Chemical and Enzymatic Fragmentation of Tetanus Toxin and Immunological Studies on Anti Tetanus Toxin and Toxoid Sera

Alecos Michaelides

Thesis submitted to the School of Graduate Studies and Research in partial fulfillment for the degree of Masters of Science in Chemistry.

University of Ottawa

ISBN 0-612-15744-X

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

This thesis describes the immunization protocols for the production of antibodies against tetanus toxin and toxoid in guinea pigs and mice. Antibodies were successfully raised against the toxin without mortalities in either species. The murine sera obtained, were isotyped by ELISA and the toxin was proven to be a superior antigen in eliciting production of IgG_{2a} and IgG_3. The two isotypes which have demonstrated antitumor activity. The anti-toxoid sera exhibited a lower reactivity towards the toxin and toxoid when compared with anti-toxin sera. The reactivity of recombinant tetanus toxin fragment C was studied and the results indicated that in the murine serum, 72% of anti-toxin or anti-toxoid antibodies were directed against epitopes on fragment C. The study of the guinea pig sera suggested that similar to mouse serum, it can develop in response to toxin as an antigen, antibodies against toxin which are mostly directed against the fragment C portion. On the other hand, guinea pigs seem to respond to the toxoid as an antigen by producing antibodies to more than fragment C.

Tetanus toxin was digested with immobilized papain cross-linked to 6% beaded agarose. Different experimental conditions were tested and satisfactory yields of fragment C were obtained. Several preparations of toxin were analysed on SDS-PAGE and were found to be highly fragmented. The preparation found to contain intact 150,000 dalton single-chain tetanus

toxin molecules was digested with papain and separated by SDS-PAGE under non-reducing conditions. The results indicated that on a larger scale it would be feasible to isolate preparative amounts of fragment C for further analysis.

Recombinant tetanus toxin fragment C was cleaved with cyanogen bromide (CNBr) at points containing methionine residues. The experimental conditions were optimized and the CNBr derived peptides were separated on SDS-PAGE. The separation of the low molecular weight polypeptides was achieved on a 20% uniform pore gel with 0.5% cross-linking and containing 10% glycerol. A gel containing the separated peptides was electrotransfered onto a nitrocellulose supporting membrane. The reaction of the peptides with the four different antisera, murine anti-toxin (MTN), murine anti-toxoid (MTD), guinea pig anti-toxin (GPTN) and guinea pig anti-toxoid (GPTD) gave different identity staining patterns.

The ELISA immunoreactivity of antisera derived from guinea pigs and mice immunized with tetanus toxin and toxoid was tested in microtitre wells coated with an octameric Multiple Antigenic Peptide (MAP) containing peptide fragment 830 to 844 from tetanus toxin. This MAP was proven to be antigenic in both species. The immmunogenic fragment 830 to 844 of tetanus toxin must contain at least one linear sequence of amino acids capable of acting as a T-cell epitope. The results from these assays also shows that, although this sequence is important for immunogenicity, it is also important for antigenicity and must contain at least one B-cell epitope.

Guinea pig sera induced by immunization with both tetanus toxin and toxoid gave a shallow response to the MAP. This is indicative of large amounts of low affinity antibodies. On the other hand, antisera from mice immunized with tetanus toxin and toxoid produced steeper curves which is characteristic of small quantity of high affinity antibodies.

ACKNOWLEDGEMENTS

I would like to thank Professor René Roy for all the guidance and support he has given me during the course of my studies. I am very appreciative of the time he has spent and the patience he has demonstrated in the course of this thesis corrections.

My thanks are extented to Dr Jose Louis Di Fabio, my co-supervisor and Dr Paul Percheson for their invaluable help and allowing me to do the work at Health Canada.

Finally, I would like to acknowledge all my family and friends for their encouragement and moral support. My gratitude goes especially to Nicole Beaudoin for all the hours she spent deligently editing this thesis.

LIST OF ABBREVIATIONS

APC	Antigen-Presenting cells
C	The constant region
CD	Cluster of differentiation antigens
CDRs	The hypervariable regions or complementarity determining regions
CFA	Freund's Complete Adjuvant
Da	Daltons
EDTA	Ethylene-diaminetetraacetate
ELISA	Enzyme Linked Immunosorbent Assay
FMDV	Foot-and-Mouth Disease Virus
GP	Guinea Pigs
GPTD	Guinea Pig anti-Toxoid
GPTN	Guinea Pig anti-Toxin
H	Heavy chain
HLA-DR	A Human Histo-Compatibility leucocyte antigen
HRPO	Horseradish Peroxidase
ICFA	Freund's Incomplete Adjuvant
IFN-γ	Gamma Interferon
Igs	Surface immunoglobulins
IL-2	Interleukin-2
IU	International Unit
K cells	Killer cells
kD	Kilo Daltons
KLH	Keyhole-limpet Haemocyanin
L	Light chain

LD_{50}	Lethal dose at 50%
Lf	Limit of flocculation
M	Murine
MAbs	Monoclonal Antibodies
MAP	Multiple Antigenic Peptide
MHC II	Class II Major Histo-Compability complex molecules
Mol wt	molecular weight
MTD	Murine anti-Toxoid
MTN	Murine anti-Toxin
MWCO	Molecular Weight Cut-Off
NC	Nitrocellulose
O.D.	Optical Density
PBS-T	Phosphate buffer saline-Tween
rTTC	Recombinant fragment C of Tetanus Toxin
SDS-PAGE	Sodium Dodecyl Sulfate (SDS)-PolyAcrylamide Gel Electrophoresis (PAGE)
SPPS	Solid-Phase Peptide Synthesis
T_c cells	Cytotoxic T cells
TCR	The T cell antigen receptor
TD	Tetanus Toxoid
T_h	Helper T cells
TIG	Tetanus Immune Globulin
TMB	3,3',5,5' Tetramethylbenzidine
TN	Tetanus Toxin
TNF-ß	Tumor Necrosis Factor-ß
T_s cells	Suppressor T cells
V	The variable region

TABLE OF CONTENTS

Chapter 1: Immunologigal Considerations 1

1.1 Introduction 1

1.2 T lymphocytes and their receptors 3

1.3 Antigenic reactivity of proteins 5

1.4 Antibodies 9

1.5 The T-cell antigen receptor 13

Chapter 2: Cancer Immunotherapy 19

2.1 Introduction 19

2.2 Tetanus toxin 22

2.3 Toxoid Production 23

2.4 Immunogenicity of tetanus toxoid 26

Chapter 3: Preparation of Antibodies against Tetanus toxin and toxoid 28

3.1 Introduction 28

3.2 Results and discussion 29

3.2.1 Isotypes 29

3.2.1.1 Introduction 29

3.2.1.2 Determination of isotype and their relative concentration in the generated murine antibodies 30

3.2.2 Reactivity of Murine and Guinea pig sera 41

3.2.2.1 Introduction 41

3.2.3 Reactivity of fragment C of tetanus toxin 41

3.3 Summary 45

3.4 Experimental 46

PART A - Immunization with tetanus toxin 46

3.4.1 Murine antibodies raised against tetanus toxin 46

3.4.1.1 Introduction 46

3.4.1.2 IMMUNIZATION PROTOCOL 47

3.4.1.3 Reagents and methods 48

3.4.2 Guinea pig antibodies raised against tetanus toxin 50

3.4.2.1 Introduction 50

3.4.2.2 IMMUNIZATION PROTOCOL 51

3.4.2.3 Reagents and methods 51

PART B - Immunization with tetanus toxoid 53

3.4.3 Murine antibodies raised against tetanus toxoid 53

3.4.3.1 Introduction 53

3.4.3.2 IMMUNIZATION PROTOCOL 53

3.4.3.3 Reagents and methods 54

3.4.4 Guinea pig antibodies raised against tetanus toxoid 55

3.4.4.1 Introduction 55

3.4.4.2 IMMUNIZATION PROTOCOL 55

3.4.4.3 Reagents and methods 56

PART C - Indirect Enzyme-Linked Immunosorbent Assay (ELISA) 57

3.4.5 Introduction 57

3.4.5.1 Principle of the test 57

3.4.5.2 General 58

3.4.5.2.1 Reagents and Equipment 58

3.4.5.2.2 Sera 61

3.4.5.3 Method 64

PART D - ELISA ASSAYS 64

3.4.6 Determination of isotype and relative concentration of the murine antibodies generated against tetanus toxin and toxoid 64

3.4.6.1 Assay protocol 64

3.4.7 Determination of the titre of murine antibodies generated against tetanus toxin and toxoid 66

3.4.7.1 Assay protocol 66

3.4.8 Determination of the titre of guinea pig antibodies generated against tetanus toxin and toxoid. Detection of *C. tetani* antitoxin 68

3.4.8.1 Assay protocol 68

Chapter 4: Enzymatic breakdown of TT with Immobilized Papain 70

4.1 Introduction 70

4.2 Schematic representation of papain digestion of tetanus toxin 72

4.3 Results and discussion 73

4.4 Summary 77

4.5 Experimental 79

PART A - Papain digestion of tetanus toxin 79

4.5.1 Introduction 79

4.5.2 Reagents and Equipment 79

4.5.3 Method 81

PART B - Separation of tetanus toxin digested with immobilized papain on SDS-PAGE 83

4.5.3 Introduction 83

4.5.3.1 Reagents and Equipment 83

4.5.3.2 Preparation of stock solutions 85

4.5.4 Method 87

4.5.4.1 Preparation of the separating gel 87

4.5.4.2 Preparation of the stacking gel 88

4.5.4.3 Sample preparation 88

4.5.4.4 Preparation of molecular weight standards 89

4.5.4.5 Electrophoretic run conditions 89

Chapter 5: Cyanogen bromide cleavage of Tetanus Toxin Fragment C at points containing Methionine residues 92

5.1 Introduction 92

5.2 Results and discussion 94

5.2.1 Introduction 94

5.2.2 SDS-PAGE of CNBr generated fragments 97

5.2.3 Western Blot of fragment C cleavage products 100

5.3 Summary 102

5.4 Experimental procedures 103

PART A - Cyanogen bromide cleavage of tetanus toxin fragment C at points containing methionine residues 103

5.4.1 Introduction 103

5.4.2 Reagents and Method 103

PART B - Separation of CNBr cleaved tetanus toxin C fragment on SDS-PAGE 105

5.4.3 Introduction 105

5.4.4 Reagents and equipment 105

5.4.5 Preparation of stock solutions 105

5.4.6 Method 106

5.4.7 Electrophoretic run conditions 107

PART C - Electrophoretic transfer of SDS-PAGE gels 109

5.4.8 Introduction 109

5.4.9 Reagents and Equipment 110

5.4.10 Preparation of Buffers 110

5.4.11 Method 111

PART D - Immunoblot on nitrocellulose membrane 113

5.4.14 Introduction 113

5.4.15 Reagents and Methods 113

Chapter 6: Multiple Antigenic Peptide 117

6.1 Introduction 117

6.2 Solid phase peptide synthesis 120

6.2.1 Introduction 120

6.3 The Direct approach 121

6.3.1 Introduction 121

6.3.2 The Polymeric support 121

6.3.3 Functionalization and anchoring 123

6.3.4 Repetitive deprotection/coupling cycle 124

6.3.5 Protection 124

6.3.6 Coupling or Activation 126

6.3.7 Deprotection 126

6.3.8 Cleavage 126

6.4 The indirect approach 127

6.4.1 Introduction 127

6.4.2 The importance of orientation 127

6.5 Limitations of synthetic peptide vaccines 128

6.6 The MAP approach 129

6.6.1 Introduction 129

6.6.2 Advantages of MAP 131

6.7 Results and Discussion 132

6.7.1 Introduction 132

6.7.2 ELISA assays 134

6.8 Summary 139

6.9 Experimental procedures 139

APPENDICES

Appendix I. Nucleotide sequence of Tetanus Toxin 140

Appendix II. BIO-RAD Protein Assay 145

REFERENCES 146

LIST OF FIGURES DIAGRAMS AND TABLES

FIGURE 1.1	T helper cell recognition of foreign protein antigen	2
FIGURE 1.2	Receptors for foreign antigens on lymphocytes	4
FIGURE 1.3	Model of epitopes on a protein	6
FIGURE 1.4	The interaction of an epitope and its homologous paratope	7
FIGURE 1.5	The general structure of immunoglobulins	11
FIGURE 1.6	B and T cell antigen recognition	14
FIGURE 2.1	T cell epitopes in fragment C of Tetanus Toxin	27
DIAGRAM 1	Indirect ELISA	32
FIGURE 3.1	Isotype determination of Murine Antibodies reacting with Tetanus Toxin	34
FIGURE 3.2	Isotype determination of Murine Anti-toxin reacting with Tetanus Toxin (TN) and Toxoid (TD)	36
FIGURE 3.3	Isotype determination of Murine Antibodies reacting with Tetanus Toxoid	37
Table 1	Percentage Values of IgG Isotypes	38
FIGURE 3.4	Isotype determination of Murine Anti-toxoid reacting with Tetanus Toxin (TN) and Toxoid (TD)	40
FIGURE 3.5	Murine Antibodies reacting with Tetanus Toxin, Toxoid and rTTC	43

FIGURE 3.6	Guinea Pig Antibodies reacting with Tetanus Toxin, Toxoid and rTTC	44
FIGURE 4.1	The papain cleavage site on tetanus toxin is at Lysine residue 865	71
FIGURE 4.2	SDS-PAGE of two preparations of tetanus toxin	75
FIGURE 4.3	SDS-PAGE separation of tetanus toxin digested with papain, tetanus toxin fragment C and undigested toxin	76
FIGURE 4.4	SDS-PAGE of two preparations of tetanus toxin under reducing and non-reducing conditions	78
FIGURE 5.1	Cyanogen Bromide action on Methionine	93
Table 5.1	Molecular weights of expected peptides	95
FIGURE 5.2	Amino acid sequence of expected peptides from cyanogen bromide cleavage of fragment C	96
FIGURE 5.3	SDS-PAGE of CNBr generated peptides	99
FIGURE 5.4	Immunoblot of Fragment C Cleavage Products	101
FIGURE 6.1	Schematic representation of the core matrix of the MAP	118
FIGURE 6.2	General Scheme of SPPS	121
Figure 6.3	Protecting group strategies in SPPS	125
Figure 6.4	General structure of 8-branch MAP core	130
Figure 6.5	A map of the major human Th epitopes of tt	132
Figure 6.6	The structure of the MAP constructed by Dr Roy *et al*	133

FIGURE 6.7 Reactivity of α-tetanus toxin Murine sera with MAP (1 μg/ml) 135

FIGURE 6.8 Reactivity of α-tetanus toxoid Murine sera with MAP (1 μg/ml) 136

FIGURE 6.9 Reactivity of α-tetanus toxin Guinea pig sera with MAP (1 μg/ml) 137

FIGURE 6.10 Reactivity of α-tetanus toxoid Guinea pig sera with MAP (1 μg/ml) 138

Chapter 1: Immunologigal Considerations

1.1 Introduction

The immune system has evolved primarily to preserve the biological identity of the individual (self) eliminating all the possible sources of foreign antigens (non-self). It is characterized by its ability to discriminate between myriad different antigens. The task of eliminating non-self antigens without attacking self antigens can be carried out properly only if the system has the ability to discriminate precisely between self and non-self antigens. This essential feature of the immune system has evolved to a sophisticated level of complexity.[1]

Recognition of antigens is mediated by T and B lymphocytes. To protect the individual, the lymphocytes must be capable of recognizing any potential pathogen such as bacteria, viruses, parasites or tumors. The basic immunological strategy has been to generate a large potential repertoire of antigen-specific receptors by somatic rearrangement of gene segments (relatively few germ line genes would give rise to many mutated genes during the lifetime of the individual), to distribute them clonally and then select against cells expressing-receptors able to recognize self antigens, allowing the differentiation of lymphocytes expressing receptors potentially able to recognize non-self antigens.

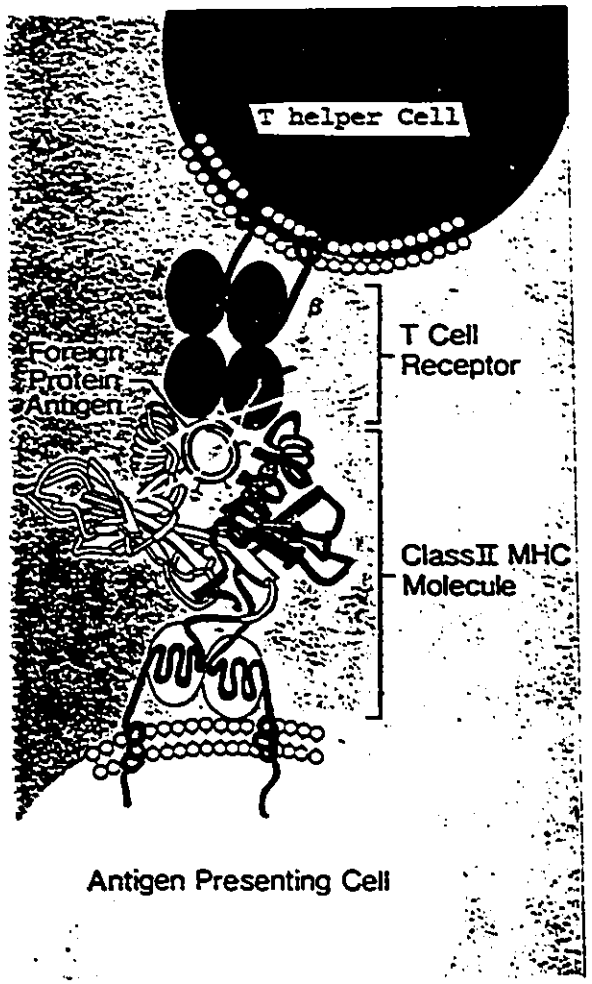


FIGURE 1.1 T helper cell recognition of foreign protein antigen[2]

1.2 T lymphocytes and their receptors

B cells can recognize antigens in their native conformation either free in solution, on membranes, or on the surface of cells, using surface immunoglobulins (Igs) as their specific antigen receptor.[3]

The T cell antigen receptor (TCR) is structurally different from antibody. It is generated by different sets of genes and most T cells can only recognize antigen on the surface of other cells. There are two types of T cell antigen receptors. The $\alpha\beta$ and the $\gamma\delta$ heterodimers. The T cell population is heterogenous with respect to both functional capabilities and cell surface phenotype. They are divided structurally into helper T cells (T_h cells): which promote cell mediated and antibody responses; cytotoxic T cells (T_c cells): which lyse antigen-bearings cells; and suppressor T cells (T_s cells) which specifically suppress immune responses. Phenotypically T_h cells are CD4+ and T_c cells are CD8+. Subsets of leukocytes can be differentiated by various proteins expressed on their surface membrane. These cell surface markers are referred to as CD (cluster of differentiation) antigens. As of 1989, seventy-eight of the human leukocyte antigens were officially recognized and given cluster designation numbers "CD1" through "CD78". The CD4 molecule is one of the first human T cell differentiation antigens that was defined by a monoclonal antibody. CD4 is found on a subset of peripheral blood T cells that is also characterized by the lack of the CD8 molecule.[5]

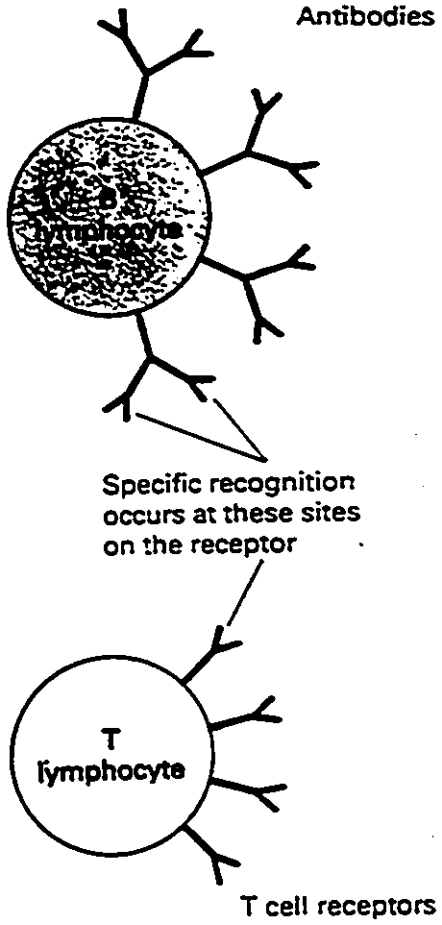


FIGURE 1.2 Receptors for foreign antigens on lymphocytes[4]

The CD4+ subset includes the T-helper cell population and is present on the helper/inducer T cell subset that comprises 45 ± 10% of normal peripheral blood lymphocytes. The CD8+ subset includes the T-suppressor cell population and is present on the cytotoxic/suppressor subset which comprises 28 ± 8% of normal peripheral blood lymphocytes.[5]

1.3 Antigenic reactivity of proteins

The antigenic reactivity of a protein is located in those regions of the molecule that are recognized by the binding sites or paratopes of certain immunoglobulins. The region of an antigen that is recognized by a paratope is called an antigenic determinant or epitope.[6] Immunochemists have traditionally divided antigenic determinants into two structural categories: (a) continuous determinants, in which all the residues in contact with antibody are contained within a single segment of the aminoacid sequence of the antigen and (b) non continuous determinants, in which residues are far apart in the sequence but are brought together by the folding of the protein in its native conformation, for example by disulfide linkages.[7]

Conformational epitopes (non continuous) correspond to the vast majority of epitopes found on proteins. Antibodies, the antigen receptors of B cells, frequently recognize conformational epitopes. When an animal is immunized with the antigen in its native conformation, the antibodies formed will

recognize the antigen only if the protein molecule is intact and its conformation is preserved. However, it is possible that approximately 10% of antibodies directed to conformational epitopes are able to react with linear peptide fragments of the protein.

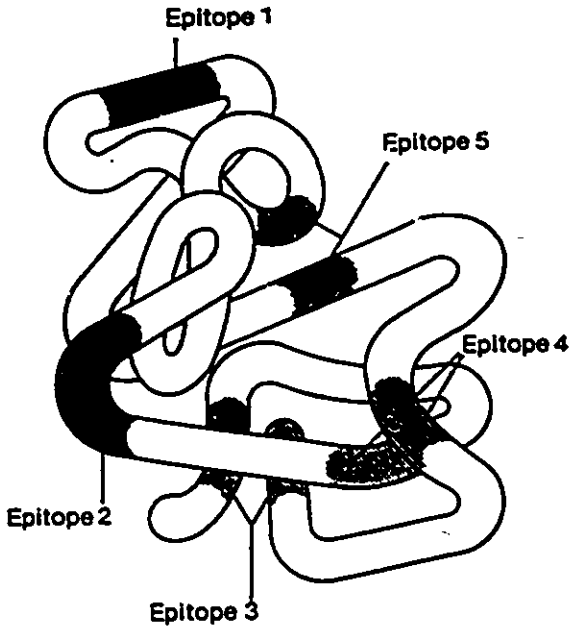


FIGURE 1.3 Model of epitopes on a protein. The shaded areas are the specific epitopes. They are composed of chain segments that are either continuous (epitopes 1 and 2) or conformational (epitopes 3-5).[8]

The binding of an antibody to its antigen results from a specific interaction between the antigen binding site of the antibody or paratope and the antigenic determinant or epitope of the antigen that elicited the immune response.

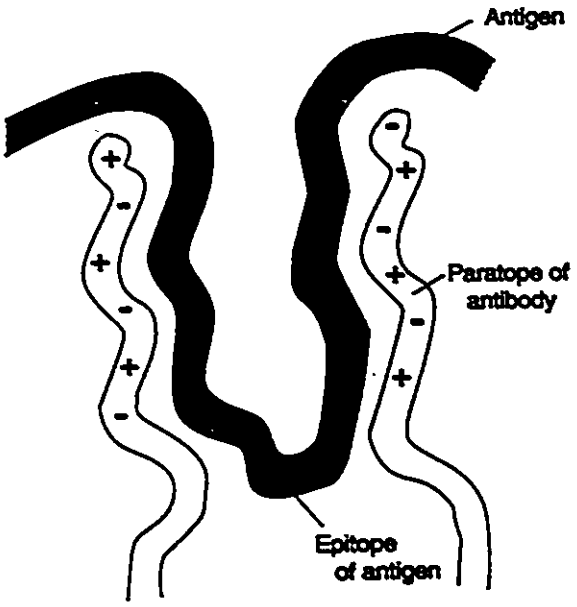


FIGURE 1.4 The interaction of an epitope and its homologous paratope. The bonds between an epitope and paratope include hydrophobic interactions, Van der Waal's forces, hydrogen bonds and ionic bonds. It is particularly notable that charged residues on the antigen are frequently neutralized by oppositely charged residues on the antibody.[8]

An epitope is not an intrinsic feature of a protein existing independently of its paratope partner, but a relational entity that can be defined only in an operational sense by the binding of a complementary paratope. Not every residue in a so-called continuous epitope is necessarily a contact residue interacting with the paratope.

H.M. Geysen *et al*[9] synthesized an immunogenic epitope of the immunologically important coat protein of foot-and-mouth disease virus (type O_1) located with a resolution of seven amino acids, corresponding to amino acids 146-152 of that protein using rapid concurrent synthesis. Then, a complete replacement set of peptides, in which all 20 amino acids were substituted in turn at every position within the epitope, were synthesized. It was found that the leucine residues at positions 148 and 151 were essential for reaction with antisera raised against intact virus. Other residues could be replaced by any amino acid, without affecting binding, and their role is probably limited to that of a scaffold presenting no direct interactions with residues of the antibody.

Evidence shows that short sequences of two or three residues can be recognized by antibodies, and it is possible that longer peptides may bind to an antibody because of the presence in the peptide of a few contact residues interspersed by noninteracting residues. The weak antigenic reactivity of short peptides can be magnified when the peptide antigen is

tested at high concentration in a solid-phase assay or in immunoblotting. Such assay formats favor the binding avidity of multivalent immunoglobulin molecules.

Many studies have established that certain antipeptide antibodies are able to neutralize the biological activity associated with the native state of proteins. For instance, in many viruses it is clear that immunization with peptides can lead to the formation of antibodies that neutralize viral infectivity.[10] These findings imply that the antipeptide antibodies recognize the native state of the viral protein present in infectious virus particles. However, such instances of cross-reactivity does not mean that the linear peptide is able to exactly reproduce the structure of the epitope present in the native protein, because a limited degree of resemblance may be sufficient to allow antibody cross-reactivity.

1.4 Antibodies[4,11-13]

Antibodies comprise a family of glycoproteins consisting of two distinct types of polypeptide chains linked by both covalent and non-covalent bonds. Two identical heavy (H) chains and two identical light (L) chains, linked by intrachain and interchain disulphide bonds. The light chain polypeptide, molecular weight (mol wt) of 25,000 Da is characteristic of this whole group of glycoproteins whereas the heavy chain (mol wt of 50,000 to 77,000 Da) is structurally distinct for each class or subclass. These chains

are linked together by covalent and non-covalent forces to give a four-chain structure based on pairs of identical heavy and light chains. Enzymatic treatment of antibodies with papain cleaves the immoglobulin molecule into three components: two Fab fragments and a third component, the Fc fragment. If the enzyme pepsin is used, a fragment containing the two Fab regions still joined together by a covalent bond is obtained. This fragment, termed $F(ab^1)2$, is able to bind and precipitate antigen. The two Fab fragments separately are able to bind but do not precipitate antigen since they are univalent. Fragment Fc appears to be similar between antibodies, suggesting that antibody variability and heterogeneity is a function of the Fab fragments.

The results of extensive amino acid sequence analysis of many immunoglobulin chains have revealed that each immunoglobulin polypeptide could be divided into two distinct regions, the variable (V) and the constant (C) regions. The variable regions is located at the amino-terminal end of each chain and the constant region lies in the C-terminal portion.

Amino acid analysis of the variable region of antibodies with different antigen specificities has shown that certain positions vary considerably more than others and that the variable amino acids are neither randomly nor uniformely distributed throughout the variable region, but are clustered in three or four particular, relatively small areas known as the hypervariable regions or complementarity determining

regions (CDRs). X-ray diffraction studies of antibody-antigen complexes, show that the sites of contact between the antibody and antigen can involve amino acid residues in the CDRs and that these are located at the extremeties of the Fab arms.

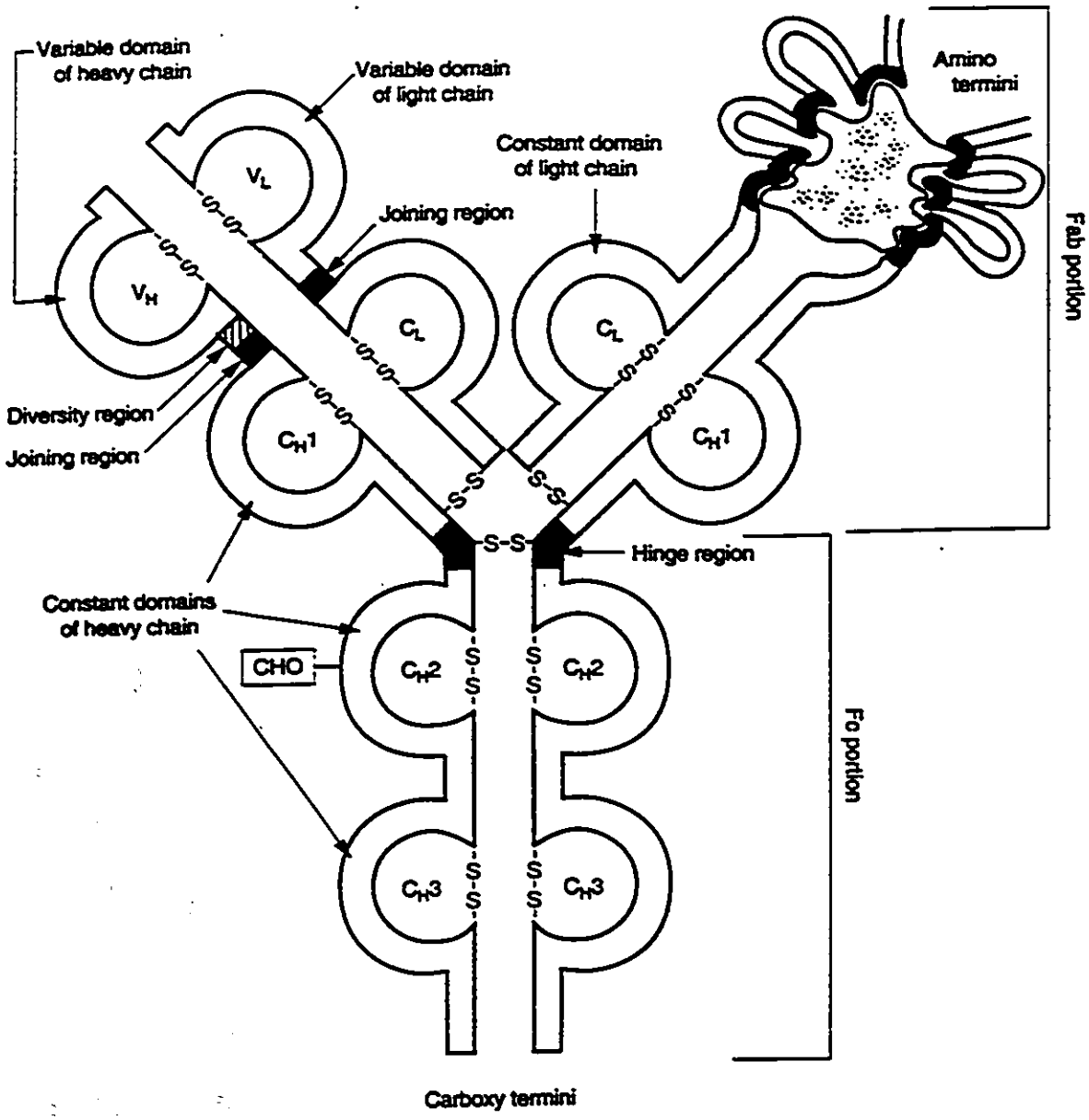


FIGURE 1.5 The general structure of immunoglobulins[8]

Immunoglobulins can be divided into different classes and subclasses depending on the antigenic determinants located on the heavy chain. Five distinct classes are recognized in most higher mammals, namely IgG, IgA, IgD, IgE and IgM based on the five classes of heavy chains (γ, α, δ, ϵ and μ). Some classes are heterogeneous, and can be further divided into subclasses. There are four subclasses of the human heavy chain called γ_1, γ_2, γ_3 and γ_4, thus the four human IgG subclasses: IgG_1, IgG_2, IgG_3 and IgG_4. There are also two subclasses of α chain, α_1 and α_2 therefore IgA_1 and IgA_2.

The genes for every class and subclass of heavy chain and for both types and all the subtypes of light chains, are present in all healthy members of a species. Each of these distinct forms is known as an isotypic variant or isotype. The immunoglobulin heavy chain classes and subclasses are responsible for the biological effector functions of antibody molecules. Light chains are of two major types (κ and λ). The type of light chain present in a particular antibody does not effect the class or subclass of the immunoglobulin. Although both κ and λ light chains are present in all species, the percentage of each type of light chain that is expressed can vary considerably. In the mouse the light chains are predominantly of the κ type, approximately 95% κ and only 5% λ, whereas in human the ratio of κ to λ is approximately 2:1.[13]

1.5 The T-cell antigen receptor

Antigen recognition by CD4+ T lymphocytes differs from that by antibodies in a number of important aspects. Whereas antibodies bind antigen molecules free in solution, CD4+ T lymphocytes recognize peptide fragments of antigens that lie in the antigen binding pocket of class II major histocompability complex (MHC) molecules expressed on antigen-presenting cells. Specificity of T cells is determined by structural features of both the MHC molecule and antigenic peptide. MHC class II amino acid sequences are highly polymorphic (large number of variants at the same gene locus) within a population, and correlate with individual differences in response to infectious agents, vaccines, tumour antigens, and autoantigens.[14] Antigen processing involves the intracellular catabolism of proteins to produce immunogenic peptides that bind to class II MHC molecules.[15] MHC class II expression occurs constitutively on classical antigen-presenting cells (APC) like Langerhans cells of the skin, dendritic, macrophages and B cells.[16] The part of the proteolytic fragment which interacts with the T cell antigen receptor (TCR) is a continuous epitope and is called a T cell epitope.

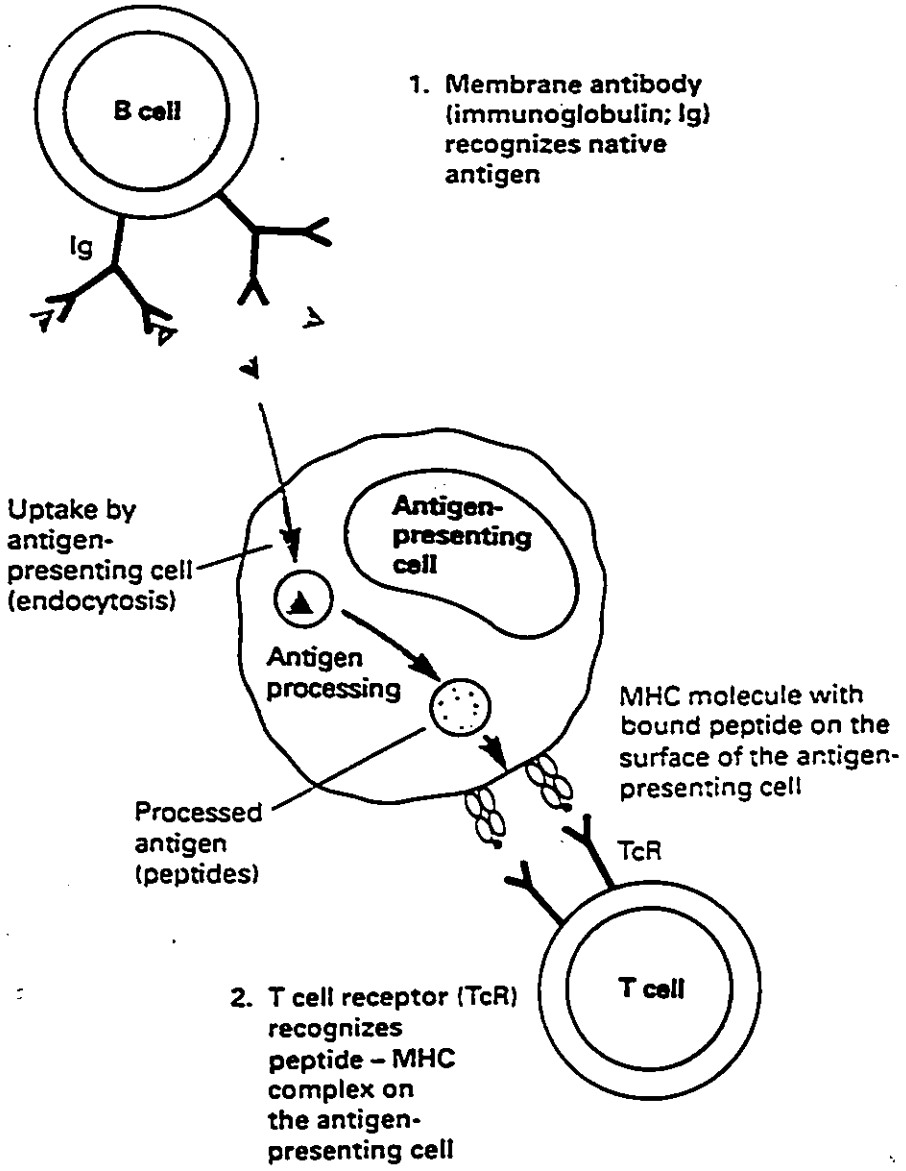


FIGURE 1.6 B cells can recognize native antigens; T cells recognize peptide-MHC complexes on antigen presenting cells.[4]

T cell antigenic epitopes in native proteins are generally unable to interact directly with MHC molecules because of their internal location or other steric hindrance constraints. The antigen-specific immune response is initiated by CD4+ T lymphocytes that are activated by the antigen bound to a relevant MHC class II protein on the surface of antigen-presenting cells (APC).[17]

Recognition of MHC proteins complexed with peptides derived from protein antigens, stimulates DNA synthesis and cell division, and causes the release of lymphokines that mediate T-cell function.[18] One of the hallmarks of class II MHC molecules is their extraordinary allelic polymorphism.

Unlike the antigen receptors, however, the polymorphism of MHC molecules is maintained in the germline MHC genes. Different MHC haplotypes vary in their ability to present different antigens and for this reason MHC genes control immune responsiveness, acting at the level of T cell antigen recognition. The ability to generate receptors which can recognize any antigen means that there must be specific mechanisms to prevent auto-immune reactions.

The effect of allelic polymorphism in T cell responses is dual: quantitative effects, epitope selection, that is the capacity for high affinity or effective binding of particular peptides and qualitative effects, T cells are unable to bind free antigen in the same way as B cells and need to see antigen on the surface of accessory cells, forming a

trimolecular complex. Two highly related MHC molecules that differ by a single residue could both bind a particular peptide, however an individual T cell receptor will only bind to one form.[3] The same antigenic peptide may indeed be recognized bound to many different DR molecules, and it has been demonstrated for tetanus toxin peptides.[19] The structural basis for this broad binding capacity to DR molecules could be for conformational reasons. So we can not talk about T cell epitopes simply by looking at their sequences. It is in the context of the MHC molecule to which they are bound that actually determines the structure that a receptor can recognize. Similarly, a single amino acid substitution may be sufficient to drastically decrease the binding of a peptide to a class II molecule.[20,21] Thus interactions between peptides and class II molecules usually tolerate aproximately 80% to 90% of single amino acid substitutions in the peptide molecule without appreciable effects. In contrast, recognition of the peptide-MHC complex by the TCR is highly specific since it accepts only 10% to 20% of single amino acid substitutions in a given peptide.[1] Overall, the peptide-class II molecule interaction is permissive and the recognition by TCR's is specific.

It is well established that T lymphocytes are unable to bind free antigen. They recognize antigen as a ternary complex formed with MHC gene products on the membrane of accessory cells. Furthermore, the T cells must encounter that same gene

product expressed by the host during their development.[22]

Another major difference between T cell and antibody recognition of an antigen is that T cell recognition tends to be limited to a small number of immunodominant sites, whereas antibodies recognize sites all over the surface of a protein molecule.[1] Usually, only a few of the peptides that might be released by protein breakdown are immunogenic, i.e., capable of stimulating T cells and initiating an immune response. After recognition of peptide-MHC complexes, the TCR transduces signals, resulting in the production of cytokines and the entry of the cell into a proliferative cycle.[23] Human CD4+ T cells represent a functionally heterogeneous population in their profile of cytokine production. T_H1 cells produce gamma interferon (IFN-γ), IL-2, and tumor necrosis factor (TNF)-ß; these cells promote macrophage activation (which results in delayed type hypersensitivity) and production of both complement-fixing and opsonizing antibodies.

T_H2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, provide optimal help for humoral immune responses [including immunoglobulin E (IgE)], and promote both mast cell and eosinophil differentiation and activation. In the absence of a prominent differentiation to T_H1 or T_H2 cells, most CD4+ T cells produce both T_H1- and T_H2-type cytokines and are called T_H0 cells[24].

Broadly speaking, the lymphokines produced by T_H1 cells promote cell-mediated immune responses, augment IgM and IgG2 synthesis by B cells, and activate macrophages. The T_H2-derived lymphokines, on the other hand, lead to enhanced IgG1 and IgE responses and to increased numbers of local and/or circulating eosinophils (eosinophilia).[25]

Chapter 2: Cancer Immunotherapy

2.1 Introduction

An ideal vaccine should confer long-lasting, preferably life-long, protection against the disease. It should be inexpensive enough for large-scale use, stable enough to remain potent during shipping and storage, and leave no adverse effects on the recipient.[26]

The progression of cancer depends not only on the intrinsic malignant potential of the tumor, but also on the patient's immune response. As a consequence, it may be possible to increase resistance to cancer by stimulating specific anti-tumor immunity with a vaccine.[27]

Human cutaneous malignant melanomas have been demonstrated to be immunogenic in patients by *in vitro* and *in vivo* analyses. Sialylated glycosphingolipid (gangliosides) antigens are prominent constituents displayed on the surface of melanoma cells. Active specific immunotherapy with vaccines constructed of gangliosides is a conceptually attractive approach to treat and possibly prevent melanomas.[27] However gangliosides are poorly immunogenic because they do not appear to bind to class II major histocompatibility complex molecules (MHC II), which explains the T-cell independence of carbohydrate antigens.[28]

The recognition of an antigen by T lymphocytes is essential for its effective elimination by the host.

T lymphocytes of the CD4 or CD8 subsets recognize antigen but only after an internal processing event by antigen presenting cells (APC) that results in the generation of immunogenic peptides. Such peptides associate with histocompatibility molecules to form bimolecular complexes on the cell surface. The T cell receptors for antigen recognize the bimolecular complex and initiate the events that result in an inflammatory response.[29]

Ganglioside-targeted passive immunotherapy by monoclonal antibodies involves the administration of monoclonal anti-ganglioside antibodies capable of mediating antibody-dependent or complement-dependent cytotoxicity. Long-term repeated administration of murine monoclonal antibodies is not possible, since patients develop anti-murine immunoglobulins, which cause hypersensitivity against foreign proteins. Therefore, regression is dramatic but temporary, and recurrence is common. Human monoclonal antibodies against gangliosides showed no significant response, unless the patient's melanoma possessed the target antigen to which the human monoclonal antibody was directed.

Ganglioside-targeted active specific immunotherapy with purified gangliosides has been proven ineffective because gangliosides alone do not possess intrinsic adjuvanticity.[30]

There is currently a need for vaccine development to improve the immunogenicity of ganglioside epitopes, which by themselves are poorly immunogenic. Although the low

immunogenicity of gangliosides can be circumvented by linking them to highly immunogenic large carrier molecules and adjuvants, such carriers derived from current vaccines have not proven to be generally effective. The importance of carriers is their ability to stimulate T helper cell responses necessary for the generation of humoral responses which require cooperation between T and B cells. Tetanus toxoid (TT) is such a carrier molecule because it has been used for human vaccination for many years and is devoid of side-effects. However, there are two limitations in using TT as a carrier molecule. TT is usually administered in clinical situations in amounts of 5 μg onto which it is possible to couple only a small amount of gangliosides.

Several studies have also demonstrated that the immune response against a synthetic epitope conjugated to TT can be suppressed by pre-existing immunity against this same carrier.[31]

The aim of this project is to define epitopes of tetanus toxin and characterize a T helper cell epitope mapped on fragment C. This is part of a project which focuses on the production of Multiple Antigen Peptide-Carbohydrate Conjugates for cancer immunotherapy and serodiagnosis.

The T helper cell epitope will be used to construct an octameric multiple antigen peptide (MAP) which will constitute the T-cell dependent carrier core. The construction of the octameric carbohydrate-peptide cluster will overcome the

problems of low immunogenicity of gangliosides, the immunoregulatory mechanism of epitopic suppression and the problem of antibody class switching.

2.2 Tetanus toxin

Tetanus and botulism have fascinated mankind since they were described by Ἱπποκράτης (Hippocrates) cited by Major (1965) and Kierner (1817) respectively.[32] The progress in understanding these diseases started with the detection of the causative bacteria and their toxins at the end of the 19th century, namely *Clostridium tetanus* and *Clostridium botulinum*. The clostridia are anaerobic rods, are generally gram-positive and many produce exotoxins. Several species are pathogenic, and many occur as saprophytes in the soil and in the intestinal tracts of man and other animals.

Tetanus toxin is a highly potent neurotoxin produced by toxigenic strains of *Clostridium tetani*, an anaerobic organism, which is widely dispersed in nature. It binds to the presynaptic membrane at the neuromuscular junction, penetrates into the motorneuron and moves retroaxonally to reach the cytoplasm of the inhibitory interneurons of the spinal cord. Here it blocks the release of neurotransmitters, thus causing the spastic paralysis of tetanus.[33-35]

In most animal species the LD_{50} (the dose of toxin required to kill 50% of a group of animals within a specified time) is in the range of 1 to 5 ng/kg, i.e. 10^8 molecules are

sufficient to kill a mouse.[36] It has long been known that the disease is due to active secretion of the toxin and the ascent of Immunology has led to the development of toxoids and antibodies that paved the way for treatment and prophylaxis of the disease.[32] Efficient resistance to tetanus is induced by vaccination with formaldehyde-treated toxin or toxin subfragments.[37]

Tetanus toxin is a protein, containing no lipid or carbohydrate moieties and has a mol wt of 150,700 Da. It is synthesized as a single-chain polypeptide and upon release from the bacterium, it is processed by endogenous proteases into two subunits, designated light and heavy chains which are held together by a single disulfide bond. According to the new nomenclature (8th International Conference on Tetanus, Leningrad, 1987),[38] the toxin is composed of three domains, A, B, and C. Fragment A, the light chain (mol wt 52,000 Da) and fragment B.C, the heavy chain (mol wt 98,000 Da).[39] The amino acid sequence of tetanus toxin was determined simultaneously by two groups by cloning the gene that codes for the toxin (APPENDIX I).[40-41]

2.3 Toxoid Production

Tetanus toxoid is the immunizing antigen protecting against the adverse effects of infection by tetanus organisms. To produce the conventional tetanus vaccine, the highly toxinogenic Harvard strain of *C. tetani* is grown in a semisynthetic medium in a fermentor for about one week, until

the bacteria lyse and release tetanus toxin into the supernatant. The average yield obtained under these conditions is approximately 60 to 80 Lf/mL (Lf, the limit of flocculation, is equal to the amount of toxin flocculating most rapidly with one unit of antitoxin) (1 Lf = 2-2.5 μg of tetanus toxin).[42]

The culture is then filtered, and the filtrate containing the toxin is detoxified by adding formaldehyde to a final concentration of 0.5%. The pH is adjusted to 7.6 and the supernatant is then stored at 37° C for four weeks to allow the complete detoxification of tetanus toxin. During the detoxification process, formaldehyde reacts with the toxin molecules, peptones, and other proteins present in the medium.

The first reaction involves mainly the ε-amino groups of lysine, is rapid, fully reversible and results in imine formation (Schiff's base):

$$R\text{-}NH_2 + H_2CO \rightleftharpoons R\text{-}NH\text{-}CH_2OH + H^+ \rightleftharpoons R\text{-}\overset{+}{N}H{=}CH_2 + H_2O \quad (1)$$

$$R\text{-}\overset{+}{N}H{=}CH_2 + H_2NR \rightleftharpoons R\text{-}NH\text{-}CH_2\text{-}NH\text{-}R + H^+ \quad (2)$$

whereas the second reaction occurs much more slowly and involves the reaction of one of the above unstable products with a second molecule containing an amino group (1), an imidazole ring (2), or a phenol ring (3). The final result of the formaldehyde treatment of tetanus toxin involves a cross-linkage between an ε-amino group of lysine and (a) a

second amino group, (b) a histidine, and (c) a tyrosine or a tryptophan, through a stable methylene bridge (-CH_2-).

These products are present in the acid hydrolysates of toxoids and can be easily identified by amino acid analysis. Obviously, these reactions can occur (a) between amino acids of the same toxin molecule, resulting in internal cross-linking of the protein; (b) between two toxin molecules, resulting in dimerization; or (c) between a small peptide present in the medium and a toxin molecule.

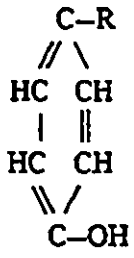
It is also likely that 3-D structures are affected, therefore making toxic conformational epitopes.

$$R\text{–}NH_2 + H_2CO \rightleftarrows R\text{–}NH\text{–}CH_2OH + H^+ \rightleftarrows R\text{–}NH{=}CH_2 + H_2O$$

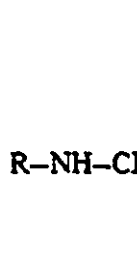
1. $R\text{–}NH_2 + R\text{–}NH\text{–}CH_2OH \rightarrow R\text{–}NH\text{–}CH_2\text{–}NH\text{–}R + H_2O$

2. $R\text{–}NH\text{–}CH_2OH + HC{=}C\text{–}R \rightarrow R\text{–}NH\text{–}CH_2\text{–}C{=}C\text{–}R + H_2O$

3. $R\text{–}NH\text{–}CH_2OH +$



$\rightarrow R\text{–}NH\text{–}CH_2\text{–}$



$+ H_2O$

A schematic representation of this process is shown in the figure of the previous page: when the formaldehyde is added to the culture supernatant containing the toxin molecules surrounded by a molar excess of peptones deriving from the culture medium, the main reaction that occurs is a cross-linkage between the peptones and the toxin molecules, such that, at the end of the process, the toxoids are coated with a number of peptides deriving from the beef used to prepare the medium. These peptides are unnecessary antigenic determinants. The purification of the toxoid is carried out in two stages. The crude toxoid is first passed through an ultrafilter and the concentrated toxoid is then subjected to fractional precipitation with ammonium sulfate.

2.4 Immunogenicity of tetanus toxoid

Vaccination with tetanus toxoid elicits immunologic memory indicating that it is a T lymphocyte-dependent antigen. This memory should be useful when conjugating T cell-independent polysaccharide antigens to tetanus toxoid.

Tetanus toxin, a protein of 1,315 amino acids, has a molecular weight of 150,700 Da. The scheme followed for the preparation of tetanus toxin peptides by specific enzymatic and chemical procedures described in this project is as follows:

T CELL EPITOPES IN FRAGMENT C OF TETANUS TOXIN

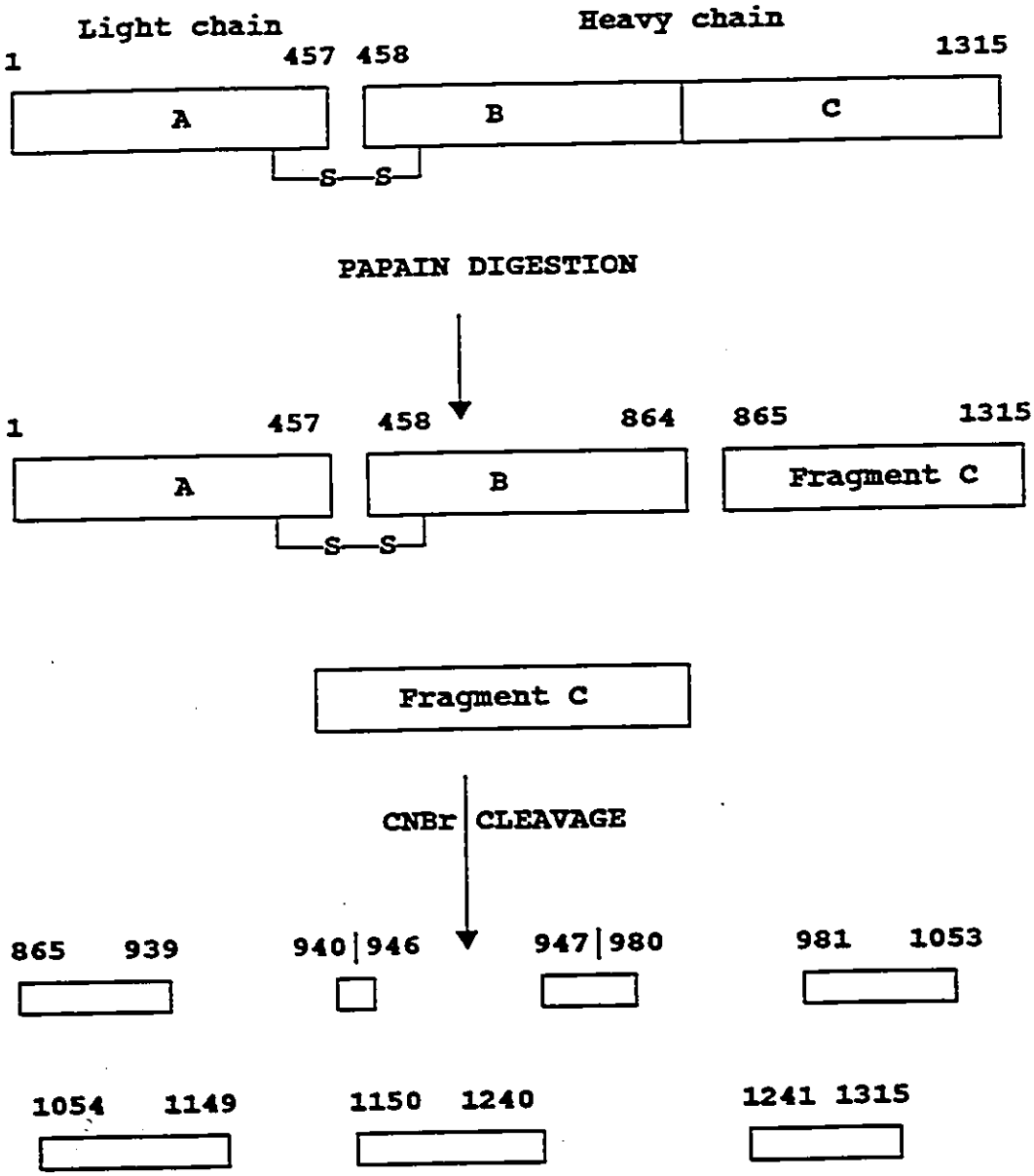


FIGURE 2.1 T cell epitopes in fragment C of tetanus toxin. 1: 916-932, 2: 937-967 and 3: 1273-1284

Chapter 3: Preparation of Antibodies against Tetanus Toxin and Toxoid

3.1 Introduction

Antibodies are serum immunoglobulins with binding specificity for particular antigens. The usual methods for eliciting antibodies involve immunization with purified or partially purified antigen preparations.[43] Immunization of mice or guinea pigs can easily be accomplished by injecting tetanus toxoid or toxin in doses that do not cause toxicity. Freund's Complete Adjuvant (CFA) was used for the generation of antibodies in both animal species. The composition of CFA is mineral oil containing heat-killed mycobacteria (*Mycobacterium tuberculosis* or *Mycobacterium butyricum*) and is used as an emulsion with aqueous antigen. The Freund's incomplete adjuvant (ICFA) is mineral oil used as an emulsion with aqueous antigen.[42] The route of administration of a mixture of antigen and Freund's adjuvant was by a subcutaneous injection.

Immunization with purified toxin is more difficult because the nervous system is more sensitive to tetanus toxin than the immune system.[44] Therefore, the animals were protected by a combined subcutaneous injection of toxin and antitoxin in the first immunization.

3.2 Results and Discussion

3.2.1 Isotypes

3.2.1.1 Introduction

It is known that protection against infectious agents is achieved by successful induction of antibodies of the appropriate class to a subset of recognizable epitopes (sequential and assembled) conventionally called protective or neutralizing epitopes.[45]

Monoclonal antibodies (MAbs) to melanoma are specific and powerful inhibitors of growth of tumors in experimental animals and in patients. Inhibition of tumor growth depends on antibody isotype. It has been shown conclusively that only the MAbs of IgG_{2a} and IgG_3 isotype were effective in melanoma tumor destruction whereas MAbs of IgG_1, IgG_{2b}, IgM and IgA isotype were inactive.[46] The ability of IgG_{2a} MAbs to inhibit tumor growth in nude mice strongly correlates with their reactivity in antibody-dependent macrophage-mediated cytotoxicity assays. IgG_{2a} MAbs that are reactive *in vivo* are also reactive in these assays *in vitro*. Complement, however, mediates lysis of target cells with MAbs of IgG_3 and IgM isotypes and not IgG_{2a}.[47]

Human IgGs consist of four isotypic subclasses which are structurally closely related and show > 95% amino acid sequence identity between homologous domains (IgG1, IgG2, IgG3 and IgG4). The differences all lie in the heavy chains which have been labelled $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ respectively. The heavy chain is responsible for the effector functions of antibody

molecules. These functions are mediated by antibody constant regions.

The most basic requirement for a cancer vaccine is that it should stimulate a strong and clinically effective antitumor immune response in humans. Evaluation of a vaccine's immunogenic potential involves measuring the antibody and cellular responses which it is able to induce in humans, and determining whether these responses are directed to tumor as opposed to unrelated antigens.[48]

Anti-Melanoma antibodies for example must have strong anti-tumor effects either by themselves or in the presence of complement or effector cells such as killer (K) cells or macrophages. Antibodies of some isotypes efficiently activate complement, bind to high affinity receptors on monocytes, and act synergistically with antibody-dependent effector cells to produce cytotoxicity such as IgG_{2a} and IgG_3.[49]

3.2.1.2 Determination of isotype and their relative concentration in the generated murine antibodies

The qualitative identification and quantitative determination of antibodies made by an immunized animal, provide useful information concerning the immune system. The speed and sensitivity of ELISAs make them the assays of choice for isotype determination.

The murine antibodies raised against tetanus toxin and toxoid (pool of 3 mice) were isotyped by ELISA using a 96-well

plate coated with Tetanus toxin and toxoid. Five concentrations of toxin and toxoid (10, 5, 1, 0.1 and 0.01 μg/ml) in carbonate buffer were absorbed at 200 μl/well for 18 hours on a microtitre plate at 4° C. The different sera produced were assayed against these concentrations and the optimal antigen concentration, based on maximal activity of positive sera and acceptable background activity of negative sera, was 1.0 μg/ml of toxin and 0.5 μg/ml of toxoid. Various dilutions of the sera were also assessed for optimal binding. The murine antibodies raised against tetanus toxin and toxoid were diluted 1:100, the optimal dilution obtained in trial assays and applied to the plates as described above for 1 hour at 37° C. After immunoglobulins reactive with the coated antigens had adhered, the wells were washed with 0.01 M Phosphate buffer saline, pH 7.2 containing 0.05% Tween 20 (PBS-T) and rabbit antibodies directed against murine IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, κ chain and λ chain were applied to identify reactive species. The incubation with a secondary goat anti-rabbit IgG (H+L)-HRP conjugated antibody and the 3,3',5,5' Tetramethylbenzidine (TMB) peroxidase substrate resulted in a color reaction which was measured at 450 nm and led to the identification of the different isotypes. As these concentrations and dilutions were found to be optimal in binding for each antigen and antibody, one can make a comparison of the extent of the binding, as measured by the O.D. of the color reaction. The O.D. values depicted in the

A diagramatic representation of the Indirect ELISA for the determination of the different isotypes is shown below:

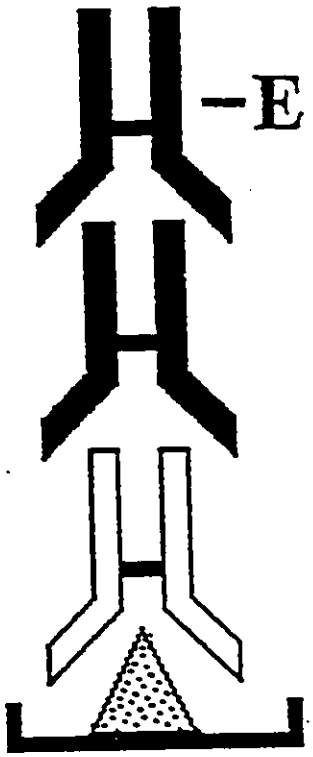


DIAGRAM 1. Indirect ELISA

Figure 3.1 is representative of results of plates coated with toxin only, but treated with both mouse anti-toxin and anti-toxoid. The highest binding of the toxin with either anti-toxin or anti-toxoid was seen to be with the IgG_1 fraction. The light chain of either antiserum was of the kappa type rather than the lambda type. Since in the mouse, the light chains are predominantly of the κ type, this finding was expected. In any one molecule light chains are of the same type and hybrid molecules do not occur.[50] IgA showed the lowest binding, and IgM moderate binding at about 28% of the IgG_1 level. The other subgroups of anti-toxin and anti-toxoid showed differential binding with the coated toxin in that IgG_{2a} and G_{2b} from the anti-toxin were fairly reactive (52% to 66% of the IgG_1) while those isotypes from the anti-toxoid serum were little reactive. The IgG_3 isotype, although of low binding, also displayed this difference. Of noteworthy interest was the observation that as an immunogen the toxin had elicited more IgG subgroups than had the toxoid. The toxoid was able to elicit IgG_1 and IgM. Considering that IgG_{2a} and IgG_3 isotypes have demonstrated antitumor activity rather than the other isotypes,[51-53] the tetanus toxin appears by the present studies, to be a superior antigen for eliciting such production. The anti-toxin displayed the strongest binding of the two sera towards the toxin. This antiserum was next examined for its binding against toxoid coated wells in comparison with those coated with toxin.

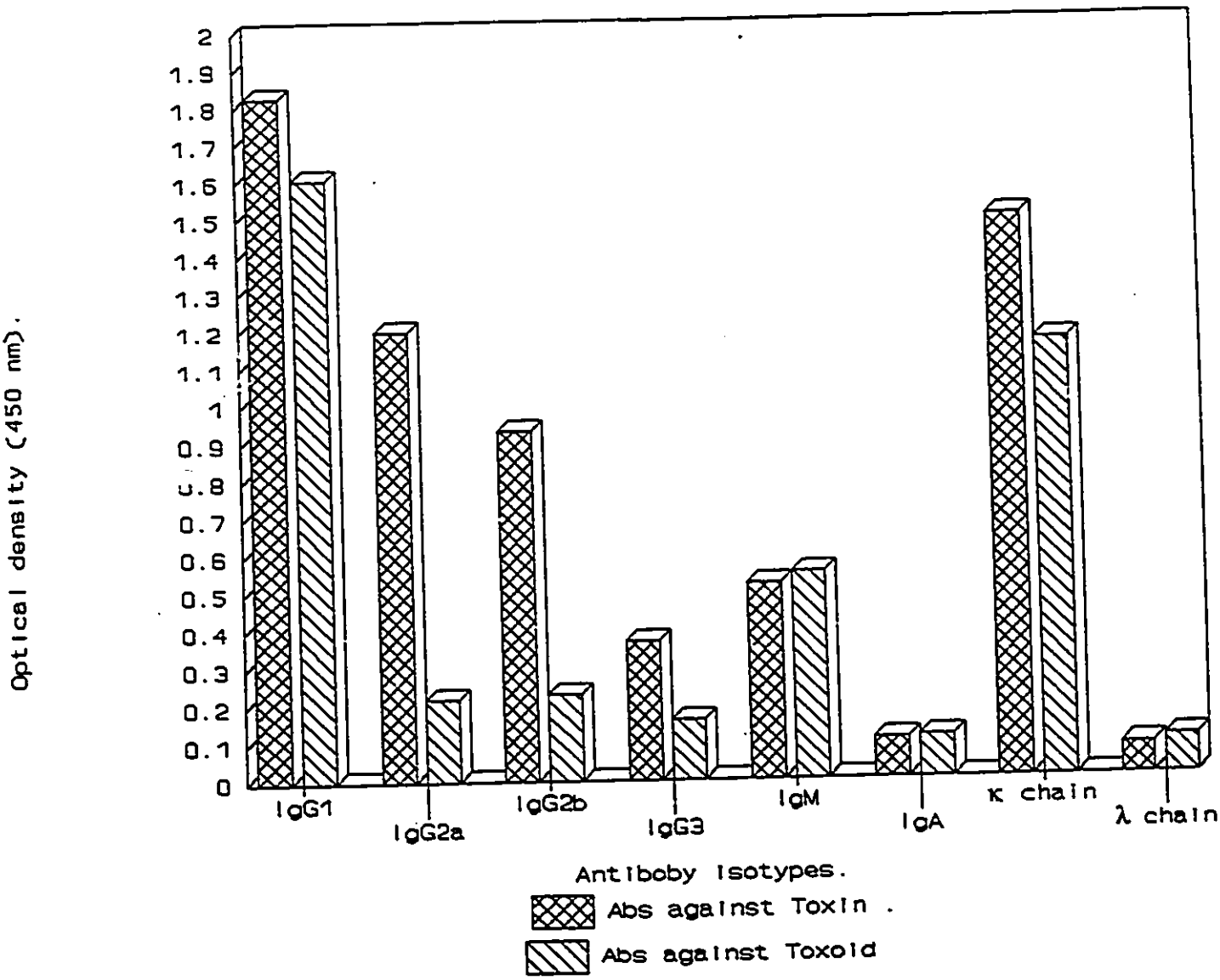


FIGURE 3.1 Isotype determination of Murine Antibodies reacting with Tetanus Toxin

Figure 3.2 represents results of plates coated with toxin and toxoid where the antiserum to the toxin was thus compared. This one antiserum showed virtually the same binding with toxin and toxoid in all isotypes. The kappa chain was again seen to be involved, while the IgG_1 showed the highest levels. The IgG_{2a} and IgG_{2b} were in the range of 52% to 66% of that level, for either antigen. The IgG_3 and IgM levels were in the 16% to 28% range of the levels of the IgG_1, while the IgA and lambda chain had minimal binding. The similarity of binding measurements of the two sera with either antigen suggested that epitopes on the toxoid were equally well recognized as those on the toxin by the Ig isotypes in the anti-toxin. This observation suggests also that the different concentrations of coating antigen did not make a quantitative difference to the binding under optimal conditions.

The binding of both anti-toxin and anti-toxoid antisera against wells coated solely with the toxoid is shown in Figure 3.3. Again the kappa chain of these antibodies was seen as responsible while the lambda chain and IgA had negligible binding. The IgM of either antiserum was slightly more reactive than IgA but at 20% to 25% of the most reactive isotype, the IgG_1. Again it was seen that the IgG_1 of either antiserum was equally reactive with toxoid.

However, the IgG_{2a}, IgG_{2b} and IgG_3 isotypes differed between antisera: the isotype derived from the anti-toxoid was little reactive.

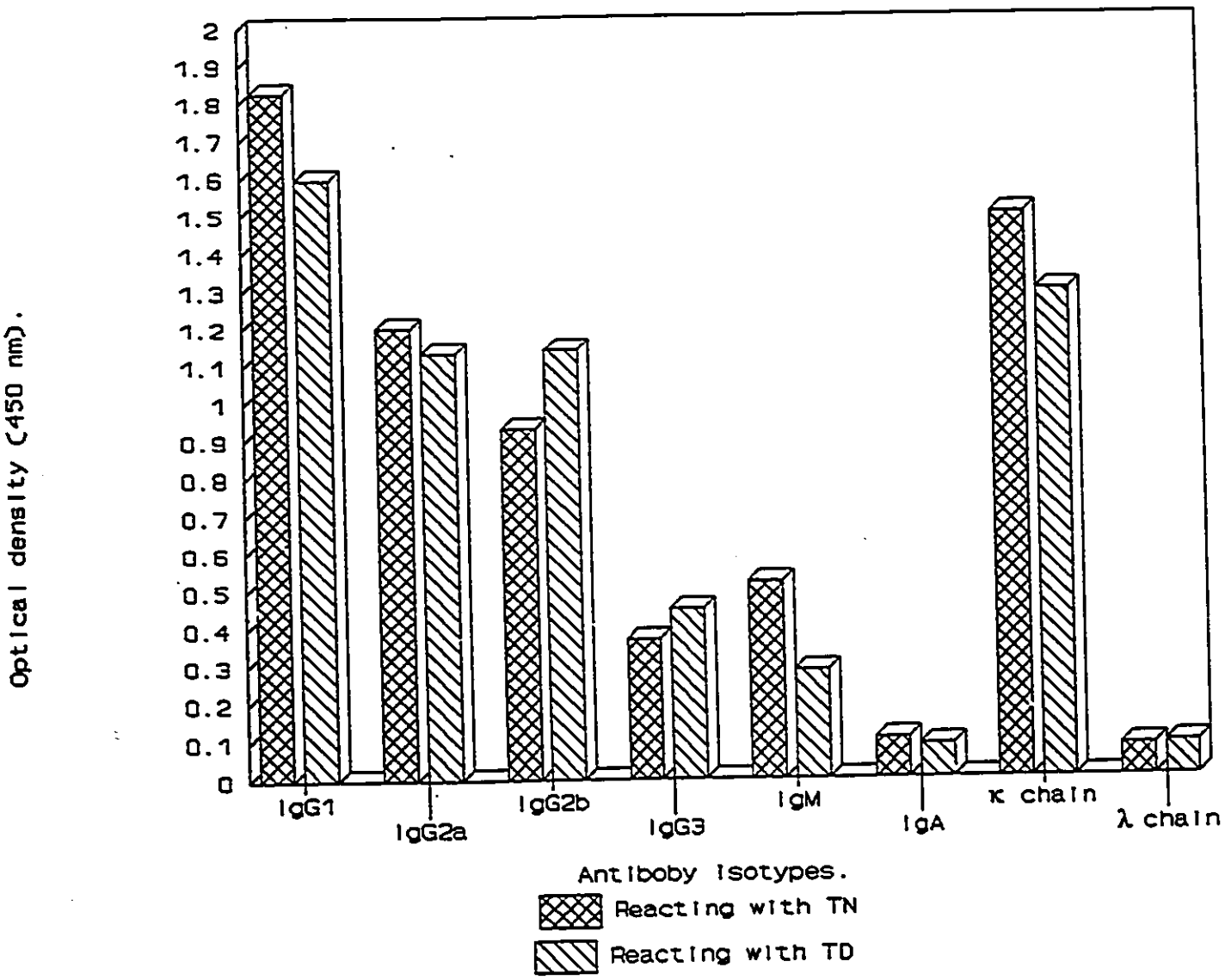


FIGURE 3.2 Isotype determination of Murine Anti-toxin reacting with Tetanus Toxin (TN) and Toxoid (TD)

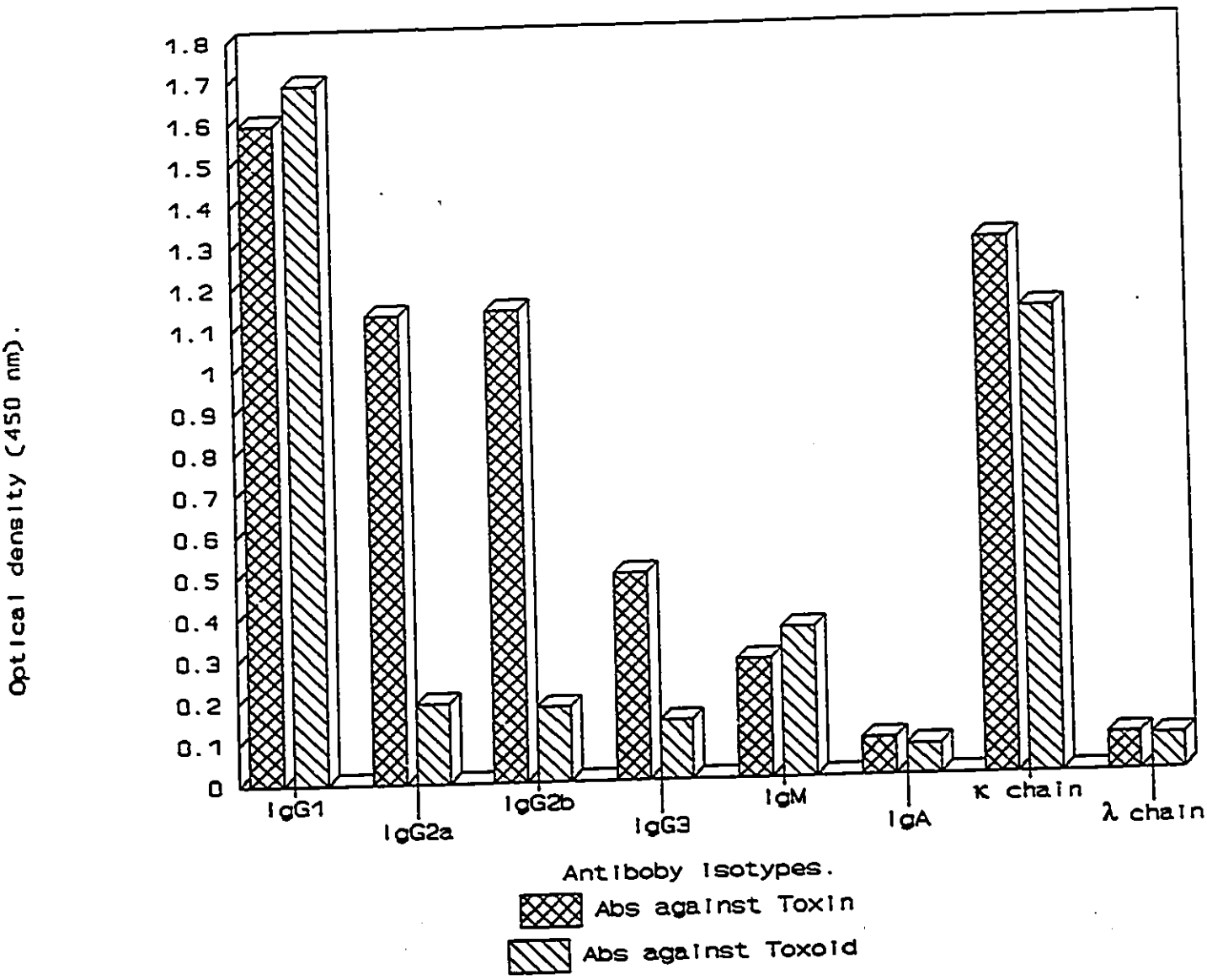


FIGURE 3.3 Isotype determination of Murine Antibodies reacting with Tetanus Toxoid

The IgG_{2a} and IgG_{2b} from anti-toxin reacted with toxoid at about 70% of the level that IgG_1 reacted. The anti-toxin IgG_3 reacted at about 25% of that. These findings have suggested that generally anti-toxoid antibodies have a limited variety of binding in comparison to anti-toxin antibodies. Figure 3.4 depicts comparative testing of the binding of anti-toxoid against both toxin and toxoid.

Low binding of the antiserum was confirmed by the low quantities of IgA, IgG_3, IgG_{2a} and IgG_{2b} against either antigen. But the antiserum did demonstrate that its main binding was in IgG_1 with either antigen, and that the kappa chain was the dominant reactive light chain. The percentages of values for the IgG isotypers in the two sera were estimated and are shown in Table 3.1. These results confirm that, the selection of antigen determines the type of isotype produced and that the antibodies produced cross-react with the toxin and toxoid.

Percentage Values of IgG Isotypes

	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3
α-Toxin vs Toxin	42.2	27.7	21.5	8.5
α-Toxin vs Toxoid	37.0	26.2	26.4	10.4
α-Toxoid vs Toxoid	76.6	8.8	8.2	6.4
α-Toxoid vs Toxin	74.7	9.9	10.4	7.1

TABLE 3.1 Binding of the murine antibodies generated against tetanus toxin and toxoid with toxin and toxoid. The percentage values of the different IgG isotypes were calculated from the absorbance maxima at 450 nm. The ELISA was performed as described earlier. The sera used were from a pool of 3 immunized mice.

Thus toxoid as antigen appears to have provoked antibodies mainly in the form of IgG_1, and of IgM at one quarter the IgG_1 binding (Figure 3.4). It is the toxin which appears to have elicited antibodies with a variety of isotypes (Figure 3.2), and for the most part these globulines reacted similarly with toxin and toxoid.

Having looked at the subfractions of the antisera to whole toxin and toxoid it became of interest to examine the binding of the whole sera with fractions of the antigens in order to map the antigenic epitopes recognized by the antibody combining sites (paratopes) of the sera. The paratopes are complementary to the structure of the epitopes.

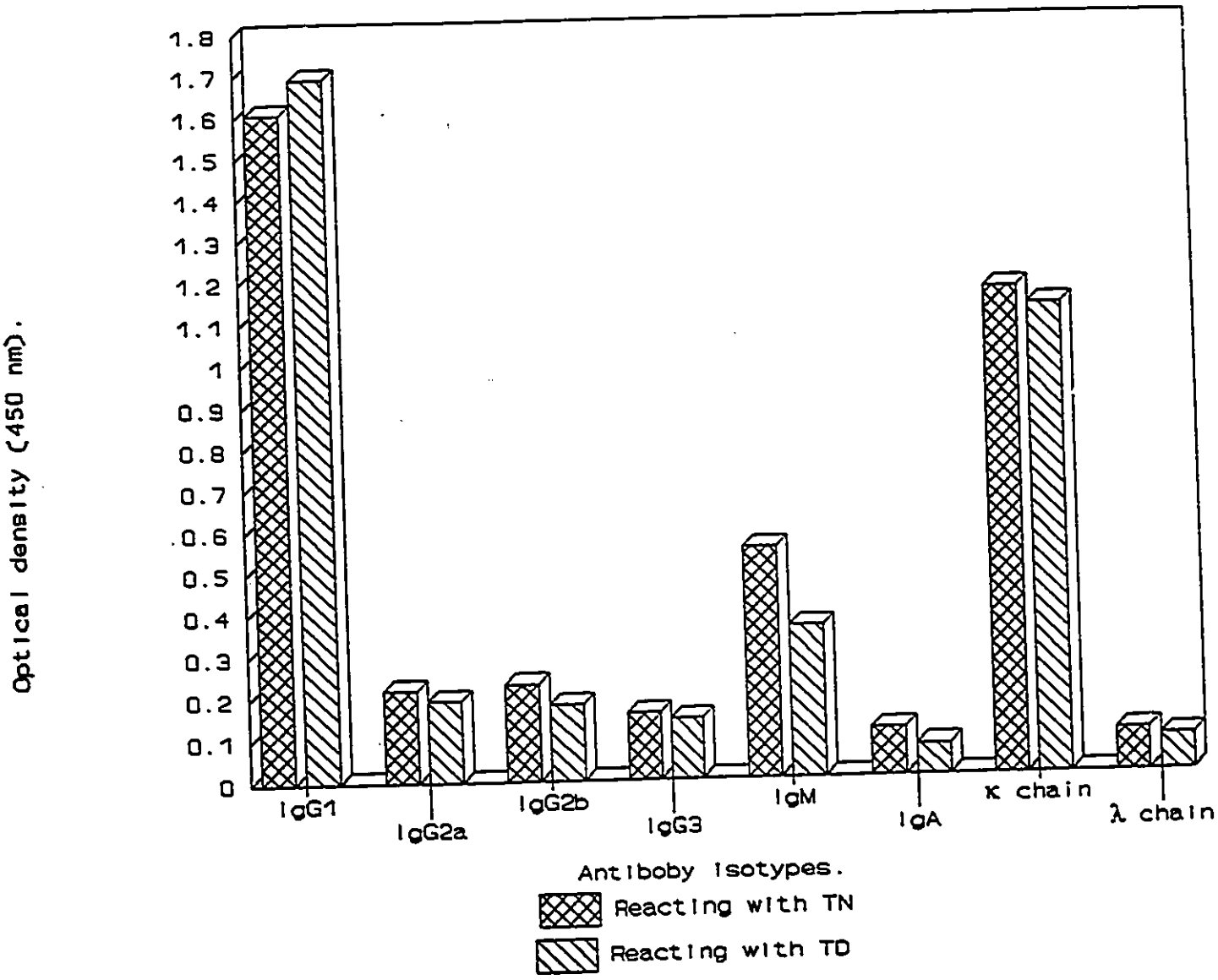


FIGURE 3.4 Isotype determination of Murine Anti-toxoid reacting with Tetanus Toxin (TN) and Toxoid (TD)

3.2.2 Binding of the Murine and Guinea pig sera

3.2.2.1 Introduction

The assay was performed essentially as described by Voller *et al.*[54] Briefly, 96-well plates were coated with Tetanus toxin or toxoid. Antibodies raised against tetanus toxin and toxoid were applied to the plate for 1 hour. Next, the plates were incubated with horseradish peroxidase conjugated goat antibodies directed against the γ chain of mouse immunoglobulins. The addition of TMB peroxidase substrate resulted in a colour reaction and the absorption was determined at 450 nm.

3.2.3 Binding of Fragment C of Tetanus Toxin

The specific fraction of the toxin known as fragment C, which is a product of papain digestion of toxin, is known to be an antigenically reactive portion, and recombinant fragment C of tetanus toxin (rTTC) is commercially available for testing. Figure 4.1 will demonstrate how fragment C can be obtained from the toxin, however, the product used for this study was a commercial sample. It was used to compare the binding of the MAP, toxin and toxoid with the antisera.

Figure 3.5 shows the binding of the two murine antisera with coated antigens, developed by enzyme linked to antibody to the γ chain of the mouse Ig. Thus, the readings should represent the sum of the binding measurements of the Ig subtypes. The antisera reacted with whole toxin at levels such that the binding was, for the anti-toxoid, 80% of the binding

with the anti-toxin. On the other hand the whole toxoid reacted less (about 50%) with its own antiserum that with the anti-toxin, which is consistent with the lower binding measurements seen in Figure 3.4 than seen in Figure 3.2. This observation indicated that toxoid, as an antigen, was less immunogenic than toxin. The rTTC reacted with anti-toxoid at about 80% of the level at which it reacted with anti-toxin. This ratio was similar to the differential binding of whole toxin with the two antisera. The lower total values for binding of either antibody with rTTC were approximately 70% of the values for the binding with the toxin. This suggested that in mouse serum, 28% of anti-toxin or anti-toxoid antibodies are directed against epitopes not found on fragment C and that the bulk of the antigenicity in both sera have their origin in the C fragment.

The latter comparison, of murine anti-toxin and anti-toxoid with toxin, toxoid and rTTC (Figure 3.5) was next retraced using the two guinea pig sera which had been produced against toxin and toxoid. Figure 3.6 indicates that the two guinea pig sera were close to each other in binding with the toxin, and the difference may not be significant, anti-toxin showing 94% the binding of the anti-toxoid, while against the toxoid anti-toxoid showed 92% the binding of the anti-toxin. Additionally there were similar levels of binding of guinea pig anti-toxin to toxin and to rTTC (the former being 95% of the latter) which suggested that most of the antibodies in

the antibodies in this anti-toxin are again directed against epitopes within the fragment C.

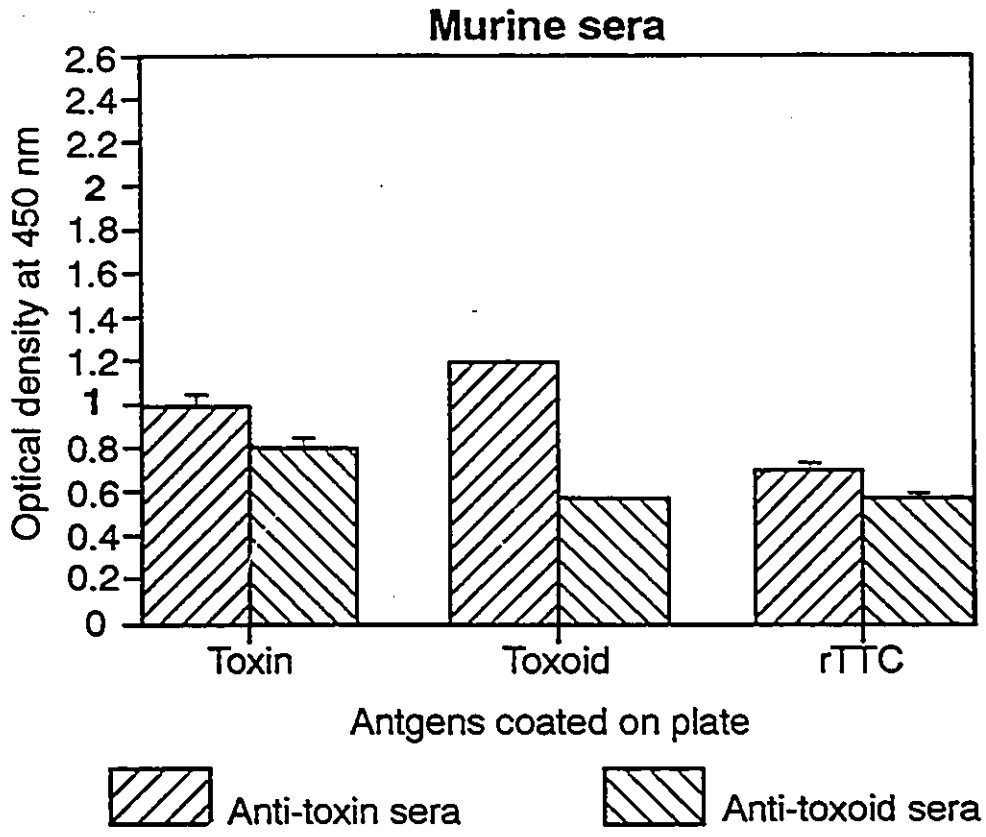


FIGURE 3.5 Murine Antibodies reacting with Tetanus Toxin, Toxoid and rTTC

Against fragment C the anti-toxoid showed only 75% of its reactivity against the anti-toxin, and a similar 75% ratio was seen when comparing the anti-toxoid reactivity against fragment C and against whole toxin. These observations suggested that unlike mouse serum, guinea pig serum can develop, in response to toxin as antigen, antibodies against toxin which are mostly directed against the fragment C portion. On the other hand, guinea pigs seem to respond to the toxoid as antigen, by producing antibodies to more than fragment C. That is, since 75% of the toxin reactivity of the anti-toxoid is against fragment C there must be antibodies against other epitopes as well.

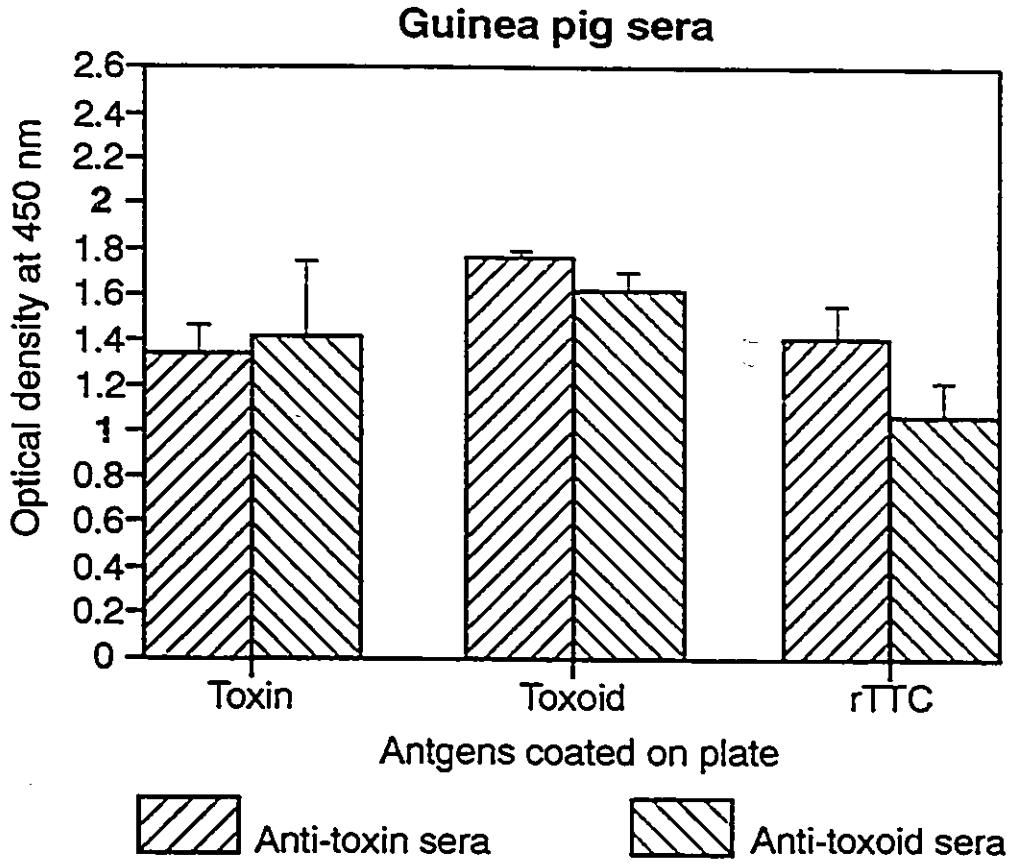


FIGURE 3.6 Guinea Pig Antibodies reacting with Tetanus Toxin, Toxoid and rTTC

3.3 Summary

In summary, immunization protocols for the production of antibodies against tetanus toxin and toxoid were established. Antibodies were successfully raised against the toxin without mortalities in either species. The murine sera obtained, were isotyped by ELISA and the toxin was proven to be a superior antigen in eliciting production of IgG_{2a} and IgG_3, the two isotypes which have demonstrated antitumor activity. The anti-toxoid sera exhibited a lower binding towards the toxin and toxoid when compared with anti-toxin sera. The binding of rTTC was studied and the results indicated that in the murine serum, 28% of anti-toxin or anti-toxoid antibodies are directed against epitopes not found on fragment C. The study of the guinea pig sera, suggested that unlike mouse serum, guinea pig serum can develop, in response to toxin as antigen, antibodies against toxin which are mostly directed against the fragment C portion. On the other hand, guinea pigs seem to respond to the toxoid as antigen, by producing antibodies to more than fragment C. That is, since 75% of the toxin binding of the anti-toxoid is against fragment C there must be antibodies against other epitopes as well.

3.4 Experimental

PART A - Immunization with Tetanus toxin

The Protein concentration of the sample of Tetanus Toxin (TT-1, Connaught Laboratories) used for immunization was determined using the BIO-RAD Protein Assay (Appendix II).

3.4.1 Murine Antibodies Raised Against Tetanus Toxin

3.4.1.1 Introduction

Fifteen μg of Tetanus Toxin (TT) and 15 IU (IU is defined as the activity contained in 0.3094 mg of the WHO International Standard for Tetanus Antitoxin) of Tetanus Immune Globulin, Human (TIG) mixed with equal volume of Complete Freund's adjuvant (CFA) were administered to each of Twenty Swiss Webster female mice, weighing between 13 and 15 grams each, subcutaneously (the antigen adjuvant mixture is injected into the loose connective tissue located between the skin and the underlying musculature). The antibodies produced during a primary response are mostly IgMs. A booster injection (secondary immunization) was administered nine weeks later consisting of 20 ng toxin alone. The secondary response to the same antigen is much faster, more potent and produces antibodies of higher affinity, predominantly of the IgG class, and higher numbers of helper T cells and memory B cells in the immune system of the primed animal. The booster injection was carried out using the Freund's incomplete adjuvant (ICFA) to avoid an intense response to the mycobacteria in the CFA.[55]

Every four weeks after that, a booster shot was given; the amount of toxin was increased tenfold and injected subcutaneously. The last injection contained 20 μg of TT and was given intraperitoneally (injection into the peritoneal cavity). The injection volume throughout the protocol was maintained at 0.1 ml. Blood collection was through exsanguination of mice. The mice were first anesthesized with ether and the heart was punctured through the fourth intercostal space at the sternal margin. The blood was collected in vacutainers and allowed to stand for 4 hours at room temperature and centrifuged.

3.4.1.2 IMMUNIZATION PROTOCOL

DAY 1	Bleeding #1 3 mice
	15 μg TT + 15 IU TIG + CFA
4 WEEKS	Bleeding #2. 3 mice
9 WEEKS	20 ng TT alone + ICFA
13 WEEKS	Bleeding #3. 3 mice
	200 ng TT alone
17 WEEKS	Bleeding #4. 3 mice
	2 μg TT alone
21 WEEKS	Bleeding #5. 3 mice
	20 μg TT alone
24 WEEKS	Bleeding #6, Bleeding of ALL MICE

3.4.1.3 Reagents and methods

A. Reagents

1. Tetanus Toxin Lot No TT-1, Connaught Laboratories, kept in 1:10,000 Merthiolate, 382 µg/ml
2. Tetanus Immune Globulin, Human (TIG), Lot No. 14VO6D, Cutter Biologigal Elkhart, IN 46515 USA 614, 393 IU/ml
3. Freund's Adjuvant, Complete (CFA), Gibco BRL, Cat. No. 660-5721AS
4. Freund's Adjuvant, Incomplete (ICFA), Gibco BRL, Cat. No. 660-5720AS
5. Saline, 0.9% NaCl

B. Methods

AT DAY 1: **Bleeding #1,** 3 mice.

15 µg TT + 15 IU TIG + CFA

Injected 20 mice **Subcutaneously** with 0.1 ml.

(1 ml TT + 972 µl TIG + 1.500 ml **CFA**)

At 4 Weeks: **Bleeding #2,** 3 mice.

At 9 Weeks: **First Booster, 20 ng TT in ICFA**

Injected 0.1 ml (20 ng TT), **Subcutaneously** into each mouse.

(100 µl TT, 4 µg/ml + 0.9 ml saline + 1 ml **ICFA**).

At 13 Weeks: **Bleeding** #3, 3 mice.

200 ng TT alone

Injected 0.1 ml (200 ng TT), **Subcutaneously** into each mouse.

(2 ml TT, 4μg/ml + 2 ml **Saline**).

At 17 Weeks: **Bleed** #4, 3 mice.

2 μg TT alone

Injected 0.1 ml (2 μg TT), **Subcutaneously** into each mouse.

(105 μl TT, 382 μg/ml + 895 μl Saline + 1 ml **CFA**).

At 21 Weeks: **Bleeding** #5, 3 mice.

20 μg TT alone

Injected 0.1 ml (20 μg TT), **Intraperitoneally** into each mouse.

(629 μl TT, 382 μg/ml + 571 μl **Saline**).

At 24 weeks: **Bleeding** #6, Bleeding of ALL Mice.

3.4.2 Guinea Pig Antibodies Raised Against Tetanus Toxin

3.4.2.1 Introduction

Fifteen μg of Tetanus Toxin and 15 IU of Human Antitoxin mixed with equal volume of Complete Freund's Adjuvant (CFA) were administered to each of three female Outbred White Laboratory Guinea pigs (GP), CRL: COBS (HA) BR, weighing 225 to 275 grams, subcutaneously. Nine weeks later the first booster injection followed with 20 ng of toxin alone (in equal volume of saline and ICFA). Every four weeks after that, a booster shot was given; the amount of toxin was increased tenfold and injected subcutaneously. The last injection contained 20 μg of TT and was given intraperitoneally. The injection volume throughout the protocol was maintained at 0.1 ml. Blood collection was through cardiac puncture and for this reason the animals were given a week of rest before the next immunization.

3.4.2.2 IMMUNIZATION PROTOCOL

DAY 1	Bleeding #1
	15 μg TT + 15 IU TIG + CFA
4 WEEKS	Bleeding #2.
9 WEEKS	20 ng TT alone + ICFA
12 WEEKS	Bleeding #3.
13 WEEKS	200 ng TT alone
16 WEEKS	Bleeding #4.
17 WEEKS	2 μg TT alone + CFA
20 WEEKS	Bleeding #5.
21 WEEKS	20 μg TT alone
24 WEEKS	Bleeding #6, FINAL BLEEDING

3.4.2.3 Reagents and methods

A. Reagents See 3.4.1.3

B. Methods

At DAY 1: Bleeding #1.

15 μg TT + 15 IU TIG + CFA

Injected 0.1 ml **Subcutaneously** into each GP.

(1 ml TT + 972 μl TIG, + 1.500 ml **CFA**).

At 4 Weeks: Bleeding #2.

At 9 Weeks: First Booster, 20 ng TT in ICFA

Injected 0.1 ml (20 ng TT), **Subcutaneously** into each GP.

(100 μl TT (4 μg/ml) + 0.9 ml saline + 1 ml **ICFA**).

At 12 Weeks: Bleeding #3.

At 13 Weeks: **Second booster, <u>200 ng TT alone in Saline</u>**

Injected 0.1 ml (200 ng TT), **Subcutaneously** into each GP.

(2 ml TT, 4 μg/ml + 2 ml **Saline**).

At 16 Weeks: **Bleeding #4.**

At 17 Weeks: **Third booster, <u>2 μg TT alone in CFA</u>**

Injected 0.1 ml (2 μg TT), **Subcutaneously** into each GP.

(105 μl TT, 382 μg/ml + 0.895 ml Saline + 1 ml **CFA.**

At 20 Weeks: **Bleeding #5.**

At 21 Weeks: **Fourth booster, <u>20 μg TT alone in Saline</u>**

Injected 0.1 ml (20 μg TT), **Intraperitoneally** into each GP.

(629 μl TT, 382 μg/ml + 571 μl **Saline**).

At 24 weeks: **Bleeding #6,** Final Bleeding.

PART B - Immunization with Tetanus Toxoid

3.4.3 Murine Antibodies Raised Against Tetanus Toxoid

3.4.3.1 Introduction

One μg of Tetanus Toxoid from Connaught Laboratories (50 μl), mixed with equal volume of Complete Freund's Adjuvant (CFA) were administered to each of 17 female Swiss Webster female mice, weighing between 13 and 15 grams each, subcutaneously. Nine weeks later the first booster injection followed with 1 μg of tetanus toxoid. Every four weeks after that, a booster shot was given with 1 μg tetanus toxoid adsorbed subcutaneously.

3.4.3.2 IMMUNIZATION PROTOCOL

DAY 1	Bleeding #1, 3 mice
	1 μg Tetanus Toxoid
9 WEEKS	Bleeding #2, 3 mice
	1 μg Tetanus Toxoid
13 WEEKS	Bleeding #3, 3 mice
	1 μg Tetanus Toxoid Adsorbed
17 WEEKS	Bleeding #4, 3 mice
	1 μg Tetanus Toxoid Adsorbed
21 WEEKS	Bleeding #5, 3 mice
	1 μg Tetanus Toxoid Adsorbed
24 WEEKS	Bleeding #6, Bleeding of ALL mice

3.4.3.3 Reagents and methods

A. Reagents

1. Tetanus Toxoid Fluid, Lot# 1307A, 50 ml, Connaught Laboratories Limited, 10 Lf/ml.

   (1 lf = 2-2.5 μg of tetanus toxin).[42]

2. Tetanus Toxoid Adsorbed (Aluminum phosphate), Lot# 2420-11, 10 ml, Connaught Laboratories Limited, 10 Lf/ml.

B. Methods

At DAY 1: **Bleeding #1,** 3 mice.

1 μg Tetanus Toxoid + CFA.

Injected 20 mice **Subcutaneously** with 0.1 ml.

(1 ml Tetanus Toxoid + 1 ml CFA)

At 9 Weeks: **Bleeding #2,** 3 mice.

First Booster, 1 μg Tetanus Toxoid + CFA.

At 13 Weeks: **Bleeding #3,** 3 mice.

Second Booster, 1 μg Tetanus Toxoid Adsorbed.

Injected 0.05 ml Tetanus Toxoid Adsorbed, **Subcutaneously** into each mouse.

At 17 Weeks: **Bleeding #4,** 3 mice.

Third Booster, 1 μg Tetanus Toxoid Adsorbed.

At 21 Weeks: **Bleeding #5,** 3 mice.

Fourth Booster, 1 μg Tetanus Toxoid Adsorbed.

At 24 Weeks: **Bleeding #6,** Bleeding of ALL Mice.

3.4.4 Guinea Pig Antibodies Raised Against Tetanus Toxoid

3.4.4.1 Introduction

Ten μg of Tetanus Toxoid (500 μl) mixed with equal volume of Complete Freund's Adjuvant (CFA) were administered to each of three female Outbred White Laboratory Guinea pigs, CRL: COBS (HA) BR, weighing 225 to 275 grams, subcutaneously. Nine weeks later the first booster injection followed with 10 μg of tetanus toxoid. Every four weeks after that, a booster shot was given with 10 μg tetanus toxoid adsorbed subcutaneously. Blood collection was through cardiac puncture that is why the animals were given a week of rest before the next immunization.

3.4.4.2 IMMUNIZATION PROTOCOL

DAY 1	Bleeding #1
	10 μg Tetanus Toxoid
8 WEEKS	Bleeding #2
9 WEEKS	10 μg Tetanus Toxoid
12 WEEKS	Bleeding #3
13 WEEKS	10 μg Tetanus Toxoid Adsorbed
16 WEEKS	Bleeding #4
17 WEEKS	10 μg Tetanus Toxoid Adsorbed
20 WEEKS	Bleeding #5
21 WEEKS	10 μg Tetanus Toxoid Adsorbed
24 WEEKS	Bleeding #6, FINAL BLEEDING

3.4.4.3 Reagents and methods

A. Reagents See 3.4.3.3 A

B. Methods

At DAY 1: Bleeding #1

10 μg Tetanus Toxoid in CFA.

Injected Guinea pigs Subcutaneously with 1 ml.

(1 ml Tetanus Toxoid + 1 ml CFA)

At 8 Weeks: Bleeding #2

At 9 Weeks: First booster, 10 μg Tetanus Toxoid in CFA.

At 12 Weeks: Bleeding #3

At 13 Weeks: Second booster, 10 μg Tetanus Toxoid Adsorbed.

Injected 0.05 ml Tetanus Toxoid Adsorbed, Subcutaneously into each GP.

At 16 Weeks: Bleeding #4

At 17 Weeks: Third booster, 10 μg Tetanus Toxoid Adsorbed.

At 20 Weeks: Bleeding #5

At 21 Weeks: Fourth booster, 10 μg Tetanus Toxoid Adsorbed.

At 24 Weeks: Bleeding #6, FINAL BLEEDING.

PART C - Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

3.4.5 Introduction

This section describes the procedure for determining the isotypes of antibodies raised in mice against tetanus toxin and toxoid and the reaction of murine and guinea pig antibodies to tetanus toxin, toxoid, fragment C and MAP.

3.4.5.1 Principle of the test

Antigen is coated on polystyrene plates. Antibodies specific for the antigen bind to this antigen and horseradish peroxidase conjugated antiglobulins bind to these antibodies.

The participation of 3,3',5,5' Tetramethylbenzidine (TMB) as an hydrogen donor in the oxidation/reduction of H_2O_2 results in colour development. The actual enzyme reaction is invisible. What we see is the oxidation of the reduced TMB to its chromogenic form. The amount of antibody in the serum preparation is proportional to the optical density as read on a multichannel photometer.

3.4.5.2 GENERAL

3.4.5.2.1 Reagents and Equipment

A. Reagents

1. Antigens and preparation of Working Antigen solution

a) Tetanus Toxoid Plain (Lot No. 77-104C, 120 Lf/vial, Connaught Laboratories).

A vial of Tetanus Toxoid Plain was reconstituted in 4.8 ml MilliQ H_2O to give a stock solution of 25 Lf/ml. The stock solution was further diluted 100 fold in CO_3 buffer, pH 9.6 (0.4 ml stock in 40 ml of buffer) to a final working concentration of **0.25 Lf/ml.**

b) Tetanus toxin, Department of Public Health, Division of Biologic Laboratories, Boston, Massachusetts CP (txn) 49, 1050 Lf/ml; 2.0 mg Protein/ml.

The stock solution was diluted 2000 fold in CO_3 buffer (8 μl x 1050 Lf/ml (16 μg Protein) in 16 ml CO_3 buffer) to a final concentration of **1 μg/ml or 0.5 Lf/ml.**

c) Tetanus toxin C fragment, recombinant (Boehringer, lyophilized 1 mg, Cat. No. 1348655).

A one mg vial was reconstituted in 1 ml H_2O to give a stock solution of 1 mg/ml. The stock solution was further diluted 1000 fold in CO_3 buffer (16 μl of 1 mg/ml in 16 ml CO_3 buffer) to a final concentration of **1 μg/ml.**

d) Multiple Antigenic Peptide, provided by Dr René Roy. Reconstituted A one mg vial was reconstituted in 1 ml H_2O to give a stock solution of 1 mg/ml. The stock solution was further diluted 1000 fold in CO_3 buffer (21 μl of 1 mg/ml in 21 ml CO_3 buffer) to a final concentration of 1 **μg/ml**.

2. **Coating Buffer:** 0.06 M Carbonate buffer, pH 9.6.

3. **Wash/Diluent Buffer:** 0.01 M Phosphate buffer saline, pH 7.2 containing 0.05% Tween 20 (PBS-T).

4. **Horseradish Peroxidase Enzyme Conjugates**

a) Goat anti-Guinea Pig IgG (H+L) HRPO labelled, Affinity purified, (Kirkegaard & Perry, USA, 14-17-06, Lot No. KB45-5).

A 0.5 mg vial was reconstituted in 1 ml H_2O. Twenty-one μl of reconstituted conjugate were added to 21 ml PBS-T to give a final dilution of 1:1000.

b) Goat anti-Mouse IgG (γ) HRPO labelled, Affinity purified, Human serum Adsorbed, (Kirkegaard & Perry, USA, 14-18-02).

A 0.5 mg vial was reconstituted in 1 ml H_2O. Twenty-one μl of reconstituted conjugate were added to 21 ml PBS-T to give a final dilution of 1:1000.

c) Goat anti-Rabbit IgG (H+L), HRPO labelled, Human serum Adsorbed, (Bio-Rad USA, 172-1019).

3.3 μl of reconstituted conjugate were added to 10 ml PBS-T to give a final dilution of 1:3000.

5. **Substrate solution:** 3,3',5,5' Tetramethylbenzidine (TMB) Peroxidase substrate solution (Kirkegaard & Perry Laboratories,USA).

The working substrate solution was prepared as per manufacture's directions.

B. Equipment

1. Nunc-Immuno plates Polysorp F96 flat bottomed (InterMed Co., Denmark)
2. Plate Washer: Biotek Instruments Inc., USA Model EL403 Automatic Microplate Washer
3. UVmax Kinetic Microplate Reader (Molecular Devices Corp., USA)
4. Plate sealers: Linbro/Titertek acetate plate sealer with adhesive backs
5. Tubes: Borosilicate glass 16 x 100 mm (Fisher Scientific)
6. Pipettors: Eppendorf, Pipetman, Brinkmann, Transferpette and Titertek Digital Multichannel Pipette

3.4.5.2.2 Sera

1. **Guinea pig sera raised against tetanus toxin and toxoid**

The following dilutions of the sera were prepared:

34 µl in 1.7 ml PBS-T,			i.e. 1:50
1 ml of	1:50	+ 1 ml PBS-T,	i.e. 1:100
1.4 ml of	1:100	+ 1.4 ml PBS-T,	i.e. 1:200
2 ml of	1:200	+ 1 ml PBS-T,	i.e. 1:300
2.4 ml of	1:300	+ 0.8 ml PBS-T,	i.e. 1:400
2.4 ml of	1:400	+ 0.6 ml PBS-T,	i.e. 1:500
2.5 ml of	1:500	+ 0.5 ml PBS-T,	i.e. 1:600
1.8 ml of	1:600	+ 0.6 ml PBS-T,	i.e. 1:800
1.6 ml of	1:800	+ 0.4 ml PBS-T,	i.e. 1:1,000
1 ml of	1:1,000	+ 1 ml PBS-T,	i.e. 1:2,000
0.4 ml of	1:2,000	+ 0.6 ml PBS-T,	i.e. 1:5,000

2. **Murine sera raised against tetanus toxin and toxoid**

Pool of 3 mice. Dilutions were prepared as per Guinea pig sera.

3. **Mouse typer isotyping panel**

Rabbit anti-mouse subclass specific antiserum to mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, κ-chain, λ-chain, (Bio-Rad USA, 172-2055). Antisera were provided reconstituted.

3.4.5.3 METHOD

1. **Antigen Coating**

   Two hundred µl of working antigen concentration were added to the appropriate wells of a microtitre plate. The plate was covered with an acetate plate sealer and incubated overnight at 4° C. At the end of the incubation period, the plate was removed from the refrigerator and washed 4 times with 300 µl PBS-T. The residual buffer was discharged by tapping onto lint-free absorbent towels.

2. **Addition of Primary Antibody**

   Two hundred µl of each diluted sera were added to appropriate wells as depicted in the template and incubate for **1 hour at 37° C.**

   At the end of the incubation period, the plate was removed from the incubator and washed 4 times with 300 µl PBS-T. The residual buffer was discharged by tapping onto lint-free absorbent towels.

3. **Addition of Enzyme Conjugate**

   Using a multichannel pipettor, 200 µl of Horseradish Peroxidase Enzyme Conjugate were added to each well and the plate was incubated for **1 hour at 37° C.** At the end of the incubation period, the plate was removed from the incubator and washed 4 times with 300 µl PBS-T. The residual buffer was discharged by tapping onto lint-free absorbent towels.

4. **Substrate Addition**

Using a multichannel pipettor, 100 µl of substrate solution were added to each well. Immediately upon addition of substrate, the reaction was stopped with the addition of 100 µl of 1 M Phosphoric acid to each well.

5. **Absorbance Determination**

The plate was shaken and the absorbance of each well read using the UVmax Elisa reader at 450 nm.

6. **Results and Calculations**

The Softmax software was used to define the microplate template with the location of blanks, standards and unknowns. The results were analysed using standard curve interpolation.

PART D - ELISA ASSAYS

3.4.6 **Determination of isotype and relative concentration of the murine antibodies generated against tetanus toxin and toxoid. Detection of *C. tetani* Antitoxin**

3.4.6.1 **Assay protocol**

1. **Microtitre plates:** NUNC-Polysorb
2. **Antigens:** a) Tetanus Toxin
   b) Tetanus Toxoid Plain

   Add 200 µl of working antigen concentration per well and incubate plates overnight at 4° C.
3. **Sera:** Murine antibodies raised against tetanus toxin and toxoid, Dilution: 1:100.

   Add 200 µl to each well and incubate for **1 hour at 37° C.**
4. **Mouse typer isotyping panel**

   Add 100 µl to the appropriate wells and incubate for **1 hour at 37° C.**
5. **Conjugate:** Goat α-Rabbit IgG (H+L), Human absorbed, HRPO.

   Add 200 µl to each well and incubate for **1 hour at 37° C.**
6. **Substrate:** TMB Peroxidase Substrate System

   Mix 5.5 ml TMB Peroxidase Substrate with 5.5 ml Peroxidase Solution B. Add **100 µl/well**, STOP the reaction with **100 µl/well** 1 M Phosphoric acid and read at OD_{450}nm.

	NO HRP 1	anti-Toxin 2	3	anti-Toxoid 4	5	6	NO HRP 7	anti-Toxin 8	9	anti-Toxoid 10	11	12	
A	PBS-T α-IgG1					PBS-T						PBS-T	A
B	αIgG2a												B
C	αIgG2b												C
D	α-IgG3		TOXIN						TOXOID				D
E	α-IgM												E
F	α-IgA												F
G	κchain												G
H	λchain												H
	1	2	3	4	5	6	7	8	9	10	11	12	

The reagents were placed in the appropriate wells as depicted in the template above.

3.4.7 Determination of the titre of murine antibodies generated against tetanus toxin and toxoid. Detection of *C. tetani* Antitoxin

3.4.7.1 Assay protocol

1. **Microtitre plates:** NUNC-Polysorb
2. **Antigens:** a) Tetanus Toxin
   b) Tetanus Toxoid Plain
   c) rTTC
   d) MAP

   Add 200 µl of working antigen concentration per well and incubate plates overnight at 4° C
3. **Sera:** Murine antibodies raised against tetanus toxin and toxoid, Dilution: 1:50, 100, 200, 300, 400, 500, 600, 800, 1,000, 2,000 and 1:5,000.

   Add 200 µl to each well and incubate for **1 hour at 37° C.**
4. **Conjugate:** Goat α-Mouse IgG (γ) HRPO, Affinity purified

   Add 200 µl to each well and incubate for **1 hour at 37° C.**
5. **Substrate:** TMB Peroxidase Substrate System

   Mix 5.5 ml TMB Peroxidase Substrate with 5.5 ml Peroxidase Solution B. Add **100 µl/well**, STOP the reaction with **100 µl/well** 1 M Phosphoric acid and read at OD_{450}nm.

anti-Toxin sera

	TOXIN 1	TOXOID 2	rTTC 3	4	M A P 5	6	7	8	9	10	11	12	
A	PBS-T	PBS-T	PBS-T	PBS-T	1:300	1:800							A
B	1:1000	1:1000	1:1000	1:50	1:400	1:1000							B
C	1:2000	1:2000	1:2000	1:100	1:500	1:2000							C
D	1:5000	1:5000	1:5000	1:200	1:600	1:5000							D
E	PBS-T	PBS-T	PBS-T	PBS-T	1:300	1:800							E
F	1:1000	1:1000	1:1000	1:50	1:400	1:1000							F
G	1:2000	1:2000	1:2000	1:100	1:500	1:2000							G
H	1:5000	1:5000	1:5000	1:200	1:600	1:5000							H
	1 TOXIN	2 TOXOID	3 rTTC	4	5 M A P	6	7	8	9	10	11	12	

anti-Toxoid sera

The reagents were placed in the appropriate wells as depicted in the template above.

The sera were placed in duplicate in the appropriate wells in diagonal quadrants in order to eliminate intra-plate variation.

3.4.8 Determination of the titre of guinea pig antibodies generated against tetanus toxin and toxoid.

Detection of *C. tetani* Antitoxin

3.4.8.1 Assay protocol

1. **Microtitre plates:** NUNC-Polysorb
2. **Antigens:** a) Tetanus Toxin
   b) Tetanus Toxoid Plain
   c) rTTC
   d) MAP

   Add 200µl of working antigen concentration per well and incubate plates overnight at 4° C.
3. **Sera:** Guinea pig antibodies raised against tetanus toxin and toxoid, Dilution: 1:50, 100, 200, 300, 400, 500, 600, 800, 1,000, 2,000 and 1:5,000.

   Add 200 µl to each well and incubate for **1 hour at 37° C.**
4. **Conjugate:** Goat α-Guinea Pig IgG (H+L) HRPO, Affinity purified

   Add 200 µl to each well and incubate for **1 hour at 37° C.**
5. **Substrate:** TMB Peroxidase Substrate System

   Mix 5.5 ml TMB Peroxidase Substrate with 5.5 ml Peroxidase Solution B. Add **100 µl/well**, STOP the reaction with **100 µl/well** 1 M Phosphoric acid and read at OD_{450}nm.

anti-Toxin sera

	TOXIN 1	TOXOID 2	rTTC 3	4	M A P 5	6	7	8	9	10	11	12	
A	PBS-T	PBS-T	PBS-T	PBS-T	1:300	1:800							A
B	1:1000	1:1000	1:1000	1:50	1:400	1:1000							B
C	1:2000	1:2000	1:2000	1:100	1:500	1:2000							C
D	1:5000	1:5000	1:5000	1:200	1:600	1:5000							D
E	PBS-T	PBS-T	PBS-T	PBS-T	1:300	1:800							E
F	1:1000	1:1000	1:1000	1:50	1:400	1:1000							F
G	1:2000	1:2000	1:2000	1:100	1:500	1:2000							G
H	1:5000	1:5000	1:5000	1:200	1:600	1:5000							H
	1 TOXIN	2 TOXOID	3 rTTC	4	5 M A P	6	7	8	9	10	11	12	

anti-Toxoid sera

The reagents were placed in the appropriate wells as depicted in the template above.

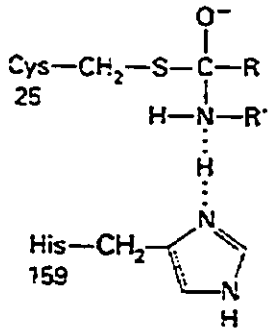
The sera were placed in duplicate in the appropriate wells in diagonal quadrants in order to eliminate intra-plate variation.

Chapter 4: Enzymatic breakdown of Tetanus Toxin with Immobilized Papain

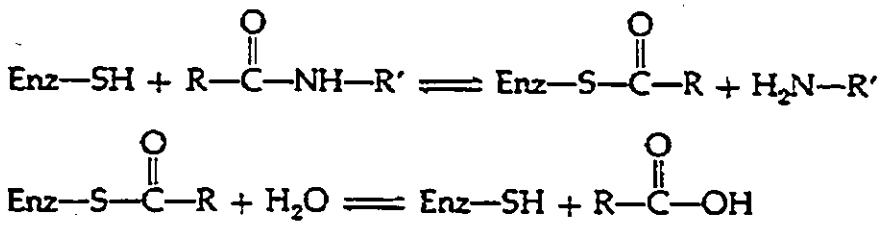
4.1 Introduction

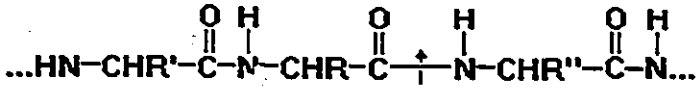
Lysosomal thiol proteases with a highly reactive Cysteine residue at the active site are abundant in living cells and play important roles in intracellular proteolysis such as the catabolism of proteins and peptides.[56] Papain is such a nonspecific thiol protease and is highly useful for enzymatic cleavage of proteins. It is composed of a single polypeptide of 212 amino acids and mol wt of 23,406 Da.[57] Maximal hydrolytic activity of papain requires activation. The sulfhydryl group of the cysteine in the active site must be in the reduced form and can be activated by a variety of thiol compounds such as cysteine and glutathione as well as by reducing agents such as sodium borohydride.[58] Reducing agents which can also act as chelators maximize papain's activity by removing cations which catalyse oxidation of -SH. Cysteine-HCl was used in these experiments with ethylene-diaminetetraacetate (EDTA) as a chelator.[59] The action of papain's active site on the substrate, TT, is described in the following:

The nucleophilicity of the -SH group in papain's active site is enhanced by a nearby histidine side chain.



Catalysis proceeds through the formation of Enzyme-bound thiol esters with the carboxyl group of the peptide bond to be cleaved.[60]





Papain hydrolyzes the amides of α-amino-substituted amino acids and the preferred cleavage site order is

Lys, Arg>His>Gly>Leu>Gln>Glu

4.2 Schematic representation of Papain digestion of Tetanus Toxin

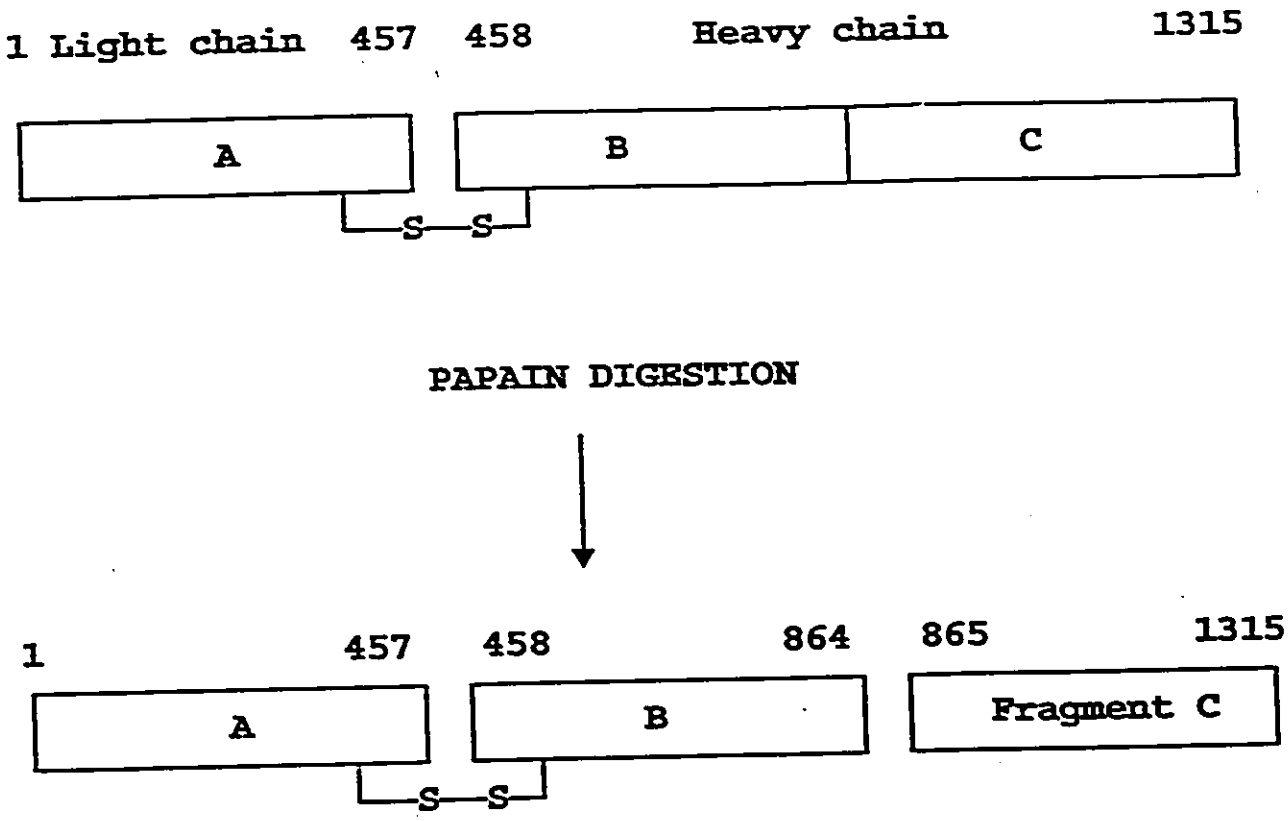


FIGURE 4.1 The papain cleavage site on tetanus toxin is at Lysine residue 865

4.3 Results and Discussion

Figure 4.2 depicts the electrophoretic separation of the components of two preparations of tetanus toxin under reducing and non-reducing conditions on SDS-PAGE. The gel was stained with a dye, Coomassie Blue, to detect the proteins, shown as sharp zones or bands. Both preparations were reduced to the heavy and light chain when compared to the high molecular mass marker proteins.

Lane A contains the reduced toxin preparation CP (txn) 54. Note the highly heterogeneous mixture of peptides with a clear band corresponding to heavy chain but without a clear band corresponding to the light chain. The light chain is contained in the doublet with apparent molecular mass between 45 and 66 kD. The band with apparent molecular mass between 116 kD and 200 kD is not the 150 kD tetanus toxin molecule because it has not been reduced.

Lane B contains the non-reduced preparation of CP (txn) 54. The band with the highest apparent molecular mass is tetanus toxin, as this band does not appear in Lane A (it was reduced). Also note the contamination with possibly light chain.

Lane C contains a mixture of proteins used as molecular markers. The proteins migrate from the top to bottom. The protein is indicated in the experimental section and the molecular mass on the side next to each band.

Lane D contains the toxin preparation CP (txn) 49 in its reduced form. Here, there are distinct bands corresponding to the heavy and light chain. The band close to 97 kD corresponds to the heavy chain while the band between 45 and 66 kD corresponds to the light chain. The inpurity on the top of the gel was not reduced again.

Lane E contains the non-reduced preparation of CP (txn) 49. The heavy band on the top, corresponds to the single-chain 150 kD tetanus toxin molecule. This band totally disappears when reduced as shown in Lane D. This preparation does not contain any impurities between 45 and 66 kD molecular mass and that is why it was used for the papain digestion experiments.

Figure 4.3 depicts the electrophoretic separation of the components of the papain digested toxin, fragment C and undigested toxin.

Lane A contains the digested toxin. Note the heterogeneous mixture of peptides with a clear band corresponding to fragment C of tetanus toxin as shown in Lane B.

Lane B contains the commercially obtained fragment C. The heavy band at the bottom of the gel corresponds to fragment C.

Lane C contains the undigested tetanus toxin. The heavy band on the top of the gel is the intact single-chain tetanus toxin molecule. As shown in Lane A, this band was totally digested with papain. This procedure demonstrates that on a larger scale it would be feasible to isolate preparative amounts of fragment C for further analysis.

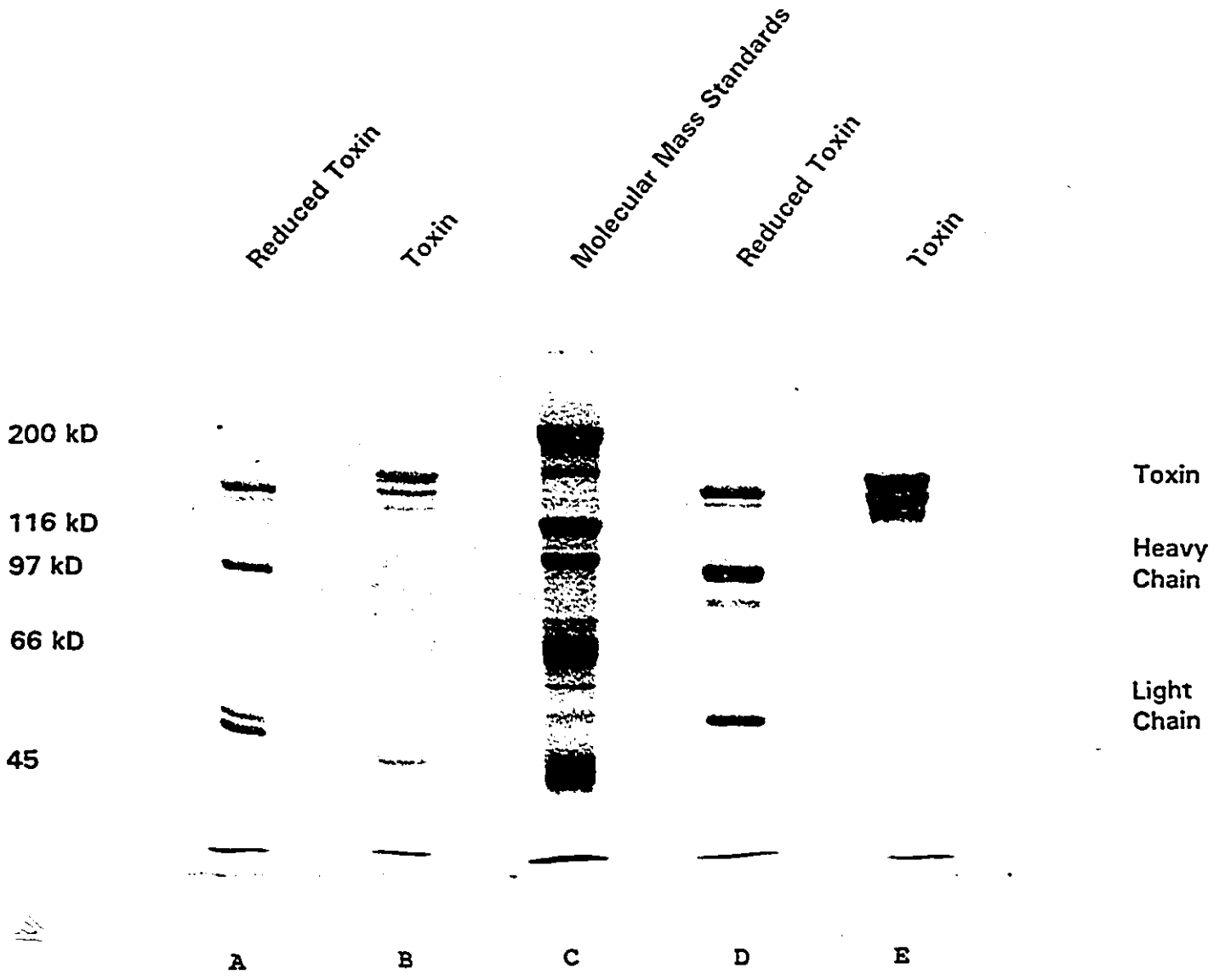


FIGURE 4.2 SDS-PAGE of two preparations of tetanus toxin. Lane A: Lot # CP (txn) 54-reduced. Lane B: Lot # CP (txn) 54. Lane C: High molecular mass marker proteins. Lane D: Lot # CP (txn) 49-reduced and Lane E: Lot # CP (txn) 49.

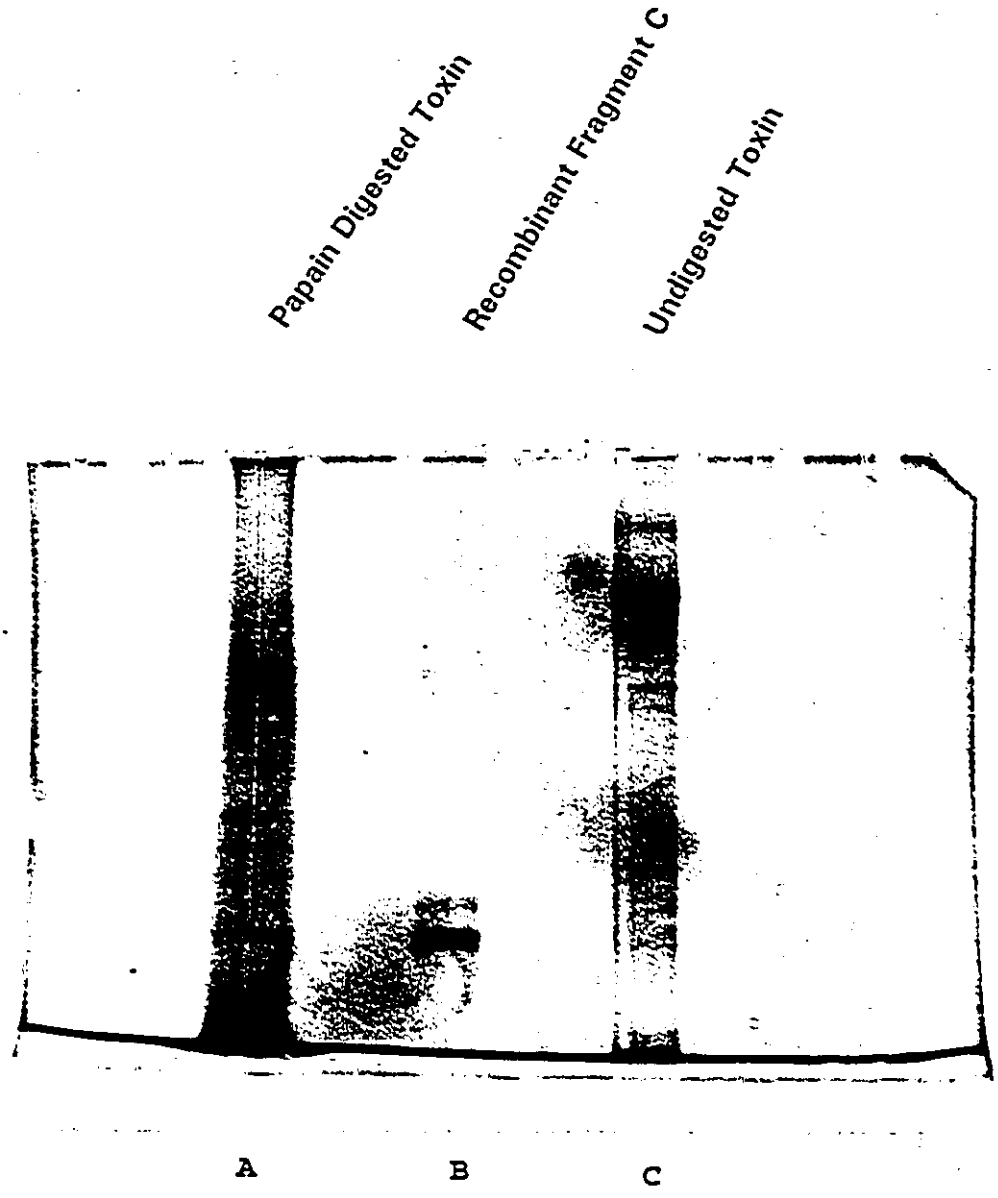


FIGURE 4.3 SDS-PAGE separation of tetanus toxin digested with papain, tetanus toxin fragment C and undigested toxin. Lane A: tetanus toxin digested with papain. Lane B: Fragment C and Lane C: undigested toxin.

Figure 4.4 shows how two tetanus toxin preparations were analyzed for purity and molecular integrity with SDS-PAGE. Both preparations contained multiple bands below 116 kD in the reduced and non-reduced form. Neither of the non-reduced preparations contained a band corresponding to the 150 kD tetanus toxin molecule.

4.4 SUMMARY

Tetanus toxin was digested with immobilized papain cross-linked to 6% beaded agarose at 0.01 IU/mg TT. Different experimental conditions were tested and satisfactory yields of fragment C were obtained at high concentrations of toxin. Incubation at 56° C for 4 hours was found to give better results than at 37° C. Several preparations of toxin from Connaught Laboratories were analysed on SDS-PAGE and were found to be highly fragmented. Purified tetanus toxin donated by Dr G. Sieber, Department of Public Health, Division of Biologic Laboratories, Boston, Massachusetts [CP (txn) 49] was found to contain the intact 150,00 Da single-chain tetanus toxin molecules. This preparation was digested with papain and separated by SDS-PAGE under non-reducing conditions using a 7.5% homogeneous gel and a 4% stacking gel. The results indicate that on a larger scale it would be feasible to isolate preparative amounts of fragment C for further analysis.

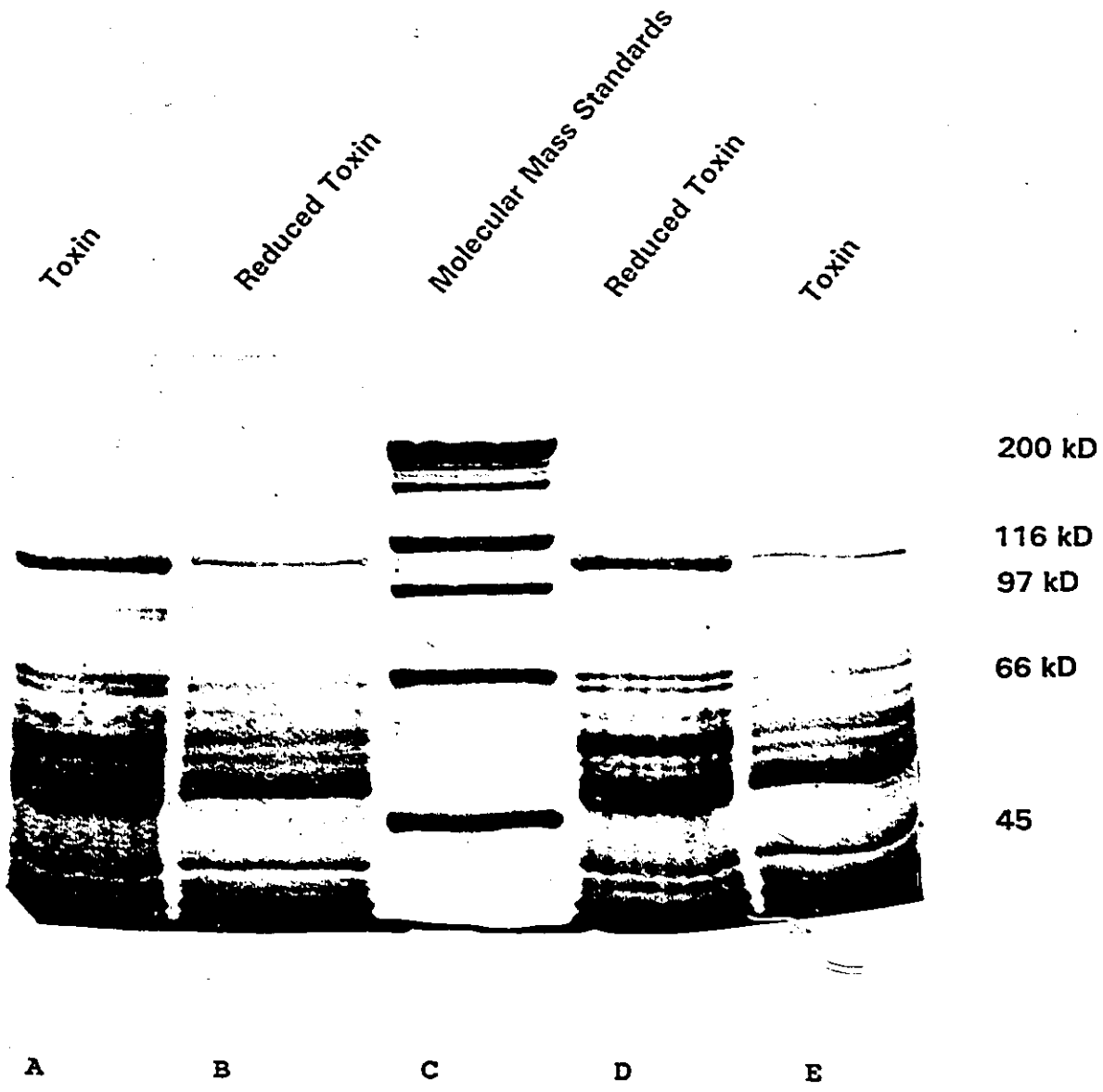


FIGURE 4.4 SDS-PAGE of two preparations of tetanus toxin under reducing and non-reducing conditions. Neither preparation contained single-chain 150 kD tetanus toxin molecules when compared to the molecular mass protein markers. Lane A: Lot # TT-1 reduced. Lane B: Lot # TT-1 non-reduced. Lane C: High molecular mass marker proteins. Lane D: Lot # TT-2 reduced and Lane E: Lot # TT-2 non-reduced.

4.5 Experimental Procedures

PART A - Papain digestion of tetanus toxin

4.5.1 Introduction

Two mg of purified tetanus toxin pre-equilibrated four times with 1 ml digestion buffer and concentrated to a volume of 0.5 ml using Centricon-30 microconcentrator (10 min at 3000 rpm) was digested with immobilized papain cross-linked to 6% beaded agarose at 0.01 IU/mg TT. Digestion was carried out with papain, prewashed with digestion buffer, at 56° C for 4 hours with occassional shaking. At the end of the incubation the mixture was centrifuged at 14,000 rpm for 5 minutes and approximately 480 µl were recovered from the 500 µl papain digest. An aliquot was made up to 5 ml with H_2O and dialysed in Spectra/Por membrane tubing MW cut-off (MWCO) 12,000-14,000. Purified tetanus toxin was donated by Dr G. Sieber, Department of Public Health, Division of Biologic Laboratories, Boston, Massachusetts. Several preparations by Connaught Laboratories were analyzed on SDS-PAGE and found to be highly fragmented. None contained intact single-chain 150 kD tetanus toxin molecules (Figure 4.4). However these preparations were found to be very immunogenic and lethal to laboratory animals.

4.5.2 Reagents and Equipment

A. Reagents

1. Purified tetanus toxin, Department of Public Health, Division of Biologic Laboratories, Boston, Massachusetts. Lot# CP (txn) 49, 1050 Lf/ml; 2.0 mg Protein/ml

2. Immobilized Papain, Pierce # 20341, 5 ml of settled gel. Supplied: 50% slurry, Activity: 7 BAEE units/ml of settled gel. [1 U correponds to the amount of enzyme which hydrolyzes 1 μmol of N-benzoyl-L-arginine ethyl ester (BAEE) per minute at pH 6.2 and 25° C]
3. Na_2EDTA, FW 372.24, BIO-RAD 161-0729
4. L-Cysteine-HCl, FW 157.6 GIBCO BRL (USA) 810-1035IM.
5. Centriprep-100 concentrator, Amicon (USA)

B. Equipment

1. Eppendorf Centrifuge 5415C
2. Water bath
3. Spectra/Por membrane tubing MW cut-off 12,000-14,000, dia:15.9 mm, Fisher Scientific

4.5.3 METHOD

1. **Preparation of digestion buffer**

   A 0.002 M Na_2EDTA (FW 372.24) solution was prepared by dissolving 0.0186 g in 25 ml H_2O.

   A 0.5 M Cysteine-HCl (FW 157.6) solution was prepared by dissolving 0.788 g in 10 ml PBS.

   The digestion buffer consisted of 10 ml 0.002 M EDTA in H_2O plus 2 ml 0.5M Cysteine HCl in PBS.

2. **Sample preparation**

   Two ml (4 mg) of purified tetanus toxin was equilibrated with 40 ml H_2O using a Centriprep-100 concentrator (10 min x 3000 rpm). The sample was finally reconstituted in 1 ml H_2O (4 mg/ml).

3. **Sample equilibration with digestion buffer**

   Two hundred and fifty microlitres (1 mg) of the pre-washed tetanus toxin was equilibrated four times with 1 ml digestion buffer and concentrated to a volume of 0.5 ml using a Centricon-30 microconcentrator (10 min at 3000 rpm).

4. **Preparation of Immobilized Papain**

   Five hundred microlitres of Immobilized papain (1.75 IU) were washed three times with freshly made digestion buffer. Finally it was made up to 1 ml (1.75 IU/ml) with digestion buffer.

5. **Digestion with Papain at 0.01 IU/mg TT**

The pre-equilibrated 1 mg of sample was digested with 6 µl (1.75 IU/ml, therefore 6 µl = 0.01 IU papain) of the pre-equilibrated papain at 56° C for 4 hours with occassional shaking in a water bath.

6. **Digested sample**

6.1 **Digested sample recovery**

At the end of the incubation the mixture was centrifuged at 14,000 rpm for 5 minutes. Approximately 480 µl of the sample was recovered from the 500 µl papain digest.

6.2 **Dialysis of recovered sample**

The digested tetanus toxin was made up to 5 ml with H_2O and dialysed in Spectra/Por membrane tubing MW Cut-off 12,000-14,000, dia:15.9 mm for 48 hours under running tap water and for additional 2 X 16 hours under dd H_2O.

6.3 **Concentration of sample**

The dialysed sample was equilibrated with 2 x 10 ml H_2O using a Centriprep-10 concentrator.

6.4 **Protein determination**

The protein Determination of the different fractions was performed according to the BIO-RAD protein assay, Publication 81-0525 (Appendix I).

PART B - Separation of Tetanus Toxin digested with Immobilized Papain on SDS-PAGE

4.5.3 Introduction

Purified tetanus toxin, recombinant Fragment C and papain digested toxin were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli[61]. SDS-PAGE was done under non-reducing conditions on BIO-RAD Mini-PROTEAN II slab cell using a 7.5% homogeneous gel.

4.5.4 Reagents and Equipment

A. Reagents

1. TRIS (Hydroxymethyl) Aminomethane, Electrophoresis Purity Reagent, Bio-Rad 161-0716.
2. SDS (Sodium Dodecyl Sulfate), Electrophoresis Purity Reagent, Bio-Rad 161-0300
3. Glycine, Electrophoresis Purity Reagent Bio-Rad 161-0717
4. Ammonium Persulfate, Electrophoresis Purity Reagent Bio-Rad 161-0700
5. 2-Mercaptoethanol, Electrophoresis Purity Reagent Bio-Rad 161-0710
6. Coomassie Brilliant Blue R-250, Electrophoresis Purity Reagent Bio-Rad 161-0400
7. Acrylamide, Electrophoresis Purity Reagent, >99.9% Bio-Rad 161-0100
8. Bis (N,N'-Methylene-bis-acrylamide) Electrophoresis Purity Reagent, Bio-Rad 161-0200

9. TEMED (N,N,N',N'-Tetra-methylethylenediamine), Electrophoresis Purity Reagent, Bio-Rad 161-0800

10. Tetanus toxin C fragment, recombinant. Boehringer, lyophilized 1 mg, Cat. No. 1348655. 1 mg reconstituted in 1 ml H_2O.

11. Methanol, J.T. Baker, HPLC Reagent 9093-03

12. Acetic Acid, J.T. Baker 9507-03

13. SDS-PAGE Molecular Weight Standards, High and Low Range, Bio-Rad 161-0303 and 161-0304

a) **Low Range**

Protein	Molecular Weight
Rabbit muscle phosphorylase b	97,400
Bovine serum albumin (BSA)	66,200
Hen egg white ovalbumin	45,000
Bovine carbonic anhydrase	31,000
Soyabean trypsin inhibitor	21,500
Hen egg white lysozyme	14,400

b) **High Range**

Protein	Molecular Weight
Myosin	200,000
E. coli ß-galactosidase	116,250
Rabbit muscle phosphorylase b	97,400
Bovine serum albumin (BSA)	66,200
Hen egg white ovalbumin	45,000

B. Equipment

1. BIO-RAD Mini-PROTEAN II dual slab cell

4.5.4.2 Preparation of Stock Solutions

1. **Monomer Solution,** (30%T 2.7%C_{bis})

   29.2 g Acrylamide and 0.8 g Bis were dissolved in 100 ml H_2O and stored in the dark at 4° C.

2. **4X Running Gel Buffer,** (1.5M tris-HCl pH 8.8) 18.5 g Tris were dissolved in 100 ml H_2O and the pH was adjusted to pH 8.8 with HCl.

3. **4X Stacking Gel Buffer,** (0.5M tris-HCl pH 6.8) 1.5 g Tris were dissolved in 25 ml H_2O and the pH was adjusted to pH 6.8 with HCl.

4. **10% SDS**

   25 g of SDS were dissolved, by warming at 100° C.

5. **Initiator,** 10% ammonium persulfate

   0.05 g of Ammonium persulfate were dissolved in 0.5 ml H_2O. Made fresh on day of analysis.

6. **Running Gel Overlay,** (0.375M tris-HCl pH 8.8, 0.1% SDS)

   12.5 ml of Tris solution (1.2.2) was mixed with 0.5 ml SDS solution (1.2.4) and made up to 50 ml with H_2O.

7.A **2X Treatment Buffer, SDS-REDUCING Buffer,**

   (0.125M tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol)

   2.5 ml Tris Solution (1.2.3), 4.0 ml SDS Solution (4.), 2 ml Glycerol and 1 ml 2-mercaptoethanol were mixed and made up to 10 ml with H_2O. The solution was stored frozen in 1 ml aliquots.

7.B **2X Treatment Buffer, NON-REDUCING Buffer**

(0.125M tris-HCl pH 6.8, 4% SDS, 20% glycercl)

2.5 ml Tris Solution (1.2.3), 4.0 ml SDS solution (4.) and 2 ml Glycerol were mixed and made up to 10 ml with H_2O. The solution was kept frozen in 1 ml aliquots.

8. **Tank Buffer, 0.025M tris, 0.192M glycine, 0.1% SDS**

6 g Tris, 28.8 g Glycine and 20 ml SDS solution (4.) were mixed and made up to 2.0 litres with H_2O. There is no need to adjust the pH of this solution (pH 8.3).

9. **Stain Stock, 1% Coomassie Blue R-250**

1.0 g of Coomassie Blue R-250 was dissolved in 100 ml H_2O, stirred and filtered through Watman #1 filter paper.

10. **Stain, 0.125% Coomassie Blue R-250 50% methanol, 10% acetic acid**

31.25 ml of Coomassie Blue R-250 (Stain stock 9.), 125 ml methanol and 25 ml acetic acid were mixed and made up to 250 ml with H_2O.

11. **Destaining Solution I, 50% methanol, 10% acetic acid**

250 ml methanol and 50 ml acetic acid were mixed and made up to 500 ml with H_2O.

12. **Destaining Solution II, 7% acetic acid, 5% methanol**

140 ml acetic acid and 100 ml methanol were mixed and made up to 2 litres with H_2O.

13. **Water-Saturated n-Butanol**

25 ml n-butanol combined with 2.5 ml of H_2O in a bottle, shaken well and the top phase was used to overlay the gels.

4.5.4 METHOD

4.5.4.3.1 Preparation of the Separating Gel

1. The MINI-PROTEAN II Slab Cell Unit was assembled in the casting mode, using 1 mm spacers.
2. 5.025 ml of H_2O, 3.75 ml of 4X Running Gel Buffer and 6 ml 30%T 2.7%C_{bis} (B.1.2.1) were mixed in a 125 ml side arm vacuum flask.
3. The flask was stoppered and vacuum was applied for 10 minutes with occassional shaking. This ensured the degassing of the solution.
4. 150 µl 10% SDS (B.1.2.4), 75 µl ammonium persulfate and 7.5 µl TEMED were added to the flask and swirled gently to ensure mixing. Care was taken not to generate bubbles.
5. This solution was pipeted into the sandwich to a level about 2 cm from the top.
6. The slaps were overlayed with about 0.3 ml water-saturated n-butanol and allowed to polymerize for 30 minutes.
7. The casting stand was tilted to pour off the overlay.
8. The surfaces of the gels were rinsed three times with distilled water.
9. About 0.5 ml of Running Gel Overlay Solution was added to the gel.
10. The gels were allowed to sit for 45 minutes.

4.5.4.3.2 Preparation of the Stacking Gel

1. The Running Gel Overlay Solution was poured off from the surface of the gels.
2. Mix 3.05 ml H_2O, 1.25 ml 4X Stacking Gel Buffer and 0.65 ml 30%T 2.7%C_{bis}(B.1.2.1) in a 50 ml side arm vacuum flask.
3. This solution was deaerated by vacuum as before.
4. 50 μl 10% SDS (B.1.2.4), 25 μl ammonium persulfate and 5 μl TEMED were added to the flask and swirled gently to ensure mixing. Care was taken not to generate bubbles.
5. 1-2 ml of stacking gel solution were added to each sandwich in order to rinse the surface of the gels. The casting stand was rocked and the liquid was poured off.
6. Each sandwich was filed with stacking gel solution.
7. A ten well comb was inserted into each sandwich. Care was taken not to trap any bubbles below the teeth of the combs. Oxygen inhibits polymerization and causes a local distortion in the gel surface at the bottom of the wells.
8. The gel was allowed to sit for at least 30 minutes.

4.5.4.3.3 Sample preparation

1. Equal parts of protein sample and 2X Treatment Buffer were combined in a 2 ml test tube.
2. The tubes were placed in a boiling water bath for 5 minutes.
3. The samples were removed, centrifuged and put on ice until they were loaded on the gel. The treated samples can be kept frozen for future electrophoretic runs.

4.5.4.3.4 Preparation of molecular mass standards

1. Combine 1 μl of High Molecular mass standards with 19 μl sample buffer and 4 μl of 5% bromophenol blue in a test tube and put in a boiling water bath for 5 minutes.
2. Combine 1 μl of Low Molecular mass standards with 19 μl sample buffer and 4 μl of 5% bromophenol blue and put in a boiling water bath for 5 minutes.

4.5.4.3.5 Electrophoretic run conditions

1. Introduction

Purified tetanus toxin, recombinant Fragment C and papain digested toxin were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli.[61] SDS-PAGE was done under non-reducing conditions on BIO-RAD Mini-PROTEAN II slab cell using a discontinuous gel of 7.5% separating gel and 4% stacking gel.

2. Sample preparation

2.1 Tetanus toxin Lot# CP 49, 1050 Lf/ml; 2.0 mg Protein/ml 20 μl + 20 μl NON-Reducing buffer.
Load 18 μl in lane #1.

2.2 Tetanus toxin digested with 0.01 IU/mg TT immobilized papain for 4 hours at 56° C.
20 μl + 20 μl NON-Reducing buffer.
Load 18 μl in lane #3.

2.3 Tetanus toxin C fragment, recombinant (rTTC)
5 μl rTTC + 15 μl H_2O + 20 μl NON-Reducing buffer.
Load 10 μl in lane #5

3. **Separating gel: 7.5%, 15 ml**

H_2O	7.275 ml
Tris pH 8.8 (2)	3.75 ml
Acrylamide/Bis (30%)	3.75 ml
10% SDS	0.150 ml
Ammonium PerSulfate	0.075 ml
TEMED	0.0075 ml

4. **Stacking gel: 4%, 5 ml**

H_2O,	3 ml
Tris pH 6.8 (3)	1.25 ml
Acrylamide/Bis (30%)	0.670 ml
10% SDS	50 µl
Ammonium PerSulfate	25 µl
TEMED	5 µl

5. **Loading the samples**

The samples were loaded on the gel in the appropriate wells as depicted in the template below using a 25 µl Hamilton syringe.

1	TT + PAPAIN NON-RED 3		rTTC NON-RE 5		TT NON-RED 7	9
	18 µl		10 µl		5 µl	

6. **Power conditions**

The lid was placed on top of the buffer chamber and 100 V constant voltage was applied to the cell and electrophoresis began. When the sample bands were concentrated, as they were entering the running gel, approximately 12 minutes, the power was interupted. The power was changed to 200 V constant voltage and the gel was allowed to run for another 45 minutes.

7. **Removal of the gel**

After electrophoresis was completed, the power supply was turned off and the electrical leads disconnected. The gel was removed from the glass plates and placed in a petri dish containing fixative solution (40% methanol/10% acetic acid).

8. **Staining and destaining**

The gel was placed in a petri dish containing 0.125% Coomassie Blue Stain (1.1.10) and shaken gently for 1 hour followed by Destaining Solution I and Destaining Solution II.

Chapter 5: Cyanogen bromide cleavage of Tetanus Toxin Fragment C at points containing Methionine residues

5.1 Introduction

The single chain of Tetanus Toxin Fragment C contains six methionine residues. Cyanogen bromide (CNBr) is capable of cleaving thioethers. The action of cyanogen bromide upon proteins is unique in its selective attack on methionine. The nucleophilic nature of the sulfur atom on methionine causes it to react with cyanogen bromide. The reaction is greatly facilitated by the strong neighbouring group effect exerted by the carboxyl group. Sulfur displaces the bromide anion to form the cyano-sulfonium bromide. The particular stereochemistry of the Met side chain favors the intramolecular rearrangement of the sulfonium salt. This leads to the elimination of methylthio-cyanate and the formation of the iminolactone of homoserine. The iminolactone is unstable in the aqueous, acidic medium, and readily hydrolyze by water to cleave the polypeptide chain with the liberation of the amino acid that followed methionine. The methionine on the N-terminal fragment is converted to homoserine which becomes the -COOH terminal amino acid of the peptide fragment as shown in Figure 5.1.[62] Gross and Witkop originally carried out the reaction in 0.1-0.3 N HCl.[63] Corradin and Harbury performed the reaction in 70% formic acid and by controlling the time and the concentrations obtained a methionine residue.[64]

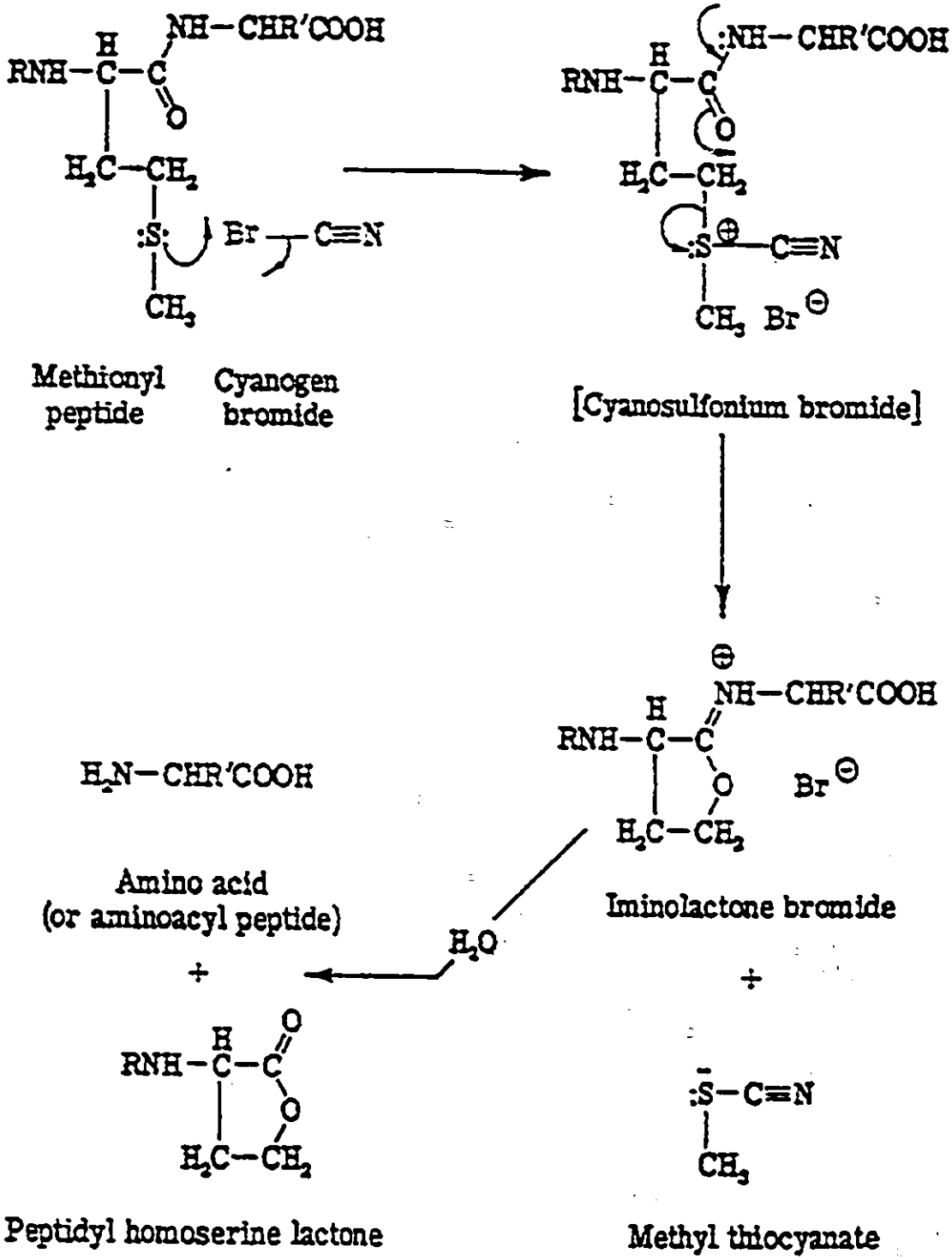


FIGURE 5.1 Cyanogen Bromide action on Methionine.[62]

5.2 Results and Discussion

5.2.1 Introduction

Gross and Witkop showed that the methionyl peptide bond could be cleaved with cyanogen bromide under mild conditions.

Cyanogen bromide cleavage is the method of choice since the average number of methionine residues in a protein is relatively low (ca 2%) and the cleavage goes to completion.[65]

Since the sequence of tetanus toxin fragment C contains six methionine residues (Figure 5.2) a CNBr digest should yield seven distinct fragments and some partially digested material.[66] The high vapour pressure of CNBr favours its delivery via gas phase. The reaction can be carried out in any closed container but it is advantageous to keep the volume small.[67]

Recombinant tetanus toxin C fragment (rTTC) was dissolved in 70% formic acid and incubated with CNBr in the dark for 24 hours in a closed autosampler vial. Formic acid protonates the amine functional groups (inactive) thereby leaving only the electron rich Sulfur center to react with CNBr. The reaction was quenched by evaporating the formic acid in a desiccator containing NaOH pellets under vacuum.[65]

Under the experimental conditions described above, CNBr will cleave Met-X bonds on tetanus toxin fragment C in near quantitative yield, to leave peptides with a C-terminal methionine.[68] The expected peptides are shown by increasing size in Table 5.1 and their amino acid sequence in Figure 5.2

	# AA	MOLECULAR WEIGHTS
PEPTIDE #2:	7	809
PEPTIDE #3:	34	3,820
PEPTIDE #7:	76	8,122
PEPTIDE #4:	73	8,139
PEPTIDE #1:	75	8,168
PEPTIDE #6:	91	9,377
PEPTIDE #5:	96	11,064
FRAGMENT C:	452	49,499

Table 5.1 Molecular weights of expected peptides (see Figure 5.2 below for the amino acid sequence of the peptides).

865
Lys Asn Leu Asp Cys Trp Val Asp Asn Glu Glu Asp Ile Asp Val Ile Leu Lys Lys Ser
Thr Ile Leu Asn Leu Asp Ile Asn Asn Asp Ile Ile Ser Asp Ile Ser Gly Phe Asn Ser
Ser Val Ile Thr Tyr Pro Asp Ala Gln Leu Val Pro Gly Ile Asn Gly Lys Ala Ile His
Leu Val Asn Asn Glu Ser Ser Glu Val Ile Val His Lys Ala Met 939

75 a.a

940
Asp Ile Glu Tyr Asn Asp Met 946

7 a.a

947
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu
Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile Ile Ser Ser Met 980

34 a.a

981
Lys Lys His Ser Leu Ser Ile Gly Ser Gly Trp Ser Val Ser Leu Lys Gly Asn Asn Leu
Ile Trp Thr Leu Lys Asp Ser Ala Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro
Asp Lys Phe Asn Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr Ile Thr Asn Asp Arg
Leu Ser Ser Ala Asn Leu Tyr Ile Asn Gly Val Leu Met 1053

73 a.a

1054
Gly Ser Ala Glu Ile Thr Gly Leu Gly Ala Ile Arg Glu Asp Asn Asn Ile Thr Leu Lys
Leu Asp Arg Cys Asn Asn Asn Asn Gln Tyr Val Ser Ile Asp Lys Phe Arg Ile Phe Cys
Lys Ala Leu Asn Pro Lys Glu Ile Glu Lys Leu Tyr Thr Ser Tyr Leu Ser Ile Thr Phe
Leu Arg Asp Phe Trp Gly Asn Pro Leu Arg Tyr Asp Thr Glu Tyr Tyr Leu Ile Pro Val
Ala Ser Ser Ser Lys Asp Val Gln Leu Lys Asn Ile Thr Asp Tyr Met 1149

96 a.a

1150
Tyr Leu Thr Asn Ala Pro Ser Tyr Thr Asn Gly Lys Leu Asn Ile Tyr Tyr Arg Arg Leu
Tyr Asn Gly Leu Lys Phe Ile Ile Lys Arg Tyr Thr Pro Asn Asn Glu Ile Asp Ser Phe
Val Lys Ser Gly Asp Phe Ile Lys Leu Tyr Val Ser Tyr Asn Asn Asn Glu His Ile Val
Gly Tyr Pro Lys Asp Gly Asn Ala Phe Asn Asn Leu Asp Arg Ile Leu Arg Val Gly Tyr
Asn Ala Pro Gly Ile Pro Leu Tyr Lys Lys Met 1240

91 a.a

1241
Glu Ala Val Lys Leu Arg Asp Leu Lys Thr Tyr Ser Val Gln Leu Lys Leu Tyr Asp Asp
Lys Asn Ala Ser Leu Gly Leu Val Gly Thr His Asn Gly Gln Ile Gly Asn Asp Pro Asn
Arg Asp Ile Leu Ile Ala Ser Asn Trp Tyr Phe Asn His Leu Lys Asp Lys Ile Leu Gly
Cys Asp Trp Tyr Phe Val Pro Thr Asp Glu Gly Trp Thr Asn Asp End 1315

76 a.a.

FIGURE 5.2 Amino acid sequence of expected peptides

from cyanogen bromide cleavage of fragment C.

5.2.2 SDS-PAGE of CNBr generated fragments

The most commonly used protein electrophoresis system is that described by Laemmli.[61] Laemmli gels by including SDS in the gel formulation and by treating the sample with SDS, separate polypeptides on the basis of molecular mass alone. These gels are useful for the separation of large and medium weight size polypeptides but will not resolve small peptides below 15 daltons. Attempts were made to separate the CNBr generated fragments on a 20% homogeneous and a 4-20% gradient gels but were unsuccesful. Fourteen percent acrylamide gels containing 4M urea were also used but in our Mini-Protean system excess heat was generated and the gels were literally cooked on the glass plates.

The SDS system found to be the most effective in separating the CNBr generated peptides was a 20% uniform pore gel with 0.5% crosslinking containing 10% glycerol. The pH of the separating buffer of this system is 9.3 instead of 8.8 as in the Laemmli system. This system was published by Guilian *et al* (1985)[69] and is described in Section B Experimental.

The cleavage products of fragment C were separated by SDS-PAGE electrophoresis in comparison with markers of molecular mass, and with the uncleaved fragment C.

Figure 5.3 indicates that fragment C was broken into several peptides ranging from apparenr molecular mass 45 kD to well below 14.4 kD. The separation of these CNBr fragments in pure form is not easily achieved, due to the tendency to aggregate.[66]

The peptides of higher apparent molecular mass than 14.4 kD could also be the result of partial digestion.

However, two distinct bands appear below 14.4 kD. The band of approximately 11 kD could be the expected high molecular mass peptide # 5. The band below could be any of the expected medium molecular mass peptides # 6, # 1, # 4 and # 7.

These peptides could then be transferred to a nitrocellulose supporting membrane for staining and identification.

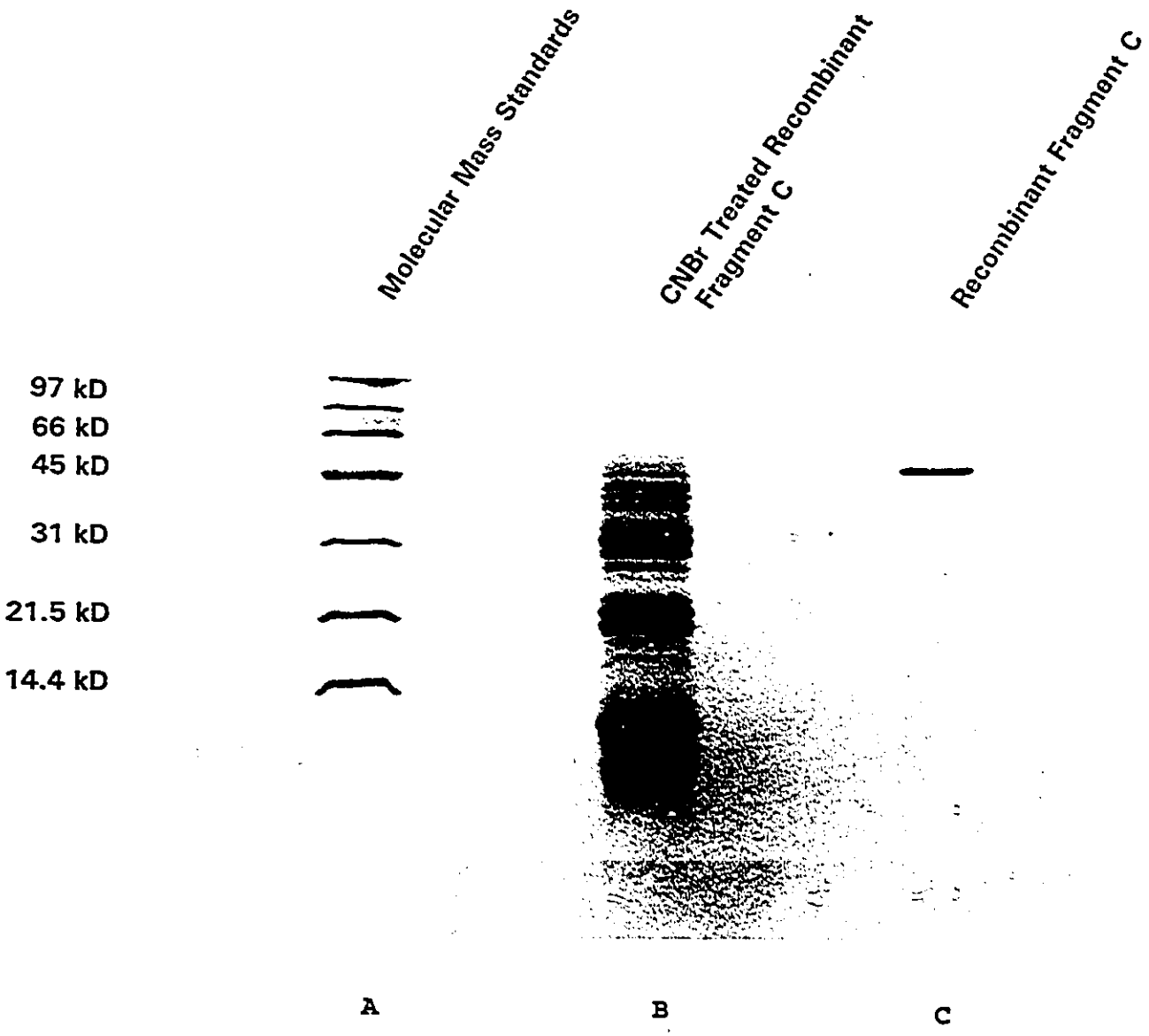


FIGURE 5.3 CNBr generated peptides of fragment C separated by SDS-PAGE and stained with Coomassie blue. Lane A: Molecular mass markers. Lane B: rTTC cleaved by CNBr and Lane C: rTTC.

5.2.3 Western Blot of fragment C cleavage products

The gel containing the separated peptides of the CNBr cleaved fragment C was sandwiched with an immobilizing membrane. When an electric field was applied across this sandwich, the molecules moved from the gel to the nitrocellulose (NC) membrane where they bound. At the end of the transfer, the NC membrane was washed extensively to remove SDS, which otherwise would denature the peptides and interfere with antibody binding. Once bound, the peptides were incubated with the four different antisera, namely the murine anti-toxin (MTN), murine anti-toxoid (MTD), the guinea pig anti-toxin (GPTN) and guinea pig anti-toxoid (GPTD) in order to test their binding and specificity. These antisera gave different identity staining of the peptides.

Figure 5.4 shows that GPTN exibited reactivity to as many as 8 dissimilar peptides. The antibody binding sites localized by immunoblotting correspond to most of the bands shown on Figure 5.3, both high and low molecular mass fragments, some of which were more strongly indicated than others. This strong reactivity of the anti-toxin guinea pig sera confirms the ELISA findings in Chapter 3 where most of the GPTN sera reacted with epitopes found within fragment C. GPTD reacted with 10 bands but only 4 of which were commonly matched with the GPTN reactants. MTN indicated 3 strong bands which seemed to align with bands seen among the GPTD reactants but not strongly identified among the GPTN reactants.

MTD showed very weak identity of the bands altogether. This light staining may reflect few helical epitopes recognized by the MTD antibodies remaining after SDS-PAGE and electroblotting.[70]

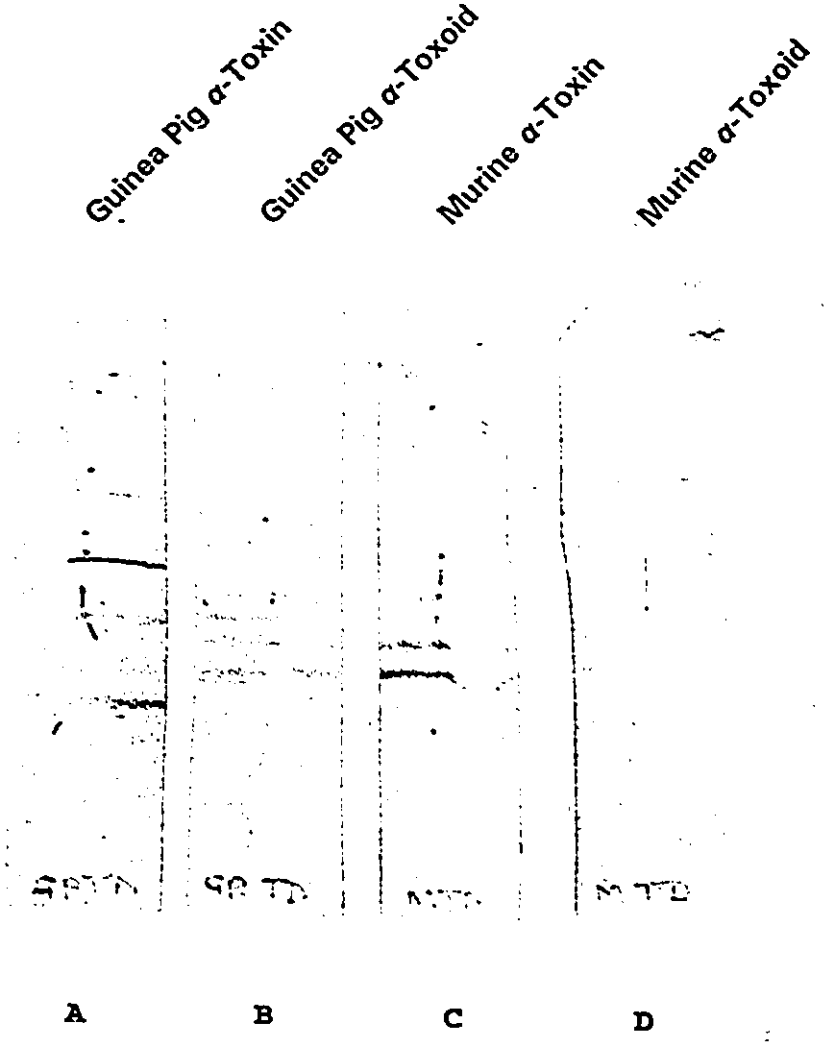


FIGURE 5.4 Immunoblot of fragment C cleavage products. Lane A: Guinea pig α-Toxin. Lane B: Guinea pig α-Toxoid. Lane C: Murine α-Toxin and Lane D: Murine α-Toxoid.

5.3 Summary

Recombinant tetanus toxin C fragment (rTTC) was cleaved with cyanogen bromide at points containing methionine residues. The experimental conditions were optimized and the CNBr derived peptides were separated on SDS-PAGE. The separation of the low molecular mass polypeptides was achieved on a 20% uniform pore gel with 0.5% crosslinking and containing 10% glycerol. A gel containing the separated peptides was electrotransferred onto a nitrocellulose supporting membrane. The reaction of the peptides with the four different antisera, namely the murine anti-toxin (MTN), murine anti-toxoid (MTD), the guinea pig anti-toxin (GPTN) and guinea pig anti-toxoid (GPTD) gave different identity staining patterns.

5.4 Experimental procedures

PART A - Cyanogen bromide cleavage of tetanus toxin fragment C at points containing methionine residues

5.4.1 Introduction

Cyanogen bromide cleaves proteins at points containing methionine residues. The experimental conditions were optimized and the reaction was carried out in 70% formic acid. Under the conditions described below, CNBr will cleave Met-X bonds on tetanus toxin fragment C in near quantitative yield, to leave peptides with a C-terminal methionine.

5.4.2 Reagents and Method

A. Reagents

1. Cyanogen Bromide F.W. 105.3 Fisher Scientific, Reagent Grade, 0-2080.

   A 1M solution can be prepared by dissolving 10.6 mg in 100 μl Acetonitrile.

   9.6 mg CNBr were weighted and dissolved in 906 μl acetonitrile to give a one molar solution.

2. Acetonitrile, HPLC grade, J.T. Baker
3. Auto sampler vials (2ml) with teflon septa, Varian Scientific (USA)
4. 88% Formic Acid

   7.955 ml of 88% formic acid were mixed with 2.045 ml H_2O to give a 70% solution.

5. Tetanus toxin C fragment, recombinant (rTTC) lyophilized. Boehringer Mannheim Biochemica 1 mg Cat. No. 1348655. One mg vial rTTC was reconstituted in 1 ml H_2O to give 1 mg/ml concentration.

B. Method

1. One hundred μl of 1 mg/ml(=100 μg) rTTC were evaporated to dryness under Nitrogen in a 2 ml auto sampler vial.
2. The dryed rTTC was dissolved in 50 μl of 70% Formic acid and mixed with 2 μl of 1M CNBr (molar excess of CNBr). This corresponds to approximately 200 μg CNBr/100 μg of protein.
3. The top of the solution was flushed with Nitrogen and the vial was closed with a polypropylene open screw cap and a teflon septum.
4. The sample was covered completely with aluminum foil and incubated in the dark and at room temperature for 24 hours.
5. At the end of the incubation period the formic acid was diluted to 7% by adding 450 μl H_2O.[62]
6. The reaction was quenched by evaporating the formic acid in a desiccator containing a beaker of NaOH pellets under vacuum.
7. Steps 2.5 and 2.6 were repeated.
8. The sample was reconstituted with 100 μl H_2O.

PART B - Separation of CNBr cleaved tetanus toxin C fragment on SDS-PAGE

5.4.3 Introduction

The electrophoretic separation of the low molecular mass polypeptides obtained from the CNBr cleavage of rTTC was performed on SDS-PAGE according to the method of Giulian, G. G., *et al.*[69]

5.4.4 Reagents and Equipment

See Chapter 4 Experimental Section

5.4.5 Preparation of Stock Solutions

1. **Separating Gel Acrylamide Stock,** (37.7%T 0.5%C_{bis})
   37.5 g Acrylamide and 0.1875 g Bis were dissolved in 100 ml H_2O and stored in the dark at 4° C
2. **Stacking Gel Acrylamide Stock,** (39.4%T 4.8%C_{bis})
   37.5 g Acrylamide and 0.1875 g Bis were dissolved in 100 ml H_2O and stored in the dark at 4° C.
3. **Separating Gel Buffer Stock,** (3 M Tris pH 9.3)
   16.1 g trizma base and 2.7 g trizma HCl were dissolved by warming in 50 ml H_2O and stored at room temperature.
4. **Stacking Gel Buffer Stock,** (0.5M Tris pH 6.8)
   0.075 g trizma base and 3.85 g trizma HCl were dissolved by warming in 50 ml H_2O and stored at room temperature.
5. **10% Sodium Dodecyl Sulfate**
   Ten grams of SDS were disolved by warming in 100 ml H_2O.

6. **10% Ammonium Persulfate (AMPS)**

One hundred mg of AMPS were disolved by warming in 1 ml H_2O. This solution was prepared just prior to use.

7. **Bromophenol Blue, (0.5% w/v in 10% EtOH)**

Fifty mg of bromophenol blue were disolved in 10 ml 10% ethanol.

8. **Tank Buffer, 0.05 M Tris, 0.38 M glycine, 0.1% SDS**

10.264 g Trizma base, 2.456 g Trizma-HCl, 57.8 g Glycine and 20 ml SDS solution (1.2.5) were mixed and made up to 2.0 litres with H_2O.

The pH of this solution was not checked (pH 8.4).

NOTE Reagents used below but their preparation not described here are found in Chapter 4.

5.4.6 Method

1. **Preparation of the Separating Gel**

The procedure for preparing the separating gel is the same as in Chapter 4 Section B except for the composition of the gel which is shown below.

Separating gel 20%T, 0.5%C, 17 ml

Acrylamide Stock (1.2.1)	9.07 ml
H_2O	1.75 ml
Glycerol (99.9%)	1.7 ml
Separating Gel Buffer (1.2.3)	4.24 ml
SDS (1.2.5)	0.170 ml
Ammonium PerSulfate (1.2.6)	0.048 ml
TEMED	0.024 ml

2. **Preparation of the Stacking Gel**

The procedure for preparing the stacking gel is the same as in Chapter 4 section B except for the composition of the gel which is shown below.

Stacking gel 10%T, 4.8%C, 5 ml

Acrylamide Stock (1.2.2)	1.26 ml
H_2O	1.91 ml
Glycerol (99.9%)	0.5 ml
Stacking Gel Buffer (1.2.4)	1.25 ml
SDS (1.2.5)	0.050 ml
Ammonium PerSulfate (1.2.6)	0.015 ml
TEMED	0.012 μl

3. **Sample preparation** - see Chapter 4 Section B.

4. **Preparation of Molecular weight standards** - see Chapter 4 Section B.

5.4.7 Electrophoretic run conditions

1. **Introduction**

Tetanus toxin fragment C, recombinant, tetanus toxin fragment C, recombinant cleaved by cyanogen bromide and low molecular mass standards were separated on SDS-PAGE by the method of Laemmli[61] as modified by Giulian *et al.*[69] SDS-PAGE was done under non-reducing conditions on BIO-RAD Mini-PROTEAN II slab cell using a discontinuous gel of 20 % separating gel and 10 % stacking gel.

2. **Sample preparation**

2.1 Tetanus toxin fragment C, recombinant (rTTC)

5 μl rTTC + 15 μl H_2O + 20 μl **NON-Reducing buffer.**

Load 10 μl in lane #5

2.2 Tetanus toxin fragment C, recombinant (rTTC) cleaved with cyanogen bromide.

10 μl rTTC + 30 μl **NON-Reducing buffer.**

Load 10 μl in lane #5

2.3 **Loading the samples**

The samples were loaded on the gel in the appropriate wells as depicted in the template below using a 25 μl Hamilton syringe.

1	LOW MOL WEIGHT STDS NON-RED 3		rTTC CNBr CLEAVED NON-RED 5		rTTC NON-RED 7	9
	18 μl		10 μl		5 μl	

3. **Power conditions**

The lid was placed on top of the buffer chamber and 100 V constant voltage was applied to the cell and electrophoresis began. When the sample bands were concentrated, as they were entering the running gel, in approximately 12 minutes, the power was interrupted. The power was changed to 200 V constant voltage and the gel was allowed to run for another 45 minutes.

4. **Removal of the gel**

After electrophoresis was completed, the power supply was turned off and the electrical leads disconnected. The gel was removed from the glass plates and staining proceeded.

5 **Staining and destaining**

The gel was placed in a petri dish containing 0.125% Coomassie Blue Stain (1.1.10) and shaken gently for one hour followed by one hour each of Destaining Solution I and Destaining Solution II.

PART C - Electrophoretic transfer of SDS-PAGE gels

5.4.8 Introduction

The electrophoretic elution of proteins from polyacrylamide gels was first described by Towbin *et al.*[71] The peptides generated by CNBr cleavage of fragment C, were separated on SDS-PAGE as described in Section B. The electrophoretic transfer of the gels was performed on the BIO-RAD Mini-Trans-Blot Electrophoretic Transfer Cell. Following electrophoresis, the gels were equilibrated for 30 minutes in blotting buffer. They were then placed in a gel holder cassette, sandwitched between a nitrocellulose membrane, filter and fiber pads. The transfer was performed at 30 V constant voltage for 16 hours. The peptides generated by CNBr cleavage of fragment C, were separated on SDS-PAGE as described in Section B, but with different loading arrangement on the gel as shown below.

Tetanus toxin fragment C cleaved by Cyanogen Bromide Non Reduced
100 µl

5.4.9 Reagents and Equipment

1. BIO-RAD Mini Trans-Blot Electrophoretic Transfer Cell
2. Nitrocellulose membrane (0.20 micron), BIO-RAD 162-0112
3. Filter paper, Whatman #1 Qualitative (England)
4. Gelatin, EIA grade, BIO-RAD 170-6537
5. For additional information, see Chapter 4

5.4.10 Preparation of buffers

1. **Preparation of Blotting Buffer, (0.25 M Tris, 192 mM glycine, 20% v/v methanol, pH 8.3)**

   3.03 g Trizma base, 14.4 g glycine and 200 ml of methanol were mixed and made up to 1.0 litre with H_2O. The pH of this solution was not adjusted (pH 8.4).

2. **Preparation of 10 % Gelatin Solution (Stock), (10% Gelatin in 0.01M TBS-T, pH 8.0).**

   Ten grams of gelatin were dissolved by warming in 100 ml TBS-T.

3. **Preparation of Blocking Solution, (1% Gelatin in 0.01M TBS-T, pH 8.0).**

   One percent Gelatin was prepared fresh on day of analysis by thoroughly mixing 9 ml TBS-T buffer with 1 ml of 10% gelatin solution (stock) in TBS-T.

5.4.11 Method

1. **Equilibration of the gel, filter and fiber pads**

1.1 The gel was placed in a glass petri dish containing blotting buffer and allowed to equilibrate for 30 minutes.

1.2 Nitrocellulose of 0.2 micron porosity and pre-cut to the dimensions of the gel was placed in a glass petri dish containing blotting buffer and allowed to soak for 15 minutes.

1.3 Pre-cut Filter paper and Fiber Pads were completely saturated by soaking them in blotting buffer in a plastic dish.

1.4 The Mini Trans-Blot electrode was installed in the buffer chamber and the buffer tank was filled half full with blotting buffer. A 1-inch magnetic stir was placed at the bottom of the unit.

2. **Assembly of the gel holder cassette**

2.1 The gel holder cassette was opened and placed in a shallow glass dish so that the grey panel is flat on the bottom of the vessel. The grey panel is the cathode (-) side and the clear panel is the anode (+) side.

2.2 A pre-soaked Fiber Pad was placed on the grey panel of the cassette and a piece of saturated Filter Paper was placed on top.

2.3 The surface of the Filter Paper was saturated with additional 2-3 ml blotting buffer. The equilibrated gel was placed on top of the paper.

2.4 The surface of the gel was flooded with blotting buffer and the pre-wetted nitrocellulose membrane was lowered on top of the gel. A glass test tube was rolled over the top of the membrane to ensure that no air bubbles were trapped between the gel and the membrane.

2.5 The surface of the membrane was flooded with buffer and the sandwich was completed by placing a piece of saturated Filter Paper on top of the membrane followed by a saturated fiber pad on top of the Filter paper.

2.6 The cassette was closed by holding firmly so the sandwich would not move and the latch was secured.

2.7 The gel holder was placed in the buffer tank so that the grey panel (-) is facing the grey cathode electrode panel.

2.8 The buffer tank was set on top of a magnetic stirrer and was filled with buffer to just above the level of the top row of circles on the gel holder cassette. The magnetic stirrer was turned on and the lid was put in place with the electrode wires attached to the appropriate pins of the electrode module.

2.9 The power supply was turned on and set at 30 V constant voltage for an overnight transfer.

2.10 At the end of the transfer, the membranes were removed from the gel holder cassette.

PART D - Immunoblot on nitrocellulose membrane

5.4.12 Introduction

The tansferred peptide bands were visualized and identified by immunoassay techniques described below.

5.4.13 Reagents and Methods

A Reagents

1. Sera and preparation of working serum dilution

1.1 Guinea Pig #1 Antibodies, anti-tetanus toxin (Final bleeding). Ten μl of neat serum was diluted in 5 ml TBS-T to give a working dilution of 1:500.

1.2 Guinea Pig #1 Antibodies, anti-tetanus toxoid (Final bleeding). Ten μl of neat serum was diluted in 5 ml TBS-T to give a working dilution of 1:500.

1.3 Murine Antibodies (Pool of 3 mice), anti-tetanus toxin (Final bleeding). Ten μl of neat serum was diluted in 5 ml TBS-T to give a working dilution of 1:500.

1.4 Murine Antibodies (Pool of 3 mice), anti-tetanus toxoid (Final bleeding). Ten μl of neat serum was diluted in 5 ml TBS-T to give a working dilution of 1:500.

2. **Conjugates and preparation of Working Conjugate dilution**

2.1 Goat α-Guinea Pig IgG (H+L) Horseradish Peroxidase (HRPO) labelled, Affinity purified (Kirkegaard and Perry Laboratories, USA). K&P 14-17-06.

A 0.5 mg vial was reconstituted in 1 ml H_2O.

Ten µl was diluted in 5 ml in TBS-T to give a working dilution of 1:500.

2.2 Goat α-Mouse IgG (H+L) Alkaline phosphatase (AP) labelled, 1 ml Zymed (California USA) 62-6522.

Ten µl were diluted in 10 ml to give a working dilution of 1:1000.

3. **Substrates/Chromogens**

3.1 **Horseradish Peroxidase Substrate**

4-chloro-1 naphthol (CNS), Sigma 115F-3680.

Fifteen mg of CNS were dissolved in 15 ml methanol to give a Stock solution of 3 mg/ml.

One ml of CNS (stock) was mixed with 5 ml TBS pH 7.4 and 20 µl of 3% H_2O_2 to. This working concentration was prepared 5 minutes prior to use in an amber container.

3.2 **Alkaline Phosphatase Substrate**

BIO-RAD Alkaline Phosphatase Conjugate Substrate Kit, Catalog No. 170-6432

Contents: - Color reagent A (contains nitroblue tetrazolium in aqueous dimethylformamide (DMF), containing magnesium chloride).

- Color reagent B (contains 5-bromo-4-chloro-3-indolyl phosphate in DMF)
- AP color development buffer.

3.3 **Preparation of working solution**

One hundred µl of A, 100 µl of B and 9.8 ml of colour development buffer were mixed and allowed to warm to room temperature.

3.4 For additional information, see Chapter 4.

B. Method

1. Transfer power conditions

Overnight transfer: At start 30 V and 91.7 mA;

At finish 30 V and 117 mA.

2. Blocking with 1% Gelatin

At the end of the transfer, the membranes were removed from the gel holder cassette, and cut into five equal strips. Four strips were placed in a glass petri dish and allowed to incubate for 4 hours in blocking solution.

3. Wash

The four strips were washed twice with TBS-T pH 8.0.

4. Sera

4.1 The four nitrocellulose strips were properly labelled and incubated for one hour at 25 °C with the appropriate sera.

4.2 At the end of the serum incubation the strips were washed twice with TBS-T pH 8.0

5. **Conjugates**

5.1 The two nitrocellulose strips incubated with guinea pig sera were incubated for one hour at 25° C with the goat α-Guinea Pig IgG (H+L) HRPO labelled conjugate.

5.2 The two nitrocellulose strips incubated with murine sera were incubated for one hour at 25° C with the goat α-Mouse IgG (H+L) Alkaline phosphatase labelled conjugate.

5.3 At the end of the conjugate incubation the strips incubated with the HRPO conjugate were washed twice with TBS pH 7.4 and the strips incubated with the AP conjugate were washed twice with TBS-T pH 8.0 and once with the AP colour development buffer at room temperature.

6. **Substrates/Chromogens**

6.1 The strips incubated with the AP conjugate were incubated with the AP substrate.

6.2 The strips incubated with the HRPO conjugate were incubated with the HRPO substrate.

GUINEA PIG α-TOXIN	GUINEA PIG α-TOXOID	MURINE α-TOXIN	MURINE α-TOXOID

Chapter 6: Multiple Antigenic Peptide

6.1 Introduction

Conventional methods for the production of vaccines involve the conjugation of synthetic peptide antigens to protein carriers to enhance the low immunogenicity of the peptides. However, these methods have several limitations. The molecular excess of the carrier may supress the B cell response to the epitope of interest.[72] Conjugation to the carrier may radically modify the antigenic epitopes of the peptide. The peptide antigen represents only a minor fraction of the total molecular weight of the peptide-antigen conjugate thus the humoral immunity would be directed mostly against other epitopes of the complex.[73]

The Multiple Antigenic Peptide (MAP) method for peptide-based vaccines overcomes such limitations. The protein carrier is replaced with a small immunogenically inert core matrix comprised of lysine residues with α- and ϵ- amino groups for anchoring multiple copies of the same or different synthetic peptides.[74-75]

Lysine is a trifunctional amino acid which has two amino groups (N^{α} and N^{ϵ}) available as reactive ends. The first level of lysine will produce two reactive ends; the second level, consisting of three lysines, contains four amino groups; the third level consisting of seven lysines, contains eight amino acids groups, and so on.[76]

A schematic representation of the core matrix of the MAP is depicted in Figure 6.1.

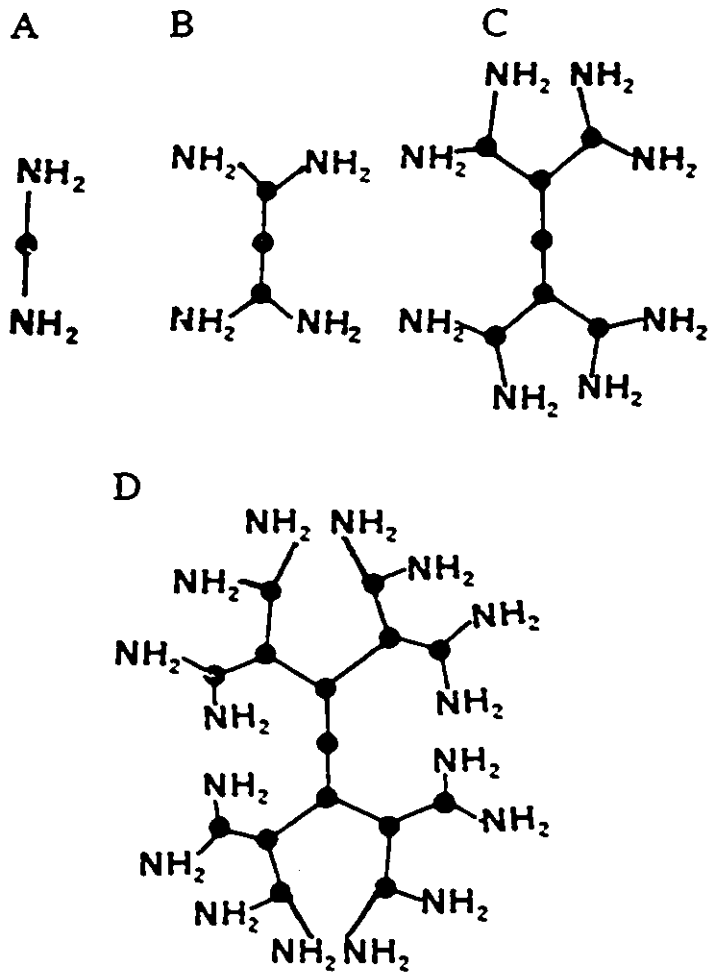


FIGURE 6.1 Schematic representation of the core matrix of the MAP. (A) First level, divalent; (B) second level, tetravalent; (C) third level, octavalent ; and (D) fourth level, hexadecavalent.[74]

A typical example of MAP consists of a core matrix with a heptalysine that links to eight dendritic arms of peptides containing antigenic peptides. This approach is flexible and allows preparation of immunogens consisting of various epitcpes in different arrangements. Because of the close packing of these antigens in a scaffolding, MAPS may have the advantage of providing a tertiary structure that allows stabilization of secondary structures of peptide antigens.

Each arm of peptide may consist of 10 to more than 20 amino acids resulting in a very immunogenic macromolecular structure with a high antigen content (about 80%). This macromolecular structure provides a high density of surface peptide antigen while the small lysine backbone, representing a minor portion of the total octameric structure is non immunogenic.[77]

The MAP containing the peptides can be synthesized as a single unit by the direct step-wise solid-phase (SPPS) method of Merrifield (1963).[78] Another approach is the indirect modular which synthesizes the activated peptide antigen and the carrier separately, and then coupled to each other to form the MAP. This procedure allows one to keep the carrier in stock and to couple one or more synthetic antigens to it when desired.[72]

6.2 Solid phase peptide synthesis

6.2.1 Introduction

Solid Phase Peptide Synthesis (SPPS) is based on sequential addition of α-amino and side chain protected amino acid residues to an insoluble polymeric support (Figure 6.2). The acid-labile Boc group or base labile Fmoc-group is used for N-α-protection. After removal of this protecting group, the next protected amino acid is added using either a coupling reagent or pre-activated protected amino acid derivative. The resulting peptide is attached to the resin, via a linker, through its C-terminus and may be cleaved to yield a peptide acid or amide, depending on the linking agent used. Side chain protecting groups are often chosen so as to be cleaved simultaneously with detachment of the peptide from the resin.

Cleavage of the Boc protecting group is achieved by trifluoroacetic acid (TFA) and the Fmoc protecting group by piperidine. Final cleavage of the peptide resin and side chain deprotection requires strong acid such as hydrogen fluoride (HF) or trifluoromethanesulfonic acid (TFMSA) in the case of Boc chemistry and TFA in Fmoc chemistry. Dichloromethane (DCM) and N,N-dimethylformamide (DMF) are the primary solvents used for resin deprotection, coupling and washing.

Unlike regular peptide synthesis, there is no need for purification of the crude cleavage products other than desalting using a Sephadex column.[79]

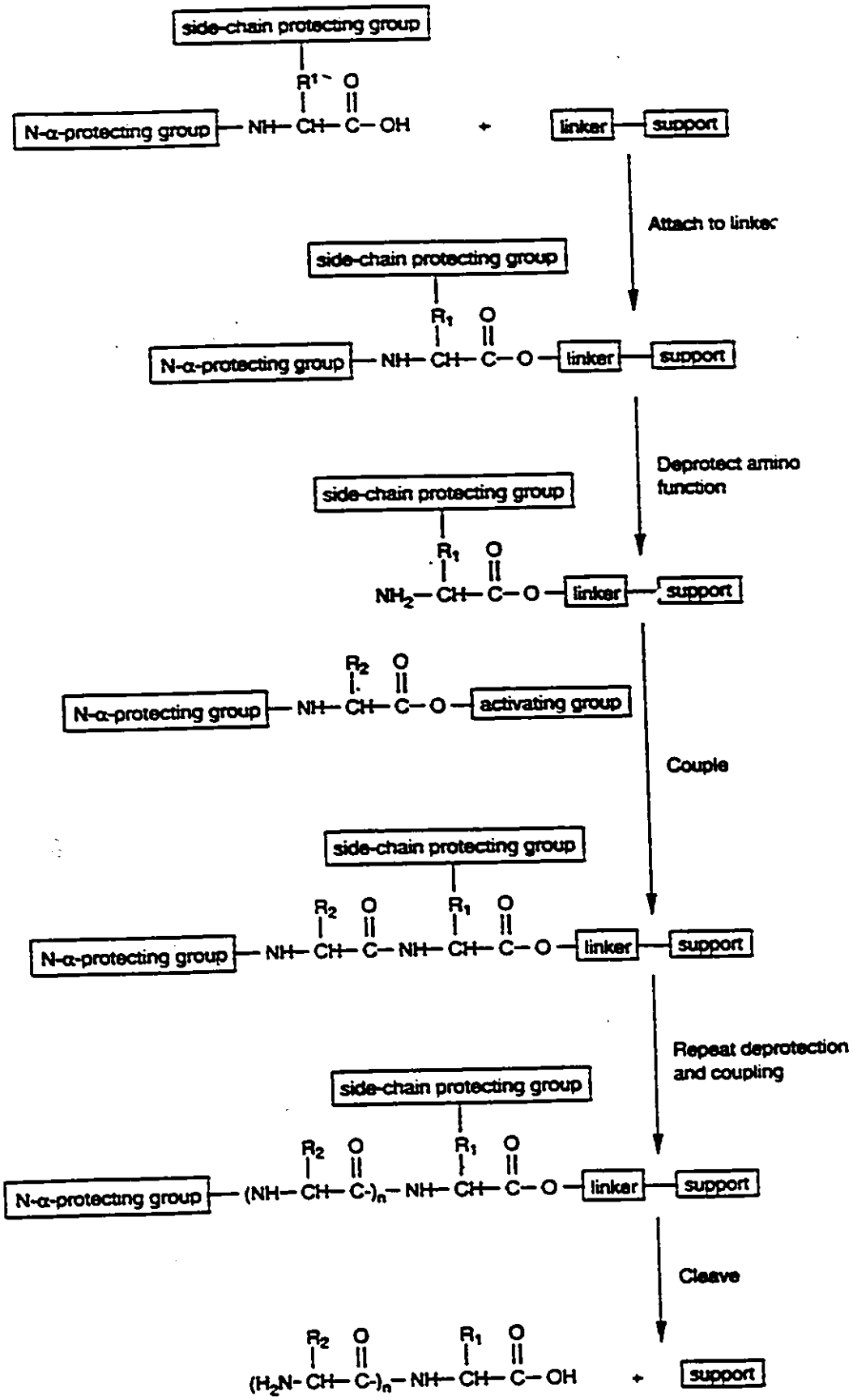


FIGURE 6.2 General Scheme of SPPS

6.3 The direct approach

6.3.1 Introduction

A peptide antigen and the lysine core are synthesized as a single unit on a resin support.

6.3.2 The polymeric support

The first requirement of solid phase peptide synthesis is a suitable solid support. The most commonly used support still remains a cross-linked polymeric resin, produced as a suspension copolymer of styrene-divinylbenzene (s-dvb) in the form of beads approximately 50 microns in diameter. This peptide resin because of its physical and chemical properties not only does not have detrimental effects on the synthesis but actually has beneficial effects in certain instances. One of the well recognized difficulties with the classical synthesis in homogeneous solution is insolubility of some intermediates.[80] The swollen peptide-resin exerts a strong solvating effect on the covalently attached peptide chains. The net result is that peptide-resin beads swell to an ever greater extent as the protected peptide chain is elongated. The highly solvated state of the peptide resin should be maintained throughout the synthetic cycle in order to maximize reaction yields. Maximal swelling also allows unhindered diffusion of molecules between the inside and the outside of the swollen beads and is essential for efficient washing of the peptide resin.[81]

6.3.3 Functionalization and anchoring

Originally the mode of attachment of the peptide to the resin was through formation of a benzyl ester bond between the carbonyl-terminal amino acid and the solid support. This bond is often derived from chloromethyl-resin and is not completely stable under the acidic conditions required for the removal of the protecting group. The resin is chloromethylated by a substitution of a chloromethyl group $-CH_2Cl$ for a benzene ring hydrogen using $ZnCl_2$ as catalyst and further modified by nitration. A more acid-stable anchoring bond is needed for the differential stability toward acid for the α-amino and α-carbonyl groups. Such a group was synthesized, known as the BOC-aminoacyl-OCH_2-pam-resin, it is 100-fold more stable than the conventional benzyl ester-resin linkage. In short, this greater acid-resistant pam-resin support reduces heterogeneity of peptide products, results in much higher yields, and is essential for the synthesis of complex and long peptides.[82-85]

The introduction of functional groups suitable for further elaboration is called functionalization or priming with an active group.

Many other supports have also been examined such as polymeric membranes, cellulose paper disks that can be packed into a column, polyethelene film and polyacrylamides. Only polyacrylamides have seen widespread use.[86]

The process begins with a resin support containing a simple amino acid such as glycine, which serves as an internal

standard for calculating the molar ratio of other amino acids. The carboxyl terminal residue of the polypeptide enters the reaction attached to the polymer by an ester bond. This process is called anchoring.

6.3.4 Repetitive deprotection/coupling cycle

The first level of the core matrix contains a diprotected lysine, usually BOC-lys (BOC) (t-butyloxycarbonyl). Since the protection scheme of the α- and ϵ-amino groups are similar, deblocking the BOC groups followed by coupling a new round of BOC-lys (BOC) will furnish the next level of the core matrix containing two lysines, one at the α-amino position and the other at the ϵ-amino position. This second level of branching will give a tetrameric map containing four amino groups. Similarly, the third-level branching will produce four lysines, eight amino groups, and an octameric map.

The incorporation of the peptide antigen could begin at either the second level of the tetrameric map or the third level of the octameric map. In this way, the peptide immunogen is amplified four or eight fold during the synthesis.[73]

6.3.5 Protection

In the assembly of peptide chains by chemical means it is necessary to protect reactive functionalities other than those involved in peptide bond formation.

Such protection must be reversible without damage to the assembled peptide chain. In SPPS at least two types of protection are necessary: temporary protection of the α-amino group, removable after formation of each peptide bond; and, side chain protection removable after assembly of the complete peptide chain. Current methods fall into two categories, depending on the nature of the group used to block reversibly the amino groups of the amino acids that are being added. The classical method uses the *tertiary*-butyloxycarbonyl [$(CH_3)_3C$-O-CO-, BOC] group which can be removed by acid. The recent method uses the *Fmoc* (fluorenylmethoxycarbonyl) group as the temporary protecting group and the permanent side chain is by the acid-labile *tertiary* butyl-derived protecting groups.[83]

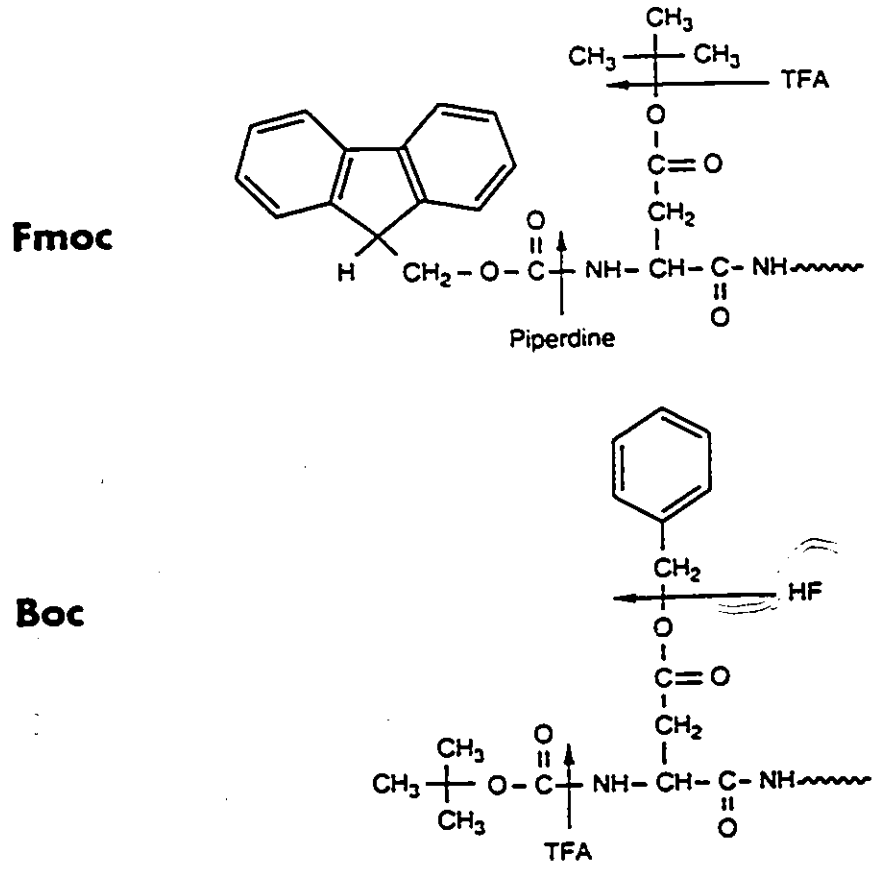


Figure 6.3 Protecting group strategies in SPPS

6.3.6 Coupling or activation

Peptide bond formation between a carbonyl and an amino group does not occur spontaneously under most conditions. One of these groups must be converted into a more reactive form. Inspite of many alternatives, essentially all peptide bonds are currently formed using the coupling agent dicyclohexylcarbodiimide, either "in situ" activation of the carbonyl component in the presence of the amino component, or through the prior formation of a symmetric anhydride or an active ester.[88]

6.3.7 Deprotection

The amino group must now be deprotected to allow for the formation of a peptide bond with the next amino acid. Incubation of the resin with a strong solution of HBr in anhydrous acetic acid removes the protecting group.

The desired peptide chain is synthesized by successive stepwise amino acid addition and deprotection reactions.

6.3.8 Cleavage

At the completion of the synthesis, the peptide immunogen is usually capped with an acetyl group. The necessity for acetylation depends on the origin of the peptide in the protein sequency. Capping is appropriate for peptide derived from the internal sequences since it would remove the charged amino group. The whole unit is then cleaved from the resin

support by a strong acid. The product may be purified by dialysis or the usual chromatographic techniques, such as gel permeation chromatography, and could be used directly for immunization.

6.4 The indirect approach

6.4.1 Introduction

This approach requires the conjugation of two purified components: a chloroacetylated oligomeric lysine core matrix and a synthetic peptide containing cysteine at either the carboxyl or amino terminus. The resulting MAP is structurally unambiguous and contains a quantifiable amount of peptide antigens. Furthermore, this method also provides a flexible strategy to link a peptide antigen to the core matrix at the desirable orientation to mimic the native molecule.[89]

6.4.2 The importance of orientation

The importance of the correct orientation of a peptide immunogen attached to a protein carrier has been examined when the same peptide is coupled through a Gly-Gly-Cys linker at either the N- or C-terminal end. From these results it was clear that orientation of the immunizing peptide has a profound effect on the magnitude and specificity of antibody responses when raising antipeptide antibodies.[90] In general, the orientation of the peptide immunogen should mimic the protein structure, which would also govern the orientation of the peptide antigen attached to the core matrix by the MAP

approach. For peptide immunogens that are derived from the amino terminal fragments or the internal positions of protein molecules, the direct approach of linking the carboxyl end of these fragments to the core matrix would provide the best results. In such cases, the flexible end of a peptide immunogen would be at the amino terminus. For peptide immunogens that are carboxyl fragments of protein molecules, the direct approach would give an incorrect orientation because the free and flexible carboxyl end is attached to the core matrix. The indirect approach of preparation would overcome this deficiency. By positioning a cysteine at the NH_2 terminus of a peptide, the peptide antigen can be attached to the chloroacetylated lysine core matrix at its NH_2 end to give the correct orientation. When the immunogenicity of a T cell stimulatory mycobacterial epitope (65-85) conjugated to poorly immunogenic sites from foot-and-mouth disease virus was studied, it was established that the 65-85 can provide T cell help for antibody response to adjacent peptide linked on the N-terminus, but not on the C-terminus.[91]

6.5 Limitations of synthetic peptide vaccines.[73]

1. The feasibility of large-scale production.
2. Selectivity, stability, and safety of an idealized synthetic vaccine.
3. Use of protein carrier is a major limitation. Large compared to peptide antigen.

4. The desirable antibodies may represent only a minor fraction of the total amount of antibodies produced.
5. Protein carrier: many irrelevant epitopes that cause carrier toxicity and epitopic suppression for human vaccines.
6. Chemical ambiguity of the antigen-protein carrier conjugate. No consistent batch-to-batch production of vaccines.
7. Low immunogenicity induced by the peptide-protein carrier conjugate, partly because the amount of desired antigens attached to the protein carrier is usually small.
8. Limited to the incorporation of one epitope to the protein carrier.

6.6 The MAP approach

6.6.1 Introduction

As the name implies, the MAP approach, using a small core matrix comprised of oligomeric lysine, is a unique presentation system of amplifying peptide antigens into multiple copies. As a result, this approach provides a very high density of peptide antigens at the surface and a nonprotein core matrix as a scaffolding that supports the peptide antigens. This approach replaces the conventional approach of using a protein carrier and thus eliminates many

of the disadvantages associated with its use.

The map approach: Replacing the protein carrier, with a small structural unit that can amplify peptide antigens without the disadvantages of a protein carrier.

This small core matrix must be:

1. Nonimmunogenic
2. Able to amplify the peptide antigens to a macromolecule
3. Flexible to incorporate multiple epitopes
4. Accessible to design and chemical synthesis

A core matrix consisting of branching trifunctional amino acids such as lysine satisfies these requirements. The core matrix, is comprised of oligomeric branching rather than polymeric lysine (Figure 6.4)

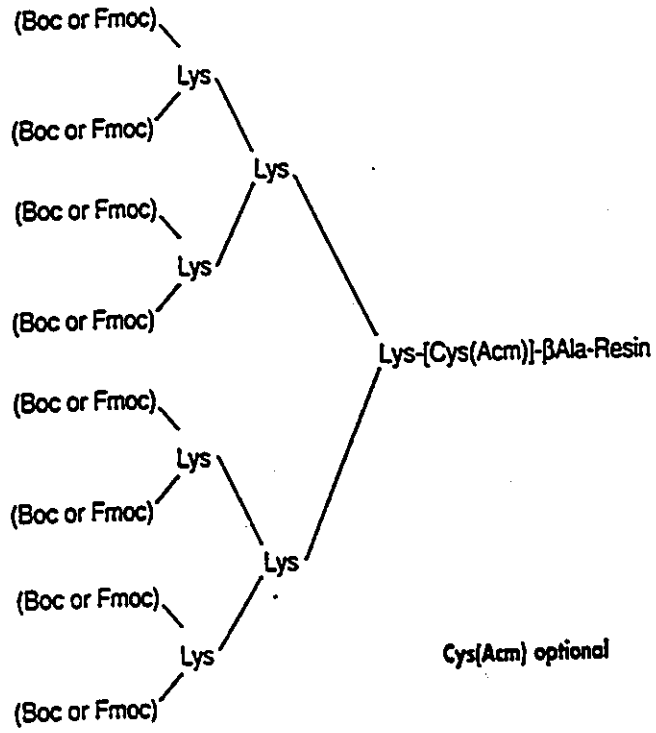


Figure 6.4 General structure of 8-branch MAP core

For example, a foot-and-mouth disease virus (FMDV) 20-residue attached to an octameric branching core matrix consisting of seven lysines will give a MAP molecule with a mol wt of about 23,000 Da. However the same immunogen when is conjugated to a protein carrier such as KLH (mol wt > 1,000,000) will give a low density of peptide antigens randomly distributed on the protein carrier surface. The FMDV MAP elicited protective levels of neutralizing antibody without the use of a carrier protein.[92]

6.6.2 Advantages of MAP

1. The map structure can be unequivocally represented by a chemical formula and can be verified with great precision by analytical methods such as amino acid analysis, sequencing, and mass spectrometry. This property is essential for quality control and consistent batch-to-batch production.
2. There is no need for conjugation to a protein a carrier that may cause such undesirable immunological reactions as epitopic suppression.
3. Finally, there is the advantage of flexibility that allows the design and engineering of multiple B- and T-cell epitopes.

This flexibility is possible due to the sophistication of peptide synthetic methodology to chemically distinguish

between the α and ε amino groups and its ability of selective attachment of different epitopes of the B- and T-cell origin. The inclusion of T-cell epitopes that could enhance the immunogenicity or elicit cell mediated immune responses is an important consideration in the design of vaccines.

In short, the MAP strategy provides many of the desirable features of the peptide based vaccine.

6.7 Results and Discussion

6.7.1 Introduction

T helper cell epitopes within tetanus toxin have been located by generating protein fragments by various methods, including enzymic or chemical fragmentation. A number of these peptides are universally immunogenic, since they are recognized by all primed donors irrespective of their MHC haplotypes.[89] The ability of these T cell epitopes to interact with many different HLA alleles means they may potentially be very useful "universal carrier molecules" in synthetic vaccines. A map of the major human T helper cell epitopes of tetanus toxin was constructed by Geysen and his group as depicted in Figure 6.5.[90]

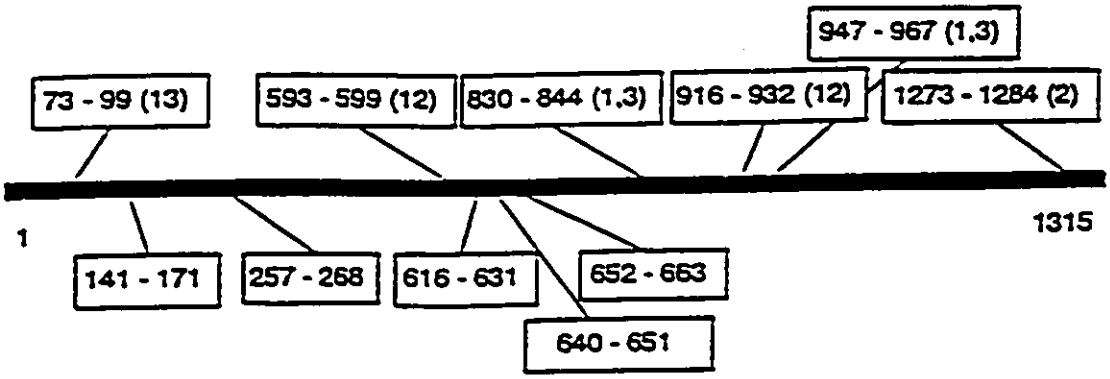


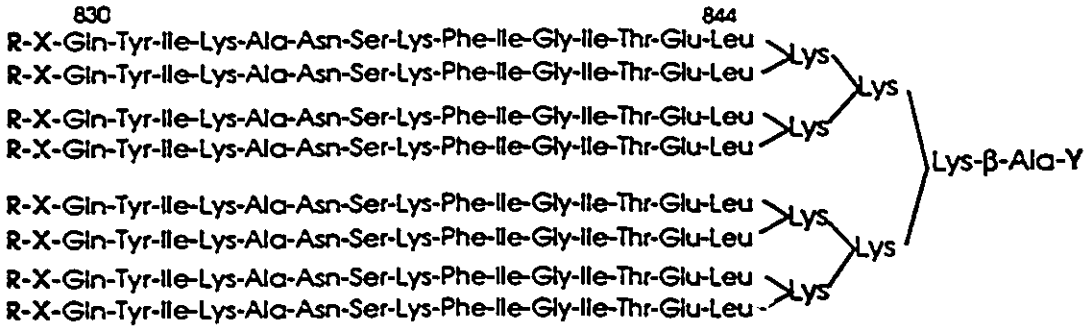
FIGURE 6.5 A map of the major human Th epitopes of tt[90]

One such promiscuous epitope is located on fragment 830-844. This epitope was used by Dr. Roy and his collaborators to construct a MAP.[91] Different sialic acid-containing antigens (B cell epitopes) were conjugated to the promiscuous T cell epitope. The immunogenicity of carbohydrates is very poor since they do not trigger a T-cell dependent response and hence memory B cells and antibody class switching. This was observed repeatedly when gangliosides were used as vaccines. Preliminary results with BALB/c mice indicated that the MAP induced IgG_{κ} and IgM antibodies.

The amino acid sequence of fragment 830-844 is:

830 844
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu

The antigenic reactivity of this MAP was tested with anti-toxin and anti-toxoid antibodies in ELISA assays.



R = Sialosides : Sialic Acid. Sialyl- α-(2.3)-Lactose. poly- α-(2.8)-Sialic Acid
X = -S-Cys-Gly- or - S-CH_2CO-Gly-Gly-
Y = -NH_2 or -OH

Figure 6.6 The structure of the MAP constructed by Dr Roy *et al*[91]

Another such promiscuous epitope is 947-967, which is found in fragment 947-980, obtained by the CNBr cleavage of tetanus toxin fragment C as described in Chapter 5.

947
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val
967
Ser Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile
980
Ile Ser Ser Met

6.7.2 ELISA assays and Discussion

The ELISA immunoreactivity of antisera derived from guinea pigs and mice immunized with tetanus toxin and toxoid was tested in microtitre wells coated with 1 μg/ml MAP. The MAP was donated Dr Roy but without the carbohydrate moiety attached. The immmunogenic fragment 830 to 844 of tetanus toxin must contain at least one linear sequence of amino acids capable of acting as a T-cell epitope. The results from these assays also show that although this sequence is important for immunogenicity, it is also important for antigenicity and must contain at least one B-cell epitope.

Guinea pig sera induced by immunization with both tetanus toxin and toxoid gave a shallow response to the MAP (Figures 6.9 and 6.10). This is indicative of large amounts of low affinity antibodies. On the other hand, antisera from mice immunized with tetanus toxin and toxoid produced steeper curves which is characteristic of small quantity high affinity antibodies.[96]

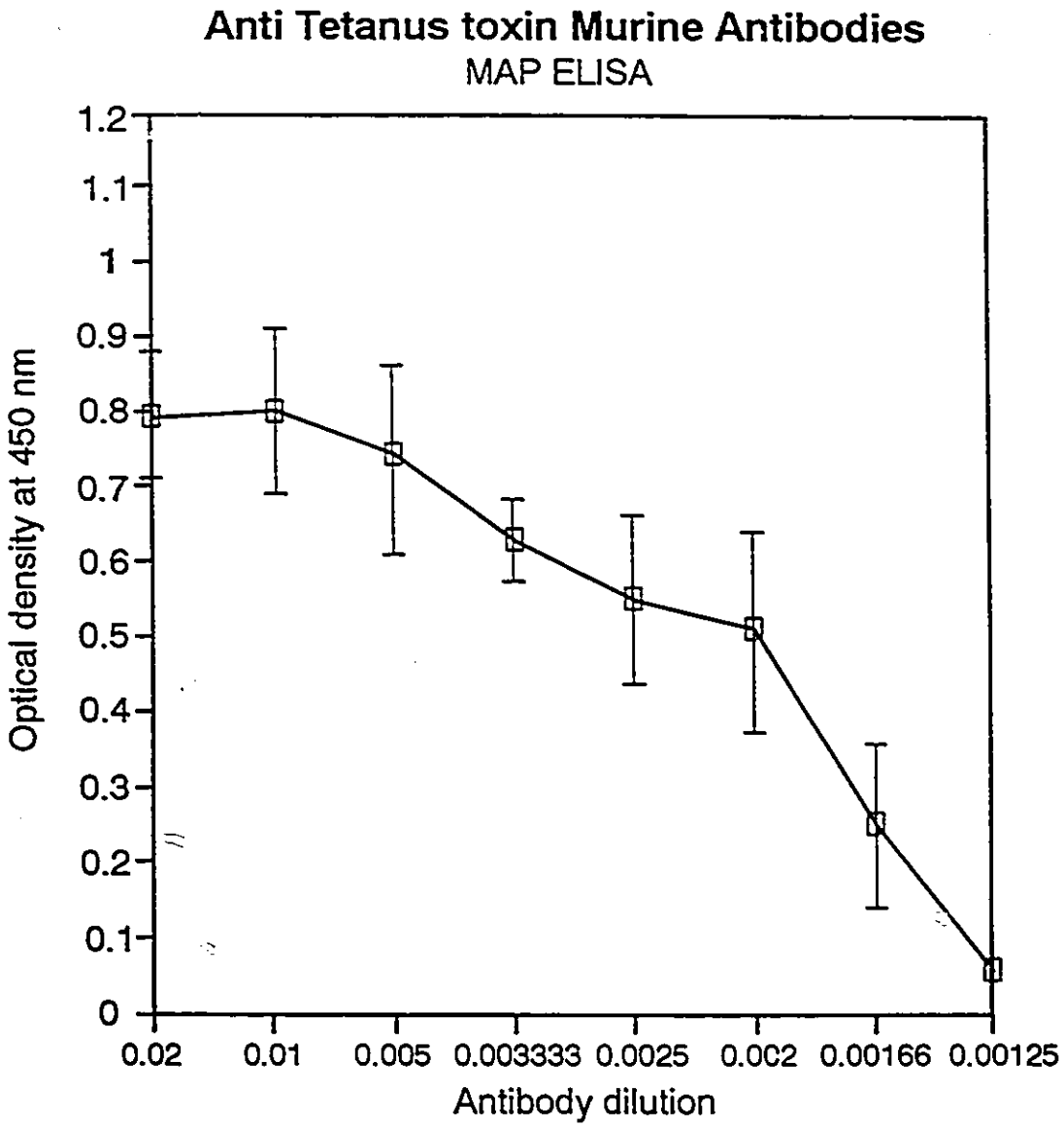


Figure 6.7 Binding of α-tetanus toxin Murine sera with MAP (1 µg/ml)

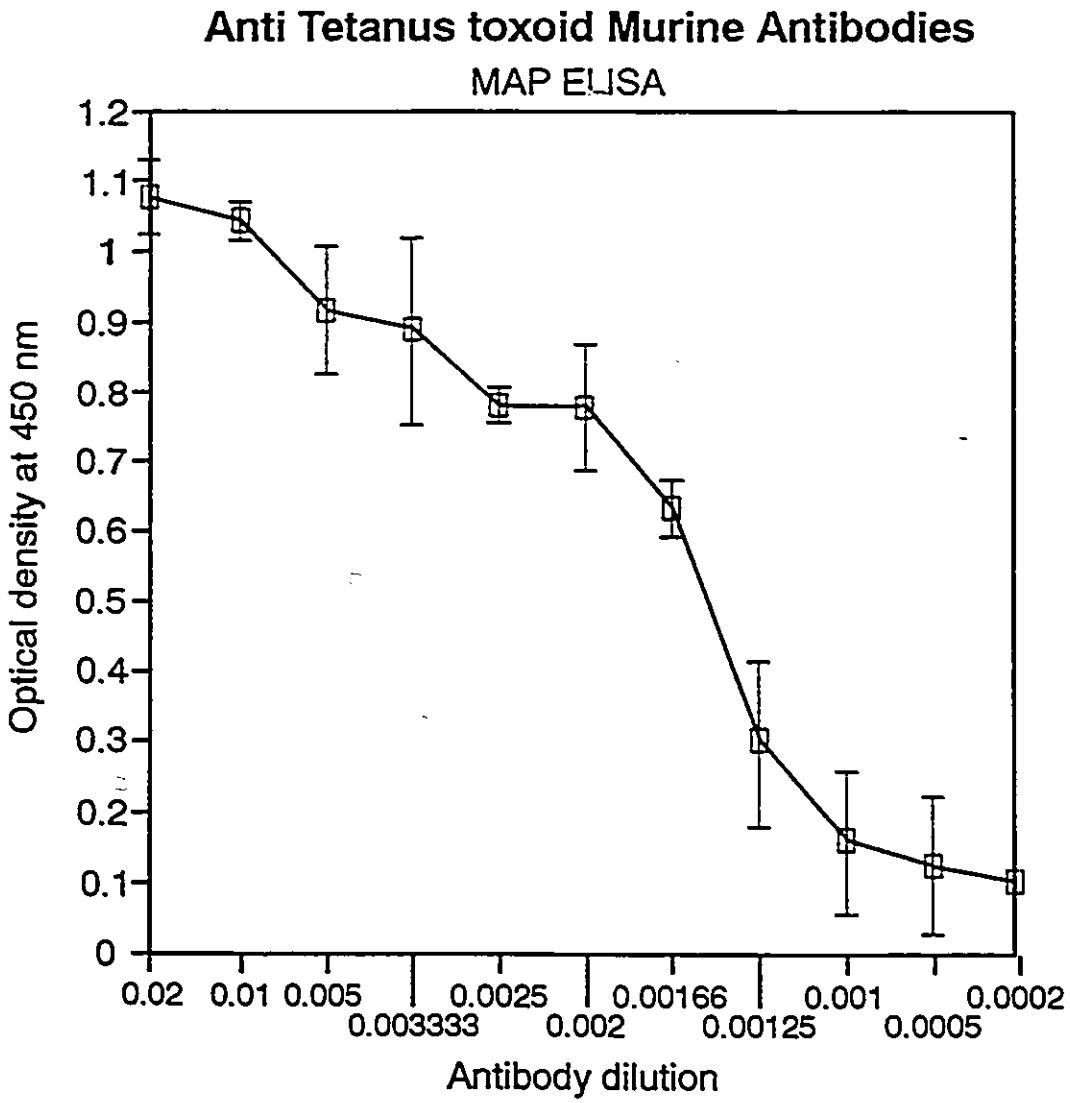


Figure 6.8 Binding of α-tetanus toxoid Murine sera with MAP (1 µg/ml)

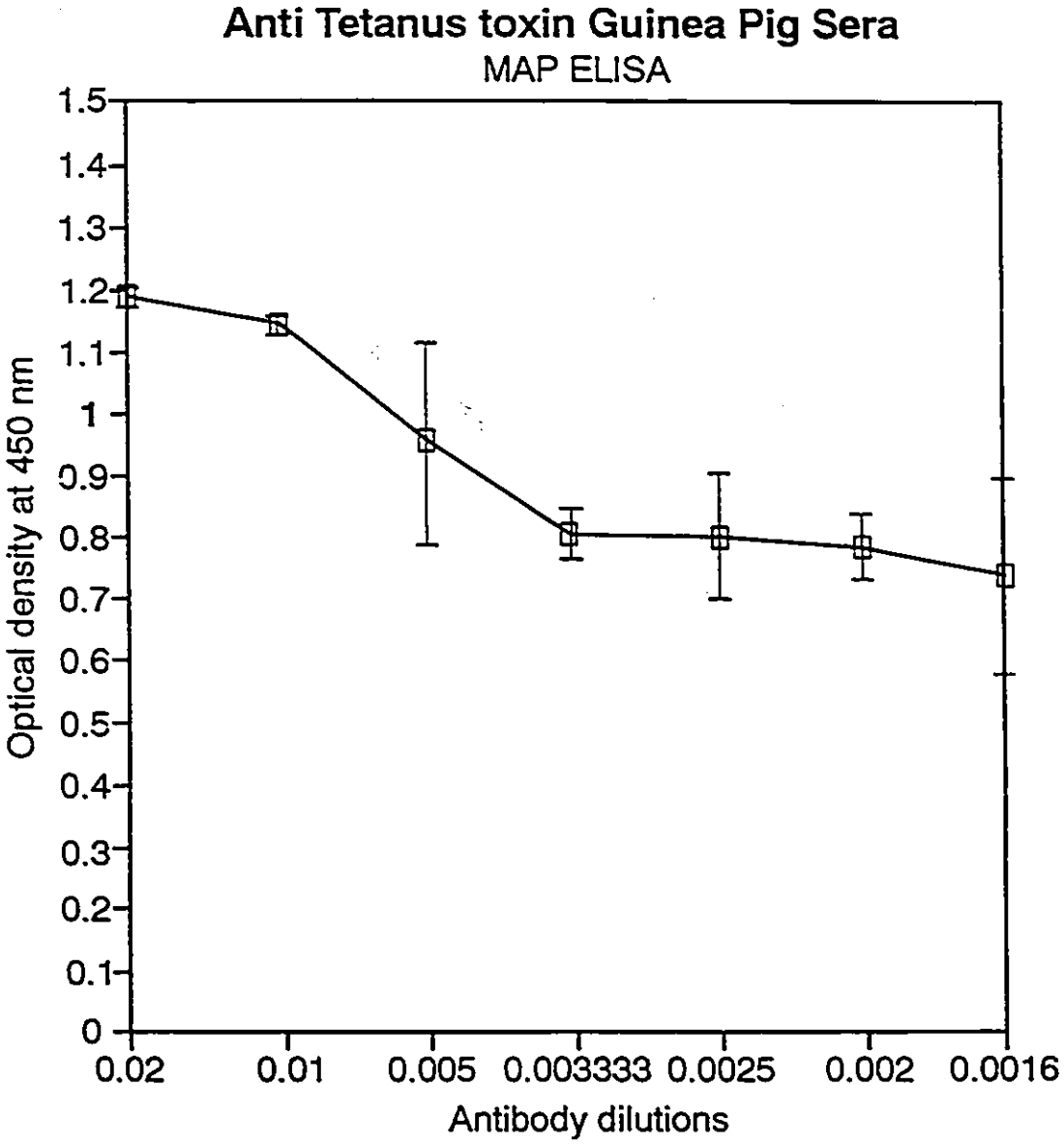


Figure 6.9 Binding of α-tetanus toxin Guinea pig sera with MAP (1 µg/ml)

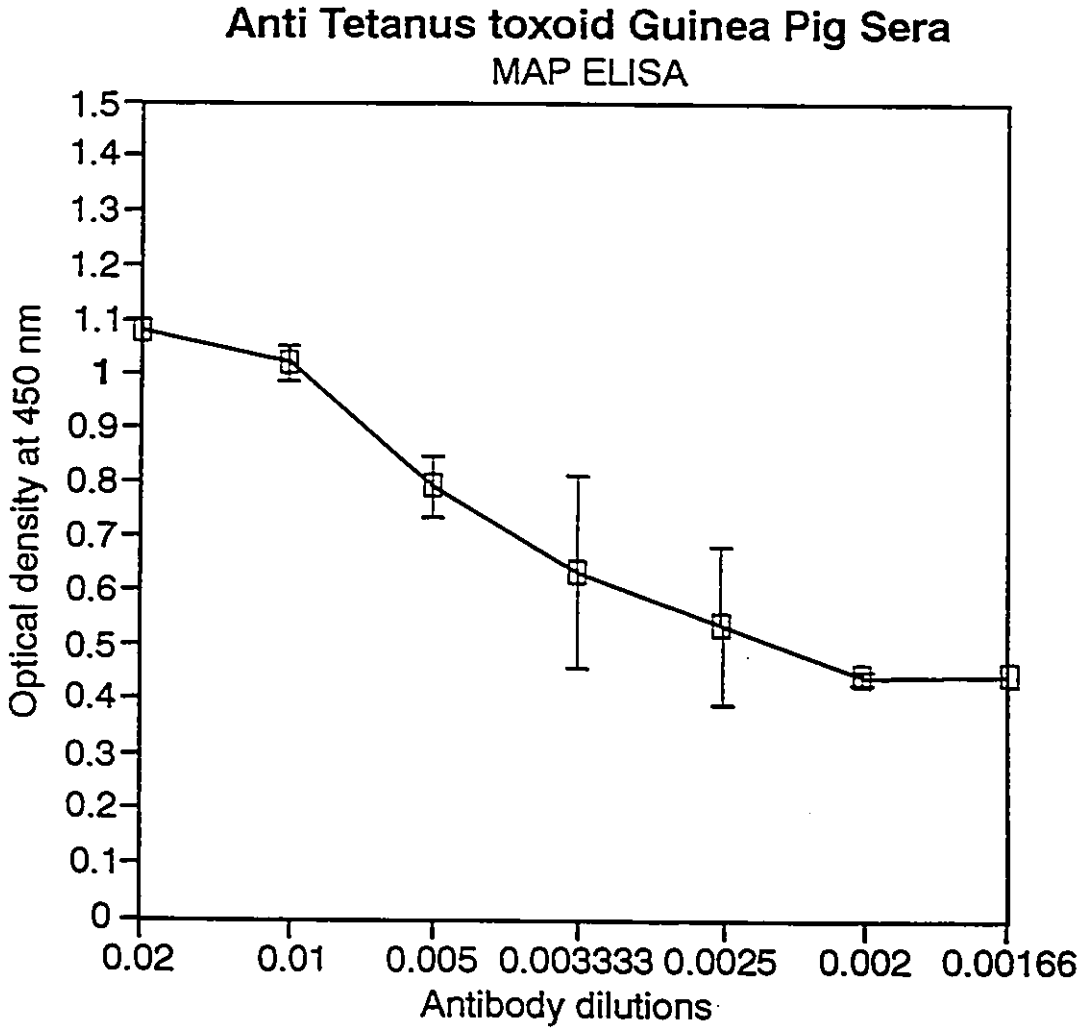


Figure 6.10 Binding of α-tetanus toxoid Guinea pig sera with MAP (1 µg/ml)

6.8 Summary The reactivity of the four different antisera, namely the murine anti-toxin, murine anti-toxoid, the guinea pig anti-toxin and guinea pig anti-toxoid was tested in ELISA assays with the MAP. The MAP used in these experiments was not sialylated. However this MAP was proven to be antigenic in both species. This indicates that the 830 to 844 sequence contains at least one B-cell epitope. The Guinea pig sera induced by immunization with both tetanus toxin and toxoid appeared to contain antibodies of low affinity towards the MAP. On the other hand, antisera from mice immunized with tetanus toxin and toxoid produced antibodies of high affinity towards the MAP.

6.9 Experimental Procedures

For details of the ELISA assays please see Chapter 4.

APPENDIX I

Nucleotide sequence of the tetanus toxin gene and the deduced amino acid sequence. The arrow ↓ between Ala(457) and Ser(458) indicates the start of the heavy chain. ▼ indicates the papain cleavage site.[40,41]

```
+1                                                                  30                                                                  60
ATG CCA ATA ACC ATA AAT AAT TTT AGA TAT AGT GAT CCT GTT AAT AAT GAT ACA ATT ATT
Met Pro Ile Thr Ile Asn Asn Phe Arg Tyr Ser Asp Pro Val Asn Asn Asp Thr Ile Ile

                                                                    90                                                                 120
ATG ATG GAG CCA CCA TAC TGT AAG GGT CTA GAT ATC TAT TAT AAG GCT TTC AAA ATA ACA
Met Met Glu Pro Pro Tyr Cys Lys Gly Leu Asp Ile Tyr Tyr Lys Ala Phe Lys Ile Thr

                                                                   150                                                                 180
GAT CGT ATT TGG ATA GTG CCG GAA AGG TAT GAA TTT GGG ACA AAA CCT GAA GAT TTT AAC
Asp Arg Ile Trp Ile Val Pro Glu Arg Tyr Glu Phe Gly Thr Lys Pro Glu Asp Phe Asn

                                                                   2I0                                                                 240
CCA CCA TCT TCA TTA ATA GAA GGT GCA TCT GAG TAT TAC GAT CCA AAT TAT TTA AGG ACT
Pro Pro Ser Ser Leu Ile Glu Gly Ala Ser Glu Tyr Tyr Asp Pro Asn Tyr Leu Arg Thr

                                                                   270                                                                 300
GAT TCT GAT AAA GAT AGA TTT TTA CAA ACC ATG GTA AAA CTG TTT AAC AGA ATT AAA AAC
Asp Ser Asp Lys Asp Arg Phe Leu Gln Thr Met Val Lys Leu Phe Asn Arg Ile Lys Asn

                                                                   330                                                                 360
ATT GTA GCA GGT GAA GCC TTA TTA GAT AAG ATA ATA AAT GCC ATA CCT TAC CTT GGA AAT
Asn Val Ala Gly Glu Ala Leu Leu Asp Lys Ile Ile Asn Ala Ile Pro Tyr Leu Gly Asn

                                                                   390                                                                 420
TCA TAT TCC TTA CTA GAC AAG TTT GAT ACA AAC TCT AAT TCA GTA TCT TTT AAT TTA TTA
Ser Tyr Ser Leu Leu Asp Lys Phe Asp Thr Asn Ser Asn Ser Val Ser Phe Asn Leu Leu

                                                                   450                                                                 480
GAA CAA GAC CCC AGT GGA GCA ACT ACA AAA TCA GCA ATG CTG ACA AAT TTA ATA ATA TTT
Glu Gln Asp Pro Ser Gly Ala Thr Thr Lys Ser Ala Met Leu Thr Asn Leu Ile Ile Phe

                                                                   510                                                                 540
GGA CCT GGG CCT GTT TTA AAT AAA AAT GAG GTT AGA GGT ATT GTA TTG AGG GTA GAT AAT
Gly Pro Gly Pro Val Leu Asn Lys Asn Glu Val Arg Gly Ile Val Leu Arg Val Asp Asn

                                                                   570                                                                 600
AAA AAT TAC TTC CCA TGT ABA GAT GGT TTT GGC TCA ATA ATG CAA ATG GCA TTT TGC CCA
Lys Asn Tyr Phe Pro Cys Arg Asp Gly Phe Gly Ser Ile Met Gln Met Ala Phe Cys Pro

                                                                   630                                                                 660
GAA TAT GTA CCT ACC TTT GAT AAT GTA ATA GAA AAT ATT ACG TCA CTC ACT ATT GGC AAA
Glu Tyr Val Pro Thr Phe Asp Asn Val Ile Glu Asn Ile Thr Ser Leu Thr Ile Gly Lys

                                                                   690                                                                 720
AGC AAA TAT TTT CAA GAT CCA GCA TTA CTA TTA ATG CAC GAA CTT ATA CAT GTA CTA CAT
Ser Lys Tyr Phe Gln Asp Pro Ala Leu Leu Leu Met His Glu Leu Ile His Val Leu His
```

```
                                        750                                 780
GGT TTA TAC GGA ATG CAG GTA TCA AGC CAT GAA ATT ATT CCA TCC AAA CAA GAA ATT TAT
Gly Leu Tyr Gly Met Gln Val Ser Ser His Glu Ile Ile Pro Ser Lys Gln Glu Ile Tyr

                                        810                                 840
ATG CAG CAT ACA TAT CCA ATA AGT GCT GAA GAA CTA TTC ACT TTT GGC GGA CAG GAT GCT
Met Gln His Thr Tyr Pro Ile Ser Ala Glu Glu Leu Phe Thr Phe Gly Gly Gln Asp Ala

                                        870                                 900
AAT CTT ATA AGT ATT GAT ATA AAA AAC GAT TTA TAT GAA AAA ACT TTA AAT GAT TAT AAA
Asn Leu Ile Ser Ile Asp Ile Lys Asn Asp Leu Tyr Glu Lys Thr Leu Asn Asp Tyr Lys

                                        930                                 960
GCT ATA GCT AAC AAA CTT AGT CAA GTC ACT AGC TGC AAT GAT CCC AAC ATT GAT ATT GAT
Ala Ile Ala Asn Lys Leu Ser Gln Val Thr Ser Cys Asn Asp Pro Asn Ile Asp Ile Asp

                                        990                                1020
AGC TAC AAA CAA ATA TAT CAA CAA AAA TAT CAA TTC GAT AAA GAT AGC AAT GGA CAA TAT
Ser Tyr Lys Gln Ile Tyr Gln Gln Lys Tyr Gln Phe Asp Lys Asp Ser Asn Gly Gln Tyr

                                       1050                                1080
ATT GTA AAT GAG GAT AAA TTT CAG ATA CTA TAT AAT AGC ATA ATG TAT GGT TTT ACA GAG
Ile Val Asn Glu Asp Lys Phe Gln Ile Leu Tyr Asn Ser Ile Met Tyr Gly Phe Thr Glu

                                       1110                                1140
ATT GAA TTG GGA AAA AAA TTT AAT ATA AAA ACT AGA CTT TCT TAT TTT AGT ATG AAT CAT
Ile Glu Leu Gly Lys Lys Phe Asn Ile Lys Thr Arg Leu Ser Tyr Phe Ser Met Asn His

                                       1170                                1200
GAC CCT GTA AAA ATT CCA AAT TTA TTA GAT GAT ACA ATT TAC AAT GAT ACA GAA GGA TTT
Asp Pro Val Lys Ile Pro Asn Leu Leu Asp Asp Thr Ile Tyr Asn Asp Thr Glu Gly Phe

                                       1230                                1260
AAT ATA GAA AGC AAA GAT CTG AAA TCT GAA TAT AAA GGA CAA AAT ATG AGG GTA AAT ACA
Asn Ile Glu Ser Lys Asp Leu Lys Ser Glu Tyr Lys Gly Gln Asn Met Arg Val Asn Thr

                                       1290                                1320
AAT GCT TTT AGA AAT GTT GAT GGA TCA GGC CTA GTT TCA AAA CTT ATT GGC TTA TGT AAA
Asn Ala Phe Arg Asn Val Asp Gly Ser Gly Leu Val Ser Lys Leu Ile Gly Leu Cys Lys

                                       1350                  HEAVY CHAIN   1380
AAA ATT ATA CCA CCA ACA AAT ATA AGA GAA AAT TTA TAT AAT AGA ACT GCA TCA TTA ACA
Lys Ile Ile Pro Pro Thr Asn Ile Arg Glu Asn Leu Tyr Asn Arg Thr Ala Ser Leu Thr
                                                              457 458

                                       1410                                1440
GAT TTA GGA GGA GAA TTA TGT ATA AAA ATT AAA AAT GAA GAT TTA ACT TTT ATA GCT GAA
Asp Leu Gly Gly Glu Leu Cys Ile Lys Ile Lys Asn Glu Asp Leu Thr Phe Ile Ala Glu

                                       1470                                1500
AAA AAT AGC TTT TCA GAA GAA CCA TTT CAA GAT GAA ATA GTT AGT TAT AAT ACA AAA AAT
Lys Asn Ser Phe Ser Glu Glu Pro Phe Gln Asp Glu Ile Val Ser Tyr Asn Thr Lys Asn

                                       1530                                1560
AAA CCA TTA AAT TTT AAT TAT TCG CTA GAT AAA ATT ATT GTA GAT TAT AAT CTA CAA AGT
Lys Pro Leu Asn Phe Asn Tyr Ser Leu Asp Lys Ile Ile Val Asp Tyr Asn Leu Gln Ser

                                       1590                                1620
AAA ATT ACA TTA CCT AAT GAT AGG ACA ACC CCA GTT ACA AAA GGA ATT CCA TAT GCT CCA
Lys Ile Thr Leu Pro Asn Asp Arg Thr Thr Pro Val Thr Lys Gly Ile Pro Tyr Ala Pro
```

1650 1680
GAA TAT AAA AGT AAT GCT GCA AGT ACA ATA GAA ATA CAT AAT ATT GAT GAC AAT ACA ATA
Glu Tyr Lys Ser Asn Ala Ala Ser Thr Ile Glu Ile His Asn Ile Asp Asp Asn Thr Ile

1710 I740
TAT CAA TAT TTG TAT GCT CAA AAA TCT CCT ACA ACT CTA CAA AGA ATA ACT ATG ACT AAT
Tyr Gln Tyr Leu Tyr Ala Gln Lys Ser Pro Thr Thr Leu Gln Arg Ile Thr Met Thr Asn

1770 1800
TCT GTT GAT GAC GCA TTA ATA AAT TCC ACC AAA ATA TAT TCA TAT TTT CCA TCT GTA ATC
Ser Val Asp Asp Ala Leu Ile Asn Ser Thr Lys Ile Tyr Ser Tyr Phe Pro Ser Val Ile

1830 1860
AGT AAA GTT AAC CAA GGT GCA CAA GGA ATT TTA TTC TTA CAG TGG GTG AGA GAT ATA ATT
Ser Lys Val Asn Gln Gly Ala Gln Gly Ile Leu Phe Leu Gln Trp Val Arg Asp Ile Ile

1890 1920
GAT GAT TTT ACC AAT GAA TCT TCA CAA AAA ACT ACT ATT GAT AAA ATT TCA GAT GTA TCC
Asp Asp Phe Thr Asn Glu Ser Ser Gln Lys Thr Thr Ile Asp Lys IIe Ser Asp Val Ser

1950 1980
ACT ATT GTT CCT TAT ATA GGA CCC GCA TTA AAC ATT GTA AAA CAA GGC TAT GAG GGA AAC
Thr Ile Val Pro Tyr Ile Gly Pro Ala Leu Asn Ile Val Lys Gln Gly Tyr Glu Gly Asn

2010 2040
TTT ATA GGC GCT TTA GAA ACT ACC GGA GTG GTT TTA TTA TTA GAA TAT ATT CCA GAA ATT
Phe Ile Gly Ala Leu Glu Thr Thr Gly Val Val Leu Leu Leu Glu Tyr Ile Pro Glu Ile

2070 2100
ACT TTA CCA GTA ATT GCA GCT TTA TCT ATA GCA GAA AGT AGC ACA CAA AAA GAA AAG ATA
Thr Leu Pro Val Ile Ala Ala Leu Ser Ile Ala Glu Ser Ser Thr Gln Lys Glu Lys Ile

2130 2160
ATA AAA ACA ATA GAT AAC TTT TTA GAA AAA AGA TAT GAA AAA TGG ATT GAA GTA TAT AAA
Ile Lys Thr Ile Asp Asn Phe Leu Glu Lys Arg Tyr Glu Lys Trp Ile Glu Val Tyr Lys

2190 2220
CTA GTA AAA GCA AAA TGG TTA GGC ACA GTT AAT ACG CAA TTC CAA AAA AGA AGT TAT CAA
Leu Val Lys Ala Lys Trp Leu Gly Thr Val Asn Thr Gln Phe Gln Lys Arg Ser Tyr Gln

2250 2280
ATG TAT AGA TCT TTA GAA TAT CAA GTA GAT GCA ATA AAA AAA ATA ATA GAC TAT GAA TAT
Met Tyr Arg Ser Leu Glu Tyr Gln Val Asp Ala Ile Lys Lys Ile Ile Asp Tyr Glu Tyr

2310 2340
AAA ATA TAT TCA GGA CCT GAT AAG GAA CAA ATT GCC GAC GAA ATT AAT AAT CTG AAA AAC
Lys Ile Tyr Ser Gly Pro Asp Lys Glu Gln Ile Ala Asp Glu Ile Asn Asn Leu Lys Asn

2370 2400
AAA CTT GAA GAA AAG GCT AAT AAA GCA ATG ATA AAC ATA AAT ATA TTT ATG AGG GAA AGT
Lys Leu Glu Glu Lys Ala Asn Lys Ala Met Ile Asn Ile Asn Ile Phe Met Arg Glu Ser

2430 2460
TCT AGA TCA TTT TTA GTT AAT CAA ATG ATT AAC GAA GCT AAA AAG CAG TTA TTA GAG TTT
Ser Arg Ser Phe Leu Val Asn Gln Met Ile Asn Glu Ala Lys Lys Gln Leu Leu Glu Phe

2490 2520
GAT ACT CAA AGC AAA AAT ATT TTA ATG CAG TAT ATA AAA GCA AAT TCT AAA TTT ATA GGT
Asp Thr Gln Ser Lys Asn Ile Leu Met Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly

2550 2580
ATA ACT GAA CTA AAA AAA TTA GAA TCA AAA ATA AAC AAA GTT TTT TCA ACA CCA ATT CCA
Ile Thr Glu Leu Lys Lys Leu Glu Ser Lys Ile Asn Lys Val Phe Ser Thr Pro Ile Pro

▼ PAPAIN

```
                                2610                                2640
TTT TCT TAT TCT AAA AAT CTG GAT TGT TGG GTT GAT AAT GAA GAA GAT ATA GAT GTT ATA
Phe Ser Tyr Ser Lys Asn Leu Asp Cys Trp Val Asp Asn Glu Glu Asp Ile Asp Val Ile
          864 865

                                2670                                2700
TTA AAA AAG AGT ACA ATT TTA AAT TTA GAT ATT AAT AAT GAT ATT ATA TCA GAT ATA TCT
Leu Lys Lys Ser Thr Ile Leu Asn Leu Asp Ile Asn Asn Asp Ile Ile Ser Asp Ile Ser

                                2730                                2760
GGG TTT AAT TCA TCT GTA ATA ACA TAT CCA GAT GCT CAA TTG GTG CCC GGA ATA AAT GGC
Gly Phe Asn Ser Ser Val Ile Thr Tyr Pro Asp Ala Gln Leu Val Pro Gly Ile Asn Gly

                                2790                                2820
AAA GCA ATA CAT TTA GTA AAC AAT GAA TCT TCT GAA GTT ATA GTG CAT AAA GCT ATG GAT
Lys Ala Ile His Leu Val Asn Asn Glu Ser Ser Glu Val Ile Val His Lys Ala Met Asp

                                2850                                2880
ATT GAA TAT AAT GAT ATG TTT AAT AAT TTT ACC GTT AGC TTT TGG TTG AGG GTT CCT AAA
Ile Glu Tyr Asn Asp Met Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys

                                2910                                2940
GTA TCT GCT AGT CAT TTA GAA CAA TAT GGC ACA AAT GAG TAT TCA ATA ATT AGC TCT ATG
Val Ser Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile Ile Ser Ser Met

                                2970                                3000
AAA AAA CAT AGT CTA TCA ATA GGA TCT GGT TGG AGT GTA TCA CTT AAA GGT AAT AAC TTA
Lys Lys His Ser Leu Ser Ile Gly Ser Gly Trp Ser Val Ser Leu Lys Gly Asn Asn Leu

                                3030                                3060
ATA TGG ACT TTA AAA GAT TCC GCG GGA GAA GTT AGA CAA ATA ACT TTT AGG GAT TTA CCT
Ile Trp Thr Leu Lys Asp Ser Ala Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro

                                3090                                3120
GAT AAA TTT AAT GCT TAT TTA GCA AAT AAA TGG GTT TTT ATA ACT ATT ACT AAT GAT AGA
Asp Lys Phe Asn Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr Ile Thr Asn Asp Arg

                                3150                                3180
TTA TCT TCT GCT AAT TTG TAT ATA AAT GGA GTA GTT ATG GGA AGT GCA GAA ATT ACT GGT
Leu Ser Ser Ala Asn Leu Tyr Ile Asn Gly Val Leu Met Gly Ser Ala Glu Ile Thr Gly

                                3210                                3240
TTA GGA GCT ATT AGA GAG GAT AAT AAT ATA ACA TTA AAA CTA GAT AGA TGT AAT AAT AAT
Leu Gly Ala Ile Arg Glu Asp Asn Asn Ile Thr Leu Lys Leu Asp Arg Cys Asn Asn Asn

                                3270                                3300
AAT CAA TAC GTA TCT ATT GAT AAA TTT AGG ATA TTT TGC AAA GCA TTA AAT CCA AAA GAG
Asn Gln Tyr Val Ser Ile Asp Lys Phe Arg Ile Phe Cys Lys Ala Leu Asn Pro Lys Glu

                                3330                                3360
ATT GAA AAA TTA TAC ACA AGT TAT TTA TCT ATA ACC TTT TTA AGA GAC TTC TGG GGA AAC
Ile Glu Lys Leu Tyr Thr Ser Tyr Leu Ser Ile Thr Phe Leu Arg Asp Phe Trp Gly Asn

                                3390                                3420
CCT TTA CGA TAT GAT ACA GAA TAT TAT TTA ATA CCA GTA GCT TCT AGT TCT AAA GAT GTT
Pro Leu Arg Tyr Asp Thr Glu Tyr Tyr Leu Ile Pro Val Ala Ser Ser Ser Lys Asp Val

                                3450                                3480
CAA TTG AAA AAT ATA ACA GAT TAT ATG TAT TTG ACA AAT GCG CCA TCG TAT ACT AAC GGA
Gln Leu Lys Asn Ile Thr Asp Tyr Met Tyr Leu Thr Asn Ala Pro Ser Tyr Thr Asn Gly

                                3510                                3540
AAA TTG AAT ATA TAT TAT AGA AGG TTA TAT AAT GGA CTA AAA TTT ATT ATA AAA AGA TAT
Lys Leu Asn Ile Tyr Tyr Arg Arg Leu Tyr Asn Gly Leu Lys Phe Ile Ile Lys Arg Tyr
```

```
                                    3570                                3600
ACA CCT AAT AAT GAA ATA GAT TCT TTT GTT AAA TCA GGT GAT TTT ATT AAA TTA TAT GTA
Thr Pro Asn Asn Glu Ile Asp Ser Phe Val Lys Ser Gly Asp Phe Ile Lys Leu Tyr Val

                                    3630                                3660
TCA TAT AAC AAT AAT GAG CAC ATT GTA GGT TAT CCG AAA GAT GGA AAT GCC TTT AAT AAT
Ser Tyr Asn Asn Asn Glu His Ile Val Gly Tyr Pro Lys Asp Gly Asn Ala Phe Asn Asn

                                    3690                                3720
CTT GAT AGA ATT CTA AGA GTA GGT TAT AAT GCC CCA GGT ATC CCT CTT TAT AAA AAA ATG
Leu Asp Arg Ile Leu Arg Val Gly Tyr Asn Ala Pro Gly Ile Pro Leu Tyr Lys Lys Met

                                    3750                                3780
GAA GCA GTA AAA TTG CGT GAT TTA AAA ACC TAT TCT GTA CAA CTT AAA TTA TAT GAT GAT
Glu Ala Val Lys Leu Arg Asp Leu Lys Thr Tyr Ser Val Gln Leu Lys Leu Tyr Asp Asp

                                    3810                                3840
AAA AAT GCA TCT TTA GGA CTA GTA GGT ACC CAT AAT GGT CAA ATA GGC AAC GAT CCA AAT
Lys Asn Ala Ser Leu Gly Leu Val Gly Thr His Asn Gly Gln Ile Gly Asn Asp Pro Asn

                                    3870                                3900
AGG GAT ATA TTA ATT GCA AGC AAC TGG TAC TTT AAT CAT TTA AAA GAT AAA ATT TTA GGA
Arg Asp Ile Leu Ile Ala Ser Asn Trp Tyr Phe Asn His Leu Lys Asp Lys Ile Leu Gly

                                    3930                    3948
TGT GAT TGG TAC TTT GTA CCT ACA GAT GAA GGA TGG ACA AAT GAT TAA
Cys Asp Trp Tyr Phe Val Pro Thr Asp Glu Gly Trp Thr Asn Asp End
```

APPENDIX II

Determination of protein concentration of tetanus toxin by the BIO-RAD PROTEIN ASSAY

1.0 Introduction

The Protein concentration of the sample of tetanus toxin used for immunization was determined using the BIO-RAD Protein Assay. The BIO-RAD Protein Assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The extinction coefficient of a dye albumin complex solution is constant over a 10-fold concentration range. Thus Beer's Law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

2.0 Sample

Tetanus Toxin Lot No TT-1, Connaught laboratories, kept in 1:10,000 Merthiolate, 382 µg/ml.

3.0 Reagents

Bio-Rad Dye Reagent Concentrate, Catalog 500-006

Albumin, Bovine (BSA), Sigma No. A-2153.

4.0 Method

4.1 A solution of 10 mg/100 ml H_2O of Bovine Serum Albumin was used to prepare a standard curve, of OD_{595} vs protein concentration. The concentration of the sample was extrapolated from the standard curve.

4.2 Standard Curve: Slope=0.013942, Intercept=0.067

Sample: 19.08 µg, Analysed 50µl therefore 0.382 mg/ml.

REFERENCES

1 Adorini L. *Short Analytical Review - Antigen Presentation and Self-Nonself Discrimination.* Clinical Immunology and Immunopathology. 1990;55:327-36.

2 Srivastava R., Ram B.P., Tyle P. *Molecular Mechanisms of Immune Regulation.* New York: VCH Publishers Inc. 1991. Cover.

3 Owen M.J., Lamb J.R. *Immune Recognition.* Washington, DC: IRL Press 1989:1-16.

4 Austyn J.M., Wood K.J. *Principles of Cellular and Molecular Immunology.* Oxford: Oxford University Press. 1993.

5 Lightstone E.B., Marvel J. *CD45RA is detected in all thymocyte subsets defined by CD4 and CD8 by using three-colour flow cytometry.* Immunology 1990;71:467-72.

6 Arnon R., Van Regenmortel M.H.V. *Structural basis of antigenic specificy and design of new vaccines.* FASEB J 1992;6:3265-74.

7 Mariuzza R.A., Phillips S.E.V., Poljak R.J. *The structural basis of antigen-antibody recognition.* Ann Rev Biophys Biophys Chem 1987;17:139-59.

8 Thorpe R. *Producing Antibodies.* In: Kerr M.A., Thorpe R. eds. *Immunochemistry LabFax.* Oxford: Academic Press. 1994:63-70.

9 Geysen H.M., Meloen R.H., Barteling S.J. *Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid.* Proc Natl Acad Sci USA 1984;81:3998-4002.

10 Van Regenmortel M.H.V. *Molecular dissection of protein antigens.* In: Van Regenmortel, M.H.V. ed. *Structure of Antigens.* Boca Raton, Florida: CRC Press. 1992;1:1-28.

11 Roitt I., Brostoff J., Male D. *Immunology.* London: Gower Medical Publishing. 1986.

12 Roitt I. *Essential Immunology.* London: Blackwell Scientific Publications. 1994.

13 Abbas A.K., Lichtman A.H., Pober J.S. *Cellular and Molecular Immunology*. Philadelphia: W.B. Saunders Company. 1994.

14 Sinigaglia F., Hammer J. *Rules for peptide binding to MHC class II molecules*. APMIS 1994;102:241-48.

15 Harding C., Roof R.W., Allen P.M., Unanue E.R. *Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules*. Proc Natl Acad Sci 1991;88:2740-44.

16 Wyss-Coray T., Brander C., Frutig K., Pichler W.J. *Discrimination of human CD4 T cell clones based on their reactivity with antigen-presenting T cells*. Eur J Immunol 1992;22:2295-2302.

17 Schwartz R.H. *T-lymphocyte recognition of antigen in association with gene products of the MHC-complex*. Annu Rev Immunol 1985;3:237-61.

18 Watson J., Gillis S., Marbrook J., Mochizuki D., Smith K.A. *Biochemical and biological characterisation of lymphocyte regulartory molecules. I. Purification of a class of murine lymphokines*. J Exp Med 1979;150:849-61.

19 Demotz S, Lanzavecchia L, Eisel U, Niemann H, Widmann C, Corradin G. *Delineation of several DR-restricted tetanus toxin T cell epitopes.* The Journal of Immunology 1989;142:394-402.

20 Suhrbier A, Rodda SJ, Ho PC, Csurhes P, Dunckley H, Saul A, Geysen HM, Rzepcsyk CM. *Role of single amino acids in the recognition of a T cell epitope.* The Journal of Immunology 1991;147:2507-13.

21 Grey HM, Sette A, Buus S. *Structural characteristics of peptides required for their interaction with IA^d.* In: Bona CA. *Molecular basis of the immune response.* New York: The New York Academy of Sciences 1988:72-79.

22 Germain R.N. *Antigenicity versus Immunogenicity: Signifigance for T-cell-dependent Vaccine Development.* In: Vaccines 92. Cold Spring Harbor Laboratory Press 1992:1-8.

23 Evavold BD, Allen PM. *Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand.* Science 1991;252:1308-10.

24 Maggi E, Mazzetti M, Ravina A, Annunziato F, De Carli M, Piccinni MP, Manetti R, Carbonari M, Pesce AM, Del Prete G, Romagnani S. *Ability of HIV to promote a T_H1 to T_H0 shift and to replicate preferentially in T_H2 and T_H0 cells.* Science 1994;265:244-48.

25 Stites DP, Terr AI, Parslow TG. *Basic and Clinical Immunology.* 8th edition. Connecticut: Appleton & Lange. 99-101.

26 LCDC. *Canadian Immunization Guide.* 4th Edition. 1993.

27 Bystryn J.-C. *Tumor vaccines.* Cancer and Metastasis Reviews. 1990;9:81-91.

28 Harding C.V., Kihlberg J., Elofsson M., Magnusson G., Unanue E.R. *Glycopeptides Bind MHC Molecules and Elicit Specific T Cell Responses.* The Journal of Immunology. 1993;151:2419-25.

29 Unanue E.R., Cerottini J.-C. *Antigen presentation.* The FASEB Journal. 1989;3:2496-2502.

30 Morton D.L., Ravindranath M.H., Irie R.F. *Tumor gangliosides as targets for active specific immunotherapy of melanoma in man.* In: Svennerholm L., Asbury A.K. Reisfeld R.A., Sandhoff K., Suzuki K., Tettamanti G., Toffano G. eds. *Biological Function of Gangliosides.* Progress in Brain Research. Volume 101. Amsterdam. 1994:251-75.

31 Ho P.C., Mutch D.A., Winkel K.D., Saul A.J., Jones G.L., Doran T.J., Rzepczyk C.M. *Identification of two promiscuous T cell epitopes from tetanus toxin.* Eur J Immunol 1990;20:477-83.

32 Habermann E., Dreyer F. *Clostridial Neurotoxins: Handling and Action at the Cellular and Molecular Level.* Current Topics in Microbiology and Immunology 1986;129:94-179.

33 Rossetto O., Schiavo G., Polverino de Laureto P., Fabbiani S., Montecucco C. *Surface topography of histidine residues of tetanus toxin probed by immobilized-metal-ion affinity chromatography.* Journal of Biochemistry 1992;285:9-12.

34 Niemann H. In: Alouf J.E., Freer J.H. eds. *Bacterial Protein Toxins; a Source Book*. London: Academic Press 1991;303-48.

35 Simpson L.L. ed. *Botulinum Neurotoxin and Tetanus Toxin*. London: Academic Press 1989.

36 Montecucco C. *How do tetanus and botulinum toxins bind to neuronal membranes?* TIBS 11 1986. Amsterdam: Elsevier Science Publishers B.V.

37 Andersen-Beckh B., Binz T., Kurazono H., Mayer T., Eisel U., Hiemann H. *Expression of Tetanus Toxin Subfragments in Vitro and Characterization of Epitopes*. Infection and Immunity. 1989;57:3498-505.

38 The Eighth International Conference on Tetanus, Leningrad, Union of Soviet Socialist Republics, 24-28 August 1987. Editorial. The Journal of Trauma 1987;29:268-71.

39 Matsuda M., Lei D.-L., Sugimoto N., Ozutsumi K., Okabe T. *Isolation, purification, and characterization of fragment B, the NH_2-terminal half of the heavy chain of tetanus toxin*. Infection and Immunity. 1989;57:3588-593.

40 Eisel U., Jarausch W., Goretzki K., Henschen A., Engels J., Weller U., Hudel M., Habermann E., Niemann H. *Tetanus toxin: primary structure, expression in E. coli, and homology with botulinum toxins*. The EMBO Journal 1986;5:2495-2502.

41 Fairweather N.F., Lyness V.A. *The complete nucleotide sequence of tetanus toxin*. Nucleic Acids Research 1986;14:7809-12.

42 Rappuoli R. *New and Improved Vaccines Against Diphtheria and Tetanus*. In: Woodrow G.C., Levine M.M., eds. *New Generation Vaccines*. New York: Marcel Dekker, Inc. 1990:251-68.

43 *Production of antibodies*. Current Protocols in Immunology 1992;Suppl 4:2.4.1-7.

44 Habermann E., Goretzki K. *Monoclonal antibodies against tetanus toxin and tetanus toxoid*. Monoclonal antibodies against bacteria 1985;I:191-205.

45 Geysen H.M. *Molecular technology: peptideepitope mapping and the pin technology*. Southeast Asian J Trop Med Public Health 1990;21(4):523-33.

46 Lloyd K.O. *Tumor antigens known to be immunogenic in man.* Annals of the New York Academic of Sciences 1993; 690:50-58.

47 Herlyn M., Koprowski H. *Melanoma antigens: immunological and biological characterization and clinical significance.* Ann Rev Immunol 1988;6:283-308.

48 Bystryn J.-C. *Tumor vaccines.* Cancer and Metastasis Reviews 1990;9:81-91.

49 Allison, Byars. *Adjuvants for new generation vaccines.* In: Woodrow G.C., Levine M.M. eds. *New generation vaccines.* New York: Marcel Dekker Inc. 1990;130-37.

50 Reeves W.G., Holborow E.J. *Immunology in Medicine.* London: Grune and Stratton. 1983;38.

51 Thurin J., Thurin M., Kimoto Y., Herlyn M., Lubeck M.D., Elder D.E., Smereczynska M., Karlsson K.A., Clark W.M. Jr., Steplewski Z., Koprowski H. *Monoclonal antibody-defined correlations in melanoma between levels of GD2 and GD3 antigens and antibody-mediated cytotoxicity.* Cancer Research 1987;47:1229-33.

52 Cheresh D.A., Honsik C.J., Staffileno L.K.,Jung G., Reisfeld R.A. *Disialoganglioside GD3 on human melanoma serves as a relevant target antigen for monoclonal antibody-mediated tumor cytolysis*. Proc Natl Acad Sci USA 1985;82:5155-59.

53 Houghton A.N., Mintzer D., Cordon-Cardo C., Welt S., Fliegel B., Vaghan S., Carswell E., Melamed M.R., Oettgen H.F., Old L.J. *Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma*. Proc Natl Acad Sci USA 1985; 82:1242-46.

54 Voller A., Bidwell D. *Enzyme-Linked Immunosorbent Assay*. In: Rose N.R., Friedman H., Fahey J.L. eds. *Manual of Clinical Laboratory Immunology*. 3rd edition. Washington, D.C.: American Society for Microbiology. 1986:99-109.

55 Marchalonis J.J., Warr G.W. *Antibody as a tool*. Chichester: John Wiley and Sons. 1982;30-42.

56 Yamamoto A., Tomoo K., Doi M., Ohishi H., Inoue M., Ishida T., Yamamoto D., Tsuboi S., Okamoto H., Okada Y. *Crystal Structure of Papain-Succinyl-Gln-Val-Val-Ala-Ala-p-Nitroanilide Complex at 1.7-Å Resolution: Noncovalent Binding Mode of a Common Sequence of Endogenous Thiol Protease Inhibitors*. Biochemistry 1992;31:11305-309.

57 Fersht A. *Enzyme Structure and Mechanism*. Second edition. New York: W.H. Freeman and Company 413-16.

58 Glazer A.N., Smith E.L. *Activation of Papain*. In: Boyer P.D. ed. *The Enzymes*. 3rd edition. Volume III. New York: Academic Press. 1971:511-25.

59 *Serine, zinc, thiol, and carboxyl proteases are the major families of proteolytic enzymes*. In: Stryer L. ed. *Biochemistry*. 3rd edition. New York: W.H. Freeman and Company. 227.

60 White A., Handler P., Smith E.L., Hill R.L., Lehman I.R. *Principles of biochemistry*. 6th edition. Chapter 9. New York: McGraw-Hill Book Company 1978;92-95.

61 Laemmli U.K. *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature 1970;227:680-85.

62 Gross E. *The Cyanogen Bromide Reaction.* Methods in enzymology 1967;11:238-55.

63 Gross E, Witkop B. *Selective cleavage of the methionyl peptide bonds in ribonuclease with cyanogen bromide.* J Am Chem Soc 1961;83:1510-11.

64 Corradin G., Harbury H.A. *Cleavage of cytochrome C with cyanogen bromide.* Biochimica et Biophysica Acta 1970;221;489-96.

65 Matsudaira P. *Limited N-terminal sequence analysis.* In: *Methods in enzymology.* 1990;182:602-13.

66 Morrison J.R., Fidge N.H., Grego B. *Studies on the formation, separation, and characterization of cyanogen bromide fragments of human al apolipoprotein.* Analytical Biochemistry 1990;186:145-52.

67 Frank G., Redweik U., Rumbeli M., Schoedon G., Blau N. *Cyanogen bromide cleavage of proteins on blots and subsequent separation of the fragments by polyacrylamide gel electrophoresis directly from those blots.* In: Imahori K., Sakiyama F. eds. *Methods in Protein Sequence Analysis.* New York: Plenum Press. 1993:79-85.

68 Aitken A., Geisow M.J., Findlay J.B.C., Holmes C., Yarwood A. *Peptide preparation and characterization*. In: Findlay J.B.C., Geisow M.J. eds. *Protein sequencing - a practical approach*. Oxford: IRL Press. 43-68.

69 Giulian G.G. *et al. Resolution of low molecular weight polypeptides in a non-urea sodium dodecyl sulfate slab polyacrylamide gel system*. Fed Proc 1985;44:686.

70 Srinivas G.R., Barrach H.J., Chichester C.O. *Quantitative immunoassays for type II collagen and its cyanogen bromide peptides*. Journal of Immunological Methods 1993;159:53-62.

71 Towbin H., Staehelin T., Gordon J. *Electrophoretic transfer of proteins from ployacrylamide gels to nitrocellulose sheets: procedures and some applications*. Proc Natl Acad Sci U.S.A. 1979;76:4350.

72 Schutze M-P., Leclerc C., Jolivet M., Audibert F., Chedid L. *Carrier-induced epitopic suppression, a major issue for future synthetic vaccines*. The Journal of Immunology 1985;135:2319-22.

73 Tam J.P. *Chemically Defined Synthetic Immunogens and Vaccines by the Multiple Antigen Peptide Approach*. In: W.C. Koff, editor. *Vaccine Research and Developments*. New York: Marcel Dekker Inc., 1992:51-87.

74 Tam J.P. *Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system*. Proc Natl Acad Sci USA 1988;85:5409-13.

75 Posnett D.N., McGrath H., Tam J.P. *A Novel Method for producing Anti-peptide Antibodies - Production of Site-specific Antibodies to the T cell Antigen Receptor ß-Chain*. Journal of Biological Chemistry 1988;263:1719-25.

76 Baleux F., Dubois P. *Novel version of Multiple Antigenic Peptide allowing incorporation on a cysteine functionalized lysine tree*. Int J Peptide Protein Res 1992;40:7-12.

77 Tam J.P., Lu Y.A. *Vaccine engineering: enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes*. Proc Natl Acad Sci USA 1989;86:9084-88.

78 Merrifield B. *Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide*. J Am Chem Soc 1963;85:2149-54.

79 Pessi A., Bianchi E., Bonelli F., Chiappinelli L. *Application of the Continuous-flow Polyamide Method to the Solid-phase Synthesis of a Multiple Antigen Peptide (MAP) based on the Sequence of a Malaria Epitope*. J. Chem. Soc., Chem. Commun., 1990;1:8-9.

80 Barany G., Merrifield B. *Solid Phase Peptide Synthesis*. In: Gross E., Meinhofer J. eds. *The Peptides*. New York: Academic Press. 1980:3-255.

81 Kent S.B.H. *Chemical synthesis of peptides and proteins*. Anu Rev Biochem 1988;57:957-89.

82 Merrifield B. *Solid Phase Synthesis*. Science 1986;232:341-47.

83 Tam J.P. *Solid Phase Synthesis of Type-α Transforming Growth Factor*. Methods in Enzymology 1987;146:127-143.

84 Mitchell A.R., Erickson B.W., Ryabtsev M.N., Hodges R.S., Merrifield B. *tert-Butoxycarbonylaminoacyl-4-(oxymethyl)-phenylacetamidomethyl-resin, a more acid-resistant support for solid phase peptide synthesis*. Journal of the American Chemical Society 1976;98(23):7357-62.

85 Mitchell A.R., Kent S.B.H., Engelhard M., Merrifield R.B. *A new synthetic route to tert-butyloxycarbonylaminoacyl-4-(oxymethyl)-phenylacetamidomethyl-resin, an improved support for solid-phase peptide synthesis*. J Org Chem 1978;43:2845-52.

86 Plaue S., Muller S., Briand J.P., Van Regenmortel M.H.V. *Recent advances in solid-phase peptide synthesis and preparation of antibodies to synthetic peptides*. Biologicals 1990;18:147-57.

87 *Peptide synthesis*. In: Creighton T.E. ed. *Proteins - Structures and Molecular Properties*. 2nd ed. New York: W.H. Freeman and Company 1993.

88 *Solid phase peptide synthesis*. In: Bodanszky M. ed. *Peptide chemistry - A practical textbook*. New York: Springer-Verlag. 1988:147-68.

89 Lu Y.A., Clavijo P., Galantino M., Shen Z.-Y., Liu W., Tam J.P. *Chemically unambiguous peptide immunogen: preparation, orientation and antigenicity of purified peptide conjugated to the multiple antigen peptide system.* Molecular Immunology 1991;28:623-30.

90 Dyrberg T., Oldstone M.B.A. *Peptides as antigens - Importance of Orientation.* J Exp Med 1986;164:1344-49.

91 Cox J.H., Ivanyl J., Young D.B., Lamb J.R., Syred A.D., Francis M.J. *Orientation of epitopes influences the immunogenicity of synthetic peptide dimers.* Eur J Immunol 1988;18:2015-19.

92 Francis M.J., Hastings G.Z., Brown F., McDermed J., Lu Y.-A., Tam J.P. *Immunological evaluation of the multiple antigen peptide (MAP) system using the major immunogenic site of foot-and-mouth disease virus.* Immunology 1991;73:249-54.

93 Reece J.C., McGregor D.L., Geysen H.M., Rodda S.J. *Scanning for T helper epitopes with human PBMC using pools of short synthetic peptides.* J Immunol Methods 1994;172:251-54.

94 Reece J.C., Geysen H.M., Rodda S.J. *Mapping the major human T helper epitopes of tetanus toxin. The emerging picture.* J Immunol 1993;151:6175-84.

95 Roy R., Zanini D., Romanowska A., Meunier S.J., Park W.K.C., Gidney M.A., Harrisson B., Bundle D.R., Williams R.W. *Synthesis of sialylated multiple antigen peptides having tetanus toxin T-cell epitopes.* XVIIth International Carbohydrate Symposium, Ottawa, Canada July 17-22, 1994; page 305, Abstract B2-31.

96 Kemeny D.M. ed. *A practical guide to ELISA.* Oxford: Permagon Press. 1991;61.