INVESTIGATION OF THE EPITOPE SPECIFICITIES OF ANTIBODIES TO ISLET β-CELL AUTOANTIGENS

by

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DEDICATION

This thesis is dedicated to my parents, to whom I owe so much,

who have been a constant source of encouragement.

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ABSTRACT

Glutamic acid decarboxylase-65 (GAD-65) and the tyrosine phosphatase-like protein IA-2 are major targets of autoimmunity in type 1 diabetes mellitus (type 1 DM), stiff-man syndrome (SMS) and autoimmune polyendocrine syndrome (APS). In this study, the precise epitopes in GAD-65 of three different mouse monoclonal antibodies (MoAbs) (N-terminal MoAb within amino acid residues 4-17, C-terminal MoAb within amino acid residues 572-585 and GAD-6), human monoclonal antibody (b96.11 huAb) and polyclonal antibodies (SMS patients' sera) were investigated. The precise epitopes in IA-2 of two different MoAbs (76B and 76F) were also investigated. These precise epitope investigations were performed using two different phage-displayed random peptide libraries with different characteristics (T7 phage library gene X C9C and linear 9-mers, and M13 filamentous phage library gene III C7C and linear 12-mers and gene VIII 5C4C4).

Sequencing of N-terminal and C-terminal MoAb reactive peptides, which were obtained from the successful biopanning using M13 gene III linear 12-mers phage library and were selected by high affinity binding in immnuo-blotting assay using nitro-cellulose membranes and in capture ELISA, revealed that the most significant motif recognised was P-G-x-x-x-W-S-F and F-L-I-x-E-I/V/L-D-x-L, respectively, which showed conservative substitutions and may correspond to the position 4-10 amino acids (aa) of GAD-65 (P-G-S-G-F-W-S-F) and to the position 573-581 aa of GAD-65 (F-L-I-E-E-I-E-R-L), respectively. To further define the N-terminal MoAb epitope, sequencing of N-terminal MoAbs reactive peptides, which were obtained from the successful biopanning using M13 gene VIII 5C4C4 phage library and were

selected by high affinity binding in immnuo-blotting assay using nitro-cellulose membrane and in capture ELISA, revealed a motif of S-T-P, which does not correspond to 4-17 aa of GAD-65 and does not overlap with the previous motif of the N-terminal MoAb, i.e. P-G-S-G-F-W-S-F (4-10 aa) of GAD-65. Therefore, the M13 pVIII 5C4C4 worked with N-terminal MoAb by expressing a relevant sequence for the N-terminal MoAb but which is unlike its epitope in GAD-65. To further define the N-terminal MoAb epitope, sequencing of N-terminal MoAb reactive peptides, which were obtained from the successful biopanning using T7 gene X linear 9-mers phage library and were selected by high affinity binding in immnuo-blotting assay using nitro-cellulose membrane, revealed a motif of P-X-X-G which may correspond to 4-7 aa of GAD-65 (P-G-S-G), which overlaps with the previous motif P-G-S-G-F-W-S-F (4-10 aa).

Sequencing of GAD-6 MoAb reactive peptides, which were obtained from the successful biopanning using T7 gene X C9C phage library and were selected by high affinity binding in immnuo-blotting assay using nitro-cellulose membrane and in ELISA, revealed two different motifs of R/K-L/A/I-x-K and M-x-x-A, which showed conservative substitutions and may correspond to the position 525-528 aa of GAD-65 (R-L-S-K) and to the position 523-526 aa of GAD-65 (M-S-R-L), respectively, which overlap with each other. To further define the GAD-6 epitope, sequencing of GAD-6 reactive peptides, which were obtained from the successful biopanning using T7 gene X linear 9-mers phage library and were selected by high affinity binding in immnuo-blotting assay using nitro-cellulose membrane, revealed a motif of R-x-x-K, which may correspond to 525-528 aa of GAD-65 (R-L-S-K) and overlaps with the previous motif of the GAD-6 selected from T7 gene X C9C phage library. To further define the

GAD-6 epitope, sequencing of GAD-6 reactive peptides, which were obtained from the successful biopanning using M13 gene VIII 5C4C4 phage library and were selected by high affinity binding in immnuo-blotting assay using nitro-cellulose membrane and in capture ELISA, revealed a motif of M-x-x-A which may correspond to 523-526 aa of GAD-65 (M-S-R-L), and overlaps with the previous motif selected from T7 gene X C9C phage library. Thus, the overall motif of GAD-6 may correspond to 523-528 aa of GAD-65 (M-S-R-L-S-K). To further define the GAD-6 epitope, sequencing of GAD-6 reactive peptides, which were obtained from the successful biopanning using M13 gene III C7C and linear 12-mers phage libraries, did not show a clear motif and did not show reactivity with GAD-6 by capture ELISA. A possible explanation for this is that the peptides, which are specific to GAD-6, are not present in these M13 pIII phage libraries.

Sequencing of b96.11 huAb reactive peptides, which were obtained from the successful biopanning using M13 gene III linear 12-mers phage library and were selected by moderate affinity binding in immnuo-blotting assay using nitro-cellulose membrane, revealed two different motifs of I/V-T/S-A/G/L-T/S-A/L and S-T/S-G/A/L/I, which showed conservative substitutions and may correspond to the position 332-336 aa of GAD-65 (V-S-A-T-A) and to the position 338-340 aa of GAD-65 (T-T-V), respectively. Thus, the overall motif of b96.11 might correspond to 332-340 aa of GAD-65.

In a capture ELISA system for the detection of GAD-65 specific antibodies, b78 huAb bound slightly better with GAD-6 (rather than N-terminal MoAb) as the capture MoAb, but b96.11 bound much better with GAD-6 as the capture MoAb. This might

suggest that GAD-6 does interfere with b78 huAb binding to GAD-65 more than it does with b96.11 huAb. However, it must also suggest that the GAD-6 and b78 huAb epitopes are not directly overlapping.

Sequencing of SMS sera reactive peptides, which were obtained from the successful biopanning using M13 gene III linear 12-mers phage library and were selected by moderate affinity binding in immnuo-blotting assay using nitro-cellulose membrane, revealed two different motifs of L/A-A-x-T/S-R/H/K and of T/S-T-V/I/L-F-E-L/G/I/V/A-H/K-L/G-x-K/R, which showed conservative substitutions and may correspond to the position 371-375 aa of GAD-65 (L-L-M-S-R) and to the position 463-472 aa of GAD-65 (T-T-G-F-E-A-H-V-D-K), respectively, as public epitopes of SMS patients' sera.

Sequencing of 76B and 76F MoAbs reactive peptides, which were obtained from the successful biopanning using T7 gene X C9C and M13 gene III linear 12-mers phage libraries and were selected by high affinity binding in immnuo-blotting assay using nitro-cellulose membrane and in ELISA, revealed that the most significant motif recognised was D-x-K-P-L-S and F-x-Y-Q, respectively, which may correspond to the position 477-482 aa of IA-2 (D-Q-K-P-L-S) and to the position 626-629 aa of IA-2 (F-E-Y-Q), respectively.

The studies described in this thesis have shown that the epitope mapping of different antibodies on GAD-65 and IA-2 may help to understand the relationship between antigenicity and structure in these autoantigens which are targets in type 1 DM and related disorders (e.g. SMS and APS).

CHAPTER ONE

GENERAL INTRODUCTION

1. Introduction

1.1 Autoimmunity

1.1.1 The Spectrum of Autoimmune Diseases

Autoimmune disease occurs when a specific adaptive immune response is directed against self. Human autoimmune diseases are divided into two major groups, organ- or tissue-specific, and systemic. In organ- or tissue–specific autoimmune diseases, autoimmune responses are directed against autoantigens present in a single organ or tissue [e.g., type 1 diabetes mellitus (type 1 DM)]. On the other hand, in systemic autoimmune diseases, autoantigens that are widespread throughout the body are the target of autoimmune responses [e.g., nuclear antigens in systemic lupus erythematosus (SLE)] (Table 1.1). Some autoimmune diseases are characterised by autoimmune responses against both systemic and organ- or tissue-specific autoantigens (e.g., Sjögren's syndrome).

Autoantibodies or T cells can cause tissue damage in autoimmune diseases. Autoantibodies to autoantigens on cell surfaces (e.g., autoimmune haemolytic anaemia) or extracellular matrix (e.g., Goodpasture's disease) lead to tissue damage. T cells specfic to autoantigens can damage tissue cells directly or by activating macrophages (e.g., in type 1 DM). Also, autoantibodies to receptors cause disease by stimulating (e.g., thyroid-stimulating hormone receptor in Graves' disease) or blocking (e.g., acetylcholine receptor in myasthenia gravis) receptor function. On the

other hand, chronic generation of immune complexes causes tissue damage in systemic autoimmune diseases (e.g., SLE).

Table 1.1 Classification of Autoimmune Diseases, Target Autoantigen Associations.

Autoimmune Diseases	Autoantigens
Organ- or Tissue-Specific Diseases	
Type 1 Diabetes Mellitus	Islet β cells
Autoimmune Thyroiditis	Thyroglobulin, Thyroid Peroxidase
Graves' Disease	Thyroid Stimulating Hormone Receptor
Myasthenia Gravis	Acetylcholine Receptor
Pernicious Anaemia	Intrinsic Factor
Primary Biliary Cirrhosis	Bile Ducts of Liver
Goodpasture's Disease	Glomerular Basement Membrane
Pemphigus Vulgaris	Epidermal Cadherin
Bullous Pemphigoid	Epidermal Basement Membrane
Addison's Disease	Secretory Cells of Adrenal Cortex
Systemic Diseases	
Systemic Lupus Erythematosus	DNA, Histones, Ribosomes
Systemic Sclerosis	Nucleoli
Autoimmune Haemolytic Anaemia	Red Blood Cells, Rhesus Antigen
Dermatomyositis	Soluble Nuclear Proteins
Rheumatoid Arthritis	IgG
Idiopathic Thrombocytopenic Purpura	Platelet Integrin

1.1.2 Mechanisms of Self-Tolerance Induction

There are two mechanisms involved in the induction of self-tolerance: primary and secondary (regulatory).

1.1.2.1 Primary Mechanisms

Primary mechanisms include clonal deletion, clonal anergy and clonal indifference (ignorance) which influence lymphocyte development and shaping of the repertoire.

1.1.2.1.1 Clonal Deletion

T and B-cell lineages can be eliminated during their development and maturation in central and peripheral lymphoid tissues. Also, clonal deletion includes receptor editing (escaped deletion), resulting in synthesis of receptors lacking specificity for the original self antigen; and propriocidal killing: deletion by apoptosis when the lymphocytes are exposed to self antigen either early in development or when the self antigen is present in multivalent form or at high concentrations of self antigens, deleting particularly those lymphocytes with high affinity for the antigen.

1.1.2.1.1.1 B cells

B cells, like T cells, are susceptible to deletion at an early stage in their development. B cells obtained from tolerant double transgenic mice expressing multivalent self antigens [e.g. major histocompatibility (MHC) class I or membrane bound hen egg lysozyme (HEL)] are eliminated from peripheral lymphoid tissues in the absence of signal 2 for cell-cell interactions (Hartley et al., 1991). Also, inducing tolerance in B cells with specificity for natural self antigens by deletion has been confirmed by using transgenic models expressing the genes for autoantibodies to double stranded DNA (Chen et al., 1995), erythrocytes (Murakami et al., 1992) and CD8 (Brombacher et al., 1991). In the case of the erythrocyte model, the Ig transgenic B cells correlated to the B-1 rather than the B-2 lineage, suggesting that the B-1, like B-2, cells are susceptible to deletion, and the deletion can occur in the periphery as well as the bone marrow. Furthermore, in the case of the CD8 model, mature peripheral B cells may also be eliminated.

Self-reactive B cells may also be eliminated by receptor editing (Tiegs et al., 1993; Gay et al., 1993). Following engagement of Ig receptors on immature B cell, recombinase activating genes are reinduced leading to receptors lacking specificity for the original self antigen.

Administration of large amounts of soluble antigen to normal or transgenic mice leads to apoptotic death in germinal centre B cells, particularly those with high affinity for the inducing antigen, by interfering with the interaction of B cells with the follicular dendritic cell network (Han et al., 1995; Pulendran et al., 1995); this resembles propriocidal killing of acutely activated T cells. This process is T cell and Fas independent and inhibited by bcl-2.

1.1.2.1.1.2 T cells

Thymic stromal cells which include endogenous thymic epithelial cells mainly control positive selection whereas bone marrow-derived stromal cells like macrophages and dendritic cells mainly control negative selection (deletion) (Pardoll and Carrera, 1992; Hugo et al., 1993).Early precursor thymocytes, which lack either T cell receptor (TCR) or accessory molecules, mature into TCR+ CD4+ CD8+ (double positive) cells with the ability to interact with antigen-presenting stromal cells. The double positive cells with higher avidity for self-peptide-MHC complexes are eliminated, while those with lower avidity survive and differentiate into single positive CD4+ CD8- or CD4- CD8+ T cells with anti-foreign specificity (Scollay and Godfrey, 1995).

Intrathymic deletion of TCRαβ+ single positive T cells recognising self peptide on thymic APC is very efficient (Sprent, 1995). There is evidence that γδ T cells are also

partially intrathymically deleted (Allison, 1993). Some αβ TCR+ double negative T cells are positively selected in the thymus and migrate to peripheral lymphoid tissues and the bone marrow (Dejbakhsh-Jones et al., 1995). Within the thymus, apoptosis can occur in double positive thymocytes via a Fas dependent pathway (Arase et al., 1994), while within the periphery those expressing the NK1.1 marker appear to play an immunoregulatory role through release of large amounts of Th2 cytokines (IL-4 and IL-10) (Bendalac, 1995).

T-cell deletion is complete when massive doses of antigen are used. For example, inoculation of a peptide from lymphocytic choriomeningitis virus (LCMV) in TCR transgenic mice causes complete deletion of LCMV-specific T cells (Moskophidis et al., 1993). In addition, Fas may play a role in maintaining self-tolerance in humans as evident from the development of autoimmune lymphoproliferative syndromes in patients with Fas gene mutations (Fisher et al., 1995). Fas-related molecules, such as tumour necrosis factor receptor (TNFR), control the deletion of CD8+ T cells whereas the Fas pathway also controls the deletion of CD4+ T cells (Zheng et al., 1995).

1.1.2.1.2. Clonal Anergy

Clonal Anergy is characterised in both T- and B-cell lineages by a failure to proliferate in response to antigen , down regulation of the antigen receptor complex and/or cytokine receptors and costimulatory molecules and reversibility on exposure to appropriate second signals.

1.1.2.1.2.1 B cells

Both mature and immature B cells can be rendered anergic. Thus, this mode of tolerance occurs in peripheral lymphoid tissue as well as the bone marrow (Goodnow et al., 1989). Although anergic B cells, which are removed from the tolerant environment, fail to respond to T-cell help or T-independent stimuli such as lipopolysaccharide (LPS), removal of the anergic B cells to an environment that lacks tolerogenic concentrations of antigen leads to upregulation of IgM and antibody production (Adams et al., 1990). Thus, the anergic state is reversible: it does not commit the B cell to death and may allow the development of autoimmunity to soluble (oligovalent) self antigens. Anergic B cells from soluble HEL double transgenic mice can retain the capacity to process antigen and to proliferate and differentiate into antibody producing cells following exposure to CD40 ligand and the cytokines IL-4 and IL-5. On the other hand, this state is associated with downregulation of the Ig receptor function and a reduction in tyrosine kinase activity and calcium flux (Eris et al., 1994).

1.1.2.1.2.2 T cells

T-cell anergy occurs when IL-2 is lacking, as for example, when the interaction between pairs of costimulatory molecules like B7/CD28 is blocked. T-cell anergy is associated with inhibition of proliferation and secretion of IL-2 and with downregulation of T cell receptors for antigen and IL-2 or coreceptors. Also, it is associated with a block in the transcription of the IL-2 gene by AP-1, the activity of which depends on signaling through the TCR-CD3 and CD28 pathways (Kang et al., 1992). A failure in activation of the Ras protein may block the production of IL-2,

since the Ras protein transmits signals along the TCR-CD3 pathway to AP-1 and influences early events in the CD28 pathway (Li et al., 1996).

Less antigen results in anergy whereas more antigen leads to deletion (Fazekas de St. Groth et al., 1992). Murine and human CD4+ T-cell clones can be rendered anergic in vitro on encounter with peptide in the absence of CD28/B7 signals (DeSilva et al., 1991), whereas naïve T cells (e.g., from TCR transgenic mice) remain indifferent. Blockade of the interaction between CD28 and B7 in vivo does not necessarily lead to anergy; so the T cells either remain indifferent (naïve) or if blockade is incomplete, partial activation may occur which may shift the cytokine profile from Th1 to Th2 (Kearney et al., 1995).

Exposure of CD4+ T cells to intravenous peptide or superantigen is accompanied by an anergic state, because the avidity of binding to the TCR receptor does not reach that required for deletion.

1.1.2.1.3 Clonal Indifference (Ignorance)

T and B cells expressing anti-self receptors may persist in the host but fail to interact with antigen. There are three reasons why these cells remain indifferent to self antigens. The first reason includes antigen related factors such as low concentration and valency or inaccessibility (sequestration). The second reason is cellular factors including low precursor frequency of naïve T cells, low receptor avidity of preswitched B cells or low functional activity of neonatal (early in life) dendritic cells and naïve unprimed T cells which lack adhesion molecules required for penetration of nonlymphoid tissues. The third reason includes host or tissue related factors such as

low or absent expression of MHC, lack of costimulatory molecules or lack of cytokine secretion.

1.1.2.2 Secondary Mechanisms

1.1.2.2.1 Interclonal Competition and Follicular Exclusion

In the case of B cells, interclonal competition may occur between existing and newly formed clones of cells for a range of rate-limiting processes, such as growth factors. On the other hand, follicular exclusion is a way of refining the anti-foreign B-cell repertoire which can occur for two reasons: first, in HEL double transgenic mice, the presence of immune complexes within peripheral lymphoid tissue may block antigen binding Ig receptors leading to interference by these immune complexes with normal B-cell signalling (Fulcher and Basten, 1997). Second, a lack of T-cell help leads to the lack of the second signals and the failure of self-reactive B cells to enter the follicles (Fulcher et al., 1996).

1.1.2.2.2 Suppression

Suppression can be mediated under different conditions by CD4+, CD8+ and double negative CD4- CD8- T-cell subpopulations (Adelstein et al., 1990). CD4+ T cells suppression is not entirely clear although it can be associated with induction of a Th2 cytokine profile. Also, double negative T cells, including those expressing NK1.1, secrete large amounts of IL-4 and IL-10 and very little amounts of IL-2 or IFNγ (Bendalac, 1995). Suppression by this subset may be due to preferential induction of a Th2 response and immune deviation. In addition, CD8+ T cells exert their suppressive

effect on development of experimental allergic encephalomyelitis (EAE) via release of the inhibitory cytokine, transforming growth factor-β (TGF-β) (Miller et al., 1992).

1.1.2.2.3 Immune Deviation

Immune deviation describes the reciprocal relationship between T-cell reactivity [delayed type hypersensitivity (DTH)] and antibody production which can occur following immunization with antigen in different physical forms or concentrations. For example, administration of a small dose of antigen leads to a strong DTH response with little or no antibody, whereas large doses lead to significant antibody production and less DTH. Also, strong DTH responses occur when Th1 cells secreting IL-2 and IFNγ are activated under the influence of macrophage derived IL-12. On the other hand , production of most classes of antibodies is stimulated by Th2 derived IL-4. Moreover, Th2 and Th1 cells are inhibited by IFNγ and IL-4/IL-10, respectively (Mosmann et al., 1991) .

1.1.2.2.4 Vetoing

Human and mouse CD8+ T cells can recognise self peptide-MHC class I complexes present on other CD8-expressing T cells which then kill the original cell by apoptosis (Miller, 1986). Vetoing depends on an interaction between the CD8 molecule on the veto cell and the α3 domain of the MHC class I molecule on the recognising cell (Fink et al., 1983). Human, not mouse, CD4+ T cells can act as veto cells by expressing HLA class II (Schwartz, 1993).

1.1.2.2.5 Idiotype Network

Perinatal administration of certain anti-idiotype antibodies can lead to prolonged inhibition of the corresponding idiotypes later in life by cross-linking of Ig receptors on immature B cells, which are then deleted in the absence of second signals (Keaney and Vakil, 1986). The efficacy of pooled Ig in the treatment of certain autoimmune diseases, such as immune thrombocytopenic purpura, may be related to its content of anti-idiotype antibodies (Berkman et al., 1990).

1.1.3 Mechanisms of Induction of Autoimmunity

1.1.3.1 Release of Anatomically Sequestered Antigens

Antigens associated with peripheral tissues, especially those sequestered behind anatomical barriers, may not be exposed to the developing T cell repertoire and, therefore, tolerance may normally be unnecessary. However, evidence indicates that exposure of previously sequestered antigens as a cause of organ-specific autoimmunity can occur. For example, in sympathetic ophthalmia, damage to one eye leads to a response to released sequestered antigen causing autoimmune damage to the other eye.

1.1.3.2 Cryptic Epitopes

Some tissue proteins are normally processed and presented to lymphocytes by antigen presenting cells (APCs), with tolerance being established to the dominant epitopes. If, however, circumstances arise which cause the same components to be degraded in an unusual or excessive manner, peptide fragments may be generated

which would normally be cryptic, and to which the immune system is not tolerant. Lymphocytes can react against these cryptic epitopes, generating an autoimmune response. Epitope crypticity may result from various factors, including ineffective processing or dominance of a flanking epitope that competes for binding to the same human leukocyte antigen (HLA) molecule.

1.1.3.3 Peripheral Tolerance and Immunological Ignorance

Mature resting T cells specific for extrathymic antigens, which are presented by non-professional APCs (other than dendritic cells and macrophages), may be induced to undergo anergy due to the absence of appropriate second signals or costimulatory factors (Schwartz, 1990). An alternative possibility is that there is no induction of anergy, but that the mature T cells are unable to receive appropriate signals and/or help possibly due to the low amount of antigen presented. Therefore, the T cell will ignore such antigens. Thus, if adequate antigen presentation and costimulation occurs through professional APCs, then these T cells may be activated and cause tissue damage.

1.1.3.4 Molecular Mimicry

Many peptide fragments of infectious agents are homologous with host proteins [e.g., coxsackie virus P2-C protein and glutamic acid decarboxylase (GAD) (Kaufman et al., 1992)]. Thus, the immune responses against these fragments of infectious agents may cross-react with self antigens. Autoreactive T cells may be stimulated initially by cross-reactive foreign antigens, not by the autoantigens themselves, due to the way in which the foreign antigens are presented, i.e. their immunogenicity. The foreign

antigens are normally presented by professional antigen presenting cells (APCs) (i.e. dendritic cells and macrophages) which express HLA class II and costimulatory adhesion molecules. Once T cells are activated by foreign antigens, these effector T cells may react against cross-reactive autoantigens.

1.1.3.5 Molecular Modification

Some antigenic determinants are created on somatically mutated antibodies during the maturation of the immune response. For example, in rheumatoid arthritis, rheumatoid factors (RFs) (anti-IgG Fc autoantibodies) may be induced because modified antigenic determinants are exposed on antigen-complexed IgG. Although these low affinity autoantibodies (RFs) react better with multimeric IgG, they can bind to monomeric IgG. However, glycosylation defects may also play a role in RF induction (Tsuchiya et al., 1993).

In addition, the carrier effect enables autoreactive B cells to receive T cell help when a foreign determinant, e.g. drug or virus, becomes covalently linked to a self-determinant which is recognised by the receptor (Ig) of autoreactive B cell. TCR of the helper T cells recognise the foreign determinant which is processed and presented by MHC of self-reactive B cells. Then, these helper T cells cooperate with self-reactive B cells to induce proliferation of these B cells and production of autoantibodies (Reeves and Todd 1996).

1.1.3.6 Failure of Natural Immune Tolerance to Autoantigens

Errors in central or peripheral tolerance at the T- or B-cell level have also been suggested as causes of autoimmunity, which might be due to interference with apoptosis during negative selection of reactive clones. Interactions between Fas and Fas ligand (FasL) are required for the initiation of apoptosis, and SLE-prone mice, including lpr (lymphoproliferation) and gld (generalised lymphoproliferative disease) mice, are defective in the apoptosis-promoting Fas and FasL, respectively. The SLE-prone MRL-lpr mice, rendered transgenic for TCR-recognising antigens in the context of MHC class I or class II, show defects in peripheral T-cell tolerance with no defects in thymic negative selection (Singer and Abbas 1994). Fas-deficient autoimmune lymphoproliferative syndrome (FD-ALPS), also known as the Canale-Smith syndrome, is the human equivalent of the lymphoproliferative autoimmune disease of MRL-lpr mice which is associated with inherited genes encoding defective versions of the Fas protein.

1.1.3.7 Polyclonal Activation

Polyclonal T- and/or B-cell activation has also been suggested as an initiating mechanism of autoimmunity, particularly in systemic diseases. Polyclonal B-cell activators may induce autoantibodies because of the existence of non-deleted self-reactive B cells and/or stimulating arrested anergised B cells which may become active. Polyclonal T-cell activators may also induce autoantibodies indirectly. The T cells, which react with HLA class II-bound antigenic self-peptides on B cells, may stimulate self-antigen specific B cells, thereby leading to production of autoantibodies (Theofilopoulos, 1993).

1.1.3.8 Immunoregulatory Disturbances

Changes in T-cell subsets and their cytokines may be involved in initiating autoimmune diseases. For example, in type 1 DM, CD4+ T helper 1 cell (TH1)-derived IFN-γ activates macrophages and CD8+ cytotoxic T cells (TC) and inhibits TH 2 cells, which might otherwise have suppressed the autoimmune response.

1.2 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (type 1 DM) is an autoimmune disease which is characterised by destruction of insulin-producing pancreatic islet β cells. This autoimmune response to the islet β cells can damage these cells following cellular infiltration of the islets by Th1 cells, macrophages and CD8+ cytotoxic T cells (TC). Also, autoantibodies, e.g. to glutamate decarboxylase (GAD), are produced to the autoantigens of the islet β cells, and these can be used as markers of the pathogenesis of type 1 DM. These autoantibodies can also be produced in other diseases, such as autoimmune polyendocrine syndrome (APS) and stiff-man syndrome (SMS).

There are two types of APS, type I (APS I) and type II (APS II). APS I is a rare disorder with an autosomal recessive inheritance. The main common features of APS I are mucocutaneous candidiasis, hypoparathyroidism, adrenal failure and 5% of patients develop type 1 DM. APS II is a rare disorder with an autosomal dominant inheritance. The main common features of APS II are thyroid disease, gastric autoimmunity, adrenal failure and up to 50% of patients develop type 1 DM (Neufeld et al., 1980).

SMS is a rare neurological disorder characterized by progressive rigidity and spasms of skeletal muscles and a deficiency of gamma-amino-butyric acid (GABA). This is associated with the binding of autoantibodies to GAD, which is responsible for the synthesis of GABA from glutamic acid in the brain and peripheral neurons and islet β cells (Solimena et al., 1990). About 30% of SMS patients develop type 1 DM, but most patients with type 1 DM do not have SMS (Solimena et al., 1990).

Environmental factors, such as specific viruses, and genetic factors, primarily HLA, are associated with the development of type 1 DM.

1.2.1 Immunopathogenesis of Type 1 Diabetes Mellitus

Islet β cells may express many autoantigens (for example, insulin and GAD). The autoimmune reponse against these autoantigens involves humoral mediators (autoantibodies) and cellular mediators, i.e. TC, TH, macrophages and other APCs, such as dendritic cells (Benoist and Mathis 1997).

Islet β cell autoantigens are processed and presented by macrophages and/or other APCs (particularly dendritic cells) in association with HLA class II molecules, which are present on the surface of APCs. Ag-HLA class II complexes and co-stimulatory adhesion molecules (B7), which are present on the surface of macrophages, may activate TH1. Also, macrophages secrete interleukin-12 (IL-12) to further activate TH1. Then, TH1 produce IL-2 and interferon-γ (IFN-γ). IFN-γ activates macrophages and TC, while IL-2 activates TC only (Rabinovitch, 1994). Also, IFN-γ inhibits TH2 (Lord et al., 1996) and may have direct cytotoxic effects on the islet β cells (Awata et al., 1994). Furthermore, IFN-γ upregulates the adhesion molecules on the islet β cells (Awata et al., 1994) and enhances their HLA class I expression to promote the interaction of TC with islet β cells (Awata et al. 1994; Hussain et al., 1996). IFN-γ in combination with either tumour necrosis factor (TNF)-α or -β or lymphotoxin induces HLA class II expression on the islet β cells (Pujol-Borrell et al., 1987). The macrophages, which are activated by IFN-γ, secrete IL-1α, IL-1β, TNF-α (Hussain et

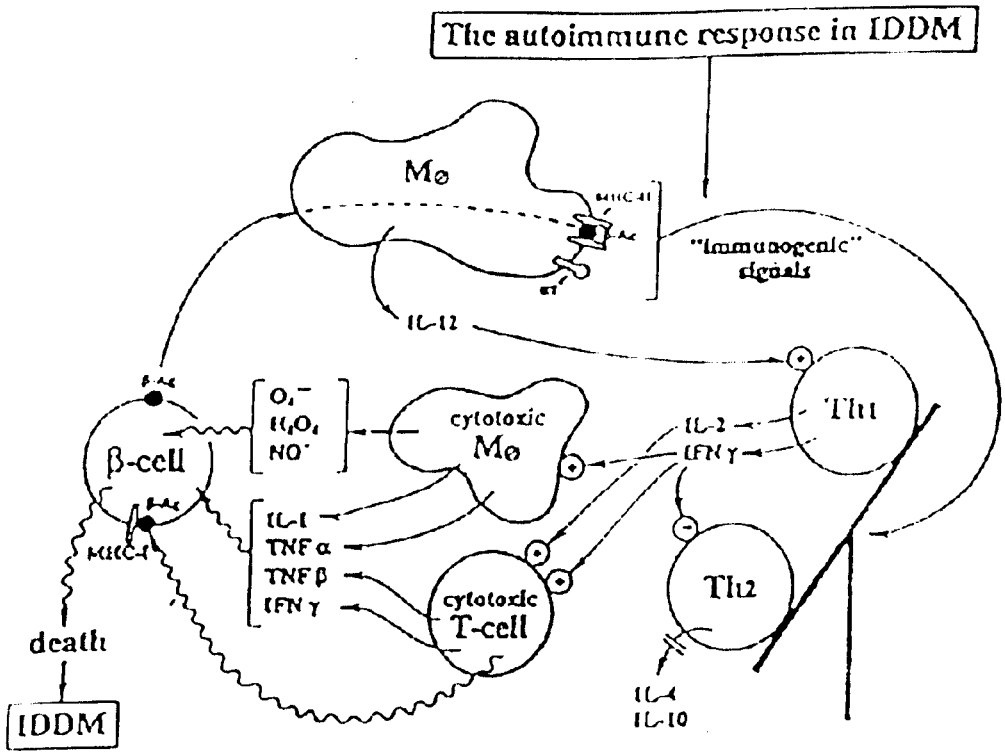
al., 1996), IL-6 (Benoist and Mathis 1997), oxygen free radicals (O_2^- and H_2O_2) and nitric oxide (NO) which are cytotoxic to islet β cells (Rabinovitch, 1994). In contrast, TC can be elicited through bystander activation, but can home to and destroy the islet β cells, by recognition of antigens (Benoist and Mathis, 1998). There is evidence that perforin plays its role in islet β cell destruction (Benoist and Mathis 1997). Also, the interaction between Fas on islet β cells and Fas ligand on infiltrating cells might trigger selective apoptotic β-cell death in inflamed islets (Moriwaki et al., 1999). Macrophage-derived IL-1β enhances expression of Fas (CD95) on islet β cells (Yamada et al., 1996) which facilitates Fas-Fas ligand interactions: Fas ligand (CD95L) is already expressed on islet β cells of normal individuals: interaction between Fas and Fas ligand both expressed on islet β cells may induce apoptosis of islet β cells (Loweth et al., 1998). In another study in NOD mice, FasL was detected on TH and TC and Fas expression was detected on β cells of syngeneic islet grafts leading to autoimmune destruction of islet β cells. Fas expression correlates with expression of IL-1α, TNFα and IFNγ in islet grafts (Suarez-Pinzon, et al., 1999). Islet β cell destruction and infiltration by leukocytes in autoimmune diabetes in NOD mice involves FasL-mediated mechanisms (Suarez et al., 2000). Also, TNF/TNFR1 interaction may play a role in the destruction of islet β cells (Pakala et al., 1997).

Vascular endothelial cells may also respond to IL-1, TNF and IFN-γ by expressing HLA class II molecules to present islet β cell autoantigens to TH1 (Rabinovitch, 1994), and adhesion molecules to enhance the accumulation of mononuclear cells in the islets (Hanninen et al., 1992).

TC secrete IFN-γ and TNF-β, which are cytotoxic to islet β cells, and the TC interact with Ag-HLA class I complexes, which are present on the surface of islet β cells (Rabinovitch, 1994) (Fig. 1.1).

Further damage to the islet β cells may enhance the exposure of some proteins, which are normally kept sequestered within the islet β cells, such as heat-shock proteins, proinsulin and cytoplasmic gangliosides. The appearance of these proteins stimulates the inflammatory T cells, particularly TC, and the secretion of inflammatory mediators (TC-derived IFN-γ), which promotes the attraction of macrophages. The macrophages will present them to TH1. The remaining healthy β cells must overwork to supply the needed insulin. This hyperactivity exhausts the islet β cells and may increase the release of the autoantigens and the killing of these islet β cells. Very few islet β cells remain to supply insulin to the body, and the symptoms of type 1 DM will appear (Atkinson and Maclaren 1990).

Figure 1.1 The role of TH1 and TH2 subsets in autoimmune response in type 1 diabetes mellitus (Rabinovitch 1994).



1.2.2 Environmental Factors

1.2.2.1 Geographical Distribution and Incidence

The incidence of type 1 DM has been shown to be increasing. However, the incidence of type 1 DM differs between populations, with a 60-fold difference between the highest and the lowest rates (Rewers et al., 1988; Green et al., 1992; Karvonen et al., 1997). The highest incidence is found in Caucasoid populations, particularly in northern Europe, and the lowest rates are found in Asia and South America (Karvonen et al., 1993; Karvonen et al., 1997).

The incidence of type 1 DM has increased by about 2% per year during the last 10 years (Bingley and Gale 1989; Dahlquist et al., 1991). During 1993-1995, the incidence of type 1 DM was 8.1 per 100,000 a year, ranging in-between lower rates in southern European countries and higher rates in northern Europe (Rosenbauer et al., 1999). In the Oxford region, the annual increase of the incidence of type 1 DM in children aged 0-4, 5-9 and 10-14 was 11%, 4% and 1%, respectively, between the year 1985-1996 (Gardner et al., 1997). In addition, in children aged under 5 years, the incidence of type 1 DM was increased from 4.2 to 9.9 per 100,000 per year between 1973-4 and 1988, in England (Metcalfe and Baum 1991). In Finnish children aged under 15 years, the annual increase in the incidence of type 1 DM was 2.4% between the year 1965-1984 (Tuomilehto et al., 1992). By the year 2010, it is estimated that the incidence of type 1 DM will be 50 per 100,000 per year in Finland and it will exceed 30 per 100,000 per year in many other populations (Onkamo et al., 1999). The incidence of type 1 DM in Sardinia is almost as high as in Finland, 36.5 per 100,000 per year in children aged ≤14 years between the year 1990-1994 (Karvonen et al.,

2000), whereas most southern European countries have a lower incidence (Dorman et al., 1990; Muntoni et al., 1992). Generally, a very high incidence of type 1 DM amongst children aged ≤14 years between the year 1990-1994 (≥20 per 100,000 per year) was found in Sardinia, Finland, Sweden, Norway, Portugal, the U.K., Canada and New Zealand (Karvonen et al., 2000).

The differences in geographical incidence, as seen in type 1 DM, are rare amongst severe chronic diseases of childhood (Diabetes Epidemiology Research International Group 1988).

1.2.2.2 Viruses as Important Environmental Factors

A range of viruses has been implicated in type 1 DM. Enterovirus infections, particularly with coxsackie virus B4 (CVB4), may play a role in activating the immune response against islet β cells. Increased islet β cell autoantibody (ICA)/insulin autoantibody (IAA) levels are associated with enterovirus infections. The levels of virus-specific IgM, IgG and IgA are increased in enterovirus infections and the level of CVB-specific IgM is increased in newly diagnosed type 1 DM patients (Hyoty et al., 1995; Kaufman et al., 1992). Proliferative lymphocyte responses to antigens from other viruses, such as CVB1, B6 and adenovirus with homologies to GAD-65 are observed in newly diagnosed type 1 DM (Jones and Crosby 1996). The prevalence of coxsackie B serum antibodies is increased in recent-onset type 1 DM (Helfand et al., 1995) and in mothers of infants who developed early-onset type 1 DM (Dahlquist et al., 1995). Furthermore, the elevated levels of CVB-specific IgM persist for a few months after infection. This has led to the suggestion that CVB infection may precipitate the onset of type 1 DM in a pre-

diabetic individual within a few months. In contrast, diabetes may develop many years after exposure to the virus. For example, initiation infections, such as congenital rubella infection, occurs in utero but diabetes develops 5-20 years later. In culture, the CVB4 and mumps viruses can infect human islet β cells, leading to overexpression of HLA class I (Parkkonen et al., 1992).

There are six amino acids, PEVKEK (260-265 amino acid residues of GAD), identical in P2-C protein of CVB4 and GAD65 (the 65 kDa isomer of GAD), while they differ in only one amino acid, PEVKTK (Glu→Thr), between P2-C protein of CVB4 and GAD67 (the 67 kDa isomer of GAD) (Hou et al., 1994). The high similarity of the amino acid sequences of GAD65/67 to the P2-C protein of CVB4, is consistent with the possibility that molecular mimicry plays a role in the pathogenesis of type 1 DM. Thus, it suggests that the immune response against P2-C protein of CVB4 may cross-react with GAD of islet β cells, leading to more destruction of islet β cells and further release of GAD from islet β cells (Kaufman et al., 1992; Hou et al., 1994). Interestingly, MICA-10, human monoclonal antibody derived from a diabetic patient, recognises an amino acid residue (E264) within this amino acid sequence of GAD, PEVKEK. However, MICA-10 does not recognise the P2-C protein itself, indicating that the linear homology region in P2-C does not acquire a conformation similar to that of GAD-65 (Schwartz et al., 1999).

In contrast to the above, Horwitz and colleagues showed that infection with CVB4, which shows similarity with GAD, rapidly provoked diabetes in TCR transgenic mice specific for a different islet autoantigen. This suggests that CVB4 induces diabetes as an indirect result of local infection leading to inflammation, tissue damage and the

release of sequestered islet antigens causing the stimulation of resting autoreactive T cells. Thus, CVB4 may induce type 1 DM through bystander damage and activation (Horwitz et al., 1998).

Congenital rubella infection may induce autoimmune islet β cell destruction and subsequently the development of type 1 DM (Pack et al., 1988). 20% of individuals with congenital rubella syndrome (CRS) develop type 1 DM while no case has been reported of type 1 DM following postnatal rubella infection (Eisenbarth et al., 1994). Pancreatic islet cell surface autoantibodies (ICSA) are found in 20% of the total CRS population. Also, the presence of HLA-DR3 and the absence of HLA-DR2 are associated with the CRS patients who develop diabetes (Feellner et al., 1984). Furthermore, there are significant correlations between T-cell clone responses to rubella virus peptide V3 (RVE1) (157-176 amino acid residues) and a determinant of GAD67 (274-286 amino acid residues) in patients with recent onset type 1 DM and the response of another T cell clone cross-reactive with RVE1 and a determinant of GAD67 (212-226 amino acid residues) in patients with late onset type 1 DM. Also, there is significant correlation between T-cell clone responses to RVE2 (87-107 amino acid residues) and GAD65 (274-286) (Ou et al., 2000).

CVB infection and type 1 DM are more strongly associated with HLA-DR4 than HLA-DR3, while congenital rubella infection and type 1 DM are more strongly associated with HLA-DR3 than HLA-DR4 (Eberhardt et al., 1985). In addition, a tandem repeat of DRB1*0404-A11(3) and/or a tandem repeat of DRB1*0404-B35(15) might predispose CRS patients to development of type 1 DM (Ou et al., 2000).

Cytomegalovirus (CMV) may play a role in the pathogenesis of type I DM by inducing islet β cell destruction (Hiltunen et al., 1995). High titres of CMV-specific IgG are associated with ICAs in healthy siblings of diabetic children (Hiltunen et al., 1995; Nicoletti et al., 1990). Also, anti-CMV antibodies can cross-react with a 38 kilodalton autoantigen (38 kDa), which is expressed on the surface of islet β cells, and subsequently may be implicated in the autoimmune response to islet β cell destruction (Hiltunen et al., 1995; Pack et al., 1990). The molecular mimicry of amino acid sequences of CMV proteins with islet β cell autoantigens may induce the islet β cell destruction by molecular mimicry (Buesa-Gomez et al., 1994).

It has recently been shown that monoclonal and polyclonal GAD65 (248-257 or 88-99 amino acid residues)-specific T cells from patients with type 1 DM could be stimulated by viral (human herpes virus) and bacterial peptides with little apparent sequence homology with autoantigenic epitopes (Bach et al., 1998).

1.2.2.3 Dietary Factors

It has been proposed that breast milk may be important either to diminish exposure of breast-fed infants to an unidentified diabetogenic agent, which may or may not be a virus (Elliott and Martin 1984; Stuart et al., 1984), or may be important for the transmission of agents that prevent islet β cell destruction. Maternal IgA may prevent viral infections which contribute to islet β cell destruction (Kassim et al., 1987; Hjelt et al., 1985). Also, in breast-fed infants, there is evidence of increased IgA production (Prentice 1987), increased IFN response to respiratory syncytial virus (Chiba et al., 1987), and increased B cell proliferation (Juto 1985). On the other hand, the

transmission of such viruses via breast milk may prevent the development of type 1 DM by T-lymphocyte subset (CD4+ TH cells) depletion, i.e. the tropism of the virus may alter the function of CD4+ TH cells which cause the autoimmune response (Oldstone 1988). In addition, many anti-inflammatory agents, such as free radical scavengers, are present in human breast milk (Mayer et al., 1988); this may be relevant since oxygen free radicals are cytotoxic to islet β cells (Rabinovitch 1994).

Alternatively, early cessation of breast milk and replacement with cows milk introduces foreign protein antigens into the immature infant. Interestingly, bovine serum albumin (BSA) is recognised by IgG and IgA anti-BSA antibodies, which are detected in all diabetic children. The ABBOS peptide, which is a region of the albumin protein, shares a sequence homology with the ICA69 autoantigen (Dotta et al., 1994).

1.2.2.4 Other Environmental Factors and Hygiene

The increasing incidence of diabetes in many countries may be attributed to improved standards of hygiene (Kolb and Eliot 1994). Children in Northern Ireland aged under 15 years in areas with a high population density and household crowding were observed to have the lowest incidence rates of type 1 DM, during the period 1989-1994 (Patterson et al., 1996). Early contact with microbial antigens prevents diabetes in animal models (Patterson et al., 1994). Social mixing in early infancy appears to confer protection against the development of childhood diabetes. This may be mediated through exposure to infectious agent(s). This indicates that early infectious exposure may play a role in the development of immunoregulatory mechanisms which protect against diabetes (McKinney et al., 2000), such as modification of the

lymphocytic response to late immunological challenges (Schwimmbeck et al., 1988) and/or stimulation of the production of immunoregulatory cytokines that prevent diabetes (Bach 1994). The 'hygiene hypothesis' postulates that reduced exposure to common microbial infections in early life may increase the risk of type 1 DM in childhood.

Chemical toxins, such as alloxan, Chlorotocin, certain pesticides (Assan and Larger 1993) and nitrosamines, such as streptozotocin , (Dulin and Soret 1978) can poison pancreatic β cells directly or trigger an immune response which further damages these cells. The incidence of childhood type 1 DM in northern England is associated with higher nitrate levels in domestic drinking water (Parslow et al., 1997). Nitrate is reduced to nitrite, the chemical precursor of nitrosamines (Choi 1985). Furthermore, the water does not contain nitrosation inhibitors, such as vitamin C (Virtanen et al., 1994).

1.2.3 Genetic factors

1.2.3.1 Human Leukocyte Antigens (HLA)

It has been estimated that about 50-60% of the risk for type 1 DM is genetic, the rest being environmental. Family studies indicate that 60% of attributable genetic risk is due to HLA (Van der Auwera et al., 1995). The HLA complex is a region of 3,500 kilo bases (kb) on the short arm of chromosome 6 (6p21.3).

In HLA class I, HLA-B7, -B8, -B15 and -B18 are also positively associated with type 1 DM. There is a significant increase in the frequency of the HLA-B7 allele in the early-onset type 1 DM patients rather than in the intermediate and late-onset type 1 DM patients (Demaine et al., 1995).

The presence of an aspartate residue (Asp) at position-57 of the DQβ chain (DQβ57) confers resistance to type 1 DM (Badenhoop et al., 1989; Routsias and Papadopoulos 1995), while the presence of serine (Ser), alanine (Ala) or valine (Val) at DQβ57 confers susceptibility to type 1 DM (Badenhoop et al., 1989). Also, the presence of arginine at DQα52 confers susceptibility to type 1 DM, while the absence of arginine at DQα52 confers resistance to type 1 DM (Ilonen et al., 1996) (Table 1.2).

In HLA class II, HLA-DR3-DQ2 and HLA-DR4-DQ8, which have DQβ57 Ala+ (Owerbach and Gabbay et al., 1996) and DQα52 Arg+ (Ilonen et al., 1996), are strongly associated with type 1 DM (Demaine et al., 1995; Erlich et al., 1996). The presence of HLA-DR3 and HLA-DR4 in individuals with autoantobodies to GAD65 (GAA65+) and complement-fixing islet cell autoantibodies (CF-ICA+) will increase the relative risk of type 1 DM (Aanstoot et al., 1994).

HLA-DR1-DQ5, which has DQβ57 Ser+ (Demaine et al., 1995), and HLA-DR9 are positively associated with type 1 DM (Liparota et al., 1995). In contrast, the HLA-DR2-DQ6, which has DQβ57 Asp+ and DQα52 Arg- (Toxi et al., 1994); HLA-DR7, which has DQα52 Arg-; and HLA-DR5 are negatively associated with type 1 DM (Toxi et al., 1994). The protective allele (HLA-DR2-DQ6) is associated with high titres of GAAs. It has been suggested that the high titres of GAAs may correlate with

a CD4+TH2 cell response. This indicates that the activation of a CD4+TH2 cell response may be associated with the presence of HLA-DR2-DQ6. Also, there is no association between ICA and HLA-DR2-DQ6 (Pugliese et al., 1995). In addition, the DPB1*0301 allele is positively associated with type 1 DM and may interact with HLA-DR4 to increase the overall risk of type 1 DM (Erlich et al., 1996).

Some protective alleles, such as HLA-DRB1*0403 or 407, have DRβ74 Glu+, while the susceptible alleles, such as HLA-DR4, have DRβ74 Ala+ or Arg+ (Auwera et al., 1995).

Table 1.2 Susceptible and Protective Amino Acid Residues in HLA Class II Loci

HLA class II locus	Susceptible amino acid residue	Protective amino acid residue
DQβ57	Serine; e.g. HLA-DR1-DQ5 Alanine; e.g. HLA-DR3-DQ2 HLA-DR4-DQ8 Valine	Aspartate; e.g. HLA-DR2-DQ6
DQα52	Arginine; e.g. HLA-DR3-DQ2 HLA-DR4-DQ8	Absence of arginine; e.g. HLA-DR2-DQ6
DRβ74	Arginine; e.g. HLA-DR4-DQ8 Alanine; e.g. HLA-DR4-DQ8	Glutamine; e.g. HLA- DRB1*0403 or 407

The HLA-DQB1*0201 allele is associated with high risk of diabetes among stiff-man syndrome (SMS) patients, while the HLA-DQB1*0602 allele (HLA-DR2-DQ6) might be associated with a reduced prevalence of diabetes in SMS patients (Pugliese et al., 1993; Pugliese et al., 1994).

DR3 and DR4 have been shown to confer susceptibility to type 1 DM, while DR2 has been shown to confer protection from type 1 DM. As indicated above, this is due to linkage disequilibrium of these alleles (DR2, DR3, and DR4) to various DQ alleles, including DQ6, DQ2 and DQ8 (Owerbach et al., 1983). An attractive hypothesis

states that the diabetogenic peptide(s) binds tighter to the resistant than to the susceptible DQ molecules, leading to elimination of strongly autoreactive T-cell clones in the thymus. In the periphery, the protective DQ molecules compete with the susceptible DQ molecules for binding to the peptide(s) (Nepom 1990). Other DQ molecules are not capable of binding such diabetogenic peptide(s). It has been postulated that DQ molecules are the mediators of immunosuppression (Salgamme et al., 1991). The susceptible DQ molecules bind to diabetogenic peptides and can initiate the immune response by the presence of a hydrophilic first pocket in the antigen-binding groove, a hydrophobic or amphiphilic β49-56 dimerisation patch that allows for spontaneous or T-cell receptor-induced dimerisation, and the Arg-Gly-Asp cell adhesion loop (Routsias and Papadopoulos 1995).

The inheritance of HLA haplotypes influences which peptides are selected for presentation to TCR. The susceptiblity model depends on the binding of diabetogenic peptides to particular HLA molecules. If an individual inherits a disease associated HLA gene, susceptibility occurs when the product of this gene is the most efficient binder of diabetogenic β cell peptides amongst the other class II molecules in that individual. Conversely, an individual with disease associated HLA genes is resistant to type 1 DM when the products of other HLA class II genes are more efficient binders of the diabetogenic β cell peptides (Nepom 1990).

An alternative theory is the protective model which depends on the binding of tolerogenic peptides to particular HLA molecules. Alleles which are negatively associated with type 1 DM, such as DR2, have a high affinity for tolerogenic β cell peptides which are necessary to induce tolerance, whereas those alleles which are

positively associated with type 1 DM have a low affinity for these peptides, or bind them in the wrong orientation (Sheehy 1992).

The TAP2A (transporters associated with antigen processing) allele is positively associated with HLA-DR3+ and HLA-DR4+ type 1 DM patients. In contrast, the TAP2B allele is negatively associated with type 1 DM patients (Esposito et al., 1995).The TAP1, TAP2 and LMP2 (low molecular-weight proteins) loci show little effect on age at onset of type 1 DM (Liparota et al., 1995).

In HLA class III, C4 allotypes are associated with type 1 DM, such as C4A (Badenhoop et al., 1989; Demaine et al., 1995). The HLA A1-B8-DR3 and HLA B62-DR4 are associated with the 5.5 and 10.5 kb TNF-α gene alleles, respectively (Badenhoop et al., 1989). Also, TNF-β polymorphism is associated with type 1 DM (Awata et al., 1994). HSP70 locus shows little effect on age at onset of type 1 DM (Liparota et al., 1995).

The key genetic factor in the development of autoimmune diabetes in the non-obese diabetic (NOD) mouse is the presence of major histocompatibility complex (MHC) class II molecule, I-A (Todd et al., 1991). In a transgenic NOD mouse, the expression of I-Aβ transgenes (mouse HLA-DQB equivalent), which has Asp57 or Pro56 instead of the normal Ser57 or His56, respectively, prevents diabetes (Demaine et al., 1995). T cells from NOD mice proliferate to the I-A (86-101 amino acid residues) and GAD65 (509-524 amino acid residues) peptides, since this peptide of I-A is similar to that peptide of GAD65 (Xu et al., 1999).

1.2.3.2 Insulin gene

The insulin gene is present on chromosome 11p15.5. VNTR (variable numbers of tandem repeats) polymorphism at the 5′ end of the insulin gene has been shown to be associated with type 1 DM (Lucassen et al., 1993; Davies et al., 1994). A 4.1 kb region, including the insulin (INS) gene, which contains ten candidate polymorphisms, are associated with HLA-DR4+ individuals (Undlien et al., 1995; Undlien et al., 1994). Insulin is the islet β cell-specific autoantigen expressed early in the development of diabetes. A small population of cells in the thymus express insulin in mice (Smith et al., 1997) and in humans (Pugliese et al., 1997).) In Caucasian populations, INS VNTR class I alleles predispose to diabetes. In contrast, individuals who have INS VNTR class III alleles and have higher levels of INS mRNA in the thymus and lower levels in the pancreas are protected. The higher levels of INS mRNA may indicate greater levels of expression and enhance tolerance to preproinsulin in the thymus (Pugliese et al., 1997).

It has been suggested that the INS VNTR class I alleles are not equally associated with type 1 DM. The λHI-1 allele is associated with type 1 DM while the 698-VNTR class I allele, which is identical in size to the λHI-1, is not associated with type 1 DM. It is not known whether the class II VNTR alleles, such as 81 repeat elements, are associated with type 1 DM or not. Furthermore, some of the class III VNTR alleles, such as HUMTH01 allele Z-8, are strongly protective against type 1 DM, while the other HUMTH01 allele is weakly protective against type 1 DM (Owerbach et al., 1996).

1.2.3.3 Association of other Polymorphisms

The IFN-γ3 and 6 alleles and IL-β polymorphisms are associated with type 1 DM (Awata et al., 1994). Also, mitochondial gene mutations are associated with type 1 DM (Awata et al., 1993). Furthermore, CTLA-4 is present on chromosome 2q31-33 and has also been linked to type 1 DM (Nistico et al., 1996). The CTLA-4, which is expressed on activated T cells, is thought to be a negative regulator of T cell function. The CTLA-4 exon 1 polymorphism (49A/G) confers genetic susceptibility to type 1 DM (Hayashi et al., 1999).

The CD3 ε locus may be associated with type 1 DM in female subjects. Also, the CD4* A4/A4 genotype is significantly increased in type 1 DM patients. On the other hand, the CD3*91 allele of the δ subunit may protect against type 1 DM (Ghabanbasani et al., 1994).

1.2.4 Autoantibody Associations

Although the autoimmune destruction of islet β cells is T cell mediated, circulating autoantibodies can often be detected years before the diagnosis of type 1 DM and can be used in the prediction of type 1 DM.

1.2.4.1 Islet Cell Autoantibody (ICA)

Islet cell autoantibodies (ICA) were first demonstrated by the indirect immunofluorescence test on frozen pancreatic sections (Bottazzo et al., 1974). The ICAs have been divided into two types, non-restricted ICAs (NR-ICAs), which recognize all islet cells (α, β, δ and pp cells), and restricted ICAs (R-ICAs), which

recognize only islet β cells. The NR-ICAs do not react with brain GAD, whereas the R-ICAs react with brain GAD (Palmer et al., 1994; Dotta et al., 1994)). The R-ICAs are not predictive (Dotta et al., 1994; Christie et al., 1994), or less predictive, of diabetes development than NR-ICAs (Palmer et al., 1994).

The availability of standard ICA+ sera has allowed the reporting of results as JDFU (juvenile diabetes foundation unit) based on an end point titration. In R-ICA+ sera, the GAAs are present in 89% of type 1 DM patients (Petersen et al., 1994). In addition, the ICAs are associated with anti-37 kD autoantibodies (Bonifacio et al., 1995), insulin autoantibodies (IAA) (Verge et al., 1994) and anti-40 kD autoantibodies (anti-ICA512bdc/IA-2 autoantibodies) (Fig. 1.2) described below. The risk of diabetes in family members with ICA ≥40 JDFU and IAAs is 77% within five years while in those with ICA ≥40 JDFU only is 42% (Eisenbarth et al., 1994). This indicates that the IAAs are associated with other autoantibodies and/or genetic susceptibility. The risk of diabetes in relatives with IAAs, GAAs and anti-IA-2 autoantibodies can be as high as 100% within five years (Verge et al., 1996). Higher predictive value for type 1 DM among first degree relatives may be obtained if only individuals with high titre ICAs or double or triple ICA/IAA, GAA or IA-2 (insulinoma-associated antigen-2 autoantibodies) are considered (Hagopian et al., 1995; Verge et al., 1996). Furthermore, the ICAs are present in some SMS and autoimmune polyendocrine syndrome (APS) patients (Bosi et al., 1991) (see below).

The ICAs are detected in 90% of newly diagnosed diabetic patients (Genovese et al., 1992). The ICA titres in first-degree relatives and co-twins are higher than in diabetic children without a family history of type 1 DM (Bingley et al., 1994; Marchal et al.,

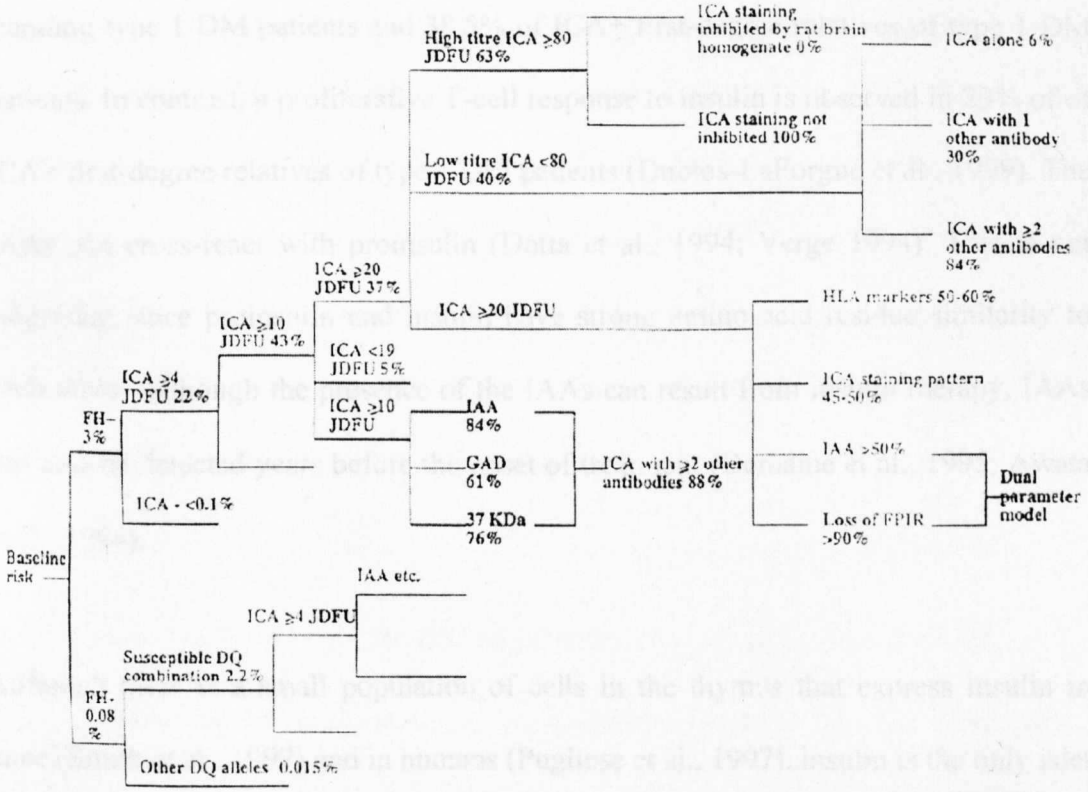
1995). This indicates that the ICAs are tightly associated with genetic susceptibility (Fig. 1.2). The risk of type 1 DM is 33% in ICA+ relatives with HLA-DQβ57 Asp- and is <5% in ICA+ individuals without a family history of type 1 DM (Eisenbarth et al., 1994).

The abrupt-onset type 1 DM patients have high titres of ICAs which decrease after the first year of type 1 DM. In contrast, the slow-onset type 1 DM patients have low titres of ICAs which continue for a long period after diagnosis (Urakami et al., 1995). This suggests that the autoimmune islet β-cell damage in abrupt-onset type 1 DM patients is much higher than in slow-onset type 1 DM patients. This strong islet β-cell damage, in abrupt-onset type 1 DM, may cause more production of ICAs against the islet β-cells, and loss of all islet β-cells will decrease ICA titres. In contrast, in slow-onset type 1 DM patients, the slow rate of islet β-cell damage may cause production of ICAs in lower amounts than abrupt-onset type 1 DM patients. In addition, the remaining islet β-cells, in slow-onset type 1 DM patients, will continue to stimulate ICA production for a long period after diagnosis. Furthermore, some ICAs have the ability to fix complement (CF-ICA), which may kill the cells by formation of the membrane attack complex, or may simply represent high titre of autoantibodies (Yamada et al., 1997; Bosi et al., 1994).

64% of type 1 DM sera can stain mouse islets (which contain little or no GAD) and therefore recognise other islet cell components in addition to GAD, while 32% can not stain mouse islets but can react with human islets following absorption of sera with GAD. Thus, mouse islet are able to differentiate between two groups of non-GAD reactive ICA (Richter et al., 1993c). Some islet cell autoantigens do not seem to

be affected by proteolysis and may represent a monosialoganglioside (lipid) migrating between the GM2 and GM1 standards (Dotta et al., 1993) (discussed below).

Figure 1.2 Decision tree for predicting type 1 diabetes mellitus in individuals with a family history (FH) of disease (Bingley et al., 1994; Genovese et al., 1992); (FPIR=First Phase Insulin Realease).



1.2.4.2 *Proinsulin and Insulin Autoantibodies (IAA)*

Proinsulin is a major autoantigen in type 1 DM. A proliferative T-cell response to proinsulin is observed in 7.7% of recent-onset type 1 DM patients, 16.7% long-standing type 1 DM patients and 38.5% of ICA+ first-degree relatives of type 1 DM patients. In contrast, a proliferative T-cell response to insulin is observed in 23% of of ICA+ first-degree relatives of type 1 DM patients (Dubios-LaForgue et al., 1999). The IAAs can cross-react with proinsulin (Dotta et al., 1994; Verge 1994). This is not surprising since proinsulin and insulin have strong amino acid residue similarity to each other. Although the presence of the IAAs can result from insulin therapy, IAAs can also be detected years before the onset of treatment (Demaine et al., 1995; Awata et al., 1994).

Although there is a small population of cells in the thymus that express insulin in mice (Smith et al., 1997) and in humans (Pugliese et al., 1997), insulin is the only islet β cell-specific autoantigen found in diabetes, and it is expressed early in development.

The IAA fluid phase radioimmunoassay has been standardised (Palmer et al., 1990). Standardisation could not be achieved in solid phase assays [such as, enzyme-linked immunosorbent assay (ELISA)], indicating that the IAA epitope is conformational (Kuglin et al., 1990).

At onset of diabetes, 65% of subjects aged >10 years have IAAs whereas 93% of subjects aged ≤10 years have IAAs. This indicates that the occurrence of IAAs is inversely related to age. Furthermore, the frequency of the IAAs is higher in males than females (Zimmet et al., 1994).

High levels of IAAs are associated with HLA-DR4 and some DQA1 alleles, such as DQA1*0102, DQA1*0201 and DQA1*0301. In contrast, the IAAs titres are very low in HLA-DR3+ subjects (Dotta et al., 1994).

In the non-obese diabetic (NOD) mouse, the peptide region of the insulin B chain (15-23 amino acid residues), which is recognised by previously isolated pathogenic CD4+ Th1 cells, is also recognised by highly pathogenic CD8+ TC cells (Wong et al., 1999). In addition, there is evidence that the insulin B chain peptide (9-23 amino acid residues) and GAD-65 are not primary diabetogenic autoantigens in the type 1 DM of the Bio-Breeding (BB) rat (Bieg et al., 1999).

1.2.4.3 Autoantibodies to Tryptic Fragments of 64 kD and to IA-2/IA-2β

Autoantibodies to 64kDa autoantigens are detected as early as ICA but persist in the circulation longer after diagnosis (Christie et al., 1990a). The 64 kD is a combination of autoantigens expressed by islet β cells that are cleaved by trypsin to 50 kD, 40 kD and 37 kD proteolytic fragments. Ninety three percent of diabetic patients have autoantibodies to the 50 kD fragment (81%) and to protein tyrosine phosphatase (PTP)-like proteins (37 kD/40 kD fragment) (77%) (Christie et al., 1990b). The anti-50 kD correlate with the level of GAA, indicating that the 50 kD fragment is derived from GAD65 (Christie et al., 1993). Also, the 40 kD fragment is derived from IA-2 or its fragment ICA-512bdc. The 37 kD fragment (ICA-related PTP) is derived from IA-2β, which is an insulin granule component named phogrin. IA-2 and IA-2β both being transmembrane proteins within the secretory granule membrane of neuroendocrine cells (Solimena et al., 1996; Wasmeier and Hutton 1996). The PTP-like domain of IA-2 has no phophatase activity detected, while IA-2β has weak phophatase activity

detected (Cui et al., 1996; Lu et al., 1994). In addition, IA-2 and IA-2β have structural similarities to tyrosine phosphatases. Autoantibodies to IA-2 and IA-2β are detected by immunoprecipitation and autoradiography (Christie et al., 1992, 1993).

The anti-50 kD autoantibodies, which react with a 50 kD fragment of GAD, can be detected in most type 1 DM patients, in SMS and in all ICA+ APS patients with or without diabetes (Hehmke et al., 1995).

The anti-37 kD (anti-IA-2β/anti-phogrin) autoantibodies are detected in acute-onset diabetic patients, in 66.7% of SMS patients with diabetes and in 90% of ICA+ APS patients who have acute-onset diabetes. Also, the anti-37 kD autoantibodies are more strongly associated with concordance for diabetes in identical twins than are GAAs (Christie et al., 1994; Notkins et al., 1996). Furthermore, the anti-37 kD autoantibodies are strongly associated with ICAs but not associated with GAAs (Bonifacio et al., 1995). The anti-37 kD autoantibodies are better predictive markers of type 1 DM onset than ICA or GAA in the general population (Ongagna and Levy-Marchal, 1995) and in pre-diabetic twins (Christie et al., 1992). The major antigenic determinant of phogrin is localized within 640-922 amino acid residues (Kawasaki et al., 1998).

The anti-40 kD autoantibodies (specific for ICA-512bdc/IA-2) are associated with ICA+ patients (Notkins et al., 1996; Bingley et al., 1993). The prevalence of anti-IA-2 AAs is higher in acute onset type 1 DM than in slowly progressive type 1 DM (Yamada et al., 1997). The anti-IA-2 AAs are present in 56% type 1 DM patients, 47% APS II patients with type 1 DM, 14% SMS patients, 4% APS II patients without

type I DM (Morgenthaler et al., 1997). Also, the IA-2 autoantibodies are associated with HLA-DR4 (Genovese et al., 1996).

Figure 1.3 Epitopes of Type 1 DM Autoantibodies on IA-2.

IA-2 consists of an intracellular cytoplasmic domain (604-979 aa), which is recognized by autoantibodies, and a luminal ecto-domain (31-577 aa), which is not recognized by autoantibodies. The anti-IA-2 AAs and anti-phogrin AAs recognize different epitopes in a cytoplasmic domain of IA-2 [56% of sera to juxtamembrane (JM) region (601-691 aa), 83% to PTP-like domain (692-979 aa) and 39% of sera to both these regions (JM region and PTP-like domain)] (Lampasona et al., 1996). In contrast, the anti-IA-2 AAs and anti-phogrin AAs recognize only the PTP-like domain of phogrin (640-1015 aa) (Kawasaki et al., 1998). The major antigenic determinant of IA-2 is localized within 762-887 aa, while the minor antigenic determinant of IA-2 is localized within 601-762 aa (Fig. 1.3), which is highly homologous between IA-2 and phogrin. In addition, it has been suggested that some of the anti-IA-2 AAs recognize a conformational epitope(s) associated with the C-terminal region of native IA-2 (949-979 aa) (Kawasaki et al., 1998). Furthermore, the PTP-like domain of phogrin shares 80% amino acid sequence identity to IA-2 (Lampasona et al., 1996; Kawasaki et al., 1998). The JM domain shows 50%

homology between IA-2 and IA-2β. Whereas, the luminal ecto-domains of both IA-2 and IA-2β share less than 10% homology (Lampasona et al., 1995). IA-2 and IA-2β probably share common epitopes but also show distinct epitopes; 50-80% of type 1 DM sera that react with IA-2 also recognize IA-2β. Alternatively, even 95% of type 1 DM sera reacting with IA-2β also recognize IA-2 (Notkins et al., 1998). In all anti-IA-2 AAs/anti-phogrin AAs positive sera, the binding to phogrin is completely blocked by preincubation with recombinant IA-2, while the binding to IA-2 is partially blocked by preincubation with recombinant phogrin (Lampasona et al., 1996; Kawasaki et al., 1998). This suggests that the type 1 DM AAs may develop predominantly to IA-2 rather than phogrin.

Human monoclonal antibodies (huAbs) have been produced from newly diagnosed type 1 DM patients against IA-2. For example, huAb 76/12 recoginzes 794-845 aa of IA-2 and 741-1033 aa of IA-2β, huAb 96/3 recognizes 780-979 aa of IA-2 and 741-1033 aa of IA-2β, huAb 96/4 recognizes 687-776 aa of IA-2, huAb 96/5 recognizes 890-979 aa and huAb 103/5 recognizes 603-686 aa of IA-2. Interestingly, the huAb 96/3 inhibits the binding of 10 out of 14 of newly diagnosed type 1 DM sera to IA-2 (Kolm-Litty et al., 2000). Sera of type 1 DM patients respond to three peptides 831-850 aa, 841-860 aa and 751-770 aa of IA-2 (Hawkes et al., 2000).

Proliferation of peripheral blood mononuclear cells (PBMCs) in response to IA-2 has been observed (Ellis et al., 1998; Durinovic-Bello et al., 1996). T-cell lines have also been generated to epitopes on IA-2 (Hawkes et al., 2000; Honeyman et al., 1998).

1.2.4.4 Glutamic acid decarboxylase autoantibodies (GAA)

GAD is present in the cytoplasm and microsecretory vesicles of islet β cells and neurons secreting γ-aminobutyric acid (GABA) (Daw et al., 1996). GAD catalyzes the conversion of glutamate to GABA. GABA is a major inhibitory neurotransmitter of the central nervous system, but its function in islet β cells remains to be clarified.

There are two non-allelic isomers of GAD, GAD65 and GAD67. GAD65 is the smaller amphiphilic form and consists of 585 amino acid residues, which is encoded on human chromosome 10. In contrast, GAD67 is the larger soluble hydrophilic form and consists of 594 amino acid residues, which is encoded on human chromosome 2. GAD65 and 67 are present in GABA-secreting neurons and in the cytosol of rat pancreatic islet β cells, whereas only GAD65 is present in human islet β cells and little GAD65 is present in mouse islet β cells (Ujihara et al., 1994). Furthermore, GAD65 and 67 are highly conserved. 97% identity exists between rat and human GAD67, while 96% identity exists between rat and human GAD65. In human, GAD65 and 67 share 76% identity and 87% similarity throughout 174-585 amino acid residues, but they differ within the N-terminus. In the first 95 aa, they share 22% identity and ~61% similarity (Hahmke et al., 1995). Whereas in the 96-173 amino acid residues, they share 49% identity.

GAD autoantigen can be detected by different assays, such as radioligand binding assay for recombinant GAD65 (Falorni et al., 1995), solid phase assays (ELISA or immunoblotting) for GAA65 in SMS sera which are specific to linear epitopes (Kim et al., 1994) and immunoprecipitation assay which is specific to conformational epitopes (Baekkeskov et al., 1982).

However, APS (diabetic or not diabetic), SMS and about 10% of diabetes sera recognise both isoforms of GAD (GAD65 and GAD67) (Tuomi et al., 1996).

1.2.4.4.1 Type I Diabetes Mellitus Associations with GAD

GAD autoantibodies (GAAs) are present in 70-80% of newly diagnosed diabetic and prediabetic patients (Christie et al., 1994), 20% of nondiabetic identical twins who are at low risk of diabetes (Christie et al., 1994), 80% of relatives of diabetic patients who themselves developed type 1 DM (Bingley et al., 1993) and 59% ICA+ diabetic patients (Morgenthaler et al., 1997).

GAAs can also be associated with other autoantibodies such as anti-DNA/RNP autoantibodies, rheumatoid factor (Petersen et al., 1994), anti-smooth muscle cells autoantibodies and anti-parietal cell autoantibodies.

As discussed earlier, genetic and environmental factors and other autoantibodies may contribute to GAAs associations. GAAs are significantly associated with HLA-DR3 phenotypes (Verge et al., 1996; Genovese et al., 1996; Hagopain et al., 1995) and not associated with HLA-DR4 (Daw et al., 1996). The PEVKEK sequence is identical between P2-C protein of CVB4 and GAD65. The high similarity of the amino acid sequences of GAD65/67 to the P2-C protein of CVB4, suggests that the immune response against P2-C protein of CVB4 may cross-react with GAD of islet β cells, leading to more destruction of islet β cells and further release of GAD from islet β cells. The frequency of GAAs is higher in females than males (Verge et al., 1994; Verge et al., 1996; Yu et al., 1994). Also, the frequency of GAAs is not associated

with the age differences among children (Verge et al., 1994). High levels of GAAs are negatively associated with IAAs (Yu et al., 1994).

High levels of GAAs are detected in newly diagnosed type 1 DM patients, but the GAA levels decrease by 50% within two years after diagnosis. By six years after diagnosis, the GAA levels in type 1 DM and healthy individuals are the same (Kaufman et al., 1992). While in SMS patients with long-standing type 1 DM, the GAAs do not disappear (Velloso et al., 1993).

The GAAs of type 1 DM sera, which are present at high titre, recognize predominantly conformational epitopes on GAD65 [middle(M)-region epitope (240-435 amino acid residues; aa, type 1 DM E1) and carboxy(C)-terminal epitope (451-570 aa, type 1 DM E2), as the major antigenic determinants (Fig. 1.4), and amino(N)-terminal epitope, as a minor antigenic determinant] and rarely on GAD67 (Lampasona et al., 1997; Daw et al., 1995). The GAD-67 reactivity is thought to be mainly against epitopes shared with GAD-65 (Hagopian et al., 1993). It has been suggested that the humoral autoimmune response to GAD-65 in GAA+ offspring of diabetic parents is initially against epitopes within the middle portion of GAD-65 (96-444 amino acid residues), as a primary target, and spreads to epitopes in other regions of GAD-65 (1-95, 96-444 and 445-585 amino acid residues) and GAD-67 (Bonifacio et al., 2000). Also, the GAAs of a rare group of type 1 DM patients who have high titre of GAAs65 and protective allele HLA-DR2 can recognize a linear epitope in 421-442 aa (Kim et al., 1994). Furthermore, some ICAs can react with the middle (M) (361-442 amino acids) and carboxy (C)-terminal region (443-585 amino acids) of GAD65 or with the

amino (N)-terminal region (1-195 amino acids) of both GAD65 and 67 (Ujihara et al., 1994).

Conformational epitopes of GAD-65, 245-450 and 450-585 amino acid residues, are common targets of GAAs in type 1 DM (50%), APS without diabetes (71%), APS with diabetes (90%) and SMS (100%). There are two dominant linear epitopes: first, 7-124 amino acid residues which are recognised by GAAs from patients of type 1 DM, SMS, APS without diabetes and APS with diabetes. Second, amino acid residues 535-585 which are recognised by GAAs from patients of APS without diabetes, APS with diabetes and SMS (Sohnlein et al., 2000).

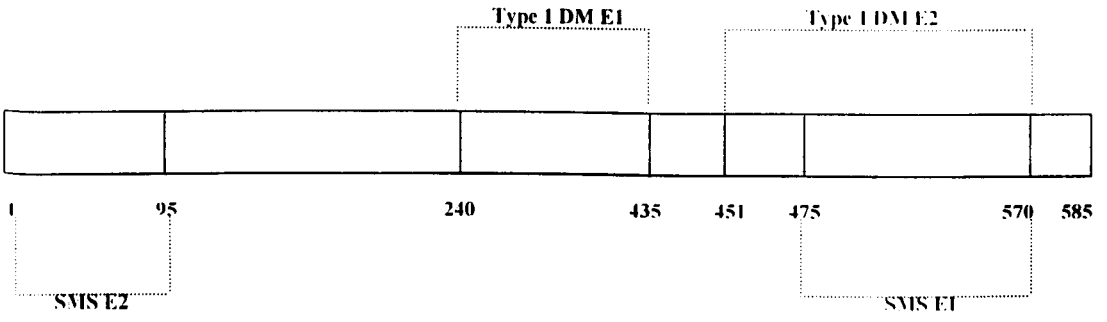
Complete suppression of pancreatic islet β cell GAD expression can block the generation of diabetogenic T cells and protects islet grafts from autoimmune injury in NOD mice. Thus, islet β cell-specific GAD expression is required for the development of autoimmune diabetes in NOD mice (Yoon et al., 1999).

Some studies have detected lymphocyte-mediated immunity in type 1 DM patients directed to the region of GAD65 (247-279 amino acid residues) (Karlsson and Ludvigsson 1998) or (250-273 amino acid residues) (Ellis et al., 1996; Atkinson, et al., 1994). This indicates that these regions of GAD-65 (247-279 or 250-273 amino acid residues), which includes the similar amino acid sequence of the P2-C protein of CVB4 (PEVKEK), are involved in the development of type 1 DM.

More than 50% of recent-onset type 1 DM patients have T cell responses to GAD (Atkinson et al., 1992; Harrison et al., 1993). In addition, T cells of patients with type

I DM respond to other GAD65 fragments, including 161-243 and 473-555 amino acid residues (Lohmann et al., 2000). Also, other studies in humans and NOD mice have detected T cell reactivity to the C-terminus of GAD65 (amino acids 524-541 and 521-535) (Schloot, et al., 1997; Patel et al., 1997). Furthermore, the T cells of type 1 DM patients have been reported to recognise a peptide 379-390 amino acids out of a range of different GAD65 peptides spanning the region of 379-450 amino acid residues (Rharbaoui, et al., 1999).

Figure 1.4 Epitopes of Type 1 DM and SMS Autoantibodies on GAD65.



1.2.4.4.2 SMS Associations with GAD

SMS results from an impairment of GABA-ergic inhibitory input to α-motor neurons (Yu et al., 1994). This may be induced by reaction of GAAs with GAD in GABA-secreting neurons (Baekkeskov et al., 1990). GAAs are detectable in 89% of SMS diabetic patients (Morgenthaler et al., 1997).

In 1/100 dilution of SMS sera, the GAAs can react with full length GAD, GAD fragments, denatured GAD and GAD peptides; but in 1/10,000 dilution of SMS sera, the GAAs react with full length GAD only. In contrast, in type I DM sera, the GAAs cannot bind with GAD fragments, denatured GAD or GAD peptides but they bind with full length GAD (Ujihara et al., 1994; Daw et al., 1996; Daw et al., 1995). This suggests that the GAAs in IDDM sera recognize conformational epitopes on GAD65 since these conformational epitopes are lost in GAD fragments.

In SMS sera, the GAAs recognize GAD65 and GAD67 by immunoprecipitation assay and recognize only GAD65 on western blots. In contrast, in type I DM sera, the GAAs can recognize only GAD65 by immunoprecipitation assay but not on western blots, and only 10-20% of type I DM sera recognize GAD67 (Kim et al., 1994). This suggests that the GAAs in SMS sera can recognize linear and conformational epitope on GAD65 and only conformational epitope on GAD67. However, in both type 1 DM and SMS sera, GAAs recognize GAD65 lacking the first 244 aa by the immunoprecipitation assay. This suggests that the GAAs in type 1 DM and SMS sera recognize conformational epitopes in the carboxy-terminal region of GAD65 (Kim et al., 1994).

In SMS and APS I sera, the GAAs are present at high titre. In contrast, in type 1 DM sera, the GAAs are present at lower titre (Bjork et al., 1994). The titre of GAAs in SMS sera is 100-500 fold higher than GAAs in type 1 DM sera. The GAAs in SMS sera bind to GAD65 and 67 at titres ≥10,000, whereas the GAAs in type 1 DM sera bind to GAD65 only at titres 1/100 (Daw et al., 1996). This indicates that the affinity

of GAAs of SMS sera is higher than those in type 1 DM sera and/or are present at higher concentrations in SMS sera. Also, as mentioned above, the GAAs of SMS, which are present at high titre, are less dependent on the conformation of the GAD and commonly target GAD67 as well as GAD65 (Lampasona et al., 1997).

Preincubation of SMS sera with 188-442 aa/GAD65 can block the binding of GAAs67 with GAD67, whereas preincubation with GAD67 cannot block the binding of GAAs65 with GAD65 (Daw et al., 1996). This indicates that the GAAs in SMS sera recognize a specific epitope in GAD67, which is highly homologous to 188-442 aa/GAD65, whereas other GAAs65 recognize epitopes which are not present in GAD-67. Furthermore, preincubation of SMS sera with 354-368 aa/GAD65 inhibits the binding of SMS sera with GAD65. In the 354-365 aa/GAD65, there are four amino acid residues K-KI-M which differ from E-NL-L of GAD67 (Li et al., 1994). This suggests that these four amino acid residues (K-KI-M) may contribute the binding of SMS sera with GAD65.

In SMS sera, GAAs recognize in GAD65 either an N-terminal epitope 1-8 aa (Kim et al., 1994) and/or 1-95 aa (SMS E2) (Hagopain et al., 1995; Bjork et al., 1994) as a major antigenic determinant, a middle region epitope 390-403 aa (Daw et al., 1996; Li et al., 1994), and a C-terminal epitope 475-585 aa (SMS E1) (Butler et al., 1993; Kim et al., 1994) as a minor antigenic determinant (Fig. 1.4). The SMS GAAs recognize SMS E1 (475-585 aa) (Hagopain et al., 1995) and SMS E2 (1-95 aa) on western blots (Kim et al., 1994). This indicates that the GAAs in SMS sera recognize linear epitopes in both N- and C-terminal regions of GAD. In human and rat GAD, SMS E1

shares 97% identity and 100% similarity (Butler et al., 1993). Thus, the human and rat GAD react equally with SMS sera.

The SMS sera may block the active site of GAD, which contains the pyridoxal 5-phosphate (PLP) binding site. GAD67 is fully saturated with PLP but GAD65 is partially saturated with PLP. The middle region of GAD65 (390-403 aa), which is recognized by SMS sera, includes the PLP binding site. The interchange, Leu→Pro 401, in 390-403 aa peptide inhibits the binding of SMS sera with this peptide. Also, the preincubation of SMS sera with the PLP binding site of GAD67 (399-413 aa peptide) inhibits the binding of SMS sera with GAD65 PLP binding site (Li et al., 1994).

T cells derived from a non-diabetic SMS patient, recognise two peptides of GAD65 (amino acids 331-350 and 341-360), as immunodominant epitopes. This region (amino acids 331-360) is 100% identical in human, mouse and rat GAD65. Also, this region of GAD65 (amino acids 339-352), which is recognised by the T cells of a non-diabetic SMS patient, has no reactivity with T cells of newly diagnosed type 1 DM patients. In addition, these T cells of a non-diabetic SMS patient recognised peptides close to the N-terminal part of GAD65 (amino acids 61-90 and 191-220) and close to the C-terminus (amino acids 491-520) of GAD-65 (Schloot et al., 1999). In addition, the T cells of SMS patients recognise two regions of GAD-65 (81-171 and 313-403 amino acid residues), including regions previously reported to be immunodiminant (Lohmann et al., 2000).

1.2.4.4.3 APS Associations with GAD

GAAs are present in 89% of APS II patients with type I DM and 21% of APS patients without type I DM (Morgenthaler et al., 1997). Also, in APS I sera, the GAAs recognize GAD65 on western blots (Bjork et al., 1994). This suggests that the GAAs in APS I recognize linear epitopes on GAD65. The GAAs of APS, which are present at high titre, are less dependent on the conformation of the GAD and commonly target GAD67 as well as GAD65 (Lampasona et al., 1997).

Using GAD65/67 chimeras, to maintain conformation-dependent epitopes of GAD65, that the APS II human monoclonal antibodies (b35, b78 and b96) target amino acids 270-359 (type I DM-E1) and 443-585 (type I DM-E2) and do not target the N-terminal third of GAD65. Interestingly, b78 and b96 require both amino acid regions 514-528 and 529-570, not only 514-528 alone (Powers et al., 1999).

Furthermore, the GAAs of diabetic APS patients' sera recognise a linear epitope within 7-124 aa of GAD65. This region of GAD65 (7-124 aa) is also recognised by other patients' sera (type I DM, non-diabetic APS and SMS), but at a lower frequency than diabetic APS as a dominant linear epitope (Sohnlein et al., 2000).

1.2.4.4.4 Monoclonal Antibodies to GAD

Human monoclonal anti-islet cell antibodies (MICA) have been produced that react with GAD. The MICA 4/6 and MICA 10 recognize epitopes in the middle region of GAD (245-450 aa). The MICA 2 epitope (506-531 aa) is very similar to MICA 1/3 epitope (450-570 aa), but the MICA 2 recognizes a linear epitope (on western blots) in the C-terminal region of GAD whereas the MICA 1/3 recognize a conformational

epitope in C-terminal region of GAD (Richter et al., 1993; Syren et al., 1996). MICA 7 recognizes a similar epitope to MICA 1/3 and 2. In addition, MICA 8 and 9 recognize the N-terminal region of GAD (Syren et al., 1996).

The mouse monoclonal antibody (GAD-6) recognizes an epitope on GAD65, 475-585 aa (SMS E1) (Hagopian et al., 1995). The GAAs of IDDM, SMS and APS II (diabetic and non-diabetic) sera recognize the region of the GAD-6 epitope on GAD-65 (Davenport et al., 1997). In addition, the extreme C-terminal sixteen amino acid residues of GAD65 are not involved in epitopes of GAAs of SMS and APS II sera and the epitope of GAD-6 (Davenport et al., 1997). Furthermore, GAD-6 binds only to GAD65, not to GAD67 (Daw et al., 1995).

1.2.4.4.5 The Three-dimensional modeling of GAD-65

GAD65-specific residues have been mapped for one linear and 13 conformational epitopes recognised by 16 human monoclonal antibodies (M1-10, b78, b96, DPA, DPB, DPC and DPD) derived from four type 1 DM patients and one ICA+ individual with APS. The three-dimensional modeling of GAD65 predicts that all the epitopes are within charged hydrophilic patches on the surface of the native molecule, and together cover most of the surface of the middle and C-terminal regions of GAD65 (Schwartz et al., 1999).

The sequence of the PLP-binding region (middle region) (Myers et al., 2000; Schwartz, et al., 1999) and C-terminal domain (Schwartz, et al., 1999) of human GAD65 was threaded onto the PLP-binding domain of ornithine decarboxylase (1ORD) (ORD residues 161-425/GAD65 residues 211-460) and the C-terminal

domain of dialkylglycine decarboxylase (2DKB) (DKB residues 320-432/GAD65 residues 461-585), respectively, as templates for the model. In the absence of proteins of known structure with significant homology to the N-terminal domain of GAD65, the secondary structure of this region was predicted using the algorithm by Chandonia and Karpulus 1999 (Fig. 1.5). The first 46 amino acid residues of the N-terminal region of GAD65 may be buried in the folded molecule (Schwartz, et al., 1999).

The three-dimensional model of the middle region of a GAD65 dimer (211-460 amino acid residues) was built by using 1ORD template (Myers et al., 2000; Schwartz, et al., 1999). The PLP-binding middle region consists of a seven-stranded β sheet surrounded by seven α-helices. Two monomers and one monomer of GAD65 are shown in the model of Schwartz et al. (1999) (Fig 1.6a) and in the model of Myers et al. (2000) (Fig. 1.6b), respectively.

The three-dimensional model of the C-terminal region of a GAD65 dimer (461-585 aa) was built by using 2DKB template. It predicts an α/β fold composed of a four-stranded β sheet and three amphipathic α-helices (Fig. 1.7), with localization of hydrophobic residues toward the β-strands and residues involved in epitope recognition on the charged face of these helices (Schwartz, et al., 1999).

Figure 1.5 Space-filling model of GAD65. The C-terminal domain (gray) and the middle domain (white) are shown. Approximate locations of the amino acids required for human monoclonal antibody recognition are coloured as follows: MICA8 and MICA9 (green), DPC (pink), the C-terminal epitopes (blue, red, orange) and the middle region epitopes (purple). Within the C-terminal epitopes, MICA7 (blue) binds across the face of two helices (helices R and T) forming a blocking group with MICA8 and MICA9 (green), as well as, MICA2, MICA5 and b78 (orange, red) which bind to an exposed helix (helix S). Within the middle domain, MICA1, MICA3 and DPA bind the backside of helix R, as well as, MICA4, MICA6 and MICA10 which bind to helix M. Two murine monoclonal antibodies (65-1 and 65-2), which block MICA7, bind the bottom region of the dimer and span both the C-terminal and middle domains. The N-terminal domain of GAD-65 is predicted to be located at the top of this model (Schwartz et al., 1999).

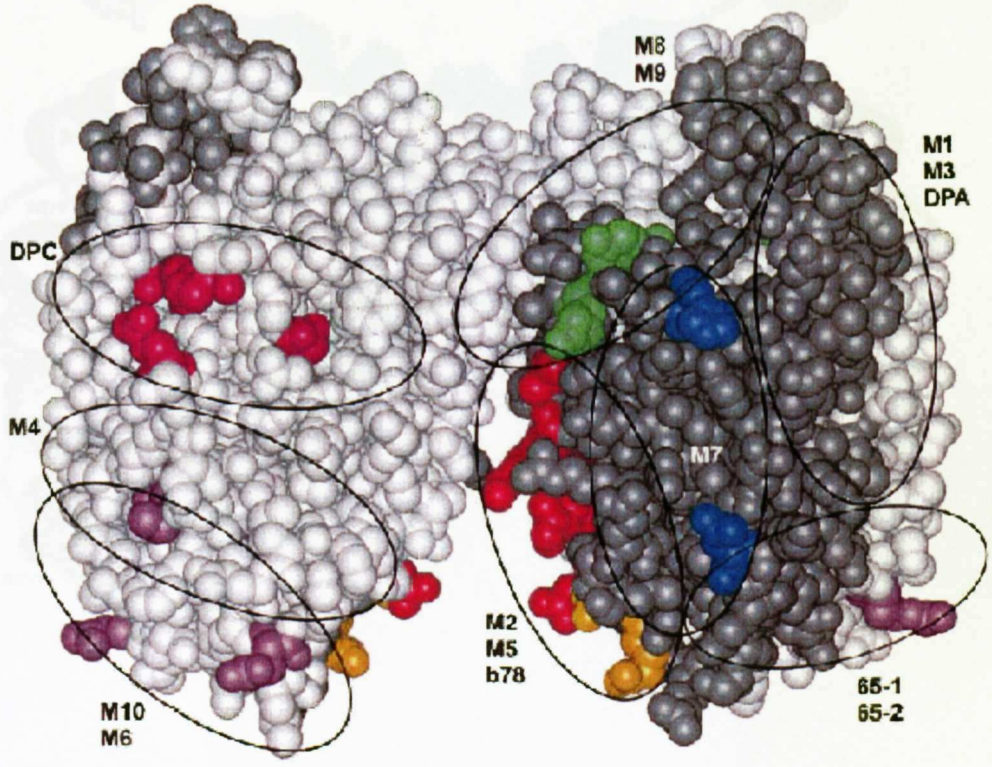


Figure 1.6 Three-dimensional model of the middle region (PLP-binding region) of a GAD-65 dimer. **a,** Two monomers of GAD65 are shown. PLP molecules are modeled in red colour. Helix J contains E264 (purple) which is essential for MICA10 binding and involved in MICA6 binding. K358 (purple) at the C-terminal end of helix M is essential for the MICA4 epitope. R317 (purple) in helix L is essential for MICA4, b96 and M10. P231 and S234 (pink) are involved in the DPC epitope. W375 and E411 (white) differ between GAD65 and GAD67 (Schwartz et al., 1999). **b,** The PLP-binding region of GAD-65 is shown highlighting the regions to which MICA3 binding peptides [residues 262-270, which constitutes the PEVKEK loop, is coloured yellow, α-helixes (residues 285-296 and 215-334) are coloured red, and residues 373-395 are coloured blue] (Myers et al., 2000).

a)

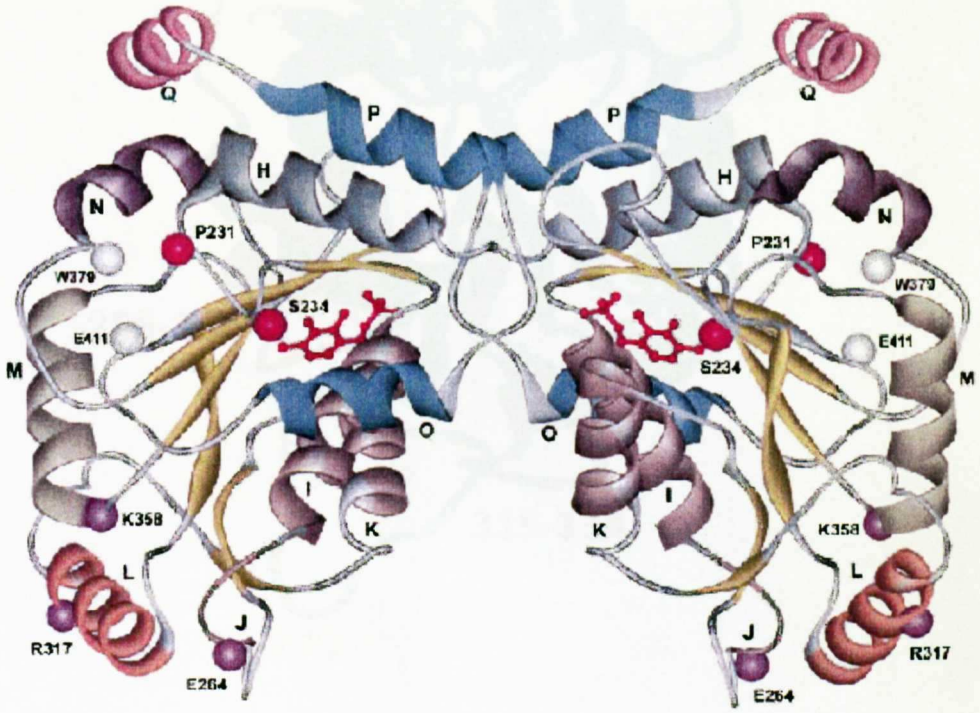


Figure 1.6-b)

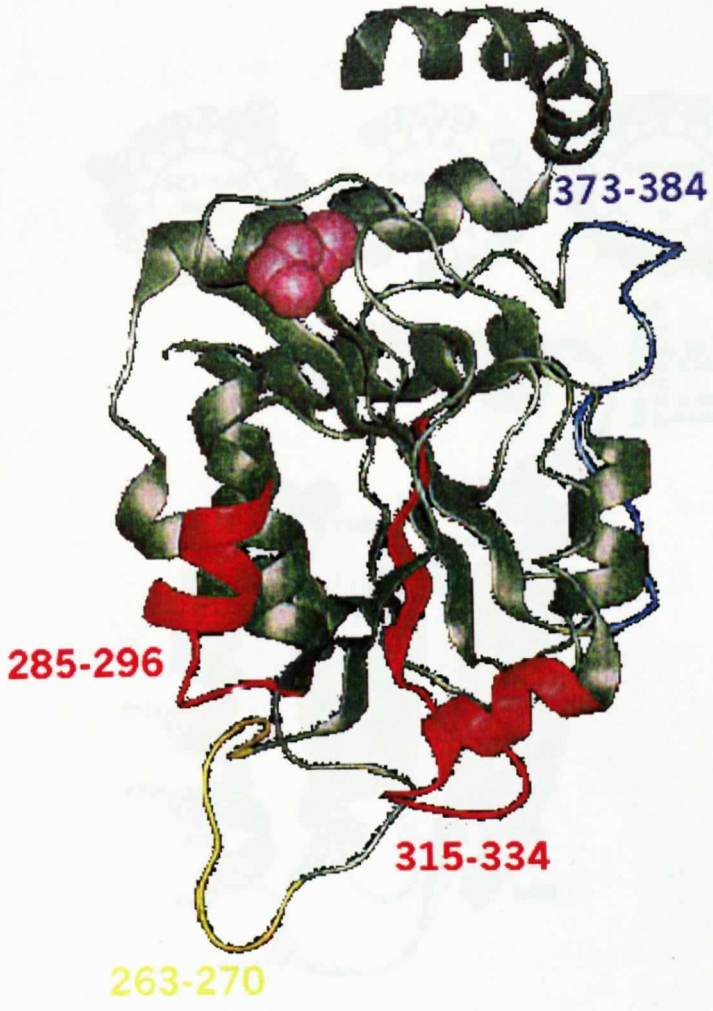
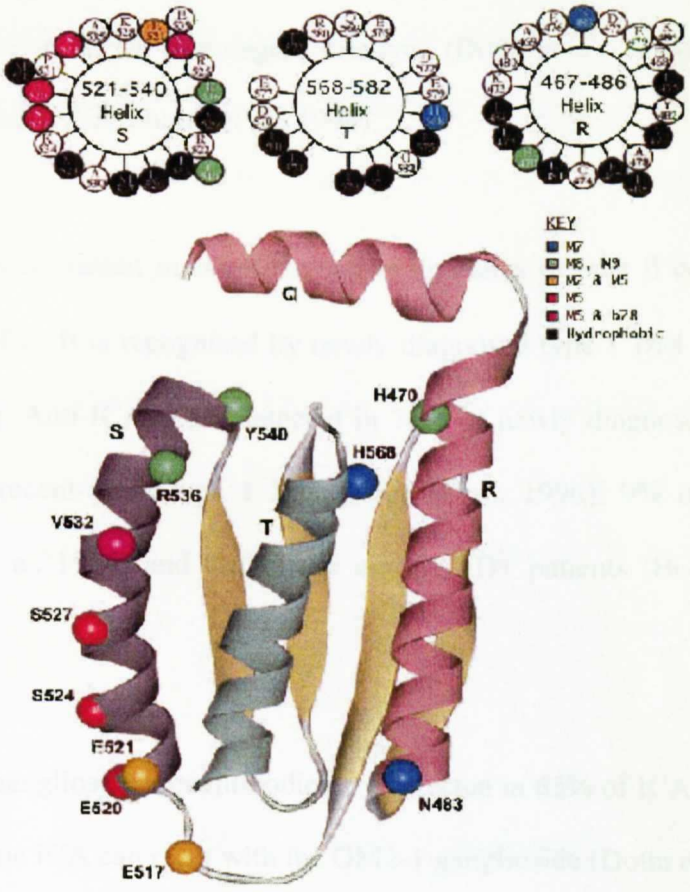


Figure 1.7 Helix wheel projections and a three-dimentional model of the C-terminal domain of a GAD-65 monomer. Helix wheel projection are viewed from the N-terminal end of the 3 α-helices in the C-terminal region of GAD-65. Hydrophobic amino acid residues are shown in black (Schwartz et al., 1999).



1.2.4.5 Other Autoantibodies

Carboxypeptidase-H (CPH) is a 52 kD glycoprotein, which is expressed by islet β cells and other neuroendocrine cells. In islet β cells, CPH is present in secretory granules to enhance the conversion of proinsulin to insulin. Anti-CPH autoantibodies are present in 25% of ICA+ first-degree relatives (Dotta et a., 1994) and 30% of prediabetic individuals (Eisenbarth et al., 1994).

38 kD autoantigen is present in insulin secretory granules of islet β cells and other neuroendocrine cells . It is recognised by newly diagnosed type 1 DM patients' sera (Dotta et a., 1994). Anti-ICA69 are detected in 70% of newly diagnosed type I DM patients, 25% of recent-onset type 1 DM (Roep et al., 1996), 9% of first-degree relatives (Dotta et a., 1994) and GAAs+ or anti-37 kD+ patients (Bonifacio et al., 1995).

Anti-GM2-1 islet ganglioside autoantibodies are detected in 65% of ICA+ first-degree relatives. Also, some ICA can react with the GM2-1 ganglioside (Dotta et a., 1994).

Islet cell surface autoantibodies (ICSAs) are weakly cytotoxic for islet β cells. ICSA have been detected using cultured rodent or human fetal islet cells and it is possible that these specificities are distinct from ICA specificities. Also, the IgM insulin receptor autoantibodies (IRAs) react with insulin receptor in newly diagnosed type 1 DM patients (Eisenbarth et al., 1994).

IgG and IgA anti-β-lactoglobulin autoantibodies (anti-β-LG) are increased in type 1 DM patients <3 years of age and in siblings. Among siblings, the levels of anti-β-

lactoglobulin are the same either before or after the clinical onset of type 1 DM (Iorini et al., 1994).

Anti-DNA Topoisomerase type II (anti-TopII) autoantibodies are detected in type I DM patients. The anti-TopII autoantibody epitopes are 1-47 aa, 286-472 aa and C-terminal third of DNA TopII. There is evidence that the anti-TopII autoantibodies may crossreact with other islet β cells autoantigens, such as GAD, insulin, CPH and HSP65 (Chang et al., 1996).

Anti-steroid 21 hydroxylase (P450c21) autoantibodies are present in type I DM patients. 86% of those patients have HLADQB1*0201 (Peterson et al., 1997). Anti-pancreatic disialo-ganglioside GD3 autoantibodies are present in newly diagnosed type 1 DM patients but not in IAA and/or ICA+ relatives of type 1 DM patients (Tiberti et al., 1995).

To determine the extent of gluten associated autoimmunity in type 1 DM, autoantibodies to tissue transglutaminase C (tTGA), a major autoantigen in coeliac disease, were measured in patients with new-onset type 1 DM. Interestingly, an increased prevalence of coeliac disease in patients with type 1 DM is well established. The prevalence of IgA to tTGA is about 8% and IgG to tTGA is about 32% in newly diagnosed type 1 DM. This suggests that the high prevalence of autoimmunity to tTGA may be due to an involvement of the gut in the pathogenesis of type 1 DM or to release of tTGA from destroyed pancreatic beta cells (Lampasona et al., 1999; Lorini et al., 2000).

Gastric parietal cell antibodies (PCA), which are a marker for iron deficiency and pernicious anaemia and atrophic gastritis (Baekkeskov et al., 1990; Riley et al., 1982), are highly prevalent in type 1 DM, especially in patients with ICA+ ≥3 years after diagnosis. The PCA are associated with GAD autoantibodies and/or HLA DR5 haplotype (De Block et al., 2000).

1.3 Phage-displayed Random Peptide Library

A random peptide library reveals a large repertoire of peptides expressed as fusions with a coat protein of bacteriophage, resulting in display of the fused proteins on the surface of the virions, while the DNA encoding the fusions resides within the virions. Some of the expressed peptides may bind to the selector molecule (such as antibody, enzyme, cell surface receptor, etc.) and often display a common consensus amino acid sequence. In some cases, this sequence (motif) shows similarity with a region of the natural protein binding to the selector molecule (Parmley and Smith 1988).

Phage-displayed random peptide libraries (PPL) have been used in a number of applications (Cortese et al., 1993), including epitope mapping (Scott and Smith 1990), mapping protein-protein contacts (Hong and Boulanger 1995), and identification of peptide mimics of non-peptide ligands (Devlin et al., 1990). Bioactive peptides have been identified either by panning against immobilised purified receptors (O'Neil et al., 1992) or against intact cells (Doorbar and Winter 1994). Larger proteins [antibody fragments (Barbas, S., and Barbas, C., 1994) , hormones (Lowman et al., 1991), protease inhibitors (Roberts et al., 1992), enzymes (Soumillion et al., 1994) and DNA binding proteins (Choo and Klug 1995)] have been displayed on phage, and variants with altered affinity or specificity have been isolated from libraries of random mutants.

The uses of combinatorial libraries include the definition of high affinity ligands both for T cell and antibodies, the application of relevant peptides for use as potential preventative and therapeutic vaccines, the requirements for TCR interactions with peptide-MHC complexes in immunogenicity, and the establishment of new principles

regarding the level of cross-reactivity in immunological recognition (Pinilla et al., 1999). The B cell epitopes are classified as either discontinuous, which recognise only native, folded structures, or continuous (Pinilla et al., 1999).

Regarding T cell epitopes, MHC-peptide complex formation has been achieved by the presence of anchor amino acids which mediate contact to the specificity pockets of MHC class I or class II molecules (Rammensee et al., 1997). The recognition of this ligand by the TCR requires contacts with polymorphic and nonpolymorphic residues of the α-helical domains of the MHC molecule which flank the peptide-binding groove (Madden et al., 1995) and also requires specific amino acids of the peptide that interact with the TCR (Kersh and Allen 1996). Class-I-restricted T cell clones show higher antigen affinities and the class I binding groove accommodates shorter peptides (8-10 amino acid residues) than class II (Rotzschke et al., 1990). Thus, relatively less complex peptide libraries can be used to determine class-I-restricted specificities. By contrast, Class-II-restricted T cell clones show lower antigen affinities (Davis et al., 1998) and the class II binding groove accommodates longer peptides (10-25 amino acid residues) (Pinilla et al., 1999). Thus, more complex peptide libraries can be used to determine class-II-resricted specificities, resulting in lower concentrations of individual peptides (complexity increases by 20-fold with each additional position) and T cell specific peptides with lower affinities.

Furthermore, the combinatorial library can be used to identify the number of different peptide ligands that can associate with the appropriate MHC molecule and stimulate a single CD4+ T cell clone (T cell degeneracy) (Pinilla et al., 1999).

The range of applications of phage technology has been extended to include the search for peptides binding to cell receptors (Cortese et al., 1995). For example, SrcSH3 domain is an intracellular receptor that has been used to select binding peptides (Sparks et al., 1994). Interleukins can also be displayed on phage and preserve their capacity to interact with their receptors (Gram et al., 1993). Random phage display peptide libraries and affinity selection methods were used to isolate small peptides that bind to and activate the receptor for the cytokine erythropoietin (EPO) (Wrighton et al., 1996). This discovery may form the basis for the design of small molecule mimetics of EPO.

Phagotopes are phage peptides selected for their ability to bind to antibody, as do anti-idiotypes. Also, phagotopes can induce antibodies (anti-anti-antibodies) that are specific for the antigen recognised by the first antibody, which can be used as a vaccine. For example, mimotopes (selected peptides) selected with an anti- human immunodeficiency virus (anti-HIV) gp120 monoclonal antibody can induce a specific humoral immune response to gp120 (Keller et al., 1993). This indicates that it is possible to develop vaccines based on mimotopes identified by their capacity to bind to patients' antibodies. An example is provided by mimotopes selected with the serum of HbsAg immunised individuals: these mimotopes were able to induce an antibody response against HbsAg in experimental animals (Folgori et al., 1994).

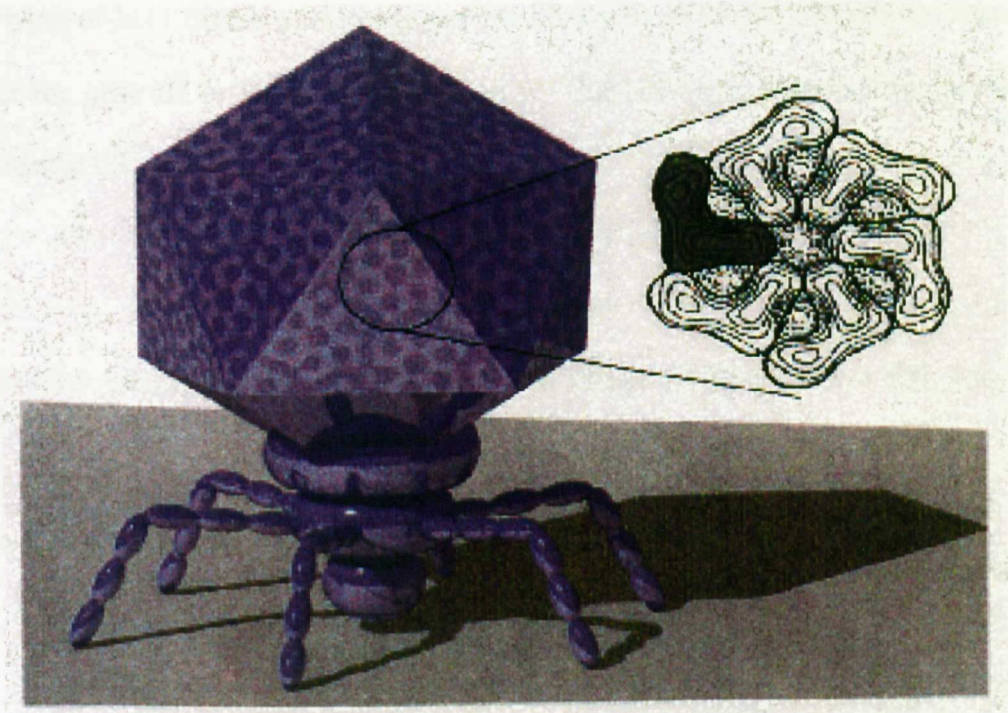
Fab antibody fragments have also been displayed on the surface of filamentous phage M13 (Barbas and Lerner 1991) by linking the heavy or light chain to a coat protein and secreting the other chain into the bacterial periplasm where the two chains are

associated (Better et al., 1988). With filamentous phage, it is also possible to mimic the expression of soluble antibody from the plasma cell (Hoogenboom et al., 1991).

1.3.1 T7 Phage Peptide Library

A constrained T7 phage peptide library C9C, (constrained 9-mers with cystine-cystine loop) (Fig. 1.8), was constructed by Dr Paddy Tighe (Division of Immunology, University of Nottingham). T7 is a double stranded DNA phage with the capability of lysing E.coli (strain BL 21). It expresses the inserted peptides as 415 copies on the surface coat protein encoded by gene X. The displayed peptide 9-mers are expressed at the C-terminus of gene X, between the EcoRI and HindIII restriction sites. Furthermore, it is easy to grow and its replication is very rapid in comparison to the filamentous phage. Also, it is very robust and stable to harsh conditions that inactivate other phage. It can display peptides and proteins do not need to be capable of export through the periplasm and the cell membrane, as is necessary with filamentous phage (Rosenberg et al., 1997).

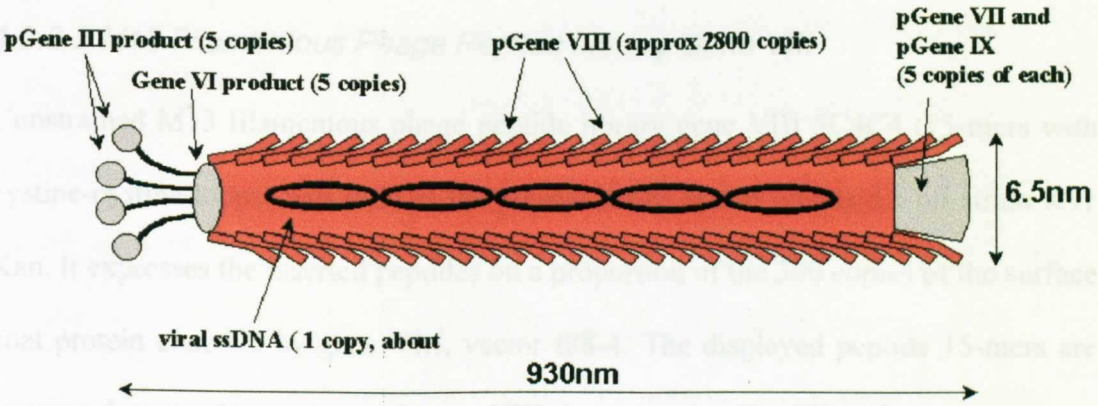
Figure 1.8 Structure of the T7 Phage Paticle (Rosenberg et al., 1997).



1.3.2 M13 Filamentous Phage Peptide Library

M13 is a single stranded DNA phage that infects specific strains of E.coli. There are two types of M13 filamentous phage peptide library in which the peptides are fused to either the gene III or VIII proteins (Fig. 1.9). Both of them express their displayed peptides at the amino terminus of the gene.

Figure 1.9 Structure of the M13 Filamentous Phage. Different Genes (III, VI, VII, VIII and IX) are shown.



1.3.2.1 *M13 Filamentous Phage Peptide Library Gene III*

Two types of M13 filamentous phage peptide library gene III (pIII) where used in the present study: a library of unconstrained 12-mers (without cystine-cystine loops) and a constrained library C7C (7-mers with cystine-cystine loops).These can infect E.coli strain called ER 2537. They express the inserted peptides as 3-5 copies on the surface coat protein encoded by gene III. The displayed peptide 12-mers are expressed at the N-terminus of pIII, between the KpnI and EagI restriction sites. The pIII is responsible for attachment of the phage to the bacterial F-pilus and infection (Marks et al., 1992).

1.3.2.2 M13 Filamentous Phage Peptide Library Gene VIII

Constrained M13 filamentous phage peptide library gene VIII 5C4C4 (15-mers with cystine-cystine loops) was used in the present study. It can infects E.Coli strain K91 Kan. It expresses the inserted peptides on a proportion of the 300 copies of the surface coat protein encoded by gene VIII, vector f88-4. The displayed peptide 15-mers are expressed at the N-terminus of gene VIII, between the HindIII and PstI restriction sites. The pVIII is responsible for coating the double stranded DNA (Marks et al., 1992).

1.4 Rationale and Aims of the Project

The aim of these studies was to determine the epitopes in GAD65 of three different mouse monoclonal antibodies: N-terminal monoclonal antibody within 4-17 amino acids (aa), C-terminal monoclonal antibody within 572-585 aa and GAD-6 (Fig. 1.10); to determine the epitopes in IA-2 of two different mouse monoclonal antibodies 76B and 76F (Fig. 1.11); and to determine the epitopes of two different human monoclonal antibodies, b78 and b96.11, on GAD-65 (Fig. 1.10). Two different types of random peptide phage libraries were used: T7 and M13 libraries. The different libraries may yield complementary information which helps in locating epitopes with more confidence. Previous studies showed that the GAD-6 epitope is present in the C-terminal region of GAD, but did not reveal definitively the specific amino acid residues of the GAD-6 epitope. Also, previous studies showed that the 76B and 76F epitopes are present in the extracellular domain of IA-2 and in the N-terminal region of cytoplasmic domain of IA-2, respectively, but also did not reveal definitively the specific amino acid residues of the 76B and 76F epitopes. In addition, previous studies showed that the b78 and b96.11 epitopes are present in 532-540 aa and 308-365aa, respectively, on GAD-65 (Schwartz et al., 1999), but did not reveal definitively the specific amino acid residues of the b78 and b96.11 epitopes. The determination of epitopes recognized by these monoclonal antibodies might help us to understand the antigenic nature of the GAD65 and IA-2 autoantigens.

Since epitope mapping is generally simpler with monoclonal antibodies than with polyclonal antibodies, these studies should indicate how best to use the random phage peptide libraries to determine the epitope specificities of patients' serum

autoantibodies. Thus, a further aim of these studies was to determine the immunodominant epitopes (public epitopes) in GAD65 of SMS patients' serum autoantibodies.

Figure 1.10 Epitopes of Mouse (N- and C-terminus and GAD-6) and Human (b96.11 and b78) Monoclonal Antibodies on GAD65.

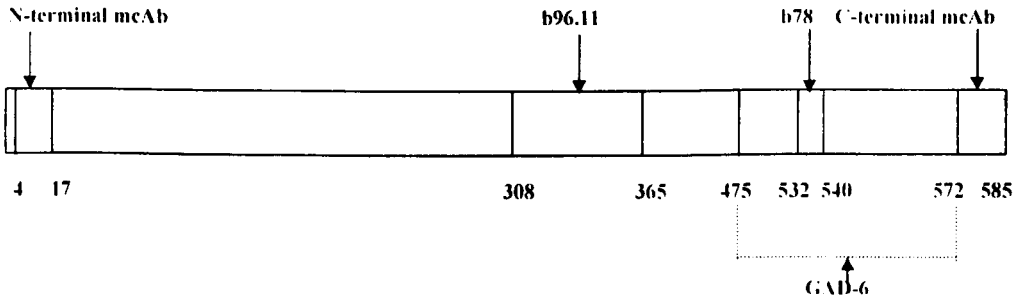
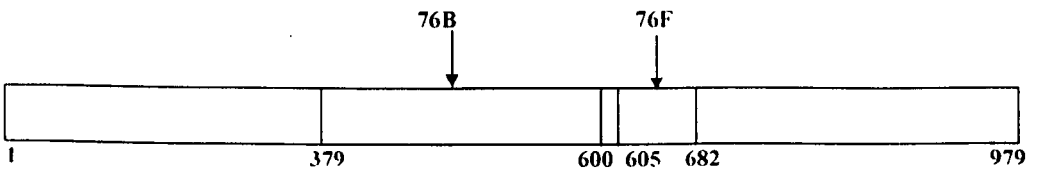


Figure 1.11 Epitopes of Mouse Monoclonal Antibodies (76B and 76F) on IA-2.



An aim of these studies was also to investigate the influence of particular features of the random peptide libraries on their usefulness for epitope mapping. The variable features included the importance of using peptides with a cysteine-cysteine loop (constrained library) or linear peptides, and the importance of using different libraries with different features [peptide copies, expression, infection, peptide numbers of amino acids per peptide (7, 9, 12 or 15-mers) and coat protein on which peptide expressed (gene pIII, pVIII or pX)] in epitope recognition.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2 General Materials and Methods

2.1 T7 (C9C or linear 9-mers) Phage Peptide Libraries

2.1.1 General Preparations

In this section, standard procedures are described which were employed in the protocols described in detail in the following sections.

2.1.1.1 E.coli BL 21 in LB Agar Plate

LB agar Petri dishes were inoculated with E.coli BL 21 strain (Novagen, US) using a sterile wire loop, and incubated at 37°C overnight.

2.1.1.2 E.coli BL 21 Overnight Culture

A single colony of E.coli BL 21 was removed from an LB agar plate using a sterile wire loop, added to 10mls of LB broth in a conical flask and incubated on a shaker at 37°C overnight.

2.1.1.3 Mid-log Phase E.coli BL 21 Culture

0.5ml of E.coli BL 21 overnight culture was added to 20-30mls of LB broth and then incubated on a shaker at 37°C for 2-3 hours until the optical density at 600 nm (OD_{600})=0.6-0.8 as determined by spectrophotometry.

2.1.1.4 Serial Dilution of Non-amplified Eluate

Ten microlitres of the non-amplified eluate (described in section 2.1.2) were serially diluted by adding 990µl LB broth and then mixed, giving a 10^{-2} dilution. Then, 100µl of 10^{-2} diluted eluate were added to 900µl LB broth and then mixed giving a 10^{-3} dilution. Then, 100µl of 10^{-3} diluted eluate were added to 900µl LB broth and then mixed, giving a 10^{-4} dilution; and so on.

2.1.1.5 Plating the Diluted Non-amplified Eluate

One hundred microlitres of a dilution of non-amplified eluate were added to 100µl of mid-log phase E.coli BL 21 culture and then mixed with 3mls of top agarose (0.7% agarose and 0.1% $MgCl_2 \cdot 6H_2O$ in LB broth), which were pre-warmed to 45°C, in a Pijuo tube. The mixture was poured on to an LB agar Petri dish and incubated at 37°C for 3 hours.

2.1.1.6 Lysate-treated Membrane

One hundred microlitres of T7 wild-type phage were added to 20mls of mid-log phase E.coli BL 21 culture in a conical flask and incubated on a shaker at 37°C for 45 minutes to induce lysis of the E.coli by the phage (termed the lysate), then centrifuged at 4°C and 1,500 g for 20 minutes. Then, the pellet was collected and resuspended in 2mls of TBS. The lysate was poured on a nitro-cellulose membrane and incubated for 30 minutes at room temperature. Then, the lysate-treated membrane was washed with 0.1% TBS-T for 15 minutes then with TBS for 5 minutes and then blocked with 5% BSA/TBS and incubated on a rotator for one hour at room temperature.

2.1.1.7 Loading of PCR Product and Gel Electrophoresis

Five microlitres of PCR product or 0.5µl of 100 base pair (bp) standard DNA ladder were added to 2µl of loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficol (type 400, Pharmacia) in water]. The agarose gel [2% agarose in Tris-borate/EDTA (TBE) buffer (90mM Tris, 90mM borate and 2mM EDTA pH 8.3) with ethidium bromide 0.2µl/ml] was placed in the gel electrophoresis tank, immersed with TBE and loaded with the samples. The gel was run at 80 volts for 45 minutes, observed under an (ultra-violet) UV transilluminator and photographed.

2.1.1.8 5× Buffers B and C

Buffer B was prepared by mixing 300mM Tris HCl pH 8.5, 75mM $(NH_4)_2SO_4$ and 10mM $MgCl_2$. While buffer C was prepared by mixing 300mM Tris HCl pH 8.5, 75mM $(NH_4)_2SO_4$ and 12.5mM $MgCl_2$.

2.1.2 Bio-panning of Antibody with T7 (C9C or Linear 9-mers) Phage Peptide Libraries

One millilitre (ml) of diluted mouse monoclonal antibody (mAb), [10 micrograms/millilitre, (10µg/ml)] in 0.05M sodium carbonate/sodium bicarbonate buffer pH 9.6 (coating buffer), was coated onto a Nunc immuno-tube at 4°C overnight on a rotator. The tube was washed 5 times with 25 millimolar (25mM) Tris-buffered saline (150mM NaCl) pH 7.4 (TBS) containing 0.1% Tween-20 (0.1% TBS-T); each time the tube was incubated at room temperature for 3 minutes with the TBS-T. The tube was then blocked with 5% bovine serum albumin (BSA) in TBS (5% BSA/TBS) (blocking solution) at room temperature for one hour on a rotator. Ten microlitres of

the T7 phage library [1×10^{10} plaque forming units (pfu)/10µl] in 1ml of blocking solution, was added to the mAb-coated tube and incubated at 4°C for 20 minutes on a rotator.

The tube was then washed ten times in 0.1% TBS-T to remove unbound phage and 1ml of a mid-log phase E.coli BL 21 culture OD_{600}=0.6-0.8 was added and incubated on a rotator at room temperature for 5 minutes. This suspension of E.coli infected with the phage which had bound to the mAb was termed the non-amplified eluate.

Ten microlitres of the non-amplified eluate were taken for dilution and plating (as described in sections 2.1.1.4 and 2.1.1.5, respectively) to be confident that the specific phage had been eluted. Thus, the phage formed lysis plaques on the plate and the number of plaques of each round were compared with the number of plaques of the previous rounds as an indication of the specificity of the biopanning. The remainder of the eluate was added to 20-30mls of the mid-log phase E.coli BL 21 culture in a conical flask and incubated on a shaker at 37°C for 1-3 hour(s) for amplification, thus forming the amplified eluate.

Following amplification, the T7 phage lysed the E.coli BL 21 causing clotted strands of DNA to become visible. 250µl of chloroform were then added and mixed to complete lysis of the E.coli BL 21 and release of the phage. The enriched phage (amplified eluate) were centrifuged at 4°C and 9,500 g for 10 minutes. The supernatant containing the phage was transferred to a fresh tube and subjected to a second cycle of affinity selection following the procedure described above. Thus, the

enriched phage of the preceding round of biopanning were used for the next round. Four rounds were carried out in this way for the biopanning of the T7 phage library.

2.1.3 Detection of Antibody-specific T7 (C9C or Linear 9-mers) Phage Peptide Clones by Immuno-blotting Assay

The phage of the final round (fourth round) of biopanning were plated out (as described in section 2.1.1.5) to obtain 100-200 plaques per Petri dish. A millipore nitro-cellulose membrane (0.45μM pore size and 82mm area) (Millipore, UK) was placed onto the plate and incubated for 30 minutes at room temperature. The membrane was then blocked with 5% BSA/TBS and incubated on a rotator for one hour at room temperature. Ten millilitres of diluted mouse monoclonal antibody, (10μg/ml, diluted in 2.5% BSA /TBS-T) was added to the membrane and incubated on a rotator for 2 hours at room temperature. The membrane was washed with 0.1% TBS-T for 15 minutes and then with TBS for 5 minutes.

Sheep anti-mouse IgG (whole molecule) antibody was depleted of reactivity against T7 phage and E.coli prior to addition to the membrane in order to reduce the non-specific background staining. This was achieved as follows: 10mls of diluted sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) (diluted 1:1000 in 2.5% BSA/TBS-T) was adsorbed with lysate-treated membrane (prepared as described in section 2.1.1.6) for 30 minutes at room temperature on a rotator. It was then added to the membrane that had the selected phage clones and antibody bound, and incubated at room temperature for one hour. The membrane was washed as above and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) substrate (Sigma) in de-ionised water with 5mM levamisole was added to the

membrane and incubated for 30 minutes at room temperature. Following the appearance of the blue spots, the membrane was washed with TBS, then with water and dried.

Antibody-specific phage clones, which developed as blue spots on the membrane, were selected from the original plate using a wire-loop and each of them mixed with 1ml of mid-log phase E.coli BL 21 culture and incubated on a shaker at 37°C for 3 hours for amplification (amplified eluate). Following amplification and lysis of E.coli by the T7 phage, 25µl of chloroform were added to each clone to complete lysis. The amplified eluates were centrifuged at 4°C and 9,500 g for 10 minutes. The supernatants containing the phage were transferred to fresh tubes and used for polymerase chain reaction (PCR), sequencing and enzyme-linked immunosorbent assay (ELISA).

2.1.4 PCR and Sequencing of Antibody-specific T7 (C9C or Linear 9-mers) Phage Inserts

0.5µl of each clone of specific phage was added to 25µl of PCR mixture [5µl of 5× buffer B or C (described in section 2.1.1.8), 0.25µl of 1% tween, 0.5µl of 10mM dNTPs (deoxynucleoside triphosphates), 0.5µl of 20µM T7 A primer (5'-ACA ACG TTA TCG GCC TGT TC-3'), 0.5µl of 20µM T7 B primer (5'-TAC CGG AGG TTC ACC GAT AG-3'), 1µl of 0.5U/µl of ampliTaq or Hotstar Taq DNA polymerase and 17.5µl of sterile de-ionised water]. The samples were transferred to a Hybaid Omnigene PCR machine, which was preheated to 75°C, and run on a PCR reaction program [(94°C, 15 minutes)×1; (94°C, 50 seconds; 50°C, 1 minute; 72°C, 1

minute)×35; (72°C, 5 minutes)×1]. One aliquot was left with no phage to be used as a negative control.

Five microlitres of PCR product were used for loading and gel electrophoresis (as desribed in section 2.1.1.7) to confirm the appearance of the band of the PCR product at 150 base pair (bp) length after the PCR reaction.

To prepare the sample for the sequencing reaction, 0.5µl of Exonuclease I (10U/µl) and 1µl of shrimp alkaline phosphatase (SAP) (1U/µl) were added to 5µl of PCR product, which showed a bright band at 150bp length, and run on the enzyme treatment program (37°C, 15 minutes; 80°C 15 minutes) in the Omnigene PCR machine. One microlitre of enzyme treated PCR product was then added to 4µl BigDye Terminator, which is labelled with the following dRhodamine acceptor dyes: dR6G to give green colour for terminator A, dROX to give red colour for terminator C, dR110 to give blue colour for terminator G and dTAMRA to give black colour for terminator T (Perkin-Elmer Applied Biosystems), 0.15µl of 10µM T7 sequencing primer (5'-TTA AGC TGC GTG ACT TGG C-3') and 5µl of sterile de-ionised water and run on a sequencing reaction program [(96°C, 30 seconds; 50°C, 15 seconds; 60°C, 4 minutes)×25; (28°C, 1 minute)×1].

Ten microlitres of sequencing reaction product was purified by adding 124µl sequence cleaning mixture (ethanol: water: 3M sodium acetate pH 4.6-5.2, in a dilution 25:5:1). The samples were centrifuged at 4°C and ≥21,000 g for 15 minutes. The supernatant was removed and the pellet cleaned by adding 300µl of 70% ethanol

PAGE
MISSING
IN
ORIGINAL

sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (diluted 1:1000 in 1% BSA/TBS-T) (Sigma) was added to all wells (100µl/well) and shaken at room temperature for one hour. Wells were washed 3 times in TBS-T and substrate [1µg/ml p-nitrophenyl-phosphate (pNPP) substrate (Sigma) in diethanolamine buffer, 25mM $MgCl_2$, 15mM NaN_3, pH 9.8] was added to all wells (100µl/well) and incubated at room temperature. Plates were read at optical density (OD) 405 nm after 60 minutes at room temperature and overnight at 37°C on a microtitre plate reader (Molecular Devices). The mean OD of the antigen-coated wells was corrected by subtracting the mean OD of the equivalent blank wells.

2.1.5.2 ELISA with Maleic Anhydride Activated Polystyrene Plates

The detection of specific T7 phage peptides was confirmed by using maleic anhydride activated polystyrene (MAAP) 96-well plates (Pierce and Warriner, UK). The selected phage, T7 wild-type (negative control) (diluted 1:20 in 0.2N carbonate/bicarbonate buffer pH 9.7) was coated onto wells (100µl/well) of the MAAP plate and incubated for one hour at room temperature on a shaker. Wells were washed 3 times with washing buffer (0.1% BSA and 0.05% TBS-T), and blocked with blocking buffer (3% BSA and 0.05% TBS-T) at room temperature for one hour. Blank wells, which were not coated with phage, were also blocked with blocking buffer, as above. Following 3 washes, mouse monoclonal antibody (1µg/ml in washing buffer) or a negative control antibody (1µg/ml in washing buffer) was applied (100µl/well) in duplicate to antigen-coated and blank wells and shaken at room temperature for two hours. Following 3 washes, sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (diluted 1:1000 in washing buffer) was

added to all wells (100µl/well) and shaken at room temperature for one hour. Wells were washed 3 times in TBS-T and pNPP substrate was added to all wells (100µl/well) and incubated at room temperature for one hour. Plates were read after 60 minutes at OD 405 nm on a microtitre plate reader (Molecular Devices). The mean OD of the antigen-coated wells was corrected by subtracting the mean OD of the equivalent blank wells.

2.1.6 Construction of T7 Linear 9-mers Phage Peptide Library

2.1.6.1 Preparation of Double-stranded DNA from Degenerate Oligonucleotides

The random peptides of the T7 library were encoded by a double stranded DNA insert assembled from synthetic degenerate oligonucleotides and cloned into gene X of the vector (T7select415-1) (Bioscience, Cambridge, UK). Oligonucleotide sequences were as follows: (9lin3'), 5´-GAT CAC CGA AGC TTC AAG AGC-3´ (15 nmol) (21bp) and (9lin), 5´-GCT GCT TAT CTA GGA ATT CC $(NNK)_9$ TGA GGC TCT TGA AGC TTC GGT GAT C-3´ (10.3 nmol) (72bp) (Cruachem, Paisley, Scotland). The oligonucleotides were annealed to produce double-stranded DNA and extended by combining the following components: 40µl of buffer C, 8µl of 5mM dNTPs, 2µl of 1% tween, 4µl of upper primer (9lin3'), 4µl of lower primer (9lin), 2µl of 0.5U/µl Taq DNA polymerase and 140µl sterile de-ionized water; followed by running the following program: 94°C, 10 minutes; 57°C, 2 minutes; 72°C, 20 minutes; into the Omnigene PCR machine.

The DNA was extracted by adding an equal volume (200µl) of 50:50 phenol:chloroform to the PCR product, mixed and centrifuged at 4°C and 21,000 g for 30 seconds. The supernatant (DNA) was transferred to a fresh microcentrifuge tube and precipitated by adding 2.5 volume of 100% ethanol and 0.1 volume of 3M Na-acetate pH 5, incubated at -20°C for 10 minutes, centrifuged at 4°C and 21,000 g for 20 minutes and cleaned by adding 0.5ml of 70% ethanol and centrifuged at 4°C and 21,000 g for 5 minutes. The supernatant was discarded, while the pellet (DNA) was suspended in 20µl of 10× OPA (One Phor All) (Pharmacia, UK) and mixed with 160µl of sterile de-ionized water. Then, the suspended DNA was transferred to 4 fresh microcentrifuge tubes as follows: 20µl without digestion, 20µl digested by adding 2µl of EcoRI, 20µl digested by adding 2µl of HindIII or 120µl digested by adding both 6.5µl of EcoRI and 6.5µl of HindIII and incubated at 37°C overnight.

The whole amount of undigested and digested DNA (EcoRI, HindIII and EcoRI+HindIII) or 0.5µl of 10bp standard DNA ladder were added to loading buffer and loaded into a 20% polyacrylamide gel in TBE. The gel was run at 10W and 10mA for 3 hours. The DNA was visualized by ethidium bromide staining under UV and photographed. The band of the DNA insert, which was digested with EcoRI+HindIII, was visualized at 45bp; and excised and crushed into a fine paste. The gel was suspended in 3ml of 0.5M ammonium acetate, incubated on a shaker at 37°C overnight and centrifuged at 4°C and 1,500 g for 1 minute. The supernatant was filtered using mobitec syntered plastic disc to remove residual polyacrylamide fragments of the gel. The supernatant was treated with 1-butanol extractions [by adding an equal volume of 1-butanol, mixing and centrifugating at 4°C and 21,000 g

for 30 seconds, then the upper phase was removed while the aqueous (lower) phase was retained for the next extraction]. The 1-butanol extractions were repeated to reduce the volume to 0.5ml containing the DNA insert.

The DNA insert was extracted by adding an equal volume (0.5ml) of 50:50 phenol:chloroform, mixed and centrifuged at 4°C and 21,000 g for 30 seconds. The supernatant (DNA) was transferred to a fresh microcentrifuge tube and precipitated by adding 2.5 volume of 100% ethanol and 0.1 volume of 3M Na-acetate pH 5.2, incubated at -20°C for 10 minutes, centrifuged at 4°C and 21,000 g for 20 minutes and cleaned by adding 0.5ml of 70% ethanol and centrifuged at 4°C and 21,000 g for 5 minutes. The supernatant was discarded, while the pellet (DNA) was air dried and re-suspended in 20-30µl of de-ionized water. The insert was aliquoted and stored at -70°C.

2.1.6.2 Preparation of Linearized Vector DNA

The vector (T7 wild type phage) (50ml) had been amplified in E.Coli BL21 strain. The vector was treated with DNAaseI (1µg/ml) and RNAaseA (100µg/ml) and incubated for 15 minutes at room temperature. Then, 2M ZnCl (20µl/ml) was added to the treated vector and incubated for 5 minutes at 37°C. After centrifugation at 4°C and 9,500 g for 1 minute, the pellet was suspended in TES buffer (0.1M Tris-HCl, pH 8; 0.1M EDTA and 0.3% SDS) (500µl/ml) and incubated for 15 minutes at 60°C. Then, 3M potassium acetate pH 5.2 (60µl/ml) was added, mixed and incubated on ice for 10-15 minutes. After centrifugation at 4°C and 13,000 g for 1 minute, the supernatant was collected. An equal volume of isopropanol was added, mixed and

incubated on ice for 5 minutes. After centrifugation at 4°C and 13,000 g for 1 minute, the pellet was cleaned by adding 70% ethanol and centrifuged at 4°C and 13,000 g for 5 minutes. The supernatant was discarded, while the pellet (DNA) was air dried and re-suspended in TE (10mM Tris-HCl, pH:8; 1mM EDTA) buffer (20-100µl/ml).

The suspended DNA vector was extracted by adding an equal volume (600µl) of 50:50 phenol:chloroform, mixed and centrifuged at 4°C and 21,000 g for 30 seconds. The supernatant (DNA) was transferred to a fresh centrifuge tube and precipitated by adding 2.5 volume of 100% ethanol and 0.1 volume of 3M Na-acetate pH 5, incubated at -20°C for 10 minutes, centrifuged at 4°C and 21,000 g for 20 minutes and cleaned by adding 0.5ml of 70% ethanol and centrifuged at 4°C and 21,000 g for 5 minutes. The supernatant was discarded, while the pellet (DNA) was suspended in 110µl of 10× OPA and mixed with 890µl of sterile de-ionized water. The suspended DNA (1ml) was transferred to a fresh microcentrifuge tube and digested by adding both 50µl of EcoRI and 50µl of HindIII and incubated at 37°C overnight. The DNA vector was then de-phosphorylated by adding 10µl of shrimp alkaline phosphatase (1-3U/µg of DNA) and incubated at 37°C for 30 minutes.

The DNA vector was extracted by adding an equal volume (1,110µl) of 50:50 phenol:chloroform, mixed and centrifuged at 4°C and 21,000 g for 30 seconds. The supernatant (DNA) was transferred to a fresh centrifuge tube and precipitated by adding 2.5 volume of 100% ethanol and 0.1 volume of 3M Na-acetate pH 5.2, incubated at -20°C for 10 minutes, centrifuged at 4°C and 21,000 g for 20 minutes and cleaned by adding 0.5ml of 70% ethanol and centrifuged at 4°C and 21,000 g for

5 minutes. The supernatant was discarded, while the pellet (DNA) was air dried and re-suspended in 100µl of de-ionized water.

2.1.6.3 Ligation of the vector and insert DNA

The ligation reactions were set up by assembling the following components: 0.04pmol vector, 0.12pmol insert, 0.5µl of 10× ligase buffer, 0.5µl of 10mM ATP, 0.5µl of 100mM DTT and 1µl of 0.4-0.6 Weiss units of T4 DNA ligase. Then, 5µl of ligation reaction were mixed gently by pipetting up and down and then incubated 3-16 hours at 16°C. The 5µl of ligation reaction was then added to 25µl of T7Select packaging extract (Bioscience, Cambridge, UK) and incubated at room temperature for 2 hours. The reaction was stopped by adding 270µl LB medium.

One microlitre of the non-amplified eluate was taken for dilution and plating (as described in sections 2.1.1.4 and 2.1.1.5, respectively), giving 1×10^{10} pfu/ml. The bulk of the phage were added to 200-400mls of the mid-log phase E.coli BL 21 culture and incubated on a shaker at 37°C for 1-3 hour(s). Following the lysis, 1ml of chloroform was added and mixed to complete lysis the E.coli BL 21 and release of the phage. The amplified phage were centrifuged at 4°C and 9,500 g for 10 minutes. The supernatant containing the phage was transferred to a fresh tube, which was termed the T7 (linear 9-mers) phage peptide library.

2.2. M13 pIII (C7C or Linear 12-mers) Filamentous Phage Peptide Libraries

2.2.1 General Preparations

In this section, standard procedures are described which were employed in the protocols described in detail in the following sections.

2.2.1.1 Minimal Plate

Five hundred millilitres of 2X M9 salts, 500ml of 3% agar, 20ml of 20% glucose, 2ml of 1M $MgSO_4$, 0.1ml of 1M $CaCl_2$, 1ml of 10mg/ml thiamine were sterilized separately, combined together at temperature <70°C and poured onto Petri dishes.

2.2.1.2 E.coli ER 2537 in Minimal Plate

A minimal Petri dish was inoculated with E.coli ER 2537 strain (New England Biolabs, UK) using a sterile wire loop, and incubated at 37°C overnight.

2.2.1.3 E.coli ER 2537 Overnight Culture

A single colony of E.coli ER 2537 was removed from a minimal plate using a sterile wire loop, added to 10mls of LB broth in a conical flask and incubated on a shaker at 37°C overnight.

2.2.1.4 Mid-log Phase E.coli ER 2537 Culture

0.2ml of E.coli ER 2537 overnight culture was added to 20mls of LB broth and then incubated on a shaker at 37°C for 3 hours until the OD_{600}=0.5 as determined by spectrophotometry.

2.2.1.5 Early-log Phase E.coli ER 2537 Culture

0.2ml of E.coli ER 2537 overnight culture was added to 20mls of LB broth and then incubated on a shaker at 37°C for 15 minutes until the OD_{600}=0.05 as determined by spectrophotometry.

2.2.1.6 Serial Dilution of Non-amplified Eluate

One microlitre of the non-amplified eluate (described in section 2.2.2) was serially diluted by adding 99µl LB broth and then mixed, giving a 10^{-2} dilution. Then, 10µl of 10^{-2} diluted eluate were added to 90µl LB broth and then mixed, giving a 10^{-3} dilution. Then, 100µl of 10^{-3} diluted eluate were added to 90µl LB broth and then mixed, giving a 10^{-4} dilution; and so on.

2.2.1.7 Plating the Diluted Non-amplified Eluate

Ten microlitres of a dilution of non-amplified eluate were added to 200µl of a mid-log phase E.coli ER 2537 culture and then mixed and incubated at room temperature for 1-5 minute(s) (to allow the M13 pIII phage to infect the E.coli ER 2537) and then mixed with 3mls of top agarose (0.7% agarose and 0.1% $MgCl_2 \cdot 6H_2O$ in LB broth), which were pre-warmed to 45°C, in a Pijuo tube. The mixture was poured onto a LB agar Petri dish and incubated at 37°C overnight.

2.2.1.8 Concentration and Purification of Amplified Eluate from Bio-panning

The amplified phage were centrifuged at 4°C and 9,500 g for 10 minutes. The supernatant containing the phage was transferred to a fresh tube and re-centrifuged. One sixth volume of PEG/NaCl [20% (w/v) polyethylene glycol in 2.5M NaCl] was added to the supernatant to precipitate the phage, and incubated at 4°C overnight. The precipitated phage were centrifuged at 4°C and 9,500 g for 15 minutes. The supernatant was removed and the pellet suspended in 1ml TBS. The suspension was transferred to a microcentrifuge tube and centrifuged at 4°C and 9,500 g for 5 minutes to pellet residual cells. The supernatant was transferred to a fresh microcentrifue tube, and re-precipitated with 1/6 volume of PEG/NaCl and incubated on ice for 15-60 minutes. The precipitated phage were centrifuged at 4°C and 9,500 g for 10 minutes. The supernatant was removed and the pellet was re-suspended in 200µl of TBS, 0.02% NaN_3. The suspension was centrifuged at 4°C and 9,500 g for one minute to pellet any remaining insoluble matter. The supernatant (the amplified eluate) was transferred to a fresh microcentrifuge tube.

2.2.1.9 Lysate-treated Membrane

One hundred microlitres of M13 pIII wild-type (M13K07) phage were added to 20mls of early-log phase E.coli ER 2537 culture (OD_{600}=0.05) in a conical flask and incubated on a shaker at 37°C for 4.5 hours to infect the E.coli by the phage (termed the lysate), then centrifuged at 4°C and 1,500 g for 20 minutes. Then, the pellet was collected and resuspended in 2mls of TBS. The lysate was poured on a nitro-cellulose membrane and incubated for 30 minutes at room temperature. Then, the lysate-treated membrane was washed with 0.1% TBS-T for 15 minutes then with TBS for 5 minutes

and then blocked with 5% BSA/TBS and incubated on a rotator for one hour at room temperature.

2.2.1.10 Lysis of E.coli ER 2537 Overnight Culture by Sonication

Forty millilitres of E.coli ER 2537 overnight culture (described in section 2.2.1.3) were centrifuged at 4°C and 1,500 g for 20 minutes. The pellet was collected and suspended in 4mls of TBS, in ice to avoid secretion of E.coli enzymes which may cause proteolysis of human serum antibodies. Then, the suspension was sonicated for 5 minutes at level 10 in ice to lyse the bacteria. The lysate was filtered using a filter (pore size=0.2μM) and stored at -20°C.

2.2.2 Bio-panning of Antibody with M13 pIII (C7C or Linear 12-mers) Phage Peptide Library

Mouse monoclonal antibody (10μg/ml), in 0.05M sodium carbonate/sodium bicarbonate buffer pH 9.6 (coating buffer), was coated onto a Nunc immuno-tube at 4°C overnight on a rotator. The tube was washed 5 times with 50mM Tris-buffered saline (150mM NaCl) pH 7.5 (TBS) containing 0.1% Tween-20 (0.1% TBS-T); each time the tube was incubated at room temperature for 3 minutes with TBS-T. The tube was then blocked with 0.5% BSA in TBS containing 0.02% NaN_3 (blocking solution) at room temperature for one hour on a rotator. Following 6 washes, 10μl of the M13 pIII phage library (2×10^{11} pfu/10μl) (New England Biolabs, UK) in 1ml of 0.1% TBS-T were added and incubated at 4°C for 30 minutes on a rotator. The tube was washed 10 times and 1ml of elution buffer (0.1% BSA in 0.2M glycine-HCl pH 2.2) was added and incubated ar room temperature for 10 minutes on a rotator. This

non-amplified eluate was removed and neutralized with 150µl of neutralizing buffer (1M Tris-HCl buffer pH 9.1).

One microlitre of the non-amplified eluate was taken for dilution and plating (as described in sections 2.2.1.6 and 2.2.1.7, respectively). While the rest of the eluate was added to 20mls of the early-log phase E.coli ER 2537 culture (OD_{600}=0.05) and incubated on a shaker at 37°C for 4.5 hours for amplification.

The amplified eluate, which was concentrated and purified (as described in section 2.2.1.8), was subjected to a second cycle of affinity selection following the same procedure described above. Thus, the enriched phage of the preceding round of biopanning were used for the next round. Three rounds were carried out in this way for the biopanning of the M13 pIII phage library.

2.2.3 Detection of Antibody-specific M13 pIII (C7C or Linear 12-mers) Phage Peptide Clones by Immuno-blotting Assay

This was performed as described in section 2.1.2 for the T7 phage library. The phage of the final round (third round) of biopanning were plated (as described in section 2.2.1.7) to obtain 100-200 plaques per Petri dish. A millipore nitro-cellulose membrane (0.2µM pore size) was placed onto the plate and incubated for 30 minutes at room temperature. The membrane was then blocked with 5% BSA/TBS. Ten millilitres of diluted mouse or human monoclonal antibody, (10µg/ml. diluted in 2.5% BSA /TBS-T) was added to the membrane and incubated on a rotator for 2 hours at room temperature. The membrane was washed with 0.1% TBS-T. Ten millilitres of

diluted sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (diluted 1:1000 in 2.5% BSA/TBS-T) was blocked with lysate-treated membrane then added to the membrane that had the selected phage clones and antibody bound, and incubated for one hour at room temperature. The membrane was washed, as above, and BCIP/NBT substrate in de-ionised water with 5mM levamisole was added to the membrane. Following the appearance of the blue spots, the membrane was washed and dried.

Antibody-specific phage clones, which developed as blue spots on the membrane, were selected and each of them mixed with 1ml of early-log phase E.coli ER 2537 culture and incubated on a shaker at 37°C for 4.5-5 hours for amplification (amplified eluate). The amplified eluates were centrifuged at 4°C and 9,500 g for 30 seconds. The supernatants containing the phage were transferred to fresh tubes and used for purification, sequencing and ELISA.

2.2.4 Purification and Sequencing of Antibody-specific M13 pIII (C7C or Linear 12-mers) Phage Inserts

Five hundred microlitres of phage clones were precipitated by adding 200µl of PEG/NaCl, mixed and incubated at room temperature for 10 minutes. The precipitated phage were centrifuged at 4°C and 9,500 g for 10 minutes. The supernatant was removed and the pellet suspended in 100µl of Iodide buffer (10mM Tris-HCl pH 7.5, 1mM EDTA and 4M NaI) and 250µl of 100% ethanol, and incubated at room temperature for 10 minutes to precipitate single-stranded phage DNA. The precipitated phage DNA was centrifuged at 4°C and 9,500 g for 10

minutes. The supernatant was removed and the pellet washed by adding 350µl of 70% ethanol and dried briefly under vacuum. Then, the pellet were re-suspended in 30µl of TE buffer (Tris-HCl pH 8 and 1mM EDTA), as purified phage.

To perform the sequencing, 4.4µl of the purified product was added to 4µl BigDye Terminator and 1.6µl of 1pM/µl of -96 gIII sequencing primer (5`-CCC TCA TAG TTA GCG TAA CG-3`, 1pmol/µl) and run on the sequencing reaction program. Ten microlitres of sequencing reaction product was purified, dried and prepared for cycle sequencing, as described in section 2.1.4 for T7 phage library.

2.2.5 Detection of Antibody-specific M13 pIII (C7C or Linear 12-mers) Phage Peptide Clones by Capture ELISA

Rabbit anti-fd IgG (Sigma) (diluted 1:100 in coating buffer) was coated onto wells (100µl/we2ll) of maxisorp ELISA plates on a shaker. The plates were incubated at 4°C overnight. Wells were washed 3 times (200µl/well) in 0.1% TBS-T, and blocked with 3% BSA/TBS (120µl/well) at room temperature for one hour. The selected phage, unselected phage or helper M13 phage as a negative control (diluted 1:20 in 1% BSA/TBS-T) were applied (100µl/well) to test wells, while 1% BSA/TBS-T was applied to blank wells, and shaken at room temperature for 2 hours. Following 3 washes, the mouse or human monoclonal antibody or a negative control antibody, (1µg/ml in 1% BSA/TBS-T) was applied (100µl/well) in duplicate to antigen-coated and blank wells and shaken at room temperature for 2 hours. Following 3 washes, sheep anti-mouse IgG (whole molecule) or goat anti-human IgG (Fc specific) alkaline phosphatase conjugate (diluted 1:1000 in 1% BSA/TBS-T) was added to all

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2.3 M13 pVIII (5C4C4) Phage Peptide Library

2.3.1 General Preparations

In this section, standard procedures are described which were employed in the protocols described in detail in the following sections.

2.3.1.1 Minimal/Kanamycin Plate

Five hundred millilitres of 2X M9 salts, 500ml of 3% agar, 20ml of 20% glucose, 2ml of 1M $MgSO_4$, 0.1ml of 1M $CaCl_2$, 1ml of 10mg/ml thiamine were sterilized separately, combined together with 100μg/ml of kanamycin at temperature <70°C and poured onto Petri dishes.

2.3.1.2 E.coli K91BluKan in Minimal/Kanamycin Plate

A minimal/kanamycin Petri dish was inoculated with E.coli K91BluKan strain (Kanamycin-resistant work-horse strain) (New England Biolabs, UK) using a sterile wire loop and incubated at 37°C overnight.

2.3.1.3 E.coli K91BluKan Overnight Culture

A single colony of E.coli K91BluKan was removed from a minmal/kanamycin plate using a sterile wire loop, added to 10mls of LB broth with 100μg/ml of kanamycin in a conical flask and incubated on a shaker at 37°C overnight.

2.3.1.4 Late-log Phase E.coli K91BluKan Culture

Forty micolitres of E.coli K91BluKan overnight culture was added to 4mls of terrific broth and then incubated on a shaker at 37°C for 3-4 hours until the OD_{600}=2 as determined by spectrophotometry, followed by re-incubation on a gentle (slow) shaker at 37°C for 5 minutes to allow sheared F pili to regenerate.

2.3.1.5 Amplified E.coli K91BluKan culture

Two millilitres of the late-log phase E.coli K91BluKan culture was added to 20mls of LB broth/0.2μg/ml of tetracycline, followed by addition of 40μl of 10mg/ml tetracycline

2.3.1.6 Serial Dilution of Non-amplified Eluate

One microlitre of the non-amplified eluate (described in section 2.3.2) was serially diluted by adding 99μl LB broth/0.2μg/ml of tetracycline and then mixed, giving a 10^{-2} dilution. Then, 10μl of 10^{-2} diluted eluate were added to 90μl LB broth/0.2μg/ml of tetracycline and then mixed, giving a 10^{-3} dilution. Then, 100μl of 10^{-3} diluted eluate were added to 90μl LB broth/0.2μg/ml of tetracycline and then mixed, giving a 10^{-4} dilution; and so on.

2.3.1.7 Plating the Diluted Non-amplified Eluate

Ten microlitres of a dilution of non-amplified eluate were added to 90μl of a late-log phase E.coli K91BluKan culture, mixed and incubated at room temperature for 5 minutes (to allow the M13 pVIII phage to infect the E.coli ER 2537), and then mixed

with 500µl of LB broth/0.2µg/ml of tetracycline and incubated on a shaker vigorously at 37°C for 35 minutes. Two hundred microlitres from that mixture were poured onto LB agar Petri dishes containing 40µg/ml of tetracycline and 100µg/ml of kanamycin and incubated at 37°C overnight. The number of phage was corrected by multiplication by 3, because 1/3 volume (200µl) of the mixture (10+90+500=600µl) were poured onto the Petri dishes.

2.3.1.8 Lysate-treated Membrane

One hundred microlitres of M13 pIII wild-type (M13K07) phage were added to 2mls of the late-log phase E.coli K91BluKan culture (OD_{600}=2) in a conical flask and then incubated on a slow shaker at 37°C for 15 minutes. Then, 20mls of LB broth/0.2µg/ml of tetracycline were added and incubated on a shaker vigorously at 37°C for 35 minutes. This was followed by addition of 40µl of 10mg/ml of tetracycline and incubation on a shaker vigorously at 37°C overnight to allow infection of the E.coli by the phage (termed the lysate). The lysate was centrifuged at 4°C and 1,500 g for 20 minutes. The pellet was collected and resuspended in 2mls of TBS. The lysate was poured on a nitro-cellulose membrane and incubated for 30 minutes at room temperature. Then, the lysate-treated membrane was washed with 0.1% TBS-T for 15 minutes then with TBS for 5 minutes and finally blocked with 5% BSA/TBS and incubated on a rotator for one hour at room temperature.

2.3.2 Bio-panning of Antibody with M13 pVIII Phage Peptide Library

Mouse monoclonal antibody (10μg/ml), in 0.05M sodium carbonate/sodium bicarbonate buffer pH 9.6 (coating buffer), was coated onto a Nunc immuno-tube at 4°C overnight on a rotator. The tube was washed 5 times with 50mM Tris-buffered saline (150mM NaCl) pH 7.5 (TBS) containing 0.1% Tween-20 (0.1% TBS-T); each time the tube was incubated at room temperature for 3 minutes with the TBS-T. The tube was then blocked with 0.5% BSA in TBS containing 0.02% NaN_3 (blocking solution) at room temperature for one hour on a rotator. Following 6 washes, 10μl of the M13 pVIII (5C4C4) phage library (1×10^{13} pfu/10μl), which was kindly provided by Dr George Smith (Missouri, USA), in 1ml of 0.1% TBS-T were added and incubated at 4°C for 30 minutes on a rotator. The tube was washed 10 times and 1ml of elution buffer (0.1% BSA in 0.2M glycine-HCl pH 2.2) was added and incubated at room temperature for 10 minutes on a rotator. The non-amplified eluate was removed and neutralised with 150μl of neutralising buffer (1M Tris-HCl buffer pH 9.1).

One microlitre of the non-amplified eluate was serially diluted and plated (as described in sections 2.3.1.6 and 2.3.1.7). While the rest of the phage were added to 2mls of the late-log phase E.coli K91BluKan culture (OD_{600}=2) in a conical flask and then incubated on a slow shaker at 37°C for 15 minutes. Then, 20mls of LB broth/0.2μg/ml of tetracycline were added and incubated on a shaker vigorously at 37°C for 35 minutes, followed by addition of 40μl of 10mg/ml of tetracycline and then incubated on a shaker vigorously at 37°C overnight.

The amplified phage were concentrated and purified (as described in section 2.2.1.8). The amplified eluate was subjected to a second cycle of affinity selection following

the same procedure. Thus, the enriched phage of the preceding round of biopanning were used for the next round. Four or five rounds were carried out in this way for the biopanning of the M13 pVIII (5C4C4) phage peptide library.

2.3.3 Detection of Antibody-specific M13 pVIII (5C4C4) Phage Peptide Clones by Immuno-blotting Assay

The phage of the last round (fourth or fifth round) of biopanning were plated out (as described in section 2.3.1.7) to obtain 50-100 plaques per Petri dish. Some phage clones were selected by using a wire-loop and each of them was mixed with 0.5ml of amplified E.coli K91BluKan culture and incubated on a shaker at 37°C overnight for amplification. The amplified phage of each clone were centrifuged at 4°C and 9,500 g for 30 seconds. The supernatant was transferred to a fresh tube and subjected to imuno-blotting, PCR, sequencing and ELISA.

The immuno-blotting was performed as described in section 2.1.2 for the T7 phage library. The amplified clones were placed onto a millipore nitro-cellulose membrane using sterile tips on a multi-channel pipette and incubated for 5 minutes at room temperature to dry. The membrane was then blocked with 5% BSA/TBS. Ten millilitres of diluted mouse monoclonal antibody, (10μg/ml, diluted in 2.5% BSA /TBS-T) were added to the membrane and incubated on a rotator for 2 hours at room temperature. The membrane was washed with 0.1% TBS-T. Ten millilitres of diluted sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (diluted 1:1000 in 2.5% BSA/TBS-T) were adsorbed with lysate-treated membrane then added to the membrane that had the selected phage clones and antibody bound, and incubated for one hour at room temperature. The membrane was washed, as above,

and BCIP/NBT substrate in de-ionised water with 5mM levamisole was added to the membrane. Following the appearance of the blue spots, the membrane was washed and dried.

2.3.4 PCR and Sequencing of Antibody-specific M13 pVIII (5C4C4) Phage Inserts

The PCR and sequencing of M13 pVIII (5C4C4) phage inserts were performed as described in section 2.1.4 for T7 phage library. 0.5µl of each clone of specific phage was added to 25µl of PCR mixture [5µl of 5× buffer B or C, 0.25µl of 1% tween, 0.5µl of 10mM of dNTPs, 0.5µl of 20µM M13 gene VIII primer (forward) (5'-GTA AAA CGA CGG CCA GT-3'), 0.5µl of 20µM of M13 gene VIII primer (reverse) (5'-GGA AAC AGC TAT GAC CAT G-3'), 1µl of 0.5U/µl of ampliTaq or Hotstar Taq DNA polymerase and 17.5µl of sterile de-ionised water]. The samples were transferred to a Hybaid Omnigene PCR machine, which was preheated to 75°C, and run on a PCR reaction program [(94°C, 15 minutes)×1; (94°C, 45 seconds; 55°C, 45 seconds; 72°C, 1 minute)×25; (72°C, 10 minutes)×1]. One aliquot was left with no phage to be used as a negative control.

Five microlitres of PCR product were used for loading and gel electrophoresis to confirm the appearance of the band of the PCR product at 150 bp length after the PCR reaction.

Finally, 0.5µl of Exonuclease I (10U/µl) and 1µl of SAP (1U/µl) were added to 5µl of PCR product and run on the enzyme treatment program. One microlitre of enzyme

treated PCR product was then added to 4μl BigDye Terminator, 0.15μl of 10μM M13 gene VIII sequencing primer (5'-TTC TTA ATG GAA ACT TCC TC-3') and 5μl of sterile de-ionised water and run on a sequencing reaction program. Ten microlitres of sequencing reaction product was purified, dried and prepared for cycle sequencing, as described in section 2.1.4 for the T7 phage library.

2.3.5 Detection of Antibody-specific M13 pVIII (5C4C4) Phage Peptide Clones by Capture ELISA

This was performed as described in section 2.2.5 for M13 pIII phage library. Rabbit anti-fd IgG (diluted 1:100 in coating buffer) was coated onto wells of maxisorp ELISA plates and then incubated at 4°C overnight. Wells were washed 3 times in 0.1% TBS-T, and blocked with 3% BSA/TBS. The selected phage, unselected phage or helper M13 phage as a negative control (diluted 1:20 in 1% BSA/TBS-T) was applied (100μl/well) to test wells, while 1% BSA/TBS-T was applied to blank wells, and shaken at room temperature for 2 hours. Following 3 washes, the mouse monoclonal antibody or a negative control antibody, (1μg/ml in 1% BSA/TBS-T) was applied to antigen-coated and blank wells and shaken at room temperature for 2 hours. Following 3 washes, sheep anti-mouse IgG (whole molecule) IgG alkaline phosphatase conjugate (diluted 1:1000 in 1% BSA/TBS-T) was added to all wells and shaken at room temperature for one hour. Wells were washed 3 times and pNPP substrate was added to all wells and incubated at room temperature for one hour. Plates were read after 60 minutes at OD 405 nm on a microtitre plate reader. The mean OD of the antigen-coated wells was corrected by subtracting the mean OD of the equivalent blank wells.

CHAPTER THREE

MOUSE MONOCLONAL ANTIBODIES

3 Mouse Monoclonal Antibodies

3.1 N-terminal and C-terminal Specific Mouse Monoclonal Antibodies

3.1.1 Introduction

Amino-terminal specific mouse monoclonal antibody (N-terminal MoAb), GC 3208 (clone 11), is a mouse IgG1 monoclonal antibody. This N-terminal MoAb reacts weakly with GAD65 on blots, and also reacts with native GAD65.

A non-obese diabetic mouse was immunized with complete Freund's adjuvant at day 6 and a single dose of pancreatic islet β-cell toxin streptozotocin at day 3 before the fusion was performed, to trigger an immune response against the animal's own GAD released from the damaged islet β cells. Lymphoctes from the spleen of the immunized mouse were fused with mouse myeloma cells. The fusion mixture was seeded in 96-well culture plate containing mouse peritoneal cells prepared from a BALB/c mouse one day before the fusion (Ziegler et al., 1994).

Epitope mapping has shown that the N-terminal MoAb recognizes a linear epitope at the N-terminus of rat GAD-65. Full abolition of N-terminal MoAb activity, as determined by Western blotting on whole rat brain lysate, has been achieved using the N-terminal peptide [rat/human GAD-65 (4-17) amino acid (aa) residues]. The MoAb was purchased from Affiniti Research Products (ARP), UK. Interestingly, non-diabetic SMS serum (DH) can inhibit the binding of the N-terminal monoclonal

antibody, in a competition ELISA (data not shown). This indicates that GAAs of some SMS sera recognize epitopes within the extreme N-terminus of GAD-65.

Carboxy-terminal mouse monoclonal antibody (C-terminal MoAb), GC 3108 (clone 111), is a mouse IgG1 monoclonal antibody (Ziegler et al., 1996). This C-terminal MoAb reacts strongly on blots, but may not react with native GAD65.

BALB/c mouse was immunized with full-length human recombinant GAD-65 (obtained from a baculovirus expression system), which was emulsified in incomplete Freund's adjuvant, intraperitoneally (i.p) and subcutaneously (s.c) at day 0 and also after 2 months. Three days before the fusion experiment, the mouse was immunized with GAD-65, which was dissolved in saline only, as one s.c and i.p injection. Lymphoctes from the spleen of the immunized mouse were fused with mouse myeloma cell line SP2/0 (Ziegler et al., 1996).

Full abolition of C-terminal MoAb activity, as determined by Western blotting on whole rat brain lysate, has been achieved using the C-terminal peptide [rat/human GAD-65 (572-585) aa residues]. The MoAb was purchased from Affiniti Research Products (APR), UK.

The N-terminal and C-terminal MoAb, whose epitopic regions on GAD-65 were already closely defined (4-17 and 572-585 aa on GAD65, respectively), were screened with different phage peptide libraries to act as controls for the screening of other monoclonal antibodies (GAD-6, 76B, 76F and b96.11) for which the specific amino acid residues of their epitopes were less closely defined. Thus, it was hoped

that screening these two mouse MoAbs (N-terminal and C-terminal MoAbs) with different types of phage peptide libraries (M13 pIII, M13 pVIII and/or T7 pX) might help to indicate the usefulness of these different libraries for screening other mouse or human MoAbs to determine their epitopes on GAD65 or on other protein(s).

3.1.2 Results

3.1.2.1 M13 pIII (linear 12-mers) Phage Peptide Library

3.1.2.1.1 Sequences of Phagotopes Isolated by Bio-panning of M13 pIII Phage Peptide Library (linear 12-mers) and Immuno-blotting Assay with N-terminal or C-terminal Specific Mouse Monoclonal Antibodies.

M13 pIII phage library (linear 12-mers) was screened by 3 rounds of immunopanning against N-terminal or C-terminal MoAb as described in section 2.2.2. The number of plaques obtained in each round during plating increased consecutively, indicating that the biopanning rounds were successful (Table 3.1).

The specific plaques of the third round were selected by blotting assay as described in section 2.2.3. Sheep anti-mouse (whole molecule) alkaline phosphatase conjugate was used to detect the binding of the N-terminal or C-terminal MoAb to the phage clones. About 20% or 30% of these plaques (clones) showed high affinity, i.e. showed blue spots on the nitrocellulose membranes, to the N-terminal or C-terminal MoAb, respectively.

Thirteen and fifteen clones specific to the N-terminal or C-terminal MoAb, respectively, were sequenced successfully as described in section 2.2.4. All sequences have been aligned with the relevant portions of GAD-65 (Fig. 3.1 and 3.2).

Eight out of these 13 clones (10,13,18,22,25,26,28 and 30), which were specific to the N-terminal MoAb, showed a motif of proline followed by glycine followed by 3 amino acids followed by tryptophan followed by serine. Also, 4/13 clones (1,24,27 and 29) showed a motif of proline followed by 4 amino acids followed by tryptophan followed by serine followed by phenylalanine. Furthermore, 1/13 clones (21) showed a motif of proline followed by glycine followed by serine. Thus, the main motif of N-terminal mouse monoclonal antibody is P-G-X-X-X-W-S-F, corresponding to amino acid residues 4-11 of GAD-65 (P-G-S-G-F-W-S-F) (Fig. 3.1).

The 15 clones which were specific to the C-terminal MoAb, which were sequenced successfully, showed a motif of Phenylalanine (11/15 clones) or tryptophan (1/15 clones) followed by leucine (14/15 clones) or valine (1/15 clones) followed by isoleucine (11/15 clones) followed by an amino acid followed by glutamate (14/15 clones) followed by valine (11/15 clones), isoleucine (1/15 clones) or leucine (1/15 clones) followed by aspartate (14/15 clones) followed by an amino acid followed by leucine (12/15 clones). Thus, the main motif of C-terminal MoAb is F-L-I-X-E-I/V/L-D-X-L, corresponding to amino acid residues 573-581 of GAD-65 (F-L-I-E-E-I-E-R-L), which showed conservative substitutions. Isoleucine, valine and leucine have aliphatic side chains and thus are similar residues. Aspartate and glutamate have acidic side chains and thus are similar residues (Fig. 3.2).

3.1.2.1.2 Testing of N-terminal or C-terminal Specific Mouse Monoclonal Antibody-specific Phagotopes of M13 pIII Phage Peptide Library (linear 12-mers) by Capture ELISA

Sequenced phagotopes of M13 pIII (linear 12-mers) phage library were selected for investigation of their binding to N-terminal or C-terminal MoAb by capture ELISA as described in section 2.2.5. Mouse IgG1 kappa myeloma protein was used as a negative control antibody (Sigma) (1µg/ml in 1% BSA/TBS-T). Sheep anti-mouse (whole molecule) alkaline phosphatase conjugate was used to detect the binding of the N-terminal or C-terminal MoAb or negative control antibody (mouse IgG1 kappa) to the phage clones. Wild-type M13K07 phage was used as a negative control phage.

Significant binding with the N-terminal or C-terminal MoAb, respectively, was shown by 12 out of 13 clones (not clone number 1) selected with the N-terminal MoAb, and 16 out of 18 clones (not clone numbers 1 and 12) selected with the C-terminal MoAb, compared to the negative controls (mouse IgG1 kappa and M13K07 phage) (Fig. 3.3 and 3.4, respectively).

Table 3.1 Number of Plaques Obtained in Each Round of Biopanning with Different Phage Peptide Libraries

Antibody	Phage Peptide Library	No. of Plaques per ml in the First Round	No. of Plaques per ml in the Second Round	No. of Plaques per ml in the Third Round	No. of Plaques per ml in the Fourth Round	No. of Plaques per ml in the Fifth round
N-terminal MoAb	•T7 pX linear 9-mers.	2×10^5	3×10^6	7×10^7	4×10^8	——
	•M13 pIII linear 12-mers.	2×10^4	3×10^6	6×10^8	——	——
	•M13 pVIII 5C4C4.	6×10^3	2×10^5	4×10^6	7×10^8	——
C-terminal MoAb	•M13 pIII linear 12-mers.	3×10^5	1×10^7	2×10^9	——	——
GAD-6 MoAb	•T7 pX C9C.	7×10^4	2×10^6	2×10^7	9×10^7	——
	•T7 pX linear 9-mers.	4×10^4	5×10^6	1×10^7	2×10^8	——
	•M13 pIII linear 12-mers.	4×10^4	3×10^6	3×10^9	——	——
	•M13 pIII C7C.	3×10^4	1×10^6	4×10^9	——	——
	•M13 pVIII 5C4C4.	2×10^2	4×10^3	2×10^5	5×10^6	2×10^8
76B MoAb	•T7 pX C9C.	3×10^6	3×10^9	4×10^9	2×10^7	——
	•M13 pIII linear 12-mers.	1×10^4	1×10^6	6×10^8	——	——
76F MoAb	•T7 pX C9C.	2×10^6	2×10^9	1×10^9	1×10^7	——
	•M13 pIII linear 12-mers.	5×10^5	1×10^8	5×10^9	——	——
b96.11 huAb	•M13 pIII linear 12-mers.	1×10^5	5×10^6	4×10^8	——	——
SMS patient' sera.	•M13 pIII linear 12-mers.	3×10^4 (JO)	2×10^3 (PT)	5×10^2 (DH) *	1×10^6 (LB)	8×10^2 (PM)

* Indicates that the eluate of only the third round of biopanning with SMS sera was amplified.

Figure 3.1 Sequences of Peptides Selected from the Filamentous M13 pIII (linear 12-mers) Phage Peptide Library by Binding to N-terminal Specific Mouse Monoclonal Antibody.

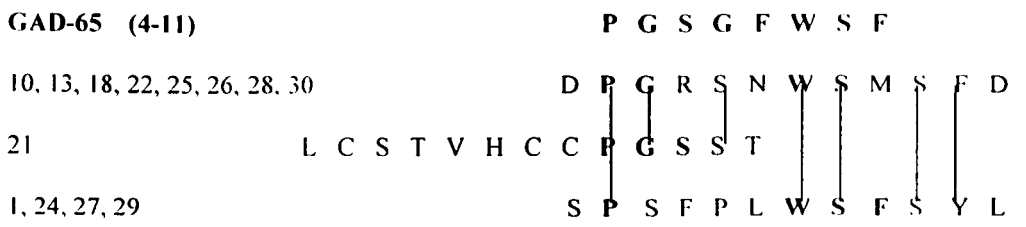


Figure 3.2 Sequences of Peptides Selected from the Filamentous M13 pIII (linear 12-mers) Phage Peptide Library by Binding to C-terminal Specific Mouse Monoclonal Antibody.

Figure 3.3 Capture ELISA: Binding of N-terminal Specific Mouse Monoclonal Antibody to M13 pIII (linear 12-mers) Phage Clones Bound to ELISA Maxisorp Plates via Rabbit anti-fd IgG.

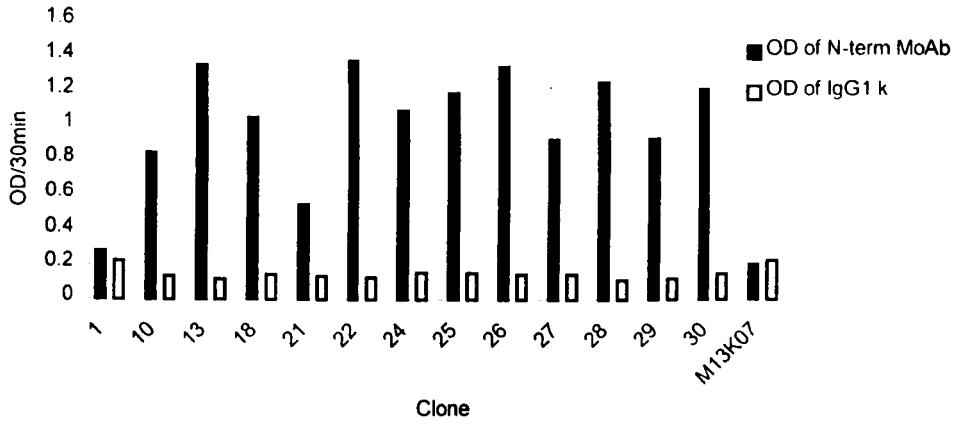
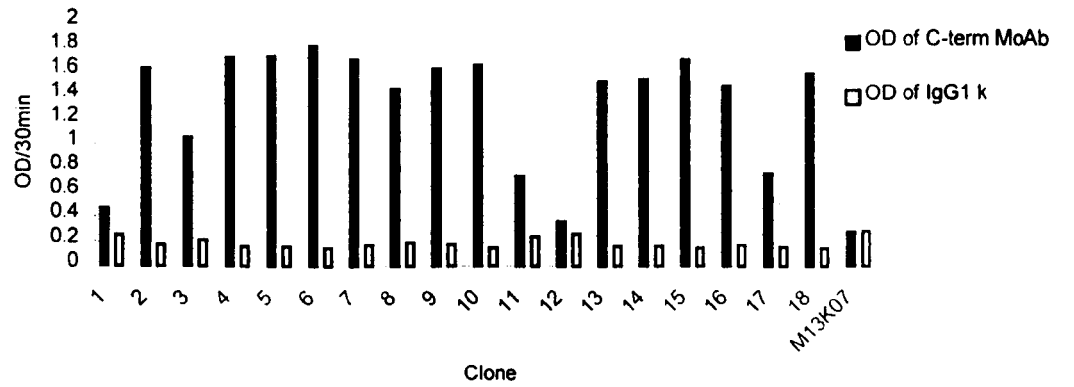


Figure 3.4 Capture ELISA: Binding of C-terminal Specific Mouse Monoclonal Antibody to M13 pIII (linear 12-mers) Phage Clones Bound to ELISA Maxisorp Plates via Rabbit anti-fd IgG.



3.1.2.2 T7 (linear 9-mers) Phage Peptide Library

3.1.2.2.1 Sequences of Phagotopes Isolated by Bio-panning of T7 Phage Peptide Library (linear 9-mers) and Immuno-blotting Assay with N-terminal Specific Mouse Monoclonal Antibody

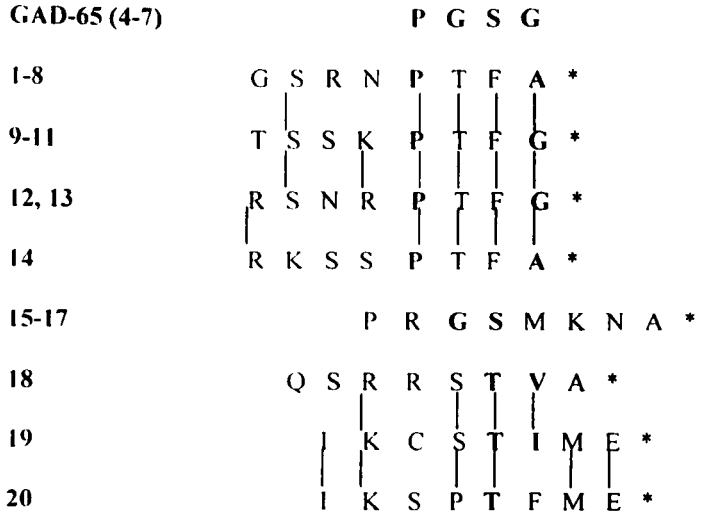
T7 phage library (linear 9-mers) was screened by 4 rounds of immunopanning against N-terminal MoAb as described in section 2.1.2. The number of plaques obtained in each round during plating increased consecutively, indicating that the biopanning rounds were successful (Table 3.1).

The specific plaques from the fourth round of immunopanning the T7 phage library (linear 9-mers) with the N-terminal MoAb were selected by blotting assay as described in section 2.1.3. About 70% of these plaques (clones) showed high affinity to the N-terminal MoAb, giving blue spots on the nitrocellulose membranes.

Twenty clones specific to the N-terminal MoAb were sequenced successfully (Fig. 3.5). Stop codons were found in all the clones at the end of the inserted portions. All sequences have been aligned with the relevant portions of GAD-65. Fourteen out of these 20 clones (1-14) showed a motif of proline followed by 2 amino acids followed by either glycine (5/14) or alanine (9/14). Thus, the main motif of the N-terminal MoAb is P-X-X-G, corresponding to amino acid residues 4-7 of GAD-65 (P-G-S-G), which overlaps with P-G-S-G-F-W-S-F motif (4-11 amino acid residues). However, this motif showed conservative substitutions. Glycine and alanine have aliphatic side chains and thus are similar residues. Also, three out of these 20 clones (15-17) showed a motif of glycine followed by serine corresponding to amino acid residues 5-6 of GAD-65 (G-S). In addition, two out of these 20 clones (18 and 19) showed a

motif of threonine followed by valine or isoleucine corresponding to amino acid residues 6-7 of GAD-65 (S-G), which showed conservative substitutions. Glycine, valine and isoleucine have aliphatic side chains and thus are similar residues. Also, serine and threonine have aliphatic hydroxyl side chains and thus are similar residues (Fig. 3.5).

Figure 3.5 Sequences of Peptides Selected from the T7 (linear 9-mers) Phage Peptide Library by Binding to N-terminal Specific Mouse Monoclonal Antibody.



3.1.2.3 M13 pVIII (5C4C4) Phage Peptide Library

3.1.2.3.1 Sequences of Phagotopes Isolated by Bio-panning of M13 pVIII Phage Peptide Library (5C4C4) and Immuno-blotting Assay with N-terminal Specific Mouse Monoclonal Antibody

M13 pVIII phage library was screened by 4 rounds of immunopanning against N-terminal MoAb as described in section 2.3.2. The number of plaques obtained in each round during plating increased consecutively, indicating that the biopanning rounds were successful (Table 3.1).

The plaques specific for N-terminal MoAb from the fourth round of M13 pVIII phage screening were selected by blotting assay as described in section 2.3.3. About 40% of these plaques (clones) showed high affinity to the N-terminal MoAb, giving blue spots on the nitrocellulose membranes.

Fourteen clones of M13 pVIII phage library were sequenced successfully (Fig. 3.6). All the clones showed a motif of serine followed by threonine followed by either proline (12/14) or valine (2/14). Thus, the motif obtained was S-T-P, which does not correspond to amino acid residues 4-17 of GAD-65 and does not overlap with the previous motif of the N-terminal MoAb, i.e. P-G-S-G-F-W-S-F (4-10 amino acids).

3.1.2.3.2 Testing of N-terminal Specific Mouse Monoclonal Antibody-specific Phagotopes of M13 pVIII Phage Peptide Library (5C4C4) by Capture ELISA

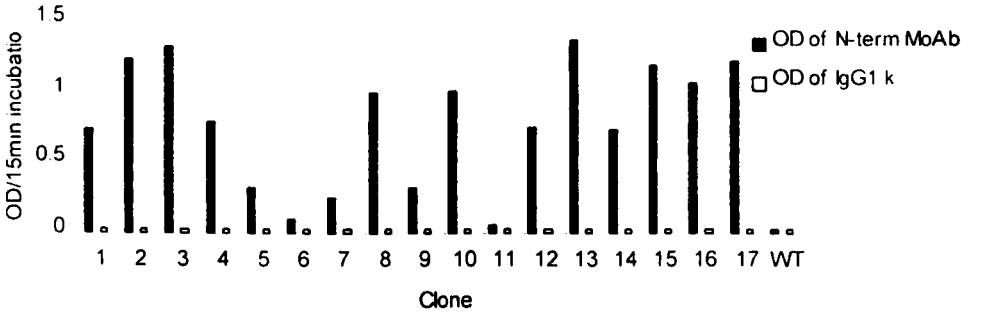
Sequenced phagotopes of M13 pVIII phage library were selected for investigation of their binding to the N-terminal mouse monoclonal antibody by capture ELISA as

described in section 2.3.5. Mouse IgG1 kappa myeloma protein was used as the negative control antibody (1μg/ml in 1% BSA/TBS-T). All the fourteen clones showed strong binding with the N-terminal mouse monoclonal antibody, compared to the negative controls (mouse IgG1 kappa and WT phage) (Fig. 3.7).

Figure 3.6 Sequences of Peptides Selected from the Filamentous M13 pVIII (5C4C4) Phage Peptide Library by Binding to N-terminal Specific Mouse Monoclonal Antibody.

1	A	I	N	T	I	C	S	T	P	L	C	W	N	E	A
2	A	T	N	R	P	C	S	T	P	M	C	M	G	S	Y
3	A	S	H	D	S	C	S	T	P	M	C	S	T	P	R
4	T	T	R	E	I	C	S	T	P	F	C	T	T	N	V
5	A	M	N	P	Y	C	S	T	P	L	C	G	M	I	Q
6	A	D	N	P	F	C	S	T	V	H	C	S	T	T	A
7	A	N	S	P	M	C	S	T	P	N	C	M	Y	G	T
8, 9	A	N	Q	K	Q	C	S	T	P	S	C	H	N	Y	S
10	T	N	R	D	R	C	S	T	P	T	C	N	G	Q	S
11	A	P	P	M	A	C	S	T	V	H	C	G	I	M	T
12	A	T	K	T	I	C	S	T	P	V	C	N	N	W	V
13	A	D	S	V	A	C	S	T	P	N	C	P	Q	T	G
14	A	S	E	M	K	C	S	T	P	F	C	G	S	L	S

Figure 3.7 Capture ELISA: Binding of N-terminal Specific Mouse Monoclonal Antibody to M13 pVIII (5C4C4) Phage Clones Bound to ELISA Maxisorp Plates via Rabbit anti-fd IgG.



3.1.3 Discussion

Thirteen clones, which were obtained from the third round of successful biopanning of the M13 pIII (linear 12-mers) phage library and were positively immunostained in the blotting assay (high affinity) with the N-terminal MoAb, showed a motif of P-G-X-X-X-W-S-F (Fig. 3.1). This corresponds to amino acid residues 4-11 of GAD-65 (P-G-S-G-F-W-S-F), as the epitopic determinant of the N-terminal MoAb.

All these 13 clones specific to the N-terminal MoAb obtained from the M13 pIII (linear 12-mers) phage library were screened in ELISA. Twelve of these clones showed significant binding with the N-terminal MoAb (Fig. 3.3).

Twenty clones, which were obtained from the fourth round of successful biopanning of the T7 linear 9-mers phage library and were positively immunostained in the blotting assay (high affinity) with the N-terminal MoAb, showed a motif of P-X-X-G, corresponding to amino acid residues 4-7 of GAD-65 (P-G-S-G), which overlaps with P-G-S-G-F-W-S-F motif (4-10 amino acid residues). However, this motif showed conservative substitutions. Glycine and alanine have aliphatic side chains and thus are similar residues. Also, three out of these 20 clones (15-17) showed a motif of glycine followed by serine corresponding to amino acid residues 5-6 of GAD-65 (G-S). In addition, two out of these 20 clones (18 and 19) showed a motif of threonine followed by valine or isoleucine corresponding to amino acid residues 6-7 of GAD-65 (S-G), which showed conservative substitutions. Glycine, valine and isoleucine have aliphatic side chains and thus are similar residues. Also, serine and threonine have aliphatic hydroxyl side chains and thus are similar residues. Only one clone did not show a significant motif (Fig. 3.5)

Stop codons were present in T7 phage peptide library (linear 9-mers), at the C-terminal region of the insert. It is possible that the full length of the insert expressed in some clones, but the MoAb only binds when the insert is present at the extreme C-terminus of the surface coat protein. Another possibility is that the insert within the surface coat protein is unstable and not expressed, except at the extreme C-terminus.

Fourteen clones, which were obtained from the fourth round of successful biopanning of the M13 pVIII (5C4C4) phage library, and were positively immunostained in the blotting assay (high affinity) with the N-terminal MoAb, showed a motif of S-T-P (Fig. 3.6). This does not correspond to amino acid residues 4-17 of GAD-65 and does not overlap with the motif of the N-terminal MoAb, i.e. P-G-S-G-F-W-S-F (4-10 amino acid residues).

Sequenced phagotopes of M13 pVIII phage library were selected for investigation of their binding to the N-terminal MoAb by capture ELISA. All the 14 clones showed strong binding with the N-terminal MoAb, compared to the negative controls (mouse IgG1 kappa and WT phage) (Fig. 3.7).

Thus, the overall motif of the N-terminal MoAb screened with these three different phage peptide libraries (M13 pVIII, M13 pIII and T7 pX) is P-G-S-G-X-W-S-F, corresponding to amino acid residues 4-11 of GAD-65 (P-G-S-G-F-W-S-F). This indicates that the epitope, which is recognized by the N-terminal MoAb, is probably not a helix in GAD-65 due to the recognition of most of the amino acid residues of the region. Also, this indicates that the phenylalanine, which is in

the middle of that epitope and not present in the phage peptides recognized by the N-terminal MoAb, may be buried in the GAD-65 structure.

The sequences of the peptides selected by using the T7 pX (linear 9-mers) and M13 pIII (linear 12-mers) phage peptide libraries showed some similarity possibly due to similarity in expressing *linear* displayed peptides (non-constrained), although they expressed their displayed peptides at the C-terminus of gene X and N-terminus of gene III, respectively. The differences in the sequences of the peptides selected by using the M13 pVIII (5C4C4) and T7 pX (linear 9-mers) phage peptide libraries, may be due to the differences in their capability to express their displayed peptides either at the N-terminus of gene VIII and at the C-terminus of gene X, respectively, and/or due to the exposed peptides being constrained (cystine-4-cystine loop, conformational epitope) and non-constrained (linear epitope), respectively, although they express a similar number of copies of inserted peptides on the phage surface. This highlights how different peptide libraries screened with the same antibody can give very different results, which may or may not correspond to the epitope in the protein to which the antibody is specific.

Interestingly, SMS serum (DH) can inhibit the binding of the N-terminal MoAb, in a competition ELISA (data not shown). This indicates that GAAs of some SMS sera recognize epitopes within the extreme N-terminus of GAD-65. This consistent with other studies on the epitope specificities of anti-GAD antibodies in SMS patients at the N-terminal region of GAD-65 (Bjork et al., 1994; Hagopain et al., 1995; Kim et al., 1994). Also, 3 out of 30 sera (i.e. 10%) from type 1 DM patients specifically displaced the binding of the N-terminal MoAb from GAD-65 (Ziegler et al., 1994).

In addition, anti-GAD antibodies of a type 1 DM patient's serum are directed against residues 5-9 aa of GAD-65 (Rharbaoui et al., 1998), which overlaps with the predicted overall epitope of the N-terminal MoAb (4-11 aa). Therefore, the N-terminal region carries a continuous antigenic determinant recognized by the sera from type 1 DM patients as well as the sera from the SMS patients.

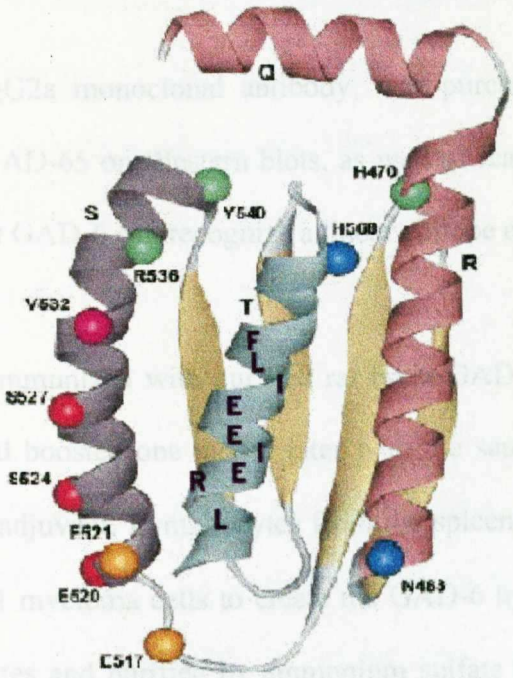
Fifteen clones, which were obtained from the third round of successful biopanning of the M13 pIII (linear 12-mers) phage library and were positively immunostained in the blotting assay (high affinity) with the C-terminal MoAb, showed a motif of F-L-I-X-E-I/V/L-D-X-L (Fig. 3.2), corresponding to 573-581 aa of GAD-65 (F-L-I-E-E-I-E-R-L), as the epitopic determinant of the C-terminal MoAb.

All these 15 clones specific to the C-terminal MoAb obtained from the M13 pIII (linear 12-mers) phage library were screened in ELISA. All of these 15 clones show significant binding with the C-terminal MoAb, comparing to all negative controls (mouse IgG1 kappa and M13K07) (Fig. 3.4).

The motif of the C-terminal MoAb, 573-581 aa, spans most of α-helix T (568-582 aa) in the three-dimensional model of the C-terminal region of GAD-65 (Schwartz et al., 1999) (Fig. 3.8). The C-terminal MoAb could clearly not interact simultaneously with all these amino acid residues as an α-helical structure, suggesting that the helix must be denatured for the MoAb to bind. Indeed, other studies in our laboratory indicate that the C-terminal MoAb binds only to denatured, and not native, GAD. It may be relevant that the C-terminal MoAb was derived from a mouse injected with GAD

emulsified in incomplete Freund's adjuvant, which will have a denaturing effect on the antigen.

Figure 3.8 The Three-dimensional Model of the C-terminal Region of GAD-65 shows that the motif of the C-terminal Specific Monoclonal Antibody, 573-581, spans most of α-helix T (Schwartz et al., 1999).



3.2 GAD-6

3.2.1 Introduction

GAD-6, a murine IgG2a monoclonal antibody, was purchased from Roche, UK. GAD-6 reacts with GAD-65 on Western blots, as well as reacting with native GAD-65. This indicates that GAD-6 can recognize a linear epitope on GAD-65.

BALB/c mice were immunized with purified rat brain GAD emulsified in complete Frends's adjuvant and boosted one month later with the same protein emulsified in incomplete Freund's adjuvant. Lymphocytes from the spleen of the immunized mice were fused with NS-1 myeloma cells to create the GAD-6 hybridoma clone. GAD-6 was produced in ascites and purified by ammonium sulfate precipitation and anion-exchange chromatography (Chang and Gottlieb 1988).

GAD-6 recognizes a specific epitope on GAD65 but not on GAD67, within amino acid residues (aa) 475-585 (SMS E1) (Butler et al., 1993). The GAAs of type 1 DM, SMS and APS II (diabetic and non-diabetic) sera recognize the region of the GAD-6 epitope on GAD-65. In addition, the extreme C-terminal sixteen amino acid residues of GAD65 do not form the epitopes of GAD65-specific GAAs of SMS and APS II sera, nor the epitope of GAD-6 since these 16 aa are identical with GAD67.

Other work from this laboratory has indicated that ICA+ non-diabetic APS II patients' sera vary in the proportion of GAAs that inhibit GAD-6 binding to GAD. There are no significant differences in levels of GAAs which inhibit GAD-6 binding to GAD between diabetic APS II patients with abrupt onset and slow onset type 1 DM

(Davenport et al., 1998). In addition, site-directed mutagenesis of the amino acid residue at position 550 of GAD65 does not inhibit the binding of GAD-6 to GAD65 (Davenport et al., 1998), indicating that this amino acid residue is not involved in the GAD-6 epitope. Furthermore, GAD67 and GAD65 differ particularly in the 515-527 amino acid residue region (Daw and Powers, 1995), indicating that this region might be recognized by GAD-6 which recognizes an epitope on GAD65 only. Also, this region might be recognized by GAAs of type 1 DM sera which recognize epitopes mainly on GAD65.

The different phage peptide libraries (T7 pX linear 9-mers, M13 pIII linear 12-mers and M13 pVIII 5C4C4) which had been screened with the N-terminal and C-terminal MoAb were also screened with GAD-6. In addition, two other libraries (T7 pX C9C and M13 pIII C7C) were screened with GAD-6.

3.2.2 Results

3.2.2.1 T7 (C9C or linear 9-mers) Phage Peptide Libraries

3.2.2.1.1 Sequences of Phagotopes Isolated by Bio-panning of T7 Phage Peptide Libraries (C9C or linear 9-mers) and Immuno-blotting Assay with GAD-6

T7 phage library, either C9C or linear 9-mers, was screened by 4 rounds of immunopanning against GAD-6 as described in section 2.1.2. The number of plaques obtained in each round during plating increased consecutively, indicating that the biopanning rounds were successful (Table 3.1).

The plaques specific to GAD-6 from the fourth round of screening the T7 phage library, either C9C or linear 9-mers, were selected by immuno-blotting assay as described in section 2.1.3. About 12% of these plaques (clones) showed high affinity to GAD-6, giving blue spots on the nitrocellulose membranes.

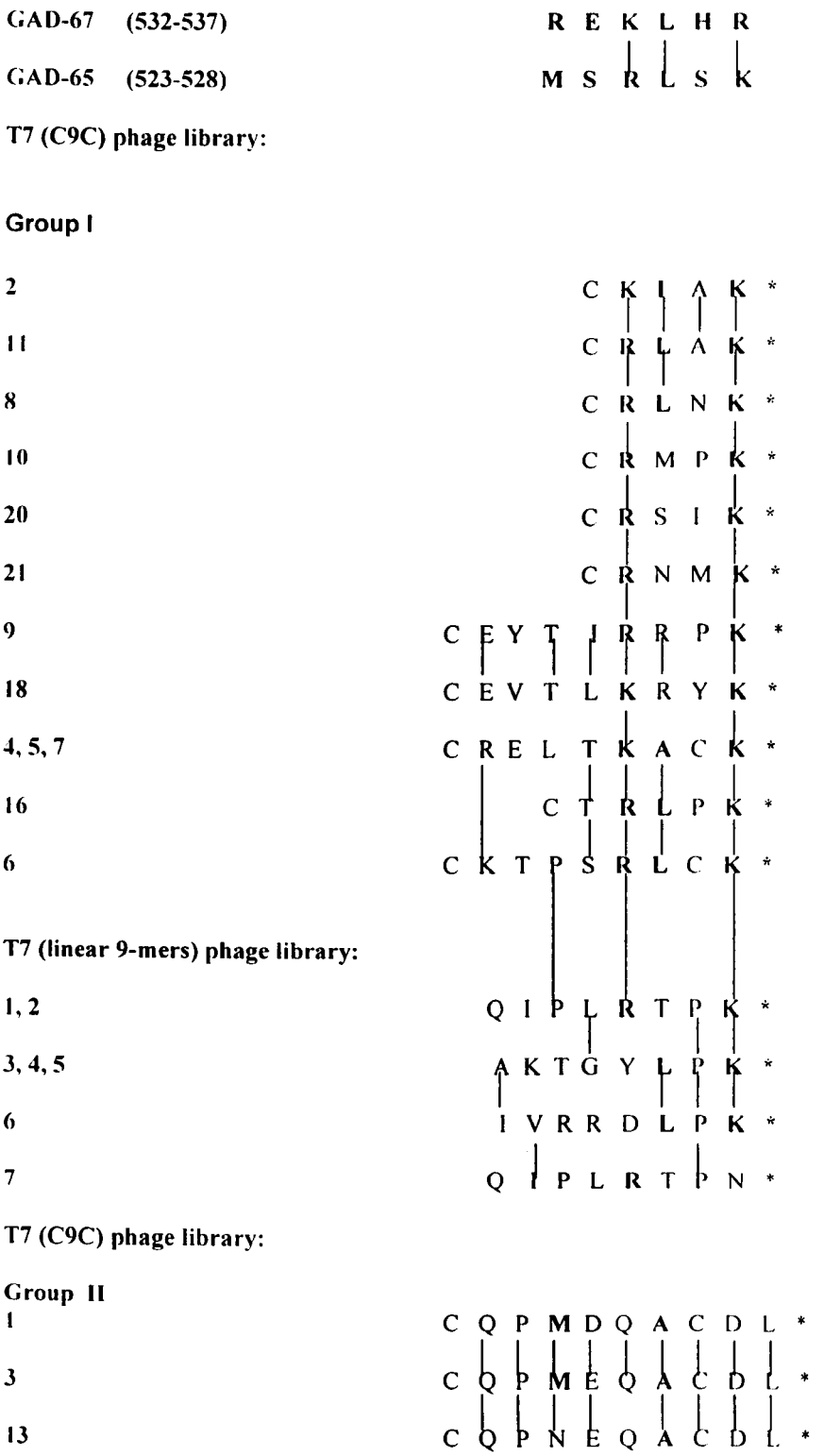
Sixteen clones of T7 (C9C) phage library and 7 clones of T7 (linear 9-mers) phage library were sequenced successfully. Stop codons were found in all clones, either in the middle of the inserted portions (in 7 out of the 16 clones of the T7 C9C phage library), or at the end of the inserted portions (in 9/16 clones of the T7 C9C phage library and in all the 7 clones of T7 linear 9-mers phage library) (Fig. 3.9).

All sequences have been aligned with the relevant portions of GAD-65, indicating sequence homologies in terms of identical residues and conservative substitutions (i.e. amino acids with biochemically similar side chains) (Fig. 3.9). In T7 (C9C)

phage library, 13/16 clones (2,4,5,6,7,8,9,10,11,16,18,20 and 21) showed a motif of either arginine (8/13) or lysine (5/13) followed by either leucine (4/13), alanine (3/13) or isoleucine (1/13) followed by an amino acid followed by lysine. Thus, the most significant motif for GAD-6 is R(or K)-L(A,I or X)-X-K. Thus, 4 out of these 13 clones (6,8,11 and 16) showed a motif of R-L-X-K, showing strong homology with amino acid residues 525-528 of GAD-65. Arginine and lysine both have basic side chains and thus are similar residues. Leucine, alanine and isoleucine have aliphatic side chains and thus are similar residues. Also, 2 clones (1 and 3) showed a motif of methionine followed by two amino acids followed by alanine. Thus, the second group of sequences showed another motif which is M-X-X-A. This shows homology to amino acid residues 523-526 (M-X-X-L) of GAD-65 which overlaps with the R-L-X-K sequence (525-528 aa) (Fig. 3.9).

Amongst the clones selected from the T7 (linear 9-mers) phage library, 2/7 clones (1 and 2) showed a motif of arginine followed by 2 amino acids followed by lysine. Thus, the most significant motif of GAD-6 is R-X-X-K, showing homology to amino acid residues 525-528 of GAD-65. Also, 4/7 clones (3,4,5 and 6) showed a motif of leucine followed by an amino acid followed by lysine (L-X-K). This shows homology to amino acid residues 526-528 of GAD-65 which overlaps with the R-L-X-K motif (525-528 aa). Clone 7 did not show a significant motif (Fig. 3.9).

Figure 3.9 Sequences of Peptides Selected from the T7 Phage Peptide Libraries by binding to GAD-6.



* Indicates stop codon.

3.2.2.1.2 Testing of GAD-6-specific Phagotopes of T7 (C9C) Phage Peptide Library by Direct and Capture ELISA

Sequenced phagotopes of T7 (C9C) phage library were selected for investigation of their binding to GAD-6 by direct ELISA. Six out of the 22 clones were screened in ELISA by using maxisorp plates as described in section 2.1.5.1. Five out of these 6 clones (1,2,3,4 and 6) showed binding with GAD-6, compared to the negative controls [mouse anti-KLH IgG2a (1μg/ml in 1% BSA/TBS-T) (Sigma), wild-type (WT) phage or unselected (US) phage] (Fig. 3.10).

All the 22 clones were screened in ELISA using maleic anhydride activated polystyrene plates as described section 2.1.5.2. All of these 22 clones showed binding with the GAD-6, compared to the mouse IgG2a kappa (negative control). However, only about eleven out of these 22 clones (1, 3-10, 12 and 15) showed binding with the GAD-6 that was higher than the binding of GAD-6 to WT phage (Fig. 3.11).

Figure 3.10 Direct binding of GAD-6 to T7 (C9C) phage clones coated on ELISA maxisorp plates.

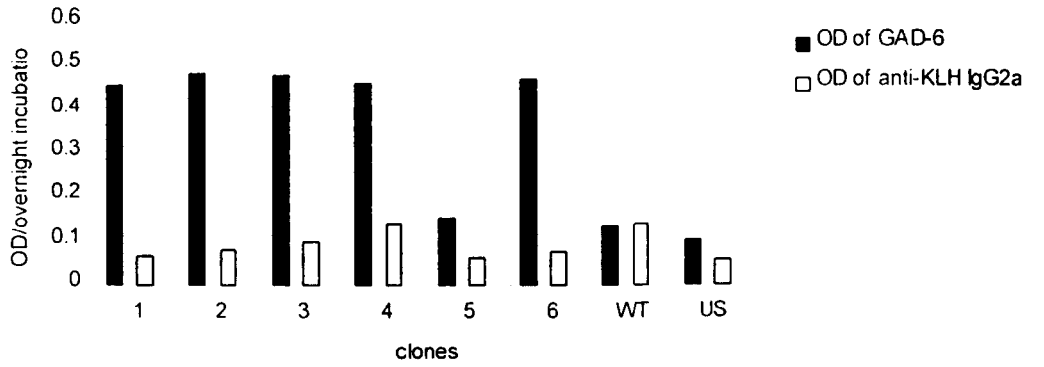
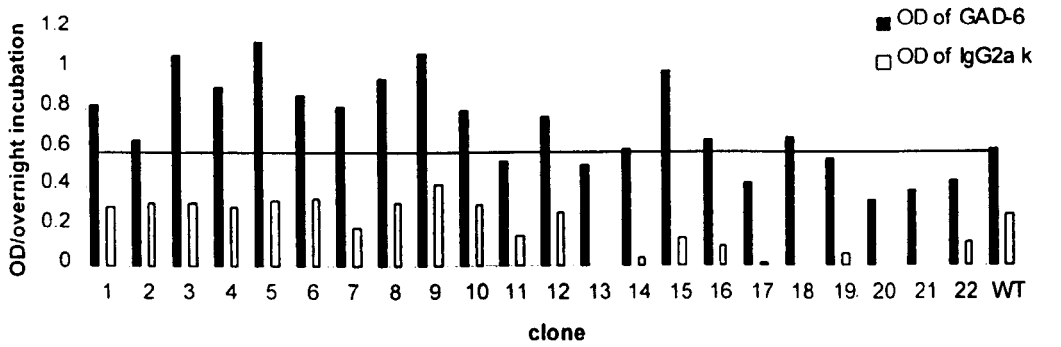


Figure 3.11 Direct binding of GAD-6 to T7 (C9C) phage clones coated on ELISA maleic anhydride activated polystyrene plates.



3.2.2.2 *M13 pIII (linear 12-mers or C7C) Phage Peptide Libraries*

3.2.2.2.1 Sequences of Phagotopes Isolated by Bio-panning of M13 pIII Phage Peptide Library (linear 12-mers or C7C) and Immuno-blotting Assay with GAD-6

M13 pIII phage libraries, linear 12-mers and C7C, were screened by 3 rounds of immunopanning against GAD-6 as described in section 2.2.2. The number of plaques obtained in each round during plating increased consecutively, indicating that the biopanning rounds are successful (Table 3.1).

The plaques from the third round of M13 pIII phage library screenings were selected by immuno-blotting assay as described in section 2.2.3. Sheep anti-mouse (whole molecule) alkaline phosphatase conjugate was used to detect the binding of the GAD-6 to the specific clones. No plaques showed high affinity to the GAD-6 (i.e. only very weak blue spots were seen on the nitrocellulose membranes). The clones which were sequenced and compared with one another did not show a clear motif and it was impossible to align them (data not shown).

3.2.2.2.2 Testing of GAD-6-specific Phagotopes of M13 pIII Phage Peptide Library (linear 12-mers or C7C) by Capture ELISA

Sequenced phagotopes of M13 pIII (either linear 12-mers or C7C) phage libraries were selected for investigation of their binding to GAD-6 by capture ELISA as described in section 2.2.5 using mouse IgG2a kappa myeloma protein (1μg/ml in 1% BSA/TBS-T) as a negative control, and sheep anti-mouse (whole molecule) alkaline

phosphatase conjugate. None of the clones showed binding with GAD-6, compared to the negative controls (mouse IgG2a kappa or WT phage) (data not shown).

3.2.2.3 M13 pVIII (5C4C4) Phage Peptide Library

3.2.2.3.1 Sequences of Phagotopes Isolated by Bio-panning of M13 pVIII Phage Peptide Library (5C4C4) and Immuno-blotting Assay with GAD-6

M13 pVIII phage library was screened by 5 rounds of immunopanning against GAD-6 as described in section 2.3.2. The number of plaques obtained in each round during plating increased consecutively, indicating that the biopanning rounds were successful (Table 3.1).

The specific plaques from the fifth round of M13 pVIII phage library screening were selected by immuno-blotting assay as described in section 2.3.3. About 90% of these plaques (clones) showed high affinity for GAD-6, showing blue spots on the nitrocellulose membranes.

Ninteen clones of M13 pVIII phage library were sequenced successfully (Fig. 3.12). Interestingly, all sequences were identical to each other. This suggests that the M13 pVIII phage library has only one peptide specific to GAD-6.

All the clones showed a motif of methionine followed by two amino acids (cysteine and tryptophan) followed by alanine. This is consistent with a motif for GAD-6 of M-

X-X-A, corresponding to amino acid residues 523-526 (M-X-X-L) of GAD-65 which overlaps with the R-L-X-K motif (525-528 aa).

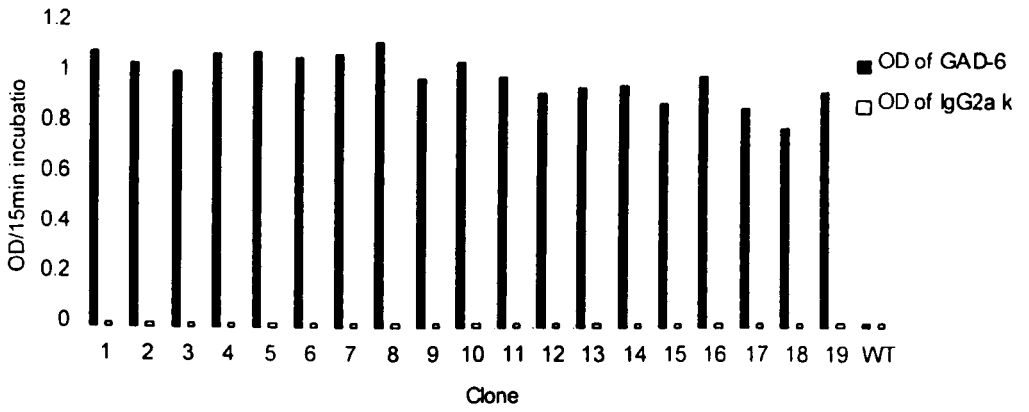
3.2.2.3.2 Testing of GAD-6-specific Phagotopes of M13 pVIII Phage Peptide Library by Capture ELISA

Sequenced phagotopes of M13 pVIII phage library were selected for investigation of their binding to GAD-6 by capture ELISA as described in section 2.3.5. All the ninteen clones showed strong binding with GAD-6, compared to the negative controls [mouse anti-KLH IgG2a (1μg/ml in 1% BSA/TBS-T), wild-type (WT) phage or unselected (US) phage] (Fig. 3.13).

Figure 3.12 Sequences of peptides selected from the filamentous M13 pVIII (5C4C4) phage library by binding to GAD-6.

GAD-67	**(532-537)**	**R E K L H R**
GAD-65	**(523-528)**	**M S R L S K**
19 clones		A R R W D C D G H M C W A Q I

Figure 3.13 Capture ELISA: binding of GAD-6 to M13 pVIII (5C4C4) phage clones bound to ELISA maxisorp plates via rabbit anti-fd IgG.



3.2.3 Discussion

Sixteen and seven clones, which were obtained from the fourth round of successful biopanning of the T7 phage libraries (C9C and linear 9-mers, respectively) and which positively immunostained in the immuno-blotting assay with GAD-6 (therefore showing high affinity), were successfully sequenced. The sequences were aligned with the relevant portions of GAD-65, showing sequence homologies in terms of identical residues and conservative substitutions (i.e. amino acids with biochemically similar side chains). Thirteen out of the sixteen clones selected from the T7 (C9C) phage library and 2/7 clones selected from the T7 (linear 9-mers) phage library expressed the motif R/K-L/A/I-X-K or R-X-X-K (Fig. 3.9), respectively, possibly corresponding to 525-528 aa of GAD-65 (R-L-S-K) as an important epitopic determinant of GAD-6. Interestingly, 4 out of these 13 clones, which were selected from the T7 (C9C) phage library, contained the motif R-L-X-K. Arginine and lysine both have basic side chains and are thus similar residues. Leucine, alanine and isoleucine have aliphatic side chains and are similar residues. The two amino acid residues on the ends of the motif (arginine and lysine) are highly conserved, suggesting that they are important in the paratope-epitope interaction between GAD-6 and GAD-65.

Another motif of GAD-6 obtained from 3 other clones, which were selected from the T7 (C9C) phage library, was M-X-X-A, possibly corresponding to 523-526 aa (M-S-R-L) of GAD-65. The presence of methionine and leucine thus appears to be important in the paratope-epitope interaction between GAD-6 and GAD-65.

Five out of 6 clones, obtained from the T7 (C9C) phage library, showed binding with GAD-6 in ELISA using maxisorp plates, compared to the negative controls (mouse anti-KLH IgG2a, wild-type (WT) phage and unselected (US) phage) (Fig. 3.10). Also, 22 clones were screened in ELISA by using maleic anhydride activated polystyrene plates. All of 22 clones showed binding with GAD-6, compared to the mouse IgG2a kappa (negative control). But only about 11 out of these 22 clones showed binding with GAD-6, compared to both mouse IgG2a kappa and WT phage (negative controls) (Fig. 3.11).

Stop codons are present in T7 phage peptide libraries, both constrained (C9C) or unconstrained (linear 9-mers), at the C-terminal region of the insert. It is possible that the full length of the insert can be expressed, but the MoAb only binds when the insert is present at the extreme C-terminus of the surface coat protein. Another possibility is that the insert within the surface coat protein is unstable and not expressed, except at the extreme C-terminus, as mentioned previously with N-terminal and C-terminal MoAb.

Other work from this laboratory has indicated that GAD-6 can recognize the motif M-X-X-L (523-526 aa of GAD-65) by aligning 7/17 clones, which were selected from a linear 15-mers M13 filamentous phage library (Davenport, 1995). Also, other work from this laboratory includes mutation studies in which M-S (523-524 aa) was changed to R-E. This did not significantly affect GAD-6 binding, but may not have affected the conformational epitope R-X-X-K and the binding of GAD-6 to the native GAD-65 (Trigwell et al., unpublished observations).

GAD-6 does not bind to GAD-67: it is therefore significant that the equivalent residues of M-S-R-L-S-K, the possible motif of GAD-6 corresponding to 523-528 aa of GAD-65, are R-E-K-L-H-R in GAD-67, which shows 5 amino acid substitutions that may disrupt the GAD-6 epitope. Also, the equivalent residues of R-X-X-K, corresponding to 525-528 aa of GAD-65 indicated by clones selected from the T7 phage libraries, are K-X-X-R in GAD-67; i.e. the opposite orientation of the motif R-X-X-K in GAD-65. This may prevent the binding of GAD-6 to GAD-67 or other changes to neighbouring amino acids may affect the binding of GAD-6 (see below).

Sixteen clones, obtained from the third round of biopanning of the M13 pIII (linear 12-mers or C7C) phage libraries were weakly immunostained in the blotting assay with GAD-6 and were successfully sequenced. None of the clones, which were sequenced and compared with one another, showed a clear motif. Also, it was impossible to align them (data not shown). None of these clones, which were screened in ELISA, showed binding with GAD-6, compared to the negative controls (mouse IgG2a kappa and WT phage) (data not shown). The reason for this may be that peptides which specifically bind with GAD-6 are not present in this type of library (M13 pIII phage library, either linear or constrained). On the other hand, the reason for this may be that the avidity is too low due to low copy number of peptides (3-5 copies) expressed by the M13 phage library on the surface coat protein encoded by gene III.

Ninteen clones, obtained from the fifth round of successful biopanning of the M13 pVIII (5C4C4) phage library, were immunostained in the immuno-blotting assay with GAD-6, and were successfully sequenced (Fig. 3.12). Interestingly, all the sequences

were identical to each other, i.e. they showed only one peptide. This indicates that the M13 pVIII phage library may contain only one peptide specific to GAD-6 under the conditions employed. All the clones showed a motif of methionine followed by two amino acids followed by alanine. Thus, the other motif of GAD-6 is M-X-X-A, possibly corresponding to amino acid residues 523-526 (M-S-R-L) of GAD-65 which overlaps with R-L-X-K motif (525-528 aa). It may be relevant that both the M13 pVIII (5C4C4) and T7 (C9C) libraries gave the M-X-X-A motif in sequences containing two cysteines and which could therefore form di-sulphide bridges. Whereas the sequences of the T7 (linear 9-mers) gave the R-X-X-K motif. It is noteworthy that the C-C bridge in the constrained libraries (M13 pVIII and T7) would bring methionine and alanine close together, since these two amino acids are present within the C-C bridge in M13 pVIII (5C4C4) and in only the three constrained clones (1, 3 and 13) of the T7 (C9C) phage library. Interestingly, the M13 pVIII (5C4C4) phage peptide library gave a motif for the N–terminal MoAb that did not correspond to its epitope in GAD-65, whereas this M13 pVIII (5C4C4) phage peptide library did work with GAD-6 by giving both the specific motif and epitope consistent with the sequences of GAD-65.

Sequenced phagotopes of the M13 pVIII phage library were selected for investigation of their binding to GAD-6 by capture ELISA. All the nineteen clones showed strong binding with the GAD-6, compared to the negative controls [mouse anti-KLH IgG2a, wild-type (WT) phage or unselected (US) phage] (Fig. 3.13).

Differences in the sequences of the peptides selected using these two different libraries (i.e. M13 pVIII filamentous phage library and T7 phage library) may be

partly due to the differences in their capability to express their displayed peptide either at the N-terminus of gene VIII protein of M13 filamentous phage or at the C-terminus of gene X protein of T7 phage, although they express similar numbers of copies of inserted peptides (300 or 415 copies, respectively) on the surface coat protein employed by the gene. Two different sequences were selected by these two different libraries which are recognized by GAD-6, indicating that the GAD-6 epitope may possess conformational, as well as linear, characteristics. The effects of conformation on epitopes of GAD-65 has been shown by the observation that replacement of Asn247Ser and Leu574Pro can inhibit the binding of human monoclonal antibodies (11 hum MoAbs) to GAD-65, although the positions of these 2 amino acid residues (Asn247 and Leu574) are not involved in the epitopes of some of these human monoclonal antibodies. For example, the replacement of Leu574Pro can inhibit the binding of some C-terminal-directed antibodies, such as MICA 1 and MICA 3 (Tree et al., 2000). In addition, the replacement of Val532Lys can inhibit the binding of MICA 5 to GAD-65, although this amino acid residue (Val532) does not form part of epitope of MICA 5 (Glu517, Glu520, Ser524 and Ser527) (Schwartz et al., 1999).

Analysis of C-terminal deletion mutants showed that the removal of 41 aa at the C-terminus of GAD65 (545-585 aa deletion mutant) abolished the binding of MICA-2 (human monoclonal antibody) and GAD-6 (Richter et al., 1993). Mapping of the MICA-2 epitope within GAD-65 using an epitope cDNA library revealed that MICA-2 recognizes an epitope between 506-531 aa of GAD65 (Richter et al., 1996), which includes the GAD-6 epitope proposed here. It has been suggested that the removal of 41 aa at the C-terminus of GAD65 (545-585 aa deletion mutant) may lead

to conformational changes of GAD-65 which abolishes the binding of MICA-2. This could also apply to the epitope of GAD-6.

There is other evidence for immunogenicity of this region of GAD-65 which may include the GAD-6 epitope (523-528 aa). In NOD mice, injection of GAD-65 peptide 524-543 aa activates diabetogenic T cells (Zekzer et al., 1998). Furthermore, T-cells in type 1 DM patients can react with 379-585 aa of GAD-65 (Rharbaoui et al., 1999).

A three-dimensional model of the C-terminal region of a GAD65 dimer (461-585 amino acid residues) was built by using the C-terminal domain of dialkylglycine decarboxylase (2DKB) as a template. It predicts an α/β fold composed of a four-stranded β sheet and three amphipathic α-helices [helix R (467-486 aa), helix S (521-540 aa) and helix T (568-582 aa)] with localization of hydrophobic residues toward the β-strands and residues involved in epitope recognition on the charged face of these helices (Schwartz et al., 1999). The proposed motif of GAD-6 (523-528 aa) is present at the N-terminal end of α-helix S (521-540 aa) in this three-dimensional model of the C-terminal region of a GAD-65 (Fig. 3.14).

In α-helix S, M523 and L526 are on the hydrophobic side while R525 and K528 are on the hydrophilic side. The M--L and R--K are facing each other and in similar positions on successive turns of α-helix S (Fig. 3.14). GAD-6 may recognize M-RL-K in linear (denatured GAD-65), but might only recognize R--K in native GAD-65 since these residues (R--K) are on the exposed hydrophilic face of the α-helix S. It is possible that the immunization of the BALB/c mouse, to produce GAD-6, with purified rat brain GAD emulsified in oil (Freund's adjuvant) as a hydrophobic

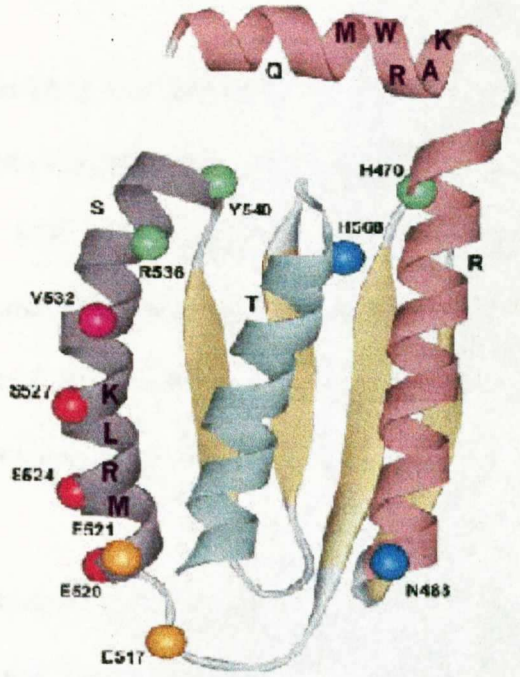
solution may have partially denatured GAD and may have exposed its hydrophobic amino acid residues.

Interestingly, others have mapped the epitope of b78 human monoclonal antibody to 532-540 aa of GAD-65 (Schwartz et al., 1999), which is very close to the proposed GAD-6 epitope (523-528 aa). In addition, the footprint pattern of GAD-6 protected trypsinized fragments of GAD-65 is similar to that given by b78 (Tremble et al., 1997). Thus, I investigated the cross-inhibition of binding to GAD-65 between GAD-6 and b78, as described in section 4.1.2.

There are other regions which could fit with our motifs, but each of these has its problems. Thus, KARMM at 534-538 aa would be MMRAK in reverse, but then presumably the 'chirality' of the side chains would be wrong. Also, R536L mutant bound to GAD-6 in the Schwartz study (legend to figure 3.14). Further, there is MWRAK at 458-462 aa, but this is outside the region 475-572 to which the Butler study mapped GAD-6.

One way to determine whether the phage peptides which were selected by immunopanning with GAD-6 were acting as true mimotopes of GAD-65 epitopes, would be to immunize mice with the GAD-6 reactive clones. Immunizing with mimotope peptides presented on filamentous phage has been shown to raise peptide-specific antibodies which may also bind to the native antigen (Meola et al., 1995).

Figure 3.14 The Three-dimensional Model of the C-terminal Region of GAD-65 shows that the motifs of GAD-6, 523-528 or 458-461, which are present at the N-terminal end of α-helix T or at the C-terminal end of α-helix Q (Schwartz et al., 1999).



3.3 76B and 76F Mouse Monoclonal Antibodies

3.3.1 Introduction

The prevalence of anti-IA-2 AAs is higher in acute onset type 1 DM than in slowly progressive type 1 DM (Yamada et al., 1997). The anti-IA-2 AAs are present in 56% type 1 DM patients, 47% APS II patients with type 1 DM, 4% APS II patients without type 1 DM and 14% SMS patients (Morgenthaler et al., 1997). A major antigenic determinant of IA-2 is localized to 762-887 aa, while a minor antigenic determinant of IA-2 is localized to 601-762 aa (Kawasaki et al., 1998).

76B (IgG; isotype unknown) and 76F (IgG2b), are two mouse monoclonal antibodies which recognize epitopes in the extracellular domain of IA-2 (378-577 aa) and N-terminal region of the cytoplasmic domain of IA-2 (605-682 aa), respectively. Hybridoma supernatants containing the MoAbs were kindly supplied by Dr Ezio Bonifacio, Milan. Interestingly, huAb 103/5 recognizes 603-686 aa of IA-2 (Kolm-Litty et al., 2000) which is similar to the region recognized by 76F MoAb.

Since the different phage peptide libraries (T7, M13 pIII and M13 pVIII) were working well with purified mouse MoAbs to GAD-65 (N-terminal and C-terminal MoAbs and GAD-6), I screened the 76B and 76F hybridoma culture supernatants containing mouse MoAb with some of these phage peptide libraries.

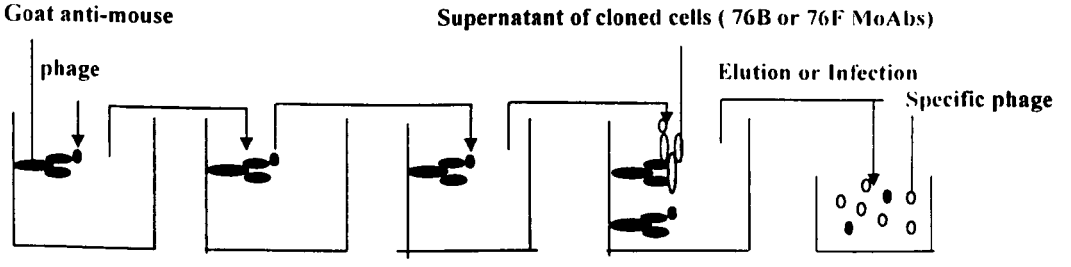
3.3.2 Materials and Methods

3.3.2.1 Screening of 76B or 76F Mouse Monoclonal Antibody with T7 (C9C) Phage Peptide Library

3.3.2.1.1 Bio-panning of 76B or 76F Mouse Monoclonal Antibody with T7 (C9C) Phage Peptide Library

Hybridoma culture supernatant was exposed to solid-phase anti-mouse IgG to selectively capture the IgG mouse monoclonal antibodies. The input phage might be recognized by anti-mouse IgG antibody as well as mouse MoAb. Thus, the input phage were depleted from anti-mouse IgG Ab-specifc phage (negative selection) by exposing the input phage to three tubes coated with anti-mouse IgG only. Then, the depleted phage were exposed to mouse MoAb, which was captured by anti-mouse IgG Ab (Fig. 3.15).

Figure 3.15 Schematic Representation of Negative Selection of Specific Phage Peptides by using Goat anti-mouse IgG Antibodies.

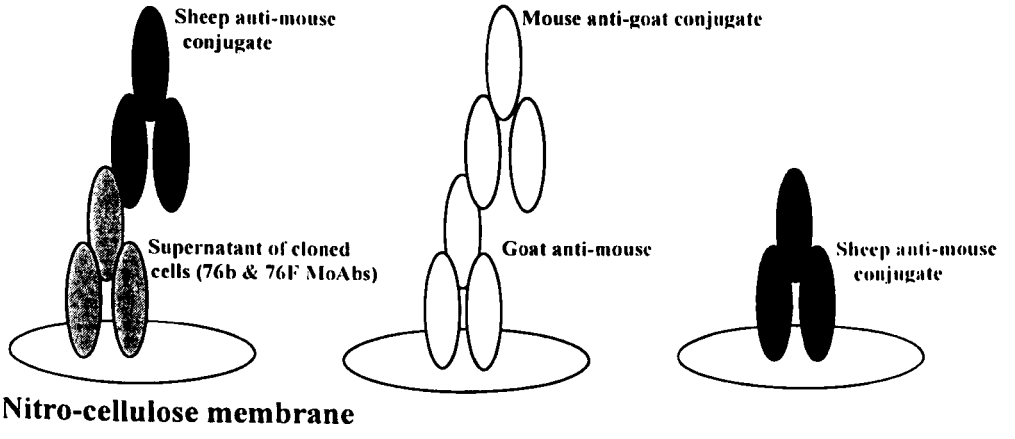


Goat anti-mouse IgG (Fc specific) (Sigma) (20μg/ml) in coating buffer, was coated onto three Nunc immuno-tubes. Ten μg/ml of the goat anti-mouse IgG in coating buffer, was also coated onto another Nunc immuno-tube. Following overnight incubation at 4°C on a rotator, all the Nunc immuno-tubes were washed 5 times in 0.1% TBS-T and blocked with 5% BSA/TBS for one hour at room temperature on a rotator. Ten microlitres of the T7 phage library (1×10^{10} pfu/10μl), as input phage, in 1ml of blocking solution, were added to one of the Nunc immuno-tubes (first tube), which had been coated with the goat anti-mouse IgG (20μg/ml), and incubated at 4°C for 30 minutes on a rotator. These pre-absorbed T7 phage were transferred from the first Nunc immuno-tube to another Nunc immuno-tube (second tube), which had also been coated with the goat anti-mouse IgG (20μg/ml), and also incubated at 4°C for 30 minutes on a rotator. The pre-absorbed T7 phage were then transferred from the second Nunc immuno-tube to another Nunc immuno-tube (third tube), which had been coated with the goat anti-mouse IgG (20μg/ml), and incubated at 4°C for 30 minutes on a rotator. Hybridoma culture supernatant containing 76B or 76F mouse monoclonal antibody, was added to the fourth Nunc immuno-tube, which had been coated with the goat anti-mouse IgG (10μg/ml), and incubated on a rotator for two hours at 4°C. Following 5 washes of the fourth Nunc immuno-tube, the pre-absorbed T7 phage were transferred from the third Nunc immuno-tube to the fourth Nunc immuno-tube (test Nunc-immuno tube) and incubated at 4°C for 30 minutes on a rotator. Following 5 washes of the test Nunc immuno-tube, 1ml of a log-phase BL 21 culture was added and incubated at room temperature for 5 minutes on a rotator. The following steps including eluate amplification, plating and purification were performed precisely as described in section 2.1.2.

3.3.2.1.2 Detection of 76B or 76F Mouse Monoclonal Antibody-specific T7 (C9C) Phage Peptide Clones by Immuno-blotting Assay

This was performed as described in section 2.1.3. The specificity of the assay involving binding of the 76B or 76F MoAbs with plaques on the membrane, was confirmed by investigating the binding of the goat anti-mouse IgG (10μg/ml in 2.5% BSA/TBS-T), which had been pre-absorbed with lysate-treated membrane, with plaques on duplicate membrane, followed by washing and adding mouse monoclonal anti-goat IgG clone GT-34 alkaline phospatase conjugate (Sigma) (diluted 1:1000 in 2.5% BSA/TBS-T). After washing, the BCIP/NBT substrate was added. Also, the specificity of the assay was further confirmed by investigating the binding of the sheep anti-mouse IgG alkaline phosphatase conjugate, which had been pre-absorbed with lysate-treated membrane, with plaques on another duplicate membrane. This was to exclude the false positive detection of plaques which showed binding with goat anti-mouse IgG and/or sheep anti-mouse IgG (Fig. 3.16).

Figure 3.16 Schematic Representation of Immuno-blotting Assay to Exclude the False Positive Binding of Ligands with non-specific Phage Peptides.



3.3.2.1.3 PCR and Sequencing of 76B or 76F Mouse Monoclonal Antibody-specific T7 (C9C) Phage Insert

This was performed as described in section 2.1.4.

3.3.2.1.4 Detection of 76B or 76F Mouse Monoclonal Antibody-specific T7 (C9C) Phage Peptide Clones by Capture ELISA

This was performed as described in section 2.1.5.2. Mouse IgG2a kappa or IgG2b kappa myeloma proteins (Sigma) were used as negative controls for 76B and 76F, respectively (1μg/ml in washing buffer).

3.3.2.2 Screening of 76B or 76F Mouse Monoclonal Antibody with M13 pIII (linear 12-mers) Phage Peptide Library

3.3.2.2.1 Bio-panning of 76B or 76F Mouse Monoclonal Antibody with M13 pIII (linear 12-mers) Phage Peptide Library

Goat anti-mouse IgG (Fc specific) (Sigma) (20μg/ml) in coating buffer, was coated onto three Nunc immuno-tubes. Ten μg/ml of the goat anti-mouse IgG in coating buffer, was also coated onto another Nunc immuno-tube. Following overnight incubation at 4°C on a rotator, all the Nunc immuno-tubes were washed 5 times in 0.1% TBS-T and blocked with 0.5% BSA containing 0.02% NaN_3 in TBS (blocking solution) for one hour at room temperature on a rotator. Following 6 washes, ten microlitres of the M13 (linear 12-mers) phage library (1×10^{11} pfu/10μl), as input phage in 1ml of blocking solution, were added to one of the Nunc immuno-tubes (first tube), which had been coated with the goat anti-mouse IgG (20μg/ml), and incubated

at 4°C for 30 minutes on a rotator. These pre-absorbed M13 phage were transferred from the first Nunc immuno-tube to another Nunc immuno-tube (second tube), which had also been coated with the goat anti-mouse IgG (20μg/ml), and also incubated at 4°C for 30 minutes on a rotator. The negative and positive selections for the next tubes were performed precisely as described in section 3.3.2.1.1. Following 10 washes of the fourth (test) Nunc immuno-tube, 1ml of elution buffer was added and incubated on a rotator for 10 minutes. The non-amplified eluate was removed and neutralized with 150μl of neutralizing buffer (Fig. 3.15). The following steps including eluate amplification, plating and purification were performed as described in section 2.2.2.

3.3.2.2.2 Detection of 76B or 76F Mouse Monoclonal Antibody-specific M13 pIII (linear 12-mers) Phage Peptide Clones by Immuno-blotting Assay

This was performed precisely as described in section 2.2.3 by using sheep anti-mouse (whole molecule) alkaline phosphatase conjugate to detect the binding of the 76B and 76F MoAb to the specific clones. The specificity of the assay was confirmed using the controls described in section 3.3.2.1.2 (Fig. 3.16).

3.3.2.2.3 Purification and Sequencing of 76B or 76F Mouse Monoclonal Antibody-specific M13 pIII (linear 12-mers) Phage Insert

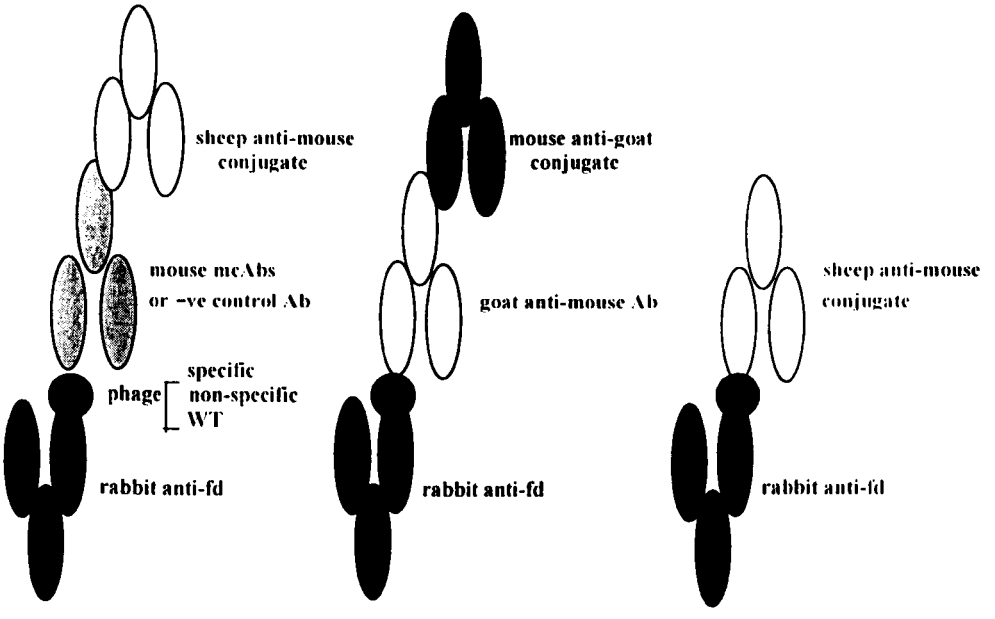
This was performed as described in section 2.2.4.

3.3.2.2.4 Detection of 76B or 76F Mouse Monoclonal Antibody-specific M13 pIII (linear 12-mers) Phage Peptide Clones by Capture ELISA

This was performed as described in section 2.2.5. The phage were bound to the plate via rabbit anti-fd IgG. Mouse IgG2a kappa myeloma protein was used as a negative control for 76B and IgG2b kappa myeloma protein (Sigma) was used as a negative control for both 76B and 76F (1μg/ml in 1% BSA/TBS-T). Sheep anti-mouse (whole molecule) alkaline phosphatase conjugate was used to detect the binding of the 76B and 76F MoAb or their negative control antibodies (mouse IgG2a or IgG2b kappa) to the specific clones.

The specificity of the assay involving binding of the 76B or 76F with the phage, was confirmed by investigating the binding of the goat anti-mouse IgG (2μg/ml in 1% BSA/TBS-T) with the phage, followed by adding mouse anti-goat IgG alkaline phospatase conjugate (diluted 1:1000 in 1% BSA/TBS-T) and the pNPP substrate was added. This was to exclude the false positive detection of clones which showed binding with goat anti-mouse IgG (Fig. 3.17). A similar control investigated whether the sheep anti-mouse conjugate bound to any of the selected phage (Fig. 3.17). Also, the specificity of the assay was further confirmed by examining the binding of the 76B MoAb with 76F-selected phage, and by binding of the 76F MoAb with 76B-selected phage. This was to exclude the non-specific binding of the 76B and 76F with 76F-selected and 76B-selected phage, respectively.

Figure 3.17 Schematic Representation of Capture ELISA to Exclude the False Positive Binding of Ligands with non-specific Phage Peptides.



3.3.3 Results

3.3.3.1 T7 (C9C) Phage Peptide Library

3.3.3.1.1 Sequences of Phagotopes Isolated by Bio-panning of T7 Phage Peptide Library (C9C) and Immuno-blotting Assay with 76B or 76F Mouse Monoclonal Antibody

T7 phage library (C9C) was screened by 4 rounds of immunopanning against 76B or 76F mouse monoclonal antibodies (MoAb) as described in section 3.3.2.1.1. The number of plaques obtained in each round during plating increased consecutively, indicating that the biopanning rounds were successful (Table 3.1).

The specific plaques from the fourth round of screening the T7 phage library (C9C) with 76B or 76F MoAb were selected by immuno-blotting assay as described in section 3.3.2.1.2. About 6% and 15% of these plaques (clones) showed high affinity, giving blue spots on the nitrocellulose membranes, to the 76B or 76F MoAb respectively. No plaques detected by the 76B MoAb showed high affinity to goat anti-mouse IgG. However, about 2% of 76F plaques showed high affinity to goat anti-mouse IgG.

Seventeen clones and nine clones, which were specific to the 76B or 76F MoAb, respectively, were sequenced successfully. Stop codons were found in all clones either in the middle of the inserted portions in 15/17 or 7/9 clones or at the end of the inserted portions in 2/17 or 2/9 clones of the 76B or 76F mouse MoAb, respectively.

All the sequences were aligned with one another and with the relevant portions of IA-2. Nine out of the 17 clones-specific to the 76B MoAb (1,2,8,9,11,12,14,15 and 18) showed a motif of lysine followed by proline followed by an amino acid followed by serine. Thus, the main motif of the 76B MoAb is K-P-X-S, corresponding to amino acid residues 479-482 of IA-2 (K-P-L-S); this precise sequence was given by 2 clones (8 and 15). Furthermore, one clone (5) showed a motif of lysine followed by proline which overlaps with the previous motif. In addition, two clones (12 and 7) showed a motif of glutamine followed by lysine (not clone 7) followed by proline, giving a motif (Q-K-P) (478-480 aa of IA-2), which overlaps with the previous motif. Thus, the overall motif of the 76B MoAb from the sequences of these clones obtained from the screening the T7 (C9C) phage library with 76B MoAb, is Q-K-P-L-S, corresponding to 478-482 aa of IA-2. The other 6 clones (3, 4, 6, 10, 13 and 16) did not show any significant motif (Fig. 3.18).

Three out of the 9 clones-specific to the 76F MoAb (3,5 and 7) showed a motif of phenylalanine (3,7) or tryptophan (5) followed by an amino acid followed by histidine followed by glutamine (7) or asparagine (3,5). Thus, the main motif of the 76F MoAb is F/W-X-H-Q/N, corresponding to amino acid residues 626-629 of IA-2 (F-E-Y-Q), which showed conservative substitutions. Phenylalanine and tryptophan have aromatic side chains and are thus similar residues. Tyrosine and histidine have amide side chains and are thus similar residues. Also, glutamine and asparagine have aromatic side chains and are thus similar residues. Furthermore, 2 clones (13,22) showed a motif of tyrosine followed by glutamine which overlaps with the previous motif. In addition, 3 clones (2, 8 and 12) showed a motif of histidine followed by

asparagine (H-N), corresponding to 628-629 aa of IA-2 (Y-Q), which showed conservative substitutions and also overlaps with previous motif. Clone 4 did not show a significant motif (Fig. 3.19).

3.3.3.1.2 Testing of 76B or 76F Mouse Monoclonal Antibody-specific Phagotopes of T7 Phage Peptide Library (C9C) by ELISA

Sequenced phagotopes of T7 (C9C) phage library were selected for investigation of their binding to the 76B or 76F MoAb by ELISA as described in section 3.3.2.1.4. Six clones specific to 76B and six specific to the 76F MoAb were screened in ELISA by using MAAP plates. All of these 76B-specific clones showed a significant binding with the 76B MoAb, compared to both negative controls [mouse IgG2a kappa (Fig. 3.20a) and WT phage, as indicated by the ratio of OD for binding of 76B to its specific clones divided by OD for binding of IgG2a to the 76B-specific clones (Fig. 3.20b)]. In contrast, all of the 76F-specific clones showed a significant binding with the 76F MoAb, compared to the negative control antibody (mouse IgG2b kappa) only (Fig. 3.21a), whereas, only some of the clones (particularly clone 4) showed binding with the 76F MoAb, compared to both negative controls (mouse IgG2b kappa and WT phage, as indicated by the ratio of OD for binding of 76B to its specific clones divided by OD for binding of IgG2a to the 76B-specific clones) (Fig. 3.21b).

Figure 3.18 Sequences of Peptides Selected from T7 (C9C) Phage Peptide Library by Binding to 76B Mouse Monoclonal Antibody.

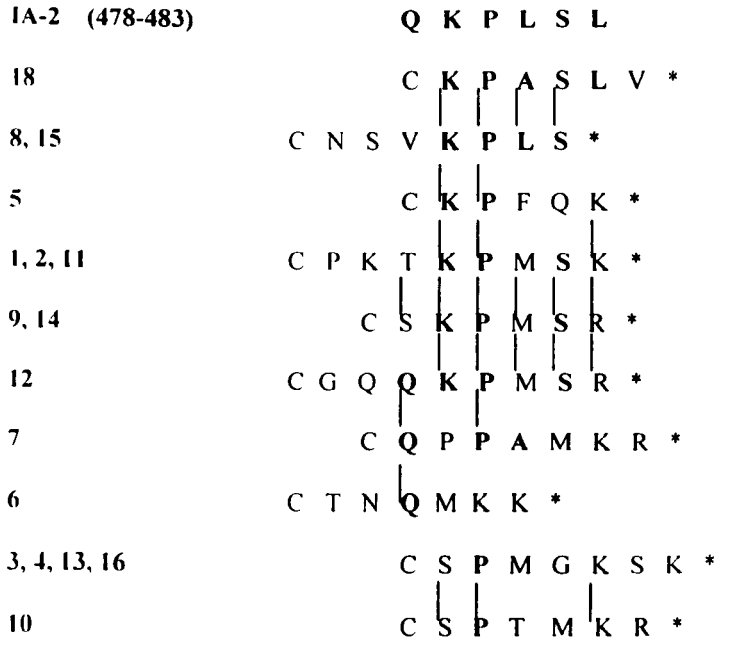


Figure 3.19 Sequences of Peptides Selected from T7 (C9C) Phage Peptide Library by Binding to 76F Mouse Monoclonal Antibody.

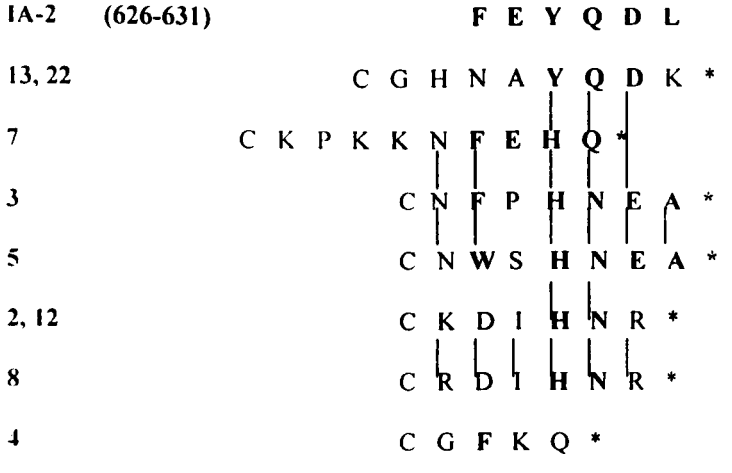
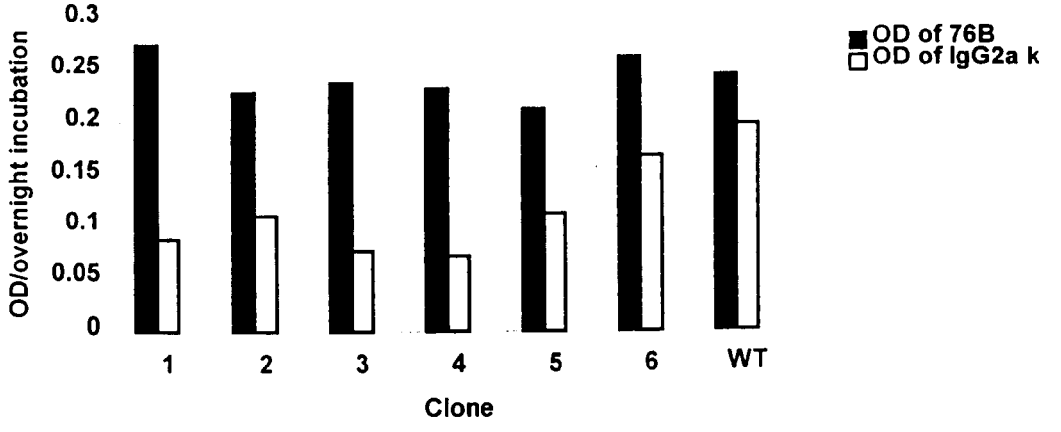


Figure 3.20 a) Direct Binding of 76B Mouse Monoclonal Antibody to T7 (C9C) Phage Clones Coated ELISA Maleic Anhydride Activated Polystyrene Plates, **b)** Ratio of OD for Binding of 76B to its Specific Clones divided by OD for Binding of IgG2a to the 76B-specific Clones.

a)



b)

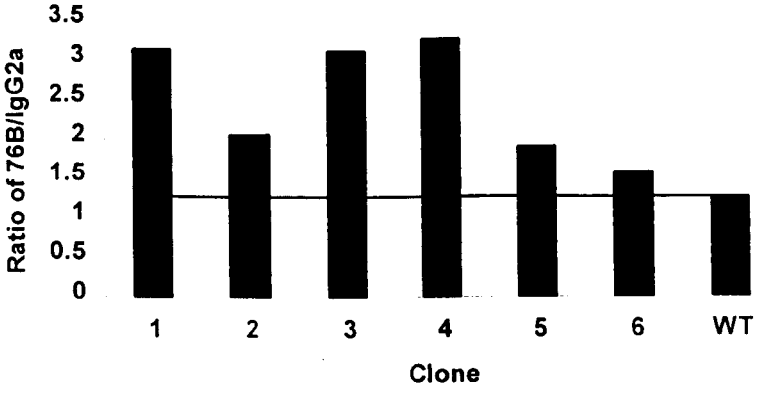
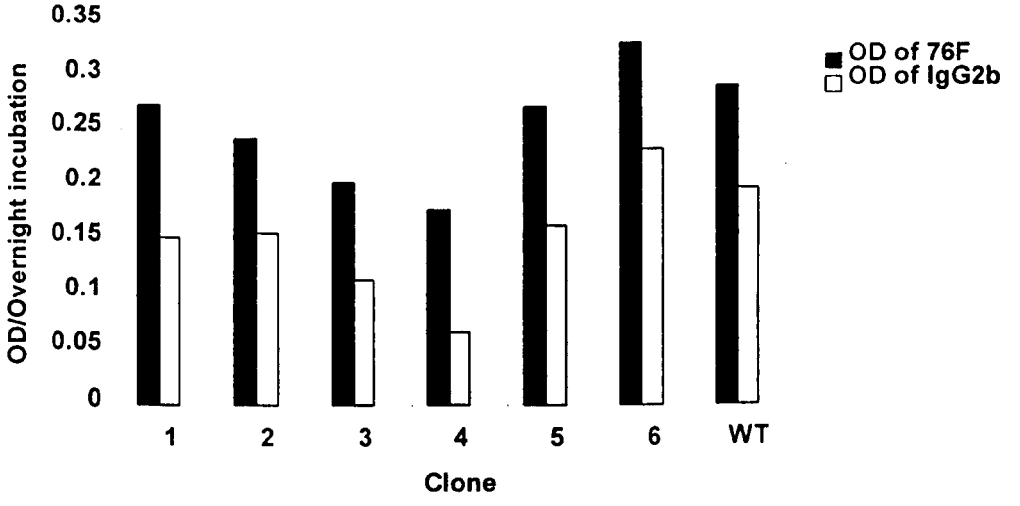
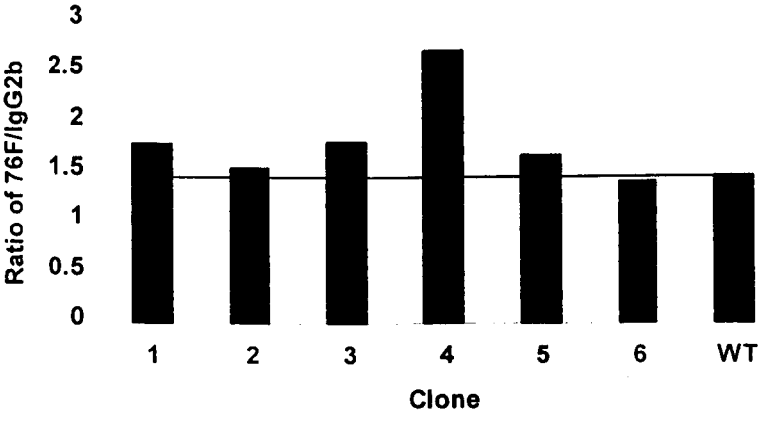


Figure 3.21 a) Direct Binding of 76F Mouse Monoclonal Antibody to T7 (C9C) Phage Clones Coated ELISA Maleic Anhydride Activated Polystyrene Plates, **b)** Ratio of OD for Binding of 76F to its Specific Clones divided by OD for Binding of IgG2b to the 76F-specific Clones.

a)



b)



3.3.3.2 *M13 pIII (linear 12-mers) Phage Peptide Library*

3.3.3.2.1 Sequences of Phagotopes Isolated by Bio-panning of M13 pIII Phage Peptide Library (linear 12-mers) and Immuno-blotting Assay with 76B or 76F Mouse Monoclonal Antibody.

M13 pIII phage library (linear 12-mers) was screened by 3 rounds of immunopanning against 76B or 76F MoAb as described in section 3.3.2.2.1. The number of plaques obtained in each round increased consecutively, indicating that the biopanning rounds were successful (Table 3.1).

The specific plaques from the third round of M13 pIII phage library (linear 12-mers) immuno-panning with 76B or 76F MoAb were selected by immuno-blotting assay as described in section 3.3.2.2.2. About 30% and 15% of these plaques (clones) showed high affinity, giving blue spots on the nitrocellulose membranes, to the 76B or 76F MoAb, respectively. About 3% of the 76B plaques showed high affinity to goat anti-mouse IgG. Also, about 3% of the 76F plaques showed high affinity to goat anti-mouse IgG.

Eleven and sixteen clones specific to the 76B or 76F MoAb, respectively, were sequenced successfully. All sequences were aligned with the relevant portions of IA-2. The 11 clones specific to the 76B MoAb showed a motif of aspartic acid followed by an amino acid followed by lysine followed by proline followed by leucine followed by serine. Thus, the main motif of the 76B MoAb is D-X-K-P-L-S, corresponding to amino acid residues 477-482 of IA-2 (D-Q-K-P-L-S), which was exactly represented in one clone (11) (Fig. 3.22). Furthermore, this motif overlaps

with the previous motif, which was obtained by using T7 (C9C) phage library (see above).

The 16 clones specific to the 76F MoAb showed a motif of phenylalanine 8/16 (3,4,10,11,12,13,16,20), tryptophan 7/16 (5,6,8,9,14,15,19) or tyrosine 1/16 (7) followed by an amino acid followed by tyrosine 15/16 or tryptophan 1/16 (7) followed by glutamine 15/16 or an amino acid 1/16 (11). Thus, the main motif of the 76F MoAb is F/W/Y-X-Y/W-Q, corresponding to amino acid residues 626-629 of IA-2 (F-E-Y-Q) (Fig. 3.23), which showed conservative substitutions. Phenylalanine, tyrosine and tryptophan have aromatic side chains and are thus similar residues. Furthermore, this motif is similar to the motif that was obtained by using T7 (C9C) phage library (see above).

3.3.3.2.2 Testing of 76B or 76F Mouse Monoclonal Antibody-specific Phagotopes of M13 pIII Phage Peptide Library (linear 12-mers) by ELISA

Sequenced phagotopes of M13 pIII (linear 12-mers) phage library were selected for investigation of their binding to 76B or 76F MoAb by capture ELISA as described in section 3.3.2.2.4. All the 11 or 20 clones, which were specific to 76B or 76F MoAb, showed a significant binding with the 76B or 76F MoAb, respectively, compared to all negative controls [mouse IgG2a kappa (negative control for 76B MoAb), mouse IgG2b kappa and helper M13 (M13K07) phage] (Fig. 3.24 or 3.25, respectively). Also, these 11 or 20 clones, which were specific to 76B or 76F mouse monoclonal antibody, respectively, showed negligible cross-reactivity with goat anti-mouse IgG.

In addition, five randomly selected clones specific to 76B MoAb showed negligible cross-reactivity with 76F MoAb, compared to the negative control (WT phage), in capture ELISA as described in section 3.3.2.2.4 (Table 3.2). Conversely, five randomly selected clones specific to 76F MoAb showed negligible cross-reactivity with 76B MoAb, compared to the negative control (WT phage) (Table 3.2).

Figure 3.22 Sequences of Peptides Selected from M13 pIII (linear 12-mers) Phage Peptide Library by Binding to 76B Mouse Monoclonal Antibody.

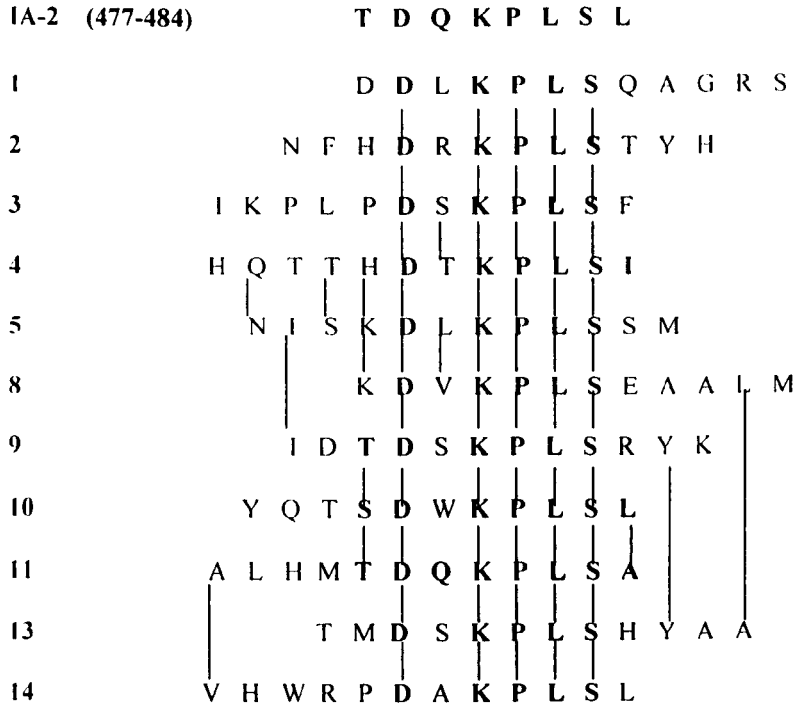


Figure 3.23 Sequences of Peptides Selected from M13 pIII (linear 12-mers) Phage Peptide Library by Binding to 76F Mouse Monoclonal Antibody.

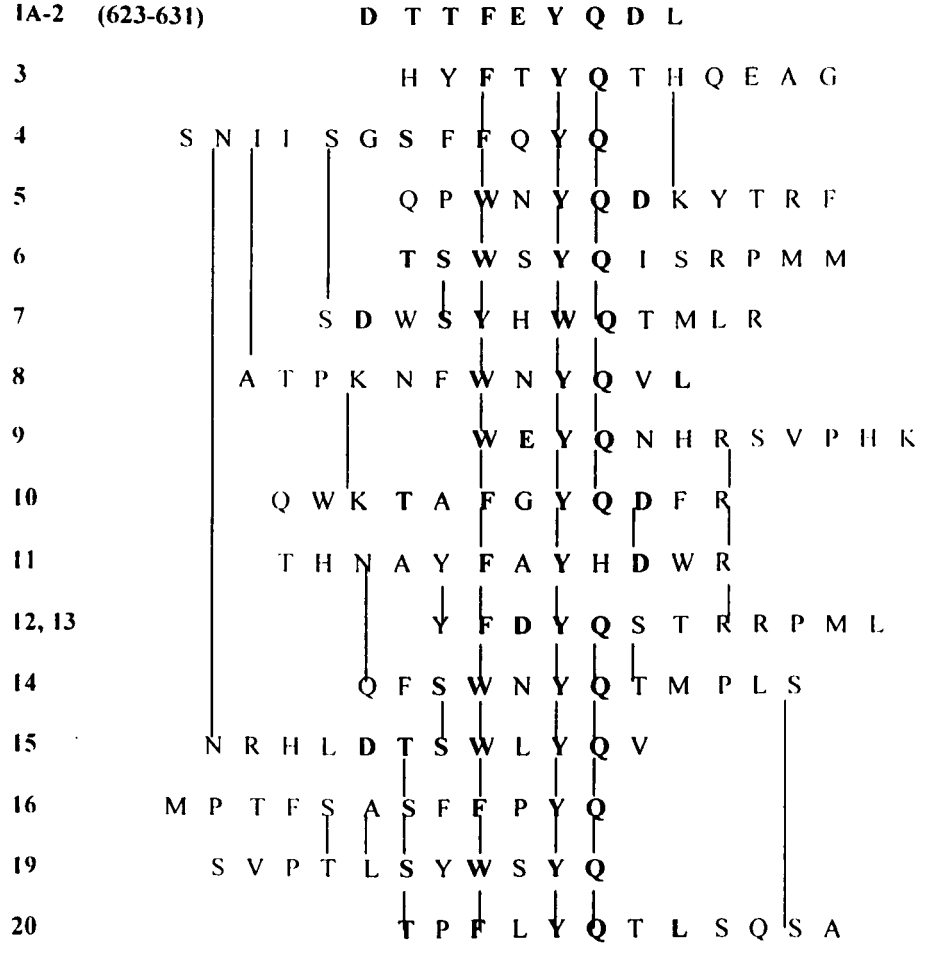


Figure 3.24 Capture ELISA: Binding of 76B Mouse Monoclonal Antibody to M13 pIII (linear 12-mers) Phage Clones Bound to ELISA Maxisorp Plates via Rabbit anti-fd IgG.

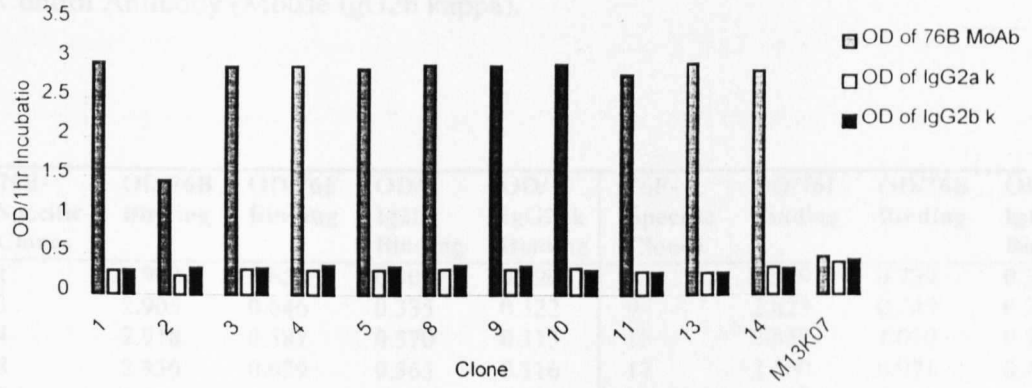


Figure 3.25 Capture ELISA: Binding of 76F Mouse Monoclonal Antibody to M13 pIII (linear 12-mers) Phage Clones Bound to ELISA Maxisorp Plates via Rabbit anti-fd IgG.

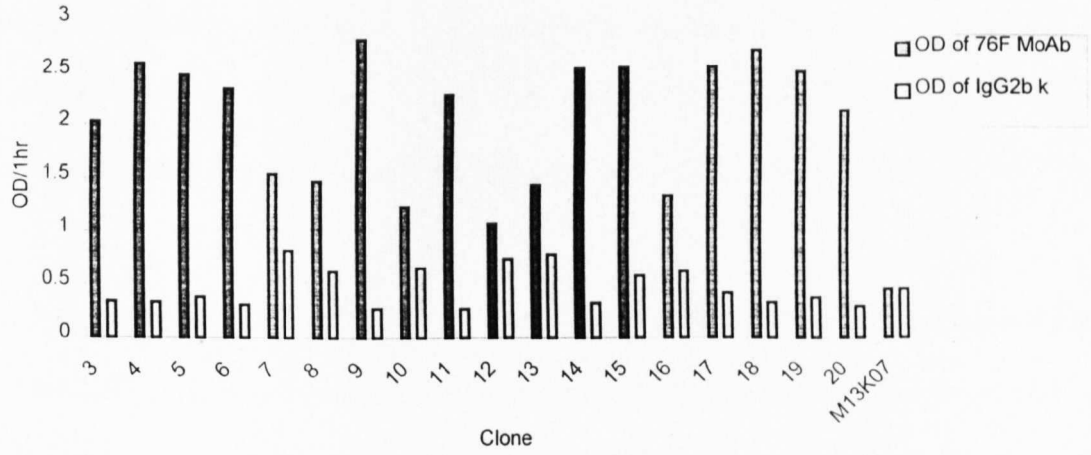


Table 3.2 Capture ELISA: Binding of 76F Mouse Monoclonal Antibody to M13 pIII (linear 12-mers) Phage Clones Specific to 76B Mouse Monoclonal Antibody Bound to ELISA Maxisorp Plates via Rabbit anti-fd IgG, compared to Wild-type Phage (WT) and Negative Control Antibodies (Mouse IgG2b and IgG2a kappa). Also, Binding of 76B Mouse Monoclonal Antibody to M13 pIII (linear 12-mers) Phage Clones Specific to 76F Mouse Monoclonal Antibody Bound to ELISA Maxisorp Plates via Rabbit anti-fd IgG, compared to Wild-type Phage (WT) and Negative Control Antibody (Mouse IgG2b kappa).

76B-Specific Clones	OD/76B Binding	OD/76F Binding	OD/ Ig2bk Binding	OD/ IgG2ak Binding	76F-Specific Clones	OD/76F Binding	OD/76B Binding	OD/ IgG2bk Binding
1	2.952	0.657	0.303	0.296	4	2.589	0.739	0.333
3	2.905	0.646	0.335	0.322	9	2.823	0.745	0.277
4	2.918	0.587	0.370	0.313	15	2.559	1.090	0.580
8	2.930	0.629	0.365	0.316	17	2.560	0.971	0.418
9	2.925	0.614	0.359	0.318	18	2.716	0.897	0.327
WT	0.941	0.918	0.871	0.824	WT	0.918	0.941	0.871

3.3.4 Discussion

Seventeen clones, which were obtained from the fourth round of successful biopanning rounds of T7 (C9C) phage library and were positively immunostained in the blotting assay (high affinity) with the 76B MoAb, were successfully sequenced. Nine out of these 17 clones showed a motif of K-P-X-S (Fig. 3.18), corresponding to 479-482 aa of IA-2 (K-P-L-S), as the epitopic determinant of the 76B MoAb. Interestingly, 2 out of these 9 clones showed the precise motif of K-P-L-S. Six of the clones were screened in ELISA by using maleic anhydride activated polystyrene plates. All of these 6 clones showed binding with the 76B MoAb, compared to both negative controls [mouse IgG2a kappa (Fig. 3.20a) and WT phage; by looking at the ratio of OD for binding of 76B to its specific clones divided by OD for binding of IgG2a to the 76B-specific clones (Fig. 3.20b)].

Stop codons are present in T7 (C9C) phage peptide library at the C-terminal region of the insert. It is possible that the full length of the insert is expressed, but the MoAb only binds when the insert is present at the extreme C-terminus of the surface coat protein. Another possibility is that the full insert within the surface coat protein is unstable and not expressed, except at the extreme C-terminus, as mentioned previously with N-terminal and C-terminal MoAb and GAD-6.

Eleven clones, which were obtained from the third round of successful biopanning of the M13 pIII (linear 12-mers) phage library and were positively immunostained in the blotting assay (high affinity) with the 76B MoAb, were successfully sequenced. All of these 11 clones showed a motif of D-X-K-P-L-S (Fig. 3.22), corresponding to 477-482 aa of IA-2 (D-Q-K-P-L-S), as an important epitopic determinant of the 76B

MoAb. Interesingly, 1 out of these 9 clones (clone 11) showed the precise motif of T-D-Q-K-P-L-S, corresponding to 476-482 aa of IA-2.

All these 11 76B-specific clones obtained from the M13 pIII (linear 12-mers) phage library, which showed negligible cross-reactivity with 76F MoAb, negative control antibodies (mouse IgG2b and IgG2a kappa) (Table 3.2) and goat anti-mouse IgG (data not shown), were screened in ELISA. All of these 11 clones showed significant binding with the 76B MoAb, compared to all negative control antibodies (mouse IgG2a and IgG2b kappa) and helper M13 (WT phage) (Fig. 3.24).

The sequences of 76B-specific clones obtained from both libraries [T7 (C9C)and M13 pIII (linear 12-mers)] showed homologies amongst themselves and with the original IA-2 sequence. Thus, the main motif of 76B is D-X-K-P-L-S, corresponding to 477-482 aa of IA-2 (D-Q-K-P-L-S).

Nine clones, which were obtained from the fourth round of successful biopanning of the T7 phage library and were positively immunostained in the blotting assay (high affinity) with 76F MoAb, were successfully sequenced. Eight out of these 9 clones showed a motif of Y/H-Q/N (Fig. 3.19), corresponding to 628-629 aa of IA-2 (Y-Q), as part of the epitopic determinant of the 76F MoAb. Tyrosine and histidine both have aromatic side chains and are thus similar residues. Also, glutamine and asparagine both have amide side chains and are thus similar residues. Interesingly, 1 out of these 8 clones (clone 7) showed a motif of F-E-H-Q, corresponding to 626-629 aa of IA-2 (F-E-Y-Q), which shows a conservative substitution. One clone did not show a clear motif of IA-2.

Six clones obtained from the T7 (C9C) phage library, were screened in ELISA by using maleic anhydride activated polystyrene plates. All of these 6 clones showed binding with 76F MoAb, compared to the negative control antibody (mouse IgG2b kappa) only (Fig. 3.21a), whereas, only certain of the clones (particularly clone 4) showed binding with the 76F MoAb, compared to both negative controls (mouse IgG2b kappa and WT phage, as indicated by the ratio of OD for binding of 76B to its specific clones divided by OD for binding of IgG2a to the 76B-specific clones) (Fig. 3.21b). This may be due to a high level of non-specific reactivity with the wild-type phage.

Sixteen clones, which were obtained from the third round of successful biopanning of the M13 pIII (linear 12-mers) phage library and were positively immunostained in the blotting assay (high affinity) with 76F MoAb, were successfully sequenced. Fifteen out of these 16 clones showed a motif of F/W/Y-X-Y/W-Q (Fig. 3.23), corresponding to 626-629 aa of IA-2 (F-E-Y-Q), as an important epitopic determinant of 76F MoAb. Phenylalanine, tryptophan and tyrosine have aromatic side chains and are thus similar residues. Interestingly, one out of these 15 clones, clone 9 showed a motif of W-E-Y-Q.

Twenty 76F-specific clones obtained from M13 pIII (linear 12-mers) phage library, which showed negligible cross-reactivity with both 76B MoAb, negative control antibody (mouse IgG2b kappa) (Table 3.2) and goat anti-mouse IgG (data not shown), were screened in ELISA. All of these 20 clones showed significant binding

with the 76F, compared to all negative control antibody (mouse IgG2b kappa) and helper M13 phage (WT phage) (Fig. 3.25).

The sequences of 76F-specific clones obtained from both libraries [T7 (C9C) and M13 pIII (linear 12-mers)] show homologies amongst themselves and with the original IA-2 sequence. Thus, the main motif of 76F is F-X-Y-Q, corresponding to 626-629 aa of IA-2 (F-E-Y-Q).

Since 76F MoAb may recognize 626-629 aa of IA-2 and huAb 103/5 recognizes 603-686 aa of IA-2 (Kolm-Litty et al., 2000) which is similar to the region recognized by 76F MoAb, both 76F MoAb and huAb 103/5 may recognize the same region on IA-2, i.e. 626-629 aa. In addition, 621-630 aa of IA-2 (epitope JM2) is one of the major epitopes recognized by sera from over 50% of patients with type 1 DM (Bearzatto et al., 2001), which overlaps with the predicted epitope of 76F MoAb on IA-2 (626-629 aa). This is also supported by a recent report identifying an epitope within IA-2 623-631 aa recognized by type 1 DM patients' sera (Notkins et al., 1997), which also overlaps with the predicted epitope of 76F MoAb. HLA-DR4 containing haplotypes are always found in relatives and type 1 DM patients with IA-2 JM2 specific antibodies. Also, HLA-DR3 and DR13 are associated with IA-2 JM2 specific antibody positive relatives. It has been suggested that B cell receptor binding to the JM2 autoantibody epitopes preferentially generates T cell epitopes which can bind to HLA class II molecules expressed in DR3/4, DR4/4, DR4/13 or DR1/4 B cells and results in T cell help for JM2 specific B cells (Bearzatto et al., 2001). Also, this region of IA-2 (626-629 aa), which may be recognized by 76F MoAb, is present within the minor antigenic determinant of IA-2 which is localized to 601-762 aa

(Kawasaki et al., 1998). Thus, it will be worthwhile to investigate competition between 76F MoAb and anti-IA-2 sera of type 1 DM patients.

Since these different phage peptide libraries (T7 and M13 pIII) were working well with hybridoma culture supernatant mouse monoclonal antibodies (76B and 76F), it encouraged me to screen these phage peptide libraries with B-cell line culture supernatant human monoclonal antibodies followed by screening these phage libraries with human polyclonal antibodies (patients' sera), as described in chapter 4.

CHAPTER FOUR

HUMAN ANTIBODIES

4 Human Antibodies

4.1 Human Monoclonal Antibodies

4.1.1 b96.11 Human Monoclonal Antibody

4.1.1.1 Introduction

Venous blood was obtained from a nondiabetic APS-II patient, who had been treated with radioiodine for Graves' disease complicated by ophthalmopathy and pretibial myxoedema. He was positive for multiple autoantibodies, including thyroid stimulating hormone (TSH) receptor, thyroid peroxidase, gastric parietal cells and ICA, and he was strongly positive for GAD-65 and GAD-67 autoantibodies. Peripheral blood lymphocytes (mononuclear cells) were isolated and immortalized with Epstein-Barr virus (EBV). The mononuclear cells were infected by overnight culture with infectious EBV supernatant from the B-95 marmoset cell line, and the IgG-secreting B cells were isolated by magnetic separation on beads coated with anti-human IgG. These IgG-secreting B cells were plated in complete medium and screened for antibodies to GAD-65, cloned and expanded (Tremble et al., 1997).

b96.11 is an IgG1 human monoclonal antibody (huAb) derived from the above patient (Tremble et al., 1997). Culture supernatant of the EBV-transformed B-cell line producing the b96.11 human monoclonal antibody, which recognizes amino acid residues between 308-365 on GAD-65 but not on GAD-67 (Schwartz et al., 1999), was kindly supplied by Dr J Paul Banga (Department of Medicine, King's College

School of Medicine, London, UK). It is noteworthy that the b96 line huAb recognizes amino acid residues between 514-570 on GAD-65 (Powers et al., 1999) and thus is different from the epitope of b96.11 clone huAb described here. The reason for this is not clear but may relate to the isolation of a cell clone with a different specificity to the original cell line.

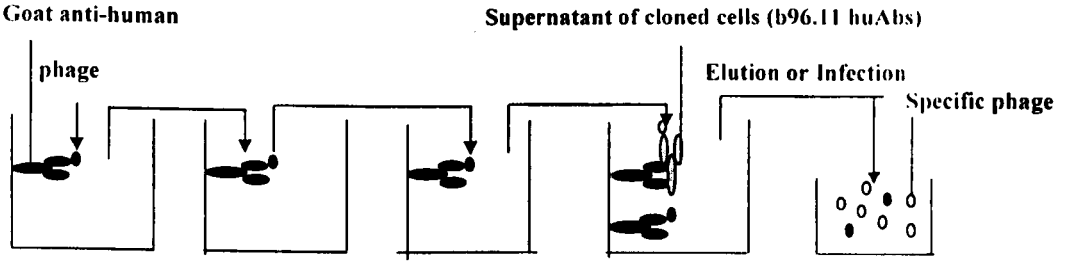
Since M13 pIII (linear 12-mers) was working well with hybridoma culture supernatant mouse monoclonal antibodies (76B and 76F: section 3.3), this library was screened with culture supernatant of the EBV-transformed B cell line producing b96.11.

4.1.1.2 Results

4.1.1.2.1 Sequences of Phagotopes Isolated by Bio-panning of M13 pIII Phage Peptide Library (linear 12-mers) and Immuno-blotting Assay with b96.11 Human Monoclonal Antibody.

Negative selection was performed as described in section 3.3.2.2.1 (Fig. 4.1). Then, M13 pIII phage library (linear 12-mers) was screened by 3 rounds of immunobiopanning against b96.11 huAb as described in section 2.2.2. Goat anti-human IgG (Fc specific) (Sigma) was used to capture b96.11 huAb. The number of plaques obtained in each round during plating increased consecutively, indicating that the biopanning rounds were successful (Table 3.1).

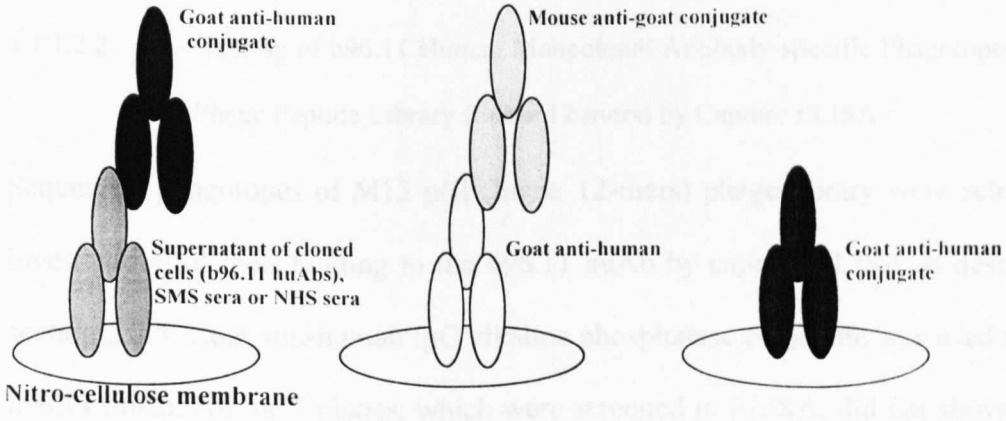
Figure 4.1 Schematic Representation of Negative Selection of Specific Phage Peptides by using Goat anti-human IgG Antibodies.



The specific plaques from the third round of M13 pIII phage library (linear 12-mers) screening with the b96.11 huAb were selected by immuno-blotting assay as described in section 2.2.3. About 12% of these showed high affinity, giving moderate blue spots on the nitrocellulose membrane, to b96.11 huAb. Goat anti-human (Fc specific) alkaline phosphatase conjugate (Sigma) was used to detect b96.11 huAb. Also, the specificity of the assay was confirmed by investigating the binding of the

goat anti-human IgG with plaques on duplicate membrane, which was detected by mouse monoclonal anti-goat IgG clone GT-34 alkaline phosphatase conjugate (Sigma) as described in section 3.3.2.2.2. Also, the specificity of the assay was further confirmed by investigating the binding of the goat anti-human IgG alkaline phophatase conjugate with plaques on another duplicate membrane. This was to exclude the false positive detection of plaques which showed binding with either conjugated and/or unconjugated goat anti-human IgG (Fig. 4.2). About 10% of these plaques (clones) showed high affinity to goat anti-human IgG, giving pale blue spots on the nitrocellulose membranes.

Figure 4.2 Schematic Representation of Immuno-blotting Assay to Exclude the False Positive Binding of Ligands with non-specific Phage Peptides.



Seven clones specific to b96.11 huAb were sequenced successfully. These clones showed a motif of isoleucine (3/7 clones) or valine (1/7 clones) followed by serine (3/7 clones) or threonine (4/7 clones) followed by alanine (1/7 clones), glycine (1/7 clones) or leucine (1/7 clones) followed by threonine (3/7 clones) or serine (4/7 clones) followed by alanine (2/7 clones) or leucine (2/7 clones). Thus, the main motif of the b96.11 huAb is V/I-S/T-A/G/L-T/S-A/L. This is similar to amino acid residues

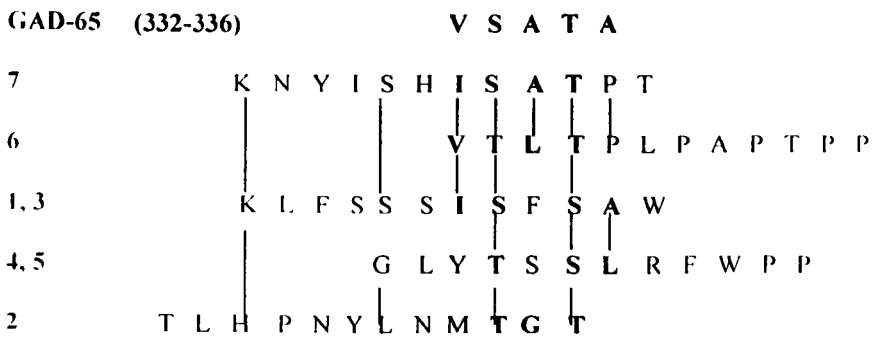
332-336 of GAD-65 (V-S-A-T-A), with conservative substitutions (Fig. 4.3a). Threonine and serine have aliphatic hydroxyl side chains and thus are similar residues. Also, alanine, glycine, leucine, valine and isoleucine have aliphatic side chains and thus are similar residues. However, these 7 clones can also be aligned to show another motif of serine (4/7 clones) followed by threonine (2/7 clones) or serine (5/7 clones) followed by glycine (1/7 clones), alanine (1/7 clones), leucine (3/7 clones) or isoleucine (2/7 clones). Thus, the other motif of the b96.11 huAb is S-T/S-G/A/L/I. This is similar to amino acid residues 338-340 of GAD-65 (T-T-V), with conservative substitutions (Fig. 4.3b). Since the other motif of b96.11 huAb is very close to the previous motif, the overall motif of b96.11 huAb may require amino acid residues 332-340 of GAD-65.

4.1.1.2.2 Testing of b96.11 Human Monoclonal Antibody-specific Phagotopes of M13 pIII Phage Peptide Library (linear 12-mers) by Capture ELISA

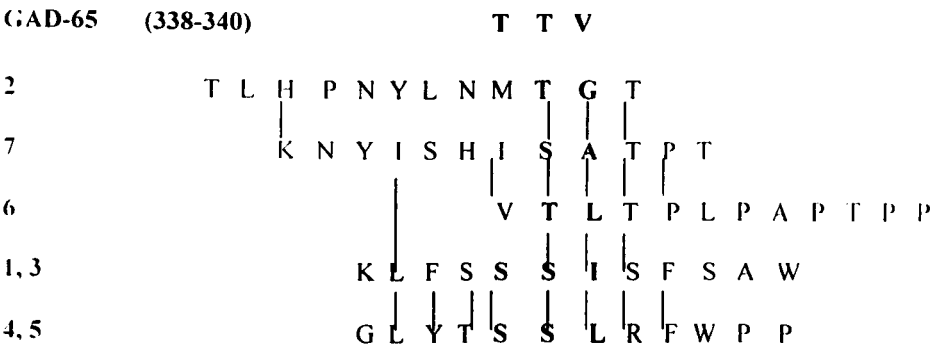
Sequenced phagotopes of M13 pIII (linear 12-mers) phage library were selected for investigation of their binding to the b96.11 huAb by capture ELISA as described in section 2.2.5. Goat anti-human IgG alkaline phosphatase conjugate was used to detect b96.11 huAb. All the 7 clones, which were screened in ELISA, did not show binding with the b96.11 huAb, compared with the negative controls (b78 huAb and WT phage) (data not shown). In particular, high optical density readings of binding of b96.11 huAb and the negative control antibody (b78 huAb) were given with WT phage as well as the selected phage clones. Thus, because of this high background reactivity with WT phage, it was not possible to demonstrate specific binding to the specific phage.

Figure 4.3 Sequences of Peptides Selected from M13 pIII (linear 12-mers) Phage Peptide Library by Binding to b96.11 Human Monoclonal Antibody.

a)



b)



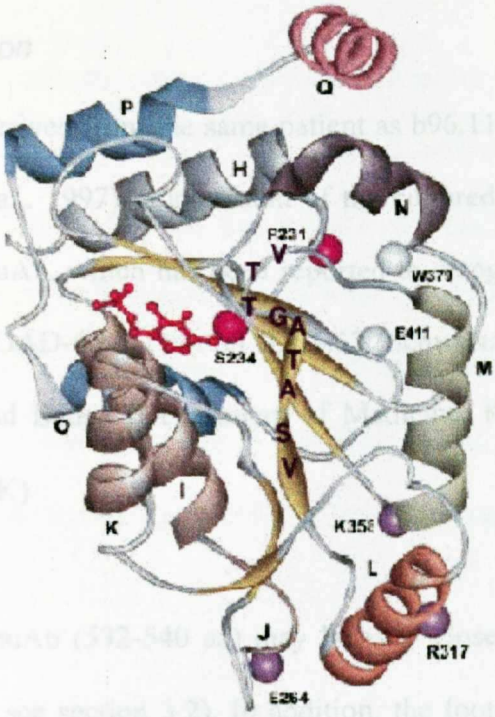
4.1.1.3 Discussion

Seven clones, which were obtained from the third round of successful biopanning of the M13 pIII (linear 12-mers) phage library and were moderately immunostained in the blotting assay with the b96.11 huAb, showed a motif of I/V-T/S-A/G/L-T/S-A/L (Fig. 4.3a), which might correspond to 332-336 aa of GAD-65 (V-S-A-T-A), and another motif of S-T/S-G/A/L/I (Fig. 4.3b), which might correspond to 338-340 aa of GAD-65 (T-T-V) as important epitopic determinants of the b96.11 huAb. Thus, the overall motif of b96.11 might correspond to 332-340 aa of GAD-65, which is within the region of GAD65 (308-365 aa) to which binding of b96.11 huAb is abolished when replaced by the GAD-67 sequence (Schwartz et al., 1999).

All these seven b96.11-specific clones obtained from the M13 pIII (linear 12-mers) phage library were screened in ELISA but b96.11 huAb gave high optical density readings with both selected phage and the negative control (WT phage). In addition, negative control antibody (b78 huAb) also bound to the phage selected with b96.11 and bound to the WT phage in this assay. The reason for this non-specific reactivity is unclear.

The three-dimensional model of the middle region of a GAD65 dimer (211-460 amino acid residues) was built by using ornithine decarboxylase (1ORD) as a template. The PLP-binding middle region consists of a seven-stranded β sheet surrounded by seven α-helices (Schwartz et al., 1999). In Schwartz's model, amino acid residues 332-340 aa, are present within a β strand between α-helix L (313-324 aa) and α-helix M (348-356 aa) (Fig. 4.4), which is also present in the same region of Myers's model (Myers et al., 2000).

Figure 4.4 The Three-dimensional Model of the Middle Region of GAD-65 shows that the motif of b96.11 Human Monoclonal Antibody, 332-340, which is present within a β strand between α-helix L and α-helix M (Schwartz et al., 1999).



4.1.2 b78 Human Monoclonal Antibody

4.1.2.1 Introduction

The b78 huAb was derived from the same patient as b96.11 huAb (see above section 4.1.1.1) (Tremble et al., 1997). Supernatant of the cultured EBV-transformed B-cell line producing b78 huAb, which has been reported to recognize amino acid residues between 532-540 on GAD-65 but not on GAD-67 (Schwartz et al., 1999), was kindly supplied by Dr J Paul Banga (Department of Medicine, King's College School of Medicine, London, UK).

The epitope of b78 huAb (532-540 aa) may be very close to the proposed GAD-6 epitope (523-528 aa; see section 3.2). In addition, the footprint pattern of protected trypsinized GAD-65 fragments with GAD-6 is similar to that with b78 (Tremble et al., 1997). Thus, cross-inhibition of binding to GAD-65 between GAD-6 and b78 was examined in terms of b78 huAb inhibition of GAD-6 binding to GAD-65 as well as the converse of GAD-6 inhibition of b78 huAb binding to GAD-65.

In circular dichroism studies, the structure of GAD seems to change significantly according to whether it has bound to pyridoxal phosphate or not (Chen, et al., 1998). Furthermore, the rat brain preparation probably contains GAD in various forms, i.e. monomeric, dimeric and also multimers (Trigwell et al., in preparation). The different monoclonal antibodies (b78 or b96.11 huAb and GAD-6) may react preferentially with different GAD fractions of the preparation. If so, cross-inhibitions may not work very well. Thus, the results of the cross-inhibition studies were confirmed by exposing

b78 or b96.11 huAb to rat brain GAD which was captured by GAD-6 in the solid phase.

It is noteworthy that I did attempt to screen the b78 huAb with M13 pIII (linear 12-mers) phage library following similar procedure as with b96.11 but no b78 huAb-specific clones were selected, since the number of phage plaques increased from round to another. The possible reason is no b78 huAb-specific peptides in this library as happened with GAD-6 screened with M13 pIII phage library. While the increasing number of the plaques is possibly due to specificity of these plaques to anti-human IgG antibody, Fc portion of b78 huAb or BSA (blocking reagent).

4.1.2.2 Materials and Methods

4.1.2.2.1 Direct Binding of b78 and b96.11 Human Monoclonal Antibodies and GAD-6 to Rat Brain GAD by Direct and Capture ELISA

4.1.2.2.1.1 Direct ELISA (Rat Brain GAD Coating)

Semi-purified rat brain GAD was prepared as previously described (Davenport et al., 1998). The GAD preparation was diluted to 40μg total protein per ml in 0.05M sodium carbonate/sodium bicarbonate buffer pH 9.6 (coating buffer), and coated onto wells (50μl/well) of maxisorp ELISA plates. Plates were incubated at 4°C overnight. Wells were washed 3 times (200μl/well) in phosphate buffer saline (PBS) containing 0.1% Tween-20 (PBS-T) (each time the plates were incubated 3 minutes at room temperature), and then blocked with 2% BSA in PBS (2% BSA/PBS) (200μl/well) for one hour at room temperature. Blank wells, which were not coated with GAD65, were also blocked with 2% BSA/PBS, as above. Then, GAD-6 (20, 10, 5, 2.5 or 1.25 μg/ml) or a supernatant of cultured EBV-transformed B-cell line producing human monoclonal antibody b78 or b96.11 (diluted 1:2, 1:4, 1:8, 1:16 or 1:32), in 1% BSA in PBS-T (1% BSA/PBS-T) was added to the wells. Each dilution was applied (50μl/well) to antigen-coated and blank wells for 2 hours at room temperature on a shaker. Following 3 washes in PBS-T, sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate or goat anti-human IgG (Fc specific) alkaline phosphatase conjugate (diluted 1:1000 in 1% BSA/PBS-T) was added to all wells (50μl/well) and incubated for one hour at room temperature on a shaker. Wells were washed 3 times in PBS-T and pNPP substrate in diethanolamine buffer was added to

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temperature on a shaker. Wells were washed 3 times in PBS-T, and pNPP substrate in diethanolamine buffer was added to all wells (100µl/well) and incubated at room temperature. Plates were read at 30 and 60 minutes at 405 nm on a microtitre plate reader (Molecular Devices). The mean OD of the test wells was corrected by subtracting the mean OD of the equivalent blank wells.

4.1.2.2.2 Competition between GAD-6 and b78 or b96.11 Human Monoclonal Antibody for Binding to GAD in ELISA

4.1.2.2.2.1 Inhibition of GAD-6 Binding by b78 or b96.11 Human Monoclonal Antibody

N-terminal MoAb (diluted 1:200) in coating buffer, was coated onto wells (100µl/well) of maxisorp ELISA plates. Plates were incubated at 4°C overnight. Wells were washed 3 times in PBS-T and then blocked with 2% BSA/PBS (200µl/well) for one hour at room temperature. Blank wells, which were also coated with N-terminal MoAb, were also blocked with 2% BSA/PBS, as above. Then, semi-purified rat brain GAD (40µg/ml) in 1% BSA/PBS-T was added to all wells and incubated for 2 hours at room temperature on a shaker. Following 3 washes in PBS-T, the supernatant of cultured EBV-transformed B-cell line producing human monoclonal antibody b78 or b96.11 (diluted 1:2), in 1% BSA/PBS-T, was applied (100µl/well) to all wells for 2 hours at room temperature on a shaker. Then, without washing the wells, GAD-6 (0.25 µg/ml) mixed with the supernatant of cultured EBV-transformed B-cell line producing human monoclonal antibody b78 or b96.11 (diluted 1:2), in 1% BSA/PBS-T, was applied (100µl/well) in duplicate to test (not blank)

wells for 2 hours at room temperature on a shaker. One percent of BSA/PBS-T was applied (100µl/well) in duplicate to the blank wells (i.e. without GAD-6) for 2 hours at room temperature on a shaker. Following 3 washes in PBS-T, rat anti-mouse IgG2a monoclonal antibody alkaline phosphatase conjugate (diluted 1:1000 in 1% BSA/PBS-T) was added to all wells (100µl/well) and incubated for one hour at room temperature on a shaker. Wells were washed 3 times in PBS-T, and pNPP substrate in diethanolamine buffer was added to all wells (100µl/well) and incubated at room temperature. Plates were read at 30 minutes at 405 nm on a microtitre plate reader (Molecular Devices). The mean OD of the test wells was corrected by subtracting the mean OD of the equivalent blank wells. The results were compared with the ODs given for binding of GAD-6 in the absence or b78 or b96.11.

4.1.2.2.2.2 Inhibition of b78 or b96.11 Human Monoclonal Antibody Binding by GAD-6

N-terminal MoAb (diluted 1:200) in coating buffer, was coated onto wells (100µl/well) of maxisorp ELISA plates. Plates were incubated at 4°C overnight. Wells were washed 3 times in PBS-T and then blocked with 2% BSA/PBS (200µl/well) for one hour at room temperature. Blank wells, which were also coated with N-terminal MoAb, were also blocked with 2% BSA/PBS, as above. Then, semi-purified rat brain GAD (40µg/ml) in 1% BSA/PBS-T was added to all wells and incubated for 2 hours at room temperature on a shaker. Following 3 washes in PBS-T, GAD-6 (0.5 µg/ml), in 1% BSA/PBS-T, was applied (100µl/well) to all wells for 2 hours at room temperature on a shaker. Then, without washing the wells, GAD-6 (0.5 µg/ml) mixed with the supernatant of cultured EBV-transformed B-cell line

producing human monoclonal antibody b78 or b96.11 (diluted 1:2), in 1% BSA/PBS-T, was applied (100µl/well) in duplicate to test (not blank) wells for 2 hours at room temperature on a shaker. One percent of BSA/PBS-T was applied (100µl/well) in duplicate to the blank wells (i.e. without b78 or b96.11) for 2 hours at room temperature on a shaker. Following 3 washes in PBS-T, goat anti-human IgG (Fc specific) alkaline phosphatase conjugate (diluted 1:1000 in 1% BSA/PBS-T) was added to all wells (100µl/well) and incubated for one hour at room temperature on a shaker. Wells were washed 3 times in PBS-T, and pNPP substrate in diethanolamine buffer was added to all wells (100µl/well) and incubated at room temperature. Plates were read at 30 minutes at 405 nm on a microtitre plate reader (Molecular Devices). The mean OD of the test wells was corrected by subtracting the mean OD of the equivalent blank wells. The results were compared with the ODs given for binding of b78 or b96.11 in the absence of GAD-6.

4.1.2.2.3 Direct Binding of b78 and b96.11 Human Monoclonal Antibodies to Rat Brain GAD Captured by GAD-6 in ELISA

GAD-6 (1µg/ml) or N-terminal MoAb as a control (diluted 1:200) in coating buffer, was coated onto wells (100µl/well) of maxisorp ELISA plates. Plates were incubated at 4°C overnight. Wells were washed 3 times in PBS-T and then blocked with 2% BSA/PBS (200µl/well) for one hour at room temperature. Blank wells, which were also coated with GAD-6 or N-terminal MoAb, were also blocked with 2% BSA/PBS. Then, semi-purified rat brain GAD (40µg/ml) in 1% BSA/PBS-T was added to all wells and incubated for 2 hours at room temperature on a shaker. Following 3 washes in PBS-T, a supernatant of cultured EBV-transformed B-cell line producing human

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4.1.2.3 Results

4.1.2.3.1 Direct Binding of GAD-6 Mouse Monoclonal Antibody or b78 or b96.11 Human Monoclonal Antibody to Native or Denatured Rat Brain GAD

Supernatants of cultured EBV-transformed B-cell lines producing human monoclonal antibodies, b78 and b96.11, did not bind to semi-purified rat brain GAD after applying these human monoclonal antibodies (huAb) to the GAD-coated wells even in low dilution (1:2) (Table 4.1). Also, there was no significant differences within the serial dilutions of b78 or b96.11 huAb. In contrast, GAD-6 bound to the GAD-coated wells even in low concentration (1.25 μg/ml) with significant differences within the serial dilutions of GAD-6 (Table 4.1). This is probably because direct binding of GAD to the plastic wells partially denatures the GAD such that the epitopes of b78 and b96.11 (but not GAD-6) are disrupted.

On the other hand, b78 and b96.11 huAb, bound to semi-purified rat brain GAD after applying these human monoclonal antibodies (huAb) to the GAD-65 that was captured by N-terminal MoAb-coated wells, even in high dilution (1:64). Also there was significant differences within the serial dilutions of b78 (not b96.11) huAb. Whereas, GAD-6 bound also to the native GAD even in very low concentration (0.625 μg/ml) (Table 4.1), also with significant differences within the serial dilutions of GAD-6. This indicates that b78 or b96.11 huAb recognize conformational epitopes at the C-terminus on native GAD since capturing the GAD with N-terminal MoAb should maintain its conformation, but not on denatured GAD. GAD-6 recognizes an epitope at the C-terminus on native GAD more efficiently than on denatured GAD. Another difference between the two ELISAs is that the capture system will purify the GAD in the rat brain preparation by binding to the N-terminal MoAb, whereas the

GAD will have to compete for binding to the wells with other brain proteins in the direct-coating system.

4.1.2.3.2 Competition between GAD-6 Mouse Monoclonal Antibody and b78 or b96.11 Human Monoclonal Antibody Directed to the C-terminus of Native Rat Brain GAD

GAD-6 did not greatly inhibit b78 and b96.11 huAb binding to native semi-purified rat brain GAD, which was captured by N-terminal MoAb: inhibition was only 14% and 13%, respectively, in a competition ELISA (Table 4.2). Conversely, b78 and b96.11 huAb did not greatly inhibit GAD-6 binding to native GAD-65 by 18% and 32%, respectively, in a competition ELISA (Table 4.2). This indicates that b78 and b96.11 may not recognize epitopes which are very close to that of GAD-6.

The different monoclonal antibodies (b78 or b96.11 huAb and GAD-6) may react preferentially with different GAD fractions of the preparation (monomeric, dimeric and also multimers). This could be an alternative explanation as to why the cross-inhibitions did not work very well. Therefore, the results of the cross-inhibition experiments were confirmed by exposing b78 or b96.11 huAb to rat brain GAD which was captured by GAD-6, to avoid false negative results. SMS serum bound equally to GAD captured either by GAD-6 or captured by N-terminal MoAb (Table 4.3). b78 huAb bound slightly better with GAD-6 as the capture MoAb, but b96.11 bound much better with GAD-6 as the capture MoAb (Table 4.3). This suggests that the GAD-6 and b78 or b96.11 epitopes are not directly overlapping.

Table 4.1 Optical Densities for Direct Binding of b78 or b96.11 Human Monoclonal Antibodies or GAD-6 to coated Rat Brain GAD and GAD Which Captured by N-terminal Monoclonal Antibody in ELISA.

Concentr-aion (μg/ml)	GAD-6 Direct Binding to GAD	GAD-6 Binding to Captured GAD	Dilution	b78 Direct Binding to GAD	b78 Binding to Captured GAD	b96.11 Direct Binding to GAD	b96.11 Binding to Captured GAD
20	0.266	0.532	1:2	0.023	0.318	0.013	0.228
10	0.184	0.514	1:4	0.015	0.284	0.006	0.232
5	0.124	0.491	1:8	0.009	0.236	0.005	0.260
2.5	0.095	0.463	1:16	0.006	0.183	0.004	0.226
1.25	0.076	0.435	1:32	0.005	0.151	0.008	0.189
0.625	___	0.412	1:64	___	0.119	___	0.208
Another Plate							
0.5		0.500	1:2		0.434		0.279
0.25		0.413					
0.125		0.343					
0.0625		0.244					
0.03125		0.141					

Table 4.2 Optical Densities for Direct Binding and Competition between GAD-6 and Human Monoclonal Antibodies (b78 or b96.11) Directed to the C-terminus of Native GAD captured by N-terminal Monoclonal Antibody in ELISA.

Monoclonal Antibody	Direct Binding to Native GAD-65	Binding Inhibited by GAD-6 (0.5µg/ml) (% Inhibition)	Binding Inhibited by b78 (Diluted 1:2) (% Inhibition)	Binding Inhibited by b96.11 (Diluted 1:2) (% Inhibition)
At 30 min Incubation				
b78 (Diluted 1:2)	0.651	0.561 (14%)	_____	_____
b96.11 (Diluted 1:2)	0.398	0.348 (13%)	_____	_____
GAD-6 (0.25µg/ml)	0.596	_____	0.486 (18%)	0.399 (32%)
At 60 min Incubation				
b78 (Diluted 1:2)	1.496	1.276 (15%)	_____	_____
b96.11 (Diluted 1:2)	0.972	0.948 (2%)	_____	_____
GAD-6 (0.25µg/ml)	1.307	_____	1.103 (16%)	1.127 (14%)

Table 4.3 Optical Densities for Direct Binding of b78 or b96.11 Human Monoclonal Antibodies or SMS Serum (DH) to Native GAD captured by either N-terminal Monoclonal Antibody or GAD-6 in ELISA.

Polyclonal Antibody	Direct Binding to Native GAD Captured by N-terminal MoAb	Direct Binding to Native GAD Captured by GAD-6 MoAb	GAD-6/N-terminal MoAb Capture Ratio for Binding
At 15 min Incubation			
SMS Serum (DH)	0.931	1.024	1.10
b78 (1:2)	0.668	0.826	1.24
b96.11 (1:1)	0.117	0.296	2.53
At 30 min Incubation			
SMS Serum (DH)	2.521	2.710	1.07
b78 (1:2)	1.665	2.201	1.32
b96.11 (1:1)	0.309	0.849	2.75

4.1.2.4 Discussion

GAD-6 recognizes the C-terminus of both native and denatured GAD-65, indicating that the GAD-6 epitope may have both conformational and linear properties. b78 and b96.11 huAb recognize the C-terminus of native (not denatured) GAD-65, indicating that these huAbs recognize conformational (not linear) epitopes on GAD-65.

GAD-6 inhibited the binding of b78 and b96.11 huAb to native GAD-65 by only 14% and 13%, respectively. Conversely, b78 and b96.11 huAb inhibited the binding GAD-6 to native GAD-65 by 18% and 32%, respectively, in a competition ELISA (Table 4.2). This indicates that b78 and b96.11 may recognize an epitope, at the C-terminus of native GAD-65, which is not very close to the GAD-6 epitope since the inhibition is not high.

According to Schwartz et al., 1999, the huAb b78 recognizes amino acid residues between 532-540 on GAD-65 which is very close to the GAD-6 epitope (523-528 aa) proposed in this thesis. In addition, the footprint pattern of protected trypsinized fragments of GAD-6 is similar to that of the b78 (Tremble et al., 1997). It is possible that the three amino acids between the epitopes of GAD-6 and b78 may give different orientations to the epitopes which do not give strong steric hindrance (only 14-18% of binding inhibition).

The b78 huAb bound slightly better with GAD-6 as the capture MoAb, but b96.11 bound much better with GAD-6 as the capture MoAb (Table 4.3). This might suggest that the GAD-6 does interfere with b78 huAb binding to GAD-65 more than it does with b96.11 huAb binding. Alternatively, it could suggest that the N-terminal MoAb

inhibits b96.11 huAb binding to some extent. However, it must also suggest that the GAD-6 and b78 huAb epitopes are not directly overlapping, or the ratio of OD for binding to GAD-6 captured GAD divided by OD for binding to N-terminal MoAb captured GAD should be well below 1.00.

The epitope of b78 huAb is present at the extreme C-terminus of α-helix S in the three dimensional model of the C-terminus of GAD-65 (Schwartz et al., 1999). It is noteworthy that the mutation V532K disrupts the binding of b78 to GAD-65, while other mutations in the same region of b78 epitope, R536L and Y540S, do not disrupt the binding of b78 to GAD-65. This indicates that V532 is a critical amino acid residue for the b78 epitope and there are neighbouring amino acid residues which are not essential for b78 binding to GAD-65. Also, it has been suggested that V532 may only be involved in a surface patch of the b78 epitope, which is formed by other amino acid residue(s) from different region(s).

4.1 Human polyclonal antibodies

4.2.1 SMS Patients' Sera

4.2.1.1 Introduction

GAAs are present in 89% of SMS sera. GAAs of SMS and APS sera, which are present at high titre, are less dependent on the conformation of the GAD than in type I DM and commonly target GAD-67 as well as GAD-65 (Tremble et al., 1997). Preincubation of GAAs of SMS sera with 188-442 aa/GAD65 can block the binding of GAAs67 with GAD67, whereas preincubation with GAD67 cannot block the binding of GAAs65 with GAD65 (Daw et al., 1996). This indicates that the GAAs in SMS sera recognize a specific epitope in GAD67, which is highly homologous to 188-442 aa/GAD65, whereas other GAAs65 recognize epitopes which are not present in GAD-67. Furthermore, preincubation of SMS sera with 354-368 aa/GAD65 inhibits the binding of SMS sera with GAD65. In the 354-365 aa/GAD65, there are four amino acid residues K-KI-M which differ from E-NL-L of GAD67 (Li et al., 1994). This suggests that these four amino acid residues (K-KI-M) may contribute to the binding of SMS sera with GAD65. In SMS sera, GAAs recognize some epitopes on GAD65 not recognized by GAAs in IDDM sera, e.g. an N-terminal epitope within 1-16 aa and/or 1-95 aa (SMS E2) (Hagopian et al., 1995; Bjork et al., 1994) as a major antigenic determinant, a middle region epitope 390-403 aa (Daw et al., 1996; Li et al., 1994), and a C-terminal epitope 475-585 aa (SMS E1) as a minor antigenic determinant. The binding of GAAs of some SMS sera with SMS E2 is much higher than with SMS E1.

An immunodominant epitope of T cell reactivity with GAD-65 in non-diabetic SMS patients is 341-351 aa residues. Also, in non-diabetic SMS patients, T cells can react with N-terminal regions (61-90 and 191-220 aa) and C-terminal regions (491-520 aa) of GAD-65 (Schloot et al., 1999).

The sequences obtained by screening M13 pIII (linear 12-mers) phage peptide library with supernatant of cultured EBV-transformed B-cell line producing b96.11 huAb, suggested that this was a good library to screen with SMS patients' sera containing human polyclonal antibodies to GAD. The protocol adopted for screening the library with SMS patients' sera is shown schematically in Fig. 4.5.

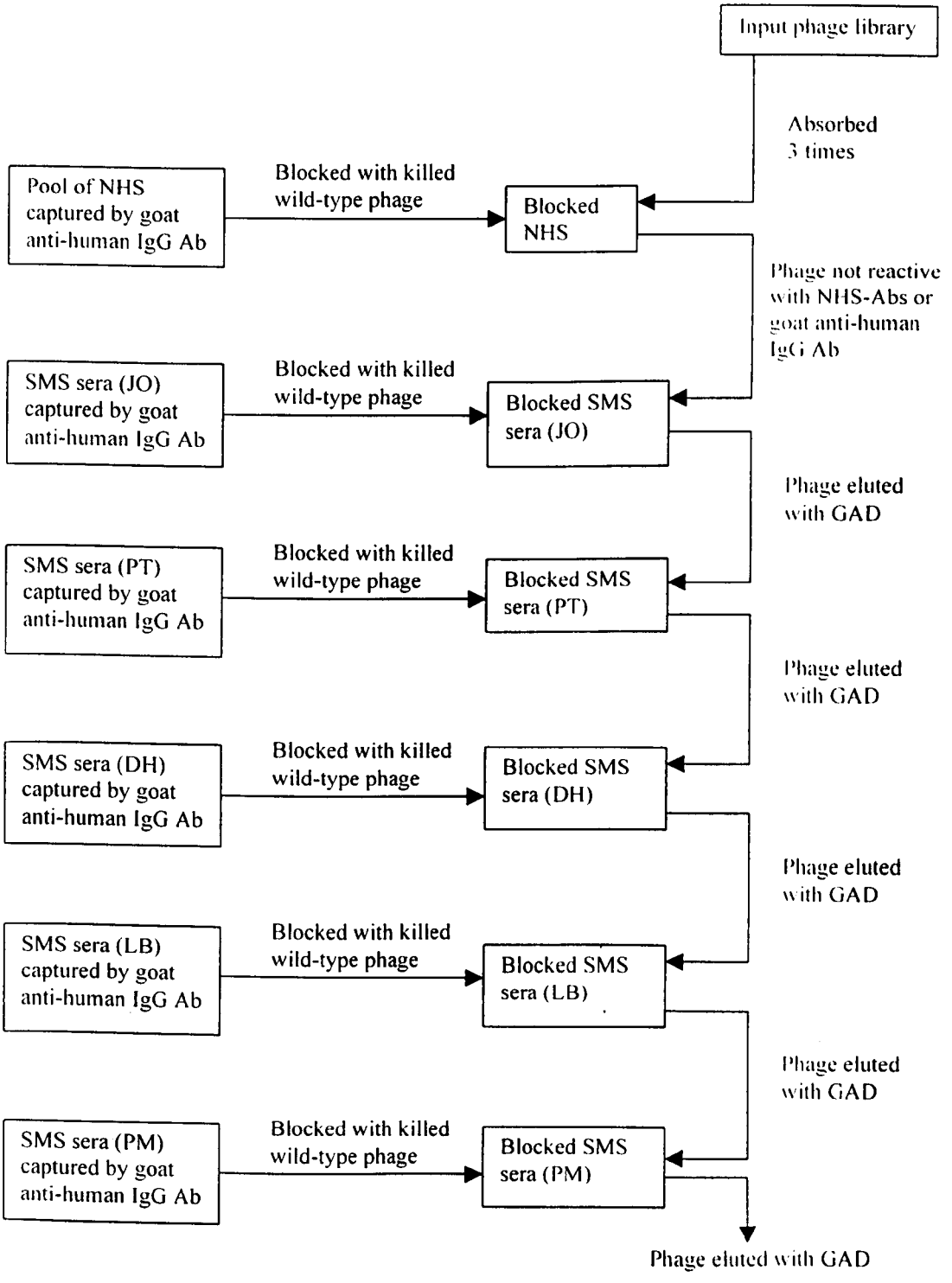
Since SMS patients' sera contain polyclonal antibodies, these sera have autoantibodies which recognize GAD but also many other antibodies which are also present in normal human sera (NHS). Some of the input phage might be specifically recognized by the GAD-65-specific autoantibodies binding to their peptide sequences, but others might be bound via their inserted peptide sequences to polyclonal antibodies which are also present in NHS. Thus, first of all, NHS pooled from different donors was incubated overnight with M13K07 killed phage (wild-type phage) to block any phage-reactive antibodies. Then, the phage-blocked NHS was exposed to the input phage library through three rounds of biopanning to deplete phage bearing peptides reactive with normal polclonal antibodies. The depleted phage were then exposed to SMS patients' sera, which had also been pre-incubated with killed wild-type phage to block the binding of phage-reactive antibodies, which could be present in patients' sera as well as NHS.

The patients' sera or NHS were captured on the immunotubes by goat anti-human IgG polyclonal antibody, which may itself recognize some phage peptides non-specifically. However, since the input phage were depleted with NHS antibodies, as described above, which were themselves captured by goat anti-human IgG, this may also have depleted input phage reactive with the goat anti-human IgG (Fig. 4.5).

Several SMS patients' sera were used in consecutive biopannings of the phage library to try to select public epitopes. DH, PM, LB, JO and PT sera were obtained from GAAs+ SMS patients. LB was the only diabetic SMS patient.

Rat brain GAD was used during biopanning to elute the bound phage (rather than acid elution) to try to increase the specificity of the selection. Also, the GAD elution was performed to select for peptides mimicking conformational epitopes.

Figure 4.5 Schematic Diagram Showing the Protocol for Immuno-panning of SMS patients' Sera with M13 pIII (linear 12-mers) Phage Peptide Library, preceded by the Negative Selection of these Sera.



4.2.1.2 Materials and Methods

4.2.1.2.1 Screening of SMS Patients' Sera with M13 pIII (linear 12-mers) Phage Peptide Library

4.2.1.2.1.1 Preparation of Killed Wild-type Phage

One hundred microlitres of M13K07 wild-type phage were added to 500mls of an early-log phase E.coli ER 2537 culture (OD_{600}=0.05) and incubated on a shaker at 37°C for 4.5 hours for amplification of the M13K07 wild-type phage. The amplified phage were precipitated and purified as described in section 2.2.1.8. The purified M13K07 phage were spotted onto a Petri-dish as drops (25µl each drop) and then exposed 3 times to UV=120,000 µJ(micro Jole)/cm^2 (Prezzi et al., 1996), to kill the phage which were then tested by plating them as described them in section 2.2.1.7.

4.2.1.2.1.2 Preparation of Semi-purified Rat Brain GAD

Rat brain homogenate was prepared as described previously (Davenport et al., 1998) and enriched for GAD by using fast protein liquid chromatography (FPLC) system (by Miss S Hyde of the Institute of Infections and Immunity, Queen's Medical Centre, Nottingham). A Q Sepharose Fast Flow column was connected to a FPLC system and washed with 20mM Tris HCl pH:7.5 at 4°C. Then, 4mls of rat brain GAD was applied to the column. A salt gradient was set up by eluting in 20mM Tris HCl pH:7.5, 1M NaCl to give 61 2mls fractions. The last 26 fractions, which show high concentration of proteins, were tested for GAD by direct ELISA (direct binding of GAD-6 to these 26 fractions, which were coated onto wells of a maxisorp ELISA

plate). The fractions, which showed the highest concentration of GAD, were pooled and used to elute the SMS patients' antibodies specific for M13 pIII phage peptides.

4.2.1.2.1.3 Bio-panning of SMS Patients' Sera with M13 pIII (linear 12-mers) Phage Peptide Library

Goat anti-human IgG (Fc specific) (Sigma) (0.5μg/ml) in coating buffer, was coated onto four Nunc immuno-tubes. Following overnight incubation at 4°C on a rotator, all the Nunc immuno-tubes were washed 5 times in 0.1% TBS-T and blocked with 0.5% BSA and 0.02% NaN_3 in TBS (blocking solution) for one hour at room temperature on a rotator. Following 6 washes, 25μl of three different pools of NHS (nine different NHS in each pool) (diluted 1:100 in 0.1% BSA/TBS) were added to three of these Nunc immuno-tubes (called first, second, or third tube), i.e one pool in each tube, and incubated at 4°C overnight on a rotator. Twenty five microlitres of a SMS patient's serum (diluted 1:100 in 0.1% BSA/TBS) were added to the fourth Nunc immuno-tube (test tube) and incubated overnight at 4°C on a rotator.

After 6 washes of the four Nunc immuno-tubes, 1μl of UV light killed phage M13K07 ($(1\times10^{12}$ pfu/1μl) (prepared as described above in section 4.2.1.2.1.1) diluted in 0.1% TBS-T/0.01% BSA was added to each of them and incubated at 4°C for 4 hours on a rotator. Ten microlitres of the M13 pIII (linear 12-mers) phage library (1×10^{11} pfu/10μl), as an input phage, were added to the first tube, coated with a pool of NHS, and incubated at 4°C overnight on a rotator. The pre-absorbed M13 pIII phage were transferred from the first Nunc immuno-tube to another Nunc immuno-tube (second tube), which was coated with a different pool of NHS and also

incubated at 4°C overnight on a rotator. The pre-absorbed M13 pIII phage were transferred from the second Nunc immuno-tube to another Nunc immuno-tube (third tube), which also was coated with a different pool of NHS, and incubated at 4°C overnight on a rotator. The pre-absorbed M13 pIII phage were transferred from the third Nunc immuno-tube to the test Nunc immuno-tube (test tube), which had SMS patient's serum coated on it, and incubated at 4°C overnight on a rotator (Fig. 4.5).

Following 10 washes of the test tube, 1ml of rat brain GAD (prepared as described previously in section 4.2.1.2.1.2) was added and incubated on a rotator at room temperature for one hour. The non-amplified eluate was collected. One microlitre of the non-amplified eluate was taken for dilution and plating (as described in sections 2.2.1.6 and 2.2.1.7, respectively). The rest of the eluate was subjected to the next (second) round of affinity selection with a different patient's serum following the same procedure, without amplification. Following a third round of affinity selection, the eluate was amplified by adding it to 20mls of early-log phase E.coli ER 2537 culture (OD_{600}=0.05) and incubated on a shaker at 37°C for 4.5 hours for amplification. The amplified eluate of the third round was concentrated and purified (as described in section 2.2.1.8), and was subjected to the next round (fourth round) of affinity selection with a different patient's serum following the same procedure without amplification. Five rounds were carried out; each round employed a different SMS serum.

4.2.1.2.1.4 Detection of SMS Patients' Sera-specific M13 pIII (linear 12-mers) Phage Peptide Clones by Immuno-blotting Assay

This was performed as described in section 2.2.3 to detect the specific phage peptide clones of the third, fourth and fifth rounds of the biopanning. Fifty microlitres of patient's serum diluted 1:100 in 5% BSA /TBS-T, which were pre-incubated with 100µl of sonicated E.coli ER 2537 lysate (see section 2.2.1.10) and 10µl of M13K07 (1×10^{13} pfu/µl) at room temperature for two hours on a rotator, were added to the membrane and detected by applying goat anti-human IgG (Fc specific) alkaline phosphatase conjugate.

Also, the specificity of the assay was confirmed by using goat anti-human IgG and NHS, which was detected by anti-goat IgG clone GT-34 alkaline phosphatase conjugate and goat anti-human IgG (Fc specific), respectively, precisely as described in section 3.3.2.2.2. This was to exclude the false positive binding of the SMS patient's serum with plaques which showed binding with either NHS, conjugated goat anti-human IgG and/or unconjugated goat anti-human IgG (Fig. 4.2).

4.2.1.2.1.5 Purification and Sequencing of SMS patients' Sera-specific M13 pIII (linear 12-mers) Phage Insert

This was performed as described in section 2.2.4.

4.2.1.2.1.6 Detection of SMS Patients' Sera-specific M13 pIII (linear 12-mers) Phage Peptide Clones by Direct ELISA

The clones of selected phage, and unselected phage clones, were amplified by adding them to 5mls of the early-log phase E.coli ER 2537 culture (OD_{600}=0.05) and incubated on a shaker at 37°C for 4.5 hours. The amplified phage were concentrated and purified as described in section 2.2.1.8.

The purified phage clones or helper M13, in coating buffer (50µl phage with 50µl coating buffer), were coated onto wells (100µl/well) of maxisorp ELISA plates (Life Technologies, UK). Plates were incubated at 4°C overnight. Wells were washed 3 times (200µl/well) in 0.1% TBS-T, each time the plates were incubated 3 minutes at room temperature. The wells were then blocked with 1% BSA/TBS (120µl/well) for one hour at room temperature. Blank wells, which were not coated with phage, were also blocked with 1% BSA/TBS, as above. The SMS patients' sera or normal sera diluted 1:100 in 1% BSA/TBS and pre-incubated with 1µl of sonicated E.coli ER 2537 lysate, see above, and 1µl of M13K07 phage (1×10^{13} pfu/µl) were applied (100µl/well) in duplicate to antigen-coated and blank wells for 2 hours at room temperature on a shaker. Following 5 washes goat anti-human IgG alkaline phosphatase conjugate (diluted 1:1000 in 1% BSA/PBS-T) was added to all wells (100µl/well) and incubated for one hour at room temperature on a shaker. Wells were washed 3 times and pNPP substrate was added to all wells (100µl/well) and incubated at room temperature for one hour. Plates were read at 60 min at 405 nm on a micromolecular plate reader (Molecular Devices).

Rabbit anti-fd, sheep anti-M13 or mouse anti-M13 IgG were not used to capture the phage due to strong cross-reaction of all human sera (either normal or patients' sera) with these antibodies (either anti-fd or anti-M13 antibodies), suggesting that there are anti-idiotypes in human sera against anti-fd or anti-M13 IgG (data not shown).

4.2.1.3 Results

4.2.1.3.1 Sequences of Phagotopes Isolated by Bio-panning of M13 pIII Phage Peptide Library (linear 12-mers) and Immuno-blotting Assay with SMS Patients' Sera.

M13 pIII phage library (linear 12-mers) was screened by 5 rounds of immunopanning with SMS patients' sera as described in section 4.2.1.2.1.3. The number of plaques obtained in each round during plating decreased consecutively. This may be due to the loss of non-amplified phage which are specific to one patient's serum, but not to another patient's serum, leading to a decrease in the number of the selected phage.

The specific plaques of 3 different rounds (third, fourth and fifth rounds) of M13 pIII phage library (linear 12-mers) to the SMS patients' sera were selected by immuno-blotting assay as described in section 4.2.1.2.1.4. About 80% of these plaques (clones) showed moderate affinity, giving pale blue spots on the nitrocellulose membranes, to the SMS patients' sera in the third, fourth or fifth round. The plaques showed high affinity neither to goat anti-human IgG nor to normal human sera.

Twenty clones derived from rounds 3, 4 or 5 of bio-panning with SMS patients' sera were sequenced successfully. The 20 clones specific to SMS patients' sera showed two possible motifs depending on how the sequences were aligned. The first motif showed leucine (13/20 clones), valine (1/20 clones), glycine (2/20 clones) or alanine (2/12 clones) followed by alanine (4/20 clones) or isoleucine (1/20 clones) followed by cystine (2/20 clones) or methionine (1/20 clones) followed by threonine (10/20 clones) or serine (7/20 clones) followed by histidine (5/20 clones), arginine (6/20 clones) or lysine (2/20 clones). Thus, one motif of these 20 clones is L/A-A-X-T/S-

R/H/K, corresponding to amino acid residues 371-375 of GAD-65 (L-L-M-S-R), which showed conservative substitutions (Fig. 4.6, group I). Alanine, valine, glycine, isoleucine and leucine have aliphatic side chains and thus are similar residues. In addition, cystine and methionine have sulphur-containing side chains and thus are similar residues. Also, threonine and serine have aliphatic hydroxyl side chains and thus are similar residues. Furthermore, arginine, histidine and lysine have basic side chains and thus are similar residues.

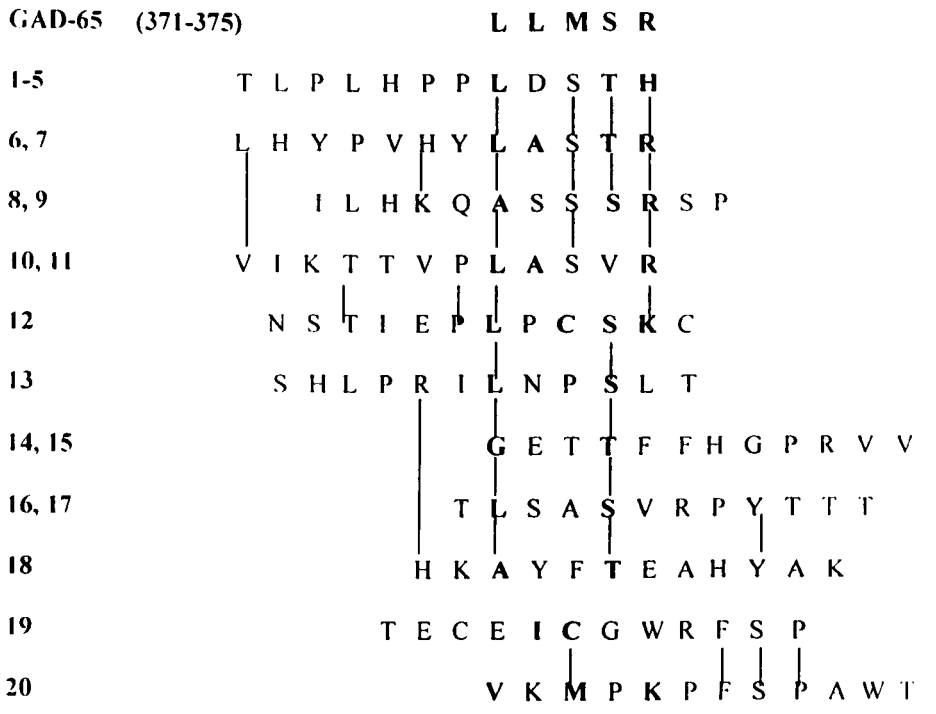
The second motif given by different alignment of the same sequences showed a motif of threonine (5/20 clones) or serine (1/20 clones) followed by threonine (12/20 clones) followed by valine (2/20 clones), isoleucine (1/20 clones) or leucine (7/20 clones) followed by phenylalanine (2/20 clones) followed by glutamate (2/20 clones) followed by leucine (10/20 clones), glycine (2/20 clones), isoleucine (1/20 clones), valine (3/20 clones) or alanine (1/20 clones) followed by histidine (11/20 clones) or lysine (1/20 clones) followed by leucine (1/20 clones) or glycine (1/20 clones) followed by an amino acid residue followed by lysine (3/20 clones) or arginine (6/20 clones). Thus, the other motif of these 20 clones is T/S-T-V/I/L-F-E-L/G/I/V/A-H/K-L/G-X-K/R, corresponding to amino acid residues 463-472 aa of GAD-65 (T-T-G-F-E-A-H-V-D-K), which showed conservative substitutions, see above. (Fig. 4.6, group II).

4.2.1.3.2 Testing of SMS Patients' Sera-specific Phagotopes of M13 pIII Phage Peptide Library (linear 12-mers) by ELISA

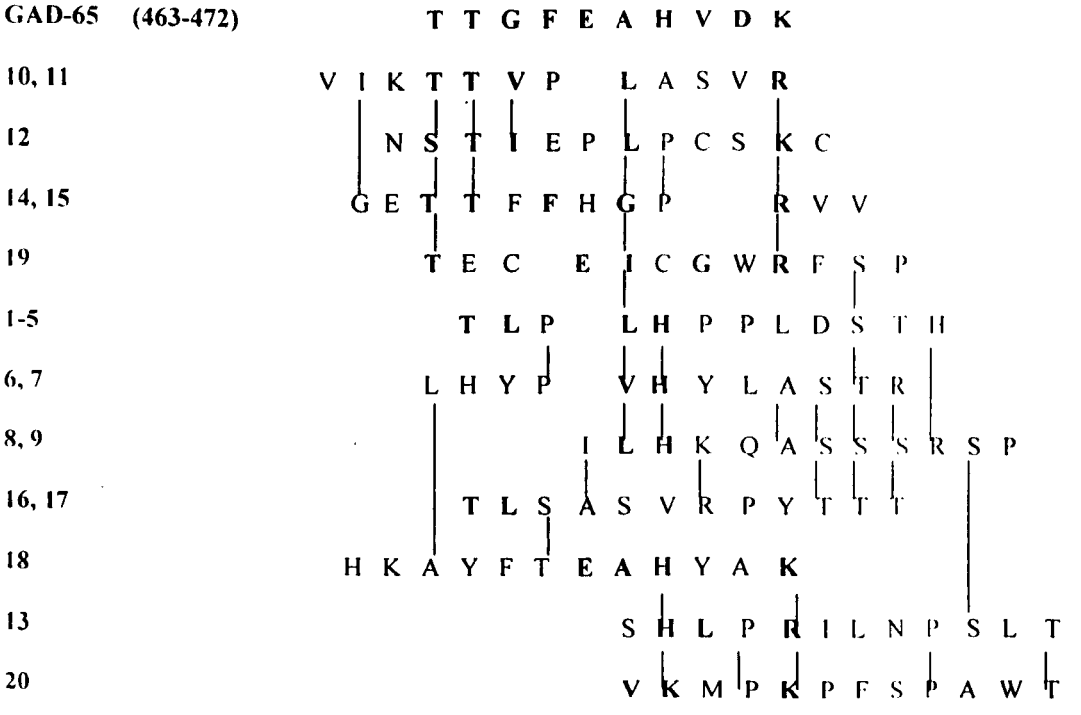
Sequenced phagotopes of M13 pIII (linear 12-mers) phage library were selected for investigation of their binding to the SMS patients' sera by direct ELISA as described in section 3.2.1.2.4. Randomly selected 5 out of these 20 clones, which were screened in ELISA, showed high binding with SMS patients' sera (JO, PT, DH and PT), not LB SMS patient's serum compared to the negative control (normal human serum) (data not shown). In particular, high optical density readings were given with negative control phage (N-terminal MoAb specific clone) as well as the selected phage clones. Thus, because of this high background reactivity with the negative control phage, it was not possible to demonstrate specific binding to the specific phage.

Figure 4.6 Sequences of Peptides Selected from M13 pIII (linear 12-mers) Phage Peptide Library by Binding to Antibodies of SMS Patients' Sera.

Group I:



Group II:



4.2.1.4 Discussion

Twenty clones, which were obtained from the third, fourth and fifth rounds of successful biopanning of the M13 pIII (linear 12-mers) phage library, were positively immunostained in the blotting assay (moderate affinity) with the SMS patients' sera.

The 20 clones showed a motif of L/A-A-X-T/S-R/H/K (Fig. 4.6, group I), which could correspond to 371-375 aa of GAD-65 (L-L-M-S-R), as a public epitope of SMS patients' sera. Also, the 20 clones showed another motif of T/S-T-V/I/L-F-E-L/G/I/V/A-H/K-L/G-X-K/R (Fig. 4.6, group II), which could correspond to 463-472 aa of GAD-65 (T-T-G-F-E-A-H-V-D-K), as another public epitope of SMS patients' sera.

Since SMS patients' sera-bound phage were eluted with rat brain GAD (native GAD) rather than with acid, the phage peptide sequences may represent a conformational, rather than linear, epitope.

Thus, a motif of SMS patients' antibodies may be 371-375 aa, which is present at the C-terminal end of α-helix N in the three-dimensional model of the middle region of a GAD-65 (Schwartz et al., 1999) (Fig. 4.7a). Alternatively, a motif of SMS patients' antibodies could be 463-472 aa, which is present at the extreme N-terminal end of α-helix R (467-486) and the loop region preceding it, in the three-dimensional model of the C-terminal region of a GAD-65 (Schwartz et al., 1999) (Fig. 4.7b, group II).

The proposed group II motif of SMS patients' sera (463-472) has a critical amino acid residue H470 which, when mutated to Q470, affects the binding of MICA-8 and

MICA-9 to GAD-65 (Schwartz et al., 1999). In addition, MICA-1, MICA-3 and DPA (huAbs of type 1 DM patients) are predicted to bind the backside of helix R (Schwartz et al., 1999) which has the predicted public epitope of SMS patients' sera (463-472). Thus, this predicted public epitope of SMS patients' sera may be a major antigenic determinant of type 1 DM patients sera as well as SMS patients' sera.

It should also be noted that the two regions of GAD-65 identified here (371-375 aa and 463-472 aa) are predicted to be very close together in the three-dimensional model of GAD-65 due to the alignment of the middle and C-terminal regions of the molecule.

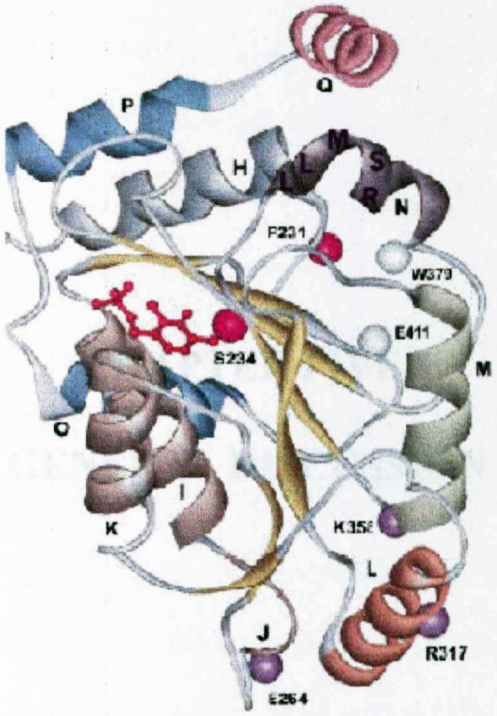
Four out five sera of SMS patients (JO, DH, PT and LB) showed higher binding than negative control serum (NHS) to the selected phage in ELISA (data not shown). However, these four SMS patients' sera gave high optical density readings with both selected phage and the negative control phage (N-terminal MoAb specific clone). The reason for this non-specific reactivity is unclear, but it is possible that the N-terminal MoAb specific clone was not the proper negative control phage in this assay since some SMS sera may recognize the N-terminus of GAD-65 as a major antigenic determinant (Bjork et al., 1994; Kim et al., 1994). Another possibility is that the selected phage may bind to GAD, since these phage were eluted by GAD; this could be tested by determining whether the selected phage bind to GAD itself. Also, it has been suggested that one NHS was not sufficient to control for the specific binding of SMS sera to the selected phage. Although, in immuno-blotting assay, 80% of clones showed specific binding to SMS sera compared to NHS, we cannot be certain that the

selected phage represent disease-specific sequences. Thus we cannot exclude that other NHS might have shown high binding to the selected phage similar to SMS sera.

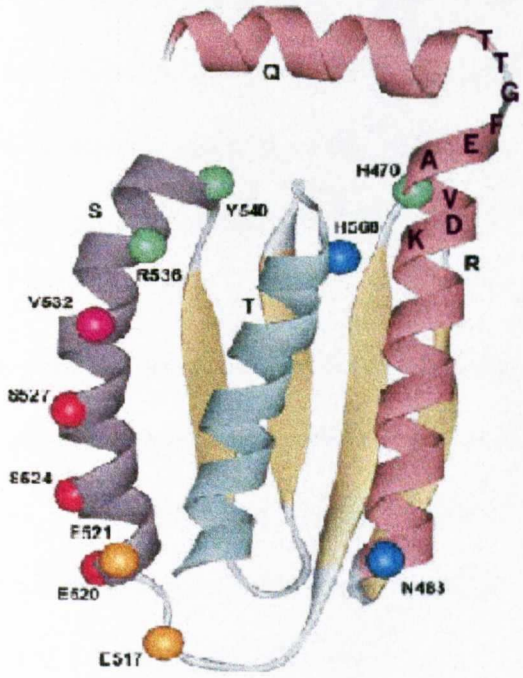
To investigate whether the selected phage represent disease-specific sequences, future work could involve immuno-precipitation assay by exposing the selected phage to the antibodies of SMS sera in a fluid phase, rather than solid phase (which may denature the exposed peptide or facilitate non-specific binding, followed by adding protein-A sepharose to precipitate the antibodies of SMS sera, including the antibody-phage immune complexes. The precipitated phage in the immune complexes could then be detected by their ability to infect E.coli and generate the plaques. Also, other future work could involve the mutation of the suggested regions of GAD to see if this affects the binding of the SMS antibodies. Also, other future work could involve raising mouse monoclonal antibodies (MoAbs) against the selected phage and see if these MoAbs bind to GAD.

Figure 4.7 The Three-dimensional Models of the Middle (a) and C-terminal (b) Regions of GAD-65 (modified from Schwartz et al., 1999) showing the suggested Public Epitopes of SMS Patients' Sera; **a)** 371-375, which is present in the C-terminal end of α-helix N and **b)** 463-472, which is present in the Loop Region at the extreme N-terminal end of α-helix R (Schwartz et al., 1999).

a)



b)



CHAPTER FIVE

GENERAL DISCUSSION

5 General Discussion

5.1 Introduction

Type 1 diabetes mellitus (type 1 DM) is believed to have an autoimmune pathogenesis with a multifactorial etiology leading to destruction of insulin-producing pancreatic islet β cells and insulin deficiency. Many genetic loci are involved in determining genetic susceptibility to the disease, the most important of which is found in the HLA, mainly HLA-DR3/DQ2 and HLA-DR4/DQ8. Environmental factors are also important, especially viruses, such as coxsackie virus B4 (CVB4). Humoral and cellular autoimmune responses to the pancreatic islet β cells can damage these islet β cells and expose many autoantigens. Both subsets of T cells, CD4+ T helper 1 (Th1) cells and CD8+ cytotoxic T cells (Tc), and macrophages are involved in the cellular autoimmune response to the pancreatic islet β cells. Autoantibodies (IgG1), mainly GAD autoantibodies (GAAs), anti-IA-2/ICA-512bdc autoantibodies (anti-IA-2 AAs), anti-IA-2β/phogrin autoantibodies (anti-IA-2β AAs) and insulin autoantibodies (IAAs), are produced to the autoantigens of the pancreatic islet β cells and can be used as markers of the pathogenesis of type 1 DM.

GAAs are important markers for type 1 DM. Individuals with other autoimmune diseases such as stiff man syndrome (SMS) and autoimmune polyendocrine syndrome (APS) also have GAAs. Diabetes is often a component of SMS and APS type II with an incidence of about 30% (Bosi et al., 1991; Solimena et al., 1990) whereas it is not a usual feature of APS type I (Riley 1992).

While there is an autoimmune response to GAD-65 in type 1 DM, SMS and APS II, a difference in the clinical frequency of diabetes suggests that the humoral and cellular autoimmune response to GAD-65 may differ in these diseases. GAAs in SMS have both similarities and differences with those found in type 1 DM (Daw et al., 1996; Kim et al., 1994) and APS II. GAAs from patients with SMS or type 1 DM both recognize determinants in the middle and carboxy (C) terminal regions of GAD65 but, predominantly in SMS patients, antibodies also recognize determinants in the amino (N) terminal region of GAD-65 (Daw et al., 1996; Kim et al., 1994). Furthermore, GAAs in patients with SMS recognize GAD on Western blots, whereas GAAs in type 1 DM fail to detect GAD on Western blots (Baekkeskov et al., 1990), indicating that GAAs of type 1 DM recognize only conformation-dependent epitopes while some GAAs of SMS recognize linear epitopes. Although the locations of epitopes of GAAs in APS type II have not yet been precisely mapped, GAAs of diabetic APS patients' sera recognise a dominant linear epitope within 7-124 amino acid residues (aa) of GAD65 which is also recognised by other patients' sera (type 1 DM, non-diabetic APS and SMS), but at a lower frequency than diabetic APS (Sohnlein et al., 2000). Thus, there is considerable heterogeneity between anti-GAD responses of these disorders, which may be due to GAD being presented to the immune system through separate pathogenic mechanisms. In addition, GAAs in SMS and APS are present at high titre while GAAs in type 1 DM are present at lower titre (Bjork et al., 1994). GAAs are present in 89% of APS II patients with type 1 DM, 21% of APS II patients without type 1 DM, 89% of SMS patients (Morgenthaler et al., 1997), 80% of newly diagnosed type 1 DM patients, 20% of non-diabetic identical twins who are at low risk of diabetes (Christie et al., 1994) and 80% of relatives of diabetic patients who themselves developed type 1 DM (Bingley et al., 1993).

T cells of SMS patients recognize different immunodominant epitopes of GAD-65 compared with T cell from patients with type 1 DM. The region of GAD65 amino acids 339-352, which is recognised by the T cells of a non-diabetic SMS patient, has no reactivity with T cells of newly diagnosed type 1 DM patients (Schloot et al., 1999). In contrast, the GAD regions, 161-243 and 473-555 aa, induce a dominant T cell response in type 1 DM, not in SMS; while the GAD regions, 81-171 and 313-403 aa, induce a dominant T cell response in SMS, not in type 1 DM (Lohmann et al., 2000).

The prevalence of anti-IA-2 AAs is higher in acute onset IDDM than in slowly progressive IDDM (Yamada et al., 1997). The anti-IA-2 AAs are present in 56% IDDM patients, 47% APS II patients with IDDM, 14% SMS patients, 4% APS II patients without IDDM (Morgenthaler et al., 1997). The major antigenic determinant of IA-2 in type 1 DM patients is localized within 762-887 aa, while the minor antigenic determinant of IA-2 is localized within 601-762 aa, which is highly homologous between IA-2 and phogrin. In addition, it has been suggested that some of the anti-IA-2 AAs recognize a conformational epitope(s) associated with the C-terminal region of native IA-2 (949-979 aa) (Kawasaki et al., 1998).

A new approach to the identification of both linear and non-linear epitopes is antibody probing of phage display peptide libraries which can reveal conformational epitopes and also linear mimotopes that mimic the shape of conformational epitopes (Scott and Smith 1990). Conformational epitopes have been identified using monoclonal antibodies (Cook et al., 1998; Felici et al., 1993; Luzzago et al., 1993) and also using

polyclonal antibodies in immune sera from both animals and humans (Folgori et al., 1994; Osman et al., 1998). However, there have been few definitive studies on polyclonal antibodies in human sera using phage display libraries.

The aim of my studies was to determine the epitopes in GAD65 of three different mouse monoclonal antibodies: GAD-6, N-terminal mouse monoclonal antibody binding within 4-17 amino acids (aa) and C-terminal monoclonal antibody binding within 572-585 aa; to determine the epitopes in IA-2 of two different mouse monoclonal antibodies 76B and 76F; and to determine the epitopes of two different human monoclonal antibodies, b78 and b96.11, on GAD-65. The determination of epitopes recognized by these monoclonal antibodies might help us to understand the antigenic nature of the GAD-65 and IA-2 autoantigens.

Furthermore, since epitope mapping is generally simpler with monoclonal antibodies than with polyclonal antibodies, it was intended that these studies should indicate how best to use the random phage peptide libraries to determine the epitope specificities of patients' serum autoantibodies. The very high levels of GAAs in SMS patients' sera, indicates that phage display technology should be applicable to the identification of mimotopes of GAD-65 that are reactive with GAAs of SMS patients' sera. Thus, a further purpose of these studies was to determine the immunodominant epitopes (public epitopes) in GAD65 of SMS patients' serum autoantibodies.

5.2 *Antigenic Nature of GAD-65 defined by Binding of N-terminal and C-terminal Specific Mouse Monoclonal Antibodies*

N-terminal MoAb, GC 3208 (clone 11), is a mouse IgG1 MoAb which recognizes the 4-17 aa residues of GAD-65. C-terminal monoclonal antibody, GC 3108 (clone 111), is a mouse IgG1 monoclonal antibody which recognizes the 572-585 aa residues of GAD-65.

In the present study the N-terminal MoAb was screened with two types of M13 filamentous phage-displayed library, gene III linear 12-mers and gene VIII 5C4C4, to derive peptide mimotopes of N-terminal MoAb on GAD-65. All the clones of phagotopes of M13, both gene III linear 12-mers and gene VIII 5C4C4 libraries, were also tested for reactivity with N-terminal MoAb by using capture ELISA, thus identifying a set of reactive phagotopes with sequences that represented mimotopes of the epitope of the N-terminal MoAb on GAD-65. All the clones of the phagotopes derived by biopanning of M13 gene VIII 5C4C4 library with N-terminal MoAb were strongly reactive with the N-terminal MoAb by ELISA.

The clones, which were obtained from the successful biopanning of the M13 pIII linear 12-mers phage library with the N-terminal MoAb, showed a motif of P-G-X-X-X-W-S-F, corresponding to 4-10 aa of GAD-65 (P-G-S-G-F-W-S-F). The presence of proline in the motif of the N-terminal MoAb may influence the binding of the N-terminal mouse monoclonal antibody with the middle region of the motif because it induces a turn in the sequence. By contrast, the clones, which were obtained from the successful biopanning of the M13 pVIII 5C4C4 phage library with the N-terminal MoAb, showed a motif of S-T-P, which does not correspond to amino acid residues 4-

17 of GAD-65 and does not overlap with the previous motif of the N-terminal MoAb, i.e. P-G-S-G-F-W-S-F (4-10 amino acid residues). Therefore, The M13 pVIII 5C4C4 worked with N-terminal MoAb by expressing a relevant sequence for the N-terminal MoAb but which is unlike its epitope in GAD-65. This shows that a clearly defined, antibody-reactive motif selected from a phage peptide library may not necessarily show sequence similarities with the epitope of the original protein antigen.

To further define the N-terminal MoAb epitope, in the present study the N-terminal MoAb was screened with the T7 linear 9-mers phage-displayed library to derive peptide mimotopes of N-terminal MoAb on GAD-65. Twenty clones, which were obtained from the successful biopanning of the T7 linear 9-mers phage library with the N-terminal MoAb, showed a motif of P-X-X-G, corresponding to amino acid residues 4-7 of GAD-65 (P-G-S-G), which overlaps with the previous motif P-G-S-G-F-W-S-F (4-10 amino acid residues). The other 6 clones did not show any significant motif. This may be the result of a heteroclytic monoclonal antibody character, which recognizes the related clones more strongly than the specific clones.

In the present study the C-terminal mouse monoclonal antibody was screened with M13 filamentous phage-displayed library gene III linear 12-mers to derive peptide mimotopes of C-terminal MoAb on GAD-65. The clones, which were obtained from the successful biopanning of the M13 pIII linear 12-mers phage library with the C-terminal MoAb, showed a motif of F-L-I-X-E-I/V/L-D-X-L, corresponding to 573-581 aa of GAD-65 (F-L-I-E-E-I-E-R-L), as an important epitopic determinant of the C-terminal MoAb.

The N-terminal MoAb reacts weakly with GAD65 on blots, but does react with native GAD65. The C-terminal MoAb reacts strongly on blots, but does not appear to react with native GAD65 (data not shown). Therefore, the α-helix T, where the C-terminal MoAb binds, must be denatured for C-terminal MoAb to bind.

5.3 *Antigenic Nature of GAD-65 defined by Binding of GAD-6 Mouse Monoclonal Antibody*

The murine IgG2a monoclonal antibody GAD-6 recognizes a specific epitope on GAD65 within 475-585 aa(SMS E1) (Butler et al., 1993). The GAAs of type 1 DM, SMS and APS II (diabetic and non-diabetic) sera recognize the region of the GAD-6 epitope on GAD-65. In addition, the extreme C-terminal sixteen amino acid residues of GAD65 are not involved in the epitope of GAD-6 (Davenport et al., 1997).

To further define the GAD-6 epitope, in the present study GAD-6 was screened with two types of T7 phage-display library of random peptides, both constrained 9-mers (C9C) and unconstrained 9-mers (linear 9-mers), expressed at the C-terminus of gene X coat protein of bacteriophage to derive peptide mimotopes of GAD-6 on GAD-65.

Thirteen out of 16 clones of phagotopes of the T7 C9C phage library or 2/7 clones of phagotopes of the T7 linear 9-mers phage library showed a motif of R/K-L/A/I-X-K or R-X-X-K, respectively, possibly corresponding to 525-528 aa of GAD-65 (R-L-S-K), as an important epitopic determinant of the GAD-6. The two amino acid residues on the ends of the motif (arginine and lysine) are highly conserved, suggesting that they are important in the paratope-epitope interaction between the GAD-6 and GAD-65. Another motif of GAD-6 obtained from 3 other clones, which were screened by

T7 (C9C) phage library, are M-X-X-A, possibly corresponding to 523-526 aa (M-S-R-L) of GAD-65. The presence of methionine and leucine also appears to be important in the paratope-epitope interaction between the GAD-6 and GAD-65.

GAD-6 does not bind to GAD-67: it is therefore significant that the equivalent residues of M-S-R-L-S-K, the motif of GAD-6 corresponding to 523-528 aa of GAD-65, are R-E-K-L-H-R in GAD-67, which shows 5 amino acid substitutions that may disrupt the GAD-6 epitope. Also, the equivalent residues of R-X-X-K, the motif of GAD-6 corresponding to 525-528 aa of GAD-65 obtained by T7 phage library, are K-X-X-R in GAD-67; as an opposite direction of the motif (R-X-X-K) in GAD-65. This may prevent the binding of GAD-6 to GAD-67 or other changes to neighbouring amino acids may affect the binding of GAD-6.

To further define the GAD-6 epitope, in the present study GAD-6 was screened with three types of M13 filamentous phage-displayed library of random peptides expressed at the N-terminus of gene III protein, both constrained 7-mers (C7C) and unconstrained 12-mers (linear 12-mers), or gene VIII protein constrained 13-mers (5C4C4) to derive peptide mimotopes of GAD-6 on GAD-65. All the clones of the two types of M13 gene III library, which were sequenced and compared with one another, did not show a clear motif. It was impossible to align them (data not shown) and they did not show reactivity with GAD-6 by capture ELISA. A possible explanation for this is that the peptides, which are specific to bind with GAD-6, are not present in this type of library, M13 pIII phage library, either linear 12-mers or C7C. In contrast, all the clones of the phagotopes selected by biopanning of M13 gene VIII 5C4C4 library with GAD-6 were strongly reactive with GAD-6 by capture

ELISA. Interestingly, all these sequences were identical to each other, i.e. they showed only one peptide. This indicates that the M13 pVIII phage library may have one dominant peptide specific to GAD-6. All the clones showed a motif of methionine followed by two amino acids followed by alanine. Thus, the motif of GAD-6 suggested from this library is M-X-X-A, which is identical to the motif selected from 3 clones of T7 (C9C) library, possibly corresponding to amino acid residues 523-526 (M-S-R-L) of GAD-65 which overlaps with the previous motif, R-L-X-K (525-528 aa). Other work from this laboratory has indicated that GAD-6 can recognize the motif M-X-X-L (523-526 aa of GAD-65) by aligning 7/17 clones, which was selected from a linear 15-mers M13 filamentous phage library (Davenport, 1995).

Analysis of C-terminal deletion mutants shows that the removal of 41 aa at the C-terminus of GAD65 (545-585 aa deletion mutant) abolishes the binding of MICA-2 (human monoclonal antibody) and GAD-6 on blots (Richter et al., 1993). However, the suggested GAD-6 epitope (523-528 aa) is present in α-helix S of the model of Schwartz et al. (1999), while the deletion of 545-585 aa removes the terminal α-helix T and 2 lengths of β-pleated sheet. Mapping of MICA-2 epitope within GAD-65 by using an epitope cDNA library reveals that the MICA-2 can recognize an epitope between 506-531 aa of GAD65 (Richter et al., 1996), which includes the GAD-6 epitope suggested from the libraries here. It is possible that the removal of 41 aa at the C-terminus of GAD65 (545-585 aa deletion mutant) may lead to a conformational change of GAD-65 which abolishes the binding of MICA-2 and GAD-6. Thus, 545-585 aa deletion mutant would remove terminal α-helix (T) and 2 lengths of β-pleated sheet, in the Schwartz model, which is likely to disrupt the conformation of α-helix S.

In NOD mice, injection of GAD-65 peptide 524-543 aa, which also includes the proposed GAD-6 epitope mapped here with phage libraries, activates diabetogenic T cells (Zekzer et al., 1998). Furthermore, T-cells in type 1 DM patients can react with 379-585 aa of GAD-65 (Rharbaoui et al., 1999). Binding of GAD-6 to this region of GAD-65 may block antigen processing which generates the peptides required for stimulation of GAD specific TH1 cells. Previous studies have shown that blocking the T cell response to GAD by tolerisation with GAD antigen inhibits the response to other islet antigens, in particular insulin and carboxypeptidase H (Kaufman et al., 1993; Tisch et al., 1993). Other studies have shown that injection of monoclonal antibody against GAD into NOD mice leads to delay in the onset of diabetes and a decrease in the severity of insulitis. The mechanism of diabetes prevention by administration of anti-GAD antibody could be associated with an interference in recognition of GAD by T cells (Menard et al., 1999). Indeed, autoreactive B cell may act as APC for the T cell activation (Noorchashm et al., 1997). Also, incubation of a T-cell hybridoma, which recognizes the GAD-65 274-286 epitope, with APC exposed to recombinant human GAD-65 complexed with GAD-65+ autoantibodies led to the stimulation of this T-cell hybridoma. This stimulation was most prominent using sera from patients with GAD-65 autoantibodies. Uptake of antibody-complexed GAD-65 was Fc receptor-mediated, i.e. macrophages and/or dendritic cells, not antigen-specific B-cells. These findings support the idea that GAD65 autoantibodies modulate presentation of GAD-65 to T cells (Reijonen et al., 2000). In addition, the NOD mouse fails to develop diabetes if the B cell compartment is inactivated, suggesting a crucial role of B cells, probably as essential antigen presenting cells. It has been suggested that the membrane bound IgG may play a crucial role in antigen uptake. Thus, B cell presentation of GAD-65 may play an important role in

immunopathogenesis of type 1 DM by shaping the autoreactive T cell repertoire (Duhindan et al., 2000).

To further determine whether the phage peptides which were selected by immunopanning with GAD-6 were acting as true mimotopes of GAD-65 epitopes, mice should be immunized with GAD-6 reactive clones. Immunizing with mimotope peptides presented on filamentous phage is an appropriate method to produce peptide-specific antibodies which also bind to the native antigen (Meola et al., 1995).

5.4 Antigenic Nature of IA-2: Binding of 76B and 76F Mouse Monoclonal Antibodies

76B (unknown IgG isotype) and 76F (IgG2b), supernatants of two mouse monoclonal antibodies, recognize epitopes in the extracellular domain and N-terminal region of the cytoplasmic domain of IA-2, respectively.

In the present study the 76B or 76F MoAb were screened with T7 C9C phage-displayed library to derive peptide mimotopes of 76B or 76F MoAb epitopes on IA-2. To further define the 76B or 76F MoAb epitopes, in the present study the 76B or 76F mouse monoclonal antibodies were also screened with M13 gene III linear 12-mers phage-displayed library to derive peptide mimotopes of 76B or 76F mouse monoclonal antibody on IA-2.

The sequences of 76B-specific clones obtained from both libraries, T7 C9C and M13 pIII linear 12-mers, showed homologies amongst themselves and with the original IA-2 sequence. Thus, the main motif of 76B is D-X-K-P-L-S, corresponding to 477-

482 aa of IA-2 (D-Q-K-P-L-S). The sequences of 76F-specific clones obtained from both libraries, T7 C9C and M13 pIII linear 12-mers, show homologies amongst themselves and with the original IA-2 sequence. Thus, the main motif of 76F is F-X-Y-Q, corresponding to 626-629 aa of IA-2 (F-E-Y-Q). Since the minor antigenic determinant of IA-2 is localized to 601-762 aa (Kawasaki et al., 1998) and huAb 103/5 recognizes 603-686 aa of IA-2 (Kolm-Litty et al., 2000) which includes the predicted region recognized by 76F huAb (626-629 aa), the 76F MoAb might be used to block antigen processing which generates the peptides required for stimulation of IA-2 specific TH1 cells by binding of 76F to this region of IA-2.

5.5 Antigenic Nature of GAD-65 by Binding of b96.11 and b78 Human Monoclonal Antibodies

b78 and b96.11, supernatant of cloned cells of two human monoclonal antibody producing cell lines derived from a human APS patient, recognize epitopes on GAD-65, 532-540 aa and 308-365 aa, respectively.

In the present study b96.11 huAb was screened with M13 gene III linear 12-mers library to derive peptide mimotopes of b96.11 huAb on GAD-65. The clones, which were obtained from the successful biopanning of the M13 pIII linear 12-mers phage library with the b96.11, showed a motif of I/V-T/S-A/G/L-T/S-A/L (Fig. 4.1a), which might correspond to 332-336 aa of GAD-65 (V-S-A-T-A), and another motif of S-T/S-G/A/L/I (Fig. 4.1b), which also might correspond to 338-340 aa of GAD-65 (T-T-V) as epitopic determinants of the b96.11 huAb. Thus, the overall motif of b96.11 might correspond to 332-340 aa of GAD-65, which is within the region of GAD65

308-365 aa. Deletion of this region is known to abolish the binding of b96.11 to GAD65 (Schwartz et al., 1999).

The b78 and b96.11 huAb recognize native (not denatured) GAD-65, indicating that these huAb, b78 and b96.11, can recognize conformational (not linear) epitopes on GAD-65.

It is noteworthy that the b78 huAb bound slightly better with GAD-6 as the capture MoAb (rather than the N-terminal MoAb), but b96.11 bound much better with GAD-6 as the capture MoAb (Table 4.3). This might suggest that the GAD-6 does interfere with b78 huAb binding to GAD-65 more than it does with b96.11 huAb. However, it must also suggest that the GAD-6 and b78 huAb epitopes are not directly overlapping.

The b78 huAb recognizes amino acid residues between 532-540 on GAD-65 (Schwartz et al., 1999) which is very close to the proposed GAD-6 epitope (523-528 aa). In addition, the footprint pattern of protected trypsinized fragments of GAD-6 is similar to that of b78 huAb (Tremble et al., 1997).

The mutation V532K disrupts the binding of b78 huAb to GAD-65, while other mutations in the same region of b78 epitope, R536L and Y540S, do not disrupt the binding of b78 to GAD-65. This indicates that V532 may be involved in the surface patch of the b78 epitope which then includes other amino acid residue(s) from different region(s).

5.6 *Antigenic Nature of GAD-65 defined by Binding of GAD Autoantibodies of SMS Patients' Sera*

To further define the epitopes of GAAs of SMS sera, in the present study SMS sera were screened with an M13 linear 12-mers phage-displayed library of random peptides to derive peptide mimotopes of SMS sera on GAD-65. All the clones of phagotopes of M13 gene III linear 12-mers library were tested for reactivity with SMS sera by capture ELISA, but they did not show reactivity.

The clones, which were obtained from the third, fourth and fifth rounds of successful biopanning of the M13 pIII (linear 12-mers) phage library and were positively immunostained in the blotting assay (moderate affinity) with the SMS patients' sera showed a motif of L/A-A-X-T/S-R/H/K. This could correspond to 371-375 aa of GAD-65 (L-L-M-S-R), as a public epitope of SMS patients' sera.

The 20 clones showed another motif of T/S-T-V/I/L-F-E-L/G/I/V/A-H/K-L/G-X-K/R. This could correspond to 463-472 aa of GAD-65 (T-T-G-F-E-A-H-V-D-K), as another public epitope of SMS patients' sera.

5.7 *The Importance of Using Different Phage Libraries with Different Features Screened with either Monoclonal or Polyclonal Antibodies*

The differences in the sequences of the peptides selected using the M13 pVIII filamentous phage library and T7 phage library with N-terminal MoAb may be due to the differences in their capacity to express their displayed peptide, either at the N-terminus of gene VIII of M13 filamentous phage library or at the C-terminus of gene X of T7 phage library. Also, the conformation of the expressed peptides may be

different between these two phage libraries due to differences in the number of amino acid residues and due to the presence of the cysteine-cysteine bridge in the M13 pVIII phage library. However, similar sequences were selected from these libraries with GAD-6.

The number of copies of the displayed peptide expressed by M13 pVIII (300 copies per phage) compared with the lower number of copies expressed by M13 pIII (3-5 copies per phage), may account for the reactivity of the N-terminal MoAb with specific clones of phagotopes derived from the M13 pVIII library rather than M13 pIII, due to the higher avidity of binding with the M13 pVIII library.

Polyclonal IgG includes a myriad of antibodies so that, even after extensive rounds of negative selection, the phagotopes will include many that react with other antibodies that are either specific or non-specific to the disease. The difficulty in discriminating between sequences that are specific or non-specific for any disease has greatly hampered the use of phage-displayed technology with polyclonal sera. In addition, some of the non-disease specific phagotope sequences would include either nuisance peptides intrinsic to the biopanning process itself, or mimotopes of bacterial or viral antigens for which antibodies were absent from the particular pool of IgG used for negative selection (data not shown).

5.8 Conclusion

In conclusion, amalgamate findings for different mouse monoclonal antibodies (GAD-6, 76B, 76F and N-terminal and C-terminal mouse monoclonal antibodies), human monoclonal (b96.11) and polyclonal antibodies (SMS patients' sera) have

been presented for the screening of different peptide libraries with different features [T7 gene X (C9C and linear 9-mers), M13 gene III (C7C and linear 12-mers) and M13 gene VIII (5C4C4)]. These peptide sequences have been compared with the known structure of immunodominant regions of the autoantigenic molecules, GAD-65 and IA-2, to locate precisely conformational epitopes on these major autoantigens in type 1 DM, SMS and APS patients.

APPENDICES

Appendix 1: Abbreviations for Amino Acids

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix 2: Amino Acids grouped according to Chemical Properties

Chemical Nature of Side Chain	Amino Acids
Aliphatic Side Chains	Glycine Alanine Valine Leucine Isoleucine
Aliphatic Hydroxyle Side Chains	Serine Threonine
Basic Side Chains	Lysine Arginine Histidine
Aromatic Side Chains	Phenylalanine Tyrosine Tryptophan
Acidic Side Chains	Aspartic Acid Glutamic Acid
Amide Side Chains	Asparagine Glutamine
Sulfur-containing Side Chains	Cysteine Methionine

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