9.
- Sjoholm, A. G., Martensson, U. & Sturfelt, G. (1997) Serial analysis of autoantibody responses to the collagen-like region of C1q, collagen type II, and double stranded DNA in patients with systemic lupus erythematosus. *J Rheumatol*, 24, 871-8.
- Smirnov, M. D., Triplett, D. T., Comp, P. C., Esmon, N. L. & Esmon, C. T. (1995) On the role of phosphatidylethanolamine in the inhibition of activated protein C activity by antiphospholipid antibodies. *J Clin Invest*, 95, 309-16.
- Smith, H. R., Hansen, C. L., Rose, R. & Canoso, R. T. (1990) Autoimmune MRL-1 pr/1pr mice are an animal model for the secondary antiphospholipid syndrome. *J Rheumatol*, 17, 911-5.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem*, 150, 76-85.
- Soulier, J. P. & Boffa, M. C. (1980) Repetitive abortions with thromboembolic accidents and anti-thromboplastin anticoagulant. Three cases. *Nouv Presse Med*, 9, 859-64.
- Spirin, A. S., Baranov, V. I., Ryabova, L. A., Ovodov, S. Y. & Alakhov, Y. B. (1988) A continuous cell-free translation system capable of producing polypeptides in high yield. *Science*, 242, 1162-4.
- Spronk, P. E., Ter Borg, E. J. & Kallenberg, C. G. (1992) Patients with systemic lupus erythematosus and Jaccoud's arthropathy: a clinical subset with an increased C reactive protein response? *Ann Rheum Dis*, 51, 358-61.
- Stalc, M., Poredos, P., Peternel, P., Tomsic, M., Sebestjen, M. & Kveder, T. (2006) Endothelial function is impaired in patients with primary antiphospholipid syndrome. *Thromb Res*, 118, 455-61.
- Statkute, L., Traynor, A., Oyama, Y., Yaung, K., Verda, L., Krosnjak, N. & Burt, R. K. (2005) Antiphospholipid syndrome in patients with systemic lupus erythematosus treated by autologous hematopoietic stem cell transplantation. *Blood*, 106, 2700-9.
- Steinkasserer, A., Cockburn, D. J., Black, D. M., Boyd, Y., Solomon, E. & Sim, R. B. (1992) Assignment of apolipoprotein H (APOH: beta-2-glycoprotein I) to human chromosome 17q23-qter; determination of the major expression site. *Cytogenet Cell Genet*, 60, 31-3.
- Steinkasserer, A., Dorner, C., Wurznner, R. & Sim, R. B. (1993) Human beta 2-glycoprotein I: molecular analysis of DNA and amino acid polymorphism. *Hum Genet*, 91, 401-2.

- Steinkasserer, A., Estaller, C., Weiss, E. H., Sim, R. B. & Day, A. J. (1991) Complete nucleotide and deduced amino acid sequence of human beta 2- glycoprotein I. *Biochem J*, 277, 387-91.
- Stohl, W., Metyas, S., Tan, S. M., Cheema, G. S., Oamar, B., Xu, D., Roschke, V., Wu, Y., Baker, K. P. & Hilbert, D. M. (2003) B lymphocyte stimulator overexpression in patients with systemic lupus erythematosus: longitudinal observations. *Arthritis Rheum*, 48, 3475-86.
- Stone, S., Pijnenborg, R., Vercruyse, L., Poston, R., Khamashta, M. A., Hunt, B. J. & Poston, L. (2005) The Placental Bed in Pregnancies Complicated by Primary Antiphospholipid Syndrome. *Placenta*, 27, 457-67.
- Stryer, L. (1988) *Biochemistry*, New York, W. H. Freeman and Company.
- Studier, F. W. & Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol*, 189, 113-30.
- Sturfelt, G., Mousa, F., Jonsson, H., Nived, O., Thysell, H. & Wollheim, F. (1990) Recurrent cerebral infarction and the antiphospholipid syndrome: effect of intravenous gammaglobulin in a patient with systemic lupus erythematosus. *Ann Rheum Dis*, 49, 939-41.
- Subang, R., Levine, J. S., Janoff, A. S., Davidson, S. M., Taraschi, T. F., Koike, T., Minchey, S. R., Whiteside, M., Tannenbaum, M. & Rauch, J. (2000) Phospholipid-bound beta 2-glycoprotein I induces the production of anti-phospholipid antibodies. *J Autoimmun*, 15, 21-32.
- Sultan, S. M., Ioannou, Y. & Isenberg, D. A. (1999) A review of gastrointestinal manifestations of systemic lupus erythematosus. *Rheumatology (Oxford)*, 38, 917-32.
- Sunden-Cullberg, J., Tedroff, J. & Aquilonius, S. M. (1998) Reversible chorea in primary antiphospholipid syndrome. *Mov Disord*, 13, 147-9.
- Swartz, J. R. (2001) Advances in Escherichia coli production of therapeutic proteins. *Curr Opin Biotechnol*, 12, 195-201.
- Tan, E. M., Cohen, A. S., Fries, J. F., Masi, A. T., Mcshane, D. J., Rothfield, N. F., Schaller, J. G., Talal, N. & Winchester, R. J. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum*, 25, 1271-7.
- Tang, L., Persky, A. M., Hochhaus, G. & Meibohm, B. (2004) Pharmacokinetic aspects of biotechnology products. *J Pharm Sci*, 93, 2184-204.
- Tektonidou, M. G., Sotsiou, F., Nakopoulou, L., Vlachoyiannopoulos, P. G. & Moutsopoulos, H. M. (2004) Antiphospholipid syndrome nephropathy in patients with systemic lupus erythematosus and antiphospholipid antibodies: prevalence, clinical associations, and long-term outcome. *Arthritis Rheum*, 50, 2569-79.
- Thiagarajan, P., Pengo, V. & Shapiro, S. S. (1986) The use of the dilute Russell viper venom time for the diagnosis of lupus anticoagulants. *Blood*, 68, 869-74.
- Thiagarajan, P., Shapiro, S. S. & De Marco, L. (1980) Monoclonal immunoglobulin M lambda coagulation inhibitor with phospholipid specificity. Mechanism of a lupus anticoagulant. *J Clin Invest*, 66, 397-405.
- Tietjen, G. E., Levine, S. R., Brown, E., Mascha, E. & Welch, K. M. (1993) Factors that predict antiphospholipid immunoreactivity in young people with transient focal neurological events. *Arch Neurol*, 50, 833-6.
- Tinahones, F. J., Cuadrado, M. J., Khamashta, M. A., Mujic, F., Gomez-Zumaquero, J. M., Collantes, E. & Hughes, G. R. (1998) Lack of cross-reaction between antibodies to beta2-glycoprotein-I and oxidized low-density lipoprotein in patients with antiphospholipid syndrome. *Br J Rheumatol*, 37, 746-9.
- Tincani, A., Allegri, F., Balestrieri, G., Reber, G., Sanmarco, M., Meroni, P. & Boffa, M. C. (2004) Minimal requirements for antiphospholipid antibodies ELISAs

- proposed by the European Forum on antiphospholipid antibodies. *Thromb Res*, 114, 553-8.
- Tincani, A., Allegri, F., Sanmarco, M., Cinquini, M., Taglietti, M., Balestrieri, G., Koike, T., Ichikawa, K., Meroni, P. & Boffa, M. C. (2001) Anticardiolipin antibody assay: a methodological analysis for a better consensus in routine determinations--a cooperative project of the European Antiphospholipid Forum. *Thromb Haemost*, 86, 575-83.
- Tonegawa, S. (1983) Somatic generation of antibody diversity. *Nature*, 302, 575-81.
- Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*, 76, 4350-4.
- Trappe, R., Loew, A., Thuss-Patience, P., Dorken, B. & Riess, H. (2006) Successful treatment of thrombocytopenia in primary antiphospholipid antibody syndrome with the anti-CD20 antibody rituximab--monitoring of antiphospholipid and anti-GP antibodies: a case report. *Ann Hematol*, 85, 134-5.
- Traynor, A. E., Schroeder, J., Rosa, R. M., Cheng, D., Stefka, J., Mujais, S., Baker, S. & Burt, R. K. (2000) Treatment of severe systemic lupus erythematosus with high-dose chemotherapy and haemopoietic stem-cell transplantation: a phase I study. *Lancet*, 356, 701-7.
- Trouw, L. A., Groeneveld, T. W. L., Seelen, M. A., Duijjs, J. M. G. J., Bejema, I. M., Prins, F. A., Kishore, U., Salant, D. J., Verbeek, J. S., van Kooten, C. & Daha, M. R. (2004) Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes. *J Clin Invest*, 114, 679-88.
- Tuhrim, S., Rand, J. H., Wu, X. X., Weinberger, J., Horowitz, D. R., Goldman, M. E. & Godbold, J. H. (1999) Elevated anticardiolipin antibody titer is a stroke risk factor in a multiethnic population independent of isotype or degree of positivity. *Stroke*, 30, 1561-5.
- Tulip, W. R., Varghese, J. N., Laver, W. G., Webster, R. G. & Colman, P. M. (1992) Refined crystal structure of the influenza virus N9 neuraminidase-NC41 Fab complex. *J Mol Biol*, 227, 122-48.
- Turiel, M., Sarzi-Puttini, P., Peretti, R., Rossi, E., Atzeni, F., Parsons, W. & Doria, A. (2005) Thrombotic risk factors in primary antiphospholipid syndrome: a 5-year prospective study. *Stroke*, 36, 1490-4.
- Tyndall, A., Fassas, A., Passweg, J., Ruiz De Elvira, C., Attal, M., Brooks, P., Black, C., Durez, P., Finke, J., Forman, S., Fouillard, L., Furst, D., Holmes, J., Joske, D., Jouet, J., Kotter, I., Locatelli, F., Prentice, H., Marmont, A. M., Mcsweeney, P., Musso, M., Peter, H. H., Snowden, J. A., Sullivan, K., Gratwohl, A. & Et Al. (1999) Autologous haematopoietic stem cell transplants for autoimmune disease--feasibility and transplant-related mortality. Autoimmune Disease and Lymphoma Working Parties of the European Group for Blood and Marrow Transplantation, the European League Against Rheumatism and the International Stem Cell Project for Autoimmune Disease. *Bone Marrow Transplant*, 24, 729-34.
- Tzschaschel, B. D., Guzman, C. A., Timmis, K. N. & De Lorenzo, V. (1996) An Escherichia coli hemolysin transport system-based vector for the export of polypeptides: export of Shiga-like toxin IIeB subunit by Salmonella typhimurium aroA. *Nat Biotechnol*, 14, 765-9.
- Ullers, R. S., Luirink, J., Harms, N., Schwager, F., Georgopoulos, C. & Genevaux, P. (2004) SecB is a bona fide generalized chaperone in Escherichia coli. *Proc Natl Acad Sci U S A*, 101, 7583-8.

- Urbanus, R. T., De Laat, H. B., De Groot, P. G. & Derksen, R. H. (2004) Prolonged bleeding time and lupus anticoagulant: a second paradox in the antiphospholipid syndrome. *Arthritis Rheum*, 50, 3605-9.
- Vaarala, O., Alfthan, G., Jauhainen, M., Leirisalo-Repo, M., Aho, K. & Palosuo, T. (1993) Crossreaction between antibodies to oxidised low-density lipoprotein and to cardiolipin in systemic lupus erythematosus. *Lancet*, 341, 923-5.
- Vaarala, O., Manttari, M., Manninen, V., Tenkanen, L., Puurunen, M., Aho, K. & Palosuo, T. (1995) Anti-cardiolipin antibodies and risk of myocardial infarction in a prospective cohort of middle-aged men. *Circulation*, 91, 23-7.
- Van Hinsbergh, V. W., Bertina, R. M., Van Wijngaarden, A., Van Tilburg, N. H., Emeis, J. J. & Haverkate, F. (1985) Activated protein C decreases plasminogen activator-inhibitor activity in endothelial cell-conditioned medium. *Blood*, 65, 444-51.
- Van Horn, G., Arnett, F. C. & Dimachkie, M. M. (1996) Reversible dementia and chorea in a young woman with the lupus anticoagulant. *Neurology*, 46, 1599-603.
- Van Lummel, M., Pennings, M. T., Derksen, R. H., Urbanus, R. T., Lutters, B. C., Kaldenhoven, N. & De Groot, P. G. (2005) The binding site in β 2-glycoprotein I for ApoER2' on platelets is located in domain V. *J Biol Chem*, 280, 36729-36.
- Vandenbergh, P., Zachee, P., Verstraete, S., Demuyne, H., Boogaerts, M. A. & Verhoef, G. E. (1996) Successful control of refractory and life-threatening autoimmune hemolytic anemia with intravenous immunoglobulins in a man with the primary antiphospholipid syndrome. *Ann Hematol*, 73, 253-6.
- Vargas-Alarcon, G., Granados, J., Bekker, C., Alcocer-Varela, J. & Alarcon-Segovia, D. (1995) Association of HLA-DR5 (possibly DRB1*1201) with the primary antiphospholipid syndrome in Mexican patients. *Arthritis Rheum*, 38, 1340-1.
- Vega-Ostertag, M., Casper, K., Swerlick, R., Ferrara, D., Harris, E. N. & Pierangeli, S. S. (2005) Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. *Arthritis Rheum*, 52, 1545-54.
- Vega-Ostertag, M., Harris, E. N. & Pierangeli, S. S. (2004) Intracellular events in platelet activation induced by antiphospholipid antibodies in the presence of low doses of thrombin. *Arthritis Rheum*, 50, 2911-9.
- Verro, P., Levine, S. R. & Tietjen, G. E. (1998) Cerebrovascular ischemic events with high positive anticardiolipin antibodies. *Stroke*, 29, 2245-53.
- Vianna, J. L., Khamashta, M. A., Ordi-Ros, J., Font, J., Cervera, R., Lopez-Soto, A., Tolosa, C., Franz, J., Selva, A., Ingelmo, M. & Et Al. (1994) Comparison of the primary and secondary antiphospholipid syndrome: a European Multicenter Study of 114 patients. *Am J Med*, 96, 3-9.
- Vinarov, D. A., Lytle, B. L., Peterson, F. C., Tyler, E. M., Volkman, B. F. & Markley, J. L. (2004) Cell-free protein production and labelling protocol for NMR-based structural proteomics. *Nature Methods*, 1, 149-153.
- Violi, F., Ferro, D., Valesini, G., Quintarelli, C., Saliola, M., Grandilli, M. A. & Balsano, F. (1990) Tissue plasminogen activator inhibitor in patients with systemic lupus erythematosus and thrombosis. *Bmj*, 300, 1099-102.
- Visvanathan, S. & Mcneil, H. P. (1999) Cellular immunity to beta 2-glycoprotein-1 in patients with the antiphospholipid syndrome. *J Immunol*, 162, 6919-25.
- Vlachoyiannopoulos, P. G., Mavragani, C. P., Bourazopoulou, E., Balitsari, A. V. & Routsias, J. G. (2004) Anti-CD40 antibodies in antiphospholipid syndrome and systemic lupus erythematosus. *Thromb Haemost*, 92, 1303-11.
- Vlachoyiannopoulos, P. G. & Samarkos, M. (2004) Peripheral vascular disease in antiphospholipid syndrome. *Thromb Res*, 114, 509-19.
- Vogel, R. A. (2001) Measurement of endothelial function by brachial artery flow-mediated vasodilation. *Am J Cardiol*, 88, 31E-34E.

- Vogt, E., Ng, A. K. & Rote, N. S. (1997) Antiphosphatidylserine antibody removes annexin-V and facilitates the binding of prothrombin at the surface of a choriocarcinoma model of trophoblast differentiation. *Am J Obstet Gynecol*, 177, 964-72.
- Wada, K., Wada, Y., Ishibashi, F., Gojobori, T. & Ikemura, T. (1992) Codon usage tabulated from the GenBank genetic sequence data. *Nucleic Acids Res*, 20 Suppl, 2111-8.
- Walport, M. J. (2000) Lupus, DNase and defective disposal of cellular debris. *Nat Genet*, 25, 135-6.
- Walton, T. A. & Sousa, M. C. (2004) Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation. *Mol Cell*, 15, 367-74.
- Wang, H. H. & Chiang, A. N. (2004) Cloning and characterization of the human beta2-glycoprotein I (beta2-GPI) gene promoter: roles of the atypical TATA box and hepatic nuclear factor-1alpha in regulating beta2-GPI promoter activity. *Biochem J*, 380, 455-63.
- Wang, X., Campos, B., Kaetzel, M. A. & Dedman, J. R. (1999) Annexin V is critical in the maintenance of murine placental integrity. *Am J Obstet Gynecol*, 180, 1008-16.
- Wasserman, A., Neisser, A. & Bruck, C. (1906) Eine serodiagnostische reaktion bei syphilis. *Deutsche Medizinische Wochenschrift*, 32, 745-6.
- Watson, K. V. & Schorer, A. E. (1991) Lupus anticoagulant inhibition of in vitro prostacyclin release is associated with a thrombosis-prone subset of patients. *Am J Med*, 90, 47-53.
- Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M. & Murphy, K. M. (2006) Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity*, 24, 677-88.
- Weber, C., Erl, W., Pietsch, A. & Weber, P. C. (1995) Aspirin inhibits nuclear factor-kappa B mobilization and monocyte adhesion in stimulated human endothelial cells. *Circulation*, 91, 1914-7.
- Weber, M., Hayem, G., Debandt, M., Palazzo, E., Roux, S., Kahn, M. F. & Meyer, O. (2000) The family history of patients with primary or secondary antiphospholipid syndrome (APS). *Lupus*, 9, 258-63.
- Weber, S. M., Chen, J. M. & Levitz, S. M. (2002) Inhibition of mitogen-activated protein kinase signaling by chloroquine. *J Immunol*, 168, 5303-9.
- Weening, J. J., D'agati, V. D., Schwartz, M. M., Seshan, S. V., Alpers, C. E., Appel, G. B., Balow, J. E., Bruijn, J. A., Cook, T., Ferrario, F., Fogo, A. B., Ginzler, E. M., Hebert, L., Hill, G., Hill, P., Jennette, J. C., Kong, N. C., Lesavre, P., Lockshin, M., Looi, L. M., Makino, H., Moura, L. A. & Nagata, M. (2004) The classification of glomerulonephritis in systemic lupus erythematosus revisited. *J Am Soc Nephrol*, 15, 241-50.
- Weihofen, A., Binns, K., Lemberg, M. K., Ashman, K. & Martoglio, B. (2002) Identification of signal peptide peptidase, a presenilin-type aspartic protease. *Science*, 296, 2215-8.
- Weiler, J. M., Yurt, R. W., Fearon, D. T. & Austen, K. F. (1978) Modulation of the formation of the amplification convertase of complement, C3b, Bb, by native and commercial heparin. *J Exp Med*, 147, 409-21.
- Weinblatt, M. E., Keystone, E. C., Furst, D. E., Moreland, L. W., Weisman, M. H., Birbara, C. A., Teoh, L. A., Fischkoff, S. A. & Chartash, E. K. (2003) Adalimumab, a fully human anti-tumor necrosis factor alpha monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the ARMADA trial. *Arthritis Rheum*, 48, 35-45.
- Weitz-Schmidt, G., Welzenbach, K., Brinkmann, V., Kamata, T., Kallen, J., Bruns, C., Cottens, S., Takada, Y. & Hommel, U. (2001) Statins selectively inhibit

- leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat Med*, 7, 687-92.
- Wilkinson, D. L. & Harrison, R. G. (1991) Predicting the solubility of recombinant proteins in *Escherichia coli*. *Biotechnology (N Y)*, 9, 443-8.
- Williams, F. M., Parmar, K., Hughes, G. R. & Hunt, B. J. (2000) Systemic endothelial cell markers in primary antiphospholipid syndrome. *Thromb Haemost*, 84, 742-6.
- Wilson, W. A., Gharavi, A. E., Koike, T., Lockshin, M. D., Branch, D. W., Piette, J. C., Brey, R., Derksen, R., Harris, E. N., Hughes, G. R., Triplett, D. A. & Khamashta, M. A. (1999) International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. *Arthritis Rheum*, 42, 1309-11.
- Wolberg, A. S., Monroe, D. M., Roberts, H. R. & Hoffman, M. R. (1999) Tissue factor de-encryption: ionophore treatment induces changes in tissue factor activity by phosphatidylserine-dependent and -independent mechanisms. *Blood Coagul Fibrinolysis*, 10, 201-10.
- Wolberg, A. S. & Roubey, R. A. (2004a) Anti-beta 2-glycoprotein I antibodies do not increase TF activity by de-encryption. *Thromb Res*, 114, 67-71.
- Wolberg, A. S. & Roubey, R. A. (2004b) Mechanisms of autoantibody-induced monocyte tissue factor expression. *Thromb Res*, 114, 391-6.
- Won, H. S., Low, L. Y., Guzman, R. D., Martines-Yamout, M., Jakob, U. & Dyson, H. J. (2004) The zinc-dependent redox switch domain of the chaperone Hsp33 has a novel fold. *J Mol Biol*, 341, 893-9.
- Wong, K. L., Chan, F. Y. & Lee, C. P. (1991) Outcome of pregnancy in patients with systemic lupus erythematosus. A prospective study. *Arch Intern Med*, 151, 269-73.
- Wong, R. C. (2004) Consensus guidelines for anticardiolipin antibody testing. *Thromb Res*, 114, 559-71.
- Wu, T. T. & Kabat, E. A. (1970) An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J Exp Med*, 132, 211-50.
- Wu, X. X., Pierangeli, S. S. & Rand, J. H. (2006) Resistance to annexin A5 binding and anticoagulant activity in plasmas from patients with the antiphospholipid syndrome but not with syphilis. *J Thromb Haemost*, 4, 271-3.
- Wurm, H. (1984) beta 2-Glycoprotein-I (apolipoprotein H) interactions with phospholipid vesicles. *Int J Biochem*, 16, 511-5.
- Xia, J., Yang, Q. D., Yang, Q. M., Xu, H. W., Liu, Y. H., Zhang, L., Zhou, Y. H., Wu, Z. G. & Cao, G. F. (2004) Apolipoprotein H gene polymorphisms and risk of primary cerebral hemorrhage in a Chinese population. *Cerebrovasc Dis*, 17, 197-203.
- Xu, C., Mao, D., Holers, V. M., Palanca, B., Cheng, A. M. & Molina, H. (2000) A critical role for murine complement regulator *crry* in fetomaternal tolerance. *Science*, 287, 498-501.
- Yamamoto, M., Takeda, K. & Akira, S. (2004) TIR domain-containing adaptors define the specificity of TLR signaling. *Mol Immunol*, 40, 861-8.
- Yasuda, S., Atsumi, T., Matsuura, E., Kaihara, K., Yamamoto, D., Ichikawa, K. & Koike, T. (2005) Significance of valine/leucine247 polymorphism of beta2-glycoprotein I in antiphospholipid syndrome: increased reactivity of anti-beta2-glycoprotein I autoantibodies to the valine247 beta2-glycoprotein I variant. *Arthritis Rheum*, 52, 212-8.
- Yasuda, S., Tsutsumi, A., Chiba, H., Yanai, H., Miyoshi, Y., Takeuchi, R., Horita, T., Atsumi, T., Ichikawa, K., Matsuura, E. & Koike, T. (2000) beta(2)-glycoprotein I deficiency: prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis. *Atherosclerosis*, 152, 337-46.

- Zahn, K. (1996) Overexpression of an mRNA dependent on rare codons inhibits protein synthesis and cell growth. *J Bacteriol*, 178, 2926-33.
- Zapun, A., Missiakas, D., Raina, S. & Creighton, T. E. (1995) Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. *Biochemistry*, 34, 5075-89.
- Zhang, J. & McCrae, K. R. (2005) Annexin A2 mediates endothelial cell activation by antiphospholipid/anti-beta2 glycoprotein I antibodies. *Blood*, 105, 1964-9.
- Zhang, S. P., Zubay, G. & Goldman, E. (1991) Low-usage codons in *Escherichia coli*, yeast, fruit fly and primates. *Gene*, 105, 61-72.
- Zhang, X. & Studier, F. W. (1997) Mechanism of inhibition of bacteriophage T7 RNA polymerase by T7 lysozyme. *J Mol Biol*, 269, 10-27.
- Zhou, X., Caligiuri, G., Hamsten, A., Lefvert, A. K. & Hansson, G. K. (2001) LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. *Arterioscler Thromb Vasc Biol*, 21, 108-14.
- Zhu, M., Olee, T., Le, D. T., Roubey, R. A., Hahn, B. H., Woods, V. L., Jr. & Chen, P. P. (1999) Characterization of IgG monoclonal anti-cardiolipin/anti-beta2GP1 antibodies from two patients with antiphospholipid syndrome reveals three species of antibodies. *Br J Haematol*, 105, 102-9.
- Zoghalmi-Rintelen, C., Vormittag, R., Sailer, T., Lehr, S., Quehenberger, P., Rumpold, H., Male, C. & Pabinger, I. (2005) The presence of IgG antibodies against beta2-glycoprotein I predicts the risk of thrombosis in patients with the lupus anticoagulant. *J Thromb Haemost*, 3, 1160-5.