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THE PRODUCTION, EXPRESSION, AND CHARACTERISATION OF INSULIN AND GAD65 RECOMBINANT FAB FOR USE IN EPITOPE MAPPING STUDIES

Carolyn Jane Padoa

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, South Africa, 2006

DECLARATION

I, Carolyn Jane Padoa, do hereby declare that this thesis is my own work. It is being submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg, South Africa. Any assistance that I received is stated in the acknowledgements. This work has not previously been submitted for any degree or examination at this, or any other, University. I certify that the protocol has been approved by the Committee for Research on Human Subjects of the University of the Witwatersrand, Johannesburg (Appendix 6; clearance certificate protocol number M020230).

Signed by: ……

Carolyn Jane Padoa

on this the …… day of November 2006.

DEDICATION

I would like to dedicate this thesis to my father, Neville Edward Padoa, who passed away suddenly on the 12th July 2004. My Dad was a firm believer in furthering one's education. He was very proud of all his three children and their accomplishments over the years.

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

PADOA CJ, BANGA JP, MADEC AM *et al*. (2003) Recombinant Fabs of human monoclonal antibodies specific to the middle epitope of GAD65 inhibit type 1 diabetes-specific GAD65Abs. *Diabetes*, **52(11):** 2689-2695.

PADOA CJ, CROWTHER NJ, THO-MAS JW *et al*. (2005) Epitope analysis of insulin autoantibodies using recombinant Fab. *Clin Exp Immunol*, **140(3):** 564-71.

RAJU R, FOOTE J, BANGA JP, HALL TR, *et al*. (2005) Analysis of GAD65 Autoantibodies in Stiff Person Syndrome Patients. Local stimulation of B cell secreting autoantibodies to GAD65 in the cerebrospinal fluid of Stiff Person Syndrome patients: predominance of enzymatic inhibitory antibodies. *J Immunol*, **175(11):** 7755-62.

HALL TR, THOMAS JW, PADOA CJ, TORN C, LANDIN-OLSSON M, ORTQVIST E HAMPE CS (2006) Longitudinal epitope analysis of insulin-binding antibodies in type 1 diabetes. *Clin Exp Immunol*, **146(1):** 9-14.

PADOA CJ and CROWTHER NJ (2006) Engineeredantibodies: A new tool for use in diabetes research. *Diabetes Research and Clinical Practice*, **74(Suppl 2):** S51-S62.

CONFERENCE PRESENTATIONS/POSTERS

Annual meeting of the Society of Endocrinology, Metabolism, and Diabetes of South Africa (Durban)

1. Padoa CJ, Crowther NJ, Gray IP (2000) The production and expression of an insulin-binding monoclonal antibody using PCR technology (oral). *J Endocrinol Metab Diab S Afr*, **5(1)**.

Annual meeting of the Federation of South African Societies of Pathology (Warmbaths)

1. Padoa CJ, Crowther NJ, Gray IP (2000) Engineering anti-insulin antibodies (oral).

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1. Hampe CS, Padoa CJ, Foote J and Lernmark Å (2001) Cloning and expression of the Fab fragment of a novel monoclonal antibody to GAD65 (poster).

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1. Padoa CJ, Crowther NJ, Foote J, Buss TN, Lernmark Å, Gray IP and Hampe CS (2002) The cloning and characterisation of a novel monoclonal antibody (1E2) to insulin (oral). *J Endocrinol Metab Diab S Afr*, **7(1):** 16.

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1. Padoa CJ, Crowther NJ, Foote J, Buss T and Hampe CS (2003) The cloning and characterisation of a novel monoclonal antibody (CG7C7) to insulin (poster). *Diabetes/Metabolism Research and Reviews*, **18(suppl 4):** S28.

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1. Padoa CJ, Crowther NJ, Foote J, Buss TN, Lernmark Å, and Hampe CS (2003) The use of a cloned FAB to study insulin binding of autoantibodies from Type 1 diabetic patients (oral). *J Endocrinol Metab Diab S Afr*, **8(1):** 32.

Annual meeting of the 43rd conference of the Federation of South African Societies of Pathology (Johannesburg)

1. Padoa CJ, Crowther NJ, Foote J, Buss TN, Lernmark Å, and Hampe CS (2003) The use of a cloned FAB to study insulin binding of autoantibodies from Type 1 diabetic patients (oral).

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1. Padoa CJ, Crowther NJ, Foote J, Buss TN, Lernmark Å, and Hampe CS (2003) The cloning and characterisation of a monoclonal antibody (CG7C7) to insulin (poster).

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1. Padoa CJ, Crowther NJ, Foote J, Buss TN, Lernmark Å, and Hampe CS (2004) The cloning and characterisation of a monoclonal antibody (CG7C7) to insulin (poster).

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1. Padoa CJ, Crowther NJ, Foote J, Buss TN, Lernmark Å, and Hampe CS (2004) The cloning and characterisation of a monoclonal antibody (CG7C7) to insulin (poster).

Annual meeting of the Society of Endocrinology, Metabolism and Diabetes of South Africa (Johannesburg)

1. Padoa CJ, Crowther NJ, Thomas JW, Hall TR, Bekris LM, Torn C, Landin-Olsson M, Ortqvist E, Palmer JP, Lernmark Å, and Hampe CS (2005) Epitope Analysis of Insulin Autoantibodies using Recombinant Fab (poster). *J Endocrinol Metab Diab S Afr*, **10(1):** 37.

5th Regional conference on the treatment of type 2 diabetes (Dubrovnik, Croatia)

1. Padoa CJ, Crowther NJ, Thomas JW, Hall TR, Bekris LM, Torn C, Landin-Olsson M, Ortqvist E, Palmer JP, Lernmark Å, and Hampe CS (2005) Epitope Analysis of Insulin Autoantibodies using Recombinant Fab (oral).

16th European Congress of Immunology (Paris)

1. Padoa CJ, Crowther NJ, Thomas JW, Hall TR, Bekris LM, Torn C, Landin-Olsson M, Ortqvist E, Palmer JP, Lernmark Å, and Hampe CS (2006) Epitope Analysis of Insulin Autoantibodies using Recombinant Fab (poster).

AWARDS

1. I was awarded the Novo Nordisk Travel Grant (R 25 000) at the 2000 SEMDSA conference. This award was put towards my trip to Seattle, Washington where I learnt the techniques required for cloning and expression of recombinant Fabs.
2. My oral presentation entitled 'The cloning and characterisation of a novel monoclonal antibody (1E2) to insulin' was recognised by SEMDSA as the best oral presentation at the 2002 SEMDSA conference.
3. My oral presentation entitled 'The use of a cloned Fab to study insulin binding of autoantibodies from Type 1 diabetic patients' was recognised by SEMDSA as the best oral presentation at the 2003 SEMDSA conference.
4. I received the SEMDSA Diabetes award (2004) for the best original research paper published in the field of diabetes for my paper entitled 'Recombinant Fabs of human monoclonal antibodies specific to the middle epitope of GAD65 inhibit type 1 diabetes-specific GAD65Abs'.
5. I received the Roche Young Researcher of the Year Award at the 44th conference of the Federation of South African Societies of Pathology in 2004.
6. At the same conference, I was awarded the Beckman Postgraduate Award.
7. I was awarded the University of the Witwatersrand Faculty Research Prize in 2005 for my paper entitled: 'Recombinant Fabs of Human Monoclonal Antibodies specific to the Middle epitope of Gad65 Inhibit Type 1 Diabetes-Specific Gad65Abs'.
8. My poster presentation entitled 'Epitope Analysis of Insulin Autoantibodies using Recombinant Fab' was recognised by SEMDSA as the best poster presentation at the 2005 SEMDSA conference.
9. I was awarded the 2005 Lilly Research Award and invited to present my research project entitled '*Epitope analysis of insulin autoantibodies using recombinant fab'* at the 5th Regional Conference on the Treatment of Type 2 Diabetes Mellitus in Dubrovnik in September 2005.

ABSTRACT

Objectives. Autoantibodies to the 65kDa isoform of glutamic acid decarboxylase (GAD65Abs) are accepted markers for type 1 diabetes and, together with autoantibodies to insulin (IAA) and a protein tyrosine phosphatase-like islet cell antigen (IA-2), predict the disease. IAA are often the first autoantibodies detected in type 1 diabetics and can be present before the onset of clinical diabetes. These autoantibodies and their epitopes are however not well characterized. We explored the use of monoclonal antibodies and their recombinant Fab (rFab) as reagents for epitope analysis.

Methods and Results. Four rFab specific for insulin were cloned from murine monoclonal antibodies (mAbs) 1E2, HB-126, HB-123, HB-127, and one rFab specific for GAD65 was cloned from human mAb IgG antibody DP-D (derived from autoimmune disease patients), to characterise insulin and GAD65 autoantibodies present in the sera of patients with type 1 diabetes. Only rFab 126 and DP-D showed insulin and GAD65 specific binding, respectively in radiobinding assays. In competition experiments with sera positive for autoantibodies to insulin the rFab 126 significantly reduced the binding to ^{125}I-insulin by sera of type 1 (n=35) and type 1.5 diabetes (or LADA) (n=14) patients ($p<0.0001$). There was no difference in the competition pattern in IAA positive type 1 diabetes patients (n=35) and IAA positive type 1.5 diabetes patients (n=14). The insulin epitope that the rFab binds to was mapped using competitive radiobinding assays with two monoclonal antibodies (mAb 1 and mAb 125) whose epitopes are on the B chain and A chain loop of insulin, respectively. We found the epitope of this recombinant antibody to be located on the A chain loop of the insulin molecule. The 3-dimensional structure of rFab 123, 126 and DP-D were determined using an automated homology modelling programme. Using the computer programme 'PatchDock' we attempted to further map the epitope that rFab 126 binds to on insulin. Of the three models generated, only one supported our findings that rFab 126 binds to the A chain loop of insulin.

The binding of GAD65Ab in 61 type 1 diabetes patients to GAD65 was analyzed by competitive radioimmunoassays with rFab DP-D to ascertain disease-specific GAD65Ab binding specificities. The median binding was reduced significantly by

rFab DP-D (80%) ($p<0.0001$). The competition pattern in type 1 diabetes patients was different from that in GAD65Ab-positive type 1.5 diabetes patients (n=44), first degree relatives (n=38), and healthy individuals (n=14) (Padoa *et al*., 2003).

Conclusions. We have shown that rFab specific for insulin and GAD65 can be generated using PCR technology and that such agents can be used to determine the insulin/GAD65 epitopes recognized by autoantibodies from type 1 and 1.5 diabetics. These novel findings with GAD65- and insulin-specific rFab support the view that type 1 diabetes is associated with disease- and epitope-specific GAD65- and insulin-autoantibodies and supports the notion that the middle epitope of GAD65 is disease-specific. These GAD65-specific rFab should prove useful in predicting type 1 diabetes. Furthermore, rFabs may be a novel method for blocking autoimmune responses against β cell autoantigens in type 1 diabetics.

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LIST OF ABBREVIATIONS

APS	:	Ammonium Persulphate
β	:	Beta
β-ME	:	Beta Mercaptoethanol
bp(s)	:	Base pair(s)
BSA	:	Bovine Serum Albumin
$CaCl_2$	:	Calcium Chloride
cDNA	:	Complementary Deoxyribonucleic Acid
CDR	:	Complementarity Determining Region
Ci	:	Curie
Cl	:	Chloride
C_L	:	Constant domain of the light chain
C_H1	:	First constant domain of the heavy chain
C_H2	:	Second constant domain of the heavy chain
C_H3	:	Third constant domain of the heavy chain
CO_2	:	Carbon Dioxide
cpm	:	Counts per minute
°C	:	Degree Celsius
δ	:	Delta
D	:	Diversity
Da	:	Dalton
ddNTP	:	Dideoxynucleoside 5'-triphosphate
DEPC	:	Diethyl Pyrocarbonate
dH_2O	:	Distilled water
DMEM	:	Dulbecco's Modified Eagle's Medium
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxynucleoside 5'- triphosphate
DTT	:	Dithiothreitol
ε	:	Epsilon
E. coli	:	*Escherichia coli*
EDTA	:	Ethylenediaminetetra-acetic Acid

ELISA	:	Enzyme Linked Immunosorbent Assay
Fab	:	Fragment antigen binding
$F(ab')_2$	:	Divalent antigen binding fragment
Fc	:	Immunoglobulin fragment of the C_H2/C_H3 of both heavy chains
FCS	:	Fetal Calf Serum
Fd	:	Variable domain plus the first constant region of the heavy chain
FDA	:	Food and Drug Administration
$FeSO_4$	:	Iron(II) Sulphate heptahydrate
Fv	:	Variable region fragment
γ	:	Gamma
g	:	Gram
GAD	:	Glutamic Acid Decarboxylase
GITC	:	Guanidine Isothiocyanate
H	:	Heavy
His-tag	:	Histidine tag
H_2O	:	Water
H_2O_2	:	Hydrogen Peroxide
H_2SO_4	:	Sulphuric Acid
HCl	:	Hydrochloric Acid
HPLC	:	High Performance Liquid Chromatography
HRP	:	Horseradish Peroxidase
IDDM	:	Insulin Dependent Diabetes Mellitus
IFG	:	Impaired Fasting Glucose
Ig	:	Immunoglobulin
IgA	:	Immunoglobulin A
IgD	:	Immunoglobulin D
IgE	:	Immunoglobulin E
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M
IGT	:	Impaired Glucose Tolerance
IMAC	:	Immobilised Metal Affinity Chromatography

IRMA	:	Immunoradiometric Assay
^{125}I	:	Iodine-125
J	:	Joining region
κ	:	Kappa
Kb(s)	:	Kilobase(s)
KCl	:	Potassium Chloride
kDa	:	kilo Dalton
kg	:	Kilogram
KH_2PO_4	:	Potassium Phosphate Monobasic Anhydrous
K_2HPO_4	:	Potassium Phosphate Dibasic Anhydrous
KOH	:	Potassium Hydroxide
K_2SO_4	:	Potassium Sulphate
λ	:	Lambda
L	:	Light
l	:	Litre
LA	:	Luria Agar
LB	:	Luria-Bertani
LN_2	:	Liquid Nitrogen
µ	:	Mu/Micro
M	:	Molar (grams per litre)
m	:	Meter
mAb	:	Monoclonal Antibody
mg	:	Milligram
$MgCl_2$	:	Magnesium Chloride
$MgSO_4$	:	Magnesium sulphate
ml	:	Millilitre
mM	:	Millimolar
mm	:	Millimeter
M-MuLV	:	Moloney Murine Leukemia Virus
MOPS	:	3-Morpholinopropanesulphonic Acid
Mr	:	Molecular weight
mRNA	:	Messenger Ribonucleic Acid
µg	:	Microgram

µl	**:**	Microlitre
µM	**:**	Micromolar
NaCl	**:**	Sodium Chloride
$NaHCO_3$	**:**	Sodium Bicarbonate
Na_2HPO_4	**:**	Sodium Phosphate Dibasic Monohydrate
NaH_2PO_4	**:**	Sodium Phosphate Monobasic Monohydrate
NaI	**:**	Sodium Iodide
NaOH	**:**	Sodium Hydroxide
ng	**:**	Nanogram
NH_4Cl	**:**	Ammonium Chloride
NHLS	**:**	National Health Laboratory Service
NIDDM	**:**	Non Insulin Dependent Diabetes Mellitus
Ni-NTA	**:**	Nickel-nitrilotriacetic Acid
OD	**:**	Optical Density
OGTT	**:**	Oral Glucose Tolerance Test
%	**:**	Percentage
PAGE	**:**	Polyacrylamide Gel Electrophoresis
PAS	**:**	Protein A Sepharose
PBS	**:**	Phosphate Buffered Saline
PC 1/3	**:**	Prohormone Convertase 1/3
PC 2	**:**	Prohormone Convertase 2
PCR	**:**	Polymerase Chain Reaction
PEG	**:**	Polyethylene Glycol
PGS	**:**	Protein G Sepharose
pmol	**:**	Picamole
RBA	**:**	Radiobinding Assay
RIA	**:**	Radioimmunoassay
RNase inhibitor	**:**	Ribonuclease inhibitor
RNA	**:**	Ribonucleic Acid
RbCl	**:**	Rubidium Chloride
rpm	**:**	Revolutions per minute
RT	**:**	*Reverse Transcriptase*
s	**:**	Seconds

SAP	**:**	Shrimp Alkaline Phosphatase
scFv	**:**	Single chain antibody fragment
SDS	**:**	Sodium Dodecyl Sulphate
Taq	**:**	*Thermus aquaticus*
TBE	**:**	Tris Borate EDTA
TBST	**:**	Tris Borate Tween 20
TE	**:**	Tris EDTA
TEMED	**:**	*N, N, N', N'* – tetramethylethylene diamine
Tris	**:**	Tris – (hydroxymethyl) - aminomethane
Tween 20	**:**	Polyoxyethylenesorbitan Monolaurate
U	**:**	Units
USA	**:**	United States of America
UV	**:**	Ultraviolet
V	**:**	Volts/Variable region
V_H	**:**	Variable domain of the heavy chain
V_L	**:**	Variable domain of the light chain

CHAPTER 1

1. INTRODUCTION

Towards the end of the 19th century von Behring and Kitasato (1890) showed that immunity to diphtheria and tetanus was associated with antibodies and could be transferred by immune serum. This discovery was the catalyst that, over the next century, highlighted the importance of the antibody molecule in immune defence and lead to many of the questions surrounding the structure, function, and genetics of the antibody molecule being answered.

1.1. THE STRUCTURE AND FUNCTION OF ANTIBODIES

Antibodies are large glycoprotein molecules produced by B lymphocytes of the immune system in higher organisms. Their primary function is to recognise and attach matter harmful to the organism, thereby marking it for destruction by other components of the immune system.

Antibodies are symmetrical molecules made up of four polypeptide chains: two identical glycosylated heavy chains of molecular weight (M_r) 50 to 75 kDa, and a pair of identical nonglycosylated light chains, approximately 25 kDa in size (Edelman and Benacerraf, 1962). The heavy chains determine all the effector functions of antibodies (e.g. complement fixation and transport across membranes), while the light chains play an important part in determining the specificity of antibodies. Disulphide bonds join the heavy chains together, and each light chain is disulphide bonded to one heavy chain; resulting in a 'Y' shaped antibody molecule (Figure 1.1). Each light and heavy chain is made up of a series of homology units of approximately 110 amino acids (Figure 1.1). Each homology unit is folded into a compact, globular structure (a domain) containing large amounts of β-pleated sheets (Poljak *et al*., 1972). Individual domains are relatively resistant to proteolytic attack. In contrast, the short linking sequences joining individual domains are often susceptible to proteolysis (Koch *et al*., 1973).

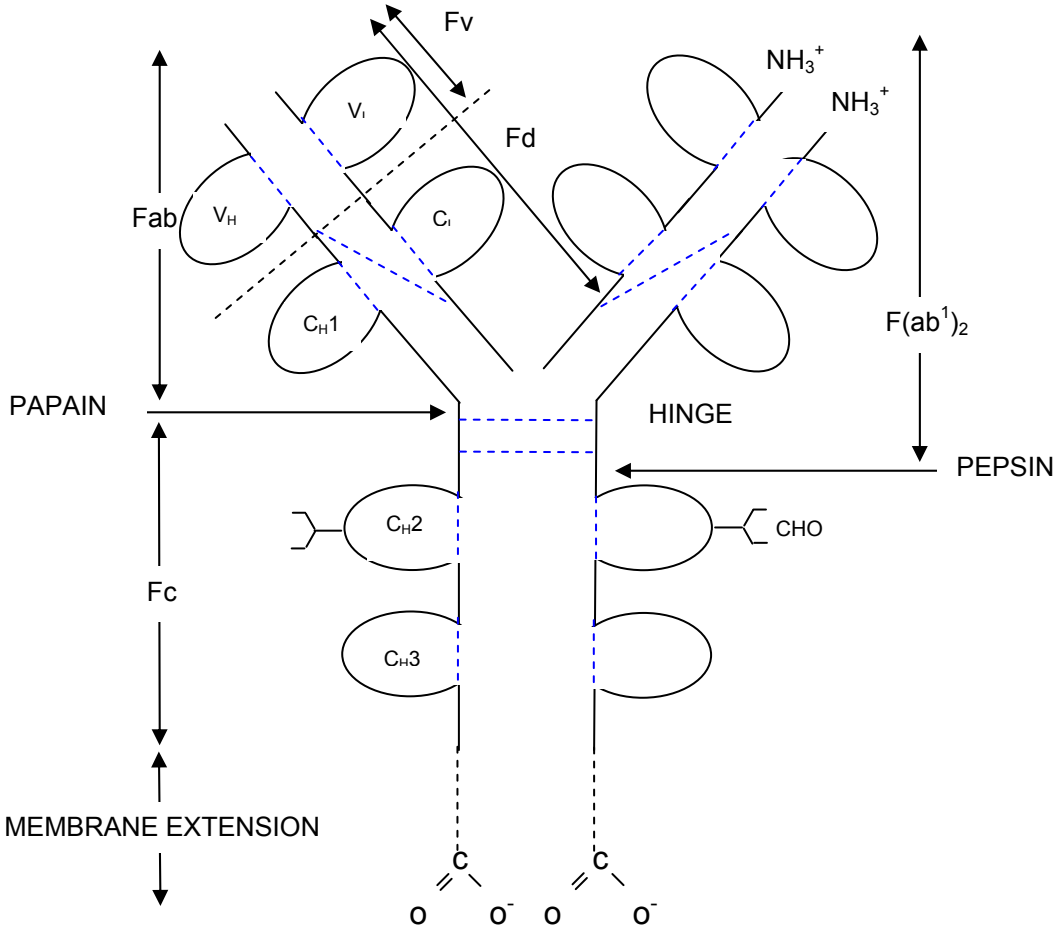


Figure 1.1: Structure of an IgG molecule (Goding, 1983).
(--- = disulphide bridges).

Each heavy chain is encoded for by variable (V_H), diversity (D), joining (J_H), and constant (C_H) segment genes; while each light chain is encoded for by V_L, J_L, and C_L segment genes (Figure 1.2) (reviewed by Milstein, 1982; reviewed by Azzazy and Highsmith, 2002). Each heavy chain is segmented into four regions: a variable domain plus three constant (C_H1, C_H2, C_H3) domains and the light chain consists of a variable domain and a single constant domain (C_L) (Kehry *et al*., 1979). The DNA and amino acid sequences of the constant region are relatively conserved within a given species. Within each variable region lie three hyper-variable segments or complementarity-determining regions (CDRs) which exhibit great sequence diversity. The V_H and V_L domains are folded in such a way that brings the hypervariable regions together to create an antigen-combining site (paratope), the specificity of which is determined by both heavy and light chains (Wu and Kabat, 1970; Kabat and Wu, 1971). Each antibody molecule has two identical antigen combining sites, except for polymeric IgM, which has ten.

An immunoglobulin molecule can be broken down into two regions, the Fc and the Fab (Figure 1.1) (Porter, 1959). The Fc portion, so called because it is the fragment of the antibody molecule that most readily crystallizes, is involved in effecting the physiological roles the antibody must play. These include antibody dependent cell-mediated cytotoxicity and initiation of the complement system. The Fc portion of the antibody also provides for the long half-life of antibodies in the blood. Two identical Fab fragments are present at the top ends of the 'Y' in every immunoglobulin structure. The Fab region is named as such because it is the fragment that contains the antibody binding site. The region between the Fab and the Fc fragment is called the hinge. This segment allows lateral and rotational movement of the two antigen binding domains.

1.1.1. Proteolytic fragmentation of immunoglobulins

Proteolytic attack of immunoglobulin G (IgG) by papain at neutral pH cleaves at the hinge (Smyth and Utsumi, 1967), generating two identical Fab fragments, each containing one intact light chain disulphide bonded to the Fd fragment (V_H and C_H1 domains) of the heavy chain (Figure 1.1). Pepsin also cleaves IgG at the hinge (pH ~4), but the pepsin cleavage site lies on the carboxy side of the inter-heavy

chain disulphide bond(s) (Figure 1.1). The divalent antigen-binding fragment produced is known as $F(ab')_2$. The heavy chain fragment in $F(ab')_2$ is slightly longer than the papain Fd fragment, and is known as Fd'. It is possible to generate an even smaller antigen-binding fragment (Fv) by proteolysis (Figure 1.1). The Fv fragment contains only the noncovalently associated V_H and V_L domains (Inbar *et al*., 1972; Kakimoto and Onoue, 1974).

1.1.2. Properties of the different immunoglobulin classes

There are five main classes of immunoglobulins: IgM, IgD, IgG, IgE, and IgA. They differ not only in their physiological roles but also in their structures (Table 1.1). The class of an immunoglobulin molecule is distinguished by its heavy chain. Thus, IgM, IgD, IgG, IgE, and IgA possess μ, δ, γ, ε, and α heavy chains respectively (Shimizu *et al*., 1982). The differences in the heavy chain polypeptides allow these proteins to function in different types of immune responses and at particular stages of the maturation of the immune response. The protein sequences responsible for these differences are found primarily in the Fc fragment. Different classes of antibodies may also vary in the number of Y-like units that join to form the complete protein. IgM antibodies, for example, have five Y-shaped units.

While there are five different types of heavy chains, there are only two types of light chains, κ or λ. One light chain always associates with one heavy chain, so the ratio of heavy to light chains is always 1:1. Any one antibody molecule will have only one type of light chain and one type of heavy chain. There are no restrictions on which types of heavy or light chains can form antibodies, so antibodies of all classes can contain either κ or λ light chains.

In the mouse and rat, over 95% of light chains are κ. In the mouse, there is only one κ light chain class, but paradoxically there are four λ chain subclasses (λ_1 – λ_4). In humans the κ:λ ratio is about 60:40, but the exact number of subclasses is unknown. Both κ and λ light chains appear to be able to take part in all biological

functions of antibodies although in some special circumstances one light chain may predominate (reviewed by Blomberg and Tonegawa, 1982).

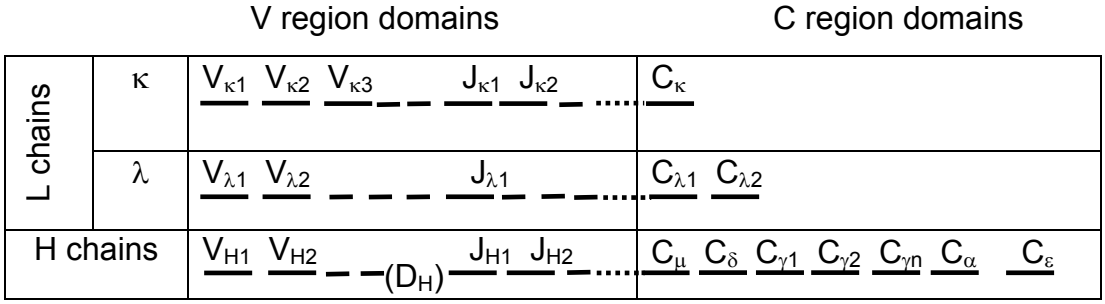


Figure 1.2: A schematic representation of the genes coding for antibodies (Milstein, 1982).

In the mouse, the κ, λ, and heavy chain genes are on chromosomes 6, 16, and 12, respectively (Swan *et al.*, 1979; D'Eustachio *et al.*, 1980; Francke *et al.*, 1982). Human κ, λ, and heavy chain genes are located on chromosome 2, 22, and 14, respectively (Shander *et al.*, 1980; Erikson *et al.*, 1981; Malcolm *et al.*, 1982). The V regions are coded by V, D, and J fragments of DNA which occur many thousands of bases apart. The number and detailed arrangements of genes in each case vary in different species. For the expression of an antibody, individual V, J, and C fragments are associated combinatorially within a horizontal array (see Figure 1.3).

Table 1.1: Properties of immunoglobulin classes.

Characteristics	IgG	IgM	IgA	IgE	IgD
Mr (kDa)	160	900	170 - 500	190	180
Heavy chain	γ	μ	α	ε	δ
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Molecular formula	$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$	$(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$	$(\alpha_2\kappa_2)_n$ or $(\alpha_2\lambda_2)_n$	$\varepsilon_2\kappa_2$ or $\varepsilon_2\lambda_2$	$\delta_2\kappa_2$ or $\delta_2\lambda_2$
Valency	2	10	2, 4, or 6	2	2
Subclasses	4	1	1	1	1
Concentration in serum	8-16 mg/ml	0.5-2 mg/ml	1-4 mg/ml	10-400 ng/ml	0-0.4 mg/ml
Serum half-life	G_1, G_{2a}, G_3 4 days G_{2b} 2 days	1 day	1 day	Short (probably less than 1 day)	Short (probably less than 1 day)
Special properties	Major class in serum. IgG-bearing lymphocytes are rare	First antibody in most responses	Major class in secretions (e.g. tears, saliva, bile, and gut)	Minor class in serum. Binds to mast cells. Responsible for allergic reactions, histamine and serotonin release	Major class on lymphocyte surface. Minor class in serum.
Function	Secondary response	Primary response	Protects mucous membranes	Protects against parasites (?)	?

Table reproduced from Goding, 1983; Harlow and Lane, 1989.

1.1.3. Antibody specificity

The immune system encounters an immense variety of foreign substances on a daily basis. To perform their crucial role as the first line of defence against any invading agent, antibodies must be extremely diverse to combat a large number of unexpected and unknown antigens. Through a complex process of gene splicing, B cells have been estimated to produce between 1 x 10^8 to 1 x 10^{10} IgG antibodies that differ in the composition of their binding sites (French *et al.*, 1989). These binding sites also take advantage of the different chemical properties of the 20 amino acids. The immune system is thus able to generate a number of antibody binding sites that can accommodate the shape, charge, and hydrophobicity of any given antigen.

1.1.4. Genetic diversity of immunoglobulin molecules

1.1.4.1. Mechanisms responsible for diversity of antigen-binding sites

Several mechanisms are responsible for the diversity of paratopes:

i. Recombinatorial diversity: created by random selection of one heavy chain V, D, and J gene, or one light chain V J gene segment out of a pool, to constitute the V_H and V_L domains, respectively.
ii. Junctional diversity: formed by the imprecise joining mechanisms and by deletion or addition of random nucleotides at the borders of the recombining V_H-D-J_H minigenes.
iii. Combinatorial diversity: generated by the assembly of the V_H and V_L domains.

Other mechanisms include alteration of the structural shape of the paratope and specific maturation of the paratope caused by a somatic hypermutation that improves the shape complementarity of the antibody with the antigen. The cumulative effects of these mechanisms determine the antigenic affinity and specificity of an antibody (Davis *et al.*, 1980; reviewed by Azzazy and Highsmith, 2002).

1.1.4.2. Formation of a functional κ gene

In germline and non-B cell DNA, the coding sequences for the V and C regions of the κ light chain are separated by several hundred kilobase pairs. The gene is

silent in these cells. There are approximately 200 different κ V regions and only one C region (Cory *et al.*, 1981; Fitzsimmons *et al.*, 1998). During the maturation of B cells, recombination brings one of the V regions and the C region together to produce a functional gene.

Studies showed that simple joining of the V and C regions did not account for all the variation that was detected. This led to the discovery of a third segment between the V and C regions called the J (joining) region. Recombination brings the V region just upstream of one of five J regions, producing a V-J-C region alignment (Weigert *et al.*, 1978). This alignment yields a functional κ gene. The recombination events do not always occur at exactly the same nucleotides, and this allows for some variation in the amino acids found at the V-J junction.

Four of the five different J regions (Sakano *et al.*, 1979) for the κ chains, found in genomic DNA sequences, can be used to synthesise functional polypeptides. J_3 has a mutated donor splice site and cannot produce a functional mRNA. Therefore, in the maturation of a functional κ light chain gene, recombination must occur between one of the approximately 200 V regions and one of the four functional J regions, yielding approximately 800 different κ chain polypeptides for homozygous mice. In humans, the κ genes contain 40 V regions and 5 J segments producing 200 possible κ light chains.

1.1.4.3. Formation of a functional λ gene

Similar types of recombination events are required to produce a functional λ light chain polypeptide. In the germ-line DNA, there are two V regions and four C regions in the λ coding region (Brack *et al.*, 1978). Each C region is paired with a single J region, and recombination occurs between the V and J regions. However, the two V regions are not found upstream of the C regions as in the κ gene. Each V region is paired with two J-C regions. Analysis of the DNA sequences around the J_4 region has shown that it carries several mutations that block the successful production of mRNA. Also, no recombination events have ever been reported that join a downstream V region to an upstream C region. This arrangement only allows the production of five possible λ light chains. In contrast, humans can

produce 120 λ light chains (30 V segments and 4 J segments). Humans can thus produce a total of 320 light chain specificities.

1.1.4.4. Formation of a functional heavy chain gene

In addition to the V, J, and C regions, heavy chains contain a fourth coding region referred to as the D region. The D region is located between the V regions and the J-C region. The production of a functional heavy chain requires two recombination events, one joining a D region to a J-C region, and another joining a V region to the D region (Figure 1.3) (Davis *et al.*, 1980). There are approximately 50 to 100 V, 12 D, and 4 J chain regions that form the heavy chain gene cluster on chromosome 12 in the mouse. The D regions produce functional proteins in all three reading frames, therefore, because the recombination events are not precise, the D regions can code for 36 different sequences. Having multiple regions helps to generate a large number of heavy chains, with at least 7 200 different possibilities for homozygous mice. Human heavy chain genes contain 65 V, 27 D, and 6 J regions producing 11 000 possible heavy chain V domains.

1.1.4.5. Creation of diverse antigen binding sites

Recombination between the V, D, and J regions produces a large number of different heavy and light chain polypeptide sequences. The V regions contain the coding sequences for CDR 1 and CDR 2 of the heavy and light chains. The third CDR is the product of the V-D-J recombination (heavy chain) or V-J joining (light chain). Because recombination is not always precise, the greatest sequence diversity is present in the third CDR.

For the mouse, there are at least 800 potential κ chains, five λ chains, and 7 200 heavy chains. If all light chains have a chance to pair with all heavy chains in the production of the antigen binding site, there are potentially 5.8×10^6 binding domains ([800 + 5] x 7 200). Similarly, the total diversity possible in a human who is homozygous for all of their antibody genes is 3.5×10^6. Diversity at the recombination junctions increases the number of possible combinations. Recombination and random assortment of the light and heavy chains create the lower limit for the number of different antigen binding sites.

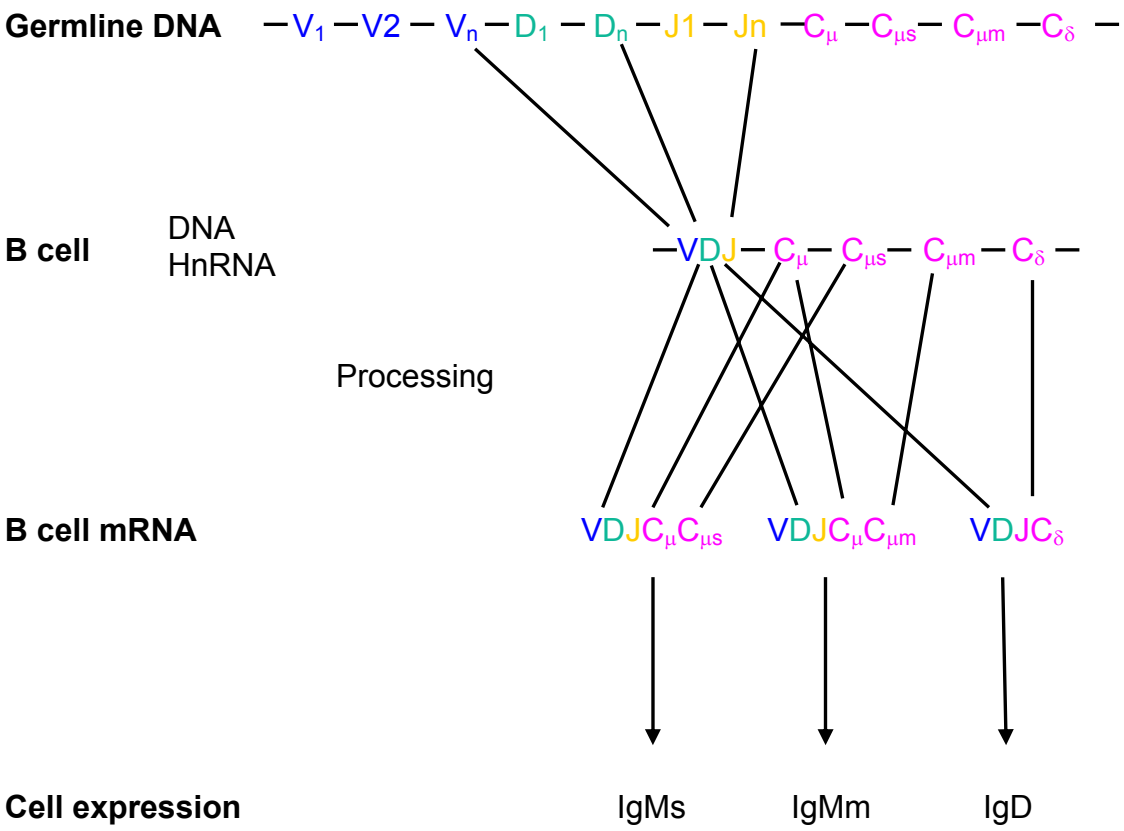


Figure 1.3: Heavy chain gene rearrangement and alternative forms of splicing of precursor RNA (Milstein, 1982).
Large deletions of germ line DNA are involved in the commitment of differentiated lymphocytes to the production of a single antibody. Alternative forms of splicing allow a single V gene to be expressed in conjunction with multiple C genes. IgMs and IgMm are the secreted and membrane bound forms.

Greater than half of the heavy or light chain V regions carry point mutations in the antigen binding regions. Although the mechanisms that generate these mutations are not known, they appear to provide a system that fine-tunes the binding site, thus creating a better fit for the antigen-antibody interaction. This process is known as affinity maturation.

1.1.5. Allelic exclusion

The recombination events that produce different heavy and light chains do not always lead to a functional gene. During B cell differentiation, light chain rearrangements begin with the κ region, and, if the first rearrangement of one of the diploid genes results in a non-functional allele, the other copy undergoes recombination. If both recombination events lead to non-functional genes, then recombination begins at the λ locus. Similar mechanisms lead to the generation of a functional heavy chain gene. Once recombination yields a functional antibody, an unknown mechanism prevents further recombination, fixing the antigen binding site for the life of the cell. This mechanism is called allelic exclusion and explains why B lymphocytes secrete antibodies with only one type of antigen binding site and why antibodies have only one type of light chain (Perry *et al.*, 1980).

1.1.6. Generation of the different classes and subclasses of antibodies

A second set of recombination events that involve the heavy chain C region were later discovered. These rearrangements do not affect the V region and do not alter the antigen binding domain. They do not contribute to the diversity of binding sites, but instead allow the replacement of one heavy chain C region with another. The rearrangement occurs downstream of the J region. Aligned here in germline DNA is a tandem array of the heavy chain C region genes: μ-chain (IgM), δ-chain (IgD), γ_3-chain (IgG_3), γ_1-chain (IgG_1), γ_{2b}-chain (IgG_{2b}), γ_{2a}-chain (IgG_{2a}), ε-chain (IgE), and α-chain (IgA). These rearrangements move the same antigen binding site onto different antibody classes and subclasses. This process is known as class switching and is one of the characteristic events of the maturation of the antibody response. The different classes and subclasses determine many of the

functional properties of antibodies. Class switching, therefore, creates an important mechanism for controlling where and how the antigen binding site will be used (Burrows *et al.*, 1980).

1.2. THE DEVELOPMENT OF HYBRIDOMA TECHNOLOGY

Genetic engineering has come to the fore in the last twenty years. It is now possible to study, control, and manipulate genes in ways that were inconceivable a few years ago. The revolution which occurred in the field of immunology was triggered in 1975 when Köhler and Milstein developed a technique for the production of monoclonal antibodies (mAbs) by fusing the nuclei of normal antibody-forming cells with those of malignant cells. It is important to acknowledge the numerous discoveries (Table 1.2) which culminated in Köhler and Milstein's achievement. Those deserving special mention were the proof of the clonal selection theory (Nossal and Lederberg, 1958), the development of cell fusion techniques (Okada, 1962; Littlefield, 1964), the artificial induction of plasmacytomas (Potter and Boyce, 1962), their adaptation to tissue culture (Horibata and Harris, 1970), and the fusion of two different plasma cell tumour lines with both antibody products being retained (Cotton and Milstein, 1973).

Table 1.2: Landmarks in the history of antibody research.

1847	Urinary protein in myeloma	Bence Jones
1890	Discovery of antibodies	von Behring and Kitazato
1900	'Side-chain' theory formulated	Ehrlich
	Discovery of ABO blood groups	Landsteiner
1955-6	Allotypes	Grubb, Oudin
1956	Classification of Bence-Jones proteins into two groups (now called κ and λ in honour of their discoverers)	Korngold and Lipari
1957	Clonal selection theory	Burnet
1958	One cell; one antibody	Nossal and Lederberg
	Cell fusion by Sendai virus	Okada
1959	Elucidation of disulphide-bonded chain	Edelman

	structure of antibodies	
1960	Discovery of spontaneous cell fusion	Barski
1962	Demonstration that Bence-Jones proteins are antibody light chains	Edelman and Gally
	Induction of plasmacytomas by mineral oil	Potter and Boyce
1962-3	Controlled proteolytic cleavage of IgG, identification of Fab and Fc; topographic relationship between light and heavy chains	Porter, Fleischman, Pain and Press
1964	Use of mutant cells and selective media to isolate hybrids	Littlefield
1965	Amino acid sequencing reveals that N-terminal half of light chains is variable; C-terminal constant	Hilschmann and Craig
	Postulate of two genes; one polypeptide	Dreyer and Bennett
1969	First complete amino acid sequence of an immunoglobulin; concept of domains	Edelman and colleagues
1970	Hypervariable regions	Wu and Kabat
	Growth of plasmacytomas in continuous culture	Horibata and Harris
1973	Fusion of mouse and rat myeloma cells with preservation of secretion of both immunoglobulins sets the stage for production of monoclonal antibodies	Cotton and Milstein
1975	Construction of hybridomas secreting antibody of predefined specificity	Köhler and Milstein
1976	Demonstration of DNA rearrangements in antibody-forming cells	Tonegawa and colleagues
	Use of polyethylene glycol for cell fusion	Pontecorvo
1977	Cloning and sequencing λ genes; J segments	Tonegawa and colleagues
1977-80	Multiple germ-line genes for V regions	Many authors
	Discovery of D segments	Group of L Hood
1980	Mechanisms of insertion of membrane IgG	Groups led by L Hood and R Wall

Table reproduced from Goding, 1983.

In an attempt to produce a general way of constructing continuous cell lines secreting antibody of known specificity, Köhler and Milstein (1975) expanded on previous experiments and fused a hypoxanthine, aminopterin, and thymidine (HAT)-sensitive variant of MOPC-21 myeloma cells with spleen cells from mice immunised with sheep red cells. The fusion was mediated by Sendai virus, and hybrids were selected by growth in HAT medium. Normal spleen cells can only survive a few days in culture and the myeloma cells are defective in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), an enzyme which enables cells to synthesise purines using an extra-cellular source of hypoxanthine as precursor. This enzyme deficiency is not normally a problem as there are a number of alternate pathways from which the cells can synthesise purines. However, in the presence of aminopterin, cells are unable to use these alternate pathways and are dependent on the HGPRT enzyme for survival. It was hoped that the two cell lines would 'complement' one another: the spleen cells providing the missing HGPRT enzyme and the myeloma cells providing the 'immortality' needed for continuous growth in culture (Figure 1.4).

The experiment was a success: a number of cloned hybrid lines secreting anti-sheep erythrocyte antibodies were produced. These mAbs can be obtained in unlimited quantities either in tissue culture or by injecting the hybridoma cells into the peritoneal cavity of mice. The injected neoplastic cells proliferate and secrete large amounts of antibody resulting in antibody-rich ascites. With the successful production of hybridomas, it now became possible to produce antibodies that are not only specific to a single antigenic site but are also a homogeneous population of molecules and, therefore, represent a chemically pure reagent.

1.2.1. Stages of hybridoma/monoclonal antibody production

1.2.1.1. Immunisation of mice

Animals are injected with an antigen preparation, and once a good humoral response has appeared, an appropriate screening procedure is developed. The sera from test bleeds are used to develop and validate the screening procedure. Animals are killed four to five days after boosting, the spleen removed, and antibody-secreting cells fused with myeloma cells.

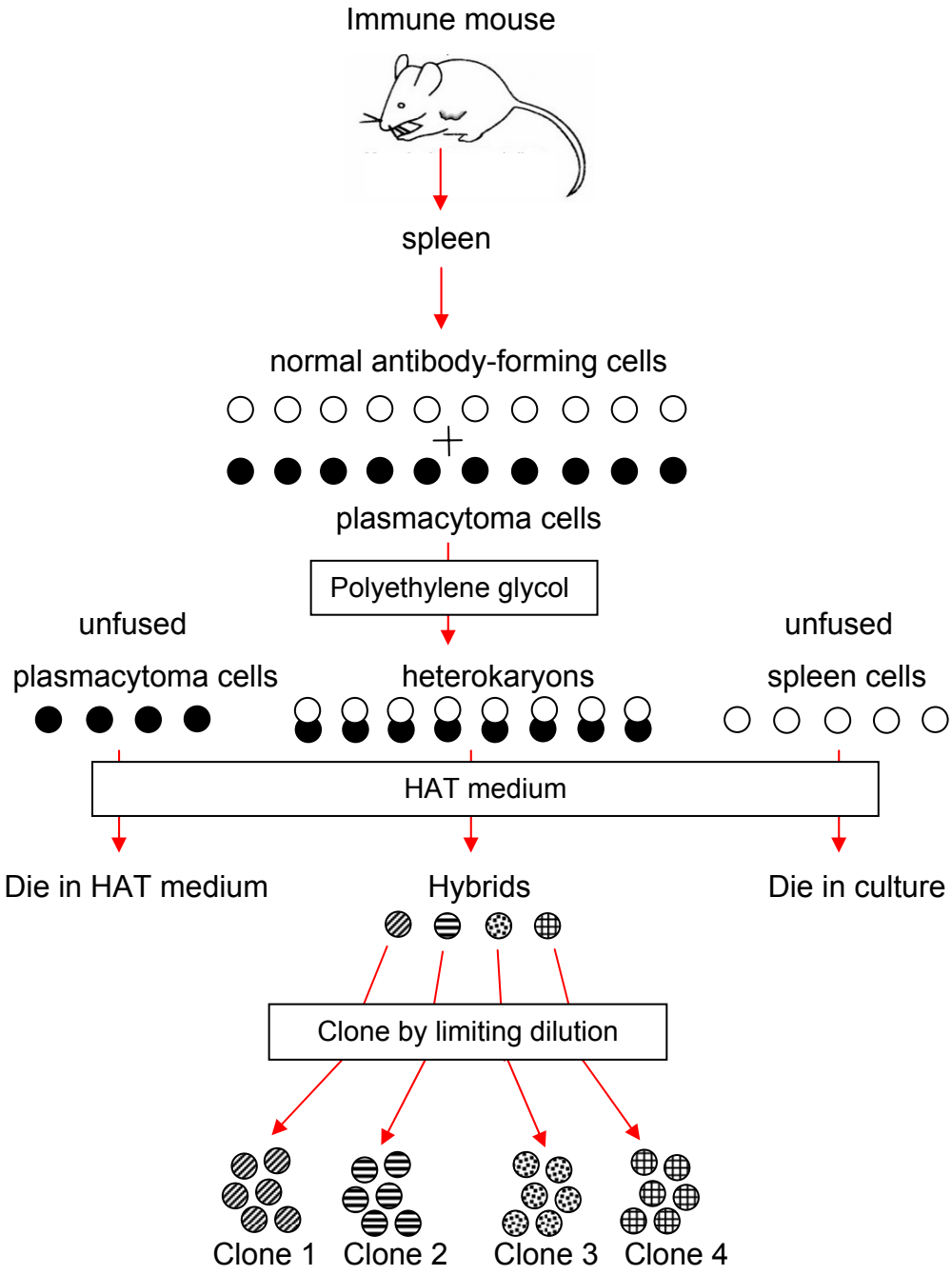


Figure 1.4: Production of hybridomas (Goding, 1983).

Spleen cells from immune mice are fused with HGPRT$^-$ myeloma (plasmacytoma) cells using polyethylene glycol. The binucleate fusion products are known as heterokaryons. At the next division, the nuclei fuse, generating hybrid cells, which grow in HAT medium. Unfused myeloma cells die in HAT medium and unfused spleen cells can only survive a few days in culture. Hybrids are tested for production of antibody of the desired specificity, and cloned by limiting dilution.

1.2.1.2. Fusion partners

Myelomas can be induced in a few strains of mice by injecting mineral oil into the peritoneum. Many of the first examples of these myelomas were isolated from BALB/c mice by Potter (1972), and these cells are referred to by the abbreviation MOPC (mineral oil plasmacytoma). Derivatives of BALB/c myelomas have become the most commonly used partners for fusions (Figure 1.5). Myelomas have all the cellular machinery necessary for the secretion of antibodies, and many secrete these proteins. To avoid the production of hybridomas that secrete more than one type of antibody, myelomas that are used for fusions have been selected for the lack of functional antibody production.

Hybridomas can be prepared by fusing myelomas and antibody-secreting cells isolated from different species, but the number of viable hybridomas increases dramatically when closely related species are used.

1.2.1.3. Fusion agents

In theory, the fusion between the myeloma cell and the antibody-secreting cell can be effected by any fusogen. In practice, hybridoma fusions became routine after the introduction of polyethylene glycol (PEG) (Pontecorvo, 1976). PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei (called heterokaryons). During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells. Because of the abnormal number of chromosomes, segregation does not always deliver identical sets of chromosomes to daughter cells, and chromosomes may be lost. There is usually a preferential loss of chromosomes from one or other cell (Tucker *et al*., 1981). If one of the chromosomes that carry a functional, rearranged immunoglobulin heavy or light chain gene is lost, production of the antibody will stop. This will result in a decrease in antibody titre and in unstable lines (Nowak, 1985). If the chromosome that is lost contains a gene used in drug selection, then the growth of the hybridoma will be unstable, and cells will continue to die during selection.

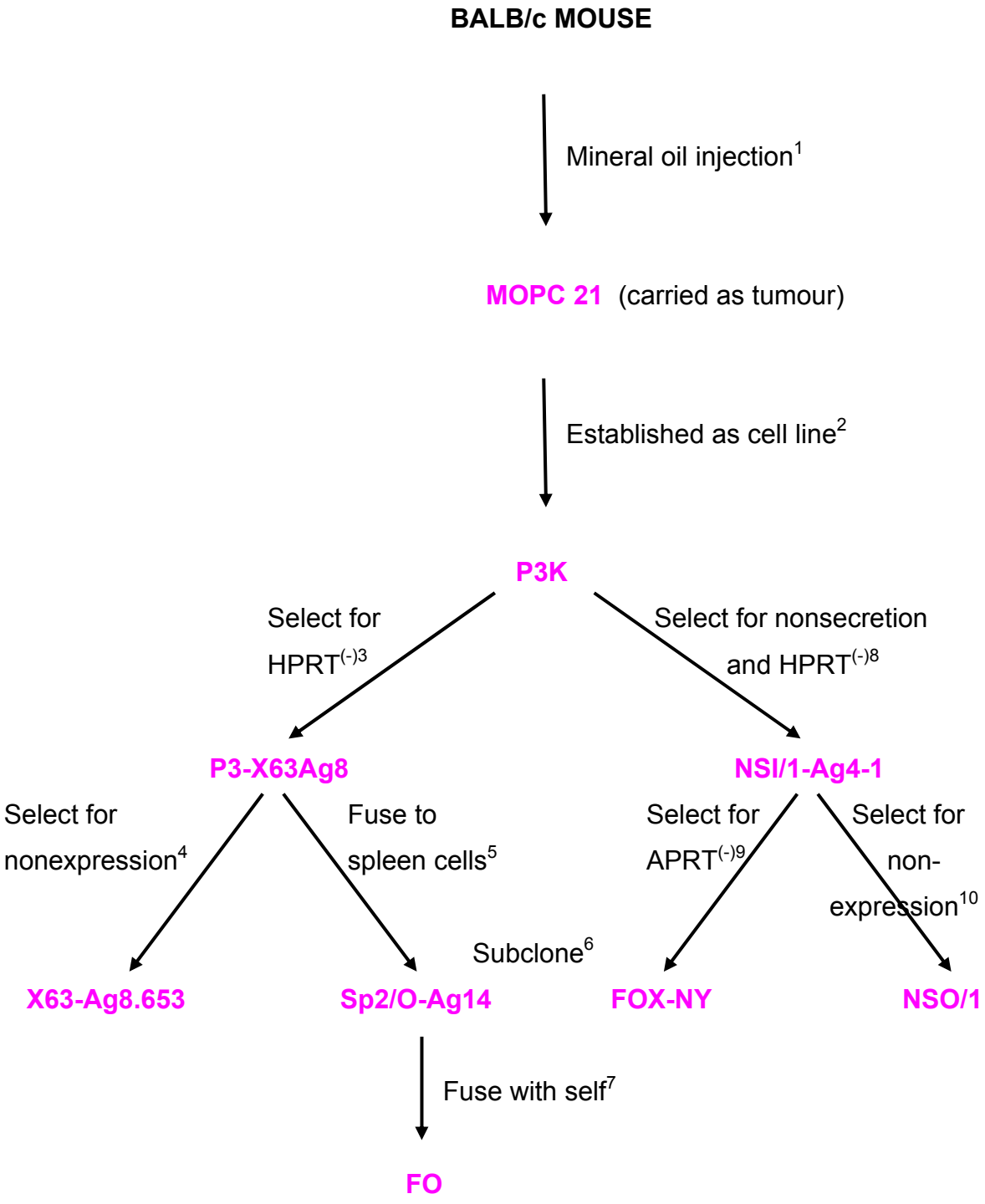


Figure 1.5: Myeloma family tree (Harlow and Lane, 1989).
[1]Potter, 1972; [2]Horibata and Harris, 1970; [3]Köhler and Milstein, 1975; [4]Kearney *et al.*, 1979; [5]Köhler and Milstein, 1975; [6]Shulman *et al.*, 1978; [7]de St. Groth and Scheidegger, 1980; [8]Köhler *et al.*, 1976; [9]Taggart and Samloff, 1983; [10]Galfre and Milstein, 1981.

1.2.1.4. Selection procedures for hybrid cells

Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused and of these about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in the culture necessitating a selection procedure to recover only fused cells. Since antibody-forming cells die quickly, it is only necessary to separate the myeloma cells. This is usually achieved by drug selection (Littlefield, 1964). Littlefield's procedure depends on the fact that when the main biosynthetic pathway for guanosine is blocked, by the folic acid antagonist aminopterin, there is an alternative 'salvage' pathway in which the nucleotide metabolites hypoxanthine or guanine are converted to guanosine monophosphate via the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT; Figure 1.6). Mutant myeloma cell lines deficient in HGPRT were used as fusion partners (Littlefield, 1964). In the absence of this enzyme the cells die in HAT medium because both the main and the salvage pathways are blocked. Thus, only hybrids which have the wild-type enzyme from the normal antibody-forming cells can actively multiply in HAT medium (Figure 1.6).

1.2.1.5. Cloning

To ensure that the antibodies are indeed monoclonal, it is essential to clone the hybrid cell lines. Köhler and Milstein (1975) used soft agar cultures to grow isolated colonies, and detected clones secreting anti-erythrocyte antibodies by overlaying with sheep red cells and complement. A zone of haemolysis surrounded colonies secreting anti-erythrocyte antibodies. Nowadays researchers prefer to clone by limiting dilution. Hybridoma lines should be cloned at least twice to make absolutely certain that each is a true clone, and also because of the relatively high probability of growth of nonproducer variants due to chromosome loss (Goding, 1983).

1.3. ANTIBODY ENGINEERING

The advent of hybridoma technology made possible the generation of a large number of antibodies that bind with high specificity and high affinity to a target molecule. The availability of mAbs has created an explosion of knowledge about

the antibody molecule, and with it widespread use in biomedical research, diagnostics, and more recently in therapeutic applications.

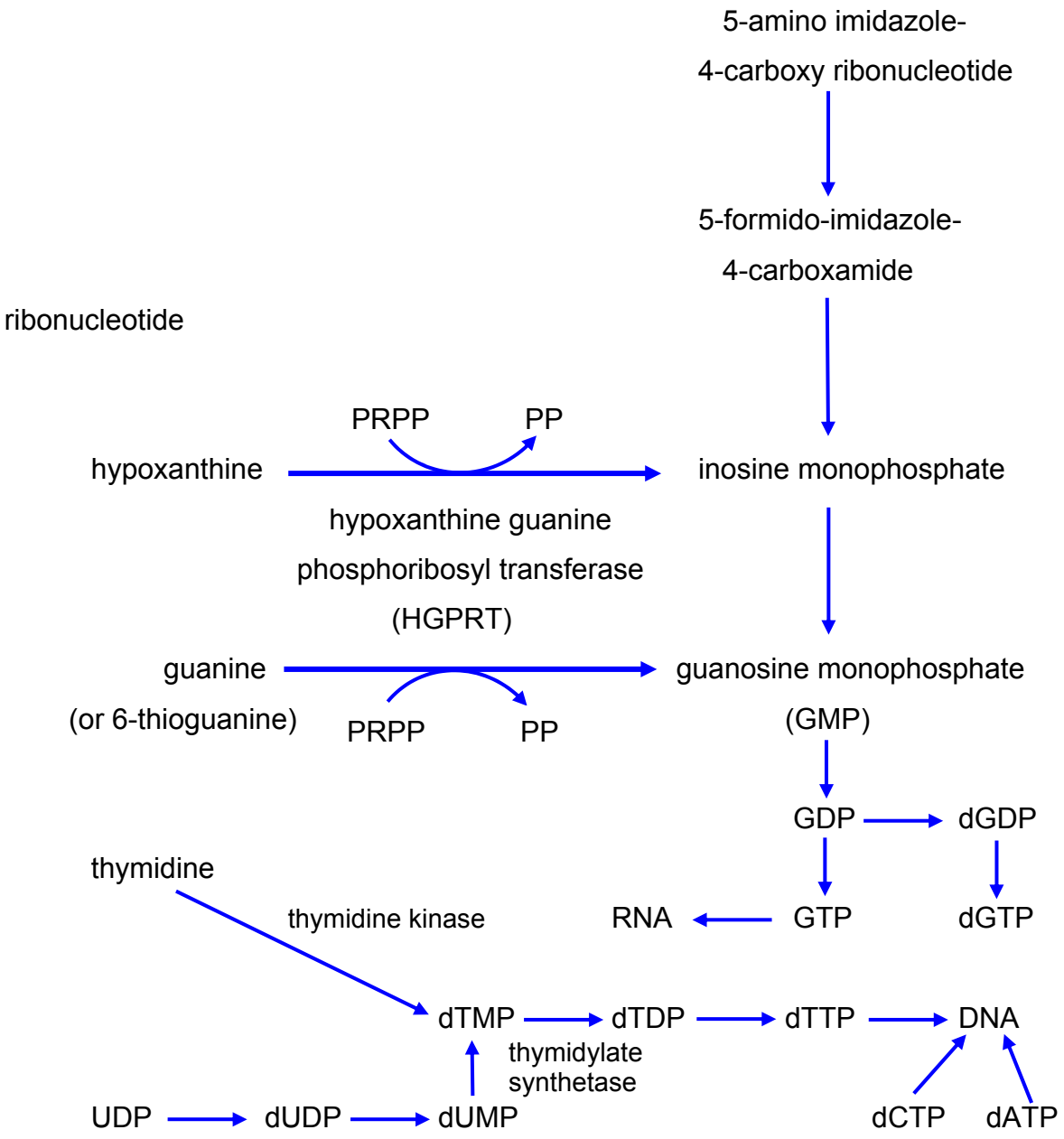


Figure 1.6: Metabolic pathways relevant to hybrid selection in medium containing HAT (Goding, 1983).

However, despite their usefulness in scientific research and as diagnostic tools, mAbs are subject to the constraints of the immune system. A few of the shortcomings of hybridoma technology include the size of mAbs, their affinity for target antigen; their effectiveness at recruiting the other cells of the human immune system; separate immunisations for each antigen, and laborious and inefficient cell fusion processes. The major hurdle, however, has proven to be similar to that of serum therapy. When the rodent mAbs are administered in multiple doses, the patient invariably raises an immune response to the mAbs. This Human Anti-Mouse Antibody (HAMA) response can occur within two weeks of the initiation of treatment and prevents long-term therapy. This greatly reduces the therapeutic potential of these mAbs (Vaughan *et al.*, 1996).

Prior to the advent of molecular biology techniques, antibody fragments (Fab or $F(ab^1)_2$) could only be obtained by proteolytic digestion of a whole immunoglobulin. Since the first description of the polymerase chain reaction (PCR) over 20 years ago, numerous discoveries have been made and new techniques developed which have transformed immunology and antibody production methods. PCR provided a simple method to amplify and rapidly clone the relevant heavy and light chain genes of an immunoglobulin into bacterial expression systems to obtain different types of fragments in fully functional form. Although the effects of designed mutations could be analysed in this system, not all proteins folded correctly in the cytoplasm. This led to the development of phage display which allowed the correct association of the engineered antibody fragments. This method bypasses hybridoma technology as well as immunisation. Phage display allows the creation of large repertoires of antibody fragments from antibody variable genes, thus making it possible to obtain 'human' antibody fragments without the laborious and often ineffective humanisation procedures applied to murine mAbs.

1.3.1. Antibody fragments

In order to address the limitations of large IgG molecules, smaller engineered mAb-based molecules were developed; namely, the antigen binding fragment (Fab) and the variable domain (Fv) fragment (Figure 1.7). These fragments can

be produced by proteolytic cleavage of antibodies or via cloning and expression of the amplified immunoglobulin genes in bacteria.

1.3.1.1. Fv and Fab antibody fragments

The 25 kDa Fv consists of a heterodimer of the V_H and V_L domains (Huston *et al.*, 1988) and is the smallest antigen binding fragment of an antibody that contains a complete binding site. Fab fragments consist of the intact light chain (V_LC_L) and the Fd fragment of the heavy chain (V_HC_H1). A disulphide bond covalently links the chains together. These antibody fragments have advantages over whole antibodies for many therapeutic uses because of their smaller size and better tissue penetration and clearance. Their smaller size also makes them more suitable for structural studies like nuclear magnetic resonance spectroscopy (reviewed by Riechmann and Muyldermans, 1999).

Specific primers are used for the amplification of V_H and V_L domains. The 5' primer is constructed from available data on the N-terminal conserved sequences of the leader and first framework regions of the antibody and the 3' primer is based on conserved constant region sequences (Larrick *et al.*, 1989). When N-terminal amino acid sequences of V_H or V_L are not available, 5' primers can be designed according to family specific degenerate primer sets available from various databases, such as the Kabat database (www.kabatdatabase.com). For construction of Fab fragments, the V_HC_H1 fragment is amplified with primers representing the leader sequence 5 to 8 amino acids 5' of the V_H framework 1 region and amino acids 205 to 220 of the C_H1 region. The V_LC_L construct is created with primers representing the leader sequence 5 to 8 amino acids 5' of the V_L framework 1 region and amino acids 205 to 220 of the C_L region (Padoa *et al.*, 2003).

Other characteristics to bear in mind when designing primers for cloning of antibody fragments include restriction enzyme sites, detection and purification tags, expression strategies, and the vector being used for cloning experiments (reviewed by Gavilondo and Larrick, 2000).

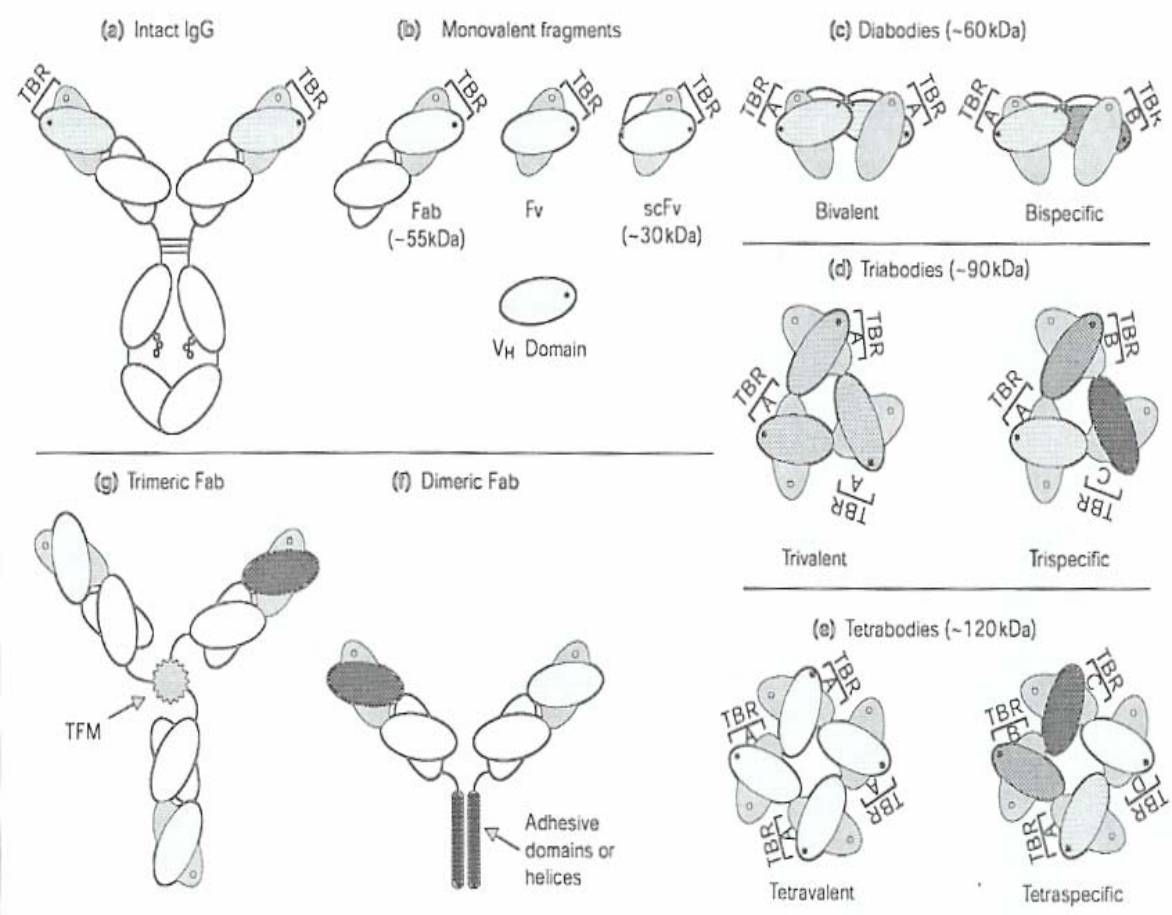


Figure 1.7: Schematic representation of recombinant antibody constructs (Hudson, 1999).

(a) Intact IgG (bivalent). (b) Monovalent immunoglobulin fragments (Fab, Fv, scFv, and a V-domain). Also shown are scFv multimers: (c) diabodies; (d) triabodies; (e) tetrabodies. V domains and C domains are represented by ovals (V_H domains are shaded grey with amino-terminal dots, V_L domains are black and C domains are white) and linkers are represented as black lines. Fv molecules are shown with the different target (antigen)-binding regions (TBRs)-A, -B, -C or –D and with different shading within the V_H domains representing different target specificity. For size comparison, also shown are (f) a domain conjugated dimeric Fab and (g) a chemically conjugated Fab trimer (using the maleiimide cross-linking reagent, TFM). Only one V domain arrangement is shown for each structure; there are obviously alternative orientations of V domains and linker polypeptides.

1.3.1.2. scFv fragments

The variable domains of Fv fragments are not associated by covalent bonds, and dissociation of the bonds at low protein concentrations has been observed (Glockshuber *et al.*, 1990). Many different approaches have been used to improve the domain association of Fv fragments. One is the construction of single chain antibodies (scFv; Figure 1.7) in which the two variable domains are linked into a single polypeptide using a short (10 to 20 residues), hydrophilic linker (Bird *et al.*, 1988; Huston *et al.*, 1988). The peptide linker should be flexible and long enough to allow pairing of the two domains to form an intact antigen binding site. The amino acid composition also has a crucial role in the design of a viable linker. Many commonly used linker peptides are rich in the small, polar glycine and serine residues $(Gly_4Ser)_3$. These residues are optimal for the linker peptides since hydrophilic amino acids allow hydrogen bonding to the solvent and glycines provide the necessary flexibility. These properties prevent the penetration of the linker peptide into the hydrophobic interface formed in the association of the V_H-V_L domains.

1.3.1.2.1. Expression of scFv and Fab fragments in bacteria

scFv and Fab fragments are produced in *E. coli* and secreted as soluble and active recombinant antibody fragments into the periplasmic space (Glockshuber *et al.*, 1990; Anand *et al.*, 1991) or they can be harvested from the bacterial culture medium (Takkinen *et al.*, 1991). These antibody fragments, derived from genes isolated from murine hybridoma cell lines, are capable of specifically binding to their target antigens with affinities ranging up to those of their parent mAbs (Denzin and Voss, 1992; Malby *et al.*, 1993). The Fab cDNA genes can be modified relatively easily before expression in bacteria. For example, modifications of the primary structure of either the Fd and/or κ chain that are useful for subsequent conjugation of imaging or therapeutic agents, or fusion to other peptides, can be introduced by site-directed mutagenesis techniques (Better *et al.*, 1988).

Gene expression of antibody fragments is controlled by inducible promoters while prokaryotic leader sequences are used to direct export of the scFv/Fab into the periplasmic space of *E. coli*. After removal of the leader peptide, the oxidising

environment in the periplasm allows correct disulphide bond formation. If the antibody fragments are expressed in the bacterial cytoplasm, inactive antibody fragment chains are produced; either in soluble form or in aggregates (inclusion bodies) that need to be refolded *in vitro* after extraction (Lilley *et al.*, 1994). scFvs are easier to express compared with recombinant Fab, where two separate proteins must be expressed and then fold together correctly to form the antigen binding pocket.

Several conventional chromatography methods have been used to purify bacterially produced antibody fragments. Immobilised metal ion affinity chromatography (IMAC) is the most widely accepted as a result of its simplicity, speed, low cost, and performance. IMAC is based on the binding affinity of metal ions such as Ni^{2+}, Cu^{2+} or Zn^{2+}, chelated to a chromatography matrix, for multiple histidine residues genetically linked to the N- or C-terminus of a scFv or one of the Fab chains (Goel *et al*., 2000; Xiang *et al*., 2002).

1.3.1.3. Valence of engineered antibodies

A major limitation of scFv molecules is their monovalent interaction with target antigen. The binding of a scFv to its target antigen can be improved by increasing its functional affinity through the creation of a multimer. A number of multivalent scFv-based structures (Figure 1.7) have been engineered, including miniantibodies (Pack and Plückthun, 1992), dimeric miniantibodies (Müller *et al.*, 1998), minibodies (Hu *et al.*, 1996), $(scFv)_2$ (Adams *et al.*, 1993), diabodies (Holliger *et al.*, 1993), and triabodies (Iliades *et al.*, 19978). These molecules span a range of valence (two to four binding sites), size (50 to 120kDa), flexibility, and ease of production.

Among the easiest constructs to engineer are the noncovalent diabody and triabody molecules. These are produced by shortening the peptide linker that connects the V_H and V_L chains of a single scFv molecule from 15 amino acids to five amino acids (diabody) or zero to three amino acids (triabody). A variety of miniantibodies have been produced by Pack and Plückthun (1992). These scFv dimers are joined by amphipathic helices that offer a high degree of flexibility. Müller *et al.* (1998) modified the miniantibody structure to create a dimeric

bispecific (DiBi) miniantibody that contains two miniantibodies (four scFv molecules) connected via a double helix (Figure 1.7).

1.3.2. Humanisation of recombinant antibodies

The most prominent aspect of antibody engineering has been the focus on the xenogeneic nature of murine mAbs. Attempts to improve the efficacy and safety of these mAbs for therapeutic applications have led to the development of a number of technologies for reducing immunogenicity of murine mAbs. An optimal technology for generation of mAbs lacking immunogenicity in humans should produce mAbs that are fully human in sequence, lacking any murine amino acid components. It should be able to generate mAbs against human antigens with the desired effector function. These mAbs should be of high affinity and specificity. The technology should be easy to use so that many researchers can generate mAbs without the need for sophisticated molecular biology techniques.

Some of the early attempts at generating human mAbs involved immortalisation of human B lymphocytes by hybridoma technology, however, numerous difficulties, including the instability of human hybridomas and a preponderance of low affinity IgM mAbs resulted in this technology seldom being used (James and Bell, 1987; Winter and Milstein, 1991). A novel technique was later described that involved infecting the human B lymphocyte with Epstein-Barr virus, thus immortalising these cells (Kudo *et al*., 1993). However, due to the difficulties in obtaining B lymphocytes producing efficient human mAbs and ethical problems finding humans immunised against target antigens, this technique was not very successful. In an attempt to overcome the problem of low efficiency, severe combined immunodeficient (SCID) mice were injected with human peripheral blood lymphocytes (Abiko *et al*., 1997). These mice show spontaneous secretion of human immunoglobulin such that a specific human antibody response is inducible following immunisation with a specific antigen.

In an effort to realise Erhlich's dream of a magic bullet with high binding affinity, reduced immunogenicity, increased half-life in the body, and adequate recruitment of effector functions, scientists have used techniques from molecular biology to

design, engineer, and express mAbs from hybridoma technology to produce humanised mAbs.

1.3.2.1. Chimeric antibodies

One approach used to reduce the immunogenicity of murine antibodies was to link the xenogeneic variable region to the corresponding human gamma and kappa (or lambda) constant regions (Morrison *et al.*, 1984), thus generating human-mouse chimeric antibodies (Figure 1.8).

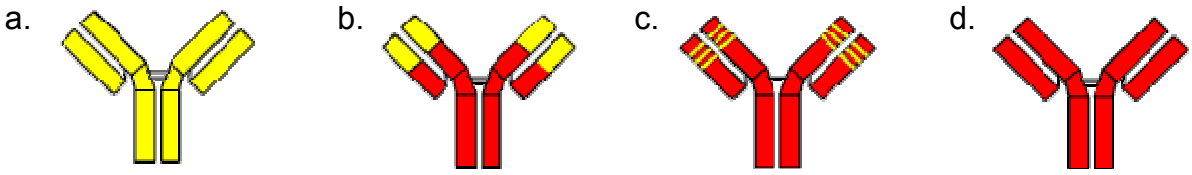


Figure 1.8: Humanised antibodies a) murine, b) chimeric c) humanised, d) human (http://www.path.cam.ac.uk/~mrc7/humanisation/index.html).

Such chimeric antibodies show the same specificity and affinity of parental murine antibodies and are capable of efficiently mediating antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent macrophage cytotoxicity (ADMC), and complement fixation in the human context. In general, substitution of the murine constant regions by human ones increases the biological half-life *in vivo*. When these antibodies were used therapeutically in clinical trial studies in humans, some still caused a human anti-chimeric antibody (HACA) response directed against the murine antibody variable regions (reviewed in Adair, 1992).

1.3.2.2. Humanised antibodies

To overcome this HACA response, scientists endeavoured to make the chimeric antibody more 'human'. This was achieved by the development of the technique of antibody 'humanisation'. This process, also known as CDR grafting (Jones *et al.*, 1986; Verhoeyen *et al.*, 1988), and reshaping (Riechmann *et al.*, 1988), involves the redesign of the variable region domain. Only the amino acids constituting the mouse CDRs are transplanted into the human antibody framework variable regions (Figure 1.8). Antibodies made this way are called humanised, reshaped, or CDR-grafted. An optimal humanised antibody is one that contains

the fewest number of mouse amino acids shifted into the human CDRs but retains the binding affinity and specificity of the original mouse antibody. Simple grafting of the rodent CDRs into human frameworks does not always reconstitute the binding affinity and specificity of the original mAb. This technology usually requires individual tailoring for each antibody, including extensive molecular modelling and manipulation of the DNA encoding the mAb. The xenogeneic CDRs of the humanized antibodies may, however, evoke an anti-idiotypic response in patients. To minimize this response, only the specificity determining residues (SDRs), the CDR residues that are most crucial in the antibody-ligand interaction, are grafted onto the human frameworks (Kashmiri *et al.*, 2005).

Human hybridoma technology has always been hampered by the fact that immunological specificity could in most cases, due to ethical reasons, not be generated by immunising human individuals. Several *in vitro* immunisation approaches have been developed that circumvent the ethical problems (Borrebaeck *et al.*, 1988; Guzman *et al.*, 1995), although immunisation of naïve human B cells *in vitro* has only generated primary immune responses. Thus, human mAbs generated by this approach are normally low-intermediate affinity IgM antibodies (Ohlin *et al.*, 1989) and not directly applicable in a clinical situation. The introduction of phage display technology (Smith, 1985), in conjunction with methods of generating antibody libraries, allowed one to bypass *in vivo* immunisations of animals or humans to produce human antibodies that can be used for therapeutic purposes. The advantage of phage display systems is that it provides a faster and more efficient selection method than traditional bacterial screening.

1.3.2.3. Phage display technology

The possibility to clone antibody genes and display the antibody fragments on the surface of filamentous bacteriophage (McCafferty *et al.*, 1990) provides a powerful tool to select specific binders from combinatorial libraries of V_H and V_L genes (panning) (Parmley and Smith, 1988; Hawkins and Winter, 1992). The phage display process involves the following steps: (1) genetically engineering phage display libraries to 'display' human scFv antibodies on the surface of the recombinant phage, (2) selecting antibodies with high affinity and specificity to any

given target by screening the antibody phage display libraries, and (3) producing and characterizing the selected antibodies (Figure 1.9).

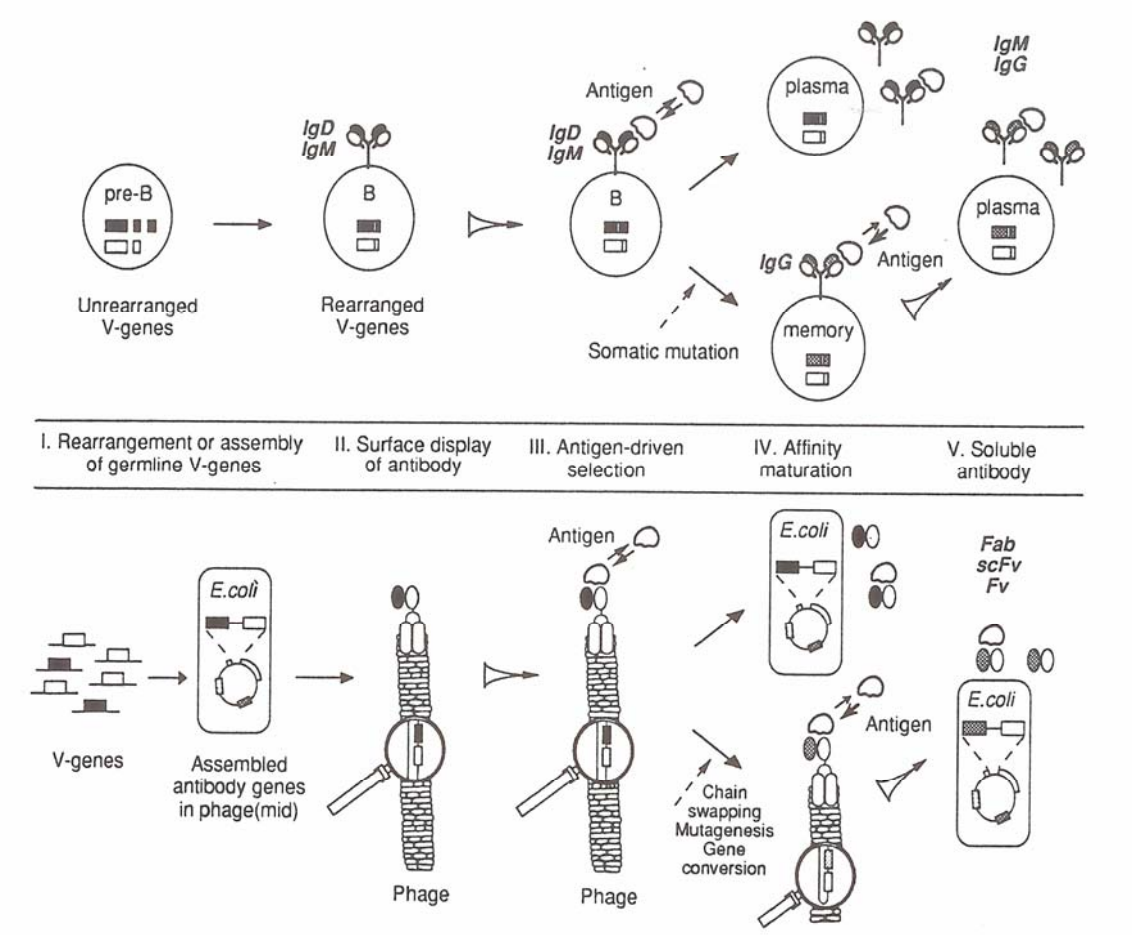


Figure 1.9: Generation of antibodies by the immune system and phage technology (Marks *et al.*, 1992).
Steps: (1) rearrangement or assembly of germline V genes; (2) surface display of antibody (fragment); (3) antigen-driven or affinity selection; (4) affinity maturation; (5) production of soluble antibody (fragment).

A pool of phage displaying a collection of diverse antibodies is called an antibody phage display library. A number of different types of phage-display libraries have been constructed. The amplification of V genes isolated from IgG-secreting plasma cells from animals or individuals immunized with antigen (Persson *et al.*, 1991), exposed to infectious agents (Burton *et al.*, 1991), with autoimmune diseases (Graus *et al.*, 1997), or with cancer (Cai and Garen, 1995) gives rise to immune libraries (Clackson *et al.*, 1991). A second category, termed the single-pot library, consists of naive, semi-synthetic, and synthetic libraries. These libraries are designed to isolate antibody fragments that bind to every possible antigen. Naive libraries are constructed from rearranged V genes from B cells (IgM) of non-immunized donors (de Haard *et al.*, 1999). Semisynthetic libraries are derived from unrearranged V genes from germline cells or from a single antibody framework with genetically randomized CDR3 regions (Pini *et al.*, 1998). The fully synthetic libraries have a human framework with randomly integrated CDR cassettes (Hayashi *et al.*, 1994). cDNA libraries are generated from RNAs derived from human bone marrow, splenic lymphocytes from splenectomized individuals, or peripheral blood lymphocytes (de Carvalho Nicacio *et al.*, 2002). The large non-immune or naïve human scFv (or Fab) libraries offer a rich source for isolation of antibodies of human origin to virtually any antigen, including self-antigens.

1.3.2.3.1. Construction of human scFv antibody phage display libraries

Libraries of antibody fragments are first constructed for the light and heavy chains by PCR using degenerate primers (Larrick *et al.*, 1989). These are combined into the phage display vector where the chains are randomly paired and can, thus, provide an additional source of variation for antibody specificity and affinity (Zebedee *et al.*, 1992). This strategy requires no immunisation, the antibody genes are cloned, and the antibody fragments express well in *E. coli* (Figure 1.9). The number and affinity of the antibodies generated to a particular antigen is a function of library size and diversity, with larger libraries yielding a greater number of high-affinity antibodies (Sheets *et al.*, 1998).

1.3.2.3.2. Display of human scFv and Fab antibodies on the surface of phage

Two methods for the display of antibody fragments on the surface of phage exist. Recombinant DNA techniques allow the pairing of the antibody fragment to one of two coat proteins of the bacteriophage, either the major coat protein encoded for by gene VIII or the minor coat protein encoded for by gene III. A system based on gene VIII will display several copies of the antibody fragment, theoretically over 2 000 copies/phage particle, leading to a multivalent selection system (Kang *et al.*, 1991). In this case, the avidity will mask the single specificity of a specific displayed fragment, thus giving good avidity as a multicopy product on the phage surface, even though the single antibody fragment might exhibit a low affinity constant. The gene III product is located at the tip of the bacteriophage particle in approximately four copies/phage (McCafferty *et al.*, 1990). In this system, each phage expresses a single, functional antibody with specificity to a particular antigen on its surface and the gene encoding the protein incorporated into its genome. It is by the gene III product that phage particles attach to the F pili of the bacteria and consequently an infectious phage must have at least one native gene III product. The system based on the gene III product is the most attractive to use in antibody engineering since it allows selection based on affinity rather than avidity (Barbas *et al.*, 1991).

1.3.2.3.3. Selection of human scFv and Fab antibodies

In addition to circumventing the limitations of human hybridoma technology, phage-expressed scFvs (or Fabs) are amenable to screening methods such as affinity enrichment that are more efficient than screening hybridoma culture supernatants by enzyme-linked immunosorbent assays (ELISA). Affinity selection methodologies typically use purified, abundant, soluble targets that can be immobilised to enrich reactive phage from large antibody display libraries. The target molecules can be purified proteins that are immobilized onto a solid surface, or blotted on nitrocellulose membranes (Skerra *et al*., 1991; Griffiths *et al*., 1994). Employing solid supports for an antigen target can alter the conformation of the antigen and lead to the isolation of antibodies that recognise only unnatural or denatured forms of the antigen. This concern can be addressed by using biotinylated antigen and recovering specific binders with streptavidin-conjugated magnetic beads (Hawkins *et al.*, 1992).

Upon incubation, the antigen specific phage antibodies will interact with the target molecule and remain attached. The remaining unbound billions of phages are washed away (Figure 1.10). The selected antigen specific phage are used to infect bacteria, one single phage per bacterium. Each infected bacterial cell grows into a single colony once spread onto agar plates. Each clone thus produces a single type of mAb. Isolated clones are then pooled and further amplified to produce the phage antibodies for the next round of screening (panning). Selection of those scFv antibodies with the strongest affinity and specificity can be achieved through multiple rounds of panning (Figure 1.10) of antibody-expressing phage particles against antigens of interest by altering selection conditions. Good candidate antibodies will go through additional testing such as ELISA and sequence analysis.

While affinity selection of phage-displayed antibodies provides a powerful method for enriching antigen-reactive scFvs (or Fabs) from large libraries, it requires multiple steps to isolate a single clone. An alternative method for rapidly screening large numbers of phage-expressed antibodies, which circumvents many of these limitations, is the plaque lift assay in which nitrocellulose is overlaid on a solid agar lawn containing bacteria infected with phage. Phage-expressed scFvs (or Fabs) are bound by the nitrocellulose, and subsequently the filter can be probed with soluble, labelled antigen to identify plaques expressing reactive antibodies (Watkins *et al.*, 1998). The plaque lift assay is complementary to affinity enrichment approaches in that it permits isolation of clones in a single step as well as the rapid characterisation of soluble scFv (or Fab) produced by phage expression systems. Finally, because plaques arise from infection by a single phage, every clone present in a library can be screened, permitting the identification and isolation of rare, nonabundant scFv (or Fab) specificities (Watkins *et al.*, 1998).

The whole phage display and selection process takes about three to four weeks to complete, with the initial identification and isolation of antibodies usually within 10 to 14 days and the genetic characterization of the antibodies within an additional 1 to 2 weeks. As in the immune system, the V genes can be subjected to random mutation, and mutants with high binding affinities may be selected.

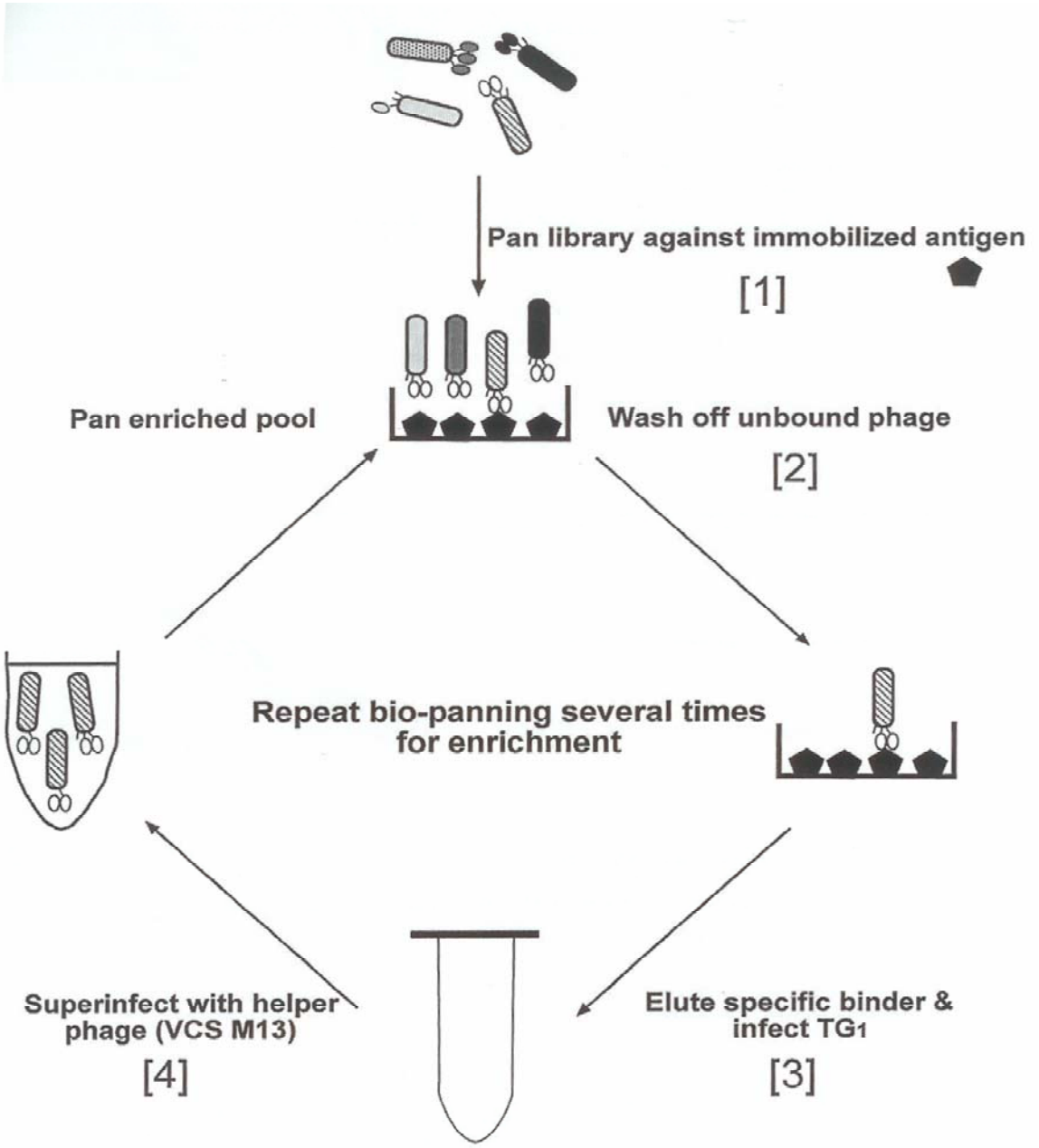


Figure 1.10: Bio-panning of phage display library (Azzazy and Highsmith, 2002). The library is screened in four steps: (1) binding of phages to the target antigen (in this case antigen immobilised on a solid support), (2) washing to remove unbound phage, (3) dissociation to recover antigen-specific phage, and (4) amplification of the antigen-specific phage by infection of *E. coli*. Caution must be exercised during the binding and washing steps to avoid low-ionic strength or other conditions that may favour adsorption of phage directly onto plastic or other matrix.

1.3.2.3.4. Affinity maturation by chain shuffling

In chain shuffling, the scFv molecule is subjected to cycles of manipulations in which the gene for one chain (e.g., V_H) is cloned into a repertoire for the second chain (e.g., V_L) (Marks *et al.*, 1992). The resulting library, that contains scFv-phage with V_H chains specific for the antigen and random V_L chains, is panned against the target antigen to identify clones with improved binding characteristics. The cycle is repeated, this time shuffling the new V_L gene into a V_H repertoire. This method provides additional diversity, particularly in the framework regions of the antibody fragments and hence, the potential for significant increases in affinity. A potential drawback associated with chain shuffling is that it can result in changes to the antigenic specificity of the scFv (Ward, 1995).

1.3.2.3.5. Affinity maturation by site-directed mutagenesis

In this method, amino acids of one or more of the CDRs are substituted, and subsequent clones with higher affinity for the antigen selected. A refinement of the above technique, termed 'parsimonious mutagenesis' (Balint and Larrick, 1993), involves screening the entire CDR sequence of an antibody fragment to identify amino acids that are actively involved in binding to the antigen. Two methods are used to improve the efficiency of the selection process. First, the number of codons introduced is limited such that each amino acid is only coded for by a single codon. Second, the amino acids at each position are manipulated to favour parental sequences, conservative changes, and those that more commonly appear in antibody CDRs (reviewed by Adams and Schier, 1999).

Protein display on filamentous phage has a broad range of applications, which include drug and target discovery, protein evolution and rational drug design. While phage display represents considerable progress over hybridoma technology, deficiencies still do exist: the size of the library is limited to approximately 10^{10}, variants may be selected against in the host environment due to toxicity to *E. coli*, and difficulties eluting phage carrying antibodies with high affinities can be encountered (Schier and Marks, 1996). Many of these limitations are circumvented by utilising a cell-free transcription, translation, and selection system in ribosome display (Schaffitzel *et al.*, 1999).

1.3.2.4. Ribosome display

Ribosome display (Hanes and Plückthun, 1997) aims for simultaneous selection and evolution of proteins from diverse libraries without any bacterial transformation. DNA (which encodes a protein library) is transcribed to mRNA which is purified and used for *in vitro* translation. The mRNA, ribosomes, and translated peptide are prevented from dissociating and this complex is then used for affinity selection on an immobilised target. Complexes that do not encode a binding peptide that specifically recognise the target antigen are removed by washing. mRNA that encodes a polypeptide similar to the target antigen dissociates from the complex and is reverse transcribed into cDNA. The cDNA is amplified and used for the following cycle of enrichment and PCR. This process involves no transformation and large libraries can, therefore, be constructed and used for selection. Additionally, library diversification is introduced via DNA shuffling (Stemmer, 1994) and error prone PCR (Cadwell and Joyce, 1992).

1.3.2.5. Transgenic mice

It is now possible to produce transgenic mice that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. Transgenic mice have been generated in which endogenous immunoglobulin heavy and light chains have been replaced by exogenous human immunoglobulin loci, so that the mice produce fully humanized antibodies (Bruggemann *et al.*, 1991; Bruggemann and Taussig, 1997).

Another way in which to produce human antibodies is with the use of severe combined immunodeficient (SCID) mice. Human lymphoid cells are stimulated *in vitro* with antigen and grafted into SCID mice. The mice are challenged with the antigen several times. Cells are harvested and used for cloning or hybridoma fusion (Duchosal *et al.*, 1992). Since SCID mice lack antibody-producing cells, the mice grafted with human cells produce human antibodies.

1.4. THERAPEUTIC USES OF MONOCLONAL ANTIBODIES

Initially, antibody engineering involved the modification of pre-existing antibodies to improve their characteristics. Now, antibody engineering encompasses the

generation of novel antibody specificities and the expression of large amounts of antibody for eventual therapeutic use. In part, the potential therapeutic utility of monoclonal antibodies stems from their specific and high affinity binding to targets coupled with their diversity of function. Antibodies are not only being developed for administration as soluble proteins, but also as intracellular molecules to alter cell function (reviewed by George, 1999).

A useful therapeutic needs to be effective and safe. The important issues relating to efficacy include the affinity and specificity, tissue penetration, clearance times, and the mode of action of the effector element of the engineered antibody. Safety concerns include inappropriate uptake by tissues and the patient reaction to the foreign protein or the pharmaceutical composition (reviewed by Adair, 1992).

Murine mAbs have inherent disadvantages as human therapeutics:

i. They require more frequent dosing to maintain a therapeutic level of mAb because of a shorter circulating half-life in humans than human antibodies.
ii. Repeated administration of murine immunoglobulin creates the likelihood that the human immune system will recognise the mouse protein as foreign, generating a HAMA response. This can cause a severe allergic reaction or result in the rapid clearance of the murine antibody upon repeated administration, and hence reduced therapeutic efficacy (Riva *et al*., 1989).

Recent advances in the design, construction, and expression of antibody fragments (Winter and Milstein, 1991) have paved the way to the production of a new range of diagnostic and therapeutic reagents. mAbs have provided reagents with unique target specificity and affinity for a wide variety of antigens and haptens. Fab and Fv fragments can be used to replace the parent mAb in situations where monovalent binding specificity is required. An advantage of using these smaller molecular fragments, apart from protein stability, is the potential to use bacterial expression systems for high-level synthesis (Glockshuber *et al.*, 1990; Hoogenboom *et al.*, 1991).

1.4.1. Engineered mAbs as pharmacological tools

Cancer is one of the leading causes of death. Considerable attention has, thus, been given to the construction of genetically engineered antibodies for use in cancer prevention/treatment. ScFv molecules have certain advantages over the use of whole mAbs in cancer immunotherapy. Whole mAbs penetrate tumours poorly due to their size. ScFvs, which are one fifth of the molecular weight of an IgG, are able to penetrate faster and deeper into tissues and clear more rapidly from the blood. scFvs also lack constant regions and are not bound by Fc receptors found in most tissues and organs, thus further reducing their side effects (Yokota *et al*., 1992; Yamaguchi *et al.*, 1998). Although IgGs penetrate tissues poorly, they are found at higher concentrations on the surface of tumours than scFvs. This is because IgGs have a longer half life and hence higher levels in the blood. The ratio of tumour to blood levels of IgG, however, remains low. In contrast, the tumour to blood ratio for scFvs is fairly high despite the low number that localise to the tumour because of their rapid clearance from the blood. A short half life is favourable for cancer imaging whereas rapid and efficient antibody uptake by the tumour is beneficial for therapeutic applications. The size of recombinant antibodies have been altered and multivalent molecules constructed for improved tumour penetration and higher affinity, respectively. These improvements will stimulate the development of new reagents for cancer imaging and therapy (reviewed by Wu and Yazaki, 2000).

1.4.2. Recombinant antibodies in clinical use

The Food and Drug Administration (FDA) has approved a number of recombinant antibody derived medicines for treatment of cancer, cardiovascular disease, Crohn's disease, rheumatoid arthritis, and for the prevention of organ rejection following transplantation (Table1.3). Many more recombinant antibodies are in late stage clinical trials for a broad range of disease indications. Antibodies now account for the single largest group of biotechnology derived molecules in clinical trials (reviewed by Green, 1999).

Table 1.3: A select list of FDA approved mAbs.

Product Name	Manufacturer	Therapeutic Applications	Antibody Type	Approved Date
Rituxan/ Rituximab	IDEC/Genentech	Non-Hodgkin's Lymphoma	Chimeric	1997
Herceptin	Genentech	Breast cancer	Chimeric	1998
Simulect	Novartis	Organ Rejection	Chimeric	1998
Remicade	Johnson & Johnson	Rheumatoid Arthritis, Crohn's Disease	Chimeric	1998
Zenapax	Roche	Organ rejection	Chimeric	1997
Mylotarg	American Home Products	Acute Myeloid Leukaemia	CDR-grafted	2000
Campath	Millenium	Chronic Myeloid Leukaemia	CDR-grafted	2001
Humira	Abbott Labs	Rheumatoid Arthritis	Phage Display	2002
Xolair	Genentech/ Novartis	Asthma	CDR-grafted	2003
Raptiva	Genentech & Xoma	Psoriasis	CDR-grafted	2003
Avastin	Genentech	Colorectal Cancer	CDR-grafted	2004
Erbitux	Imclone & Bristol-Myers Squibb	Colorectal Cancer	Chimeric	2004
Zevalin	IDEC/Schering AG	Non-Hodgkin's Lymphoma	Murine Radiolabelled	2002
Bexxar	Corixa/GSK	Non-Hodgkin's Lymphoma	Murine Radiolabelled	2003

Table reproduced from Hudson and Souriau, 2003

1.4.3. The use of recombinant antibodies in diabetes research

Recombinant antibodies can serve as tools for epitope mapping (Padoa *et al.*, 2005) and detection of epitope shifts in different stages of a disease such as type 1 diabetes and Stiff Person Syndrome (Padoa *et al*., 2003; Raju *et al*., 2005).

1.4.3.1. Autoantibodies in the sera of diabetic patients

Autoantibodies to the 65kDa isoform of glutamate decarboxylase (GAD65Abs) are accepted markers for type 1 diabetes and, together with insulin autoantibodies (IAA) and a protein tyrosine phosphatase-like islet cell antigen (IA-2), predict the disease (Vandewalle *et al*., 1995). GAD65Abs have recently been implicated in GAD65 presentation to T cells (Jaume *et al*., 2002; Reijonen *et al*., 2000) and may, therefore, play a role in the pathogenesis of type 1 diabetes. The identification and characterization of type 1 diabetes-specific GAD65 and insulin autoantibody epitopes is important to further our understanding of the pathogenic involvement of these autoantibodies in the progression of type 1 diabetes.

The identification of disease-specific GAD65Ab epitopes in type 1 diabetes has been hampered by the fact that the epitopes are conformational (Tuomi *et al*., 1994; Schwartz *et al*., 1999). Epitope identification has been attempted using GAD65/67 fusion proteins. Using these fusion proteins it has been shown that sera of type 1 diabetes patients recognize conformational epitopes located in the middle (amino acid 240-435) and the carboxy-terminal end (amino acid 451-570) of GAD65 (Daw and Powers, 1995; Hampe *et al*., 2000; Padoa *et al*., 2003). This reactivity pattern differs significantly from that of GAD65Abs in stiff-man syndrome patients (Björk *et al*., 1994; Daw *et al*., 1996), autoimmune polyendocrine syndrome type 1 patients (Björk *et al*., 1994), first degree relatives of type 1 diabetes patients, the general population, and type 1.5 diabetes patients (Hampe *et al*., 2000).

Information about conformational disease-specific GAD65Abs and their epitopes was limited (Ziegler *et al*., 1996) despite valuable data provided by the analysis of autoantibody binding patterns using GAD65/67 fusion proteins (Falorni *et al*., 2000; Hampe *et al*., 2000). Changes in GAD65Ab epitopes were observed in

prediabetic children (Bonifacio *et al*., 2000), indicating that the humoral response to GAD65 changes as an individual progresses to type 1 diabetes. Padoa and colleagues (2003) cloned rFab of well characterized GAD65-specific mAbs to characterise GAD65Abs present in the sera of patients with type 1 diabetes. The use of rFab rather than intact immunoglobulins prevents potential steric hindrance. The six GAD65-specific rFab were cloned from human mAbs b96.11, DP-C, DP-A, and DP-D (derived from autoimmune disease patients), and from murine mAbs 144 and 221-442. The epitope regions recognized by the six mAbs were spread over the length of the GAD65 molecule ensuring that different epitope specificities were represented. Although the patients' sera present a polyclonal mixture of GAD65Abs with different epitope specificities, the binding of GAD65Ab in type 1 diabetes patients to GAD65 was significantly reduced in the presence of the six rFab, supporting previous reports that major GAD65Ab epitopes in type 1 diabetes are located in the middle region and the carboxy-terminus of the molecule (Daw and Powers, 1995; Schwartz *et al*., 1999). The competition pattern in type 1 diabetes patients was different from that in GAD65Ab-positive type 1.5 diabetes patients, first degree relatives, and healthy individuals. These findings support the view that type 1 diabetes is associated with disease- and epitope-specific GAD65Abs and the opinion that the middle epitope is disease-specific. These GAD65-specific rFab should prove useful in predicting type 1 diabetes (Padoa *et al*., 2003).

Previous data has shown that GAD65Abs can modulate GAD65 processing and presentation to MHC class II-restricted T cells and thus have a pathogenic role in the development of type 1 diabetes (Jaume *et al*., 2002; Reijonen *et al*., 2000). The DP-D, DP-C, and DP-A antibodies were shown to promote the presentation of GAD65 epitopes outside their own binding sites, while inhibiting the presentation of their specific epitope to T cells (Jaume *et al*., 2002). This is in conjunction with the observation that antibodies can suppress the presentation of certain epitopes due to their high affinity binding (Simitsek *et al*., 1995; reviewed by Watts, 1997) and highlights the possible involvement of GAD65Abs in the modulation of T cell responses to GAD65. The rFab b96.11, DP-C, and other type 1 diabetes-specific rFab may represent a novel tool to modulate this processing and will be of importance in elucidating the mechanisms by which GAD65Abs may influence

disease progression. Future experiments will be needed to determine the effect of rFab b96.11 or DP-C on the action of pathogenic serum antibodies in order to reveal whether these disease-specific GAD65Abs are pathogenic or the result of epitope spreading induced by disease progression. Epitope spreading is the phenomenon whereby there is a shift in the position of the epitopes from one domain of the antigen to another as an autoimmune disease progresses. Thus, antibodies to GAD65 are initially produced against the middle and C-terminal regions of the antigen in type 1 diabetes. The autoimmune response may then undergo intramolecular epitope spreading towards epitopes on the N-terminus and further epitopes located in the mid-region of GAD65 in genetically predisposed individuals which leads to onset of the disease (Schlosser *et al*., 2005).

Raju and colleagues (2005) used these same GAD-specific rFab to study the epitopes recognised by GAD65Abs present in the sera of Stiff Person Syndrome patients. They identified a disease specific linear epitope on the GAD65 molecule in these patients. This epitope was not recognised by GAD65Abs in the sera of type 1 diabetes patients.

Similar studies have also been performed using insulin-specific mAbs and their rFab. IAA are among the first autoantibodies to appear in individuals prior to the clinical onset of type 1 diabetes (Ziegler *et al*., 1999). Their appearance is inversely correlated with age (Graham *et al*., 2002). This makes them especially important as markers for type 1 diabetes in children (Karjalainen *et al*., 1989). Little is known about the epitopes recognized by IAA. In the past, researchers have utilised naturally occurring isoforms of insulin for the analysis of IAA epitopes. In two studies, human, bovine, and porcine insulin were used to reveal differences in the binding characteristics of IAA and antibodies to exogenously administered insulin (IA), locating the major binding site of IA to the A-chain, while type 1 diabetes-associated IAA recognise a conformational epitope requiring both the A and B chain (Diaz and Wilkin, 1987; Wilkin *et al*., 1988). Differences in epitope specificities of IAA and IA were also detected using random peptide phage display (Devandra *et al*., 2003). Monoclonal antibodies and their rFab are being successfully used in the epitope analysis of other autoantibodies in type 1 diabetes (Schlosser *et al*., 1997; Schwartz *et al*., 1999; Kolm-Litty *et al*., 2000; Padoa *et al*.,

2003). This method is particularly useful in the study of conformational epitopes since the structure of the antigen remains intact (Binder *et al*., 2004).

The use of rFabs allows the detailed analysis of disease specific epitopes without disrupting the conformational structure of the antigen. In type 1 diabetes, the characterisation of disease specific GAD65 and insulin autoantibody epitopes will provide important information to further the understanding of the immunopathologic mechanisms involved in the aetiology of this disease. Characterization of possible antigenic hot spots on GAD65 and insulin may provide insights into the mechanism by which these autoantibodies are induced.

The identification of disease specific epitopes may also provide new methods for the treatment/prevention of type 1 diabetes. If a disease specific epitope is identified, patients can be treated with a peptide that binds to this epitope, effectively blocking the autoantibody from binding to the antigen. A study performed by Daniel and Wegmann (1996) showed that when NOD mice were administered with the B-(9-23) insulin fragment, either subcutaneously or intra-nasally, a delay in disease onset occurred and the incidence of diabetes decreased when compared to mice given a control peptide.

Insulin autoantibody assays have posed major problems in terms of reproducibility, sensitivity, and specificity. With the advent of antibody engineering it is envisioned that rFabs specific to insulin and its precursors can be constructed and used to standardise and improve the IAA assay. Also, the construction of rFabs which could recognise disease specific IAAs, would greatly enhance the usefulness of insulin radio-binding assays (RBAs).

1.4.3.2. Recombinant antibodies and type 2 diabetes

Type 2 diabetes is a heterogeneous and polygenic disorder resulting from the interaction of genetic and environmental factors. The disease is characterised by a failure of the pancreatic β cells to secrete sufficient quantities of insulin in the presence of reduced insulin sensitivity. Type 2 diabetes accounts for about 90% of all cases of the disease, affecting 150 million people worldwide (King *et al*., 1998).

Studies have shown that high levels of proinsulin and des-31,32 proinsulin indicate future progression to type 2 diabetes (Nijpels *et al*., 1996; Wareham *et al*., 1999). Specificity, however, poses a major problem in insulin/proinsulin assays due to the similarity in the tertiary structures of insulin and its intermediates: proinsulin and the split proinsulins contain the structures of both C-peptide and insulin. As a result, these insulin-like peptides may cross-react in immunoassays for either C-peptide or insulin. The converse also holds: C-peptide and insulin may cross-react in proinsulin assays (Sobey *et al*., 1989).

The production of antibodies against novel proinsulin epitopes may be possible and this will be extremely useful in developing specific assays for des-31,32 proinsulin since no monoclonal antibody that specifically binds to this protein exists. The only method currently available to measure this intermediate is using two assays; total proinsulin and intact proinsulin. The difference between the two assay values representing the amount of des-31,32 proinsulin (Sobey *et al*., 1989). The ability to accurately measure these molecules may provide important information on ß-cell dysfunction in type 2 diabetic patients. Similarly, these novel antibodies could be used in assays designed to measure insulin and its precursors and screen populations who have a high risk of developing type 2 diabetes. Furthermore, genetic engineering will help improve the performance of immunoassays and provide better reagents for clinical diagnosis. The production and genetic manipulation of novel mAbs that only contain the antigen binding domains of immunoglobulins will also help reduce non-specific binding (Warren *et al*., 2005).

1.4.4. Other uses of recombinant antibodies

Recombinant antibodies are also used in high-resolution X-ray crystallographic studies of antigen binding sites. The size of antibodies and the flexibility of the hinge connecting Fab arms and the Fc domain have prompted crystallographers to turn to Fab (Saul and Poljak, 1992) and Fv fragments (Boulot *et al*., 1990).

scFv can also be fused to various proteins or fluorochromes (e.g., alkaline phosphatase and fluorescein isothiocyanate) to produce coating or detection

reagents that can be used in immunoassays for direct single-step detection of target antigen (Wozniak *et al.*, 2003), double antibody sandwich ELISAs (Kerschbaumer *et al.*, 1997), or in immunofluorescence and phenotyping by flow cytometry techniques for the diagnosis of various diseases (Reimann *et al.*, 1994).

The main advantage of the use of recombinant antibodies over monoclonal and polyclonal antibodies, in traditional immunoassays, is the ability to genetically alter the antibodies binding affinity and specificity. The use of bispecific antibodies where one arm binds to a target antigen and the second to an enzyme will also be invaluable in enzyme immunoassays. Secondly, the use of recombinant antibody fragments in ELISAs provide a less time consuming screening/detection method for certain molecules (e.g., heroin) in drug monitoring than the conventional methods that are employed (thin layer chromatography, gas chromatography and high performance liquid chromatography). In addition, the traditional methods are expensive and require skilled personnel to perform the tasks (Brennan *et al.*, 2002).

Genetically engineered antibodies have similarly been used to improve the performance of immunoassays. A number of false-positive results are obtained in immunoassays due to the interference by heterophilic antibodies (naturally occurring human antibodies to immunoglobulins of animal origin). ScFvs lack the constant domain of an antibody and, therefore, interference is essentially eliminated. When such a scFv was used in an assay, it showed considerably less non-specific interference than the assay in which the parent mAb was used (Warren *et al.*, 2005).

In summary, due to technological innovations such as the production and engineering of mAbs, immunoassays have been substantially improved in terms of their sensitivity, specificity, and ease of use since the first radioimmunoassay (RIA) for the determination of plasma insulin concentration was described some 45 years ago by Yalow and Berson (1959).

1.5. INSULIN ASSAYS

1.5.1. The development of the insulin assay

Assays of insulin and its precursors are of little diagnostic use but are important tools for investigating the aetiology and pathology of diabetes. This has been the driving force behind the development of better insulin assays. The RIA for insulin described by Yalow and Berson (1959) used a polyclonal antiserum to insulin taken from human diabetes patients who had been treated with commercial mixtures of bovine and porcine insulins. The antisera was incubated with ^{131}I-labelled bovine insulin in the presence of known quantities of non-labelled bovine insulin. The antibody-bound, labelled, insulin complexes were separated from the free insulin by chromatoelectrophoresis. A future discovery that guinea pig anti-beef insulin antiserum bound human insulin led to the development of a RIA for the human hormone. Problems arose, however, due to difficulties experienced in the efficient separation of the soluble antibody-bound insulin from free insulin.

New techniques for separation were developed and the resultant assays provided new information on β cell function in type 2 diabetes patients. The importance of the immunometric measurement of insulin and its precursors has not been diminished by time. The basic assay principle has changed little, but many improvements have been made to the original insulin RIA methodology. These include the use of a more stable isotope, namely ^{125}I-insulin, simpler separation of the antibody-bound molecules from the free molecules by centrifugation, and more sensitive and specific RIAs that are easier to run and allow the measurement of many different types of biologically active molecules.

1.5.2. Problems encountered with insulin RIAs

A number of problems may be encountered with insulin RIAs. One example is the lack of standardisation for the insulin assay due to inaccurate methods. This is evidenced by disparate values for fasting plasma insulin levels and the range of results reported by different laboratories and external quality assessment schemes, respectively. A number of factors, such as the source of standards,

properties of the antisera, adsorption of insulin onto glass or plastic tubes, stability of insulin in plasma, and haemolysis are responsible for these inaccuracies (reviewed by Temple *et al.*, 1992).

1.5.2.1. Assay specificity and sensitivity

There is a great demand, both clinically and in research, for assays of insulin, proinsulin, and proinsulin-like molecules that are specific, sensitive, and precise. Specificity poses a major problem due to the similarities in the tertiary structures of insulin and its intermediates: proinsulin and split proinsulins contain the structures of both C-peptide and insulin (Figure 1.11). As a result, these insulin-like peptides may cross-react in immunoassays for either C-peptide or insulin. The converse also holds: C-peptide and insulin may cross-react in proinsulin assays. Proinsulin and split proinsulins are usually present in lower concentrations in plasma than insulin which further compounds the problems of specificity (Sobey *et al*., 1989). Assays need to be sensitive and precise especially if the results are to be used for diagnosis, monitoring treatment, or for screening purposes.

Doubts as to the specificity of human plasma insulin RIAs were raised following the finding that proinsulin-like molecules exists in human plasma (Roth *et al.*, 1968; Melani *et al*., 1970) and the demonstration that human proinsulin reacts similarly to human insulin in the insulin assays (Heding, 1972). Proinsulin-like molecules only account for 10 to 20% of immunologically insulin-like molecules in plasma from normal subjects. The poor specificity of insulin RIAs should not, therefore, be problematic when assaying insulin in normal subjects. In contrast, the lack of specificity of insulin RIAs are particularly important in the study of type 2 diabetes in which proinsulin concentrations are known to be raised in the plasma (Temple *et al*., 1989). Also, proinsulin molecules are known to cross-react (38 to 100%) in many insulin RIAs based on polyclonal antibodies. Since their biological, insulin-like activity is very much lower than that of insulin, their measurement as ‘insulin’ may lead to incorrect conclusions concerning the insulin deficiency in type 2 diabetics (Melani *et al*., 1970; Temple *et al*., 1990).

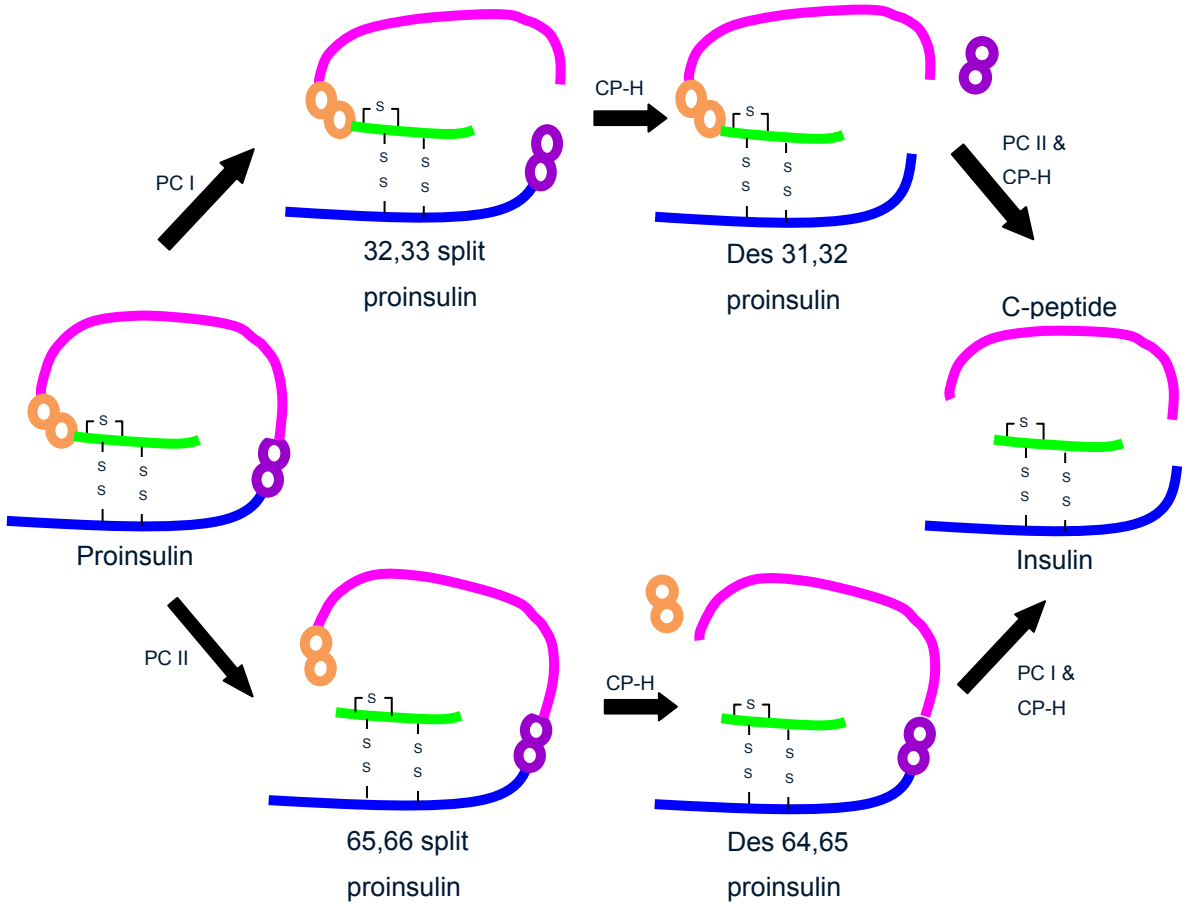


Figure 1.11: Diagrammatic representation of the insulin biosynthetic pathway.

1.5.3. Developments of insulin assays

The immunoradiometric assay (IRMA), using labelled antibody (Hales and Woodhead, 1980) was developed as a means of increasing the sensitivity of insulin assays. The two-site assay, in which one antibody is labelled and the second is coupled to a solid phase, has the advantage of improved specificity and the most convenient format. However, it was only following the production of large quantities of mAbs that scientists were able to overcome the problems of sensitivity and specificity encountered in these assays. The use of mAbs raised against conformational epitopes specific for insulin, or the initial separation of insulin from proinsulin and conversion intermediates followed by a nonspecific assay can improve specificity.

A number of groups have produced mAbs to insulin and insulin intermediates and designed two-site assays (Gray *et al.*, 1987; Chevenne *et al.*, 1994; Vieira *et al.*, 1995). However, the specificity of mAbs is not absolute. Specificity may be improved by the careful selection of pairs of mAbs to particular epitopes on the analyte of interest. Generally, the higher affinity antibody is labelled to improve sensitivity and the second antibody is attached to a solid phase. Only molecules that have epitopes that bind to both mAbs will be detected in this two-site assay. The mAbs 3B1 and 14B bind to insulin and 65,66 split proinsulin. Therefore, when these mAbs were used simultaneously in an assay, only insulin would be measured since the levels of 65,66 split proinsulin are very low in human serum. When mAb A6, which binds to proinsulin, was used in conjunction with mAb 3B1, proinsulin was measured (Sobey *et al.*, 1989). Cross-reaction with the split molecule did not interfere with the assay because the levels of this split proinsulin are very low.

A third approach was recently developed based on a new principle of exclusion between mAbs, which results in a RIA specific for insulin. This assay requires three mAbs (S1, S2, and S53) selected for their specificity, affinity, and interactive properties when mixed. S1 is directed against the B10 region of the insulin molecule, S2 to the junction between the A chain and C-peptide of proinsulin and 32, 33 split proinsulin, and S53 is directed towards the B chain, C-peptide junction of proinsulin and 65,66 split proinsulin. This assay relies on the fact that preincubation of serum samples with both S2 and S53 leaves insulin as the sole reactant with S1. This method proved that it is possible to separate insulin from proinsulin and its conversion intermediates, thus, producing a highly specific RIA for insulin (Deberg *et al.*, 1998).

Much time and effort has been spent attempting to develop insulin assays using non-isotopic labels, such as luminescent and fluorescent compounds and enzymes, to avoid the problems associated with radioisotopes (limited shelf life and biological hazards). An immunofluorometric assay (IFMA) for insulin was described by Vieira *et al.* (1995). mAbs against insulin were produced and used to develop the IFMA. One mAb was immobilised in the wells of a microtitre plate and used for capture of insulin. The second mAb was labelled with Europium and

used as the tracer antibody. This assay showed greater sensitivity and specificity than the classical RIA.

1.6. PROINSULIN ASSAYS

1.6.1. Early methods of proinsulin measurement

The original methods for the measurement of proinsulin were based on proinsulin reacting with insulin antibodies. Serum was applied to a column of Sephadex and two peaks were identified, one for insulin and the second representing the proinsulin molecules (Roth *et al.*, 1968; Melani *et al.*, 1970). Insulin RIA of the fractions revealed that proinsulin-like material consisted of about 20% of immunoreactive insulin in the fasting state in control subjects. These procedures are not suitable for the routine analysis of a large number of samples as they are time consuming, insensitive, and imprecise. In addition, an insulin RIA can only be used for the measurement of proinsulin if the recovery of proinsulin in that particular RIA has been determined.

To improve proinsulin assay specificity, a new method was developed that used an insulin-specific protease (Kitabchi *et al.*, 1971). These proteases degraded insulin in the blood. The remaining insulin-like immunoreactivity, assumed to be due to the presence of unaltered proinsulin, was then measured using an insulin RIA. Before samples were treated with the proteases, insulin levels were assessed by measuring immunoreactivity. Although this method was quick, it consistently produced higher fasting proinsulin concentrations than those obtained by gel filtration. This discrepancy was due to the fact that the proteases only partially degraded insulin, degradation was insulin concentration dependent, proinsulin was also being degraded, and the enzyme activity varied from one plasma sample to the next (Cresto *et al.*, 1974).

At the time, it was not possible to develop a direct radioimmunoassay, since production of antibodies was difficult to achieve for two reasons:

i. insufficient human pancreatic proinsulin available for large scale immunisation

ii. proinsulin antibodies produced cross-reacted with insulin and C-peptide (Melani *et al.*, 1970).

This problem was overcome with the aid of biosynthetic human proinsulin, which was available in sufficient quantities for immunisation. RIAs and IRMAs have since been developed. Indirect RIAs (Ward *et al.*, 1986; Bowsher *et al.*, 1992) require the separation of insulin or C-peptide before measuring proinsulin, and direct RIAs (Heding, 1977; Cohen *et al.*, 1985;) are based on the use of specific polyclonal antibodies to proinsulin which do not distinguish intact proinsulin from its intermediate forms. Problems with both direct and indirect RIAs include poor sensitivity and a lack of specificity. Two ELISAs, which include antibodies to insulin and C-peptide, have improved sensitivity but the specificity is still poor (Dhahir *et al.*, 1992; Kjems *et al.*, 1993). IRMAs, immunoenzymometric assays, and IMFAs have now been developed to measure proinsulin with good sensitivity and improved specificity. They do, however, still cross-react with des 64,65 proinsulin and split 65,66 proinsulin (Sobey *et al.*, 1989; Alpha *et al.*, 1992; Engling *et al.*, 1995). The concentration of des 31,32 proinsulin can only be calculated via two assays; total proinsulin and intact proinsulin. The difference between the two assay values is equal to the amount of des 31,32 proinsulin.

1.6.2. Limitations and solutions of insulin and proinsulin assays

A number of clinical studies, that have used insulin and proinsulin assays have been published. Insufficient information is frequently given concerning the assays and it is, therefore, difficult to interpret the results accurately. As a result, false conclusions are often drawn due to the lack of understanding of the limitations of the assays. It is important to include information that is necessary for validation and interpretation of clinical data obtained from an assay in publications as even minor changes to protocols can lead to significant changes in assay performance. It has also been difficult to assess the accuracy of proinsulin assays due to the lack of standardisation of assays and the lack of choice of commercially available quality control materials (reviewed by Temple *et al.*, 1992).

Future progress in assay technology will undoubtedly produce further improvements in the specificity and sensitivity of assays for insulin and its precursors, ensuring that they remain an important analytical tool for the study of the aetiology and pathology of type 2 diabetes. Advances in assay methodology have already allowed the specific measurement of insulin and proinsulin in type 2 diabetes subjects and have demonstrated β cell dysfunction as an important component in the disease process. In the near future genetic engineering may lead to the manufacture of antibodies that can differentiate between all the proinsulin-like molecules and that are sensitive enough to measure the des 64,65 proinsulin molecule.

1.7. INSULIN

The primary amino acid sequence of porcine insulin was discovered by Sanger in 1955 (Brown *et al.*, 1955) for which he was awarded the Nobel Prize in 1958. This auspicious occasion marked the first time that a protein had had the order of its amino acids (the primary sequence) determined. Fifteen years after the sequence of porcine insulin was reported, Hodgkin and her colleagues elucidated the three-dimensional structure of insulin using X-ray crystallography (Hodgkin, 1971; Blundell *et al.*, 1971). Since these initial discoveries, the primary sequence and structure for more than 70 insulin and insulin-like molecules have been unravelled.

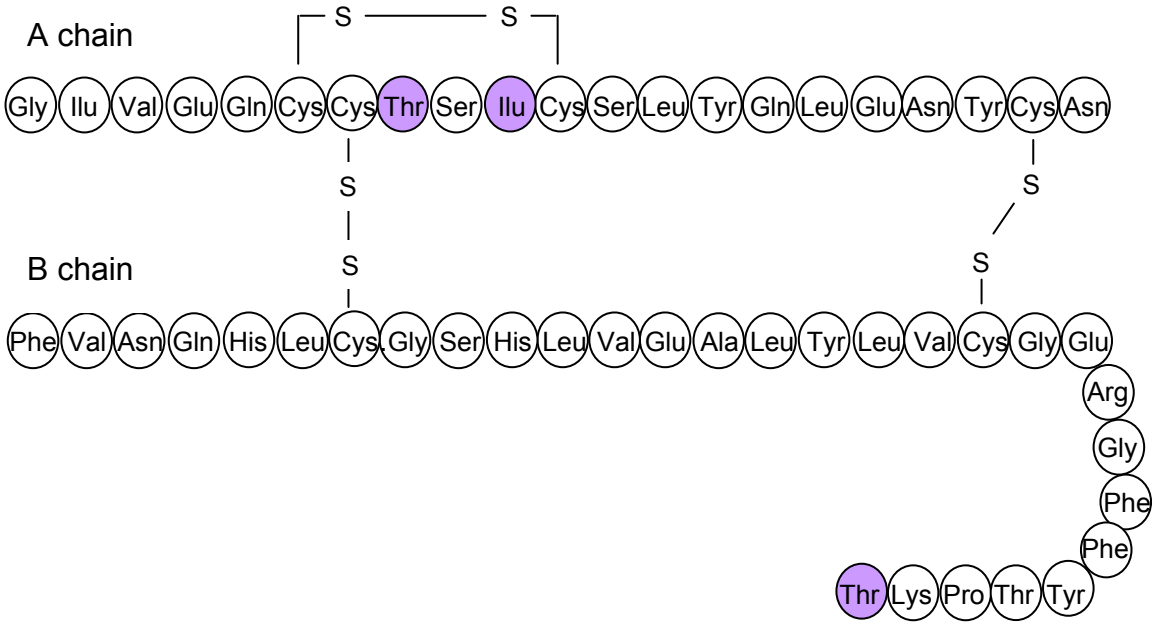
1.7.1. The structure of the insulin molecule

Insulin is a 5 808 Dalton (Da) protein composed of two peptide chains referred to as the A chain and B chain. The two chains are linked by two disulphide bonds between cysteine residues on each chain (Sanger, 1959; Figure 1.12). There is a third disulphide bridge within the A chain that links the 6^{th} and 11^{th} residues of the A chain together (Ryle *et al.*, 1955). The two chains fold together to form a compact molecule with the hydrophobic core buried within a hydrophilic surface, except for two non-polar regions. In 1967, Steiner and Oyer discovered proinsulin, a precursor of insulin. They showed that the A and B chains of insulin are initially linked via the C-peptide. In most species, the A chain consists of 21 amino acids, the B chain of 30 amino acids, and the C-peptide of 30 to 35 amino acids. The

insulin gene, located on chromosome 11p15.5 (Owerbach *et al.*, 1980; Harper *et al.*, 1981), contains 3 exons and 2 introns; exon 2 encodes the signal peptide, the B chain, and part of the C peptide, while exon 3 encodes the remainder of the C peptide and the A chain.

1.7.2. Phylogeny of the insulin molecule

Insulin is phylogenetically ancient. Not only is it found in mammals, but also in birds, reptiles, teleost and elasmobranch fish, and the very primitive hagfish (Emdin and Falkmer, 1977). The primary sequence and chemical structure of insulin from a number of species have been determined (Figure 1.13). In most species, the length and amino acid composition of the A and B chains are similar, and certain segments of the molecule are highly conserved. These include the positions of the three disulfide bridges, both ends of the A chain and the C-terminal residues of the B chain. The homology evident in the amino acid sequence of different insulins (Harris *et al*., 1956), leads to a three dimensional conformation of insulin that is very similar among species. Insulin from one animal is, therefore, very likely biologically active in other species. Thus, pig insulin can be used to replace deficient human insulin levels in diabetes patients. Nowadays, due to the mass production of human proinsulin by bacteria (recombinant insulin), porcine insulin is seldom used (Goeddel *et al*., 1979; Miller and Baxter, 1980). Human insulin differs from porcine insulin by a single amino acid at the end of the B chain (Threonine [Thr] in man, Alanine [Ala] in the pig), and from bovine insulin by substitutions at two additional residues in the A chain (positions 8 and 10: Thr and Isoleucine [Ile] in man and Ala and Valine [Val] in the ox, respectively) (Figure 1.12).



	B30	A8	A10
Human	Thr	Thr	Ile
Porcine	Ala	The	Ile
Bovine	Ala	Ala	Val

Figure 1.12: Primary structure (amino acid sequence) of human insulin (adapted from Pickup and Williams, 1991).
The highlighted residues are those which differ in porcine and bovine insulins.

A chain

Amino acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Mammalian	Gly	Ilu	Val	Glu Asp	Gln	Cys	Cys	Thr Ala	Ser Gly	Ilu Val Thr	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn	HUMAN
Hystrico-morph				Asp				Thr Ala	Ser Gly Asn	Ilu Thr Val		Ser Thr	Leu Arg	Tyr His Asn			Gln Leu Met Glu	Asn Ser Thr				Asp
Avian				Asp				His Glu	Asn	Thr Pro												
Reptilian								Glu	Asn	Thr							Gln					
Teleost			Val Leu	Glu Asp				His	Arg His Lys	Pro		Asn Asp	Ilu Lys	Phe	Asp Glx		Gln	Asp Ser Asn				
Elasmobranch					HIs			His	Asn	Thr					Asp			Gly				Gln
Cyclostome								His	Lys	Arg			Ilu		Asn		Gln					

B chain

Amino acid	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Mammalian		Phe	Val	Asn	Gln Lys	His	Leu	Cys	Gly	Ser Pro	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys Not	Thr Ala Ser	HUMAN	
Hystrico-morph		Phe Tyr		Asn Gly Ser	Gln Arg Lys	His Arg					His Asn Glu			Glu Asp	Ala Thr			Leu Ser			Gly Gln Lys Arg	Glu Asp Asn His	Arg Asp			Phe Tyr	Tyr Arg	Thr Ilu Arg		Lys Ser Asn Met	Asp Glu Ala Ser		
Avian		Ala	Ala																									Ser			Thr Ala		
Reptilian		Ala	Pro														Pho	Ilu										Ser		Arg	Thr Ser		
Teleost	Val Met	Ala	Pro	Pro Ala Asn	Gln Pro									Glu Asp														Gln Asn		Lys			
Elasmobranch		Leu	Pro	Ser														Phe				Pro	Lys			Tyr		Leu			Asx	Glx	Val
Cyclostome		Arg	Thr	Thr	Gly				Lys	Asp				Asn				Ilu	Ala			Val						Asp		Thr	Lys	Met	

Figure 1.13: The species variation in the amino acid sequence of insulin for the A- and B-chains (Hodgkin *et al.*, 1983). The sequence for human insulin is given in full. Each variation where it occurs is written below.

1.7.3. Insulin biosynthesis and secretion

1.7.3.1. The insulin biosynthetic pathway

The synthesis of insulin begins with the translation of the insulin gene, which resides on chromosome 11p13 (Figure 1.14). During translation, the two introns are spliced out of the mRNA product, which encodes a protein of 110 amino acids in length. This primary translation product is called preproinsulin and is inactive (Chan *et al.*, 1976). The first 24 amino acids form a signal peptide which is required for the protein to cross the endoplasmic reticulum membrane. Preproinsulin is rapidly transported into the lumen of the rough endoplasmic reticulum, of the pancreatic β cells, where a proteolytic enzyme cleaves the signal peptide from preproinsulin to yield proinsulin (Patzelt *et al.*, 1978). Proinsulin, a 9 kDa peptide, consists of three domains: an amino terminal B chain, a carboxyl-terminal A chain, and a small linker peptide chain - the C peptide. A major function of the C peptide is to facilitate the correct folding of the A and B chains and alignment of their disulphide bridges before eventual cleavage. Proinsulin is transported by microvesicles to the Golgi apparatus, where it is packaged into vesicles and surrounded by a membrane containing an ATP-dependent proton pump (Farquhar and Palade, 1981). The conversion of proinsulin to insulin is initiated in the Golgi complex and continues within the maturing secretory granule through the sequential action of two distinct site-specific endopeptidases (PC1/3 and PC2) and carboxypeptidase H (Steiner *et al.*, 1974; Davidson *et al*., 1988).

1.7.3.2. Detailed features of proinsulin conversion

Depending upon the conversion route taken, discrete conversion intermediates will be generated; split 32,33-, split 65,66-proinsulins; and des 31,32-, des 64,65-proinsulins (Figure 1.11) (de Haen *et al.*, 1978). If the B chain/C-peptide junction is the first site cleaved (by PC1) and trimmed, des 31,32 split proinsulin is generated as the conversion intermediate. Cleavage by PC2 at the alternative junction, linking C-peptide and the A-chain, generates des 64,65 split proinsulin (Davidson *et al.*, 1988). PC2 can also cleave at the alternative junction but with a greatly reduced efficiency. It has also been shown that PC2 only cleaves the C-peptide/A-chain junction of intact proinsulin relatively slowly, preferring des 31,32

split proinsulin as its substrate. The proinsulin conversion route via des 31,32 proinsulin appears to be the predominant pathway (Rhodes *et al.*, 1992).

In the β cell, and in the circulation, it is generally accepted that the major conversion intermediates found are des 31,32 and des 64,65 proinsulins and not the split proinsulins (Given *et al.*, 1985; Sobey *et al.*, 1989). Endoproteolytic cleavage at either of the two proinsulin junctions seems to be the rate limiting step by comparison with the very rapid and efficient subsequent cleavage of the newly exposed C-terminal amino acid by carboxypeptidase H (also known as E). The conversion intermediates recovered from granules, β cells, or encountered in the circulation, are thus typically in their trimmed state, without C-terminal basic residues (Halban, 1994). Insulin and C peptide are stored together in the granule sac and are ultimately released in equimolar amounts (Steiner *et al.*, 1971). Under normal conditions, 95% of the biosynthetic product is secreted as insulin and less than 5% as unconverted proinsulin.

1.7.3.3. Proinsulin and conversion intermediates in type 2 diabetes mellitus

Using assays that are able to distinguish between insulin, proinsulin and des 31,32 proinsulin (Sobey *et al.*, 1989), it has been shown that the ratio of proinsulin and/or des-31,32 split proinsulin to insulin is often elevated in type 2 diabetes (Temple *et al.*, 1989; Ostrega *et al.*, 1995). The explanation for these unusually elevated ratios is uncertain. The comparison of circulating levels of proinsulin and conversion intermediates in the basal and stimulated states indicates that these products, like insulin, are being released by the regulated pathway in these patients. It has been suggested that there may be a fundamental impairment of proinsulin processing in type 2 diabetes (Kahn and Halban, 1997), but the evidence is circumstantial. It seems more likely, when looking at the available data, that increased demand upon the β cell, and in particular an imbalance between rates of synthesis, intracellular degradation, and release could simply lead to the granular contents being discharged before conversion is complete. This explanation is, however, flawed as not all situations in which there is increased demand upon the β cell lead to unusual ratios of proinsulin/conversion intermediates to insulin.

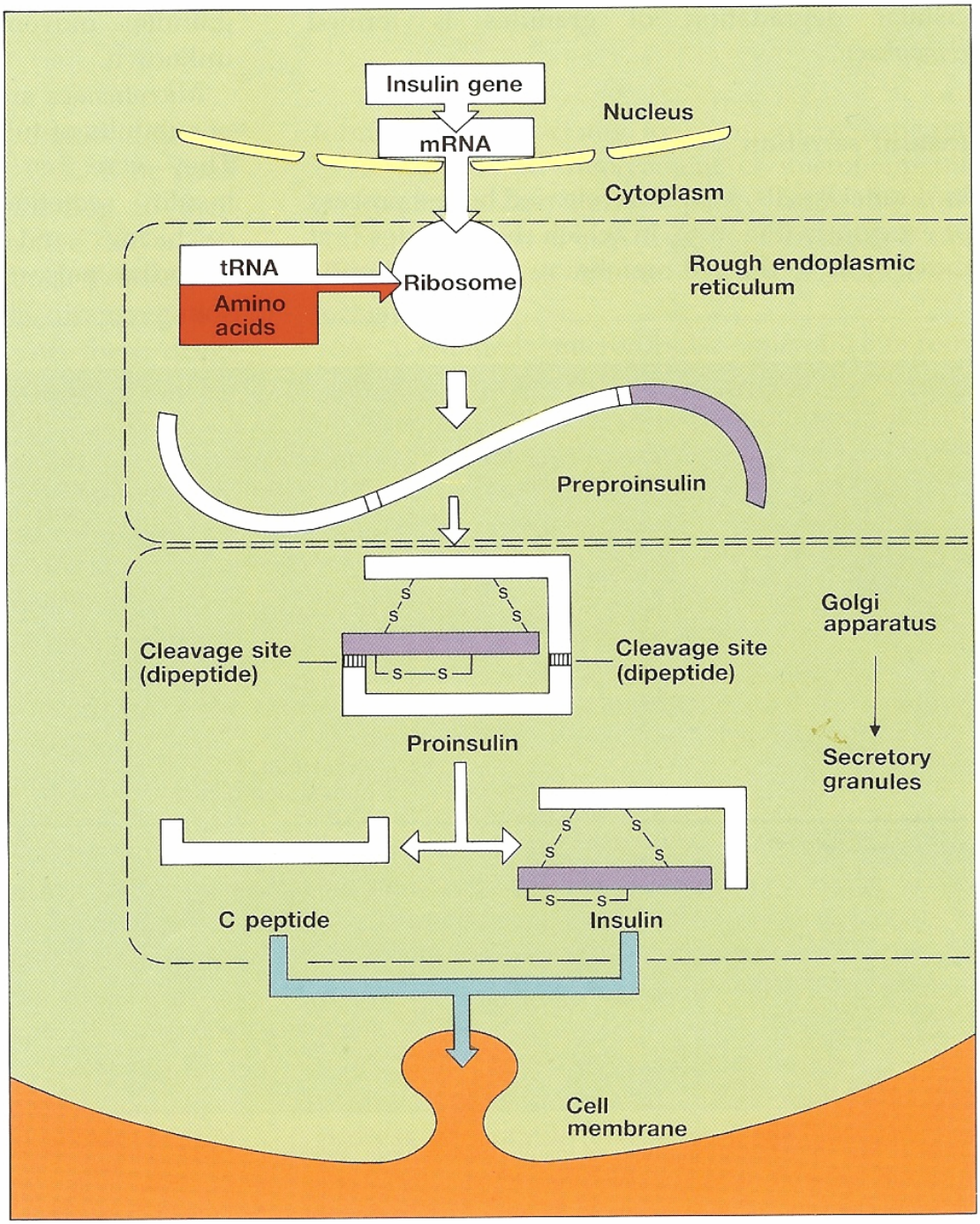


Figure 1.14: Biosynthesis of preproinsulin and its subsequent conversion to proinsulin and insulin (Pickup and Williams, 1991).

1.8. DIABETES MELLITUS

Diabetes mellitus is a chronic illness characterised by hyperglycaemia resulting from impairments in insulin secretion, defects in insulin action, or both. The chronic hyperglycaemia is associated with the development of microvascular and macrovascular complications. In the majority of cases the diagnosis can be made with certainty but the underlying aetiology and pathogenesis remain largely obscure.

1.8.1. The classification of diabetes mellitus

The classification of diabetes has evolved over the last three centuries. In 1875 diabetes was differentiated into two forms, 'diabète maigre' and 'diabète gras' which have different prognoses and require different management. Diabetes was later classified according to the age of onset of the disease, using the terms 'juvenile-onset' and 'maturity-onset'. In 1936 the disease was characterised according to the requirement of insulin as 'insulin sensitive' or 'insulin resistant'. Twenty years later the terms 'type 1' and 'type 2' diabetes emerged; this distinction was based on clinical criteria. Type 1 diabetes was later subdivided according to whether destruction of the β cells was autoimmune (type 1a), a viral infection or other agent (type 1c), or a combination of the two (type 1b) (Pickup and Williams, 1991).

In 1979, the National Diabetes Data Group (NDDG) published a provisional consensus classification, which became the basis for that recommended by the World Health Organisation (WHO) Expert Committee on Diabetes in 1980. The WHO classification represented a landmark by providing standardised diagnostic criteria for diabetes and a uniform terminology suitable for clinical and epidemiological research, which have now been widely adopted.

Over the years, our knowledge about the aetiology and pathogenesis of diabetes and about the predictive value of different blood glucose levels for development of complications has increased significantly. This has led to diabetes, and the criteria thereof, being re-examined, redefined and re-classified by both the American

Diabetes Association (ADA) and the WHO. There is currently a move away from a system where the classification of the disease was primarily distinguished by the requirement for insulin therapy, toward a system based on pathogenesis of the disease (Table 1.4) wherever possible (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). However, the impetus for the classification and diagnosis scheme proposed in 1979 still holds true. That is,

> '*the growth of knowledge regarding the aetiology and pathogenesis of diabetes has led many individuals and groups in the diabetes community to express the need for a revision of the nomenclature, diagnostic criteria, and classification of diabetes. As a consequence, it was deemed essential to develop an appropriate, uniform terminology and a functional, working classification of diabetes that reflects the current knowledge about the disease*' (NDDG, 1979).

Table 1.4: Aetiological classification of diabetes mellitus.

A. Diabetes mellitus
➢ Type 1 diabetes *(β cell destruction, usually leading to absolute insulin deficiency)*
- Autoimmune
- Idiopathic
➢ Type 2 diabetes$^{\Omega}$ *(results from a progressive insulin secretory defect on the background of insulin resistance)*
➢ Other specific types of diabetes
- Genetic defects of β cell function or in insulin action
- Diseases of the exocrine pancreas
- Endocrinopathies
- Drug- or chemical-induced
- Infections
- Uncommon forms of immune-mediated diabetes
- Other genetic syndromes sometimes associated with diabetes
➢ Gestational diabetes *(diagnosed during pregnancy)*

$^{\Omega}$ Patients with any form of diabetes may require insulin treatment at some stage of their disease. Such use of insulin does not, of itself, classify the patient. Table adapted from ADA, 2005b.

1.8.1.1. The new classification criteria

The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2003) proposed changes to the NDDG/WHO classification scheme. The main features of these changes are as follows:

i. The terms insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) have fallen away and are replaced with the terms type 1 and type 2 diabetes. Arabic numerals, rather than Roman numerals, are to be used.
ii. Type 1 diabetes encompasses cases due to the destruction of the pancreatic β cells and that are prone to ketoacidosis. Included in this category are cases due to an autoimmune process and those for which an aetiology is unknown. Subjects in whom there is no evidence of autoimmunity are classified as type 1 idiopathic.
iii. Type 2 diabetes is the most prevalent form of the disease, which results from insulin resistance with an insulin secretory defect.
iv. Malnutrition diabetes has been removed from the classification list due to the lack of convincing evidence proving that diabetes can be directly caused by protein deficiency.
v. The term IGT (impaired glucose tolerance) is retained and the similar intermediate stage of fasting glucose is named impaired fasting glucose (IFG). IGT and IFG are officially termed 'pre-diabetes'. These individuals are at increased risk of developing diabetes.
vi. Selective screening is recommended for glucose intolerance in pregnancy.
vii. Diabetes can be staged according to the severity of the metabolic abnormality.
viii. It is more important to understand the pathogenesis of the hyperglycaemia and to treat it appropriately than to assign a label to the disease i.e., type 1 or type 2 diabetes.

1.8.2. Diagnostic criteria for diabetes mellitus

The revised criteria for the diagnosis of diabetes mellitus and hyperglycaemia are shown in Table 1.5 (WHO, 1999). The major change from the previous WHO recommendation is a lower FPG (fasting plasma glucose) value ≥ 7.0 mmol/l to diagnose diabetes (previously ≥ 7.8 mmol/l). For whole blood, the proposed new

level is ≥ 6.1 mmol/l (previously ≥ 6.7 mmol/l). This recommendation was primarily based on a review of the results of more than 15 years of research which showed that a fasting blood glucose of ≥ 7.0 mmol/l is associated with an increased risk of microvascular and macrovascular complications. These complications often developed before the diagnosis of diabetes when the diagnosis was based on a blood glucose value ≥ 7.8 mmol/l. It is believed that earlier diagnosis and treatment can prevent or delay the costly and burdensome complications of diabetes.

For epidemiological or population screening purposes, the fasting or 2 h value after 75 g oral glucose may be used alone. For clinical purposes, the diagnosis of diabetes should always be confirmed by repeating the test on another day, unless there is unequivocal hyperglycaemia with acute metabolic decompensation or obvious symptoms.

An intermediate group of subjects (IFG) are recognised whose glucose levels are too high to be considered normal yet do not meet the criteria for diabetes. The categories of FPG values are as follows (ADA, 2005b):

- FPG < 5.6 mmol/l = normal fasting glucose;
- FPG ≥ 5.6 mmol/l and < 7.0 mmol/l = IFG;
- FPG ≥ 7.0 mmol/l = provisional diagnosis of diabetes.

The corresponding categories when the OGTT is used are the following:

- 2-h PG < 7.8 mmol/l = normal glucose tolerance;
- 2-h PG ≥ 7.8 mmol/l and <11.1mmol/l = IGT;
- 2-h PG ≥ 11.1 mmol/l = provisional diagnosis of diabetes.

The ADA recommends the use of FPG alone for clinical diagnosis and epidemiological studies, whereas the WHO report (1999) advocates the continued use of the OGTT.

In summary, the diagnostic criteria are now revised to avoid the discrepancy between the FPG and 2-h PG cut-off values and facilitate and encourage the use of a simpler and equally accurate test - FPG - for diagnosing diabetes.

Table 1.5: Values for diagnosis of diabetes mellitus and other categories of hyperglycaemia.

	Glucose concentration (mmol/l [mg/dl])			
	Whole blood		Plasma	
	Venous	Capillary	Venous	Capillary
Diabetes Mellitus				
Fasting	≥ 6.1 (≥ 110)	≥ 6.1 (≥ 110)	≥ 7.0 (≥ 126)	≥ 7.0 (≥ 126)
or 2 h post glucose load	≥ 10.0 (≥ 180)	≥ 11.1 (≥ 200)	≥ 11.1 (≥ 200)	≥ 12.2 (≥ 220)
or both				
Impaired glucose tolerance (IGT)				
Fasting (if measured)	< 6.1 (< 110)	< 6.1 (< 110)	< 7.0 (< 126)	< 7.0 (< 126)
and 2 h post glucose load	≥ 6.7 (≥ 120) and < 10.0 (< 180)	≥ 7.8 (≥ 140) and < 11.1 (< 200)	≥ 7.8 (≥ 140) and < 11.1 (< 200)	≥ 8.9 (≥ 160) and < 12.2 (< 220)
Impaired fasting glycaemia (IFG)				
Fasting	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 6.1 (≥ 110) and < 7.0 (< 126)	≥ 6.1 (≥ 110) and < 7.0 (< 126)
and 2 h post glucose load (if measured)	< 6.7 (< 120)	< 7.8 (< 140)	< 7.8 (< 140)	< 8.9 (< 160)

Table reproduced from WHO (1999).

1.8.3. Symptoms of diabetes mellitus

In type 1 diabetes, sudden weight loss, frequent urination, and constant thirst are the classical symptoms. In type 2 diabetes, symptoms may be more subtle and go unnoticed, or even be absent altogether (WHO, 1999). Type 2 diabetes is often diagnosed through the detection of other problems associated with having had diabetes for some years (e.g., leg pain or ulcers caused by nerve damage and/or poor circulation).

Acute, life-threatening consequences of diabetes are hyperglycaemia with ketoacidosis or the nonketotic hyperosmolar syndrome. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputation, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction (reviewed by Gerich, 1986).

1.9. TYPE 1 DIABETES MELLITUS

1.9.1. Autoimmune Diabetes Mellitus

Type 1 diabetes, previously referred to by the terms Insulin-Dependent Diabetes Mellitus (IDDM: because of the clinical requirement for insulin) or juvenile diabetes (because of the early age of onset), is widely thought to be an organ-specific autoimmune disease. Type 1 diabetes results from a deficiency of insulin due to the destruction of the insulin-producing β cells situated in the islets of Langerhans within the pancreas (WHO, 1999). The destruction process is manifested by infiltration of the pancreatic islets by mononuclear cells, and may proceed over a period of many years (Gorsuch *et al.*, 1981; Johnston *et al.*, 1989). A large proportion (60 to 80%) of the β cells are destroyed by the time clinical symptoms appear (reviewed by Notkins and Lernmark, 2001). Younger children present with more severe symptoms at diagnosis, because children younger than 7 have lost on average 80% of the islets, compared to 60% in those 7 to 14 years old and 40% in those older than 14 (Foulis *et al.*, 1986). This prediabetic period offers an opportunity to identify those individuals who are likely to become insulin-

dependent later and to start intervention aimed at delaying or preventing the manifestation of clinical disease (Kulmala *et al.*, 1998).

The rapidly progressive form is commonly observed in children and adolescents who may present with ketoacidosis as the first manifestation of the disease. The slowly progressive form generally occurs in adults who may retain residual β-cell function, sufficient to prevent ketoacidosis, for many years. These individuals eventually become dependent on insulin. This form of diabetes is referred to as latent autoimmune diabetes in adults (LADA) or type 1.5 diabetes (Tuomi *et al.*, 1993; WHO, 1999).

1.9.1.1. Idiopathic form

There are some forms of type 1 diabetes which have no known aetiology. Some of these patients have permanent insulinopenia, but have no evidence of autoimmunity. This form of diabetes is more common among individuals of African and Asian origin. Individuals with this form of diabetes suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes. This form of diabetes is strongly inherited, lacks immunological evidence for β-cell autoimmunity, and is not HLA associated. An absolute requirement for insulin replacement therapy in affected patients may come and go (WHO, 1999).

1.9.2. The epidemiology of type 1 diabetes

In 2000, it was estimated that 171 million adults ≥ 20 years of age have diabetes (Wild *et al.*, 2004). Type 1 diabetes accounts for 5 to 10% of all of the cases of the disease. Thus, roughly 10 to 20 million individuals worldwide suffer from type 1 diabetes (King *et al.*, 1998; Wild *et al.*, 2004). The majority of the patients (approximately 40%) are diagnosed and classified with type 1 diabetes within the first two decades of life, making it one of the most common, severe, chronic diseases of childhood. However, an increasing number of cases are being recognised in older individuals: 1:300 children and as many as 1:100 adults develop this disease in the United States, where 30 000 new cases are described each year (Rewers *et al.*, 1988). The prevalence of type 1 diabetes in children

aged less than 15 years ranges from 0.05 to 0.3% in most European and North American populations (Rewers *et al.*, 1988).

One of the most striking characteristics of type 1 diabetes is the large geographical variability in the incidence (Diabetes Epidemiology Research International Group, 1988; Karvonen *et al.*, 2000). Scandinavia (35/100 000 in Finland) and the Mediterranean island of Sardinia have the highest incidence rates in the world while Oriental populations have the lowest rates (1.7/100 000 per year in Japan) (reviewed in Notkins and Lernmark, 2001). In Finland, the incidence has more than tripled from 1953, when it was 12/100 000/year (reviewed by Pitkäniemi *et al.*, 2004), with an average increase of 2.4 percent per year. A recent analysis of data on published incidence trends showed that the incidence of type 1 diabetes is globally increasing by 3.0% per year, and that in 2010 the incidence of type 1 diabetes will be 40% higher than in 1998 (Onkamo *et al.*, 1999).

The incidence of type 1 diabetes varies markedly over time, both seasonally and annually. In the northern hemisphere, the incidence declines during the warm summer months; similarly in the southern hemisphere, the seasonal pattern exhibits a decline during the warm months of December and January, implicating a climatic factor (Gamble, 1980). This seasonal pattern only appears to occur in older children and adolescents (Dahlquist *et al.*, 1982, Kostraba *et al.*, 1992a), suggesting that factors triggering diabetes may be related to school attendance or viral infections.

The incidence of type 1 diabetes changes with age. The incidence rises from birth to 12 years, reaching a peak at age 11 to 13 years before falling to a much lower rate. These peaks in incidence are perhaps due to alterations in the pattern of infections or increases in insulin resistance. The incidence decreases in the third decade of life (Joner and Sovik, 1991; Bruno *et al.*, 1993), but increases again in the fifth to seventh decades of life (Christau *et al.*, 1977; Melton *et al.*, 1983).

There are noticeable racial differences in type 1 diabetes risk in multiracial populations. For example, in the United States, non-Hispanic whites are approximately one and a half times as likely to develop type 1 diabetes as African

Americans (Diabetes Epidemiology Research International Group, 1988) or Hispanics (Kostraba *et al.*, 1992a).

In most instances, there is no difference in the incidence of type 1 diabetes in males and females (Gale and Gillespie, 2001), with the pubertal peak of incidence in females preceding that in males by 1 to 2 years. Examples where the incidence may differ among the sexes are in lower risk populations, such as Japan or United States blacks, where there is a female preponderance. Conversely, in the high risk groups there is a slight male excess (Karvonen *et al.*, 1997).

1.9.3. Autoimmunity

The trigger that leads to autoimmune destruction of the pancreatic β cells is still unknown. The basis for the current thinking regarding the cause of β cell destruction comes from two discoveries made in the 1970s. The first of these was the strong linkage between type 1 diabetes and the highly polymorphic human leukocyte antigen (HLA) class II immune recognition molecules located on chromosome 6 (Singal and Blajchman, 1973; Nerup *et al.*, 1974). A number of studies have revealed many high- and low-risk HLA alleles (reviewed by Schranz and Lernmark, 1998). As a result HLA genotyping has become an important research tool for identifying individuals at risk of developing type 1 diabetes. This association supported the hypothesis that the disease has an autoimmune component because it is well known that HLA molecules play a role in antigen presentation.

Further evidence for the autoimmune nature of type 1 diabetes came from the discovery of autoantibodies in the sera of type 1 diabetic patients. Sera from type 1 diabetes patients were incubated with normal, frozen pancreatic tissue sections. Immunofluorescence revealed that type 1 diabetic patient's sera stained pancreatic islets (Bottazzo *et al.*, 1974; MacCuish *et al.*, 1974). These islet cell antibodies (ICAs) have since been widely used to study the clinical course and pathogenesis of type 1 diabetes.

Autoimmunity is defined by the presence of autoantibodies because their measurement is reliable and standardized across laboratories, in contrast to the cellular markers (reviewed by Atkinson and Eisenbarth, 2001). The initial ICA assay, using immunofluorescence and pancreatic tissue (Bottazzo *et al.*, 1974) has been notoriously difficult to standardize and has been replaced by a combination of specific β cell autoantibodies to insulin (Palmer *et al.*, 1983), GAD65 (Baekkeskov *et al.*, 1982; Baekkeskov *et al.*, 1990), and IA-2 (Passini *et al.*, 1995). These tests have been shown to be quite sensitive and predictive in relatives of type 1 diabetes patients (Bingley *et al.*, 1994; Verge *et al.*, 1996) and in the general population (Bingley *et al.*, 1997). Autoantibodies are, therefore, currently the most frequently used markers for the prediction of type 1 diabetes.

1.9.4. Autoantibodies in type 1 diabetes

The most thoroughly characterised immune phenomena associated with type 1 diabetes is the presence of circulating antibodies to various islet cell proteins. Most type 1 diabetes patients (90%) have these ICAs in their sera in the asymptomatic period that precedes clinical onset of type 1 diabetes.

1.9.4.1. Identification of autoantigens

The principal three autoantigens recognised by ICA were identified in the 1980s and early 1990s. The first autoantigen described in1982 was a 64 kDa islet cell protein. This protein was later identified as the enzyme glutamic acid decarboxylase 65 (GAD), and was shown to be one of the major antigens for autoantibodies in sera from patients with type 1 diabetes (Baekkeskov *et al.*, 1982; Baekkeskov *et al.*, 1990). The *GAD65* gene is located on chromosome 10p11 and encodes a protein of 585 amino acids (Table 1.6). There are two isoforms of GAD which share 65% homology; GAD67 with a molecular weight of 67 000 Da and GAD65, a 65 000 Da protein (Bu *et al.*, 1992). They are expressed in both neurons and pancreatic islet cells. GAD65 predominates in the latter, but its function is unknown. GAD65 plays a role in the conversion of glutamic acid to γ-aminobutyric acid (GABA), a major neurotransmitter inhibitor. Autoantibodies in sera of type 1 diabetic patients are directed primarily to the middle (amino acid

245-449) and C-terminal (amino acid 450-585) regions of the molecule (Hampe *et al.*, 2000; Padoa *et al.*, 2003).

The association of insulin autoantibodies (IAA) with type 1 diabetes was reported in 1983 when they were found in 18% of untreated, newly diagnosed diabetic patients (Palmer *et al.*, 1983). IAA are the first markers to appear during the symptomless period which precedes diabetes and they have been observed in up to 80% of patients diagnosed before the age of five years and they occur in approximately 30% of patients with clinical disease manifestation in adult age (reviewed by Potter and Wilkin, 2000). IAA appear to be inversely correlated with age in both new-onset type 1 diabetic patients and their high-risk non-diabetic relatives. A possible explanation for the production of autoantibodies to insulin before the development of diabetes could be that islet cells undergoing autoimmune lysis may release immunogenic fragments, or proinsulin, that may induce a secondary immunisation depending on differences in the HLA class II phenotypes associated with diabetes. Another possible explanation for early development of IAA could be the recognition by the immune system of insulin as antigen leading to the autoimmune response initiating islet cell lysis.

Insulin is a short polypeptide of 51 amino acids. The insulin gene is located on chromosome 11p15. Autoantibodies to insulin, in the sera of type 1 diabetic patients, are primarily directed to conformational epitopes. In the case of insulin, the epitopes map to the B chain of human proinsulin or insulin (Diaz and Wilkin, 1987).

The third autoantigen identified was a protein tyrosine phosphatase-like molecule, IA-2 (Lan *et al.*, 1996). IA-2 (Mr 106 000), also known as ICA512, is located on chromosome 2q35 (Lan *et al.*, 1996) and is a member of the transmembrane protein tyrosine phosphatase (PTP) family. Due to a critical amino acid substitution at position 911 (Asp for Ala), in the catalytic domain of the molecule, IA-2 lacks enzymatic activity. The 979 amino acid protein is expressed in secretory granules in brain and neuroendocrine cells including pancreatic islet α and β cells (Solimena *et al.*, 1996). Although IA-2 has been localised to the

secretory vesicles of endocrine and neuronal cells by immunofluorescence studies, its function remains unknown.

IA-2β, also called phogrin, is another autoantigen in type 1 diabetes and is closely related to IA-2. The aminoterminal intralumenal domains show only modest homology to each other (20%), whereas their cytoplasmic domains are closely related (80%) (Wasmeier and Hutton, 1996). IA-2β has a molecular weight of 111 000 Da and is situated on chromosome 7q36. It carries the same amino acid substitution in its catalytic domain as IA-2, and is similarly expressed in neuroendocrine tissues. Autoantibodies to IA-2 and IA-2β are directed exclusively to their intracellular domains (reviewed by Leslie *et al.*, 1999). Most sera that recognise IA-2β also recognise IA-2. Conversely, not all sera that recognise IA-2 recognise IA-2β. Thus, IA-2 is the chosen protein for most immunoassays.

International workshops have been held and extensive studies carried out in an effort to standardise the assays for autoantibodies to GAD65, IA-2 and insulin (Verge *et al.*, 1998; Mire-Sluis *et al.*, 2000). Radioligand binding assays for GAD65 and IA-2 are accepted as being precise and reproducible whereas considerable interlaboratory variation and lack of reproducibility is observed for the insulin autoantibody assays. There are numerous other autoantibodies to antigens (e.g., carboxypeptidase H, ICA69, GLUT-2, etc.) that have been reported. These have not been used in routine clinical studies due to the fact that they occur at a considerably lower frequency than the GAD65, IA-2, and insulin autoantibodies. It has been suggested that autoantibodies to GAD65, IA-2 and insulin replace ICA in the prediction of type 1 diabetes (Kulmala *et al.*, 1998).

1.9.4.2. Autoantibodies as predictors of type 1 diabetes

Autoantibodies to GAD65 are present in 70 to 80% of newly diagnosed type 1 diabetic patients and a slightly smaller percentage of patients (50 to 80%) have autoantibodies to IA-2. Overall, fewer patients appear to have autoantibodies to insulin. However, IAA are the first of the islet-related antibodies to appear during early insulitis, thus children have a markedly higher frequency of autoantibodies to insulin than teenagers or young adults (Hagopian *et al.*, 1995; Myers *et al.*, 1995).

Table 1.6: Major autoantigens in type 1 diabetes.

	GAD65	IA-2	Insulin
Amino acid length	585	979	51
Mr (Da)	65 000	106 000	6 000
Chromosome	10p11	2q35	11p15
Cell type in which expressed	Neuroendocrine, pancreatic islet cells	Neuroendocrine, pancreatic islet cells	Pancreatic islet β cells
Intracellular location	Neuron-like small vesicles	Secretory vesicles	Secretory vesicles
Function	Converts glutamic acid to GABA: inhibitory neurotransmitter	Enzymatically inactive member of PTP family	Ligand for the insulin receptor; regulation of blood glucose

Table reproduced from Notkins and Lernmark, 2001.

Some type 1 diabetics only have autoantibodies to a single autoantigen, whereas others may have autoantibodies to all three of the major autoantigens present. Up to 90% of newly diagnosed type 1 diabetic patients' have autoantibodies to one or more of these autoantigens. The percentage positivity depends on a variety of factors, including the age of the subjects, the duration of the disease, and in some instances their ethnicity. Assay variability also exists, particularly at the limit of its range of detection. The performance characteristics of the autoantibody assays have a critical impact on their predictive value. Continuous quality control of the assays is, therefore, essential (Kulmala *et al.*, 1998). In general, the presence of GAD65 autoantibodies in type 1 diabetic patients is stable. On the other hand, IA-2 autoantibodies tend to decrease with duration of disease, and the measurement of insulin autoantibodies is of no use after initiation of insulin therapy.

Initially, interest was focused on the presence of autoantibodies at the time of diagnosis and during follow-up of patients. However, it soon became apparent that these autoantibodies precede the development of diabetes by many months or years (Christie *et al.*, 1997). The presence of autoantibodies in otherwise healthy individuals can be used as a marker to identify those who are at high risk

of future development of type 1 diabetes (Mayrhofer *et al.*, 1996). Expression of two or more autoantibodies has a positive predictive value for type 1 diabetes greater than 90% among relatives of type 1 diabetics. A single autoantibody carries a lower risk of approximately 20% (reviewed by Devendra and Eisenbarth, 2003). Only about 1% of the general population have autoantibodies to GAD65, IA-2, or insulin. Thus the presence of multiple anti-islet autoantibodies will be just as predictive in the general population as it is in relatives.

1.9.4.3. Autoantibodies and latent autoimmune diabetes in adults

GAD65 and insulin autoantibodies are also found in 5 to 10% of patients classified with type 2 diabetes (Tuomi *et al.*, 1999; Pietropaolo *et al.*, 2000). Initially these patients do not require insulin treatment. However, the β cell mass in these patients will typically decline over time and necessitate insulin treatment (Groop *et al.*, 1986). This form of diabetes is often referred to as latent autoimmune diabetes in adults (LADA) (Tuomi *et al.*, 1993) or type 1.5 diabetes (Harris and Zimmet, 1992). A subgroup of LADA patients, with clinical characteristics similar to those of typical type 1 diabetes, have high titres of GAD65Ab and/or GAD65Ab directed towards C-terminal epitopes of GAD. This group of LADA patients are at high risk of becoming insulin dependent. A second group of LADA individuals are characterised by low titres of GAD65Ab, or the exclusive presence of GAD65Ab directed to the middle epitopes of the autoantigen. These patients are almost indistinguishable from GAD65Ab-negative type 2 diabetic patients (Falorni and Calcinaro, 2002).

1.9.4.4. Mapping epitopes for autoantibodies to the type 1 diabetes autoantigens GAD65, IA-2 and insulin

Autoantigenic epitope mapping represents a critical issue in autoimmune diseases. The identification of the whole spectrum of autoantibody epitopes should further the investigation of the role of these autoantigens in the pathogenesis of type 1 diabetes. While epitope mapping for GAD65Ab and IA-2Ab has been the focus of many studies using fusion proteins (Hampe *et al.*, 2000; Binder *et al.*, 2004) and monoclonal antibodies (Schlosser *et al.*, 1997; Padoa *et al.*, 2003), epitope mapping of IAA has been limited to naturally occurring insulin analogues. As a result, their epitopes are not well characterized (Castano *et al.*,

1993). Only recently phage display was used to define idiotypes of insulin antibodies (Devendra *et al.*, 2003; Devendra *et al.*, 2004).

Epitopes responsible for IA-2 autoantibody binding are mostly conformational but have only been partially defined (Farilla *et al.*, 2002). GAD65 autoantibodies present in the sera of newly diagnosed type 1 diabetic patients have been mapped to the middle and C-terminal regions of the GAD molecule. Human, bovine, and porcine insulin have been used to reveal differences in the binding characteristics of IAA and antibodies to exogenous administered insulin (IA). The major binding site of IA was located on the A chain, while type 1 diabetes-associated IAA recognized a conformational epitope requiring both the A and B chain (Diaz and Wilkin, 1987; Wilkin *et al.*, 1988).

Monoclonal antibodies and their Fab are being successfully used in the epitope analysis of other autoantibodies in type 1 diabetes (Schlosser *et al.*, 1997; Schwartz *et al.*, 1999; Padoa *et al.*, 2003). This method is particularly useful in the study of conformational epitopes since the structure of the antigen remains intact (Binder *et al.*, 2004).

1.9.5. Susceptibility

Type 1 diabetes is a heterogeneous and polygenic disorder. The onset of type 1 diabetes is attributed to both an inherited risk and external triggers, such as diet or an infection. The search for these risk factors is ongoing (Cox *et al.*, 2001).

1.9.5.1. Genetic susceptibility

The concordance for type 1 diabetes is approximately 50% for monozygotic twins (Kaprio *et al.*, 1992), and the risk to a first-degree relative is roughly 5%. The risk for offspring when the father has diabetes is about 12% compared to a 6% risk when the mother is diabetic (Warram *et al.*, 1984).

About 18 regions of the genome have been linked with influencing type 1 diabetes risk. These regions, each of which may contain several genes, have been labelled IDDM1 to IDDM18. IDDM1 (containing the HLA system) and IDDM2 (containing

the insulin gene) were both originally identified using case-control studies. The remaining type 1 diabetes susceptibility loci, IDDM3 to DM18, were mainly discovered by genome scan linkage studies (Davies *et al.*, 1994). The IDDM loci are found on several different chromosomes and contain many genes, many of which have now been identified (Figure 1.15).

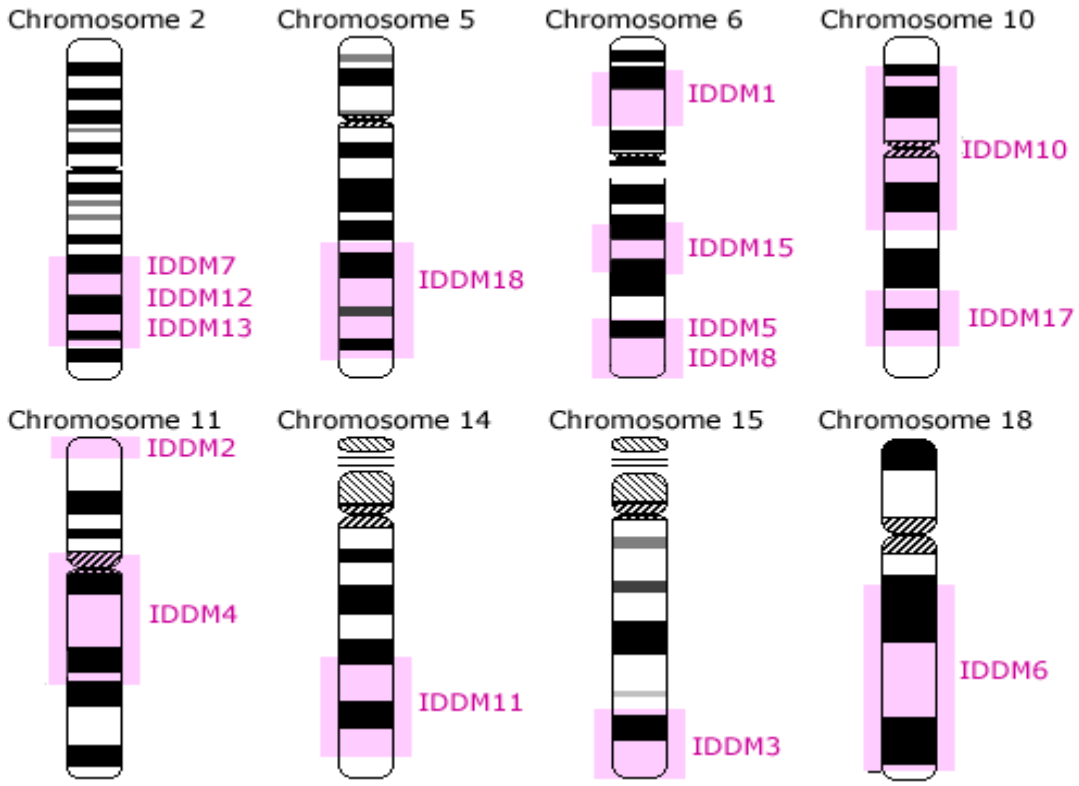


Figure 1.15: Location of the different type 1 diabetes susceptibility genes (http://www.ncbi.nlm.nih.gov/books/bookres.fcgi/diabetes/pdf_ch2.pdf).

The two major susceptibility regions that have been identified are IDDM1, the major histocompatibility complex (MHC) region on chromosome 6p21, and IDDM2, the insulin gene region on chromosome 11p15.5.

The best studied is IDDM1, which contains the HLA genes which code for HLA class II antigens; complex cell surface glycoproteins found on antigen-presenting cells. The IDDM1 locus contains many diabetes susceptibility genes (Table 1.7).

Table 1.7: Susceptibility loci for type 1 diabetes.

HLA-DR	DQA1	DQB1	DRB1	Susceptibility
DR2	0102	0602	1501	Protective
DR2	0102	0502	1601	Predisposing
DR2	0103	0601	1502	Neutral
DR3	0501	0201	0301	Predisposing
DR4	0301	0302	0401	Predisposing
DR4	0301	0302	0401	Predisposing
DR4	0301	0302	0402	Predisposing
DR4	0301	0302	0403	Neutral
DR4	0301	0302	0404	Neutral
DR4	0301	0302	0405	Neutral
DR4	0301	0301	0401	Neutral
DR4	0301	0301	0403	Neutral
DR7	0201	0303	0701	Positive
DR6	0101	0503	1401	Protective

Table reproduced from Devendra and Eisenbarth, 2003.

Fine mapping of this locus suggests that the two HLA haplotypes, DR3 and DR4, are strongly associated with type 1 diabetes susceptibility (Wolf *et al*., 1983; Herr *et al*., 2000). The vast majority (90%) of type 1 diabetics have either DR3,DQ2 (DQ2 = DQA1*0501, DQB1*0201) or DR4,DQ8 (DQ8 = DQA1*0301, DQB1*0302) haplotypes, whereas 40% to 50% of most white populations have one or the other of these haplotypes. Between 30% and 50% of patients with type 1 diabetes are heterozygotes with DR3,DQ2/DR4,DQ8 compared with only 2.4% of the general population. The DR3/DR4 haplotype is found more frequently (50%) before the age of 5 years in children with diabetes. This same haplotype is present in less than 30% of adults with type 1 diabetes (Rewers *et al*., 1996; reviewed by Devendra and Eisenbarth, 2003).

There are specific DR and DQ alleles which can protect from type 1 diabetes in an autosomal dominant manner (Platz *et al*., 1981; Redondo *et al*., 2000). One of the protective HLA haplotypes is DQA1*0102, DQB1*0602. Approximately 20% of the American and European populations have this haplotype, whereas these alleles

are found in less than 1% of children with type 1 diabetes (reviewed by Gottlieb and Eisenbarth, 1998; Redondo *et al.*, 2000). Once the diabetes disease process begins, however, the mechanism that protected these individuals from diabetes is lost, suggesting that inheriting these alleles does not prevent diabetes but may somehow delay or arrest the progression of the disease (Greenbaum *et al.*, 2000).

The IDDM2 locus accounts for about 10% of the susceptibility toward type 1 diabetes (Bennett *et al.*, 1995). This locus maps to a variable number of tandem repeat (VNTR) minisatellites upstream of the insulin gene (INS) (Bell *et al.*, 1981; Bell *et al.*, 1984; Owerbach and Gabbay, 1993; Pugliese *et al.*, 1997; Cox *et al.*, 2001). There are three classes of VNTRs in the insulin gene (Bennett and Todd, 1996):

- Class I has alleles that range from 26 to 63 repeat units.
- Class II has alleles that average around 80 repeat units.
- Class III has alleles ranging from 141 to 209 repeat units.

The class I VNTRs, ranging in size from 30 to 44 repeats, predominate (70%) in Caucasians. The remaining alleles tend to fall into the class III category. The intermediate lengths (class II) are rare. The short class I VNTR alleles predispose to type 1 diabetes, whereas the longer class III alleles have a dominant protective effect (Congia *et al.*, 1998). The presence of at least one class III allele is associated with a three-fold reduction in the risk of developing type 1 diabetes (Vafiadis *et al.*, 2001).

1.9.5.2. Environmental factors

Candidates for environmental components include viral infections (Rayfield, 1978), early introduction of cow's milk in infancy (Virtanen *et al.*, 1993), short duration of breast feeding (Virtanen *et al.*, 1992), or nitrites and nitrosamines in the diet (Dahlquist *et al.*, 1990, Kostraba *et al.*, 1992b). Conflicting reports, however, exist as to the role of these environmental agents in triggering type 1 diabetes.

1.9.6. Pathogenesis

Type 1 diabetes is caused by the progressive autoimmune destruction of the β cells. Despite numerous studies, the exact mechanism involved in the initiation and progression of β cell destruction remains unclear. Destruction of the β cells is believed to be triggered by the presentation of β cell-specific autoantigens by macrophages or dendritic cells (antigen-presenting cells) to naïve $CD4^+$ Th0 helper cells in conjunction with MHC class II molecules (Figure 1.16). Activated macrophages secrete interleukin (IL)-12 which stimulates $CD4^+$ Th0 cells to differentiate into Th1 cells, which in turn secrete interferon (IFN)-γ and IL-2. Resting macrophages are activated by IFN-γ and subsequently release other cytokines which are toxic to pancreatic β cells (e.g., IL-1β, tumour necrosis factor (TNF)-α, and free radicals). $CD8^+$ precytotoxic T cells migrate to the inflamed islets, stimulated by IL-2 and other cytokines. Upon recognition of β cell-specific autoantigen bound to MHC class I molecules, these $CD8^+$ precytotoxic T cells differentiate into cytotoxic effector T cells. These cells cause β-cell damage by releasing perforin and granzyme and by Fas-mediated apoptosis. Continued destruction of β cells eventually results in the onset of diabetes (reviewed by Kukreja and Maclaren, 1999).

Natural killer T cells can prevent β cell destruction by secreting IL-4 at the beginning stages of the differentiation of Th0 cells. Thus the Th2 type response is favoured and Th1 cells are down regulated. β cell destruction and diabetes are, therefore, prevented by the secretion of IL-4 and IL-10 by $CD4^+$ Th2 type cells and reduced secretion of IL-2 and IFN-γ (reviewed by Kukreja and Maclaren, 1999; Homann and von Herrath, 2004).

The role of β cell autoantibodies in the pathogenesis of type 1 diabetes is not understood. While in the past the autoantibodies have been perceived as a by-product of the autoimmune destruction of the β cells, recent studies indicate that GAD65Ab may be involved in antigen presentation and thus may contribute to the pathogenesis of the disease (Reijonen *et al.*, 2000; Banga *et al.*, 2004).

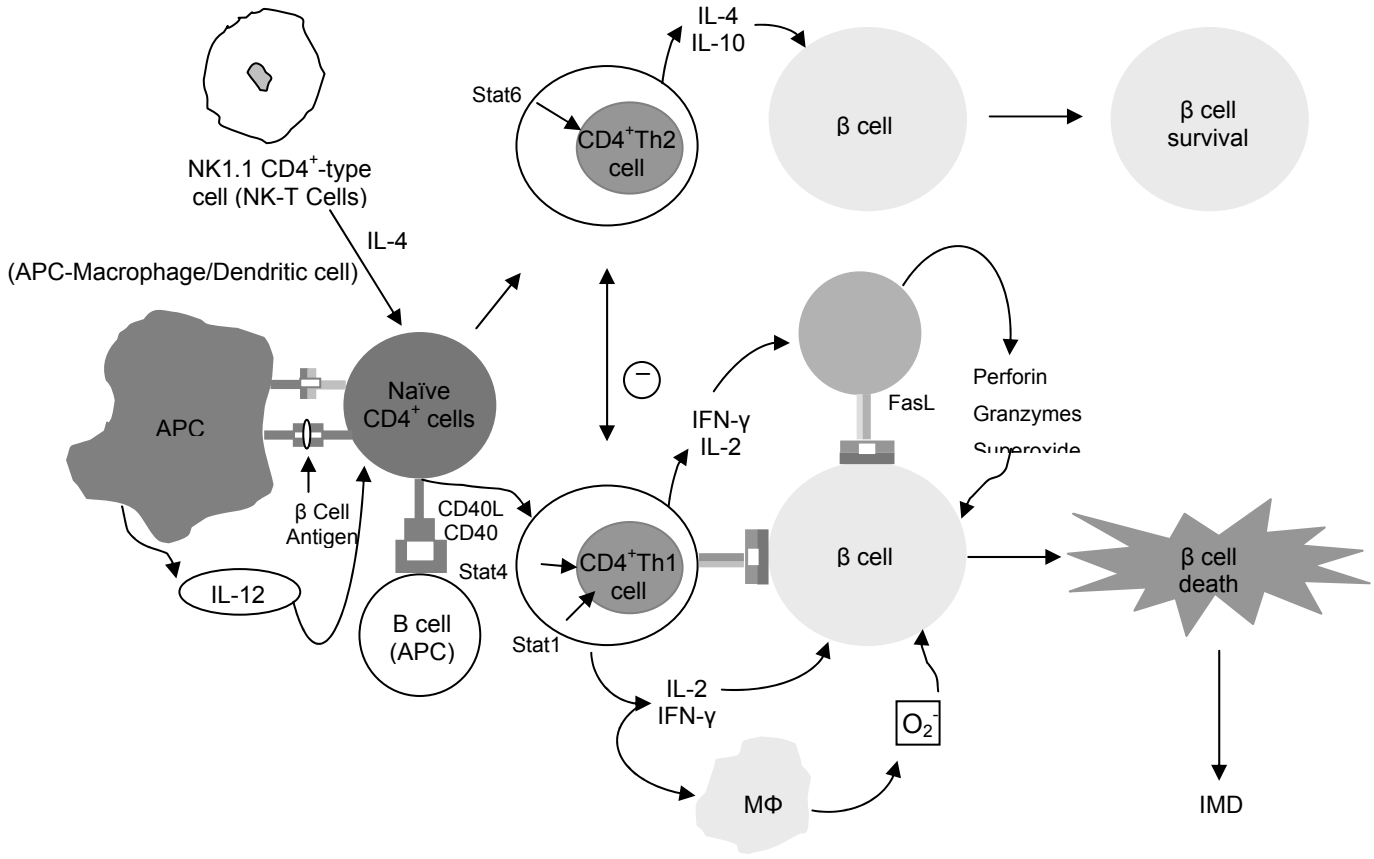


Figure 1.16: A schematic description of the roles of various subsets of T cells and their cytokines in β cell destruction, leading to diabetes (adapted from Kukreja and Maclaren, 1999).

1.9.7. Prediction of type 1 diabetes

Many reasons prevent the recommendation to test individuals routinely for the presence of any of the immune markers outside of a clinical trials setting. First, cut-off values for some of the assays for immune markers have not been completely established for clinical settings. Second, the cost effectiveness of such screening is questionable, at least until an effective therapy is available. Lastly, there is no consensus yet as to what action should be taken when a positive autoantibody test is obtained as there are no proven measures as to how the disease may be delayed or prevented. Animal models of type 1 diabetes have been invaluable for developing prevention methods.

1.9.8. Treatment of type 1 diabetes

Insulin remains the essential therapy for type 1 diabetes. Therapy has been improved by the introduction of long acting human insulin analogues and insulin pumps. Despite such advances, once most β cells have been destroyed, diabetes management is difficult, and there are multiple acute and chronic complications.

Pancreatic transplantation, and recently islet transplantation, are possible options for selected type 1 diabetes patients with severe complications such as recurrent problematic hypoglycaemia. Both can result in reversal of hyperglycaemia and prevent life-threatening hypoglycaemia. Long-term follow-up of islet transplants is lacking, but recent results with the Edmonton protocol (Lakey *et al.*, 1997) indicate that with immunosuppression, significant islet function can be achieved for one year in more than 80% of patients (Ryan *et al.*, 2002).

A new molecule that may be used in the near future as a treatment for type 1 and type 2 diabetes was recently described (Fukuhara *et al.*, 2005). This 52 kDa protein is a cytokine which is secreted by bone marrow, muscle, liver, and adipocytes. This protein has been previously described as a pre-B cell colony-enhancing factor (PBEF) (Samal *et al.*, 1994). However, as it is found abundantly in visceral fat, Fukuhara *et al.* (2005) have renamed this molecule visfatin. Examination of visfatin's biological and physiological functions revealed that it lowers plasma glucose levels in mice. The effects seen with visfatin were similar to those observed when insulin was used. Visfatin mimicked the effects of insulin in all biochemical pathways/reactions examined. Visfatin also binds to the insulin receptor at a position other than the alpha subunit. Thus, visfatin can activate the insulin receptor independently of insulin. These findings suggest that visfatin may provide a useful alternative therapy for type 1 diabetes (Fukuhara *et al.*, 2005).

1.9.9. Animal models of diabetes

The utilisation of animal models has made decisive contributions to the study of diabetes and related fields. Much of our current knowledge concerning the aetiology, pathogenesis, treatment, and prevention of human diabetes would

never have been obtained without the study of animal models of diabetes. These can be categorised as animals with spontaneously developing diabetes (BB rats, NOD mice) and as animals with induced diabetes (e.g. by virus). None of the models are perfect, but each has contributed to our present knowledge of the disease in some way.

The NOD mouse and BB rat develop diabetes at age 80 to 200, and 60 to 120 days, respectively. In the NOD mouse, the incidence of diabetes in the male is only 10% to 50%. The onset of diabetes is characterised by the appearance of the classic symptoms observed in humans: hypoinsulinaemia, hyperglycaemia, glycosuria, ketonuria, and polyuria. Both models develop insulitis before the clinical onset of the disease. In addition, BB rats develop lymphocytopenia (Poussier *et al.*, 1983). In the BB rat, islet cell antibodies can be detected up to two weeks before the onset of the disease (Dyrberg *et al.*, 1984). NOD mice also display islet cell antibodies as well as islet cell surface antibodies with a peak after age 6 months. Insulin autoantibodies are present in about 80% of the mice after 3 months of age (Reddy *et al.*, 1988). GAD antibodies and GAD-reactive T-cells have been reported in BB rats, as well as in NOD mice.

Insulin, GAD65 and the heat shock protein 65 are just a few of the autoantigens believed to initiate β cell destruction (reviewed by Roep, 1996). Of these, GAD65 and insulin have been studied the most, both in animal models and man. Prophylactic treatment with these β cell antigens has been used to specifically modify T-cell autoimmunity and diabetes development in experimental models of type 1 diabetes.

Administration of monoclonal antibodies to GAD65 has been reported to inhibit the progression of type 1 diabetes in the NOD mouse (Menard *et al.*, 1999). The authors hypothesise that the T cells do not recognise GAD65 as it is bound by the mAb and thus initiation of β cell destruction is inhibited. The administration of insulin by subcutaneous injection (Atkinson *et al.*, 1990) or oral administration (Zhang *et al.*, 1991) has similarly been shown to prevent diabetes development in the NOD mouse and the diabetes-prone BB (DP-BB) rat (Gotfredsen *et al.*, 1985).

Using intramuscular DNA vaccination for antigen administration, Karges *et al.* (2002) have shown that preproinsulin (ppIns) can adversely trigger autoimmune diabetes in the NOD and RIP-B7.1 mouse models of type 1 diabetes. Diabetes development was enhanced after ppIns DNA treatment in female NOD mice, and natural diabetes resistance in male NOD mice was diminished. In RIP-B7.1 mice, diabetes occurred in 70% of animals after ppIns vaccination. Following GAD65 treatment, however, spontaneous development of diabetes was not observed. Diabetes was characterised by diffuse CD4+ and CD8+ T-cell infiltration of pancreatic islets and severe insulin deficiency. ppIns, proinsulin, and insulin DNA were equally effective at inducing the disease (Karges *et al.*, 2002).

Based on the results found for the animal models these experiments were extrapolated to humans. A very large trial, the Diabetes Prevention Trail 1, was carried out using oral insulin as a therapeutic agent in relatives of type 1 diabetic patients. The administration of oral insulin to these subjects did not delay or prevent type 1 diabetes (Pozzilli, 2002).

1.10. TYPE 2 DIABETES MELLITUS

Type 2 diabetes (previously known as Non-Insulin Dependent Diabetes Mellitus [NIDDM], type II diabetes, or adult-onset diabetes) is characterized by insulin resistance in peripheral tissue and an insulin secretory defect of the β cells (Reaven *et al*., 1976; Turner *et al*, 1979). Insulin resistance and hyperinsulinaemia eventually lead to impaired glucose tolerance. Defective β cells become exhausted, further fuelling the cycle of glucose intolerance and hyperglycaemia.

This is the most common form of diabetes mellitus, accounting for approximately 90% of all diabetic people. Type 2 diabetes tends to develop very slowly over a period of years and may go unnoticed. If symptoms are present they are usually relatively mild. Nevertheless, such patients are at increased risk of developing macrovascular and microvascular complications. The majority of type 2 patients are obese, which itself causes some degree of insulin resistance. Many of those who are not obese by traditional weight criteria may have an increased percentage

of body fat distributed predominantly in the abdominal region (Coon *et al*., 1992; Kohrt *et al*., 1993).

Type 2 diabetes mellitus is multifactorial disease. It is often associated with a strong genetic predisposition; however, the genetics of this form of diabetes are complex and not clearly defined. Little is known about specific diabetogenic genes, other than the fact that the concordance rate among monozygotic twins is high (33 to 76%; 5 to 15 year follow up) (Medici *et al*., 1999). There is also a convincing behavioural component.

1.10.1. The epidemiology of type 2 diabetes

The prevalence of type 2 diabetes (between 5 and 7%) is now at epidemic proportions and the number of new cases being diagnosed continues to rise. In 1990, 4.9% of the American population were diagnosed with diabetes. This increased to 7.9% by the year 2001 (Mokdad *et al.*, 2001). It has been estimated that of the children born in the year 2000, a third will suffer from diabetes at some point in their lifetime (Narayan *et al.*, 2003). Diabetes is predicted to become one of the most common diseases in the world within a couple of decades, affecting at least half a billion people (King *et al.*, 1998).

In the past, type 2 was rarely seen in young individuals, hence its original name of ‘adult-onset diabetes’. Today, however, type 2 diabetes is increasingly being diagnosed in young adults and even in children. This young generation of diabetics will have many decades in which to develop the complications of the disease. The driving force behind the high prevalence of diabetes is the rise of obesity in the population. In today's society, the combination of ample food and a sedentary lifestyle, make it difficult to maintain a healthy weight.

1.10.2. Risk Factors for type 2 Diabetes

In their revised criteria the ADA (2005a) suggested that adults aged 45 years and older be tested for diabetes. If their blood glucose is normal at the first test, they should be tested at 3 year intervals. Individuals younger than 45 years of age

should be tested if they are at high risk for diabetes. These high-risk factors include:

- Obesity, being more than 20% above ideal body weight or having a body mass index ≥ 27 kg/m^2. Insulin resistance increases with body weight.
- Having a first degree relative with diabetes.
- Race, a number of populations are at increased risk of type 2 diabetes, including Hispanics, Native Americans, people of Asian or African-Caribbean descent, people of Aboriginal descent and Pacific Islander groups.
- Age, nearly all people diagnosed with type 2 diabetes are over 30 years old. Half of all new cases are aged 55 and older.
- High-fat diet - a key cause of obesity.
- Sedentary lifestyle. Insulin resistance increases with lack of exercise.
- Women who have had gestational diabetes. Previous gestational diabetes increases risk of type 2 diabetes developing later on in life.
- Giving birth to a baby weighing more than 4 kg.
- Having blood pressure at or above 140/90 mmHg.
- Having abnormal blood lipid levels, such as high density lipoprotein cholesterol less than or equal to 35 mg/dL (0.90 mmol/L) or triglycerides greater than or equal to 250 mg/dL (2.82 mmol/L).
- Having abnormal glucose tolerance when previously tested for diabetes.

As many as 2.7% of the general adult population have undiagnosed type 2 diabetes (Harris *et al.*, 1987). Undiagnosed diabetes is a serious condition as there is epidemiological evidence that complications arise many years before the clinical diagnosis of type 2 diabetes is made (Harris, 1993). These individuals are at significantly increased risk for coronary heart disease, stroke, and peripheral vascular disease. In addition, they have a greater likelihood of having dyslipidaemia, hypertension, and obesity (Klein, 1995).

Thus, early detection and consequently early treatment might well reduce the burden of type 2 diabetes and its complications. Although the relatively low prevalence of diabetes in the general population makes it unlikely that mass screening will be cost effective, testing for diabetes in people with risk factors for

type 2 diabetes or with diabetes-associated conditions is likely to result in more benefit than harm and will lead to overall cost savings.

1.10.3. Prevention strategies for type 2 diabetes

Primary approaches to preventing diabetes in a population include programmes targeting high-risk individuals in the community (such as those with IGT or obesity), high-risk ethnic groups and programmes designed to promote physical activity and healthy eating in adults or children (Simmons *et al.*, 1997; Daniel *et al.*, 1999).

1.10.4. Treatment of type 2 diabetes

The first line of attack for people who have diabetes and are overweight is usually dietary control and a weight loss programme. Physical activity and weight loss makes the body's cells more responsive to insulin. Most people are therefore treated by diet and exercise when newly diagnosed. Dietary modification that targeted a low calorie diet with reduced fat intake and moderate intensity physical activity of at least 150 minutes/week resulted in the risk for diabetes being reduced by 58% at 4 years (Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002). If the diet and exercise approach is unsuccessful then there are a number of oral medications that can be used to help treat the hyperglycaemia in type 2 diabetes; but it is seldom restored to normal. These either increase the production of insulin or help the body to use the insulin more effectively. A study using metformin significantly decreased progression to diabetes by 31% (Knowler *et al.*, 2002; The Diabetes Prevention Program Research Group, 2003). Similarly, a study using acarbose, reduced the risk of progression to type 2 diabetes by 30% (Chiasson *et al.*, 2002). The right combination of diet, exercise, and adequate medication when needed is the key to effective management of type 2 diabetes.

1.11. AIMS OF THE STUDY

Autoantibodies to GAD65 are accepted markers for type 1 diabetes and, together with IAA and IA-2, predict the disease. IAA are often the first autoantibodies detected in type 1 diabetics and can be present before the onset of clinical diabetes. These autoantibodies and their epitopes are however not well characterized. We explored the use of monoclonal antibodies and their rFab as reagents for epitope analysis. The reason for generating rFabs is that their smaller size, in comparison to the whole mAb, prevents steric hinderance. The study of antibody epitopes is, therefore, enhanced by the use of rFabs as opposed to mAbs.

The identification of type 1 diabetes-associated insulin and GAD65 autoantibody epitopes is important as this will provide us with a better understanding of the pathogenic involvement of these autoantibodies in the disease progression and also, how best to block insulin and GAD65 autoantibody binding as a possible treatment method. Approximately 17 million people worldwide have type 1 diabetes and this number is increasing. Type 1 diabetes is a serious, debilitating disease with life threatening complications. The financial burden associated with the management of this disease is enormous. Early diagnosis and intervention can, therefore, improve the morbidity and mortality.

i. To produce a gene construct containing the light chain and Fd heavy chain fragment of insulin and GAD65 binding monoclonal antibodies (mAbs).
ii. To clone the constructs into an expression vector.
iii. To test the expression products for their ability to bind insulin and GAD65.
iv. Map the epitope to which the Fab binds to on insulin by 3D computer modelling and competition assays with anti-insulin mAbs that have defined epitopes.
v. To analyse insulin and GAD epitopes recognized by autoantibodies present in the sera of newly diagnosed type 1 and type 1.5 diabetic patients.

CHAPTER 2

2. SUBJECTS AND METHODS

All reagents, suppliers, and solutions used are listed in Appendix 1.

2.1. SUBJECTS

2.1.1. Subjects used in the study of insulin rFabs

Newly diagnosed IAA-positive type 1 diabetes patients (n=16) (median age: 9 years, range: 1 - 15 years; 10 female) were part of a study conducted at the St. Görans Children Hospital, Stockholm, Sweden. The serum samples were obtained at the clinical diagnosis of diabetes. The second set of newly diagnosed type 1 diabetes patients (n=21) (median age: 22 years, range: 15 - 34 years, 5 female) used in this study were registered in 1992 to 1993 in the Diabetes Incidence Study in Sweden (DISS) and were previously identified to be IAA-positive.

Newly diagnosed type 1.5 diabetes patients (LADA) (n= 14) (median age: 42 years, range: 24 - 61 years, 6 female) were part of a screening programme in the greater Seattle area. The patients were classified with type 2 diabetes according to the 1997 American Diabetes Association criteria and were previously identified to be IAA-positive. The majority of patients (86%; 12/14) were GAD65Ab positive. All patients had been diagnosed with diabetes within 12 months of blood sampling. Characteristics of type 1.5 diabetes include:

- Adult age at diagnosis (usually over 25 years of age).
- Initial presentation masquerades as non-obese type 2 diabetes (insulin resistant; does not present as diabetic ketoacidosis).
- Initially can be controlled with diet with or without medication (not insulin).
- Insulin dependency gradually occurs, frequently within months.
- Autoantibody positivity, especially to GAD65.
- Low C-peptide levels.

- Unlikely to have a family history of type 2 diabetes (Pozzilli and Di Mario, 2001; Schernthaner *et al*., 2001).

None of the patients had been on insulin therapy prior to sampling.

2.1.1. Subjects used in the study of GAD65 rFabs

Newly diagnosed GAD65Ab-positive type 1 diabetes patients (n=61) (mean age: 10 years, range: 0 - 16 years; 33 female) were part of a study conducted at the St. Görans Children Hospital, Sweden and represented 80% of all children diagnosed in Stockholm during 1993 - 1995. The serum samples of all diabetes patients were obtained at the clinical diagnosis of diabetes.

Healthy GAD65Ab-positive first-degree relatives of type 1 diabetes patients (n=38) (mean age: 46 years, range: 8 - 74 years) were identified by screening first degree relatives in families identified in the Diabetes Incidence Study in Sweden (DISS) and the Swedish Childhood Diabetes registry. None of the GAD65Ab-positive first-degree relatives developed diabetes within 2 to 12 years of blood sampling.

Type 1.5 diabetes patients (n=44) (mean age: 51 years, range: 31 - 77 years, 16 female) were part of a screening programme in the greater Seattle area. The patients were initially classified with type 2 diabetes according to the 1997 ADA criteria and were previously identified to be GAD65Ab-positive (Hampe *et al*., 2002). All patients had been diagnosed with diabetes within 12 months of blood sampling.

Healthy individuals (n=14) (mean age: 50 years, range: 30 - 60 years, 9 female) with a GAD65Ab index above the cut-off index (0.05, using the 98^{th} percentile) were detected in a population-based screen of 2157 Swedish adults (Rolandsson *et al*., 1999). None of the GAD65Ab-positive healthy individuals developed type 1 diabetes within eight years of sample collection (Rolandsson *et al*., 2001).

All subjects in this study, their parents or legal guardians, gave informed consent. Local institutional ethics committee approval was obtained prior to collection of all serum samples.

2.2. HYBRIDOMAS

Four hybridoma cell lines that secreted anti-insulin monoclonal antibodies were used in this study. Three of these (HB-123, HB-126, HB-127) were purchased from the American Type Culture Collection (ATCC). A single article (Schroer *et al*., 1993), in which epitope mapping had been performed, was cited when a literature search was performed for insulin-specific hybridomas. From the list of 18 mAbs in this publication, six were available from the ATCC. We chose three (listed above) for our study. The fourth hybridoma cell line, termed IE2, was a kind gift from Nick Hales, Department of Clinical Biochemistry at the University of Cambridge, United Kingdom. These four monoclonal antibodies belonged to the IgG_1 heavy chain group and the kappa light chain group. The fifth mAb used in this study, which binds to GAD65, was isolated from a GAD65 autoantibody positive type 1 diabetes patient using combined EBV transformation with CD40 activation of peripheral B cells (Madec *et al.*, 1996). This monoclonal antibody was termed DP-D and binds to a conformational epitope located at the N-terminus of GAD65 (Schwartz *et al*., 1999). Monoclonal antibody N-GAD65 was raised to a peptide representing amino acid residues 4 to 22 of GAD65, and was used as a negative control (Hampe *et al.*, 2001)

The three ATCC hybridoma cell lines were produced by the fusion of BALB/c 12 – 14 day primary immune lymph node cells with Sp2/0-Ag14 myeloma cells (Schroer *et al*., 1993) whereas the 1E2 hybridoma was produced by the fusion of murine spleen cells with NS0 myeloma cells (Crowther *et al*., 1994).

Two mAbs were used for epitope analysis: mAb 125 (Schroer *et al.*, 1983) which recognizes an epitope located at the A chain loop (residues A8 - A10) of insulin, and mAb1 (BIØDESIGN, Saco, ME) which binds to the B chain with special dependency on amino acid B30.

2.2.1. Growing hybridomas

2.2.1.1. Tissue culture

The hybridoma cell lines were shipped frozen in 1 ml aliquots. The cells were thawed in a 37°C water bath. When almost thawed, the tube was wiped with 70% ethanol, and taken to the tissue culture hood. The cells were transferred to a 15 ml Nunc tube containing 10 ml Dulbecco's modified Eagle's (DME) medium with 4.5 g/l glucose which had been supplemented with fetal calf serum (FCS) to a final concentration of 10%.

The cells were centrifuged for 5 minutes at 140 x g and the supernatant carefully decanted so as not to dislodge the pellet. The cell pellet was resuspended in 2 ml of DMEM and transferred to a single well of a 6-well plate. Cells were cultured at 37°C in 5% atmospheric CO_2. Fresh medium was added to the wells two days later. A further two days later, depending on the growth of the cells, the cells were transferred to a 50 ml flask and grown in 5 ml of medium until confluent.

2.2.1.2. Subculturing hybridomas

The average doubling time of hybridomas usually depends on the myeloma fusion partner and will be between 12 and 24 hours. Therefore, most hybridomas will need to be subcultured every 2 to 4 days, depending on the dilution used in the previous passage. Fresh medium was added to the hybridomas when the concentration of cells reached approximately 10^6 cells/ml. Hybridomas do not secrete any noticeably toxic by-products, so the medium was replenished by diluting (1:10 or 1:20) growing cultures with fresh medium. Hybridoma cell lines that adhered lightly to the plastic surface were loosened by washing the plastic with a strong stream of medium from a pipette or by gently tapping the base of the flask. The flasks were examined under the microscope to ensure that all the cells had been removed. Once cells were confluent, mRNA was extracted.

2.2.1.3. Hybridoma cell line monoclonal antibody secreting test

PEG precipitation (Simionescu *et al*., 1979) of monoclonal antibodies was performed to ensure that the cells were secreting the desired antibody.

Tissue culture supernatants of confluent hybridoma cell lines were collected and tested for the presence of the desired antibody. Debris was removed from the supernatant by centrifugation at 1 000 x g for 10 minutes. For the detection of insulin mAbs, 100 µl of ^{125}I-labelled insulin (specific activity: > 15 Ci/mg Iodide; 20 000 cpm/10 µl) was incubated with either:

i. 100 µl of supernatant from hybridomas
ii. 100 µl of supernatant from hybridomas that has been diluted 10-fold
iii. 100 µl of unused DMEM (this serves as a negative control).

Samples, set up in duplicate, were incubated for at least 2 hours at room temperature followed by the addition of 1 ml of 25% PEG solution to precipitate the antibody-antigen complexes. Samples were spun for 10 minutes at 2 000 x g, the supernatant removed and the pellet counted on a γ-counter (Innotron hydragamma 16). The higher the counts obtained, the higher the concentration of anti-insulin antibodies present in the medium (Table 2.1).

Table 2.1: The relationship between number of counts and monoclonal antibody concentration.

CELL LINE SUPERNATANT	COUNTS (10 seconds)
DMEM	
- unused medium; negative control	1 154
NS0:	
- undiluted	1 105
- 10x dilution	1 043
1E2:	
- undiluted	20 369
- 10x dilution	17 153
- 160x dilution	10 835

2.2.2. Long term storage of cell lines

Hybridoma cell lines were stored by freezing cells in an appropriate solution of nutrients and a cryoprotectant such as dimethylsulfoxide (DMSO).

2.2.2.1. Freezing cells for liquid nitrogen storage

Rapidly dividing cells were gently loosened from the surface of the flask, transferred to a sterile 15 ml Nunc tube and spun at 400 x g for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 10% DMSO/90% FCS. The final cell concentration was approximately 5 x 10^6 - 5 x 10^7 cells/ml. The cell suspension was aliquoted into cryogen tubes (1 ml per tube). Vials were placed in a freezing rack at -70°C overnight. It is important to work as quickly as possible as prolonged exposure to DMSO at these concentrations is toxic to the cells. The following day the vials were transferred to LN_2. Cells stored in LN_2 should be checked for viability once a year. If the viability has dropped significantly (< 50%), the cells should be expanded, checked for continued antibody production, and refrozen.

2.2.2.2. Viability checks

To determine the percentage of viable cells within a population, the cell suspension is mixed with a vital dye and observed under the microscope. Vital dyes are excluded from living cells but stain dead cells. The most common dye used for these stains is trypan blue.

To check cell viability, the cell suspension was mixed with 0.25% (w/v) solution of trypan blue (1:1). To obtain a general impression of the viability of the cells, a drop was placed on a slide and observed under the microscope. For an exact percentage, the mixture can be transferred to a Neubauer counting chamber and the number of viable cells present in a given area counted. The formula below is used to calculate the number of viable cells present per millilitre of supernatant.

$$\frac{\text{Total number of viable cells}}{\text{Number of squares counted}} \times 2 \times 10^4 = \text{number of viable cells/ml}$$

2.2.2.3. Counting hybridoma cells

For most purposes the number of hybridoma cells can be estimated simply by observing the cells under the microscope. When an exact cell count is needed, the number can be determined using a Neubauer haemocytometer. This is a

simple device in which a special coverslip rests on supports that hold it 0.1 mm above the base of the slide. The slide is engraved with a series of lines that form a 1 x 1 mm square. By counting the number of cells within the 0.1 mm^3 chamber formed by the 1 x 1 mm square and the height of the coverslip, an accurate quantitation of cells per millilitre can be calculated (see Figure 2.1).

$$\frac{\text{Total number of cells}}{\text{Number of squares counted}} \times 10^4 = \text{number of cells/ml}$$

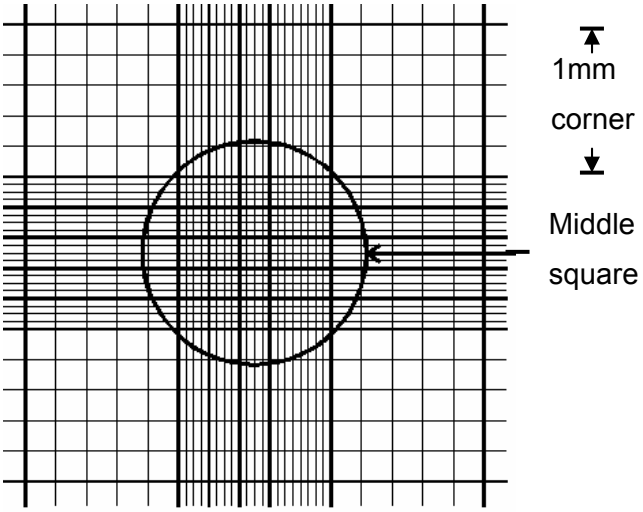


Figure 2.1: Diagrammatic representation of a Neubauer haemocytometer.

2.2.3. Single-cell cloning by limiting dilution

Cloning hybridoma cells by limiting dilution is the easiest of the single-cell cloning techniques. Even though every attempt is made to ensure that the cells are in a single-cell suspension prior to plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, limiting dilution cloning should be done at least twice to generate a clonal population.

Medium (100 µl) containing 20% FCS was aliquoted into each well of a 96-well plate. An aliquot (100 µl) of the rapidly growing hybridoma cell suspension was transferred to the top left-hand well and mixed. Dilutions (1 in 2) were performed down the left-hand row of the plate (8 wells, 7 dilution steps) followed by 1 in 2

doubling dilutions across the plate. Clones were visible by microscopy after a few days and were ready to screen after 7 to 10 days. The wells were scored by microscopy. There was a line running on a 45° diagonal that contained approximately the same number of clones per well. Wells with only one or two clones were screened. The best wells were selected and the clones grown up.

2.3. RNA ISOLATION

To protect from RNase contamination, nondisposable glassware or plasticware must be RNase-free. Glassware can be washed with DEPC-water and then autoclaved for 20 minutes. Plastic items can be soaked for 10 minutes in 0.5 M NaOH, rinsed thoroughly with water, and autoclaved. Wipe down all work surfaces, pipettes, and racks with RNase Zap (Qiagen).

2.3.1. Total RNA extraction

Total RNA was extracted from confluent cells using RNeasy Kits (Qiagen) based on the absorption of total RNA to a silica gel membrane.

2.3.1.1. The RNeasy principle and procedure

A specialised high-salt buffer system allows up to 100 µg of RNA, longer than 200 bases, to bind to the RNeasy silica gel membrane. Biological samples are first lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini column where the total RNA binds to the membrane and contaminants are effectively washed away. High-quality RNA is eluted in water.

2.3.1.2. The total RNA isolation protocol

Hybridoma cells (≤ 1 x 10^7 cells) were centrifuged for 5 minutes at 300 x g in a 15 ml Nunc tube. All centrifugation steps are performed at 20 to 25°C in a standard microcentrifuge. The supernatant was discarded and the cell pellet lysed by the addition of the appropriate volume of buffer RLT (Table 2.2).

Table 2.2: Volume of buffer RLT required for cell lysis.

Number of pelleted cells	Buffer RLT (µl)
$< 5 \times 10^6$	350
5×10^6 - 1×10^7	600

The sample was homogenised by passing it through a 20-gauge needle (0.9 mm diameter) at least 5 times. One volume of 70% ethanol was added to the homogenised lysate and mixed. A maximum of 700 µl of the sample was applied to an RNeasy mini column placed in a 2 ml collection tube. Samples were centrifuged for 15s at ≥ 8 000 x g and the flow-through discarded. If the volume exceeded 700 µl, aliquots were loaded successively onto the RNeasy column and centrifuged as above. The column was washed by the addition of 700 µl buffer RW1 and centrifuged for 15s at ≥ 8 000 x g. The column was transferred to a new 2 ml collection tube and 500 µl buffer RPE pipetted onto the column. Samples were centrifuged for 15s at ≥ 8 000 x g and the flow-through discarded. A further 500 µl buffer RPE was added to the column followed by a 2 minute spin at ≥ 8 000 x g to dry the RNase silica gel membrane. To eliminate any chance of possible buffer RPE carryover, columns were placed into a new 2 ml collection tube and centrifuged at full speed for 1 minute. Total RNA was eluted in 30 to 50 µl RNase-free water by centrifuging the column for 1 minute at ≥ 8 000 x g. To obtain a higher total RNA concentration, the second elution step was performed using the first eluate.

2.3.1.3. Determining the total RNA concentration

The concentration of the eluted RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. An absorbance of one unit at 260 nm corresponds to 40 µg of RNA per ml (Sambrook *et al.*, 1989). Once the total RNA concentration was calculated, the samples were used immediately for mRNA extraction.

2.3.2. mRNA extraction

2.3.2.1. The Oligotex principle

The Oligotex procedure for isolation, purification, and manipulation of poly A^+ RNA takes advantage of the fact that most eukaryotic mRNAs end in a poly-A tail of 20 to 250 adenosine nucleotides. In contrast, rRNAs and tRNAs, which account for over 95% of cellular RNAs, are not polyadenylated. The poly-A tail thus provides a useful tool for separation and selective isolation of eukaryotic mRNAs. Poly A^+ mRNA can be purified by hybridising the poly-A tail to a dT oligomer coupled to a solid-phase matrix. rRNA and tRNA species do not bind to the oligo-dT column and are washed away. Since hybridisation requires high-salt conditions, the poly A^+ mRNA can be released by lowering the ionic strength and destabilising the dT:A hybrids.

2.3.2.2. The Oligotex mRNA spin-column protocol

Isolation of mRNA was carried out using Oligotex mRNA spin-columns (Qiagen) according to the manufacturer's instructions. Unless otherwise indicated, all steps, including centrifugation, were performed at room temperature (20 to 30°C). All centrifugation steps were performed in a microcentrifuge at maximum speed (14 000 to 18 000 x g).

The volume of total RNA was adjusted with RNase-free water according to its starting concentration (Table 2.3).

Table 2.3: Buffer amounts for Oligotex mRNA spin-column protocol.

Total RNA concentration	RNase-free water (µl)	Buffer OBB (µl)	Oligotex suspension (µl)
≤ 0.25 mg	250	250	15
0.25 - 0.50 mg	500	500	30
0.50 - 0.75 mg	500	500	45
0.75 - 1.00 mg	500	500	55

The appropriate volume of buffer OBB (Binding buffer) and Oligotex suspension (37°C) (Table 2.3) was added and the samples incubated for 3 minutes in a 70°C

heating block to disrupt the secondary structure of the RNA. Hybridisation between the oligo dT_{30} of the Oligotex particle and the poly-A tail of the mRNA occurred in the subsequent 10 minute incubation at room temperature. The Oligotex:mRNA complex was pelleted by centrifugation for 2 minutes at maximum speed. The pellet was resuspended in 400 µl buffer OW2 (wash buffer) and transferred onto a spin column. Columns were spun for a minute, a further 400 µl buffer OW2 applied and the columns centrifuged for 1 minute. Hot (70°C) buffer OEB (elution buffer) (20 to 100 µl) was pipetted onto the column and the resin resuspended by pipetting up and down three or four times. Columns were centrifuged for 1 minute. To keep the elution volume low, the first eluate (heated to 70°C) was used for the second elution.

The isolated mRNA samples were analysed on a 1% agarose gel and visualised on a transilluminator under UV light. The mRNA samples were subsequently stored at -70°C or used immediately to synthesise cDNA for construction of the antibody fragments.

2.4. CONSTRUCTION OF A scFv FRAGMENT

The construction of a scFv polypeptide involves the amplification of the heavy and light chain variable regions (V_H and V_L) of an antibody. The carboxyl terminus of the V_L is then linked by a 15 amino acid peptide $(Gly_4Ser)_3$ to the amino terminus of the V_H fragment, or vice versa.

2.4.1. The polymerase chain reaction

In this study, PCR reactions were carried out in the Perkin Elmer Gene Amp 2400 PCR System and the PTC-200 DNA Engine thermal cycler.

2.4.1.1. cDNA synthesis

mRNA was extracted from the 1E2 anti-insulin secreting hybridoma cell line and from the NS0 cell line. NS0 was used as the fusion partner to produce the 1E2 hybridoma. First strand cDNA synthesis was carried out in a 50 µl reaction mixture containing 5 µg mRNA, 2 x Reverse Transcriptase (RT) buffer, 0.5 mM

dNTP, 0.4 pmol VH1FOR-2 (heavy chain primer) or an equimolar mix of VKFOR1, 2, 3, and 4 (light chain primers) (Orlandi *et al.*, 1989; Clackson *et al.*, 1991; Table 2.4). Samples were incubated for 5 minutes at 67°C and cooled for 15 minutes at room temperature. RNase inhibitor (40 U/µl) and Avian Myoblastosis Virus (AMV) RT (15 U/µl) were added and the reaction allowed to continue for 1 hour at 42°C. The reaction was terminated with a 3 minute incubation at 100°C. Samples were immediately quenched on ice and stored at -20°C until needed.

Table 2.4: Primers used for amplification of the V_H and V_L domains of the anti-insulin monoclonal antibody 1E2.

NAME	SEQUENCE (5' to 3')	LENGTH (bp)
HEAVY CHAIN PRIMERS:		
VH1BACK	AGGTSMARCTGCAGSAGTCWGG	22
VH1FOR-2	TGAGGAGACGGTGACCGTGGTCCC	24
LIGHT CHAIN PRIMERS:		
VK2BACK	GACATTGAGCTCACCCAGTCTCCA	24
VKFOR1	CCGTTTGATTTCCAGCTTGGTGCC	24
VKFOR2	CCGTTTTATTTCCAGCTTGGTCCC	24
VKFOR3	CCGTTTTATTTCCAACTTTGTCCC	24
VKFOR4	CCGTTTCAGCTCCAGCTTGGTCCC	24

S = C or G, M = A or C, R = A or G, and W = A or T

2.4.1.2. Amplification of the V_H and V_L domains

PCR was performed for both immunoglobulin heavy and light chain variable region genes using cDNA as a template and oligonucleotide primers listed in Table 2.4 (Orlandi *et al.*, 1989; Clackson *et al.*, 1991). PCR was performed on cDNA derived from both the 1E2 and the NS0 cell line. PCR reactions were carried out in a final volume of 50 µl. Separate reactions were set up for the heavy and light chain PCR products. Control reactions were included for each primer pair used in the PCR reaction. The PCR reaction was made up of 5 µl cDNA, 1 x buffer, 1 x Q solution, 0.25 mM dNTP, 0.2 pmol forward primer (equimolar mix of light chain primers VKFOR1, 2, 3, and 4 or VH1FOR-2), 0.2 pmol reverse primer, 0.5 µl BSA (bovine serum albumin), and 5U HotStar *Taq* polymerase.

The samples were initially denatured for 15 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 63°C for 1 minute and extension at 72°C for 2 minutes.

Following the final amplification cycle, a 10 µl aliquot of the PCR product was mixed with 5 µl of ficoll loading dye and the samples run on a 2% agarose gel. Samples were run for 45 minutes at 120 V. PCR products of approximately 350 bp and 300 bp are expected for the heavy and light chain fragment, respectively.
The PCR products obtained were purified and sequenced on an ABI 377 DNA sequencer using the forward and reverse scFv primers (Table 2.4).

2.4.1.3. Linking of the V_H and V_L domains to form a scFv polypeptide

We attempted to link the 1E2 V_H and V_L PCR products together with the $(Gly_4Ser)_3$ linker using single overlap extension based PCR. We were unable to link the two PCR fragments together to form a scFv. Therefore, to overcome this problem, we decided to amplify the light chain and Fd fragment of the heavy chain (to form a Fab fragment) of our monoclonal antibodies.

2.5. CONSTRUCTION OF A FAB FRAGMENT

2.5.1. cDNA synthesis

First strand cDNA synthesis was carried out in a final volume of 20 µl containing 1 to 500 ng of mRNA, 2 µl hexanucleotide, and 0.5 mM dNTP. Samples were incubated at 65°C for 5 minutes before adding 0.5 x RT buffer, 10 mM DTT and 40 U RNase inhibitor. Samples were incubated at 37°C for 2 minutes. 200 U of Moloney Murine Leukaemia Virus (M-MuLV) RT (Invitrogen) was added and the reaction allowed to continue for 50 minutes at 37°C followed by 15 minutes at 70°C to allow for completion of all products. cDNA was synthesised for all five mAbs.

2.5.2. Amplification of the Fd heavy chain fragment and light chain genes of the five monoclonal antibodies

PCR amplification was performed for both the Fd heavy chain fragment and light chain genes using the newly synthesised cDNA as a template. The PCR reaction was carried out in a 50 µl volume consisting of 2 µl of cDNA, 0.5 mM forward primer and 0.5 mM reverse primer (Table 2.5; primer pairs 1-10 were set up in separate reactions), 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 1 x *Taq* DNA polymerase buffer and 1 U *Taq* DNA polymerase (Qiagen). A negative control was set up for each primer pair.

The V_HC_H1 fragment is amplified with primers representing the leader sequence 5 to 8 amino acids 5' of the V_H framework 1 region and amino acids 205 to 220 of the C_H1 region. The V_LC_L construct is created with primers representing the leader sequence 5 to 8 amino acids 5' of the V_L framework 1 region and amino acids 205 to 220 of the C_L region (Padoa *et al.*, 2003). The size of the amplified PCR product is 660bp. The primers were developed to represent all mouse families. Although some primers may amplify genes in more than one family, the large number of primers is necessary to guarantee that all known families are represented.

The samples were initially denatured for 5 minutes at 94°C. This was followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 2 minutes. Following the last cycle, there was a final extension of 5 minutes at 72°C to encourage completion of partial extension products and annealing of single stranded complementary products.

PCR products were visualised on a 1% agarose gel stained with ethidium bromide. All PCR products of the expected size (± 660 bp) were cut from the gel and purified using the Geneclean kit (QBiogene).

Table 2.5: PCR primers for the initial amplification of the heavy and light chain genes.

NAME	SEQUENCE (5' to 3')*	LENGTH (bp)
LIGHT CHAIN PRIMERS:		
VKarch1	GACAGTGCACATGAAGTTGCCTGTTAGGCTGTTGG	35
VKarch2	GACAGTGCACATGGACATGAGGGCTCCTGCTCAG	34
VKarch3	GACAGTGCACAGACACACTCCTGCTATGGGTGCTGC	36
VKarch4	GACAGTGCACACTGCTCTGTGTGTCTGGTGCTCATGG	37
VKarch5	GACAGTGCACGCTCAGTTCCTTGGTCTCCTGTTGCTC	37
VKarch6	GACAGTGCACCTTGTGTTCTGGATCTCTGGAGCCA	35
VKarch7	GACAGTGCACTTCCTGCTAATCATTGTCACAGTC	34
VKarch8	GACAGTGCACATGGTATCCACACCTCAGTTCCTTG	35
VKarch9	GACAGTGCACTGCTCTGGCTTACAGGTGCCAGATGT	36
VKarch10	GACAGTGCACGCCCAGTTCCTGTTTCTGTTAGTGC	35
CKFOR	GACAACGCGTCTCATTCCTGTTGAAGCTCTTGAC	34
HEAVY CHAIN PRIMERS:		
VHarch1	GACAGTGCACATGGAATGGAGCTGGGTCTTTCTCTT	36
VHarch2	GACAGTGCACATGGAATGGAGCTGGATCTTTCTCTT	36
VHarch3	GACAGTGCACATGGGATGGAGCTGGATCTTTCTCTT	36
VHarch4	GACAGTGCACATGGGATGGAGCTGTATCATCCTCTT	36
VHarch5	GACAGTGCACATGAAGTTGTGGCTGAACTGGATTTT	36
VHarch6	GACAGTGCACATGAAATGCAGCTGGGTTATCTTCTT	36
VHarch7	GACAGTGCACATGAAGTTGTGGTTAAACTGGGTTTT	36
VHarch8	GACAGTGCACGGGCTCAGCTTGATTTTCCTTGTCCT	36
VHarch9	GACAGTGCACATGTTTAACATCATTATCTTCACAGT	36
VHarch10	GACAGTGCACCTCTTCTGCCTGGTGACATTCCCAAG	36
MOCG12FOR	GACAACGCGTCTCAATTTTCTTGTCCACCTTGGTGC	36
MOCG3FOR	GACAACGCGTCTCGATTCTCTTGATCAACTCAGTCT	36
MOCMFOR	GACAACGCGTTGGAATGGGCACATGCAGATCTCT	34

* Primer sequences were kindly given to us by Jefferson Foote from the Fred Hutchinson Cancer Research Center in Seattle, Washington

2.6. PURIFICATION OF DNA FROM AGAROSE GELS

2.6.1. Geneclean (BIO 101® Systems)

The fact that DNA binds to silica in high salt concentrations and elutes in low salt makes this method especially useful as a purification procedure. Since the DNA is eluted with a low salt buffer, it can be used immediately in subsequent reactions without precipitation or other further manipulation.

2.6.2. Geneclean protocol

The DNA band of interest was excised from the ethidium bromide-stained agarose gel, weighed [0.1 g equals approximately 100 µl] and transferred to a 1.5 ml Eppendorf tube. Tris-borate-EDTA (TBE) Modifier (0.5 volumes) and 6 M NaI (4.5 volumes) were added to a given volume of agarose. The tube was incubated in a 55°C heating block until the agarose had dissolved (approximately 10 minutes). GLASSMILK® (50 µl) was added to the tube and allowed to incubate at room temperature on a rotation wheel for 30 minutes. The silica matrix with the bound DNA was pelleted in a microcentrifuge for 30s at full speed. The supernatant was discarded. The pellet was washed 3 times with 750 µl prepared NEW™ Wash (NEW™ Wash concentrate is mixed with 280 ml distilled water and 310 ml 100% ethanol) with a 30s spin between each wash. After the final wash the tube was spun again to remove any residual NEW™ Wash. The pellet was dried for 15 minutes at room temperature. The pellet was resuspended in 50 µl of Tris-EDTA (TE), spun for 30s, and the supernatant containing the eluted DNA transferred to a new Eppendorf tube.

The purified PCR products were then cloned into a vector for sequencing. Since *Taq* polymerase was used to amplify the immunoglobulin regions, the purified PCR products were ligated into a sequencing vector (pCR®II-TOPO) with overhanging 5' thymidines so that the 3' adenosines on the PCR products could base-pair with the vector.

2.7. TOPO CLONING

TOPO TA Cloning® provides a highly efficient, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector.

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3'-ends of PCR products. The linearised TOPO vector (Figure 2.2) has single, overhanging 3'-deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. *Topoisomerase* I (covalently bound to the vector) binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand. The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of *Topoisomerase* I. The phospho-tyrosyl bond can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing *Topoisomerase* (Figure 2.3).

2.7.1. Setting up the TOPO® cloning reaction

Table 2.6 describes how to set up the TOPO® cloning reaction (6 µl) for transformation into chemically competent *Eschericia coli* (*E. coli*) cells. The reaction was incubated for 25 minutes at room temperature and placed on ice before proceeding to section 2.8.2.

Table 2.6: Reagents and their volumes required for TOPO cloning.

Reagent*	Chemically competent *E. coli*
Fresh PCR product	0.5 - 4 µl
Salt solution	1 µl
Sterile water	Add to a total volume of 5 µl
TOPO® vector	1 µl
Final volume	6 µl

*Store all reagents at -20°C when finished. Salt solutions and water can be stored at room temperature or at +4°C.

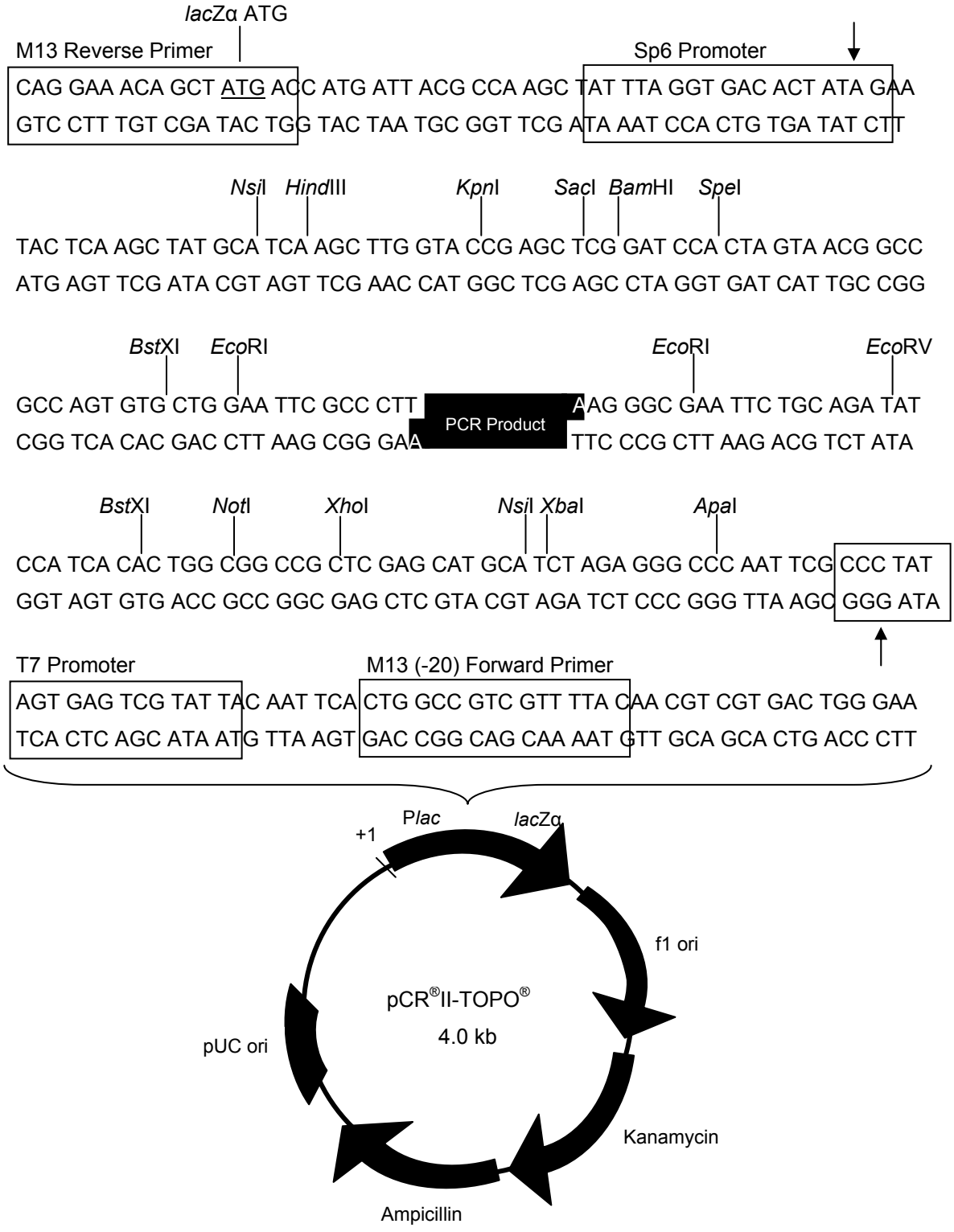


Figure 2.2: Map of pCR®II-TOPO®.

The map shows the features of pCR®II-TOPO® and the sequence surrounding the TOPO cloning site. Restriction sites are labelled to indicate the actual cleavage site. The arrows indicate the start of transcription for Sp6 and T7 polymersases.

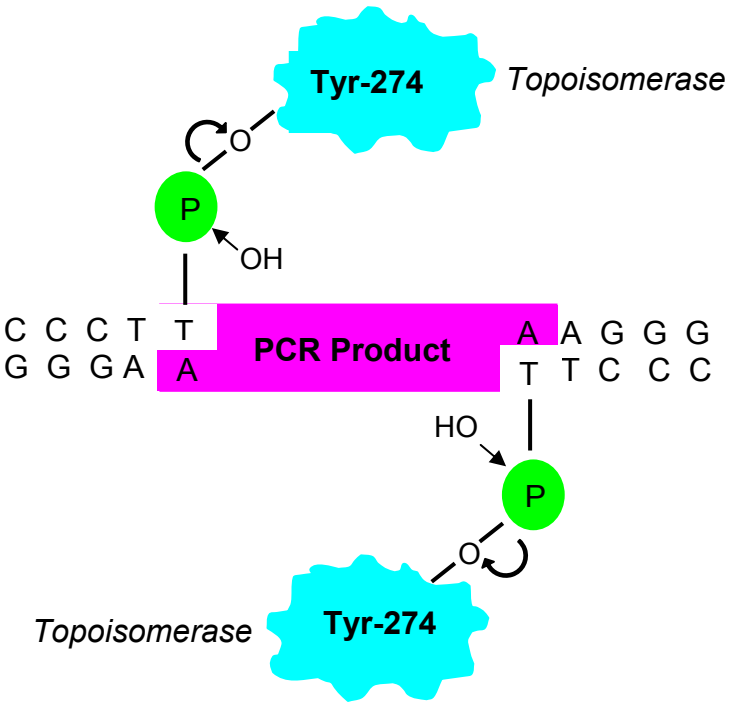


Figure 2.3: Diagrammatic representation of how a PCR product ligates with the vector in the presence of *Topoisomerase*.

2.8. TRANSFORMATION

2.8.1. Transformation guidelines

Storage conditions: Ultracompetent cells are sensitive to small variations in temperature and must be stored in a -70°C freezer.

Aliquoting cells: Keep ultracompetent cells on ice at all times and prechill any tubes that the cells are aliquoted into.

Use of Falcon 2059 Polypropylene tubes: Other tubes may be degraded by β-mercaptoethanol (β-ME) used in the transformation protocol and the incubation period during the heat-pulse step has been calculated specifically for the thickness and shape of Falcon 2059 polypropylene tubes.

Use of β-ME: β-ME increases transformation efficiency.

Length and temperature of the heat pulse: Optimal transformation efficiencies are observed when cells are heat-pulsed for 30s at 42°C.

2.8.2. One Shot® chemical transformation

A 75 µl aliquot of thawed XL 10-Gold ultracompetent cells (Stratagene) and 2 µl of β-ME were incubated on ice for 10 minutes. The TOPO® cloning reaction (2 µl; section 2.7.2) was added to the competent cells and incubated for 25 minutes on ice. Following a 30 second heat pulse at 42°C, the tubes were incubated on ice for 2 minutes. SOC medium (0.9 ml) was added to each tube and the cultures allowed to grow at 37°C for 1 hour with shaking (225 to 250 rpm). The transformation reaction (100 µl) was plated on LB (Luria-Bertani)-carbenicillin agar plates and incubated overnight at 37°C.

To ensure that the colonies contained the TOPO vector with the immunoglobulin insert, plasmids were isolated from the cultures using alkaline lysis miniprep procedures. An aliquot of plasmid DNA was digested with restriction enzymes that flank the insert site to confirm the size of the insert.

2.9. PLASMID MINIPREP

2.9.1. Growing an overnight culture

Single bacterial colonies were selected and grown overnight (300 rpm; 37°C) in 3 ml of LB medium with 0.1% carbenicillin.

2.9.2. The QIAprep principle

The QIAprep miniprep procedure consists of three basic steps:

i. preparation and clearing of a bacterial lysate,
ii. adsorption of DNA onto the QIAprep membrane, and
iii. washing and elution of plasmid DNA.

2.9.3. Plasmid Miniprep Protocol

Overnight cultures were centrifuged for 10 minutes at 2 000 x g. The pelleted bacterial cells were resuspended in 250 µl buffer P1 and transferred to a 1.5 ml

Eppendorf tube. Bacteria were lysed by the addition of 250 µl buffer P2. Proteins were precipitated by adding 350 µl buffer N3 and removed by centrifuging for 10 minutes at ≥ 10 000 x g. The supernatant containing the plasmid DNA was applied to a QIAprep column and centrifuged for 1 minute. The QIAprep spin column was washed with 750 µl buffer PE and centrifuged for 1 minute. Residual wash buffer was removed by a further 1 minute centrifugation step. Plasmid DNA was eluted (1 minute centrifugation) with 50 µl buffer EB (elution buffer; 10 mM Tris-HCl, pH 8.5).

2.9.4. Storage of plasmid DNA

Plasmid DNA can be stored in TE buffer at 4°C for several weeks or preserved for several years by storing at -20 or -70°C.

2.10. RESTRICTION ENDONUCLEASES

Restriction endonucleases are endodeoxyribonucleases that recognise specific nucleotide sequences within double stranded DNA, and cleave the double stranded DNA within or adjacent to this site. Restriction endonucleases are classified into types I, II or III. Only type II enzymes were used in this study (Sambrook *et al.*, 1989).

2.10.1. Restriction endonuclease digestion of plasmid minipreps

Restriction endonuclease cleavage is accomplished simply by incubating the enzyme(s) with the DNA in appropriate reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of the reaction will vary depending upon the specific application and enzyme being used.

The plasmid DNA was digested with *Eco*RI (this enzyme cleaves on either side of the inserted PCR fragment; refer to Figure 2.2) to ensure the presence of the PCR fragment in the TOPO vector. The digestion reaction was carried out in a 20 µl

volume consisting of 5 µl plasmid DNA, 1 x restriction enzyme buffer, and 2 U *Eco*RI. Samples were incubated in a 37°C incubator or water bath for an hour. Following restriction digest, samples were run on a 1% agarose gel (Figure 2.4).

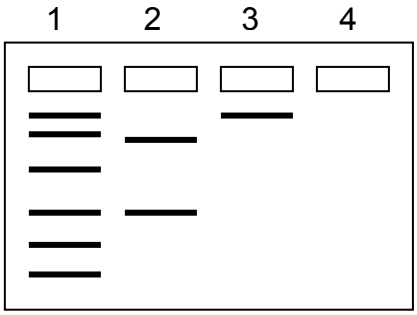


Figure 2.4: Schematic representation of the possible products obtained after restriction digest of plasmid minipreps.
Lane 1: molecular weight marker, Lane 2: digested plasmid with PCR insert, Lane 3: linearised plasmid, Lane 4: blank.

If the plasmids contained an insert, a single clone was analysed by DNA sequencing to further ensure that the inserts were immunoglobulin regions. Samples were sequenced using the TOPO-specific Sp6 and T7 primers. Sequence determination was performed using an ABI PRISM instrument and the BigDye terminator method. DNA sequences obtained were compared to those in GenBank to ensure that they were immunoglobulin regions.

2.11. SEQUENCING

Sequencing was performed on an automated ABI 377 DNA sequencer which is able to detect DNA fragments by fluorescence from four different dyes that are used to identify the adenosine (A), guanosine (G), cytosine (C), and thymidine (T) extension reactions. Four chemically related, yet distinguishable, fluorescence-tagged dideoxynucleoside triphosphates (ddNTPs) are used to label the DNA with a suitable DNA polymerase. The growing chain is, therefore, simultaneously terminated and labelled with the dye that corresponds to a particular base. Each dye emits light at a different wavelength when excited by the laser beam. All four colours can be detected and distinguished in a single gel lane by polyacrylamide

gel electrophoresis (Prober *et al.*, 1987). Analysis of the fluorescent emission of each fragment allows one to identify the terminating nucleotide and assign the sequence directly in real time.

2.11.1. Pre-treatment of PCR products for sequencing

To obtain high quality sequencing data that are easy to interpret, it is important that the PCR be as specific as possible. Poor quality sequencing data will be obtained if the PCR products contain large amounts of residual primers, nucleotides, and spurious bands. The PCR product must, therefore, be purified prior to sequencing. Depending on the purity of the amplified PCR product, one of two methods was used for purification.

2.11.1.1. Enzymatic purification of PCR products for sequencing

In the absence of spurious bands, 5 µl of PCR product was purified with two hydrolytic enzymes: exonuclease I (1 U) and Shrimp Alkaline Phosphatase (SAP; 1 U). Exonuclease I degrades residual single stranded primers and any extraneous single stranded DNA produced by the PCR. SAP dephosphorylates the excess nucleotides. The PCR product was incubated in the Hybaid omnigene PCR machine for 15 minutes at 37°C followed by a further 15 minutes at 80°C to inactivate the two enzymes.

2.11.1.2. Gel purification of PCR products

PCR products were run on a 1% agarose gel to separate and isolate the fragment of interest. The band of interest was cut out and placed in an Eppendorf tube. The Geneclean kit was used to extract the DNA from the gel (section 2.6.2). An aliquot of the purified DNA was checked on a 1% agarose gel to ensure its extraction and purity.

2.11.2. Cycle sequencing PCR amplification

Two reactions were set up for each sample being sequenced: one with the forward primer and one with the reverse primer. The reaction was carried out in a 10 µl

volume consisting of 0.5 µM stock primer (forward or reverse; Table 2.4), 200 ng of plasmid DNA, and 4 µl BigDye.

The three steps involved in the amplification of the target sequences were: denaturation at 96°C for 30s, annealing at 50°C for 15s, and extension of the PCR product at 60°C for 4 minutes. This cycle was repeated 30 times. Upon completion of the 30 cycles, the PCR was held at 4°C.

2.11.2.1. Purification of cycle sequencing PCR products

Once the PCR reaction was completed, samples were purified using a Sephadex G-50 slurry. Sephadex (2.5 g) was weighed and placed in a 50 ml conical tube. The volume was made up to 40 ml with dH_2O. The sephadex slurry (650 µl) was added to a spin column placed in an Eppendorf tube and allowed to stand at room temperature for 20 minutes. Columns were spun for 2 minutes at 770 x g and put into clean, labelled Eppendorf tubes. Samples were carefully loaded onto the centre of the resin and spun for 2 minutes at 770 x g. The purified product was dried in a speedivac at low heat for at least 20 minutes. The dried, purified samples were sent to the Howard Hughes Medical Institute & Department of Immunology DNA Sequencing Facility (University of Washington, Seattle, WA) where sequence determination was performed.

2.11.3. Subcloning of the immunoglobulin genes

Once we were certain that the chains that we had amplified were indeed immunoglobulin in nature we were ready to proceed to the cloning step. To construct the expression vector, the immunoglobulin light and heavy chain genes were reamplified by PCR using primers (refer to Table 2.7) introducing the restriction sites *Mlu*I and *Bsr*GI, and *Mlu*I and *Sph*I, respectively to allow cloning into the pCXII vector.

PCR was performed with the primers listed in Table 2.7 using the same conditions as described previously (section 2.4.1.2.). The heavy chain primers were designed to add a penta-histidine tag to the 3' end of the PCR products.

Table 2.7: Primers containing restriction sites used to clone the heavy and light chain fragments into the pAK19 expression vector.

NAME	SEQUENCE (5' TO 3')	ENZYME
DPDL5"	ACAAACGCGTATGCTGACATTGTGATGACCCAGTCTCC	*Mlu*I
DPDL3'	CTTGTGTACAAGGGCCACGATGCGTCCGGCGTAGAGGATCAGCTTAAGACT	*Bsr*GI
DPDH5'	CAAACGCGTACGCTCAGGTGCAGCTGGTGGAGTCTGGCCC	*Mlu*I
DPDH3'	CGTCGCATGCCGCGTCAGTGGTGGTGGTGGTGGTGTTCGCCCTTGCAT	*Sph*I
1E2L5'	CAAACGCGTACGCTGATATCCAGATGAC	*Mlu*I
1E2L3'	TTGTGTACAAGGGCCACGATGCGTCCGGCGTAGAGGATCAGCTTAACAACGGGTCTC	*Bsr*GI
1E2H5'	GCTACAAACGCGTACGCTCAGGTGCAGCTGCTCGAGT	*Mlu*I
1E2H3'	GTCGCATGCCGCGTTAGTGGTGGTGGTGGTGGCAGTCACGCGGAAC	*Sph*I
123L5'	ACAAACGCGTATGCTCAAATTGTTCTCACCCAGTC	*Mlu*I
123L3'	TTGTGTACAAGGGCCACGATGCGTCCGGCGTAGAGGATCAGCTTAACAACGGGTCTC	*Bsr*GI
123H5'	ACAAACGCGTACGCTCAGGTCCAGCTGCAGCAGTCTGG	*Mlu*I
123H3'	CGTCGCATGCCGCGTTAGTGGTGGTGGTGGTGGTGTTGACAACGGGTCTC	*Sph*I
126L5'	GCTACAAACGCGTATGCTGATATTGTGCTAACTCAG	*Mlu*I
126L3'	CTTGTGTACAAGGGCCACGATGCGTCCGGCGTAGAGGATCAGCTTAACAACGGGTCTC	*Bsr*GI
126H5'	GCTACAAACGCGTACGCTGAGGTCCAGCTGCAACAG	*Mlu*I
126H3'	GGGCCGTCGCATGCCGCGTCAGTGGTGGTGGTGGTGGTGTTGACAACGGGTCTC	*Sph*I

The pentahistidine tag located in the heavy 3' primer is underlined and the *Mlu*I, *Bsr*GI, and *Sph*I restriction sites are shown in red, green, and blue respectively.

An aliquot of the reamplified products were checked on a 1% agarose gel and then digested with the respective enzymes for at least four hours. Light chains were digested with *Mlu*I and *Bsr*GI while the heavy chain products were digested with *Mlu*I and *Sph*I. The digested products were run on a gel, purified and each chain ligated into a separate pCXII vector (Figure 2.5) which has similarly been digested. The heavy and light chain were individually subcloned into the pCXII vector, as opposed to the pAK19 vector because of the presence of the *Mlu*I restriction site on both immunoglobulin chains which made direct cloning of the chains into the expression vector polycloning site very difficult.

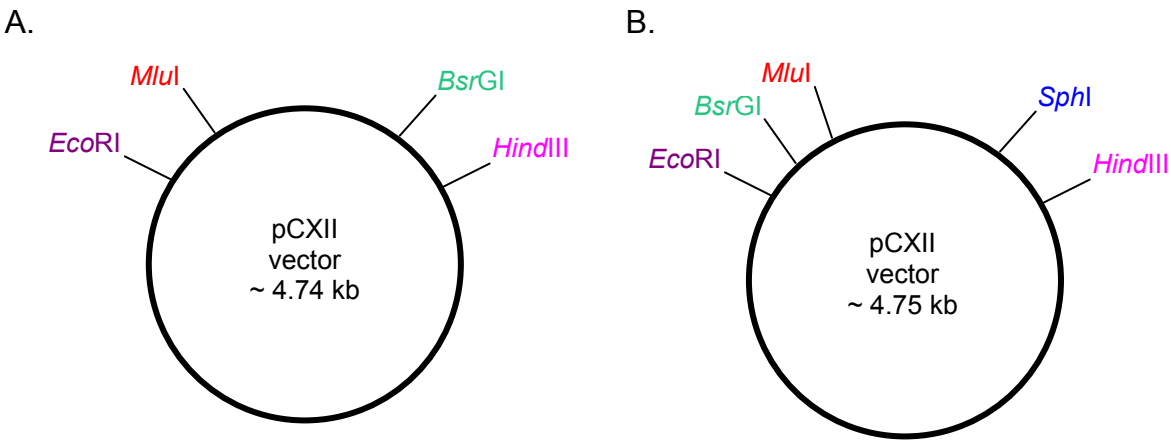


Figure 2.5: A. pCXII vector containing a light chain insert (*Mlu*I-*Bsr*GI).
B. pCXII vector containing a heavy chain insert (*Mlu*I-*Sph*I).

2.12. LIGATION AND CLONING

Two digestion reactions were set up. In one, the pCXII vector was digested with *Mlu*I and *Bsr*GI (for insertion of the light chain fragment) and in the second reaction the vector was digested with *Mlu*I and *Sph*I (for the insertion of the Fd heavy chain fragment). The vector was digested for 2 hours at 37°C. A small aliquot of the digest was run on a 1% agarose gel to ensure complete digestion of the vector and that the correct size fragments were obtained. The vector was then dephosphorylated.

2.12.1. Vector dephosphorylation

The digested vector was dephosphorylated in a 50 µl volume consisting of 0.5 U shrimp alkaline phosphatase and 1 x SAP buffer. The reaction was incubated for one hour at 37°C. The enzymes were inactivated by a 15 minute incubation at 65°C. The digested, dephosphorylated vector was run on a 1% agarose gel, cut out and purified using GLASSMILK as described above.

2.12.2. The ligation reaction

The OD_{260} of the purified, digested DNA and vector fragments were measured and the concentration of each calculated. Three ligation reactions with differing vector/insert ratios (1:1, 1:3 and 3:1) were set up (Table 2.8). For example:

Vector OD_{260} = 0.031 = 38.9 µg/ml Vk OD_{260} = 0.021 = 26.6 µg/ml

$$\frac{\text{Size (bp) insert x 100}}{\text{Size (bp) vector}} \times \frac{1}{1} = \text{ng of insert}$$

$$\therefore \frac{680 \times 100}{4070} \times \frac{1}{1} = 16.7 \text{ ng of insert}$$

Vector:
38.9 µg = 1 ml
∴ 0.1 µg = 2.57 µl

Insert:
26.6 µg = 1 ml
0.0167 µg insert = 0.63 µl

Table 2.8: Volumes required for a ligation reaction.

	Insert	Vector	Ligase	Buffer	Water
1:1	0.63 µl	2.57 µl	1 µl	1 µl	4.8 µl
1:3	0.21 µl	2.57 µl	1 µl	1 µl	5.22 µl
3:1	1.89 µl	2.57 µl	1 µl	1 µl	3.54 µl

The ligation was allowed to continue for one hour at 16°C. The products of the ligation reaction were introduced into XL 10-Gold ultracompetent *E. coli* cells (refer to section 2.8.2), and transformants identified by growth on carbenicillin plates.

DNA was prepared from the colonies by plasmid miniprep (section 2.9) and subjected to restriction endonuclease mapping (*Mlu*I/*Bsr*GI and *Mlu*I/*Sph*I for the light and heavy chain respectively) in order to determine if the desired DNA molecule was created.

The heavy chain fragment was subsequently cloned into the pCXII vector which contained the light chain insert. To clone the heavy chain into the vector containing the light chain fragment, *Bsr*GI and *Hind*III enzymes were used (refer to Figure 2.5). The products of the ligation reaction were introduced into XL 10-Gold ultracompetent *E. coli* cells, transformants identified by growth on carbenicillin plates, and DNA prepared from the colonies by plasmid miniprep. The Fab (light chain and Fd fragment of the heavy chain) was cut from the pCXII vector using the *Eco*RI and *Hind*III enzymes and cloned into the pAK19 expression vector (Figure 2.6), which had been digested with the same enzymes.

2.12.3. Choosing a plasmid vector

Some of the basic features to consider when selecting a plasmid vector include the size of the vector, its copy number, the polylinker, and the ability to select and/or screen for inserts.

The expression vector used in this study is called pAK19. The pAK19 vector is designed to co-express the light chain and heavy chain Fd fragment from a synthetic dicistronic operon (Figure 2.6) which is cloned between the *Eco*RI and *Hind*III sites of pBR322 (Bolivar *et al*., 1977). This vector uses the *E. coli* alkaline phosphatase (*pho*A) promoter, which is inducible by phosphate starvation, and heat-stable enterotoxin II (stII) signal sequences precede each antibody chain. Gene products are directed to the periplasmic space of *E. coli*, from which correctly folded, disulfide-oxidized molecules are released after cell harvest (Carter *et al.*, 1992).

2.12.4. Cloning of DNA immunoglobulin heavy and light chain fragments

The *EcoR*I and *Hind*III digested pAK19 vector was purified from the small remnant of the polycloning site by agarose gel electrophoresis. The vector was ligated to the Fab fragment cut from the pCXII vector. The resulting circular recombinant plasmids were introduced into 25F2 competent *E. coli* cells (section 2.13), and transformants identified by growth on carbenicillin plates. DNA was prepared from the colonies by plasmid miniprep and subjected to restriction endonuclease mapping (*Mlu*I and *Sph*I) in order to determine if the desired DNA molecule was ligated into the pAK19 vector.

To express the Fab, a specific strain of *E. coli* cells (25F2), needed to be transfected with the pAK19 vector containing the Fab insert.

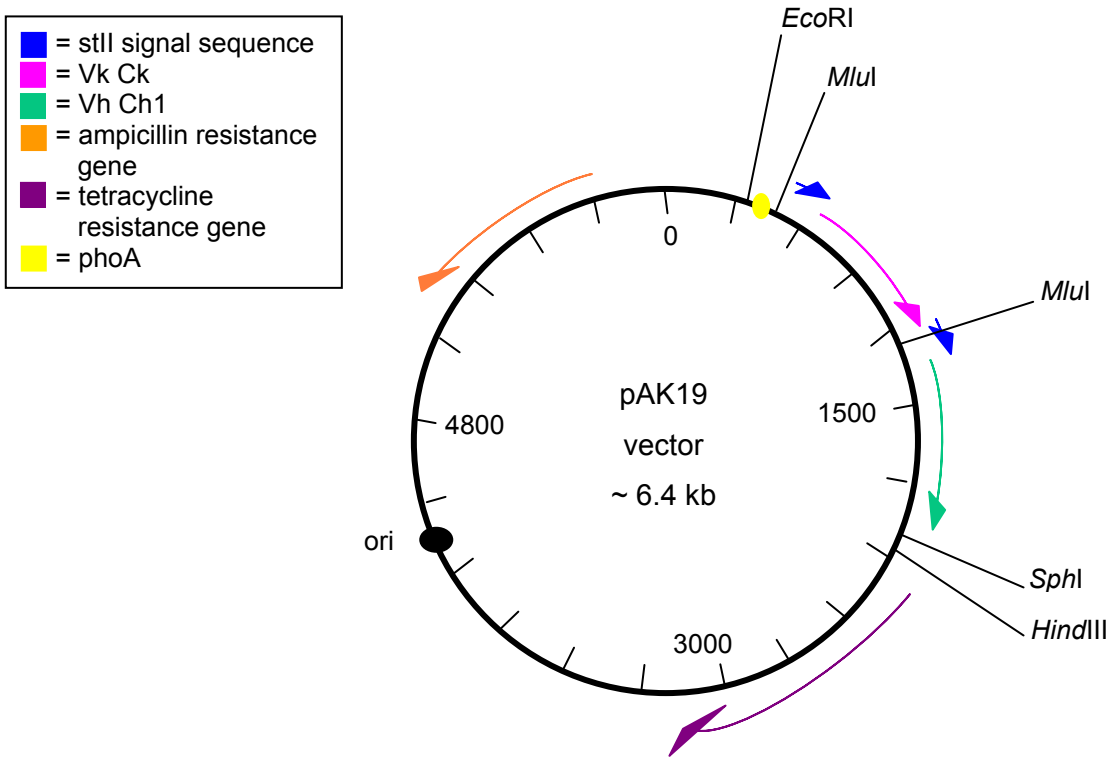


Figure 2.6: Diagrammatic representation of the pAK19 vector and the positions where the heavy and light chain fragments were inserted.

2.13. PREPARATION OF FRESH COMPETENT 25F2 *E. COLI* CELLS

Two methods were used to prepare fresh competent 25F2 *E.coli* cells in this study: calcium chloride and rubidium chloride methods.

2.13.1. Calcium chloride method for preparing competent cells

Competent cells were prepared using standard procedures (Mandel and Higa, 1970). A single colony of *E. coli* 25F2 cells was picked from a plate freshly grown for 16 to 20 hours at 37°C and transferred into 100 ml of LB broth or SOB medium in a 1 litre flask. The culture was incubated at 37°C with vigorous shaking (300 rpm) until the cells reach an OD_{600} of approximately 0.375 (~10^8 cells/ml). The cells were cooled to 0°C and recovered by centrifugation at 3 000 x g for 10 minutes at 4°C in a Beckman Model TJ-6 centrifuge. The cell pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 10 minutes. After an additional centrifugation step (3 000 x g for 10 minutes at 4°C) the cell pellets were resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. The cells were dispensed into aliquots and frozen at -70°C. The cells maintain competency under these conditions, although the transformation efficiency may drop slightly during prolonged storage.

2.13.2. Rubidium chloride method for preparing competent cells

A single colony was grown in 100 ml SOC, as for the calcium chloride method, until an OD_{600} of 0.5 was reached. The cells were cooled on ice for 10 minutes and recovered by centrifugation at 3 300 x g for 15 minutes at 4°C. The cell pellet was resuspended in 15 ml solution RF1 and put on ice for 15 minutes. Following an additional spin the cell pellet was resuspended in 4 ml solution RF2 and stored on ice for 20 minutes. The cells were aliquoted, snap frozen in liquid nitrogen and stored at -70°C.

E. coli 25F2 cells were transformed with the pAK19 vector and transformants identified by growth (37°C) on carbenicillin plates. Very few colonies were observed on the plate the following morning.

2.14. EXPRESSION OF RECOMBINANT FABS

Expression vectors are usually designed such that production of the foreign protein is tightly regulated. This is necessary because the host cell machinery is co-opted to produce large quantities of the foreign protein, the shear amount of which may be toxic to the cell, and/or the foreign protein may encode a function that will inhibit cell growth or kill the host cell.

2.14.1. Expression of secreted foreign proteins

Secretion of the foreign protein is accomplished by fusing the coding sequence to DNA encoding a signal peptide that is cleaved by signal peptidase when the protein is secreted into the periplasm located between the inner and outer membranes of *E. coli*. The two major problems encountered in secretion of foreign proteins are that yields are often low and cleavage of the signal peptide may not occur or may occur at an inappropriate position.

A single colony was picked from the agar plate and grown in 5 ml LB containing tetracycline for approximately 6 hours. The *E. coli* 25F2 cells containing the recombinant plasmids were then transferred to 500 ml complete MOPS medium with tetracycline and grown for 16 hours at 30°C (250 rpm). Cells were subcultured and grown in the absence of phosphate for 4 hours at 30°C to induce expression of the Fab and secretion into the periplasmic space.

2.14.2. Purification of the antibody recombinant Fab

The bacteria were harvested by centrifugation at 8 000 x g for 15 minutes. The pellet was subjected to a freeze (dry ice-ethanol bath)-thaw (15 minutes on ice) cycle and resuspended in lysis buffer (2 to 5 ml per gram of pellet) containing lysozyme (1 mg/ml). After a 30 minute incubation step on ice, the lysate was

sonicated using a microtip sonicator with six 10s bursts at 200 to 300 W with a 10s cooling period between each burst. Cellular debris was removed by centrifugation at 10 000 x g for 30 minutes at 4°C. The supernatant was stored at 4°C.

The rFab was isolated from the cleared supernatant by two subsequent affinity chromatography steps. Initially the rFab were bound to Ni-NTA Agarose via a histidine Tag at the C-terminus of the heavy chain. Lysate (4 ml) was added to 1 ml of Ni-NTA agarose and incubated for 1 hour at 4°C on a rotator. A column was loaded with the lysate-agarose suspension and the flow through collected. The column was washed with 8 mls of lysis buffer. The bound proteins were eluted in 1 ml fractions with step-wise increases of imidazole (10, 50, 100, and 300 mM) in lysis buffer. The eluates were examined by immunoblot (section 2.16) for the presence of rFab and the positive fractions pooled and further purified on Protein G Sepharose (PGS). rFab fractions (1 ml) were incubated with 200 µl of PGS at 4°C on a rotator for one hour. Samples were spun for 5 minutes at 2 600 x g and washed with 1 ml wash buffer. This was repeated five times. Bound rFab was eluted with 0.1 M glycine-HCl buffer (pH 2.5). Fractions (500 µl) were collected in Eppendorf tubes and immediately neutralized with 50 µl of 1 M Tris-HCl, pH 8.0. Fractions were examined by immunoblot for the presence of rFab and by radioimmunoassay (RIA) for insulin binding (section 2.17.1). Active fractions were pooled and the protein concentration determined. The yield of functional purified rFab was ~ 0.5 mg/l bacterial culture.

2.15. POLYACRYLAMIDE GEL ELECTROPHORESIS OF AFFINITY CHROMATOGRAPHY PURIFIED rFAB FRACTIONS

2.15.1. Preparation of SDS-polyacrylamide gels

The gel casting apparatus was assembled according to the manufacturer's instructions. The resolving gel [2.5 ml Tris buffer (pH 8.8), 2.3 ml dH_2O, 5 ml 30% acrylamide mix, 100 µl 10% ammonium persulphate, and 4 µl TEMED] was prepared and aliquoted between the glass plates, leaving sufficient space for the stacking gel. The stacking gel [1.25 ml Tris buffer (pH 6.8), 2.85 ml dH_2O, 820 µl

30% acrylamide mix, 25 µl 10% ammonium persulphate, and 2 µl TEMED] was pipetted directly onto the surface of the polymerised resolving gel. A comb was immediately inserted into the stacking gel solution, being careful to avoid trapping air bubbles under the teeth. The gel was allowed to polymerise at room temperature.

2.15.2. Preparation of samples for SDS-polyacrylamide gels

The Ni-NTA agarose/PGS eluted rFab protein samples were heated to 100°C for 5 minutes in 2 x SDS gel loading buffer (1:1). β-ME (10%) was added to the gel loading buffer for reducing gels. Samples were kept on ice until ready to use.

2.15.3. Running SDS-polyacrylamide gels

Protein samples were analysed on a 15% SDS polyacrylamide gel in tris-glycine buffer. Gels were run at 100 to 120 V for approximately 2 hours. A molecular weight marker (BIORAD prestained SDS PAGE standards) was included in each gel. The gel was fixed, stained with Coomassie Brilliant Blue or used to establish a Western blot.

2.15.4. Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue

Polypeptides separated by SDS-polyacrylamide gels can be stained with Coomassie Brilliant Blue R250. The gel was immersed in at least 5 volumes of staining solution and placed on a slowly rotating platform for a minimum of 4 hours at room temperature.

2.15.5. Destaining SDS-polyacrylamide gels

The stain was removed by soaking the gel in the destaining solution (methanol: water: acetic acid; 6:3:1) on a slowly rocking platform for 4 to 8 hours at room temperature. The destaining solution was changed three to four times. A piece of tissue paper was included in the normal destaining buffer as this absorbed the stain as it leached from the gel. To make a permanent record the gel was dried.

2.15.6. Drying SDS-polyacrylamide gels

The major problems encountered when a gel is dried are:

i. shrinkage and distortion, and
ii. cracking of the gel.

The first of these problems can be minimised if the gel is attached to a piece of Whatman 3MM paper before it is dried. However, there is no guaranteed solution to the second problem, which becomes more pronounced with thicker gels containing more polyacrylamide. Cracking generally occurs when the gel is removed from the drying apparatus before it is completely dried.

The gel was fixed at room temperature in 5 to 10 volumes glacial acetic acid: methanol: water (1:2:7). The bromophenol blue will turn yellow as the acidic fixing solution diffuses into the gel. Fixation was continued for 5 minutes after all of the blue colour had disappeared. The gel was washed briefly in dH_2O, placed on a piece of Glad wrap and overlayed with a piece of dry Whatman 3MM paper. The sandwich of 3MM paper/gel/Glad wrap (Glad wrap uppermost) was placed on a piece of 3MM paper on the gel dryer. The gel was dried under vacuum, with low heat (50 to 65°C) for approximately 3 hours.

2.16. IMMUNOBLOTTING OF rFAB FRACTIONS

Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. In Western blotting (Towbin *et al.*, 1979), the samples to be assayed are solubilised with detergents and reducing agents, separated by SDS-polyacrylamide gel electrophoresis, and transferred to a solid support (usually a nitrocellulose filter). The membrane is subsequently exposed to horseradish peroxidase (HRP) conjugated antibodies specific for the target protein. Finally, the bound antibody is detected by chemiluminescence.

2.16.1. Transfer of protein from SDS-polyacrylamide gels to solid supports

To transfer proteins from the polyacrylamide gel to a Millipore imobilon-P membrane a transfer stack was assembled (packing sponge, Whatman 3MM filter

paper, gel, membrane, Whatman 3MM filter paper and packing sponge). The membrane was wet in methanol and water and then soaked in transfer buffer for 5 minutes before being used. The transfer stack was assembled on the black cathode side so that molecules will migrate to the membrane. The module was positioned in the tank with the red side facing outward. The transfer was carried out at room temperature and was completed in 1 hour at 60 V.

2.16.2. Blocking binding sites for immunoglobulins on the membrane

The sensitivity of Western blotting depends on reducing non-specific binding by blocking potential binding sites with irrelevant proteins. The membrane was, therefore, incubated for at least an hour in blocking solution (1 x TBST [Tris, borate, Tween 20] with 1% BSA). The blot was rinsed three times in 1 x TBST.

2.16.3. Binding of the primary antibody to the target protein

For detection of the heavy and light chain a anti-penta-His antibody-HRP conjugate (Qiagen; 1:4 000) and a rat anti-mouse kappa antibody-HRP conjugate (Stratagene; 1:4 000) were used, respectively, in the Western blot analysis. After an hour incubation at room temperature, and extensive washing (3 x 10 minutes), the bands were visualized using standard chemiluminescence techniques, as described below.

2.16.4. Immunological detection of immobilised proteins by chemiluminescence

Chemiluminescent detection is based on the oxidation of luminol in the presence of peroxide and is catalysed by horseradish peroxidase. The oxidation of luminol emits light which is detected by exposing the blot to X-ray film.

The membrane was incubated in equal volumes (7 ml) of chemiluminescence Solution A and B (see Appendix 1) for one minute. The membrane was blotted dry and exposed to X-ray. X-ray films were developed at 10 and 60s intervals.

2.17. IMMUNOLOGICAL ASSAYS

Immunoassays are one of the most powerful of all immunochemical techniques. They employ a wide range of methods to detect and quantitate antigens or antibodies and to study the structure of antigens. With the appropriate assay, they can be remarkably quick and easy, yielding information that would be difficult to determine by other techniques.

Two types of detection systems are commonly used for immunoassays. These are iodinated reagents and enzyme-labelled reagents. Assays that use iodinated reagents are easier to quantitate than enzyme labelled reagents, while enzyme assays will often yield a quicker result. RIAs, using ^{125}I-labelled insulin and ^{35}S-labelled GAD65, were used in this project.

2.17.1. Radioimmunoassays

2.17.1.1. The GAD65 autoantibody assay

Recombinant [^{35}S]-GAD antigens were produced in an *in vitro* coupled transcription/translation system with SP6 RNA polymerase and nuclease treated rabbit reticulocyte lysate (Promega) as described previously (Grubin *et al.*, 1992). The *in vitro* translated [^{35}S]-antigen was kept at -70°C and used within 2 weeks. Binding of rFab DP-D to radiolabelled antigen was determined by a previously described RIA (Grubin *et al.*, 1992; Grubin *et al.*, 1994) using PGS as the precipitating agent.

The capacity of the rFab DP-D to inhibit GAD65 binding by human serum GAD65Abs was tested in a competitive RIA using PAS as the precipitating agent. Fab lack the C_H2 domain of the Fc region and do not bind protein A. Serum samples were first tested at a dilution of 1/25. Samples that were competed only partially were diluted to a final serum dilution of 1/100 and reanalysed. The rFab was added at the maximal concentration, as determined in competition assays (Padoa *et* al., 2003) using the intact mAb as a competitor. A reduction in binding by > 10% and < 50% was defined as partial inhibition. A reduction in binding of > 50% of the maximal binding was defined as full inhibition of antigen binding.

Human sera contain GAD65Abs of different epitope specificities, therefore, a complete inhibition of GAD65 binding (100%) was not expected.

2.17.1.2. The insulin autoantibody assay

The binding capacity of serum samples, intact mAbs, and rFab were determined in the insulin antibody radiobinding assay (RBA).

2.17.1.2.1. Preparation of antigen

The lyophilised (3-[^{125}I]iodotyrosyl^{A14})insulin product (AEC Amersham; specific activity: > 15 Ci/mg Iodide) was reconstituted to 100 µCi/ml.

The target cpm was 20 000/25 µl (or 800 cpm/µl). To calculate the amount of iodinated antigen required to reach the target cpm, the following formula was used:

(800 cpm/µl) x (1.2 µCi/2 x 10^6 cpm) x (10 µl/1 µCi) x (total volume [µl] needed for the assay) = µl of antigen to add to the buffer.

15 000 cpm A14-^{125}I-radiolabeled recombinant human insulin (> 2 000 Ci/mmol), per 35 µl buffer, was incubated overnight, at room temperature, with 7 µl of serum samples, mAb, or rFab (in triplicate).

2.17.1.2.2. Precipitation

The overnight reaction was transferred to a nitrocellulose microtitre plate (precoated with 200 µl per well of coating buffer overnight; Millipore) containing 50 µl of 40% protein A sepharose (PAS) or PGS per well. The Millipore plate had been incubated with coating buffer (200 µl/well) overnight at 4°C and washed twice with wash buffer before use. The immunocomplexes were allowed to bind to the PAS or PGS for 90 minutes at 4°C (on a shaker). The bound complexes were washed eight times (200 µl/well) on a Millipore vacuum manifold. The plate was dried for approximately 30 minutes, 50 µl of scintillation fluid added to each well and samples counted in a β-scintillation counter.

Results for rFab or human serum were expressed in arbitrary units derived from a standard curve. The standard curve was established by running dilutions of serum from an insulin autoantibody positive control patient in the IAA assay. Samples

were considered positive if they had levels above the 97.5th percentile of 50 healthy controls (0.2 units).

2.17.1.3. Competition assays using rFab 126

The capacity of the rFab to inhibit the binding of human serum IAA to labelled insulin was tested in a competitive RBA (as for section 2.17.1.2) using PAS as the precipitating agent. Fab lack the C_H2 domain of the Fc region and therefore do not bind to PAS. Serum samples were tested at a serum dilution of 1/6. The optimal concentration of rFab 126 was determined in competition assays using intact mAb 126 as the competitor. The rFab concentration determined in these experiments to achieve maximal competition of its intact IgG was used in all other competition assays. GAD65-specific rFab N-GAD65 was used to determine unspecific binding to insulin.

2.17.1.3.1. Purification of whole mAbs by adsorption to PAS

Although many techniques have been developed to purify IgG molecules, the method of choice is adsorption to, and elution from, beads coated with protein A, a component of the cell wall of *S. aureus* (Hjelm *et al.*, 1972). This protein (M_r = 42 000) binds strongly to sites in the second and third constant regions of the Fc portion of the immunoglobulin heavy chain (Deisenhofer, 1981).

The tissue culture supernatant containing the desired mAb was incubated for 1 to 2 hours with 2.5 ml of 40% PAS before being gravity loaded into an empty column. After washing the column with 10 bed volumes of PBS, the immunoglobulin was eluted with 100 mM glycine (pH 3.0). The 0.5 ml fractions were collected into Eppendorf tubes containing 50 µl of 1 M Tris-Cl (pH 8.0). The immunoglobulin-containing fractions were identified by measuring their absorbance at 280 nm. Fractions that contained immunoglobulin were pooled.

2.18. EPITOPE MAPPING

The epitope specificity of the rFab 126 was determined by competitive RBA (refer to section 2.17.1.2). Binding to insulin by mAb 1, and mAb 125 was competed with rFab 126. Competition of insulin binding by both monoclonal antibodies at

half maximal binding (0.08 and 0.04 mg/ml, respectively) by different concentrations of rFab 126 was tested.

The epitope that rFab DP-D binds to on GAD65 was mapped according to Padoa *et al*., 2003 (Appendix 7).

2.19. STATISTICAL ANALYSIS

Binding of IAA to insulin in the presence of rFab was expressed as follows: cpm of ^{125}I insulin bound in the presence of rFab/cpm of ^{125}I insulin bound in the absence of rFab x 100. The cut off for specific competition was determined as > 10% by using rFab NQ22/61.1 as a negative control (a kind gift from Dr. J. Foote, Fred Hutchinson Research Center, Seattle), specific to an irrelevant target, phenyl oxazolone, at 5 µg/ml.

All samples were analyzed in triplicate determinations and the average IAA intra-assay coefficient of variation was 5% (range: 1.6 to 9%). Similarly, all samples were run in triplicate for the GAD65 assay and the intra-assay average coefficient of variation was 7%, with the highest value being 20 and the lowest 0.1. Positive and negative controls were included on each assay plate to correct for inter-assay variations. Significance of competition within serum groups was tested using the Wilcoxon matched pair test. Differences in competition between serum groups were tested for significance with the non-parametric Mann-Whitney U-test. The significance of correlation of competition levels was analysed using Spearman's rank correlation test. A p-value < 0.05 was considered significant.

2.20. HOMOLOGY MODELLING

The 3-dimensional structure of a target protein that has not been solved empirically by X-ray crystallography or NMR can be predicted by 'comparative modelling' or 'homology modelling'. For this method to be successful the sequence of the target protein must be sufficiently similar (≥ 50% sequence identity) to a protein for which the 3-dimensional structure has been empirically determined.

The 3-dimensional structure of rFab 126 and rFab DP-D were determined using the automated ESyPred3D homology modelling programme (Lambert *et al*., 2002). Alignments are obtained by combining, weighting, and screening the results of several multiple alignment programmes. After submitting the rFab sequences, atomic coordinate PDB files were created and the final 3-dimensional structure built from these files using the Deep View Swiss-PDB Viewer modelling package software (which can be downloaded from http://au.expasy.org/spdbv/).

2.20.1. Requirements for homology modelling

Three items of input were needed to perform homology modelling:

i. The target protein sequence with unknown 3D structure.
ii. A template protein - chosen because it exhibits the highest sequence identity with the target sequence and it has a known 3D structure. The template 3D structure must be determined by reliable empirical methods. These structures are from the Protein Data Bank (http://www.rcsb.org/pdb/; Berman *et al*., 2000).
iii. An alignment between the target and template sequences.

The homology modelling software arranged the backbone of the target sequence according to that of the template protein. Sequence alignment of the two proteins determined where to position each residue. For a highly successful homology model the template must have ≥ 60% sequence identity with the target for a success rate > 70%. Below 40% sequence identity, serious errors begin to appear more frequently.

2.21. MOLECULAR DOCKING

PatchDock (http://bioinfo3d.cs.tau.ac.il/PatchDock/FAQ.html; Schneidman-Duhovny et al., 2003), an algorithm for molecular docking, was used in an attempt to further define the epitope that rFab 126 binds to on the insulin molecule. PDB files of insulin and the rFab are entered online and a list of potential complexes, sorted by shape complementarity criteria, was returned. The files were downloaded and the antigen-antibody complex viewed using the Deep View Swiss-PDB-Viewer.

CHAPTER 3

3. RESULTS

3.1. HYBRIDOMA CELL LINES

In this study, four hybridoma cell lines (1E2, HB-123, HB-126, and HB-127) that secreted monoclonal antibodies against insulin and B-cell clone (DP-D) that secretes monoclonal antibodies against GAD65 were used.

The 1E2 hybridoma cell line was chosen because the epitope that the 1E2 mAb binds to on insulin had been mapped to the N-terminus of the B chain (B1, B3, B10 and B13; Crowther *et al.*, 1994). The remaining three hybridoma cell lines that secrete mAbs against insulin were selected for epitope analysis studies due to their commercial availability. The GAD65 mAb DP-D was selected for epitope analysis of autoantibodies present in the sera of diabetic patients.

3.1.1. Fusion partners

All hybridomas were generated using the non-secreting fusion partner Sp2/0-Ag14, except for 1E2 where NS0 was used. NS0 is also a non-secreting fusion partner. Both NS0 and Sp2/0-Ag14 are mutant myeloma cells, which do not secrete their own immunoglobulins, derived from the original MOPC-21 tumour (Carroll *et al.*, 1988). Thus, the hybridoma cell lines only secrete antibodies originating from the B cell fusion partner. However, because these cells can still synthesise nontranslatable immunoglobulin RNA transcripts, the analysis of the immunoglobulin cDNA from the hybridoma cells is problematic.

3.2. ISOLATION OF RNA

3.2.1. Determination of total RNA concentration

Total RNA was extracted from the five hybridoma cell lines using the RNeasy kit from Qiagen. The concentration of total RNA (Table 3.1) was determined spectrophotometrically (OD_{260}) ([RNA] = OD_{260} x 0.04 µg/µl x dilution factor) and an aliquot run on a 1% agarose gel to confirm the presence of intact total RNA (Figure 3.1).

Table 3.1: Total RNA concentration extracted from the five hybridoma cell lines.

HYBRIDOMA CELL LINE	No. OF CELLS	RNA CONCENTRATION
1E2	~ 6.0×10^6	480 ng/µl
NS0	~ 5.6×10^6	380 ng/µl
HB-123	~ 5.4×10^6	295 ng/µl
HB-126	~ 6.0×10^6	340 ng/µl
HB-127	~ 6.4×10^6	355 ng/µl
DP-D	~ 5.7×10^6	336 ng/µl

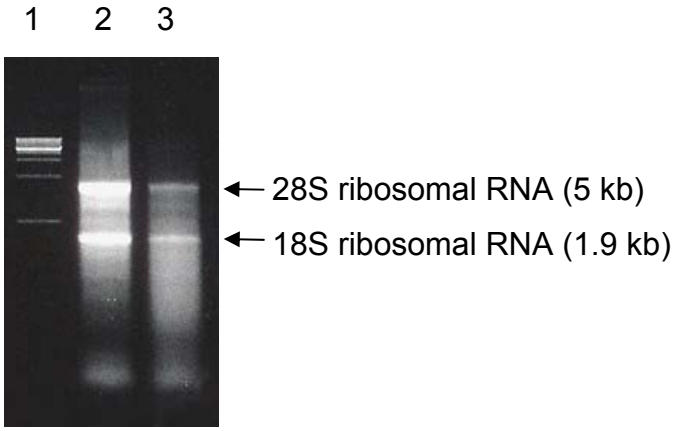


Figure 3.1: Photograph of total RNA extracted from the 1E2 and NSO (fusion partner) cell lines and run on a 1% agarose gel.
Lane 1: Molecular weight marker, Lane 2: 1E2 total RNA (15 µl), Lane 3: NS0 total RNA (15 µl).

3.2.2. Isolation and concentration determination of mRNA

mRNA was immediately isolated from the total RNA samples using Oligotex spin columns from Qiagen and the concentration determined spectrophotometrically (Table 3.2).

Table 3.2: The concentration of mRNA.

HYBRIDOMA CELL LINE	OD_{260}	mRNA CONCENTRATION
1E2	0.0297	30 ng/µl
NS0	0.0185	18.5ng/µl
HB-123	0.1514	24 ng/µl
HB-126	0.0776	12.5 ng/µl
HB-127	0.0915	14.5 ng/µl
DP-D	0.1148	18.4 ng/µl

Initially, our aim was to produce a single-chain Fv fragment that contained the antigen binding domain of an anti-insulin IgG monoclonal antibody secreted by the 1E2 cell line.

3.3. CONSTRUCTION OF THE scFv FRAGMENT

3.3.1. PCR amplification of the 1E2 anti-insulin mAb heavy and light chain variable genes

Messenger RNA was isolated from the 1E2 anti-insulin producing hybridoma cells and the NS0 cell line. NS0 was the fusion partner used for the production of the 1E2 hybridoma cell line. cDNA was synthesised from the mRNA and PCR performed using immunoglobulin V region specific oligonucleotide primers (Figure 3.2). PCR products of the amplified light and heavy chain variable region genes are in the range of 300 – 340bp. With the use of the heavy chain specific primers, two different V_H cDNA products, in the correct size range (~ 340 bp and ~ 300 bp), were obtained from the 1E2 hybridoma cell line and a single PCR product (~300 bp) was observed in the NS0 cell line (Figure 3.2A). The light chain specific primers produced a single PCR band in the correct size range (~ 330 bp) which

was present in both the 1E2 hybridoma and NS0 cell line (Figure 3.2B). Despite numerous attempts to optimise the PCR (e.g. adjusting the annealing temperature, $MgCl_2$ concentrations, use of different Taq polymerases) it was not possible to remove the spurious bands that are visible in Figure 3.2. However, because these were not in the predicted size range of the heavy and light chain gene PCR products, they did not interfere with downstream applications.

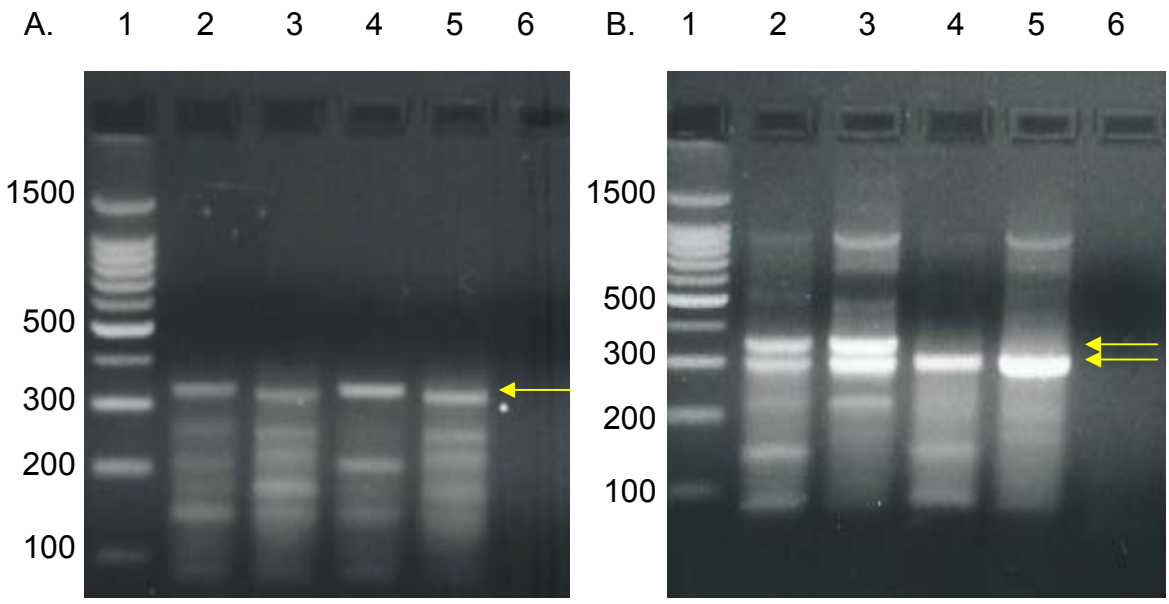


Figure 3.2: PCR of murine immunoglobulin variable regions from 1E2 and NS0.

A. Light chain variable region amplification.

Lane 1: Marker (Promega, 100bp DNA ladder; 100, 200, 300, 400, 500, 600, 700, 800, 900, 1 000, 1 500); Lanes 2-3: 1E2 light chain gene product; Lanes 4-5: NS0 light chain gene product; Lane 6: Negative control.

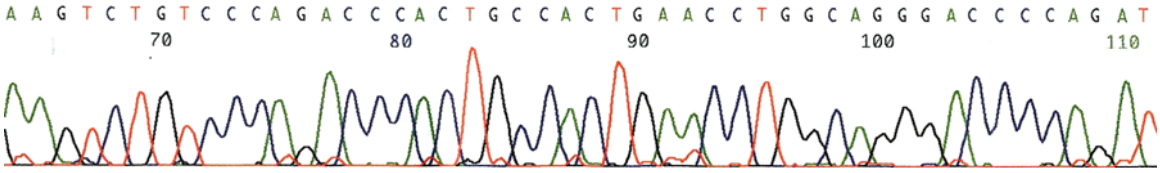
B. Heavy chain variable region amplification.

Lane 1: Marker (Promega, 100bp DNA ladder; 100, 200, 300, 400, 500, 600, 700, 800, 900, 1 000, 1 500); Lanes 2-3: 1E2 heavy chain gene product; Lanes 4-5: NS0 heavy chain gene product; Lane 6: Negative control.

Aberrant heavy (Vidarsson *et al.*, 2001) and light (Carroll *et al.*, 1988) chain mRNA transcripts have been reported in standard fusion partners derived from the original MOPC-21 tumour. In order to determine whether or not we amplified the

functional heavy and light chain genes, and not the aberrant transcripts, the PCR products (indicated by an arrow in Figure 3.2) were cut out of the agarose gel, purified, and sequenced directly (Figure 3.3). The nucleotide and deduced amino acid sequence of the variable light (Figure 3.4) and variable heavy (Figure 3.5) chains are shown for 1E2 and NS0. Results are only shown for the lower 1E2 heavy chain PCR product (Figure 3.2B) as we were unable to obtain readable sequence for the larger heavy chain band.

A.



B.

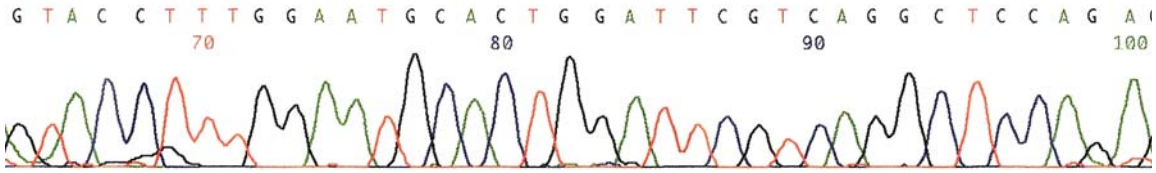


Figure 3.3: Electropherogram of sequencing results obtained for:

A. NS0 light chain (VK1FOR).

B. 1E2 heavy chain (VH1BACK).

```
cagctgacccagtctccagcttccttagctgtatctctggggcagagggccaccatctcatacagg
 Q  L  T  Q  S  P  A  S  L  A  V  S  L  G  Q  R  A  T  I  S  Y  R
gccagcaaaagtgtcagtacatctggctatagttatatgcactggaaccaacagaaaccaggacag
 A  S  K  S  V  S  T  S  G  Y  S  Y  M  H  W  N  Q  Q  K  P  G  Q
ccacccagactcctcatctatcttgtatccaacctagaatctggggtccctgccaggttcagtggc
 P  P  R  L  L  I  Y  L  V  S  N  L  E  S  G  V  P  A  R  F  S  G
agtgggtctgggacagacttcaccctcaacatccatcctgtggaggaggaggatgctgcaacctat
 S  G  S  G  T  D  F  T  L  N  I  H  P  V  E  E  E  D  A  A  T  Y
tactgtcagcacattagggagcttacacgttcggaggggggcaccaagctggaaataa
 Y  C  Q  H  I  R  E  L  T  R  S  E  G  A  P  S  W  K  *
```

Figure 3.4: Nucleotide and deduced amino acid sequence of 1E2/NS0 light chain variable region.

A single sequence is shown as the sequences obtained for 1E2 and NS0 were identical.

A.
```
ggaggcttagtgcagcctggagggtcccggaaactctcctgtgcagcctctggattcactttcagt
 G  G  L  V  Q  P  G  G  S  R  K  L  S  C  A  A  S  G  F  T  F  S
acctttggaatgcactggattcgtcaggctccagagaggggactggagtgggtcgcatacattagt
 T  F  G  M  H  W  I  R  Q  A  P  E  R  G  L  E  W  V  A  Y  I  S
agtggcagtagtgccatctactatgcagactcagtaaagggccgtttcaccatctccagagacaat
 S  G  S  S  A  I  Y  Y  A  D  S  V  K  G  R  F  T  I  S  R  D  N
cccaagaacaccctgttcctgcaaatgaccagtctaaggtctgaggacacggccatgtattactgt
 P  K  N  T  L  F  L  Q  M  T  S  L  R  S  E  D  T  A  M  Y  Y  C
gcaaga
 A  R
```

B.
```
ggaggcttagtgcagcctggagggtcccggaaactctcctgtgcagcctctggattcactttcagt
 G  G  L  V  Q  P  G  G  S  R  K  L  S  C  A  A  S  G  F  T  F  S
agctttggaatgccctgggttcgtcaggctccagagaaggggctggagtgggtcgcatacattagt
 S  F  G  M  P  W  V  R  Q  A  P  E  K  G  L  E  W  V  A  Y  I  S
agtggcagtagtaccctccactatgcagacacagtgaagggccgattcaccatctccagagacaat
 S  G  S  S  T  L  H  Y  A  D  T  V  K  G  R  F  T  I  S  R  D  N
cccaagaacaccctgttcctgcaaatgaaactaccctcactatgctatggactactggggtcaagg
 P  K  N  T  L  F  L  Q  M  K  L  P  S  L  C  Y  G  L  L  G  S  R
```

Figure 3.5: Nucleotide and deduced amino acid sequence of:

A. 1E2 heavy chain variable region (lower band ~ 300 bp)

B. NS0 heavy chain variable region.

The 1E2 and NS0 heavy chain sequences were aligned for homology comparison (Figure 3.6). The 1E2 and NS0 heavy chains were 95% homologous at the nucleotide level (Appendix 2).

```
                1       10        20        30        40        50   55
                |-------+---------+---------+---------+---------+----|
          1E2   GGLVQPGGSRKLSCAASGFTFSTFGMHWIRQAPERGLEWVAYISSGSSAIYYADS
          NS0   GGLVQPGGSRKLSCAASGFTFSSFGMPWVRQAPEKGLEWVAYISSGSSTLHYADT
    Consensus   GGLVQPGGSRKLSCAASGFTFSsFGMhW!RQAPErGLEWVAYISSGSSaihYADs

                56      65        75        85   90
                |-------+---------+---------+----|
          1E2   VKGRFTISRDNPKNTLFLQMTSLRSEDTAMYYCAR
          NS0   VKGRFTISRDNPKNTLFLQM-KLPSLCYGLLGSR
    Consensus   VKGRFTISRDNPKNTLFLQM.kLrSedta$lgca.
```

Figure 3.6: 1E2 and NS0 heavy chain amino acid sequence alignment.

These sequences were then compared to the published sequences for the aberrant kappa and heavy chain transcripts. The nucleotide sequence obtained for the 1E2/NS0 kappa chain PCR products showed 99% homology (Appendix 3)

to the kappa chain transcript reported by Carroll *et al.* (1988). The 1E2 heavy chain nucleotide sequences exhibited 81% and 86% homology (Appendix 4) to two fragments of the aberrant heavy chain transcript (AberrG7) described by Vidarsson and colleagues (2001). The NS0 heavy chain nucleotide sequence was 72% identical to the AberrG7 aberrant heavy chain transcript (Appendix 5).

Expression levels of the non-functional kappa chain transcript must have been relatively high in the 1E2 hybridoma cell line, exceeding that of the 1E2 functional kappa chain transcript. Thus, we were only able to amplify the aberrant 1E2 light chain transcript. Similarly, it appeared that we were only able to amplify the aberrant heavy chain transcript. It was, therefore, decided to construct a Fab fragment instead of the scFv.

3.4. CONSTRUCTION OF THE FAB FRAGMENTS

3.4.1. Amplification of the light chain and Fd fragment of the heavy chain of the five mAbs

The mRNA was isolated from four anti-insulin producing hybridoma cell lines (1E2, HB-123, HB-126, and HB-127), NS0 (the fusion partner for 1E2) and the DP-D B cell clone that secretes monoclonal antibodies against GAD65 (Madec *et al*., 1996). mRNA was reverse transcribed to produce cDNA which was used in the subsequent PCR reactions to amplify the heavy and light chain fragments of the anti-insulin and anti-GAD65 monoclonal antibodies. The V_HC_H1 construct was created with primers representing the leader sequence 5 to 8 amino acids 5' of the variable heavy chain framework 1 region and amino acids 205 to 220 of the first constant heavy chain region. The V_LC_L construct was created with primers representing the leader sequence 5 to 8 amino acids 5' of the variable light chain framework 1 region and amino acids 205 to 220 of the constant light chain region. Control (negative) PCR reactions were set up for each primer pair. Figure 3.7 illustrates the results obtained from the amplification of mAb 126 light and heavy chain genes. Similar results were obtained from amplification of the other four mAbs (results not shown). Table 3.3 summarises the results obtained from the

amplification of the heavy and light chain regions of the five monoclonal antibodies studied and those obtained for the NS0 myeloma cell line.

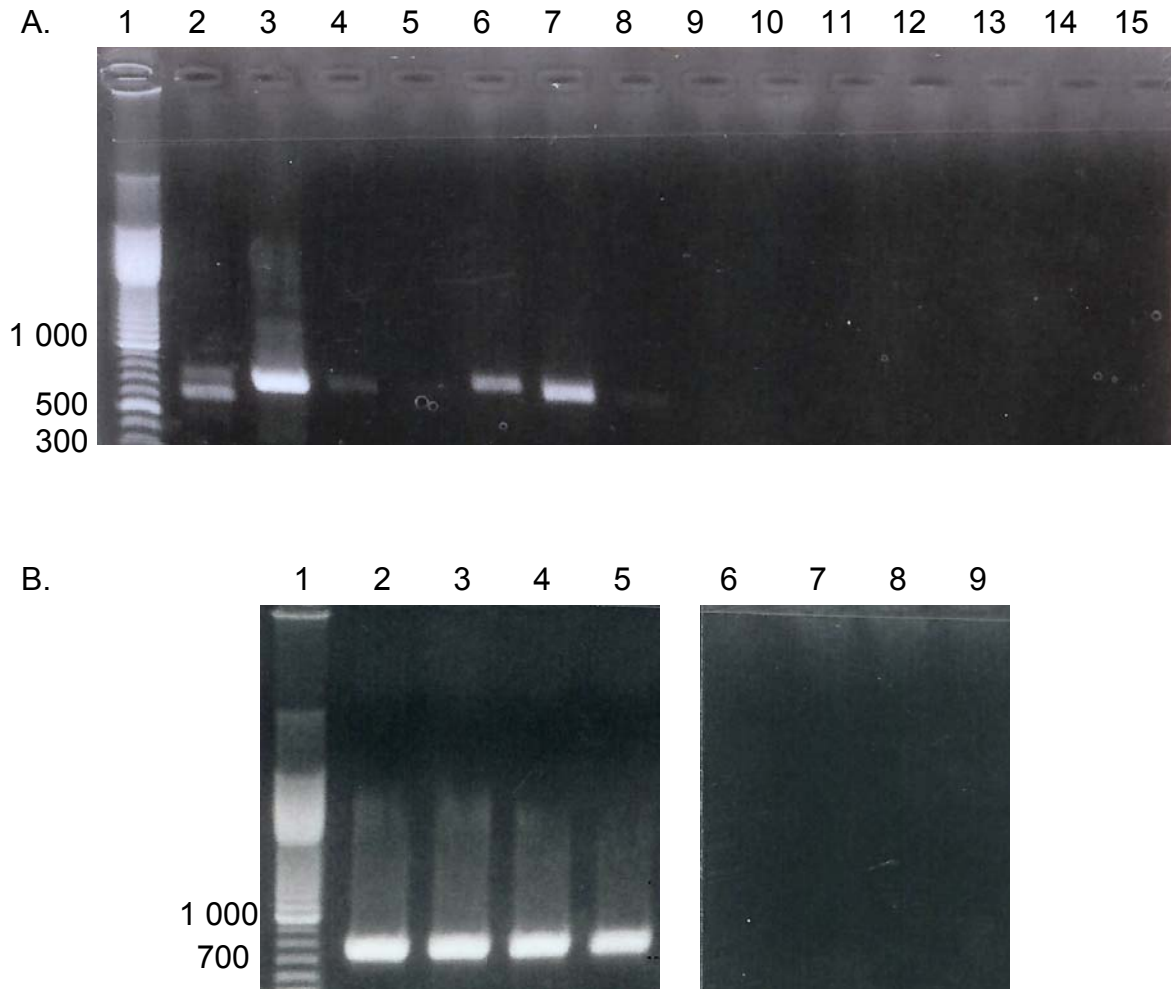


Figure 3.7: A. PCR amplification of the light chain regions from mRNA isolated from insulin-specific hybridoma cell line HB-126.

Lane 1: Marker (Promega, 100bp DNA ladder), Lanes 2-8: PCR products obtained with the light chain primers 2-6, 8, and 9, respectively; Lanes 9-15: Negative controls for the light chain primers 2-6, 8, and 9.

B. PCR amplification of the Fd heavy chain fragment from mRNA isolated from insulin-specific hybridoma cell line HB-126.

Lane 1: Marker (Promega, 100bp DNA ladder), Lanes 2-5: PCR products obtained with the heavy chain primers 1-4, respectively; Lanes 6-9: Negative controls for the heavy chain primers 1-4.

Table 3.3: PCR products amplified from the five hybridoma cell lines using ten pairs of primers specific for the heavy and the light chain genes.

CELL LINE	PCR AMPLIFICATION WITH PRIMER PAIR:									
	1	2	3	4	5	6	7	8	9	10
NS0 (L)			✓							
NS0 (H)								✓		
1E2 (L)		✓	✓	✓	✓			✓	✓	
1E2 (H)								✓		
HB-123 (L)		✓	✓	✓	✓		✓			✓
HB-123 (H)	✓	✓	✓	✓		✓				
HB-126 (L)		✓	✓	✓	✓	✓		✓	✓	
HB-126 (H)	✓	✓	✓	✓						
HB-127 (L)		✓	✓	✓	✓				✓	
HB-127 (H)	We were not able to amplify the heavy chain									
DP-D (L)	✓	✓	✓	✓	✓	✓	✓			
DP-D (H)	✓	✓	✓	✓	✓	✓				

L = Light chain, H = Heavy chain

As can be seen from Table 3.3, we again had amplification of heavy and light chain genes from the NS0 cell line. The PCR product obtained for the 1E2 light chain primer pair 3 was, as a result, not used in any further downstream applications due to the presence of an amplification product, with the same primer pair, in the NS0 cell line. A single heavy chain PCR product was amplified for the 1E2 and NS0 heavy chain (primer 8). This PCR product was sequenced and compared with that obtained for the 1E2 heavy chain when constructing the scFv (Figure 3.5). When the two sequences were aligned, they showed 100% homology for the variable region of the heavy chain gene (Figure 3.8). Due to the amplification of the aberrant 1E2 heavy chain transcript, no further experiments were carried out on this cell line.

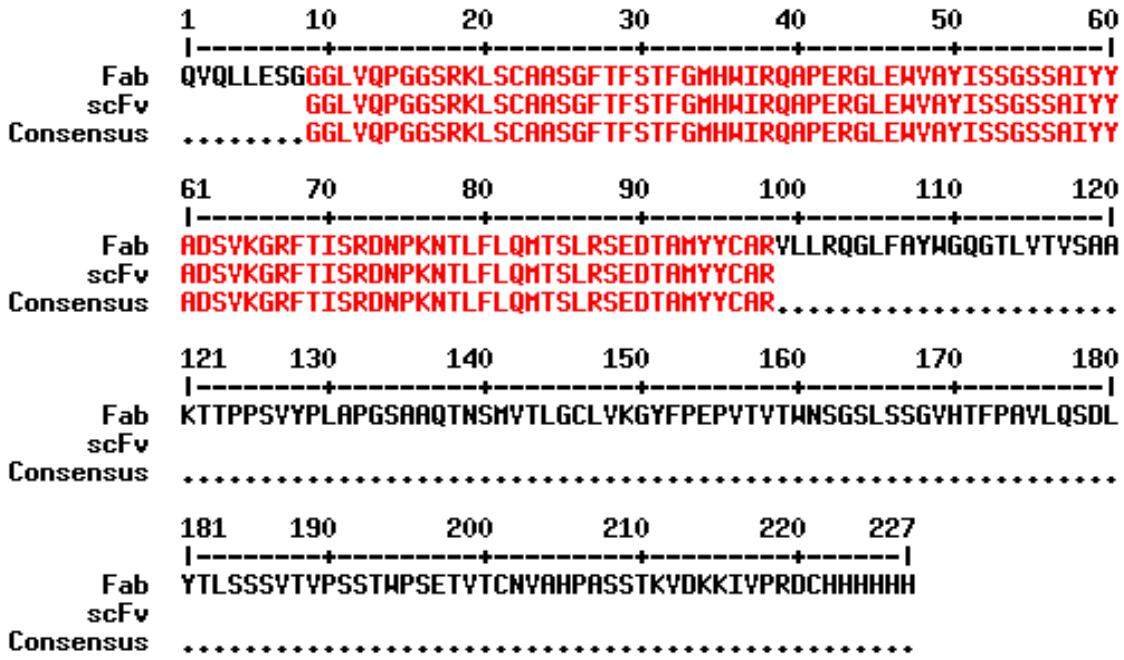
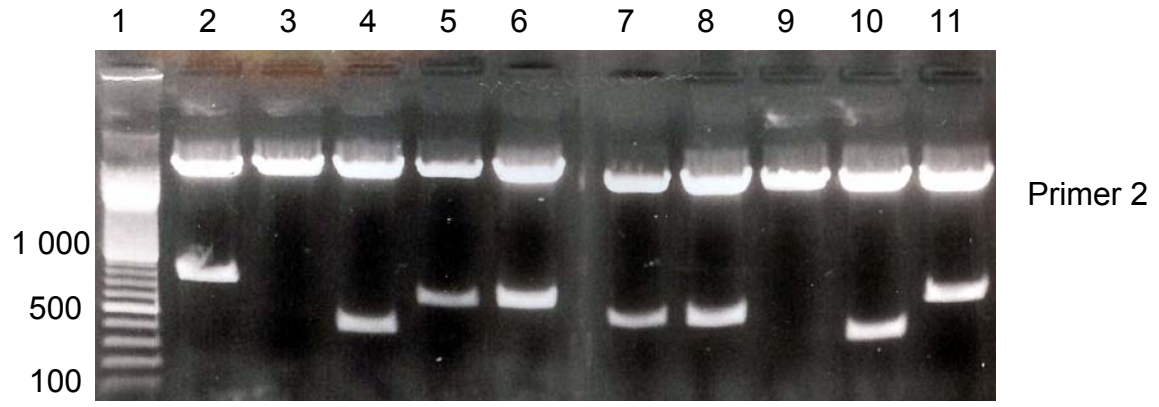


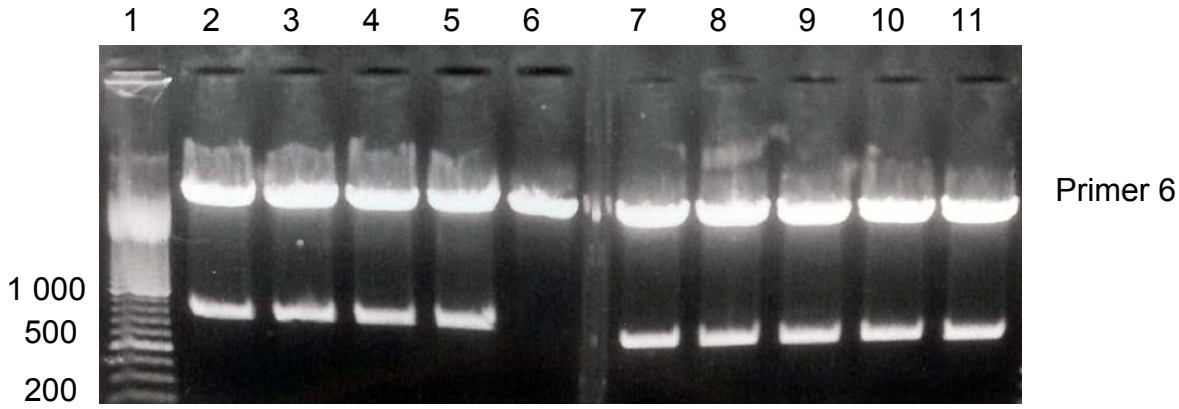
Figure 3.8: 1E2 scFv and Fab heavy chain amino acid sequence alignment.

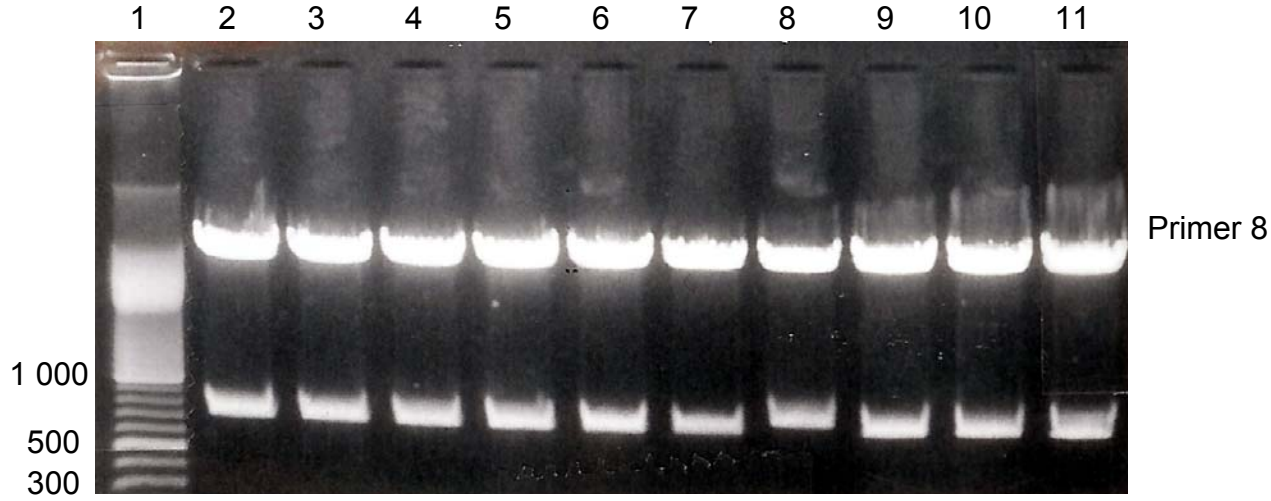
3.4.2. Subcloning of the heavy and light chains into the TOPO vector for sequencing

Following PCR amplification of the Fd heavy and light chain genes from the four cell lines (HB-123, HB-126, HB-127, and DP-D), all PCR products in the correct size range (approximately 660 bp; Table 3.3) were cut out of the gel, purified and ligated into the TOPO vector. Ultracompetent *E. coli* cells were transfected with the resultant TOPO vector and colonies allowed to grow on LA plates overnight. To ensure that the colonies contained the TOPO vector with the immunoglobulin insert, plasmids were isolated using alkaline lysis miniprep procedures and an aliquot of the plasmid DNA digested with *Eco*RI (this restriction enzyme flanks the insert site) to confirm the presence and size of the insert (Figure 3.9).

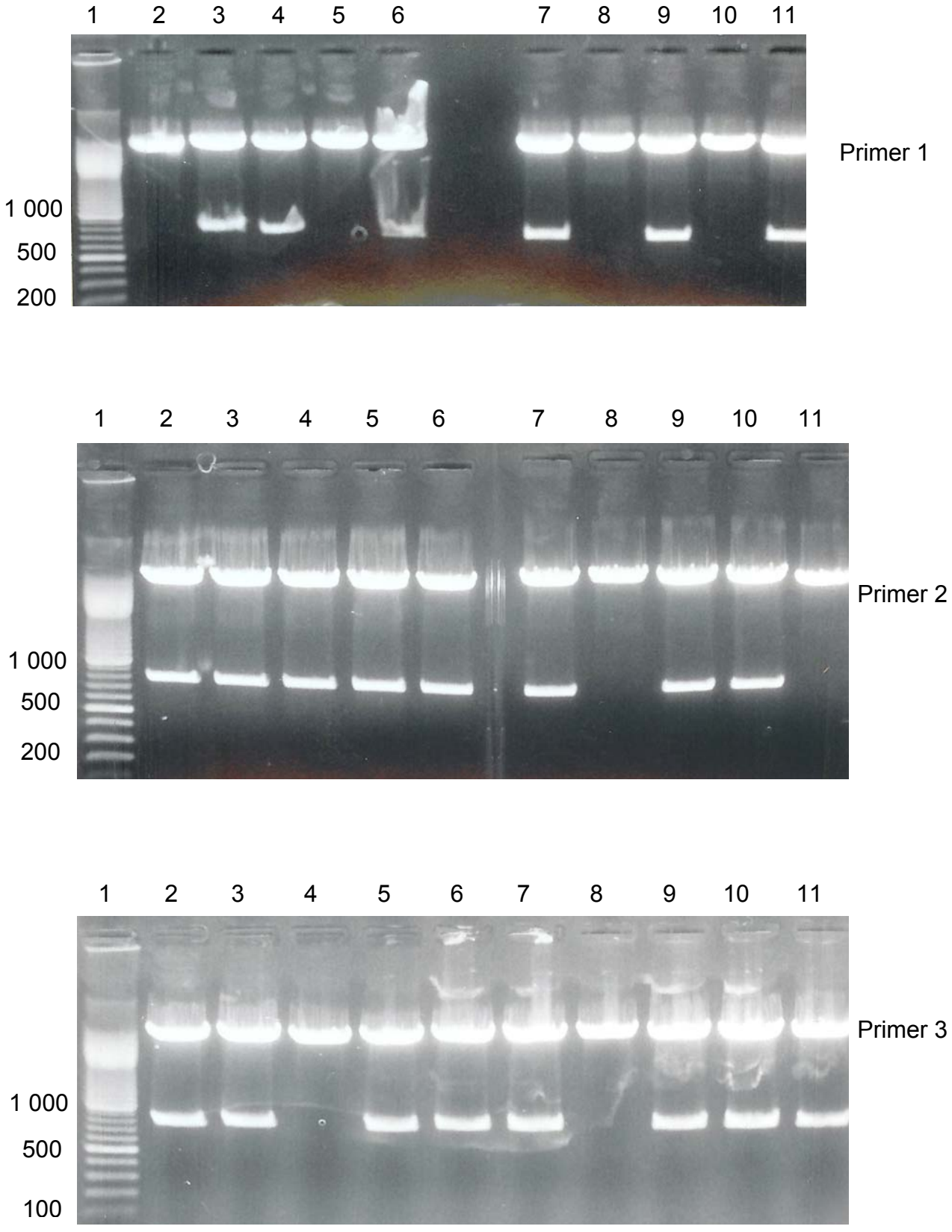
A.


1 2 3 4 5 6 7 8 9 10 11
1 000
500
100
Primer 2


1 2 3 4 5 6 7 8 9 10 11
1 000
500
200
Primer 6


1 2 3 4 5 6 7 8 9 10 11
1 000
500
300
Primer 8

B.


1 2 3 4 5 6 7 8 9 10 11
1 000
500
200
Primer 1
1 2 3 4 5 6 7 8 9 10 11
1 000
500
200
Primer 2
1 2 3 4 5 6 7 8 9 10 11
1 000
500
100
Primer 3

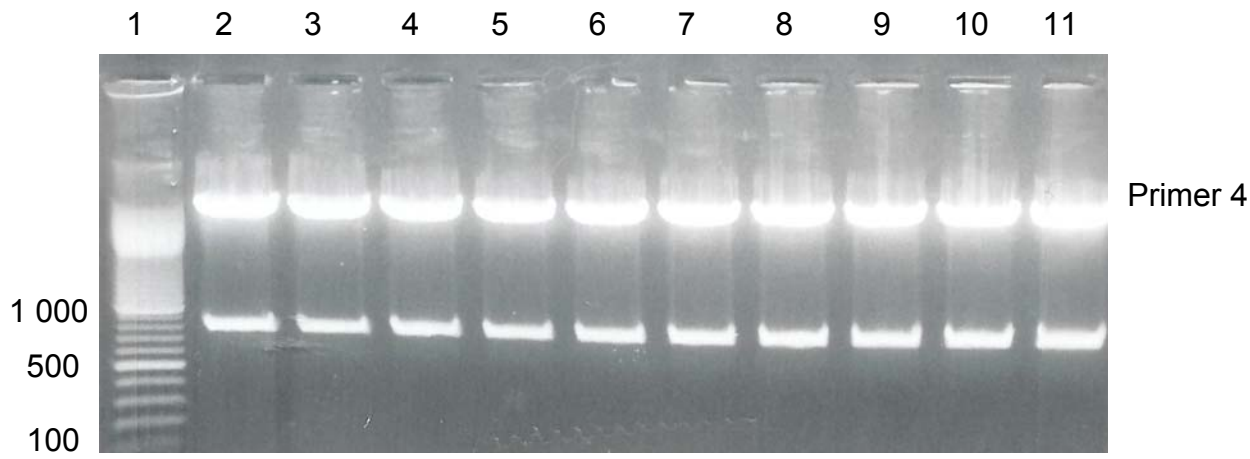


Figure 3.9: Screening of murine immunoglobulin light chain recombinant plasmids: a 1% agarose gel showing the *Eco*RI digested TOPO vector and the presence or absence of the approximately 700 bp A) HB-126 light chain insert [obtained from amplification with light chain specific primers 2, 6, and 8] and B) HB-126 heavy chain insert (obtained from amplification with heavy chain specific primers 1, 2, 3, and 4).

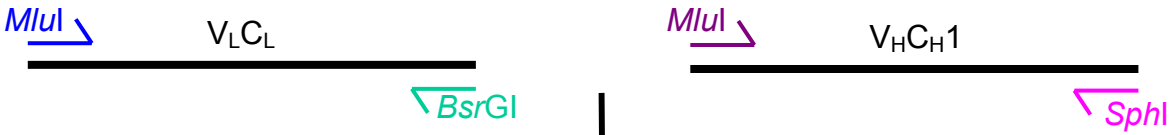
A. Lane 1: Marker (Promega, 100bp DNA ladder), Lanes 2-11: Plasmid miniprep samples digested with *Eco*RI.

B. Lane 1: Marker (Promega, 100bp DNA ladder), Lanes 2-11: Plasmid miniprep samples digested with *Eco*RI.

Recombinant plasmids with the correct size heavy/light chain insert, for each primer pair that amplified a PCR product, from each of the four cell lines (HB-123, HB-126, HB-127, and DP-D), were selected, and sequenced. All the heavy and light chain sequences for a particular mAb were aligned and analysed using the computer software programme Sequencher. A single heavy and light chain sequence, for each mAb, was selected based on the alignments. These nucleotide sequences were blasted against known sequences in GenBank to confirm the presence of immunoglobulin sequence (data not shown). A single recombinant plasmid containing the immunoglobulin light chain insert and a recombinant plasmid containing the immunoglobulin heavy chain insert, for each mAb, were selected for the production of rFab fragments.

3.4.3. Construction of expression plasmids for production of rFab fragments

The cloned mAb-derived light chains and Fd fragments of the heavy chains were reamplified with primer pairs to introduce the restriction sites necessary for directional subcloning of the PCR products into the pCXII vector (4.7 kb). The heavy chain fragment was subsequently isolated from the plasmid using *Bsr*GI and *Hind*III restriction sites flanking the insert. This was ligated into the pCXII vector containing the light chain gene insert using the same enzymes. The entire light and heavy chain construct was then cut out with *Eco*RI and *Hind*III and ligated into the expression vector pAK19 (6.4 kb). The ligated plasmids were transformed into competent *E. coli* 25F2 cells, and recombinant bacteria identified using carbenicillin resistance as a selective marker. To ensure that the colonies contained the expression vector with an intact light chain and Fd fragment of the heavy chain, vectors were isolated using alkaline lysis miniprep procedures and an aliquot of the plasmid DNA digested with *Mlu*I and *Sph*I. This digest will effectively result in the presence of two bands visible upon agarose gel electrophoresis; the *Mlu*I restriction sites flank the light chain insert and *Mlu*I and *Sph*I are situated 5' and 3', respectively, of the heavy chain insert. The construction of the pAK19 expression vector is summarised in the flow diagram below. All results shown are for the construction of the 126 Fab. Similar results were obtained for mAbs 123 and DP-D. An expression vector could not be constructed for mAb 127 as we were not able to amplify the heavy chain gene.



Heavy and light chain PCR products were reamplified with primers introducing restriction sites at the 5' and 3' ends.

PCR products were checked on an agarose gel (Figure 3.10).

PCR products were digested (Figure 3.11), purified, cloned into the TOPO vector and ultracompetent *E. coli* cells transfected.

The presence of the heavy and light chain in the TOPO vector was confirmed by restriction digest with *Eco*RI (Figure 3.12).

The pC vector was digested with *Mlu*I/*Sph*I and *Mlu*I/*Bsr*GI (Figure 3.13) for cloning of the heavy and light chain, respectively.

*Mlu*I/*Sph*I and *Mlu*I/*Bsr*GI digested heavy and light chains were cloned into the pC vector and ultracompetent *E. coli* cells

The presence of the heavy and light chain in the pC vector was confirmed by restriction digest with *Eco*RI/*Hind*III (Figure 3.14).

The pC vector with the light chain insert was digested with *Bsr*GI/*Hind*III (Figure 3.15) and the product purified.

The heavy chain was cut from the pC vector by digestion with *Bsr*GI/*Hind*III (Figure 3.16) and purified.

The heavy chain was ligated into the pC vector containing the light chain insert. *E. coli* cells were transfected and recombinant plasmids digested with *Eco*RI/*Hind*III to confirm the presence of the immunoglobulin heavy and light chains (Figure 3.17).

↓

The *Eco*RI/*Hind*III insert was purified and ligated to the similarly digested and purified expression vector pAK19 (Figure 3.18).

E. coli cells were transfected with the pAK19 vector. Plasmids were digested with *Eco*RI/*Hind*III to confirm the presence of the immunoglobulin heavy and light chains (Figure 3.19).

↓

The presence of an intact light and heavy chain insert was confirmed by restriction digest with *Mlu*I/*Sph*I (Figure 3.20).

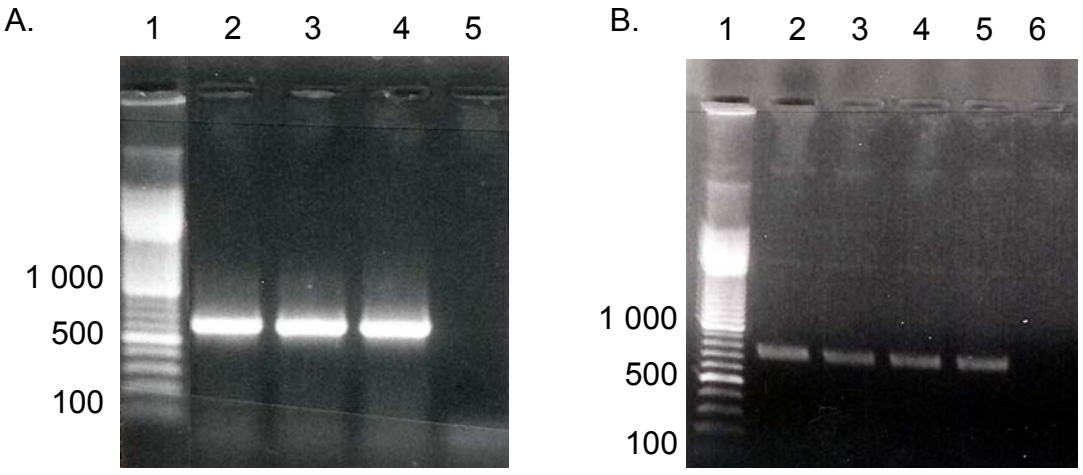


Figure 3.10: PCR products, run on a 1% agarose gel, of the reamplified heavy and light chain fragments using primers incorporating restriction sites at the 5' and 3' sites.

A. Heavy chain PCR products. Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2-4: PCR products; Lane 5: Negative control.

B. Light chain PCR products. Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2-5: PCR products; Lane 6: Negative control.

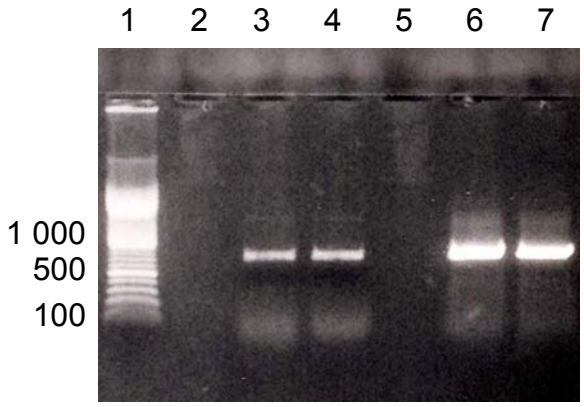
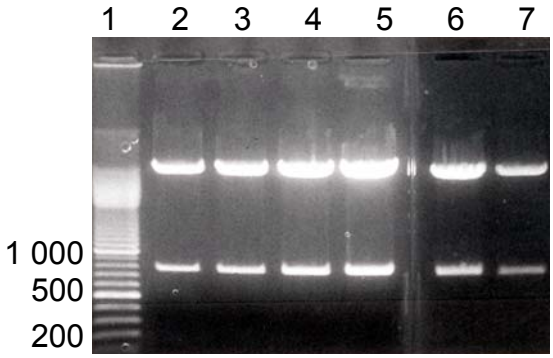


Figure 3.11: Digested heavy and light chain PCR products run on a 1% agarose gel.

Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 3-4: Light chain digested with *Mlu*I/*Bsr*GI; Lanes 6-7: Heavy chain digested with *Mlu*I/*Sph*I.

A.



B.

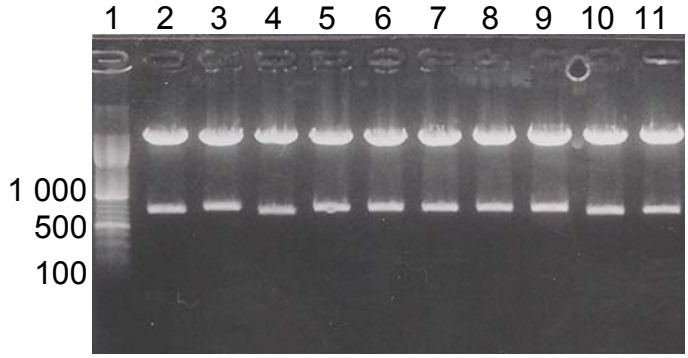


Figure 3.12: TOPO vector digested with *Eco*RI.
A. Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2-7: Heavy chain insert (~ 720 bp). B. Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2-11: Light chain insert (~ 700 bp).

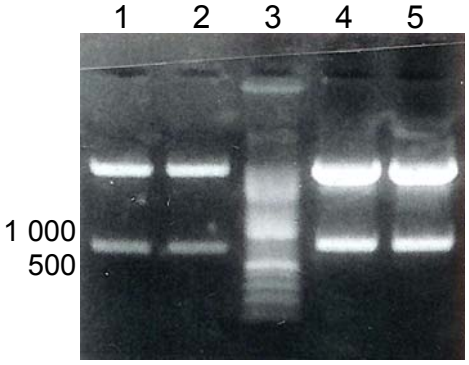


Figure 3.13: pCXII vector (containing unrelated immunoglobulin heavy and light chain) digested with *Mlu*I/*Bsr*GI and *Mlu*I/*Sph*I for cloning of the light and heavy chains, respectively.
Lanes 1-2: pCXII digested with *Mlu*I/*Bsr*GI; Lane 3: Marker (Promega, 100bp DNA ladder); Lanes 4-5: pCXII digested with *Mlu*I/*Sph*I.

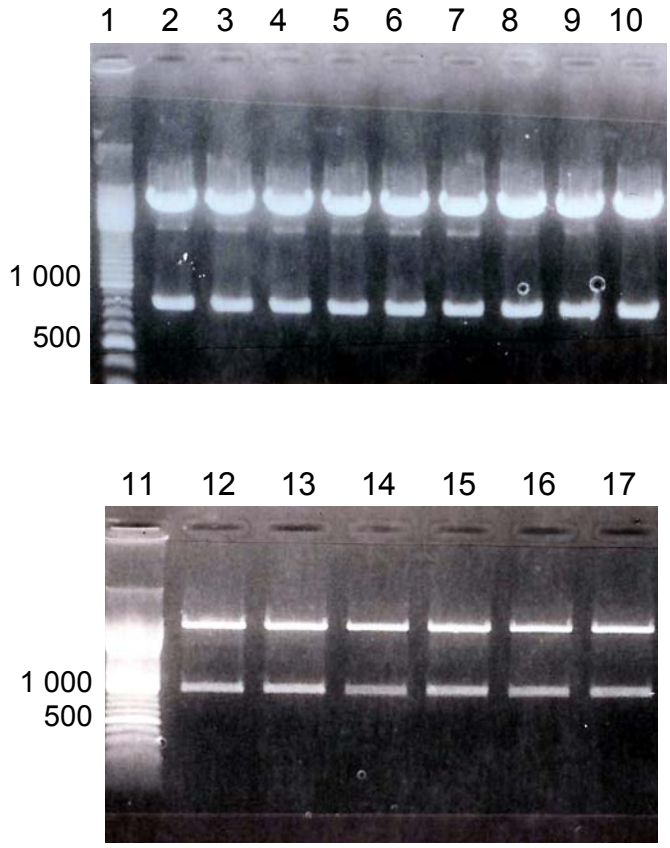


Figure 3.14: pCXII vector digested with *Eco*RI/*Hind*III.
Lane 1 and 11: Marker (Promega, 100bp DNA ladder); Lanes 2-10: pCXII vector with light chain insert; Lanes 12-17: pCXII vector with heavy chain insert.

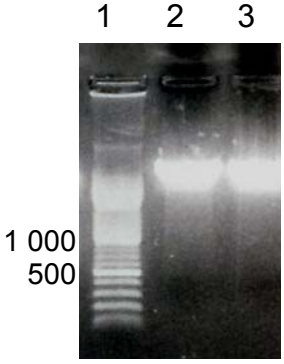


Figure 3.15: pCXII vector with light chain insert digested with *Bsr*GI/*Hind*III.
Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2-3: Digested pCXII vector.

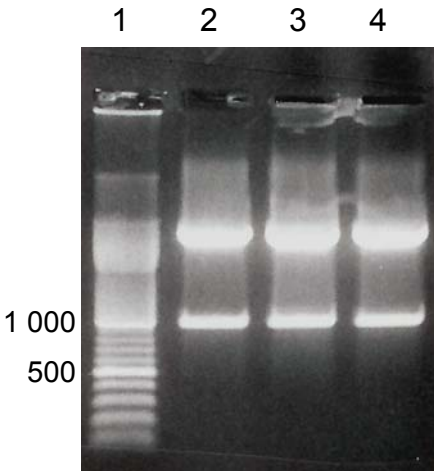


Figure 3.16: pCXII vector with heavy chain insert digested with *Bsr*GI/*Hind*III.
Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2-4: Heavy chain insert (± 1 kb) cut from pCXII.

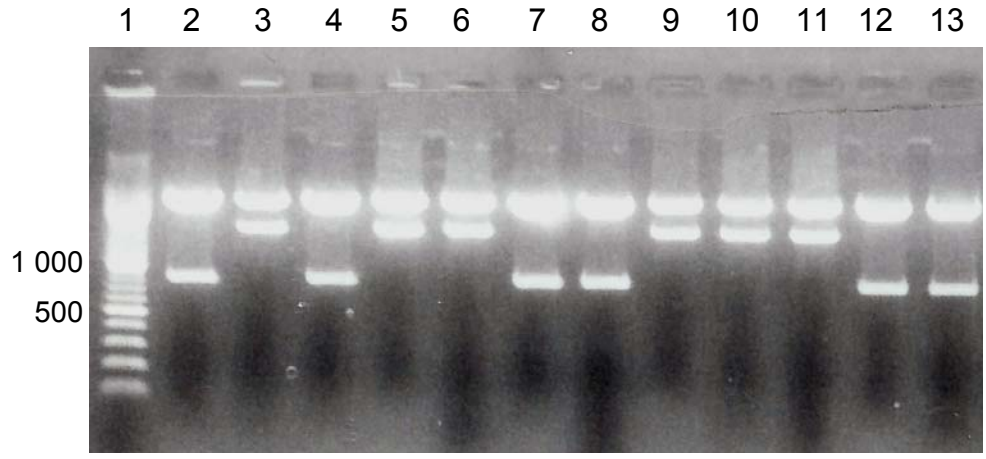


Figure 3.17: Heavy and light chain construct in pCXII vector digested with *Eco*RI/*Hind*III.
Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2, 4, 7-8, 12-13: pCXII with light chain insert only; Lanes 3, 5-6, 9-11: pCXII with heavy and light chain construct.

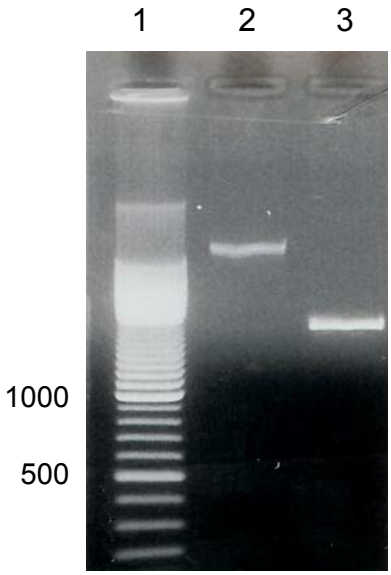


Figure 3.18: Purified heavy and light chain gene construct and pAK19 vector which have been digested with *Eco*RI/*Hind*III for cloning.
Lane 1: Marker (Promega, 100bp DNA ladder); Lane 2: Purified pAK19, Lane 3: Purified heavy chain insert.

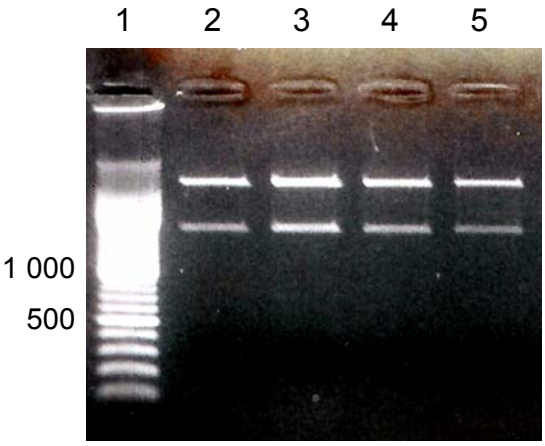


Figure 3.19: Heavy and light chain construct cloned into the pAK19 vector, digested with *Eco*RI/*Hind*III.
Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2-5: pAK19 with heavy and light chain construct.

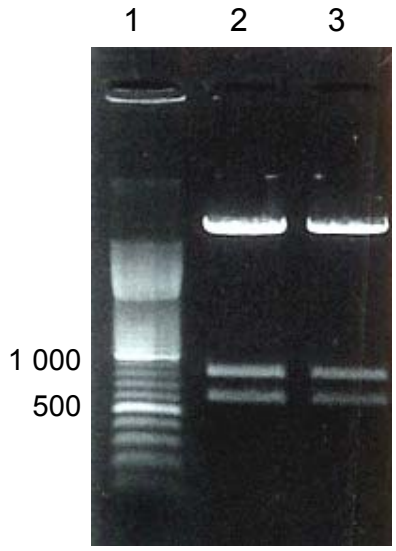


Figure 3.20: Agarose gel electrophoresis of the expression vector pAK19 which has been digested with *Mlu*I and *Sph*I.
Lane 1: Marker (Promega, 100bp DNA ladder); Lanes 2-3: The two lower bands represent the heavy and light chain fragments cut from pAK19 (upper band).

Before performing the expression experiments the heavy and light chain genes in pAK19 were sequenced to ensure that no mutations had been introduced.

3.4.4. Sequence analysis of the heavy and light chains of rFab

The nucleotide and deduced amino acid sequences of rFab 126 and DP-D, determined by cloning and sequencing of the light chain and Fd fragment of the heavy chain genes, are shown in Figure 3.21 and Figure 3.22, respectively. The heavy chain contains the entire variable region plus the first constant domain. The light chain contains the entire variable region plus the constant kappa domain. Results are only shown for those rFab which showed dose dependent binding to their respective antigens i.e., rFab 126 and rFab DP-D.

The variable region protein sequences deduced from the nucleotide sequences were aligned for comparison with germline sequences from the NCBI data bank (http://www.ncbi.nlm.nih.gov/igblast/showGermline.cgi). The nucleotide

sequences for both kappa and heavy chain genes of mAb 126 were submitted to GenBank under accession numbers AY691554 and AY691555, respectively. Sequence analysis of the 126 heavy chain-specific cDNA and comparison of the deduced amino acid sequence revealed that V_H belonged to the mouse IgG heavy chain subgroup 1. Germline Vh45.21.1 (Akolkar *et al*., 1987) displayed the highest degree of identity (94%) with the 126 heavy chain. The 20 nucleotide differences yielded seven amino acid residue replacements located mainly in CDR 1 and CDR 2. Sequence analysis of the light chain-specific cDNA and database comparison of the deduced amino acid sequence revealed that the V_L belonged to the mouse kappa light chain group 1. The germline kappa chain 23.43 (Schable *et al*., 1999) showed 100% identity on the nucleotide level. The predicted amino acid sequence of rFab 126 was aligned with the amino acid sequences from the remaining three anti-insulin secreting hybridoma cell lines; where sequences were available (Figure 3.23).

Sequence analysis of the DP-D heavy chain-specific cDNA and comparison of the deduced amino acid sequence revealed that V_H belonged to the human IgG heavy chain subgroup 1. Germline Vh4.4 displayed the highest degree of identity (95%) with the DP-D heavy chain (Matsuda *et al.*, 1998). The 14 nucleotide differences yielded six amino acid residue replacements distributed randomly in the variable region (i.e. in the framework and complementarity determining regions). Sequence analysis of the light chain-specific cDNA and database comparison of the deduced amino acid sequence revealed that the V_L belonged to the human kappa light chain subgroup 4 (Klobeck *et al*., 1985). The germline kappa chain B3 showed 97% identity on the amino acid level. The three amino acid replacements were located in CDR 1, 2 and 3. The predicted amino acid sequence of rFab DP-D was aligned with the sequences of five other GAD65 rFab (Padoa *et* al., 2003; Figure 3.24). A comparison of the overlapping amino acid sequence in the CDRs of the heavy chains showed that amino acid sequences derived from the human rFab DP-D and DP-A were closely related to each other (60% homology), but while they share the same CDR1 and CDR2 classes, they differ in the CDR3 class. The great variety in CDR classes and sequences between the antibodies suggests that the antibodies do not recognise the same epitopes on GAD65.

A.

```
          gatattgtgctaactcagtctccagccaccctgtctgtgactccaggagatagcgtcagt
           D  I  V  L  T  Q  S  P  A  T  L  S  V  T  P  G  D  S  V  S
ctttcctgcagggccagccaaagtattagcaacaacctacactggtatcaacaaaaatcacatgagtctcca
 L  S  C [R  A  S  Q  S  I  S  N  N  L  H] W  Y  Q  Q  K  S  H  E  S  P
aggcttctcatcaagtatgcttcccagtccatctctgggatcccctccaggttcagtggcagtggatcaggg
 R  L  L  I  K [Y  A  S  Q  S  I  S] G  I  P  S  R  F  S  G  S  G  S  G
acagatttcactctcagtatcaacagtgtggagactgaagattttggaatgtatttctgtcaacagagtaac
 T  D  F  T  L  S  I  N  S  V  E  T  E  D  F  G  M  Y  F  C [Q  Q  S  N
agctggcctcacacgttcggctcggggacaaagttggaaataaaacgggctgatgctgcaccaactgtatcc
 S  W  P  H  T] F  G  S  G  T  K  L  E  I  K  R  A  D  A  A  P  T  V  S
atcttcccaccatccagtgagcagttaacatctggaggtgcctcagtcgtgtgcttcttgaacaacttctac
 I  F  P  P  S  S  E  Q  L  T  S  G  G  A  S  V  V  C  F  L  N  N  F  Y
cccaaagacatcaatgtcaagtggaagattgatggcagtgaacgacaaaatggcgtcctgaacagttggact
 P  K  D  I  N  V  K  W  K  I  D  G  S  E  R  Q  N  G  V  L  N  S  W  T
gatcaggacagcaaagacagcacctacagcatgagcagcaccctcacgttgaccaaggacgagtatgaacga
 D  Q  D  S  K  D  S  T  Y  S  M  S  S  T  L  T  L  T  K  D  E  Y  E  R
cataacagctatacctgtgaggccactcacaagacatcaacttcacccattgtcaagagcttcaacaggaat
 H  N  S  Y  T  C  E  A  T  H  K  T  S  T  S  P  I  V  K  S  F  N  R  N
gagacccgttgttag
 E  T  R  C  *
```

B.

```
            gaggtccagctgcaacagtctggacctgagctggtgaagcctggggcttcagtgaag
             E  V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V  K
atatcctgcaagacttctggatacacattcactgaatacaccatgcactgggtgaagcagagccatggaaag
 I  S  C  K  T  S  G  Y  T  F  T [E  Y  T  M  H] W  V  K  Q  S  H  G  K
agccttgagtggattggaggtattaatcctaacaatggtggttctaactacaaccagaagttcaagggcaag
 S  L  E  W  I  G [G  I  N  P  N  N  G  G  S  N] Y  N  Q  K  F  K  G  K
gccacattgactgtagacaagtcctccagcacagcctacatggagctccgcagcctgacatctgaggattct
 A  T  L  T  V  D  K  S  S  S  T  A  Y  M  E  L  R  S  L  T  S  E  D  S
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 A  V  Y  Y  C  A  R [F  G  A  R  Y  Y  G  S  S  Y  G  S  W  Y  F  D  V]
tggggcgcagggaccacggtcaccgtctcctcagccaaaacgacaccccatctgtctatccactggcccct
 W  G  A  G  T  T  V  T  V  S  S  A  K  T  T  P  P  S  V  Y  P  L  A  P
ggatctgctgcccaaactaactccatggtgaccctgggatgcctggtcaagggctatttccctgagccagtg
 G  S  A  A  Q  T  N  S  M  V  T  L  G  C  L  V  K  G  Y  F  P  E  P  V
acagtgacctggaactctggatccctgtccagcggtgtgcacaccttcccagctgtcctgcagtctgacctc
 T  V  T  W  N  S  G  S  L  S  S  G  V  H  T  F  P  A  V  L  Q  S  D  L
tacactctgagcagctcagtgactgtcccctccagcacctggcccagcgagaccgtcacctgcaacgttgcc
 Y  T  L  S  S  S  V  T  V  P  S  S  T  W  P  S  E  T  V  T  C  N  V  A
cacccggccagcagcaccaaggtggacaagaaaattgagacccgttgtcaacaccaccaccaccaccactga
 H  P  A  S  S  T  K  V  D  K  K  I  E  T  R  C  Q  H  H  H  H  H  H  *
```

Figure 3.21: Nucleotide sequence and deduced amino acid sequence of the A) light and B) heavy chain of HB-126 mAb.
The CDRs according to Kabat *et al*. (1991) are boxed and the amino acid residue differences in comparison to the germline sequences are bold.

A.

```
     aacgcgtatgctgacattgtgatgacccagtctccagactccctggctgtgtctctgggcgagagg
      N  A  Y  A  D  I  V  M  T  Q  S  P  D  S  L  A  V  S  L  G  E  R
gccaccatcaactgcaagtccagccagattgttttatacagctccaacaacaagaactatttagcttggtac
 A  T  I  N  C [K  S  S  Q  I  V  L  Y  S  S  N  N  K  N  Y  L  A] W  Y
cagcagaaaccaggacagcctcctaaactgctcatttactgggcatctacccggaaatccggggtccctgac
```

```
 Q  Q  K  P  G  Q  P  P  K  L  L  I  Y [W  A  S  T  R  K  S] G  V  P  D
cgattcagtggcagcgggtctgggacagatttcactctcaccatcagcagcctgcaggctgaagatgtggca
 R  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L  Q  A  E  D  V  A
gtttattactgtcagcaatactatactactccgctcactttcggcggagggaccaaggtggagatcaaacga
 V  Y  Y  C [Q  Q  Y  Y  T  T] P  L  T  F  G  G  G  T  K  V  E  I  K  R
actgtggctgcaccatctgtcttcatcttcccgccatctgatgagcagttgaaatctggaactgcctctgtt
 T  V  A  A  P  S  V  F  I  F  P  P  S  D  E  Q  L  K  S  G  T  A  S  V
gtgtgcctgctgaataacttctatcccagagaggccaaagtacagtggaaggtggataacgccctccaatcg
 V  C  L  L  N  N  F  Y  P  R  E  A  K  V  Q  W  K  V  D  N  A  L  Q  S
ggtaactcccaggagagtgtcacagagcaggacagcaaggacagcacctacagcctcagcagcaccctgacg
 G  N  S  Q  E  S  V  T  E  Q  D  S  K  D  S  T  Y  S  L  S  S  T  L  T
ctgagcaaagcagactacgagaaacacaaagtctacgcctgcgaagtcacccatcagggcctgagctcgccc
 L  S  K  A  D  Y  E  K  H  K  V  Y  A  C  E  V  T  H  Q  G  L  S  S  P
gtcacaaagagcttcaacaggggagagtcttaa
 V  T  K  S  F  N  R  G  E  S  -
```

B.

```
caggtgcagctggtggagtctggcccaggactggtgaagccttcggagaccctgtccctcacctgc
      Q  V  Q  L  V  E  S  G  P  G  L  V  K  P  S  E  T  L  S  L  T  C
actgtttctggtggctccatcagtatttactactggagctggatccgccagcccgccgcgaagggactggag
 T  V  S  G  G  S  I  S [I  Y  Y  W  S] W  I  R  Q  P  A  A  K  G  L  E
tggattgggcgtatctataccaatgggaccaccaattacaacccctccctcaagagtcgagtctccatgtca
 W  I  G [R  I  Y  T  N  G  T  T  N  Y  N  P  S  L  K  S] R  V  S  M  S
gtagacacgtccaagaaccagttctccctgaaactgacctctgtgaccgccgcggacacggccgtatattac
 V  D  T  S  K  N  Q  F  S  L  K  L  T  S  V  T  A  A  D  T  A  V  Y  Y
tgtgcgagacaaggggcctagtgggagccagcggacggcgtgactactttgactactggggccagggaacc
 C  A  R [Q  G  G  L  V  G  A  S  G  R  R  D  Y  F  D  Y] W  G  Q  G  T
ctggtcaccgtctcctcagcctccaccaagggcccatcggtcttcccctggcaccctcctccaagagcacc
 L  V  T  V  S  S  A  S  T  K  G  P  S  V  F  P  L  A  P  S  S  K  S  T
tctgggggcacagcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaac
 S  G  G  T  A  A  L  G  C  L  V  K  D  Y  F  P  E  P  V  T  V  S  W  N
tcaggcgccctgaccagcggcgtgcacaccttcccggctgtcctacagtcctcaggactctactccctcagc
 S  G  A  L  T  S  G  V  H  T  F  P  A  V  L  Q  S  S  G  L  Y  S  L  S
agcgtggtgaccgtgccctccagcagcttgggcacccagacctacatctgcaacgtgaatcacaagcccagc
 S  V  V  T  V  P  S  S  S  L  G  T  Q  T  Y  I  C  N  V  N  H  K  P  S
aacaccaaggtggacaagaaagttgagcccaaatcttgtgacaaaactagtacatgcaagggcgaacaccac
 N  T  K  V  D  K  K  V  E  P  K  S  C  D  K  T  S  T  C  K  G  E  H  H
caccaccaccactga
 H  H  H  H  -
```

Figure 3.22: Nucleotide sequence and deduced amino acid sequence of the A) light and B) heavy chain of DP-D mAb.

The CDRs according to Kabat *et al*. (1991) are boxed and the amino acid residue differences in comparison to the germline sequences are bold.

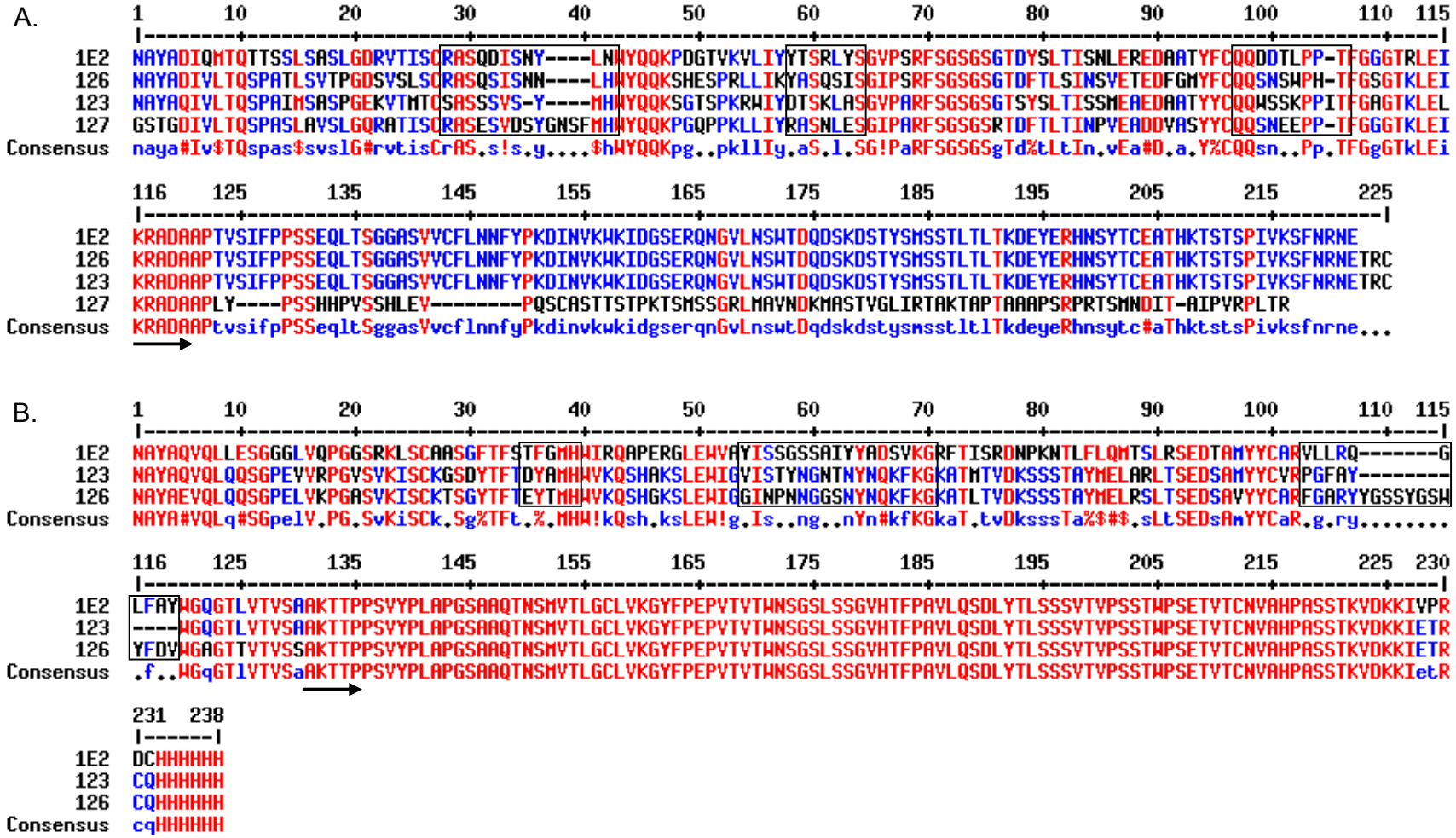


Figure 3.23: Light (A) and heavy (B) chain amino acid alignment for those cell lines for which sequence data was available. The CDRs are boxed (Kabat *et al.*, 1991) and the start of the constant regions shown by the arrow (⟶). High consensus is shown in red and low consensus in blue. Consensus symbols: ! is anyone of IV, $ is anyone of LM, % is anyone of FY, # is anyone of NDQEBZ.

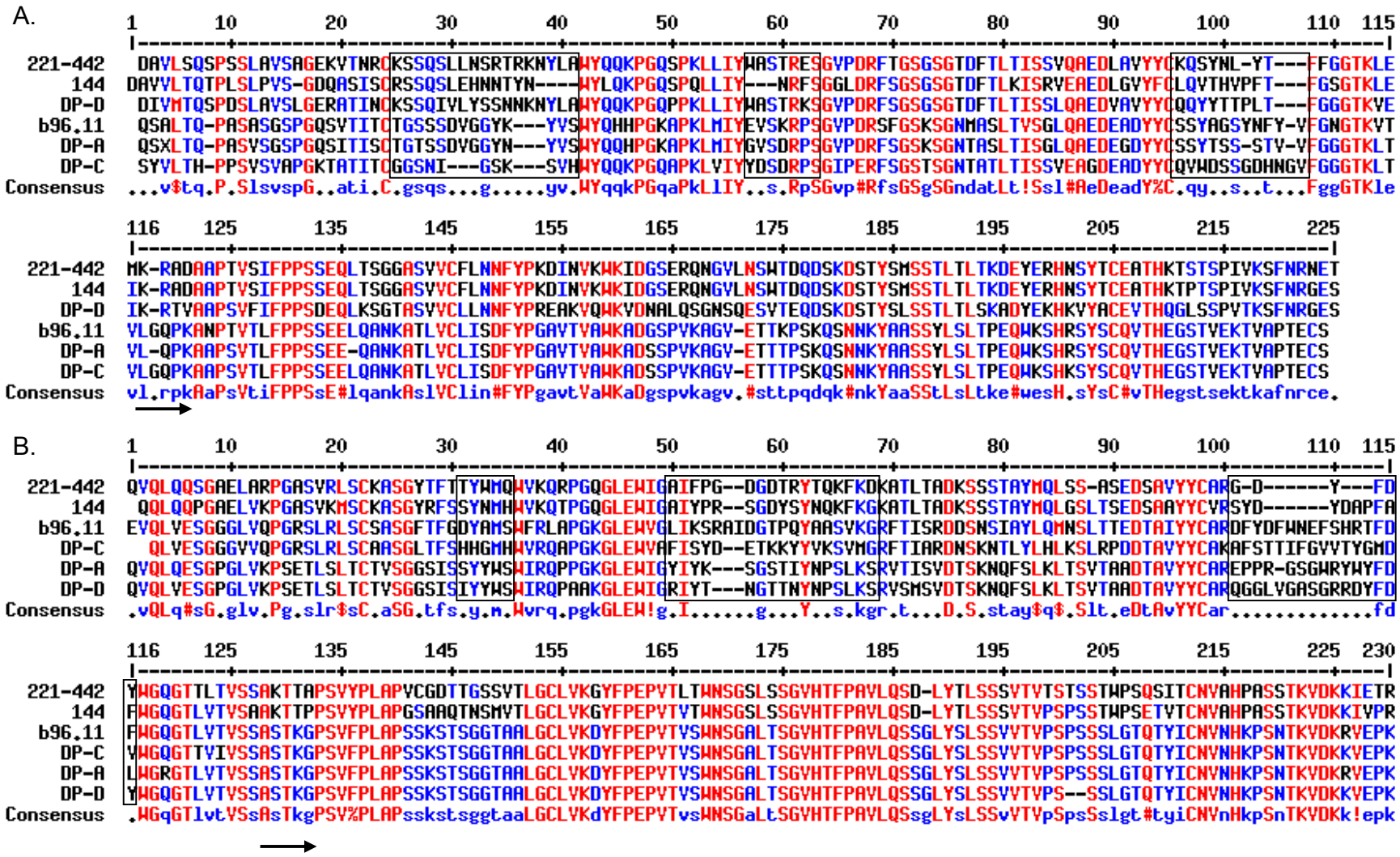


Figure 3.24: Light (A) and heavy (B) chain amino acid sequence alignment of rFabs 144, 221-442, DP-A, DP-C, DP-D, and b96.11. Boxes indicate the CDRs according to Kabat *et al.*, 1991 and the start of the constant regions are shown by the arrow (⟶).

3.5. THE EXPRESSION AND PURIFICATION OF rFAB

3.5.1. Expression of rFabs

A single recombinant colony was grown in MOPS medium and expression of the rFab (HB-123, HB-126, and DP-D) induced by phosphate starvation. rFab fragments were isolated from the periplasmic space.

3.5.2. Purification of rFabs

The rFab fragments (HB-123, HB-126, and DP-D) were subsequently purified by two affinity chromatography steps on Ni-NTA agarose and PGS. The resulting protein eluate was analysed by standard 15% SDS-polyacrylamide gel electrophoresis under reducing conditions. Heavy and light chain protein bands were identified by Western blots (Figure 3.25). In the Western blot analysis, for detection of the heavy and light chain, a penta-His HRP (Qiagen; 1:4 000) and rat anti-mouse kappa:HRP (Stratagene; 1:4 000) conjugate were used, respectively. After incubating the membrane at room temperature for an hour followed by extensive washing, the bands were visualized using standard ECL techniques. Results are shown for rFab 126. Similar results were obtained for rFab HB-123 and DP-D.

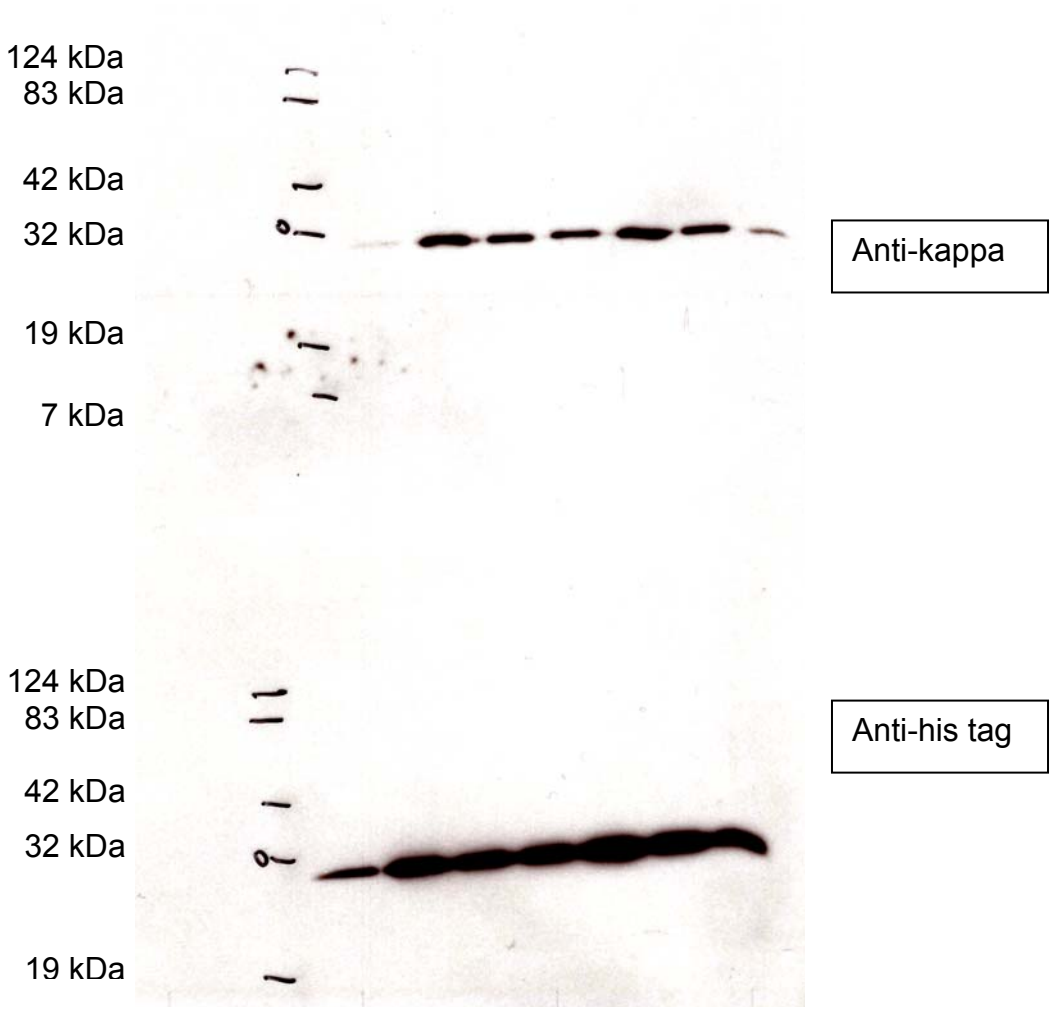


Figure 3.25: SDS-PAGE analysis of recombinant, affinity purified Fab 126. Lane 1: Molecular weight marker (Kaleidoscope prestained standards: blue-7 kDa, red-18.8 kDa, orange-32.2 kDa, violet-42.3 kDa, green-83 kDa, magenta-124 kDa, blue-206 kDa); Lanes 2-8: Eluted fractions from PGS purification of Fab 126. After reduction with β-ME, a band of ~23 kDa (free light chain; top blot) or ~25 kDa (free heavy chain Fd fragment, bottom blot) was observed.

3.6. ANTIGEN RECOGNITION OF rFAB

3.6.1. rFab 126 binds specifically to insulin

To determine whether or not the recombinant, purified Fab fragments possessed the correct conformation for antigen binding, binding of rFabs 123 and 126 to insulin, was tested in a radiobinding assay (RBA) using PGS as the precipitating agent. While rFab 126 showed dose dependent binding to ^{125}I-insulin, no binding was observed for GAD65-specific rFab 144 (Figure 3.26). Similarly, rFab 126 showed no binding to ^{35}S-GAD65 (data not shown). rFab 123 did not bind to insulin (Figure 3.27).

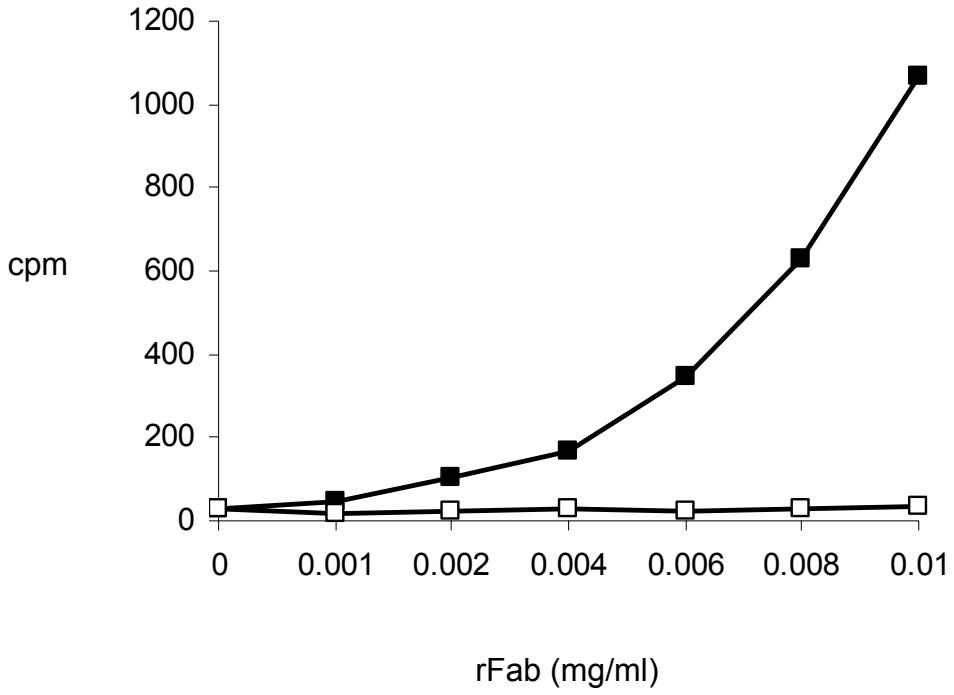


Figure 3.26: RBA of rFab 126 and GAD65-specific rFab 144 to ^{125}I-insulin. rFab 126 (black squares) and N-GAD65 rFab 144 (white squares) at the indicated concentration were incubated with ^{125}I-insulin. The immunocomplexes were precipitated using PGS. Binding to insulin is expressed as cpm units.

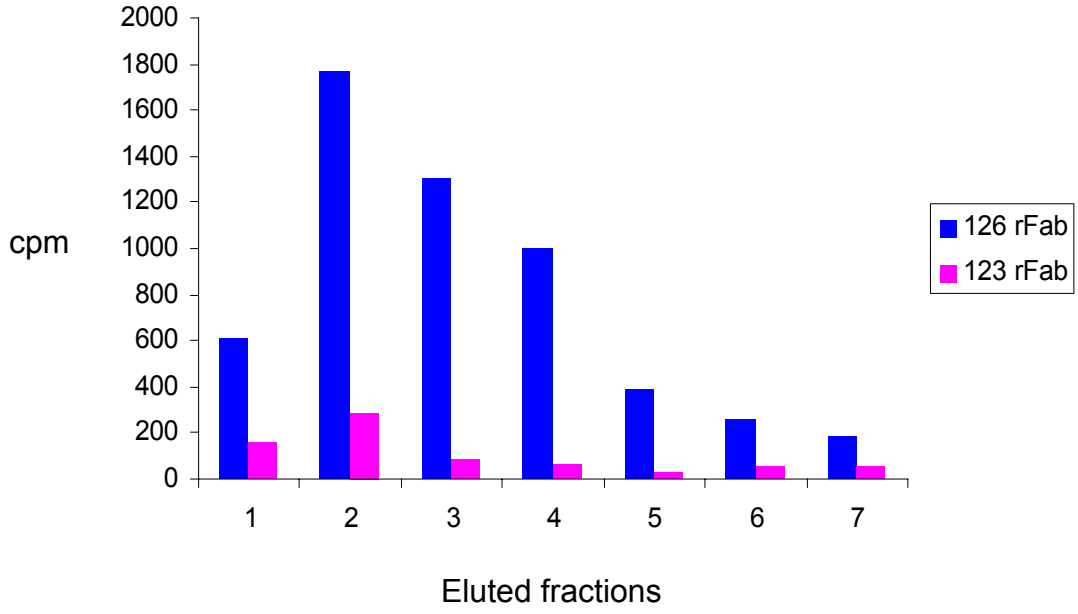


Figure 3.27: RBA of rFab 123 and rFab 126 to ^{125}I-insulin.

Ni-NTA purified fractions (1 to 6; 7 = blank) were incubated with ^{125}I-insulin. The immunocomplexes were precipitated using PGS. Binding to insulin is expressed as cpm.

The specificity of rFab 126 was further determined by competitive binding assays. Binding to ^{125}I-insulin by the intact mAb 126 at half-maximal binding concentration (0.04 mg/ml; Figure 3.28) was specifically competed with its rFab (Figure 3.29). The 126 rFab concentration (0.015 mg/ml; Figure 3.29) determined in these experiments to achieve maximal competition of the intact IgG was used in all following competition assays.

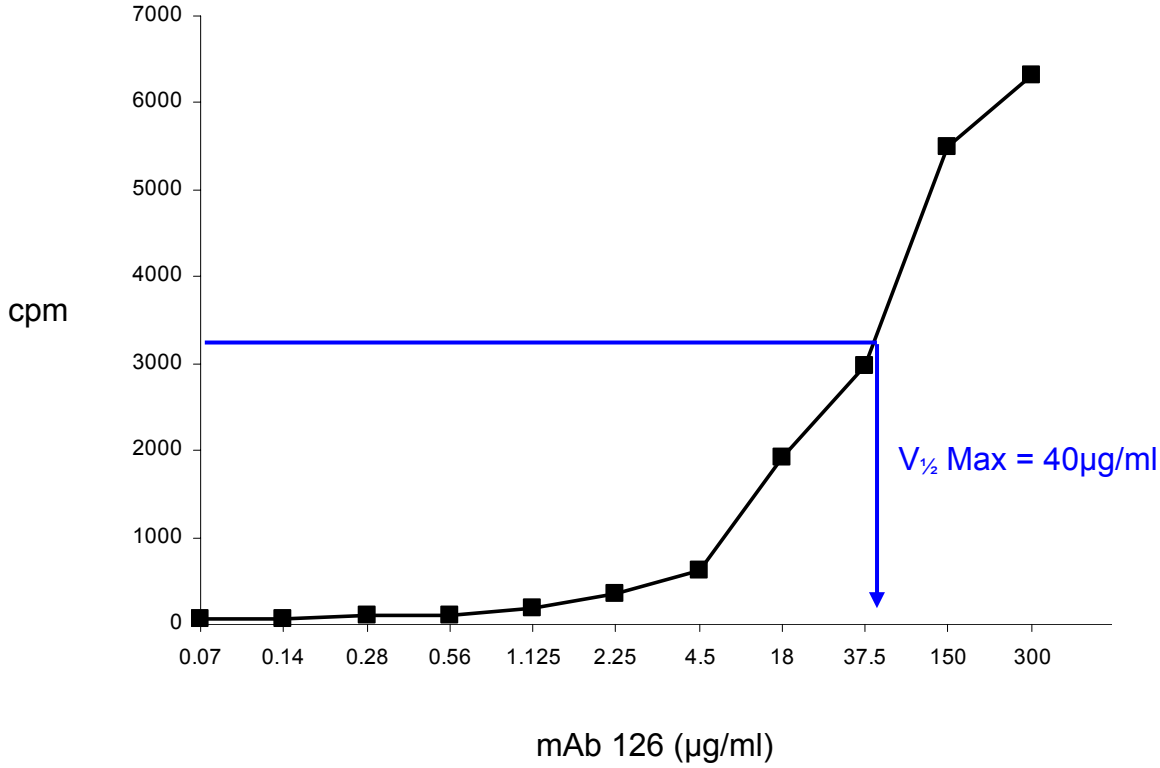


Figure 3.28: Determination of mAb 126 half-maximal binding concentration in an insulin RBA.

mAb 126 (black squares) was incubated at the indicated concentrations with ^{125}I-insulin. The immunocomplexes were precipitated using PAS.

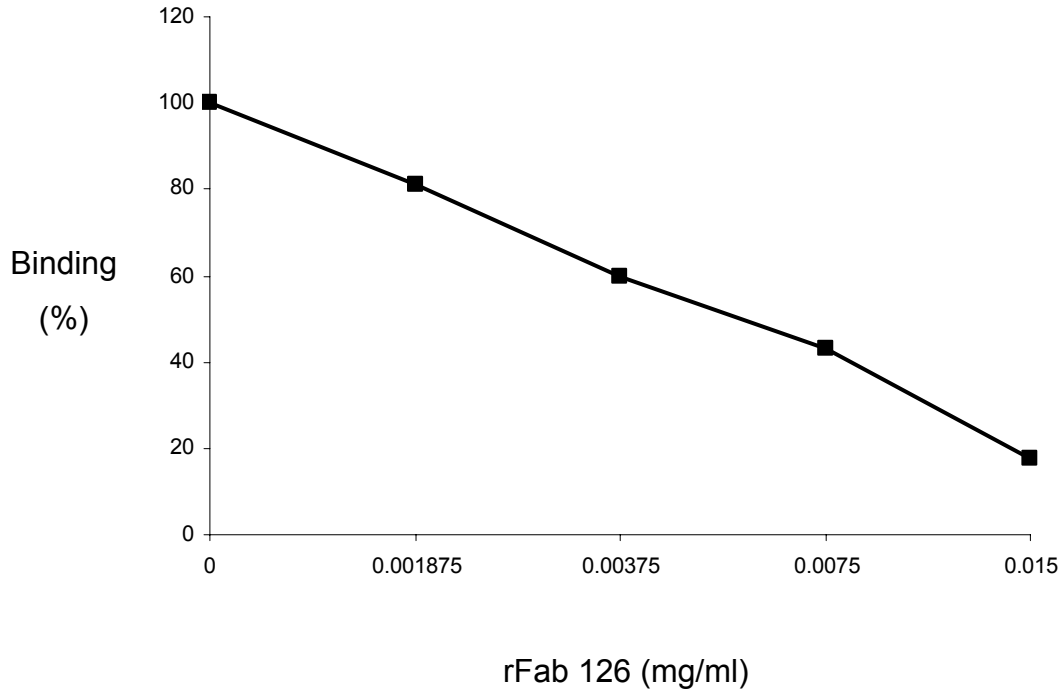


Figure 3.29: RBA of rFab 126 to ^{125}I-insulin competed by the presence of mAb 126 (0.04 mg/ml).
rFab 126, at the indicated concentrations, was incubated with ^{125}I-insulin and mAb 126. The immunocomplexes were precipitated using PAS.

3.6.2. rFab DP-D binds specifically to GAD65

To determine whether or not the recombinant, purified Fab fragment possessed the correct conformation for antigen binding, binding of rFab DP-D to GAD65 was tested in a RIA using PGS as the precipitating agent. rFab DP-D exhibited dose dependent binding to GAD65 and showed half-maximal binding at a concentration of approximately 0.1 µg/ml (Figure 3.30).

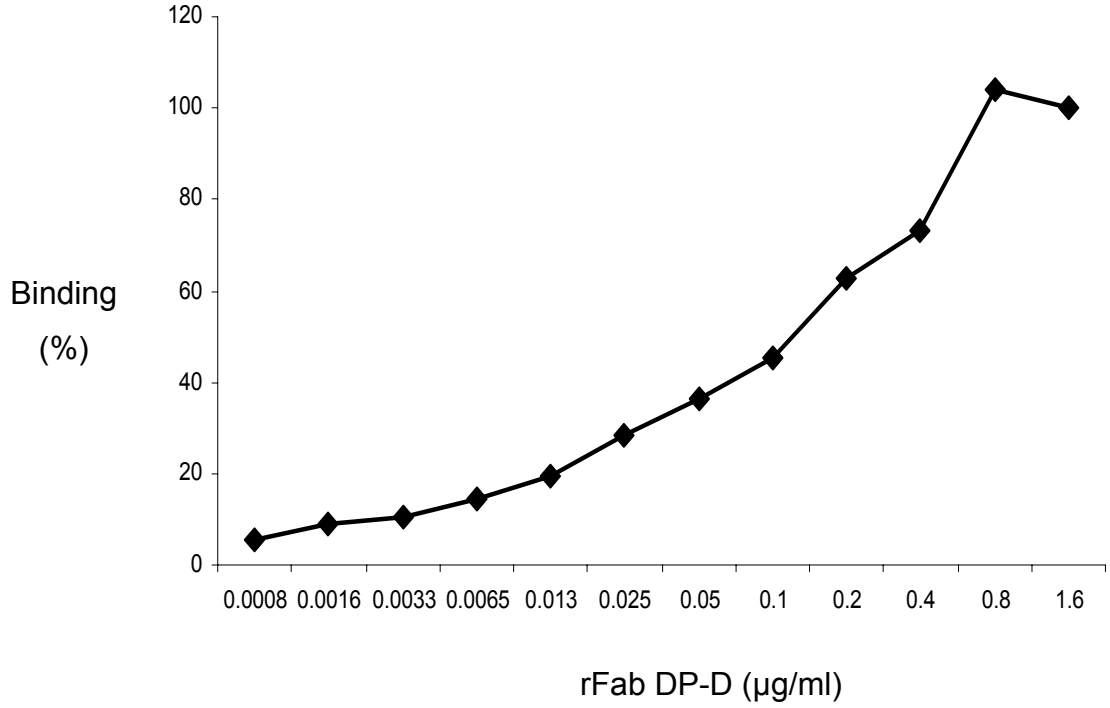


Figure 3.30: GAD65-binding and affinities.
Different amounts of rFab DP-D were incubated with radiolabelled ^{35}S-GAD65 and binding was tested in a RIA. Binding is expressed as percent binding, with maximal binding set as 100%.

3.7. EPITOPE MAPPING

3.7.1. rFab 126 recognizes an epitope located mainly at the A chain loop

In an effort to identify the epitope recognized by 126, competition assays with insulin-specific monoclonal antibodies mAb 1 (BIØDESIGN) and mAb 125 (Schroer *et al*., 1983) were performed (Figure 3.31). Both antibodies have well defined epitopes. Binding of mAb 1 is strictly dependent on amino acid residue B30, while binding of mAb 125 is directed predominantly to the A chain loop (A8-A10). Competition of insulin binding by both monoclonal antibodies at half maximal binding (0.08 and 0.04 mg/ml, respectively) by different concentrations of rFab 126 was tested. Insulin binding of mAb 125 was inhibited by 50% in the presence of 0.007 mg/ml rFab 126 and by 70% by 0.015 mg/ml rFab 126, whereas mAb 1 binding was inhibited by only 12% and 25% at these two concentrations of

competing rFab 126. From these data we conclude that the binding site of 126 overlaps with that of mAb 125.

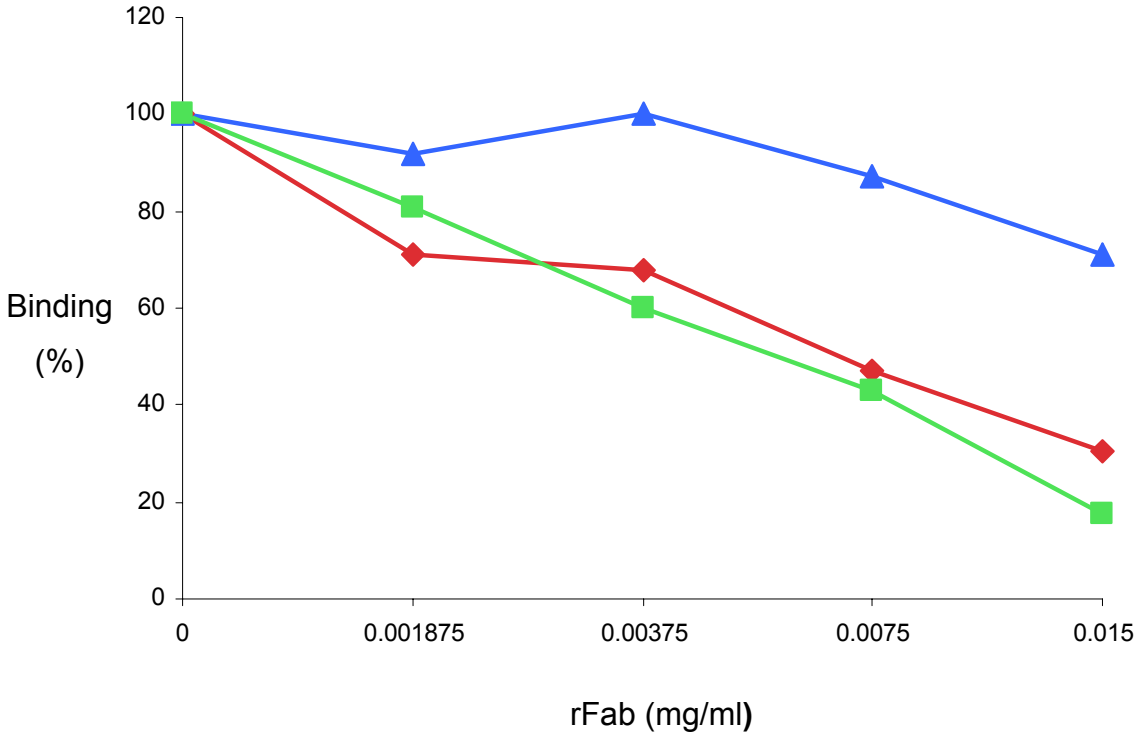


Figure 3.31: Epitope analysis of rFab 126.
Different concentrations of rFab 126 were used to compete for insulin binding by monoclonal antibodies mAb1 (blue), mAb125 (red), and mAb 126 (green) at half maximal binding. Binding to insulin is expressed as a percentage; non-competed binding is set as 100%.

3.7.2. Epitope mapping of rFab DP-D by binding to fusion proteins

The epitope that mAb DP-D binds to on GAD65 (Figure 3.32) was previously determined by domain swapping and deletion mutagenesis (Schwartz *et al*., 1999; Hampe *et al*., 2001). To verify the antigen region recognised by rFab DP-D, the binding characteristics of rFab DP-D to GAD65/67 fusion proteins (Hampe *et al*., 2000) was analysed. rFab DP-D only bound to the M and M+C fusion protein (Padoa *et al*., 2003).

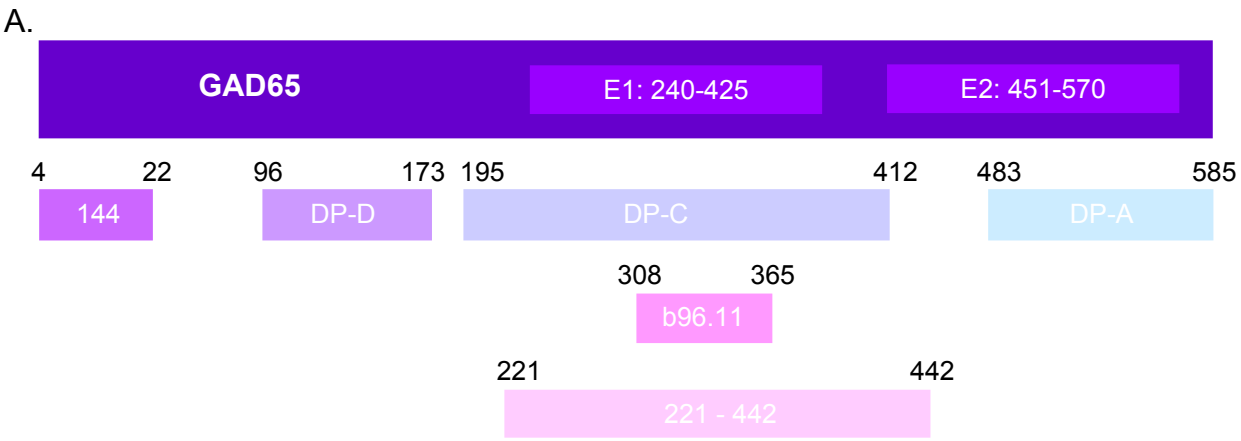


Figure 3.32: Epitope specificities of GAD mAbs.

A. The epitope specificities of mAbs 144, b96.11, DP-C, 221-442, DP-D, and DP-A are illustrated (Hampe *et al*., 2001; Schwartz *et al*., 1999). Amino acid residues bordering the epitopes are indicated for each mAb. The two major epitope regions (E1 and E2) for GAD65Abs in type 1 diabetes are shown as shaded boxes in the GAD65 molecule.

3.8. ANALYSIS OF INSULIN AUTOANTIBODIES IN DIABETIC PATIENTS

3.8.1. The autoantibody status of diabetic patients analysed with rFab 126

Table 3.4 and Table 3.5 summarise the autoantibody status of the type 1 and type 1.5 diabetes patients analysed in this study. The range of antibody levels in the control subjects was 0.12 to 0.0 for the GAD65Ab index and 0.016 to 0.0 for the IAA index. The cut-off established for the 98th percentile was 0.05 and 0.004 units (corresponding to 50cpm) for GAD65Ab and IAA, respectively. In our assays we only used sera with a cpm above 80.

Some of the patient's sera were diluted as they had very high antibody titers. Dilution of these samples ensured that the rFab/serum IgG ratio was not compromised.

Table 3.4: Autoantibody status of the analysed serum samples from patients with type 1 diabetes.

Age (years)	Gender	IAA unit	% Binding in presence of rFab 126	IA-2Ab index*	GAD65Ab index**
1	M	65	75	0.08	0.25
2	M	12	96	N/A	0.63
2	F	53	94	N/A	0.80
6	F	9	97	0.18	0.54
6	F	6	100	1.04	0.71
7	M	30	73	0.11	0.31
8		12	65	0.65	0.30
9	F	13	67	N/A	0.40
9	F	11	56	1.18	0.17
10	F	15	60	1.32	0.44
12	F	17	62	0.25	0.41
12		28	57	0.13	0.40
12	M	21	100	N/A	0.27
14	F	25	35	0.04	1.14
14	F	9	96	0.01	0.30
15	M	10	85	0.55	0.25
15	M	5	64	0.83	0.81
15	M	4	79	0.00	0.05
16	M	30	51	0.00	0.01
17	M	2	105	0.00	0.08
19	M	3	83	0.94	0.18
19	F	4	45	1.3	0.04
20	M	1	88	0.00	0.15
20	M	3	59	1.37	0.10
20	F	10	93	1.3	0.92
22	M	2	93	0.01	0.60
23	M	4	77	0.35	0.21
24	M	3	79	0.34	0.99

Age (years)	Gender	IAA unit	% Binding in presence of rFab 126	IA-2Ab index*	GAD65Ab index**
24	F	3	55	0.31	0.03
25	M	2	79	0.00	0.10
26	F	66	89	0.00	1.06
27	M	25	86	1.35	0.35
27	F	8	68	0.00	0.00
32	M	2	96	0.44	0.16
34	F	2	81	1.3	0.00

* IA-2Ab levels were measured according to the method described by Kawasaki *et al.*, 1997. ** GAD65Ab levels were measured according to the method of Grubin *et al.*, 1992; Grubin *et al.*, 1994.

Table 3.5: Autoantibody status of the analysed serum samples from patients with type 1.5 diabetes.

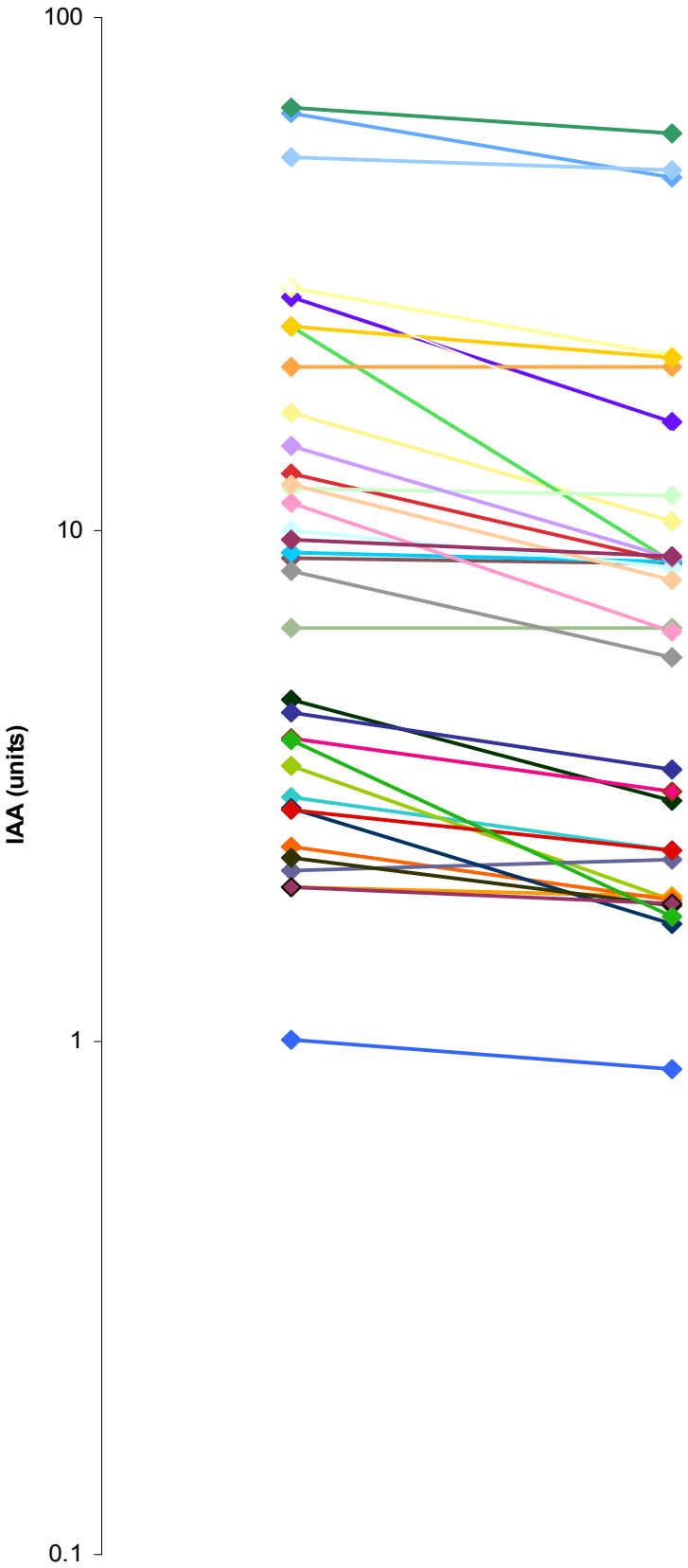
Age (years)	Gender	IAA unit	% Binding in presence of rFab 126	IA-2Ab index*	GAD65Ab index**
24	F	3	70	0.39	0.21
26	M	3	88	0.00	0.68
32	F	4	94	1.16	0.46
33	M	17	93	N/A	0.00
35	M	4	94	N/A	0.55
36	M	37	65	0.01	0.31
41	F	2	89	0.00	0.30
42	F	7	72	0.76	0.15
46	M	32	85	0.00	0.39
48	F	14	82	N/A	0.02
49	F	3	82	0.01	0.97
49	F	45	79	0.86	0.24
50	M	3	98	0.00	0.31
61	M	38	48	N/A	0.48

* IA-2Ab levels were measured according to the method described by Kawasaki *et al.*, 1997. ** GAD65Ab levels were measured according to the method of Grubin *et al.*, 1992; Grubin *et al.*, 1994.

3.8.2. Specificity of type 1 and type 1.5 diabetes associated IAAs determined with rFab 126

The relevance of the epitope recognized by rFab 126 was tested in competitive binding assays using sera (n=49) of newly diagnosed type 1 and type 1.5 diabetes patients (Figure 3.33). None of these patients had been treated with insulin prior to sampling. For the majority of the serum samples (n=35, 70%) insulin binding was competed for by more than 10% in the presence of rFab 126. The median binding of the serum samples to ^{125}I-insulin (median IAA units: 8.8, range: 1 to 67) was significantly reduced by the competing rFab to a median binding of 8 IAA units (range: 0.9 to 60) ($p < 0.0001$). No significant differences in the degree of competition in the type 1 diabetes patients to that in the type 1.5 diabetes patients was observed (Figure 3.33). Our results indicated that 70% of the analyzed serum samples contained IAA that recognized the same or similar epitopes as rFab 126.

A.



B.

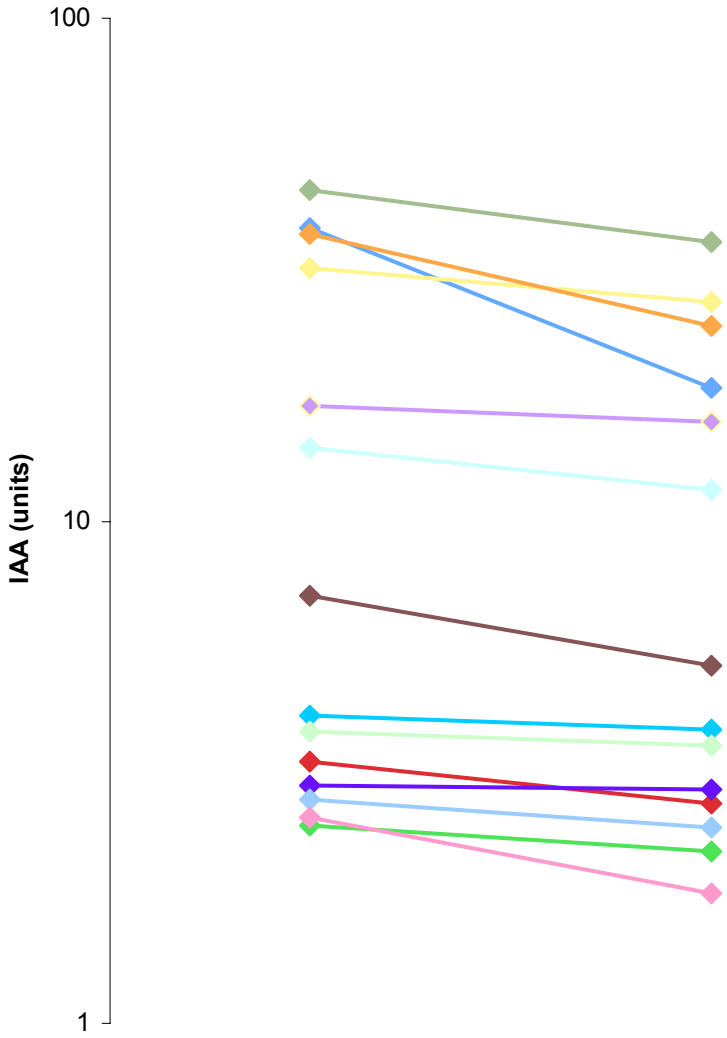


Figure 3.33: rFab 126 competes with the majority of sera from IAA-positive type 1 and type 1.5 diabetes patients.

Insulin binding of serum samples of type 1 (n=35) (panel A) and type 1.5 diabetes (n=14) (panel B) patients in the absence (left hand side diamond) and presence (right hand side diamond) of rFab 126. The data is presented on a logarithmic scale.

3.8.3. Statistical analysis

Within the serum samples that competed with rFab 126 for binding to insulin a wide range of competition was observed. Table 3.6 lists the mean/median value for each variable studied in the type 1 and type 1.5 diabetic patients. With the exception of age, there was no difference in % binding, IAA, GAD65, and IA-2 autoantibody levels between the type 1 and type 1.5 diabetic patients (Table 3.6). Similarly, no differences were found between males and females (all patients combined) when comparing the different variables (Table 3.7). No correlation was found between the degree of competition with age of onset, IAA titre, or presence of other autoantibodies (Table 3.8 and Table 3.9).

Table 3.6: The difference in age, % binding, IAA, GAD65Ab, and IA-2Ab in type 1 and type 1.5 diabetic patients.

VARIABLE	TYPE 1 PATIENTS (N=35)	TYPE 1.5 PATIENTS (N=14)	P VALUE
AGE*	16.11 ± 8.47	40.86 ± 10.39	< 0.0001
% BINDING*	76.8 ± 17.77	81.36 ± 13.73	0.3940
IAA$^+$	9 (18)	5.5 (29)	0.8944
GAD65Ab$^+$	0.3 (0.5)	0.31 (0.27)	0.8248
IA-2Ab$^+$	0.31 (1.03)	0.01 (0.76)	0.2246

* Data expressed as mean ± standard deviation; + Data expressed as median (interquartile range)

Table 3.7: The difference in age, % binding, IAA, GAD65Ab, and IA-2Ab in male and female diabetic patients.

VARIABLE	MALES (N=25)	FEMALES (N=22)	P VALUE
AGE*	23.96 ±14.17	23.5 ± 15.07	0.9146
% BINDING*	81.56 ± 15.04	75.73 ± 18.16	0.2349
IAA$^+$	4 (23.25)	9 (11)	0.4951
GAD65Ab$^+$	0.27 (0.36)	0.35 (0.56)	0.5649
IA-2Ab$^+$	0.05 (0.50)	0.39 (1.16)	0.0974

* Data expressed as mean ± standard deviation; + Data expressed as median (interquartile range)

Table 3.8: Correlation coefficient between % binding, age, IAA, GAD65Abs, and IA-2Abs using Spearman's correlation.

SPEARMAN CORRELATION						
		% BINDING	**AGE**	**IAA**	**GAD65Abs**	**IA-2Abs**
% BINDING	r		0.059	-0.237	0.191	-0.202
	p		0.6802	0.1010	0.1860	0.2019
	N		49	49	49	41
AGE	r	0.059		-0.225	-0.173	-0.161
	p	0.6802		0.1192	0.2313	0.3089
	N	49		49	49	41
IAA	r	-0.237	-0.225		0.244	0.093
	p	0.1010	0.1192		0.0914	0.5563
	N	49	49		49	41
GAD65Abs	r	0.191	-0.173	0.244		0.028
	p	0.1860	0.2313	0.0914		0.8594
	N	49	49	49		41
IA-2Abs	r	-0.202	-0.161	0.093	0.028	
	p	0.2019	0.3089	0.5563	0.8594	
	N	41	41	41	41	

Table 3.9: Correlation coefficient between % binding, age, IAA, GAD65Abs, and IA-2Abs using Pearson's correlation.

PEARSON CORRELATION						
		% BINDING	**AGE**	**IAA**	**GAD65AbS**	**IA-2AbS**
% BINDING	r		0.050	-0.147	0.096	-0.137
	p		0.7306	0.3128	0.5124	0.3926
	N		49	49	49	41
AGE	r	0.050		-0.061	-0.106	-0.093
	p	0.7306		0.6761	0.4668	0.5646
	N	49		49	49	41
IAA	r	-0.147	-0.061		0.223	-0.179
	p	0.3128	0.6761		0.1242	0.2627
	N	49	49		0.22	41
GAD65Abs	r	0.096	-0.106	0.223		-0.075
	p	0.5124	0.4668	0.1242		0.6395
	N	49	49	49		41
IA-2Abs	r	-0.137	-0.093	-0.179	-0.075	
	p	0.3926	0.5646	0.2627	0.6395	
	N	41	41	41	41	

3.9. ANALYSIS OF GAD65ABS IN DIABETIC PATIENTS

rFab DP-D competed with its intact parent mAb for binding to GAD65 (Padoa *et* al., 2003). The competition between rFab DP-D and GAD65Abs in sera of 61 type 1 diabetic patients to bind GAD65 was analysed (Figure 3.34). The median binding was significantly reduced by rFab DP-D (80%; $P < 0.0001$). rFab DP-D inhibited 3% of the samples fully and 62% partially (see section 2.17.1.1 for a definition of partial and full inhibition). The polyclonal nature of most patients' sera is by the observation that serum samples were inhibited by more than one rFab (Figure 3.35). Although samples whose GAD65 binding was competed by rFab b96.11 also showed competition by rFab DP-D (Figure 3.35, top panel), no significant correlation was observed between DP-A and rFab DP-D (Figure 3.35, bottom panel) (Padoa *et al*., 2003). This suggests that DP-A inhibited GAD65 binding to a different epitope to that recognised by b96.11 and DP-D (Padoa *et al*., 2003).

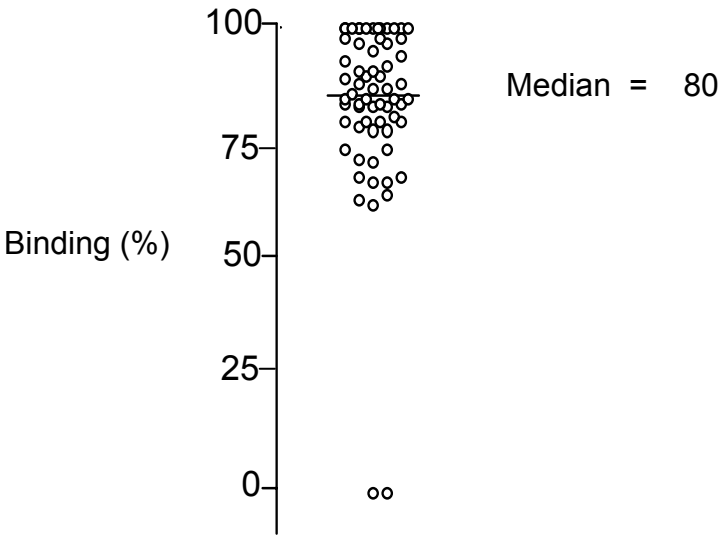


Figure 3.34: GAD65-specific rFab DP-D competes with serum GAD65Abs of type 1 diabetic patients.
Competed binding is presented as percentage binding related to uncompeted binding (100%); median binding is represented as a horizontal line.

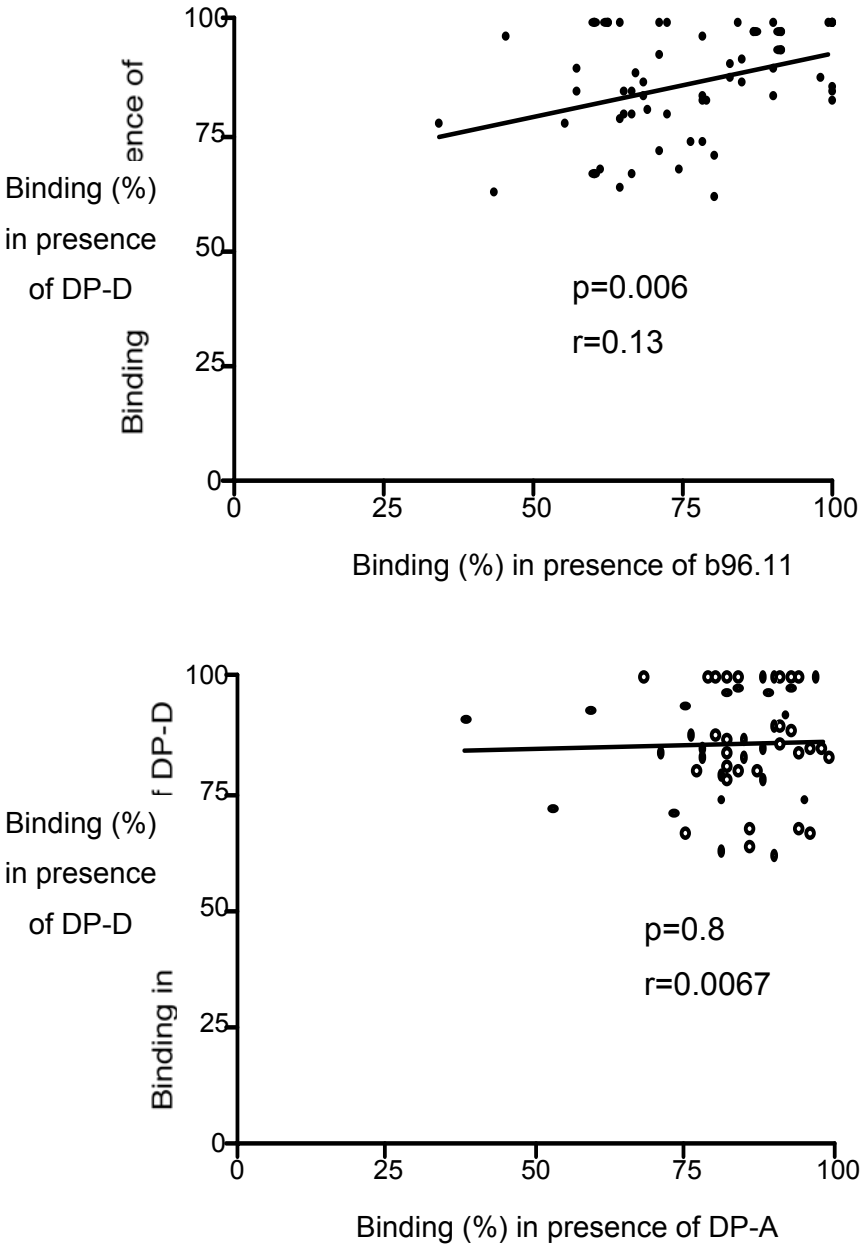


Figure 3.35: Correlation of competition.

GAD65 binding of type 1 diabetic patients' sera in the presence of rFab b96.11 (top panel) or rFab DP-A (bottom panel) is plotted against binding to GAD65 of the same samples in the presence of rFab DP-D. *P* values for the correlation (Spearman's rank correlation test) between the different competitors are indicated in the figure.

We determined the ability of rFab DP-D to compete with GAD65Abs in sera of type 1 diabetic patients (n = 61), LADA patients (n = 44), first-degree relatives of type 1

diabetic patients (n = 38), and healthy individuals (n = 14) (Figure 3.36). The differences in competition using rFab DP-D in the different serum groups was significant when type 1 diabetic patients were compared with first-degree relatives (P = 0.0002; Mann-Whitney U test) (Padoa *et al*., 2003).

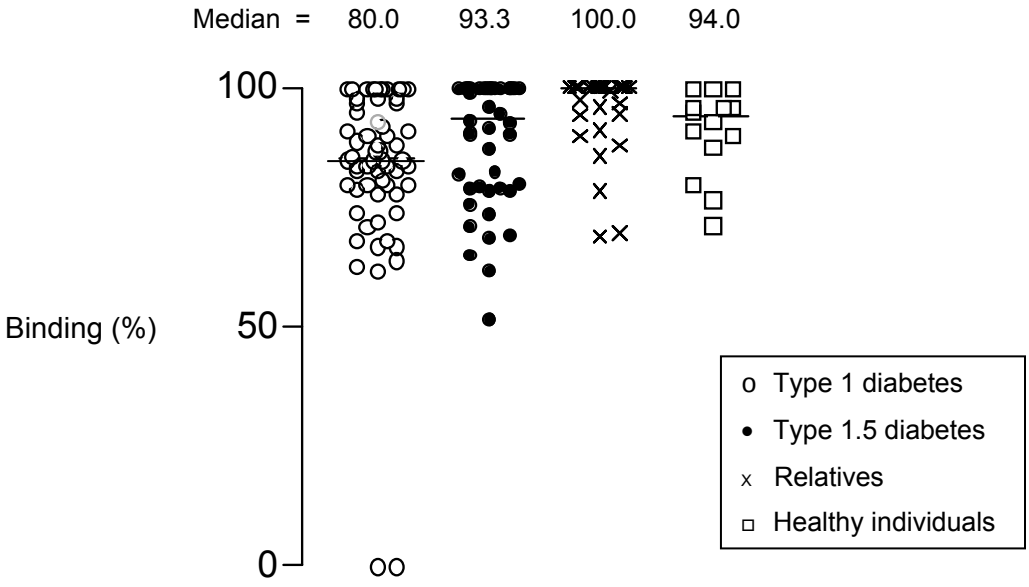


Figure 3.36: The capacity of rFab DP-D to compete with GAD65-binding of GAD65Ab-positive samples of type 1 diabetic patients, type 1.5 diabetic patients, first-degree relatives, and healthy individuals in a RIA.

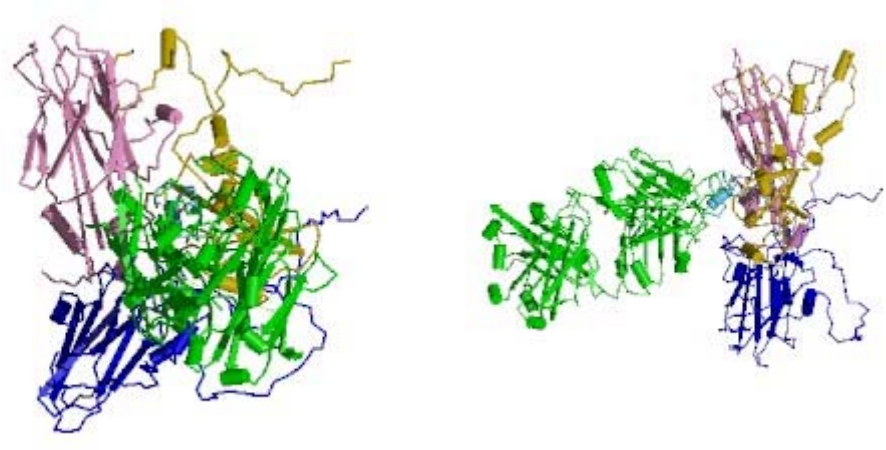
Competed binding is presented as percentage binding related to uncompeted binding (100%); median binding is indicated as a horizontal line.

3.10. HOMOLOGY MODELLING OF RECOMBINANT FAB 123, 126, AND DP-D

Using the ESyPred3D (Lambert *et al*., 2002) web server software, a 3D model of rFab 123, 126, and DP-D was built using the 3-dimensional structure 1QGC chain '4' (Fab fragment of a neutralizing antibody derived from foot and mouth disease virus; Figure 3.37) as a template (Hewat *et al*., 1997). The choice of template was decided by the computer programme and was based on closest homology to sequences of known structure. This template shares 72.2% identity with rFab 123

sequence, 71.0% identity with rFab 126 sequence, and 56.8% homology with rFab DP-D sequence. Figure 3.38 shows the 3-dimensional ribbon model structures of rFab 123, rFab 126, and rFab DP-D.

A.



Main view Bottom view

B.



Figure 3.37: A. 3-Dimensional structure of the Fab fragment of a neutralizing antibody derived from foot and mouth disease virus.
B. 3-Dimensional structure of the Fab fragment.

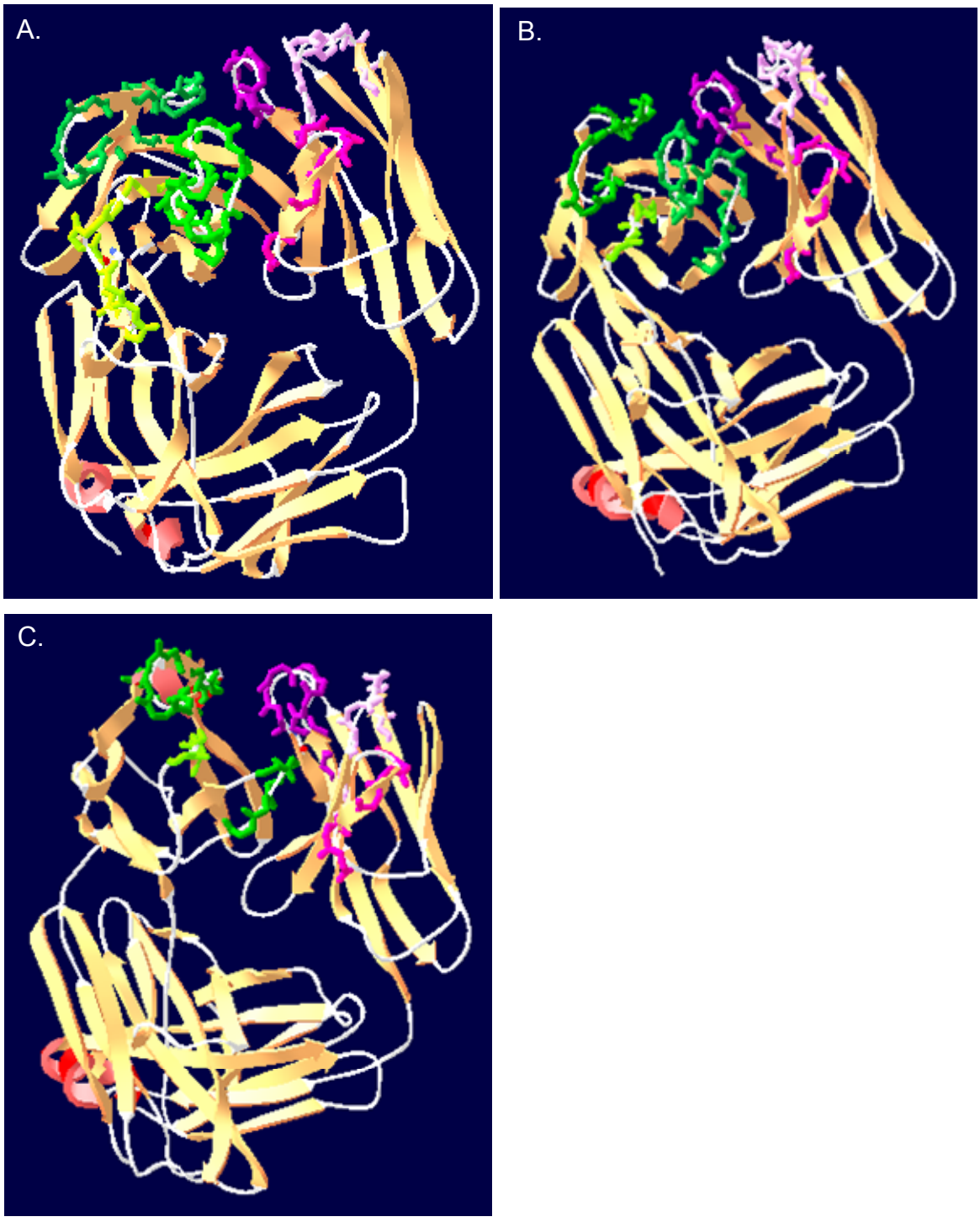
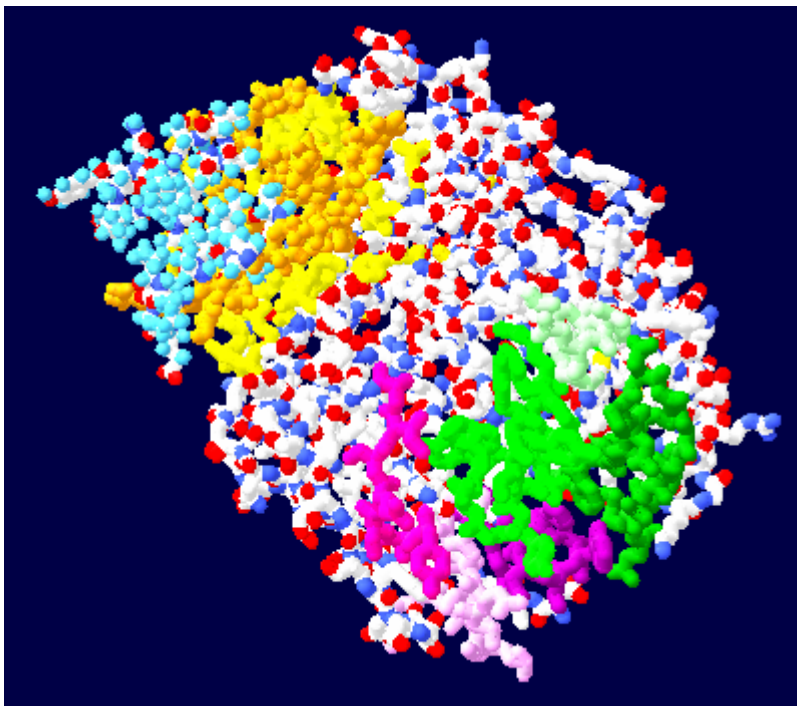


Figure 3.38: A. 3-Dimensional structure of rFab 126 (ribbon model).
B. 3-Dimensional structure of rFab DP-D (ribbon model).
C. 3-Dimensional structure of rFab 123 (ribbon model).
The CDRs are shown in pink (light chain) and green (heavy chain). The arrows indicate the β sheets and the red coils are the alpha helices.

3.11. MOLECULAR DOCKING OF THE INSULIN-RECOMBINANT FAB 126 COMPLEX

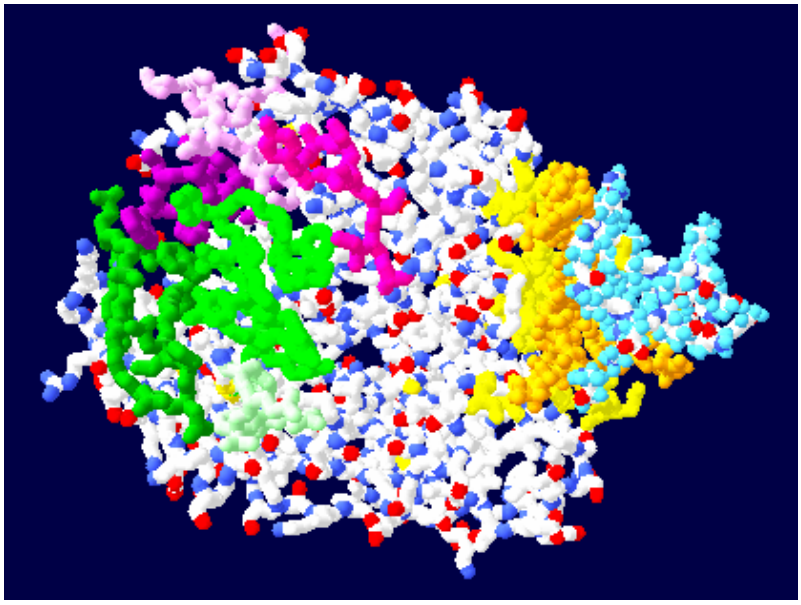
Using the computer programme 'PatchDock' we attempted to further map the epitope that rFab 126 binds to on insulin. Following the submission of the 3-dimensional structures of insulin of rFab 126 as pdb files, PatchDock returned one hundred permutations of possible insulin-Fab docking. The top three results obtained from PatchDock were analysed with Swiss-Pdb Viewer (Figure 3.39). Table 3.20 lists the different amino acid residues involved in the binding sites on insulin and rFab 126, respectively.

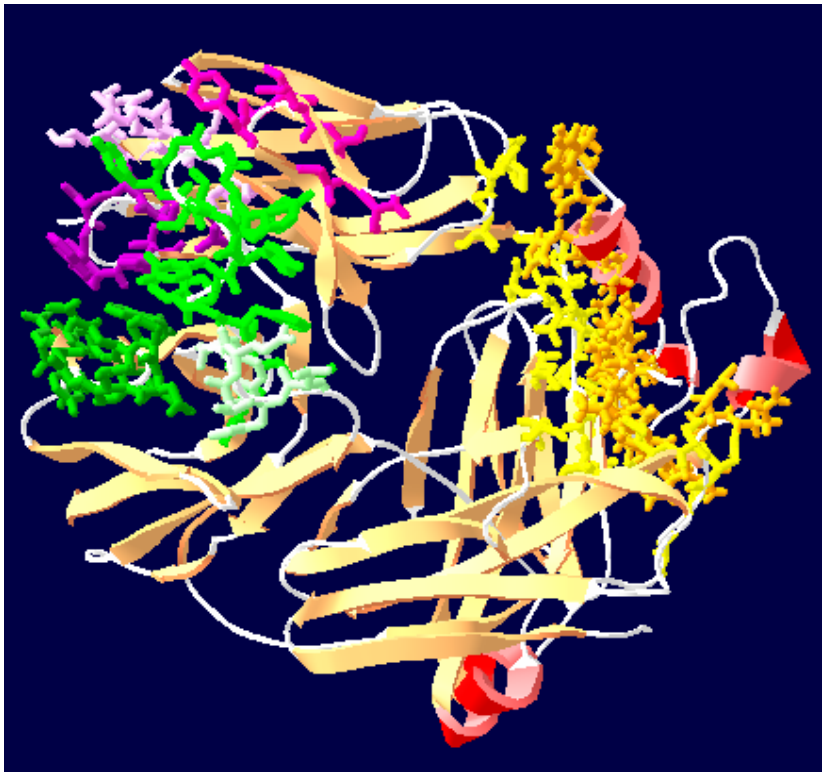
Model 1:



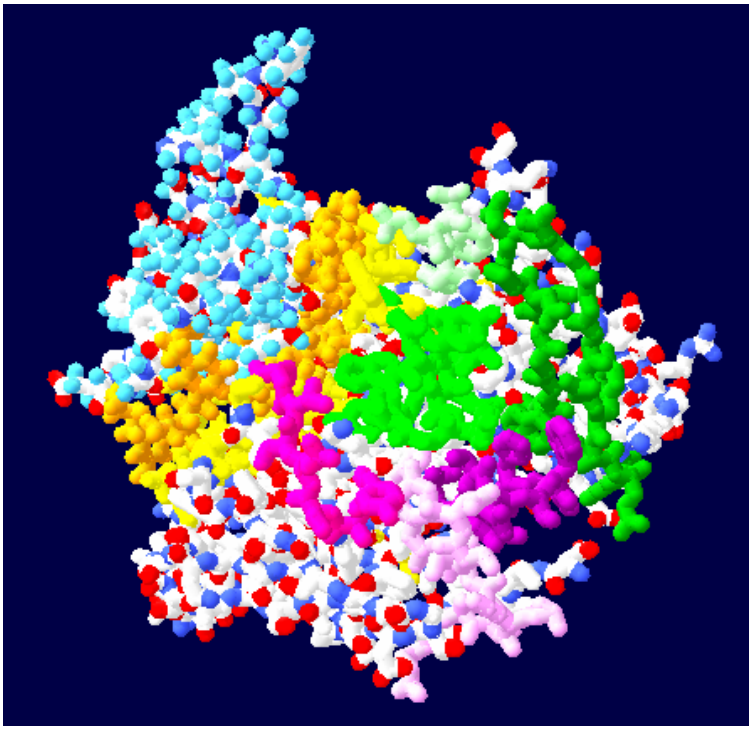


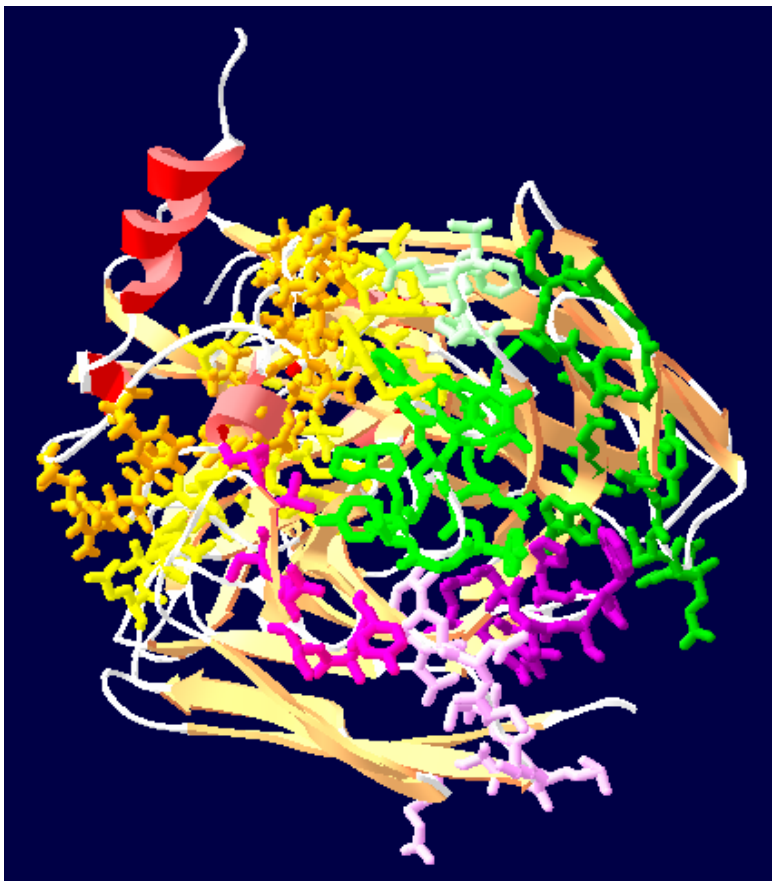
Model 2:





Model 3:





KEY:

--- Heavy chain CDR1 (244-248)

--- Heavy chain CDR21 (263-279)

--- Heavy chain CDR3 (312-328)

--- Light chain CDR1 (24-34)

--- Light chain CDR21 (50-56)

--- Light chain CDR3 (89-97)

--- insulin epitope

--- rFab 126 epitope

Figure 3.39: 3-Dimensional structure (space-fill and ribbon model) of the insulin-rFab 126 complex.

Table 3.10: Amino acid residues involved in the binding of insulin to rFab 126.

MODEL	INSULIN RESIDUES	rFAB 126 RESIDUES
Model 1	A1-A5, A21, B1-B6, B9, B13, B16, B22-B30	108-110, 112-115, 138, 169-170, 200-203, 205, 207, 356-365, 388, 390-391, 393, 411-414
Model 2	A18-A21, B1-B2, B5-B6, B8-B9, B12-B13, B16, B21-B29	15, 79, 106, 108-115, 138, 170-171, 200-207, 358, 361-364, 390-391, 411
Model 3	A4-A5, A8-A15, A17-A18, A20-A21, B22, B25, B30	39, 42, 45, 57, 59, 167-169, 216-219, 241-244, 314, 320, 385-386, 389-390, 392-394,

CHAPTER 4

4. DISCUSSION

4.1. PROBLEMS ENCOUNTERED WHEN CLONING IMMUNOGLOBULIN HEAVY AND LIGHT CHAIN GENES

4.1.1. Aberrant heavy and light chain transcripts

Both MOPC-21 and P3X63Ag8 myeloma cell lines actively secrete immunoglobulin. The 1E2 hybridoma was derived from the cellular fusion partner NS0. NS0 is a nonsecreting variant of the MOPC 21 myeloma which does not express heavy chains (Kohler and Milstein, 1976). Sp2/0, the fusion partner for HB-126, is derived by fusion of P3X63Ag8 with a splenic B-cell line. Sp2/0 has lost the ability to produce heavy and light chain proteins (Klotz *et al*., 1981). The presence of aberrant immunoglobulin chains can interfere with downstream applications. As a result, myeloma cell lines that do not secrete/produce functional immunoglobulins have been constructed to obtain specific hybrid cell lines that only produce pure mAbs (Kearney *et al*., 1979).

DNA sequencing of the cDNA product amplified from the 1E2 and NS0 cell lines, using kappa light chain specific primers, demonstrated the presence of an aberrant mRNA transcript which is present in all standard fusion partners derived from the original MOPC-21 tumour (Carroll *et al.*, 1988). The expression of this non-functional kappa transcript is variable and can be rather high in certain hybridomas, exceeding levels of the productive light chain mRNA. In such cell lines the PCR primers for amplification of the variable domain of the kappa chain will mainly amplify the aberrant kappa chain transcript (Duan and Pomerantz, 1994). The expression of this aberrant transcript can interfere with kappa mRNA sequencing by producing ambiguities in the sequencing reactions. On one or two occasions ‘dirty’ unreadable sequence was obtained when analysing the PCR derived 1E2 kappa chain products by DNA sequencing. This would indicate either improperly purified PCR products or the amplification of both the functional and

aberrant kappa chain cDNA in one reaction. If the latter were true, cDNA cloning would separate the two transcripts. The majority of the PCR derived 1E2 kappa chains that were sequenced were, however, found to be of the aberrant kappa chain transcript, suggesting that the aberrant kappa chain mRNA is far in excess of the functional kappa chain mRNA in the 1E2 hybridoma cell line.

With the use of our heavy chain specific primers, for the construction of a scFv, we consistently obtained two V_H cDNA species from the 1E2 hybridoma cell line and a single V_H cDNA species from the NS0 hybridoma cell line. Sequence determination of the PCR product present in both cell lines was homologous to the aberrant heavy chain transcript (AberrG7) reported by Vidarsson and colleagues (2001). Due to the poor quality of the sequencing results obtained for the second, smaller cDNA species amplified from the 1E2 cell line (Figure 3.2B), we were unable to rule out the possibility that this product was, in fact, the functional heavy chain gene.

There has been one report in the literature that describes the construction of an anti-insulin scFv (Lake *et al*., 1994). The heavy and light chain variable genes were amplified from a murine anti-insulin IgG 1-producing hybridoma, HB-125, which was generated using the fusion partner Sp2/0-Ag14. The authors did not report the amplification of any aberrant transcripts. This scFv was shown to bind insulin in an insulin ELISA but at a 3.5-fold lower affinity than the parent mAb. The authors attributed this difference to the fact that the parent mAb is bivalent and that because the scFv is an engineered molecule its conformation may have been altered which in turn affected its ability to bind to insulin.

In the case of mouse hybridomas, special care must be taken when designing the synthetic oligonucleotides to avoid the amplification of an aberrant kappa and heavy chain mRNA transcript that is inherited from the original MOPC-21 tumour from which most myeloma fusion partners are derived (Carroll *et al*., 1988). In an attempt to eliminate PCR contamination by endogenous aberrant kappa chain mRNA, Duan and Pomerantz (1994) designed an aberrant kappa chain CDR-specific primer set which specifically amplifies the aberrant transcript. These primers are located in the CDR1 and CDR3 regions of the aberrant kappa

transcript. Together with the immunoglobulin degenerate PCR primers, a quick and efficient selection of the correct kappa chain variable cDNAs can be achieved. Heavy and light chain genes are amplified using the immunoglobulin degenerate primers and the PCR products cloned into plasmids. Recombinant plasmids containing the aberrant light chain transcript are identified by further amplification with the aberrant kappa chain CDR-specific primer set. Only recombinant plasmids containing the aberrant transcript will show bands representing the aberrant light chain insert on an agarose gel. Lanes which do not contain a band either contain the functional kappa chain insert or do not contain any insert. This technique significantly reduces the amount of sequencing required to identify plasmids containing the functional kappa chain transcript. Duan and Pomerantz (1994) also constructed a ribozyme-based expression vector which eliminates the aberrant kappa transcript by cleaving it in the CDR1 region.

4.1.2. Problematic techniques

Although we eventually managed to perfect the cloning of the heavy and light chains of mAbs, a number of obstacles were encountered en route. Firstly, with the construction of a scFv fragment the presence of aberrant heavy and light chain transcripts interfered with the amplification of functional immunoglobulin chains. This has been confirmed by a number of researchers (Carroll *et al*., 1988; Duan and Pomerantz, 1994; Vidarsson *et al*., 2001) and new techniques/methods have been designed to overcome this issue.

When constructing the rFabs we encountered a number of difficulties with the ligases used and with the restriction enzyme sites used for cloning. The T4 DNA ligase failed to ligate the respective chains to the vector. This problem was overcome by changing the brand of ligase. The pAK19 expression vector was designed such that cloning of the heavy and light chain involved the use of *Mlu*I. As a result a ‘stuffer’ fragment had to be ligated onto the light chain to introduce a new restriction site for cloning. This, in turn, produced its own set of problems. These difficulties were eventually solved by separately subcloning the heavy and light chain fragments into the pCXII vector before finally cloning the entire Fab fragment into the pAK19 expression vector.

4.2. METHODS FOR THE CONSTRUCTION OF RECOMBINANT FABS

A number of methods exist for the production of rFab and scFv fragments; namely hybridoma cell lines, phage display, and ribosome display. In this study, mAbs were produced by the hybridoma technique described by Köhler and Milstein (1975). The genes encoding the heavy and light chains were amplified from the respective hybridoma cell lines by RT-PCR, subcloned into the pAK19 expression vector and expressed in *E. coli* 25F2 cells. Before the introduction of phage display, this was the standard method for the construction of scFv or Fab. The only differences would be the choice of expression vector, restriction sites for cloning, and the host cell for expression. The main reason for adopting the hybridoma cell line approach is that hybridomas were readily available and the method is less complex and involved than phage display, a technique which is more commonly used for the production of antibody libraries from immunoglobulin expressing cells.

An alternative approach would have been to use the phage display method. In this method, variable genes are amplified from a library of diverse antibodies isolated from IgG-secreting plasma cells from animals or individuals immunized with a specific antigen. The scFv/Fab are displayed on the surface of the bacteriophage. scFv/Fab with high affinity and specificity to the target antigen (e.g. insulin) are selected by panning. The advantage of phage display systems is that it provides a faster and more efficient selection method than traditional bacterial screening. In addition, with the hybridoma approach, a single rFab to a specific antigen is constructed whereas with the phage display method a number of rFab with differing affinities/specificities can be selected.

Some of the limitations encountered with cell-based display systems are overcome by ribosome display which is carried out *in vitro*. Protein-ribosome-mRNA complexes are formed which allows for the simultaneous isolation of protein and mRNA by affinity selection. The mRNA can then be amplified by RT-PCR and additionally PCRed, mutated, or cloned (Hanes and Plückthun, 1997).

4.2.1. Characterisation of rFab

The cDNA encoding the anti-insulin and anti-GAD65 rFab were cloned. The predicted amino acid sequences were aligned (Figure 3.22 and 3.23, respectively). rFab 126 and rFab 123 share the same classes for CDR1 and 2 but differ in their CDR3 class; the heavy chain CDRs showed 46% homology. The highest homology in the anti-insulin rFab light chains was found between rFab 126 and the 1E2 light chain sequence (53% homology). A comparison of the overlapping amino acid sequence in the CDR regions of the GAD65 rFab heavy chains showed that amino acid sequences derived from human rFab DP-D and DP-A were closely related to each other (60% homology). While these two rFab share the same CDR 1 and 2 classes, they differ in their CDR3 class. Comparison of the overlapping amino acid sequence in the CDRs of the light chain showed the highest homology when comparing b96.11 with DP-A and 221-442 with DP-D (67 and 61% homology, respectively). While b96.11 and DP-A share the same classes for CDRs 1 and 2, they differ in the CDR3 class. DP-A binds to the carboxyl-terminal of GAD65 whereas DP-D and b96.11 bind to the middle or amino-terminal end of the molecule. It is, therefore, not surprising that differences exist in CDR3 as this is where the greatest sequence diversity occurs.

4.3. AUTOANTIBODIES IN THE SERA OF DIABETIC PATIENTS

4.3.1. GAD65 autoantibodies

Autoantibodies to the 65kDa isoform of glutamate decarboxylase (GAD65Abs) are accepted markers for type 1 diabetes and, together with autoantibodies to insulin (IAA) and a protein tyrosine phosphatase-like islet cell antigen (IA-2), predict the disease (Vandewalle *et al*., 1995). Moreover, GAD65Abs have recently been implicated in GAD65 presentation to T cells (Jaume *et al*., 2002; Reijonen *et al*., 2000) and may, therefore, play a role in the pathogenesis of type 1 diabetes. Characterization of type 1 diabetes-specific GAD65 and insulin autoantibody epitopes will provide important information to further our understanding of the immunopathologic mechanisms leading to type 1 diabetes.

The identification of disease-specific GAD65Ab epitopes in type 1 diabetes has been hampered by the fact that the epitopes are conformational (Tuomi *et al*., 1994; Schwartz *et al*., 1999). Epitope identification has been attempted using GAD65/67 fusion proteins. Using these fusion proteins it has been shown that sera of type 1 diabetes patients recognize conformational epitopes located in the middle (amino acid 240-435 = epitope 1) and the carboxy-terminal end (amino acid 451-570 = epitope 2) of GAD65 (Daw and Powers, 1995; Hampe *et al*., 2000; Padoa *et al*., 2003). This reactivity pattern differs significantly from that of GAD65Abs in stiff-man syndrome patients (Björk *et al*., 1994; Daw *et al*., 1996), autoimmune polyendocrine syndrome type 1 patients (Björk *et al*., 1994), first degree relatives of type 1 diabetes patients (Hampe *et al*., 2000), the general population (Hampe *et al*., 2000), and type 1.5 diabetes patients (Falorni *et al*., 2000; Hampe *et al*., 2000).

While the analysis of antibody binding patterns using GAD65/67 fusion proteins provided valuable information about GAD65Ab binding patterns (Falorni *et al*., 2000; Hampe *et al*., 2000), information about conformational disease-specific GAD65Abs and their epitopes was limited (Ziegler *et al*., 1996). Changes of GAD65Ab epitopes were observed in prediabetic children (Bonifacio *et al*., 2000), indicating that the humoral response to GAD65 changes as an individual progresses to type 1 diabetes. To identify disease-associated GAD65Abs and their epitopes Padoa and colleagues (2003) cloned rFab of well characterized GAD65-specific mAbs. The use of rFab rather than intact immunoglobulins prevents potential steric hindrance. The six GAD65-specific rFab were cloned from human mAbs b96.11, DP-C, DP-A, and DP-D (derived from autoimmune disease patients), and from murine mAbs 144 and 221-442. The epitope regions recognized by the six mAbs were spread over the length of the GAD65 molecule ensuring that different epitope specificities were represented. Our observation that the rFabs bound most GAD65/67 fusion proteins more weakly than GAD65 confirmed earlier findings that the epitopes' conformations are changed in the fusion proteins.

Although the patients' sera present a polyclonal mixture of GAD65Abs with different epitope specificities, we were able to obtain a significant reduction in

GAD65 binding using the rFabs as competitors. rFab b96.11 and DP-C, derived from human mAbs, significantly inhibited the binding of the majority of type 1 diabetic sera to GAD65. Also, rFab DP-D and DP-A, derived from mAbs isolated from a type 1 diabetic patient (Madec *et al*., 1996), and 221-442, derived from a murine mAb (Ziegler *et al*., 1996), inhibited the GAD65Ab binding, but to a lesser extent. These findings are in agreement with previous reports that major GAD65Ab epitopes in type 1 diabetes are located in the middle region and the carboxy-terminus of the molecule (Daw and Powers, 1995; Schwartz *et al*., 1999).

The binding of GAD65Ab in type 1 diabetes patients to GAD65 was analyzed by competitive radioimmunoassay with the six rFab to ascertain disease-specific GAD65Ab binding specificities. The competition pattern in type 1 diabetes patients was different from that in GAD65Ab-positive type 1.5 diabetes patients (n=44), first degree relatives (n=38), and healthy individuals (n=14). While 87% and 72% of the type 1 diabetes sera were competed by rFab b96.11 and DP-C, respectively, only 34 % and 26% of type 1.5 diabetes patients, 18% and 25% of first degree relatives, and 7% and 28% of healthy individuals showed competition ($p<0.0001$). These novel findings (Padoa *et al*., 2003) with GAD65-specific rFab support the view that type 1 diabetes is associated with disease- and epitope-specific GAD65Ab and supports the notion that the middle epitope is disease-specific. These GAD65-specific rFab should prove useful in predicting type 1 diabetes.

Previous data has shown that GAD65Abs can modulate GAD65 processing and presentation to MHC class II-restricted T cells and thus have a pathogenic role in the development of type 1 diabetes (Jaume *et al*., 2002; Reijonen *et al*., 2000). DP-D, DP-C, and DP-A were shown to promote the presentation of GAD65 epitopes outside their own binding sites, while inhibiting the presentation of their specific epitope to T cells (Jaume *et al*., 2002). This is in conjunction with the observation that antibodies can suppress the presentation of certain epitopes due to their high affinity binding (Simitsek *et al*., 1995; reviewed by Watts, 1997) and underlines the possible involvement of GAD65Abs in the modulation of T cell responses to GAD65. rFab b96.11, DP-C, and other type 1 diabetes-specific rFab may represent a novel tool to modulate this processing and will be of importance

in elucidating the mechanisms by which GAD65Abs may influence disease-progression. Future experiments will be needed to investigate whether rFab b96.11 or DP-C can block the action of pathogenic serum antibodies in order to reveal whether these disease-specific GAD65Abs in type 1 diabetes patients' sera are pathogenic or the result of epitope spreading induced by the disease progression.

Epitope spreading is the phenomenon whereby there is a shift in the position of the epitopes from one domain of the antigen to another as an autoimmune disease progresses. For example, antibodies to GAD65 are initially produced against the middle and C-terminal regions of the antigen in type 1 diabetes. The autoimmune response may then undergo intramolecular epitope spreading towards epitopes on the N-terminus and further epitopes located in the middle in genetically predisposed individuals which leads to onset of the disease (Schlosser *et al*., 2005).

In conclusion, rFab fragments b96.11, DP-C, DP-D, DP-A, and 221-442 recognize shared epitopes with GAD65Abs in type 1 diabetes patient sera, and thus can serve as tools for epitope mapping and detection of epitope shifts in different stages of the disease.

4.3.2. Insulin autoantibodies

IAA are among the first autoantibodies to appear in individuals prior to the clinical onset of type 1 diabetes (Ziegler *et al*., 1999). Their appearance is inversely correlated with age (Graham *et al*., 2002). This makes them especially important as markers for type 1 diabetes in children (Karjalainen *et al*., 1989). While antibody epitopes for other autoantigens in type 1 diabetes have been extensively studied, little is known about IAA epitopes. In the past, researchers have utilised naturally occurring isoforms of insulin for the analysis of IAA epitopes. In two studies, human, bovine, and porcine insulin were used to reveal differences in the binding characteristics of IAA and antibodies to exogenous administered insulin (IA), locating the major binding site of IA to the A-chain, while type 1 diabetes-associated IAA recognise a conformational epitope requiring both the A and B

chain (Diaz and Wilkin, 1987; Wilkin *et al*., 1988). Differences in epitope specificities of IAA and IA were also detected using random peptide phage display (Devandra *et al*., 2003). Monoclonal antibodies and their rFab are being successfully used in the epitope analysis of other autoantibodies in type 1 diabetes (Schlosser *et al*., 1997; Schwartz *et al*., 1999; Kolm-Litty *et al*., 2000; Padoa *et al*., 2003). This method is particularly useful in the study of conformational epitopes since the structure of the antigen remains intact (Binder *et al*., 2004).

The development of a PAS-based IAA radiobinding assay greatly reduced the required amount of serum and allowed for a more comprehensive analysis of the antibodies (Williams *et al*., 1997). In this study we developed a new reagent to perform antibody epitope mapping of IAA. Monoclonal antibody 126 binds to a wide range of different isoforms of insulin and proinsulin (Schroer *et al*., 1983). We were able to localise the epitope of 126 to the A chain loop of insulin (A8-A10). We cloned and sequenced the Fab portion of mAb 126 and analysed the amino acid sequence, deduced from the nucleotide sequence, by comparing it to the closest related germline sequence available in GenBank. The heavy chain underwent somatic mutations resulting in seven amino acid residue substitutions, located in CDR1 and CDR2, while the light chain remained identical to the germline sequence.

The expressed rFab 126 binds specifically to insulin. The antigen binding site of the rFab fragment was identical to that of the intact mAb since the binding of rFab 126 to insulin was inhibited by the presence of the intact mAb 126. Significant competition was observed when IAA present in the sera of type 1 and type 1.5 diabetes patients was incubated with insulin and rFab 126. A wide range of competition was observed within the serum samples that competed with rFab 126 for binding to insulin. No correlation was found between the degree of competition with IAA titre, presence of other autoantibodies, or age of onset. Similarly, no difference was found between the degree of competition observed in the type 1 and type 1.5 diabetic patients groups. This finding differs to those observed when analysing GAD65Abs in the sera of diabetic patients. Here the competition pattern differed in the type 1 patients as compared to the type 1.5 patients. This discrepancy may be due to the small number of patients analysed for the IAAs or

the fact that the major IAA epitopes in type 1 diabetes are located at a different position on the insulin molecule i.e. not on the A chain loop where mAb 126 binds.

The lack of observed competition between rFab 126 and some of the patient sera could be due to an excess of high affinity autoantibodies in the sera of these patients which prevents the rfab from binding. The consequence of diluted patients' sera on the competitive effects of the rFab were, however, not analysed because the study by Padoa *et al*. (2003) showed that dilution of GAD65Ab positive patient sera had no influence on the degree of inhibition.

To our knowledge this is the first study describing epitope analysis of IAA using rFab. This kind of analysis should prove to be of great value in the epitope analysis of IAA and IA. While GAD65Ab and IA-2Ab radiobinding assays have been standardised and a WHO standard is available (Mire-Sluis *et al*., 2000), the IAA radiobinding assay is plagued by large inter laboratory variations (Bingley *et al*., 2003). IAA have also been isolated from healthy individuals and from patients with autoimmune diseases other than type 1 diabetes (Wilkin, 1991). Epitope mapping of IAA may improve our understanding of the underlying autoimmune response and aid in the accurate prediction of type 1 diabetes.

A study carried out on IAA positive first degree relatives of type 1 diabetes patients showed that their binding sites for insulin were similar. The epitope that these insulin autoantibodies bind to on insulin was mapped, by comparative studies using insulin from different species, to amino acids B1-B3 and A8-A13 (Castano *et al*., 1993). This study supports our finding that the binding of rFab 126 is directed to the A chain loop (A8-A10) of insulin. Although the sera studied may have antibodies recognising multiple epitopes on insulin, there was an overall inhibition of binding in the presence of rFab 126 which would suggest that a percentage of these autoantibodies recognise the same epitope as rFab 126 i.e. on the A chain loop.

The identification of disease specific epitopes may also provide new methods for the treatment/prevention of type 1 diabetes. If a disease specific epitope is identified, patients can be treated with a peptide that binds to this epitope,

effectively blocking the autoantibody from binding to the antigen. A study performed by Daniel and Wegmann (1996) showed that when NOD mice were administered with the B-(9-23) insulin fragment, either subcutaneously or intranasally, a delay in the onset and the incidence of diabetes decreased in relation to mice given a control peptide.

4.4. OTHER USES OF RECOMBINANT FAB

Type 2 diabetes is a heterogeneous and polygenic disorder resulting from the interaction of genetic and environmental factors. The disease is characterised by a failure of the pancreatic β cells to secrete sufficient quantities of insulin in the presence of reduced insulin sensitivity. Type 2 diabetes accounts for about 90% of all cases of the disease, affecting 150 million people worldwide (King *et al*., 1998).

The prevalence of type 2 diabetes is increasing in South Africa, especially in the black population. A sensitive method is required to detect those individuals who could develop the disease. Studies have shown that high levels of proinsulin and 32,33 split proinsulin indicate future progression to type 2 diabetes (Nijpels *et al*., 1996; Wareham *et al*., 1999). Assays that are currently used cannot accurately distinguish between the intermediates in the insulin pathway due to the similarity in their tertiary structure. An assay specific for 32,33 split proinsulin has not yet been produced. Nor has it been possible to detect with confidence the levels of 65,66 split proinsulin because of the low concentration of this peptide in the circulation.

Genetic engineering will help improve the performance of immunoassays and provide better reagents for clinical diagnosis. The production and genetic manipulation of novel mAbs that only contain the antigen binding domains of immunoglobulins will help reduce non-specific binding (Warren *et al*., 2005). In addition, the production of antibodies against novel epitopes may be possible and this will be extremely useful in developing assays for 32,33- and 65,66- split proinsulin since no monoclonal antibodies that specifically bind to this protein exist. The ability to accurately measure these molecules may provide important information on β-cell dysfunction in type 2 diabetic patients.

We have amplified, cloned, and expressed the hypervariable domains of the heavy and light chain of an insulin specific mAb 126. This rFab can be fused to an enzyme, for example alkaline phosphatase, to produce a reagent that can be used in immunoassays. Site-directed mutagenesis can also be used to alter the binding affinity and specificity of the rFab. These novel antibodies could be used in assays designed to measure insulin and its precursors and screen populations who have a high risk of developing type 2 diabetes. Furthermore, the methodology can be used in any assay that uses mAbs and it will, therefore, be possible to improve both the sensitivity and specificity of diagnostic assays. A study by Warren *et al.* (2005) showed that non-specific interference was considerably reduced with the use of a scFv as opposed to the parent mAb. ScFvs lack the constant domain of an antibody and, therefore, interference is essentially eliminated.

Insulin autoantibody assays have posed major problems in terms of reproducibility, sensitivity, and specificity. With the advent of antibody engineering it is envisioned that rFab specific to insulin and its precursors can be constructed and used to standardise and improve the insulin autoantibody assay. Also, the construction of rFab which could recognise disease specific IAAs, would greatly enhance the usefulness of insulin RBAs.

Engineered antibodies also have other clinical uses. For instance they can be used in cancer therapy as magic bullets for targeting and destroying tumour cells (reviewed by Merluzzi *et al*., 2000; Linenberger *et al*., 2002; Stevenson *et al*., 2002). Many antibodies raised against tumour cell surface markers are made in non-humans and are therefore not able to be used for the treatment of human cancers. However, this problem has been addressed by the production of chimeric (murine variable regions combined with human IgG constant domain) or humanised mAbs in which the hypervariable regions are isolated and placed within a human antibody framework, thus reducing the risk of immune reaction against the antibody, as in Herceptin (Morrison *et al.*, 1984; Jones *et al*., 1986; McNeil, 1998).

Other applications of recombinant antibodies include their use in epitope mapping (Padoa *et al.*, 2005), disease specific epitope mapping (Padoa *et al*., 2003) and high-resolution X-ray crystallographic studies of antigen binding sites. The smaller Fab, or scFvs, are more advantageous for epitope studies as they eliminate the possibility of steric hindrance. Similarly, the large size of immunoglobulins and the flexibility of the hinge connecting Fab arms and the Fc domain have prompted crystallographers to turn to Fab (Saul and Poljak, 1992) and Fv fragments (Boulot *et al.*, 1990) for determining the 3-dimensional structure of antigen-antibody complexes.

4.5. PROTEIN MODELLING

The 3-dimensional structures of protein complexes, determined by X-ray crystallisation methods, provide a number of insights into protein-protein interactions. These interactions play an important role when developing new drugs and treatments for disease. Although the number of proteins with defined 3-dimensional structures is increasing, the majority have not yet been crystallised. As a result, various computer software programmes have been designed to overcome this problem. The 3-dimensional models that were generated for rFabs 123, 126, and DP-D fit the desired structure of an antibody molecule as the heavy and light chain CDRs are brought together to form a neat antigen binding pocket.

Once the 3-dimensional structures of proteins have been computationally determined, protein docking experiments can be performed. The structure of a complex between two proteins is predicted based on the independently crystallized structures of the components. We used the freely available PatchDock software in an attempt to refine the epitope that rFab 126 binds to on insulin. Using this software together with Swiss PdbViewer it was possible to determine which residues on insulin are bound by the rFab 126 and *vice versa*. Of the three models generated, only model three supported our findings that rFab 126 binds to the A chain loop of insulin. This model was also the only model which showed involvement of amino acid residues in the CDRs of the rFab 126. X-ray crystallography and mutagenesis studies would be able to further optimise the models generated.

The major limitation of PatchDock is that the docking is based purely on shape complementarity; intermolecular forces are not taken into account when the models are generated. A further limitation of this method is that of the hundred or more models generated (results are ranked according to a geometric complementarity score which is supposed to measure the fit of the shape) the final model chosen is at the discretion of the user.

4.6. LIMITATIONS OF THE CURRENT STUDY

In this study we had a total of five mAbs and of these, we were only able to construct two functional rFab that showed dose dependent binding to insulin and GAD65, respectively: rFab 126 and rFab DP-D. We were unable to construct a rFab for mAb 127 and mAb 1E2 as the heavy chain gene could not be amplified for mAb 127 and we were only able to amplify the non-functional heavy chain transcript for mAb 1E2. We were able to clone and express a rFab for mAb 123 but this Fab did not exhibit binding to the insulin molecule in a RBA. This may be due to a lower yield of expressed rFab 123 and hence a lower concentration of the purified rFab, incorrect folding of the heavy and light chain in the periplasm of *E. coli*, lack of association of the two chains in the periplasm, or a decreased affinity for insulin. The latter explanation is, however, unlikely as a number of studies have shown that rFab generally have similar, or slightly lower, affinities for the antigen molecule than the parent mAb (Eriksson *et al*., 2000; Itoh *et al*., 2003). Itoh *et al*. (2001) reported a three fold lower affinity in the rFab which they attributed to a difference in valency between the rFab and intact mAb.

Although we were able to map part of the epitope that rFab 126 binds to on insulin to the A chain loop, we cannot rule out the involvement of the insulin B chain if it is a conformational epitope.

The sample size used to determine disease specific IAA epitopes in type 1 and type 1.5 diabetic subjects was relatively small which limits the conclusions that can be drawn.

4.7. FUTURE STUDIES

The clinical manifestation of type 1 diabetes is the endpoint of a long lasting immune-mediated destruction of the β-cells. Autoantibodies originating from this process can be applied in the diagnosis and clinical discrimination of autoimmune diabetes as well as in the prediction of the disease. At clinical diagnosis, between 80-90% of patients with type 1 diabetes are positive for antibodies to β-cell antigens. These antibodies can also be detected in the presymptomatic period (before onset of the disease), and can, therefore, be used to predict the disease (reviewed by Atkinson and Eisenbarth, 2001). It is important to determine the prevalence of autoantibodies in diabetic patients as this will provide valuable information on the autoimmune response in type I diabetics. Little is known about the autoimmune role in the pathology of disease in African populations. One study has shown a lower prevalence of GAD autoantibodies in Black South Africans compared to data from Caucasian type I diabetic patients (Rheeder *et al*., 2001). There is, thus, some evidence for ethnic differences in the autoimmune response. This needs to be researched using not only the measurement of GAD antibodies but also IA-2, and insulin autoantibodies. The latter two autoantibodies have never been investigated in the South African black population. The insulin epitopes will also be of interest because we can look at ethnic differences in the prevalence of insulin autoantibodies and ethnic differences in epitopes bound by such antibodies. These types of investigations have never been performed before and are important in giving clues to possible differences in autoimmune response to autoantigens in different populations. It must also be remembered that the prevalence of type I diabetes is thought to be lower in Africans than Europeans (Rewers *et al*., 1990; Karvonen *et al*., 1993; Karvonen *et al*., 2000) and there may, therefore, also be significant ethnic differences in both the aetiology and pathology of the disease.

This information will help us to characterise diabetes in the different population groups in South Africa. We believe this will provide important information on the role of the immune system in the disease aetiology, particularly in black South Africans for whom there is little such data.

The epitope to which the monoclonal antibody that we are using in this study binds to on insulin, has not been completely mapped. Initial studies suggest that the A chain loop (A8-A10) is involved in the binding of insulin to rFab 126. With the use of computer modelling and X-ray crystallography (performed on the antibody-antigen complex) we hope to be able to define which residues within the binding site of the antibody are important for determining binding affinity and specificity. Random or directed mutagenesis of the CDRs can be performed to alter the specificity of the rFab or improve the affinity of rFab 126 for insulin.

We would also like to produce a bifunctional fusion protein consisting of the rFab 126 fused to the N-terminus of alkaline phosphatase (rFab-AP). This rFab-AP can then be used in an insulin ELISA. The design of an insulin ELISA using genetically engineered Fabs (raised against insulin) may help to overcome the difficulties of standardisation of conventional insulin autoantibody assays. The production and genetic manipulation of novel monoclonal antibodies that only contain the antigen binding domains of immunoglobulins will help reduce non-specific binding. In addition, the production of antibodies against novel epitopes may be possible and this will be extremely useful in developing assays for 32,33- and 65,66- split proinsulin since no monoclonal antibodies that specifically bind these proteins exist.

4.8. CONCLUSION

The methodology of genetic engineering has made it possible to produce tailor-made antibodies which do not depend on animal vehicles. The increasing number of crystal structures of antibody combining sites, especially complexes with antigens, allow for a clearer picture of structural conservation and variability. The technology to produce mAbs led to the determination of an enormous number of antibody sequences, mostly from cloned DNA sequences. This led to the defining of consensus sequences, which allowed for rapid cloning of antibody genes by the polymerase chain reaction (PCR), thereby generating even more sequence information (Devlin *et al*., 1990). Using bacterial expression technology, different types of fragments were made in fully functional form in *E. coli* (Buchner *et al*., 1991). This expression system allowed for the rapid analysis of the effect of

designed mutations and the consequence of random changes. The applications of these rFab are widespread. The information that we are obtaining from our research on the type 1 diabetic patients using genetically engineered Fabs is extremely important in obtaining a better understanding of the disease process and how best to treat type 1 diabetes patients.

Approximately 17 million people worldwide have type 1 diabetes and this number is increasing. Type 1 diabetes is a serious, debilitating disease with life threatening complications. The financial burden associated with the management of this disease is enormous. Early diagnosis and intervention can improve the morbidity and mortality. The identification of type 1 diabetes-associated insulin and GAD65 autoantibody epitopes is important as this will provide us with a better understanding of the pathogenic involvement of these autoantibodies in the disease progression and also, how best to block insulin and GAD65 autoantibody binding as a possible treatment method.

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APPENDIX 1

1.1. LIST OF REAGENTS AND SUPPLIERS

REAGENT	SUPPLIER
30 % (w/v) acrylamide/bis solution (19:1)	Biorad
Agarose	FMC BioProducts
Ammonium chloride	Saarchem
Ammonium heptamolybdate tetrahydrate	Merck
Ammonium persulphate	USB
Bacto agar	Difco
BioMax X-ray film	Kodak
Boric acid	Merck
Bovine serum albumin	Sigma
Bromophenol blue sodium salt	BDH
Calcium chloride	uniVAR
Carbenicillin	USB
Carbon Dioxide	Affrox
Cobalt(II) chloride	Merck
Coomassie Brilliant Blue R250	Merck
Copper sulphate	Merck
p-Coumaric acid	Sigma
DMEM	Gibco
DMSO	Sigma
dNTPs	AEC Amersham
DTT	AEC Amersham
EDTA	Saarchem
Ethanol	BDH
Ethidium bromide	USB
Fetal Calf Serum	Sigma
Filter paper	Millipore/BIO-RAD
Glacial acetic acid	BDH
Glucose	Synthon

REAGENT	SUPPLIER
Glutamine	Gibco
Glycerol	Merck
Glycine	Merck
Hydrochloric acid 32 %	BDH
Hydrogen peroxide 30 %	BDH
Imidazole	Saarchem
Immobilin-P membrane	Millipore
Iodine-125	AEC Amersham
Iron(II) sulphate	Merck
Kaleidoscope Prestained Standards	BIORAD
LN_2	Affrox
Luminol	Sigma
Lysozyme	Fluka
Methanol	BDH
Magnesium chloride	Sigma
Magnesium sulphate	Sigma
Manganese (II) chloride	Merck
β-mercaptoethanol	Sigma
Molecular weight marker	Gibco
MOPS	Sigma
M-MuLV reverse transcriptase	Invitrogen
Ni-NTA agarose	Qiagen
Penicillin-streptomycin	Gibco
Penta-His HRP conjugate	Qiagen
Plasmid miniprep kit	Qiagen
Polyethylene glycol	Merck
Polyoxyethylenesorbitan monolaurate (Tween 20)	Sigma
Potassium chloride	Merck
Potassium dihydrogen phosphate	Merck
di-Potassium hydrogen phosphate	Merck
Potassium hydroxide	Merck
Potassium sulphate	Saarchem

REAGENT	SUPPLIER
Protein A sepharose	Zymed
Protein G sepharose	Zymed
Rat anti mouse kappa:HRP	Serotec
Restriction enzymes and supplied buffers: • *Bsr*GI (10 000 U/mL) • *Eco*RI (20 000 U/mL) • *Hind*III (20 000 U/mL) • *Mlu*I (10 000 U/mL) • *Sph*I (5 000 U/mL)	New England Biolabs
Ribonuclease inhibitor	AEC Amersham
SDS	Sigma
Shrimp Alkaline Phosphatase	USB
Sodium chloride	Sky chem
di-Sodium hydrogen phosphate	Merck
Sodium dihydrogen phosphate	Merck
Sodium hydrogen carbonate	Merck
Sodium hydroxide	AAR
Sodium Pyruvate	Gibco
Sterivex-GS 0.22 μm filter unit	Millipore
Sucrose	Merck
T4 DNA ligase	NEB
Taq DNA polymerase	Qiagen
Temed	USB
Tetracycline	Sigma
Thiamine-HCl	Calbiochem
Tricine	Sigma
Tris	Merck
Trypan blue solution	Sigma
Tryptone	Difco
XL10-Gold Ultracompetent Cells	Stratagene
Yeast extract	Difco
Zinc sulphate	Sigma

1.2. SOLUTIONS

Agarose gels

Weigh out agarose and dissolve in 1 x TBE buffer. Boil until clear. Add 5 μl of ethidium bromide (10 mg/ml) per 100 ml of gel

10 % Ammonium persulphate (APS)

Dissolve 1 g APS in 10 ml of dH_2O. Store at 4°C.

Blocking solution

Dissolve 1 % BSA in 1 x TBST.

Carbenicillin

Dissolve 50 mg/ml stock in dH_2O. Sterilise by filtration through a 0.22 μm filter. Aliquot and store at -20°C.

Coating buffer (IAA)

150 mM NaCl
20 mM Tris

Make up to a final volume of 500 ml and store at 4°C. Before coating the plates, add 0.5 % BSA.

Destaining solution

Mix 600 ml methanol, 300 ml water, 100 ml glacial acetic acid.

Immerse the gel in at least 5 volumes of staining solution and place on a slowly rotating platform for a minimum of 4 hours at room temperature. Remove the stain and save it for future use. Destain the gel by soaking it in the methanol/acetic acid solution (described above) on a slowly rocking platform for 4 to 8 hours at room temperature, changing the destaining solution three or four times.

DMEM stock solution

13.38 g DMEM
3.7 g $NaHCO_3$
10 ml L-glutamine (200 mM)

10 ml Sodium pyruvate (100 mM)

10 ml Penicillin-streptomycin (5 000 U/ml and 5 000 µg/ml, respectively)

Make up to 950 ml with dH_2O and pH to 7.0. Make up to 1 000 ml and filter sterilize through a 0.22 µm filter. Store at 4°C until necessary. Prior to use, measure out the volume required and supplement with FCS to a final concentration of 10 %.

ECL detection solution

250 mM luminol (5-amino-2,3-dihydro-1,4-pthalaginedione) in DMSO.

Make 1 ml aliquots and store at -20°C.

90 mM p-coumaric acid in DMSO.

Make 500 µl aliquots and store at –20°C.

a. Solution A

1 ml luminol

440 µl p-coumaric acid

10 ml 1 M Tris-HCl pH 8.5

Make up to 100 ml with dH_2O.

b. Solution B

62 µl 30 % H_2O_2

10 ml 1 M Tris-HCl pH 8.5

Make up to 100 ml with dH_2O

0.5 M EDTA pH 8.0

93.1 g EDTA

Make up to 500 ml with dH_2O. Adjust to pH 8.0 with NaOH pellets. Autoclave.

Elution buffer

50 mM NaH_2PO_4

300 mM NaCl

300 mM Imidazole

Make up to 1 000 ml with dH_2O. Adjust the pH to 8.0 using NaOH.

Ficoll loading dye

50 %	Sucrose
50 mM	EDTA (pH 7.0)
0.1 %	Bromophenol blue dye
10 %	Ficoll

Make up to volume with dH_2O. Store at 4°C.

1 M Glucose

Dissolve 9 g Glucose in 50 ml dH_2O. Filter sterilise.

1 M Glycine buffer pH 2.5

Dissolve 15 g Glycine in 200 ml dH_2O. pH to 2.5.

250 mM KCl

Dissolve 1.86 g KCl in 100 ml dH_2O.

Luria agar (pH 7.0)

10 g	Bacto-Tryptone
5 g	Bacto-Yeast extract
10 g	NaCl

Make up to 1 000 ml with dH_2O. Adjust the pH to 7.0 with approximately 1 ml of NaOH. Add 15 g agar and autoclave. Allow to cool to about 50°C to 60°C before adding 1 ml of carbenicillin (50 mg/ml). Plates can be poured directly from the flask; allow about 10 ml of medium per sterile petri dish. When the medium has hardened completely, invert the plates and store them at 4°C until needed. The plates should be removed from storage 1 to 2 hours before they are used. If the plates are fresh, they will 'sweat' when incubated at 37°C. This allows bacterial colonies or bacteriophage plaques to spread across the surfaces of the plates and increases the chances of cross contamination. This problem can be avoided by wiping off any condensation from the lids of the plates and incubating the plates for several hours at 37°C in an inverted position before they are used.

Luria-Bertani medium (pH 7.0)

10 g	Bacto-Tryptone

5 g Bacto-Yeast extract

10 g NaCl

Make up to 1 000 ml with dH_2O. Adjust the pH to 7.0 with approximately 1 ml of NaOH. Autoclave.

Lysis buffer

50 mM NaH_2PO_4

300 mM NaCl

10 mM Imidazole

Make up to 1 000 ml with dH_2O. Adjust the pH to 8.0 using NaOH.

2 M $MgCl_2$

Dissolve 19 g of $MgCl_2$ in 90 ml dH_2O. Adjust the volume to 100 ml with dH_2O and sterilise by autoclaving.

Micronutrients

3 nM $(NH_4)_6(MoO_7)_{24}$

400 nM H_3BO_3

30 nM $CoCl_2$

10 nM $CuSO_4$

80 nM $MnCl_2$

10 nM $ZnSO_4$

Make up to 1 000 ml with dH_2O.

Molecular weight marker (100bp)

250 µl Ladder (250 µg)

2.1 ml 1 x TE

125 µl Ficoll loading dye

Store at 4°C.

10 x MOPS buffer

400 mM MOPS

40 mM Tricine

Dissolve these two reagents and adjust the pH to 7.4 with KOH. Then add the following ingredients sequentially.

100 μM	$FeSO_4$
95 mM	NH_4Cl
2.76 mM	K_2SO_4
5 μM	$CaCl_2$
5.28 mM	$MgCl_2$
500 mM	NaCl
2.5 ml	Micronutrients

Finally, bring the total volume to 250 ml with dH_2O.

MOPS assembly

450 ml	autoclaved H_2O
50 ml	10 x MOPS
5 ml	20 % glucose
1 ml	tetracycline (5 mg/ml in 50 % ethanol)
500 μl	15 mM Thiamine HCl
500 μl	1 M K_2HPO_4

For the second round of growth (i.e., expression of the Fab) do not add the K_2HPO_4.

PBS (pH 7.4)

1.81 g	Na_2HPO_4
0.24 g	KH_2PO_4
8 g	NaCl
0.2 g	KCl

Adjust the pH to 7.4 and make up to a final volume of 1 000 ml. Store at 4°C.

Polyacrylamide gel solution

a. 15 % Resolving gel

2.3 ml	dH_2O
2.5 ml	1 M Tris pH 8.8 (lower tris)

5 ml	30 % acrylamide mix
0.1 ml	10 % APS
4 μl	temed

b. Stacking gel

2.85 ml	dH_2O
1.25 ml	1 M Tris pH 6.8 (upper tris)
0.82 ml	30 % acrylamide mix
25 μl	10 % APS
2 μl	temed

c. Lower SDS gel buffer

18.17 g	Tris base (1.5 M)
4 ml	10 % SDS

pH 8.8 with HCl. Make up to 100 ml with dH_2O.

d. Upper SDS gel buffer

6.06 g	Tris base
4 ml	10 % SDS

pH 6.8 with HCl. Make up to 100 ml with dH_2O.

25% Polyethylene glycol 6000

Dissolve 25 g of PEG in 100 ml of 0.9% saline.

RF1

3 g	RbCl
2.35 g	$MnCl_2.4H_2O$
7.5 ml	1 M KAc (pH 7.5)
0.375 g	$CaCl_2.2H_2O$
37.5 g	Glycerin

pH to 5.8 with 0.2 M acetic acid. Make up to 250 ml with dH_2O and filter sterilize.

RF2

5 ml	0.5 M MOPS
0.3 g	RbCl
2.75 g	$CaCl_2.2H_2O$
37.5 g	Glycerin

pH to 6.8 with NaOH. Make up to 250 ml with dH_2O and filter sterilize.

10 % SDS

10 g/100 ml

Weigh out SDS in a fume hood. Autoclave the dH_2O before adding the SDS powder.

2 x SDS gel loading buffer

0.125 M	Tris base pH 6.8
4 %	SDS (0.4 g/10 ml) (electrophoresis grade)
1 mg/10 ml	bromophenol blue
20 %	glycerol

Dissolve SDS in Tris. Then add glycerol and dissolve by heating. Add bromophenol blue. Make up to 10 ml with dH_2O.

SOC medium

20 g	bacto-tryptone
5 g	bacto-yeast
0.5 g	NaCl

Dissolve in 950 ml dH_2O. Add 10 ml of 250 mM KCl that has been sterile filtered. Adjust the pH to 7.0. Make up to 1 000 ml with dH_2O and autoclave. Add 5 ml of sterile 2 M $MgCl_2$ and 20 ml 1 M glucose that has been sterile filtered. Aliquot into sterile 15 ml Falcon tubes.

Staining solution

Dissolve 0.25 g of Coomassie Brilliant Blue R250 in 90 ml of methanol:H_2O (1:1 v/v) and 10 ml of glacial acetic acid. Filter the solution through a Whatman No. 1 filter to remove any particulate matter.

10 x TBE buffer

108 g Tris base

55 g Boric acid

7.4 g EDTA

Make up to 1 000 ml with dH_2O. Autoclave.

10 x TBST buffer (pH 7.5)

3.03 g Tris base (50 mM)

4.38 g NaCl (150 mM)

Adjust the pH to 7.5. Make up to 500 ml with dH_2O. Before use add 0.05 % Tween 20.

1 x TE buffer (pH 8.0)

10 mM Tris

1 mM EDTA

Make up to 1 000 ml with dH_2O. If necessary, pH to 8.0 with HCl.

Tetracycline

Dissolve 50 mg/ml stock in ethanol. Aliquot and store at -20°C.

Tris-glycine PAGE buffer

3.03 g Tris base

14.4 g Glycine

10 ml 10 % SDS

Make up to 1 000 ml with dH_2O.

Tris-glycine transfer buffer

6.06 g Tris base

28.8 g Glycine

Make up to 1 800 ml in dH_2O. Once all the reagents are completely dissolved add 200 ml methanol.

1 M Tris pH 8.0

Dissolve 24.22 g Tris in 200 ml dH_2O. pH to 8.0.

Wash buffer (IAA)

50 mM Tris

1 % Tween 20

Make up to 1 000 ml with dH_2O. pH to 8.0.

APPENDIX 2

Blast results for the alignment of the sequences obtained for the 1E2 and NS0 heavy chain genes.

APPENDIX 3

Blast results for the alignment of the aberrant kappa chain transcript with the sequence obtained for the 1E2/NS0 light chain.

APPENDIX 4

Blast results for the alignment of the aberrant heavy chain transcript and the sequence obtained for the 1E2 heavy chain.

APPENDIX 5

Blast results for the alignment of the aberrant heavy chain transcript and the sequence obtained for the NS0 heavy chain.

APPENDIX 6

APPENDIX 7

Published papers:

1. Recombinant Fabs of human monoclonal antibodies specific to the middle epitope of GAD65 inhibit type 1 diabetes-specific GAD65Abs (DIABETES, VOL 52, NOVEMBER 2003)
2. Epitope analysis of insulin autoantibodies using recombinant Fab (CLINICAL AND EXPERIMENTAL IMMUNOLOGY, VOL 140, JUNE 2005)