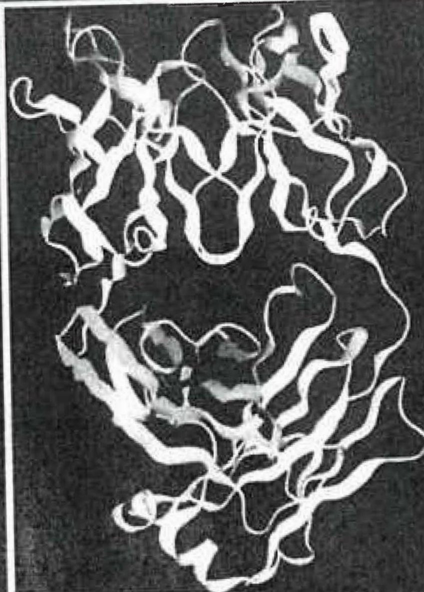
Applications and Engineering of Monoclonal Antibodies



David J King

EXHIBIT 1195 Ian A. Wilson, D.Phil.
4/21/18 Planet Depos - Tricia Rosate, RDR, CRR, CSR No. 10891

Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Applications and Engineering of
Monoclonal Antibodies

HOSPIRA EX. 1029
Page 2
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Applications and Engineering of Monoclonal Antibodies

DAVID J. KING

Celltech Therapeutics
Slough, UK

HOSPIRA EX. 1029
Page 3

Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

UK Taylor & Francis Ltd, 1 Gunpowder Square, London EC4A 3DE
USA Taylor & Francis Inc., 325 Chestnut Street, 8th Floor, Philadelphia, PA 19106

Copyright © D.J. King 1998

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, electrostatic, magnetic tape, mechanical, photocopying, recording or otherwise, without the prior permission of the copyright owner.

Every effort has been made to ensure that the advice and information in this book is true and accurate at the time of going to press. However, neither the publisher nor the author can accept any legal responsibility or liability for any errors or omissions that may be made. In the case of drug administration, any medical procedure or the use of technical equipment mentioned within this book, you are strongly advised to consult the manufacturer's guidelines.

British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library

ISBN 0–7484–0422–8 (hb)
0–7484–0423–6 (pb)

Library of Congress Cataloging Publication Data are available

Cover design by Youngs Design in Production

Typeset in Times 10/12pt by Graphicraft Limited, Hong Kong

Printed by T.J. International Ltd, Padstow, UK

HOSPIRA EX. 1029
Page 4
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Acknowledgements

I would like to thank my many colleagues at Celltech for their helpful suggestions and advice, and particularly Alastair Lawson and Martyn Robinson for their comments on the manuscript. Thanks also to Tina Jones for assistance with the figures.

And lastly, sincere thanks to my wife, Jane, for her support and patience through the long hours and weekends spent writing this book.

HOSPIRA EX. 1029
Page 5
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Contents

1 Preparation, structure and function of monoclonal antibodies *page* 1
1.1 Introduction 1
1.2 The role of antibodies in the immune response 2
1.3 Structure and function of antibodies 2
1.4 The organisation of antibody genes 11
1.5 Antigen-binding affinity and avidity 13
1.5.1 Affinity 13
1.5.2 Avidity 14
1.6 Generation of monoclonal antibodies 14
1.6.1 Hybridoma technology 14
1.6.2 Human monoclonal antibodies 18
1.6.3 Human MAbs from transgenic mice 20
1.6.4 Isolation of antibodies by phage display 21

2 Antibody engineering: design for specific applications 27
2.1 Introduction 27
2.2 Isolation of variable region genes 28
2.3 Overcoming immunogenicity 29
2.3.1 Chimeric and humanised antibodies 29
2.3.2 Antibody fragments to reduce immunogenicity 37
2.3.3 Chemical modification to reduce immunogenicity 38
2.3.4 Immunosuppressive therapy 40
2.4 Antibody fragments 40
2.4.1 Antibody fragments from proteolysis of IgG 40
2.4.2 Recombinant antibody fragments 42
Fab-based fragments 43
Fv-based fragments 45
Multivalent antibody fragments 48
2.5 Antibodies with multiple specificities 50
2.6 Engineering effector functions 54

HOSPIRA EX. 1029
Page 6
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

2.6.1 Engineering natural effector functions 54
2.6.2 Attachment of diagnostic or therapeutic agents 58
Chemical conjugates 58
Site-specific attachment 63
Fusion proteins 65
2.7 Engineering pharmacokinetics and biodistribution 67
2.7.1 Pharmacokinetics of IgG 68
2.7.2 Pharmacokinetics of antibody fragments 71
2.7.3 Clearance 74
2.7.4 Chemical modification 75
2.7.5 Fc region to extend half-life 75

3 Monoclonal antibodies in research and diagnostic applications 77
3.1 Introduction 77
3.2 Immunoassays in diagnostics and research 77
3.2.1 Radioimmunoassay 78
3.2.2 Immunoradiometric assay 79
3.2.3 Non-isotopic immunoassays 83
3.2.4 Improving sensitivity 85
3.2.5 Assay formats 89
3.2.6 Advantages of monoclonal antibodies in immunoassay 91
3.3 Immunosensors 92
3.3.1 Mass-detecting immunosensors 93
3.3.2 Electrochemical immunosensors 94
3.3.3 Optical immunosensors 95
3.4 Immunocytochemistry 97
3.5 Flow cytometry and cell sorting (FACS) 99
3.6 Western blotting (immunoblotting) 100
3.7 Immunopurification 102
3.8 Antibodies in structural biology 106
3.9 *In vivo* diagnostics 106
3.9.1 Radioimmunodetection of human tumours 107
Tumour-associated antigens 107
Form of antibody 109
Radioisotopes 110
Two- and three-step targeting approaches 114
3.9.2 Radioimmunoguided surgery 116
3.9.3 Non-tumour radioimmunodetection 116

4 Monoclonal antibodies in therapeutic applications 119
4.1 Introduction 119
4.2 Cancer 120
4.2.1 Cancer therapy with unmodified (naked) antibodies 121
4.2.2 Anti-idiotype antibodies 123
4.2.3 Bispecific antibody-mediated effector cell targeting 125
4.2.4 Other approaches to recruit the immune system using MAbs 127
4.2.5 Radioimmunotherapy 128
Form of antibody 129
Radioisotopes 132

HOSPIRA EX. 1029
Page 7
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Clearance mechanisms and two-step targeting 136
Clinical results with RIT 137
4.2.6 Immunotoxins 139
4.2.7 Drug conjugates 142
4.2.8 Antibody-directed enzyme prodrug therapy (ADEPT) 144
4.2.9 Vascular targeting 146
4.3 Infectious disease 147
4.3.1 Antiviral antibodies 147
4.3.2 Bacterial sepsis 148
4.4 Cardiovascular disease 149
4.4.1 Inhibition of platelet aggregation 149
4.4.2 Thrombolysis 150
4.5 Disorders of the immune system/inflammatory diseases 151
4.5.1 The inflammatory response 151
4.5.2 Blocking inflammatory mediators 153
Anti-TNF antibodies in rheumatoid arthritis and inflammatory bowel disease 153
Anti-C5 154
4.5.3 Blocking adhesive interactions 154
4.5.4 Antibodies which directly inhibit T cell activation and proliferation 155
4.5.5 Antibody treatment of allergy 158

5 Production of monoclonal antibodies 161
5.1 Introduction 161
5.2 Expression of antibodies in mammalian cells 161
5.2.1 Transient expression systems 162
5.2.2 Stable expression systems 163
5.2.3 Expression of antibody fragments in mammalian cells 167
5.3 Expression in *Escherichia coli* 167
5.3.1 Intracellular expression of antibody fragments in *E. coli* 168
5.3.2 Secretion of antibody fragments from *E. coli* 170
5.4 Expression in other microbial systems 172
5.5 Expression in plants 173
5.6 Production in transgenic animals 174
5.7 Expression in insect cells 175
5.8 Production of monoclonal antibodies – cell culture 175
5.9 Purification of monoclonal antibodies 176
5.9.1 Purification of IgG 176
5.9.2 Purification of IgM 181
5.9.3 Purification of monoclonal antibody fragments 181
5.9.4 Purification for therapeutic use 185

6 Prospects for engineered antibodies in biotechnology 187
6.1 Gene therapy 187
6.1.1 Intracellular antibodies 187
6.1.2 Other applications of MAbs in gene therapy 188
6.2 Applications of antibodies in plants 189
6.3 Catalytic antibodies 189

HOSPIRA EX. 1029
Page 8
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

6.4 Towards drug design 190
6.5 Improving affinity 191
6.6 Summary and prospects 192

7 **References** 195

Index 241

HOSPIRA EX. 1029
Page 9
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

1

Preparation, Structure and Function of Monoclonal Antibodies

1.1 Introduction

Antibodics are proteins produced by an individual in response to the presence of a foreign molecule in thc body. These foreign molccules are known as antigens, and they usually result from invading organisms such as bacteria, fungi or viruses. Antibodies bind to antigens and elicit a range of effector mechanisms to dcstroy the invading organism. Therefore, the generation of an antibody response is a kcy step in the immune system which has evolved to protect individuals from invading pathogenic organisms. However, antibodies are not restricted in specificity to pathogens, but can be formed to a huge variety of antigens including proteins, carbohydrates and organic compounds, including totally novel structures.

In 1975 Kohler and Milstein described a method for the 'production of antibodies of predefined specificity'. This technical breakthrough allowed, for the first time, the production of antibody molecules of a single specificity which could be characterised and defined. Such monoclonal antibodies immediately became valuable research tools, and applications in the diagnosis and therapy of human disease began to be widely investigated. Monoclonal antibodies (MAbs) have become increasingly important as diagnostic agents allowing precise molecular structures to be mapped and analysed. However, initial enthusiasm for their development as therapeutic agents was premature and many problems limited their use in humans (see Chapter 4). A second technical revolution has now arrived in the ability to manipulate antibody genes and to design and produce antibody molecules tailor-made for their application. Such redesigned antibody molecules are now rapidly becoming valuable reagents for therapy of human diseases as well as improved diagnostics and research reagents. The scope of these applications, and how antibody molecules are redesigned, or engineered, in the most suitable form for a particular application is the subject of this book. To understand the nature of this process requires an understanding of antibody structure and function and of how antibody specificities are generated.

HOSPIRA EX. 1029
Page 10
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

1.2 The role of antibodies in the immune response

The immune system can be considered in two parts. Innate immunity is mediated by a variety of physical and biochemical barriers and by cells responding non-specifically to the foreign organism or molecule. Innate immunity is characterised by a similar reponse on re-exposure to the same foreign agent. Adaptive immunity, the second part of the immune system, is mediated by cells termed lymphocytes and is characterised by improved efficiency on re-exposure to the same foreign agent. It is in this system that antibodies play a major role.

Two major types of lymphocytes are involved in the adaptive immune response, T lymphocytes and B lymphocytes. T lymphocytes can be subdivided into cytotoxic T lymphocytes and 'helper' T lymphocytes. Cytotoxic T lymphocytes bind to foreign or infected cells through a surface antigen receptor, the T cell receptor, and lyse them. Helper T lymphocytes play a regulatory role in controlling the response of both T and B lymphocytes. B lymphocytes exert their effect through producing antibody molecules which bind to the foreign agent and invoke specific mechanisms for its elimination.

Antigen is recognised by B lymphocytes through the use of antibody molecules on the surface of the cell. Each B lymphocyte carries antibody molecules on its surface with a single specificity as a consequence of the rearrangement of immunoglobulin genes by the individual cell during its development in a random, antigen-independent process. Each individual will have many millions of B cells at one time thus comprising a 'polyclonal' population. When an antigen enters the body it will be recognised by any B cell which has antibody molecules able to bind to antigenic determinants (epitopes) present on the antigen. This recognition leads to activation of the cell leading to proliferation and differentiation (Figure 1.1).

In the case of protein antigens, bound antigen molecules are internalised into the B cell and degraded into peptides. Some of these antigen peptides are then bound to major histocompatibility complex (MHC) class II molecules to form a complex which is transported to the cell surface and 'presented' by the B cell. Although B cells are relatively weak at antigen presentation compared to other cell types such as dendritic cells, these complexes can be recognised by receptors present on T lymphocytes, the T cell receptors. If appropriate recognition takes place then the T cell may deliver 'help' in the form of signals back to the B cell to stimulate proliferation and differentiation. This process therefore gives rise to a 'T-cell-dependent B-cell response'.

Proliferation results in a clone of identical cells which can differentiate to form either plasma cells capable of secreting large amounts of soluble antibody of the same specificity as the original activated B cell, or memory cells which mount an accelerated immune response on re-exposure to the original antigen. The memory cell-mediated 'secondary' response results in the production of higher affinity antibodies due to hypermutation in the immunoglobulin gene loci followed by antigen driven selection, a process known as affinity maturation. This process of B cell clonal selection and the generation of antibody responses is covered in more detail in an excellent review (Rajewsky, 1996).

1.3 Structure and function of antibodies

Antibody molecules have two principal functions, firstly to bind to antigen and secondly to trigger its elimination from the body. Antibodies are therefore 'adaptor' molecules which have both the ability to bind to the antigen molecule and the ability to bind and

HOSPIRA EX. 1029
Page 11
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

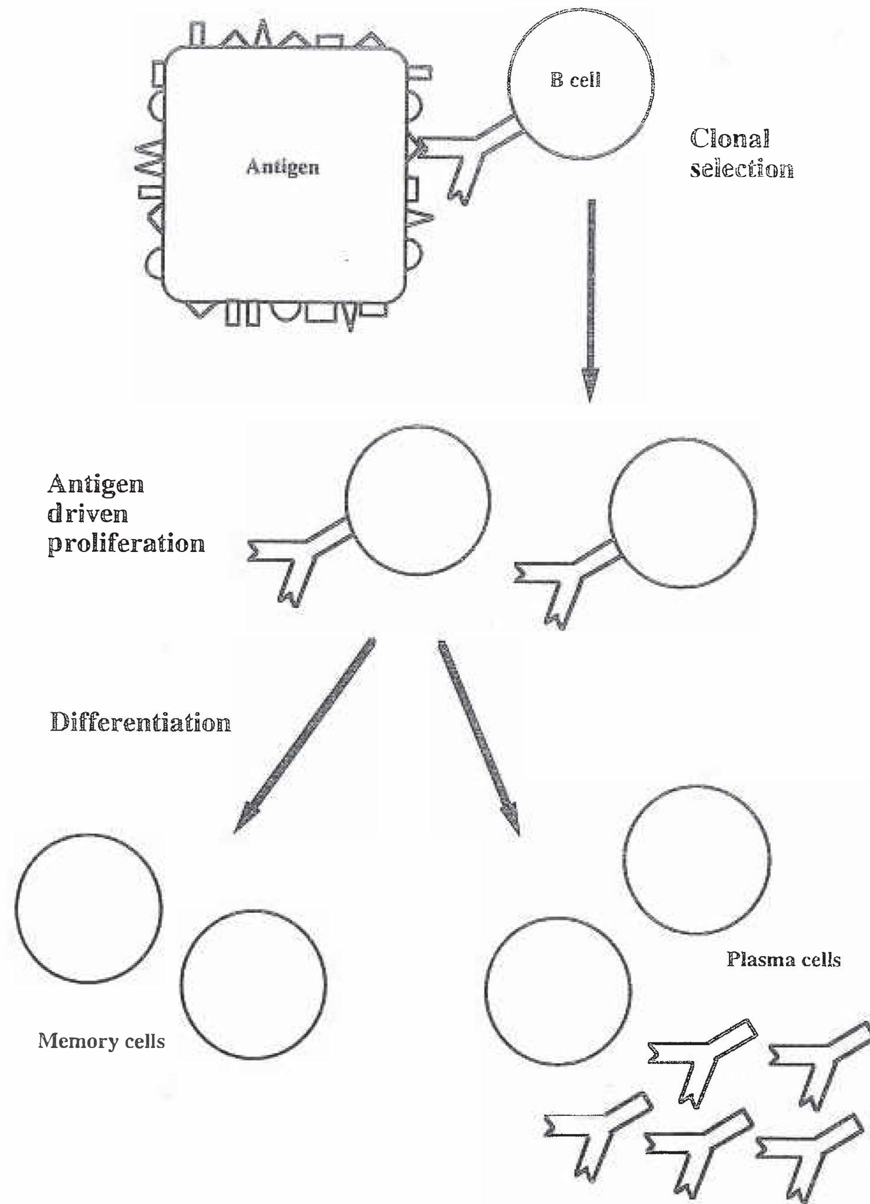


Figure 1.1 Differentiation of B cells to antibody-secreting plasma cells

bring into action molecules of the effector system which can then remove the foreign material. For effective defence systems to operate it is essential that an individual is able to recognise a wide variety of foreign material and thus antibody molecules of many different binding specificities are required. However, in each case the antibody must also

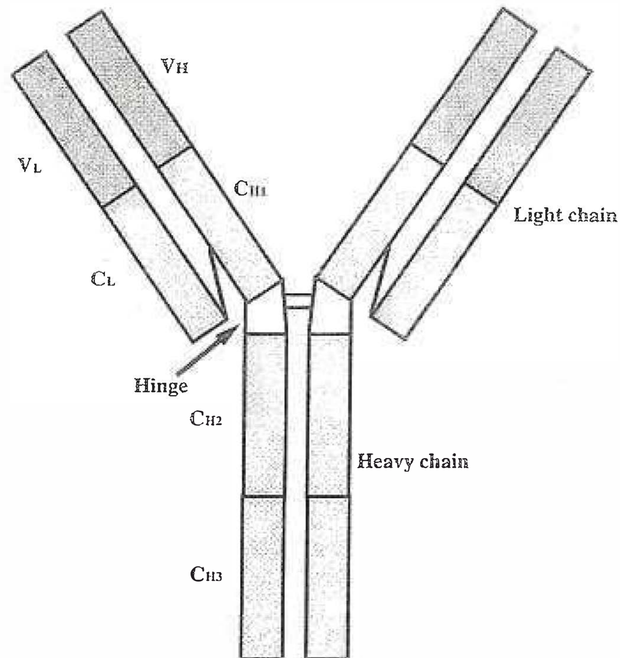


Figure 1.2 Representation of the organisation of protein chains of an IgG molecule

be able to trigger the same effector systems. To achieve this the antibody molecule has evolved variable regions which vary in protein sequence and structure to accommodate the development of binding specificities to a wide range of different antigens, and constant regions which are largely the same in each antibody. The constant regions maintain a common structure of the molecule and allow interaction with the effector systems such as complement binding and binding to Fc receptors on macrophages to activate phagocytosis.

Antibody molecules are also known as immunoglobulins. The term 'immunoglobulin' applies to the antibody protein whether or not the binding specificity of the molecule is characterised, whereas an antibody is an antigen-specific immunoglobulin. In practice the two terms are usually used interchangeably.

Higher mammals have five classes (isotypes) of immunoglobulin, termed IgG, IgM, IgA, IgE and IgD. The most abundant of these, which is used in most applications of antibodies, is the IgG class, the main class of antibody generated by the secondary immune response. The IgG molecule consists of four polypeptide chains, two heavy chains of approximately 50 kDa and two light chains of approximately 25 kDa (Figure 1.2). Each of these is divided into discretely folded structural domains of approximately 110 amino acids stabilised by an internal disulphide bond. These are linked together by short regions of comparatively flexible protein chain which allow movement of the domains relative to one another. Each light chain comprises a variable domain (VL) and a constant domain (CL), and each heavy chain a variable domain (VH) and three constant domains (CH1, CH2 and CH3). The heavy and light chain variable domains associate to form the antigen-binding site. The IgG molecule thus has two antigen-binding sites and is capable of

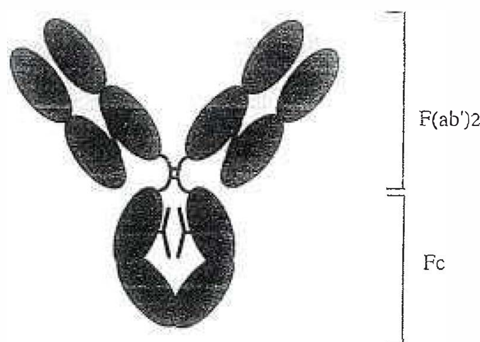


Figure 1.3 Domain structure of IgG, demonstrating the antigen-binding $F(ab')_2$ region and the Fc region with carbohydrate attached at the CH2 domain

binding antigens divalently to allow high binding avidity (see Section 1.5). The heavy and light chains are linked by a disulphide bond between the CL and CH1 domains. A flexible region, known as the hinge, links the CH1 and CH2 domains of each heavy chain and it is at this point that the two heavy chains are linked by disulphide bonds. The CH2 domain is normally glycosylated and the CH3 domains of each heavy chain associate with each other by non-covalent interactions.

Much early work on the structure and function of antibodies made extensive use of proteolysis of the antibody protein and this has led to commonly used terminology for the different regions of the antibody (Figure 1.3). The area around the antibody hinge is more susceptible to proteolysis than the tightly folded domains and thus this is the point at which proteolytic cleavage usually takes place. Proteolysis above the disulphide bonds in the hinge region results in monovalent Fab (fragment antigen-binding) fragments which comprise light chain together with the N-terminal two domains of the heavy chain. The Fab fragment thus contains one functional antigen binding site. Proteolysis immediately below one or more of the hinge disulphide bonds results in the divalent $F(ab')_2$ fragment. The CH2 and CH3 domains together make up the Fc fragment (fragment crystalline) which contains the sites for binding effector molecules. These fragments were the starting points for crystallographic determinations of antibody structure, as the flexibility of the intact IgG molecule prevented crystallisation until fairly recently. Thus while there are more than 50 structures for Fab fragments in the literature and several for hinge deleted immunoglobulins (reviewed by Padlan, 1994) there is only one fully defined structure of an intact IgG (Harris *et al.*, 1997).

Crystallographic structure determinations of many Fab fragments and several Fc fragments of antibodies have revealed that the folded domains have similar overall structures. This structure comprises two stacked β-sheets twisted into a characteristic fold, termed the immunoglobulin fold, and stabilised by a disulphide bond (Poljak *et al.*, 1973). In the constant domains one sheet has three and the other four antiparallel beta strands, while in the variable domains there are nine strands.

The primary sequences of several thousand antibody molecules are known, representing the largest number of known sequences in one protein family. Analysis of these sequences has revealed that the variability of antibody variable domains is largely restricted to three 'hypervariable' regions in each of the heavy and light chain variable domains, with comparatively little variation in the intervening 'framework' regions (Kabat

HOSPIRA EX. 1029
Page 14
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

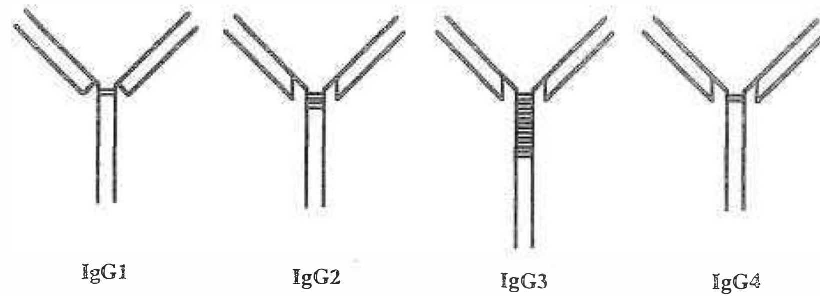
et al., 1991). These hypervariable regions, also known as complementarity determining regions, or CDRs, form loops at the tip of the Fab structure making up an antigen-binding surface of approximately 2800 Å^2. Structures of several Fab:antigen complexes have confirmed that antigen binding takes place at this surface; the hypervariability of these loops is therefore responsible for the different binding specificities of different antibody molecules. The CDR loops vary in both amino acid sequence and length between antibodies. This results in the variety of antigen-binding surfaces, some of which contain grooves or clefts in the surface to accommodate antigen binding whereas others are comparatively smooth. In some cases three-dimensional structures have been determined for antibody fragments with and without bound antigen which show that the antigen-binding site is not always completely rigid and may move to allow tight antigen binding in an 'induced fit' mechanism (Bhat *et al.*, 1990; Rini *et al.*, 1992). In some cases this may also lead to some conformational changes at sites in the antibody further away from the antigen-binding region (Guddat *et al.*, 1994).

There are four different subclasses (or isotypes) of IgG, called IgG1, IgG2, IgG3 and IgG4 in humans and IgG1, IgG2a, IgG2b and IgG3 in mice. Although all within the IgG class, these subclasses vary to some extent in their structure and function. The subclasses have different heavy chains termed γ1, γ2, γ3 and γ4 in humans and each of these can use light chains from one of the two light chain isotypes, κ or λ. The structures of the subclasses vary in their pattern of disulphide bonding (Figure 1.4). The number of disulphide bonds between the heavy chains varies from two in human IgG1 and IgG4 to 15 in human IgG3. Also, the position of the disulphide bond between the heavy and light chains varies from between the CL and CH1 region to between the CL and VH/CH1 interdomain region.

The antibody effector functions are mediated through the constant regions. The effector functions can be mediated through complement activation or by cellular interactions through specific Fc receptors expressed on a range of cell types, which can result in the process of antibody-dependent cellular cytotoxicity (ADCC). Complement activation via the classical complement cascade is initiated through binding of the IgG to the complement component C1q. This is subject to conformational restraints which are only partially understood, and requires two molecules of IgG bound to an antigenic surface to bind C1q efficiently. The binding site for C1q on IgG has been known for some time to be localised to the CH2 domain, with evidence that the N-terminal region of CH2 is important for binding C1q in the case of human IgG1 (Morgan *et al.*, 1995) and also that the C-terminal region of CH2 is required for efficient complement lysis (Tao *et al.*, 1993; Greenwood *et al.*, 1993). Mutagenesis studies have suggested that three amino acid residues on the surface of the CH2 domain of murine IgG2b, numbers 318, 320 and 322, are involved in the binding interaction (Duncan and Winter, 1988), although these residues are also present on antibodies which cannot activate complement, suggesting that these residues are necessary but not sufficient to activate complement. Mutagenesis studies with human IgG1 have also suggested that C1q binding alone is not sufficient for complement activation but that interactions of the IgG molecule with other steps in the complement pathway are also required (Tao *et al.*, 1993).

Fc receptors have been identified for all the classes of immunoglobulin, IgG, IgM, IgD, IgE and IgA. Of these the best characterised are those for IgG and IgE. Three types of Fc receptors have been identified for IgG in humans; FcγRI (also known as CD64), FcγRII (also known as CDw32), and FcγRIII (also known as CD16). These receptors are structurally related and distributed on various blood cell types. FcγRI is a high affinity receptor, capable of binding monomeric IgG, which plays a key role in ADCC and is

Human IgG



Murine IgG

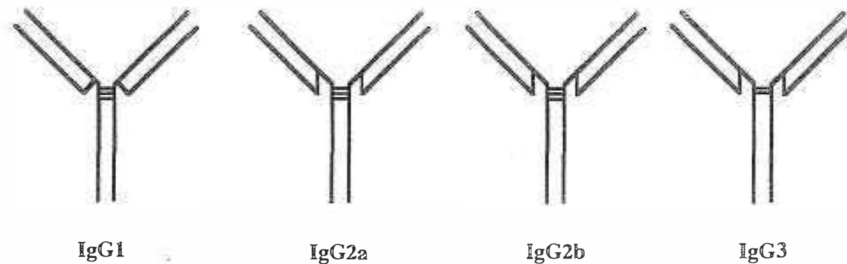


Figure 1.4 Variation in the disulphide bonds between heavy chains in IgG subclasses

found on macrophages, monocytes and neutrophils. FcγRII and FcγRIII are low affinity receptors which bind aggregated IgG. FcγRII is found on most leukocytes including monocytes, macrophages and neutrophils and FcγRIII is found on neutrophils, macrophages and NK cells. These low affinity receptors are also capable of eliciting ADCC and phagocytosis. The binding of a single antibody to Fc receptor is reversible and does not elicit a response. If several antibodies are clustered together Fc receptor clustering will take place and a cellular response will be elicited (Figure 1.5). All three Fcγ receptors recognise sites on the lower hinge/CH2 domain of IgG but not in an identical fashion. In particular, the sequence of heavy chain residues at positions 234–237 is involved in the FcγRI receptor binding site (Duncan *et al.*, 1988; Sarmay *et al.*, 1992).

The ability to elicit effector functions varies between isotypes of IgG (Table 1.1), and this may reflect important differences in the functions of the individual isotypes. For example, human IgG1 and IgG3 are highly active isotypes with respect to complement activation and elicitation of ADCC responses whereas IgG2 and IgG4 are relatively inactive, being only poorly able to activate complement through C1q binding with little binding to Fc receptors.

The presence of carbohydrate attached to the CH2 domain has been shown to be required for both Fc receptor binding and complement activation (Tao and Morrison,

HOSPIRA EX. 1029
Page 16
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

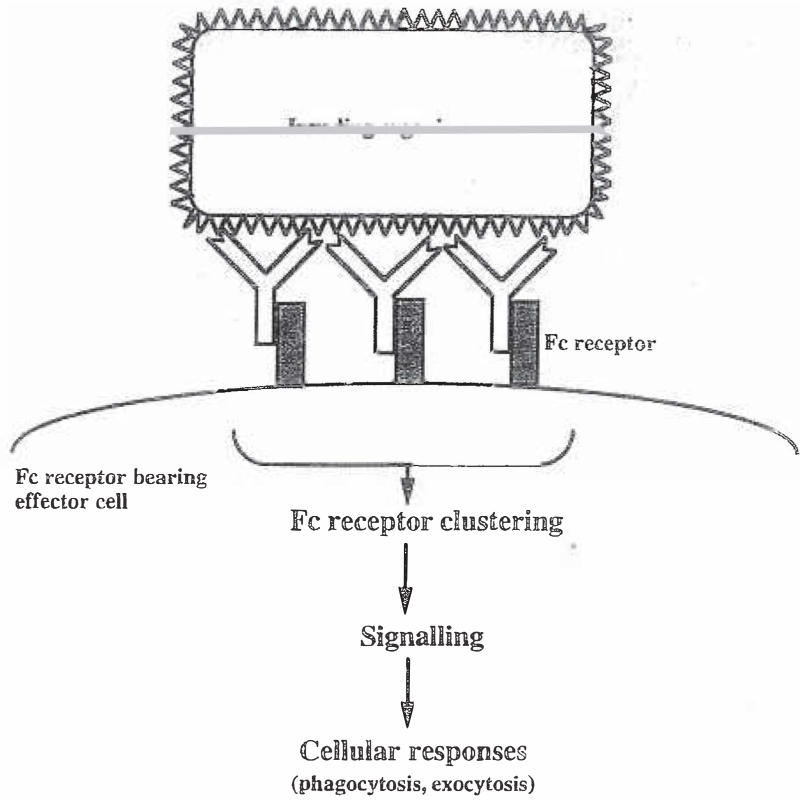


Figure 1.5 Fc receptor clustering due to multiple antibody molecules binding to antigen leading to cellular responses

Table 1.1 Effector functions of human and mouse IgG subclasses

	Human IgG				Mouse IgG			
	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2a	IgG2b	IgG3
Complement activation	+++	+	++	—	+	++	+++	++
Human FcγRI (CD64)	+++	—	+++	+	—	++	—	++
Human FcγRII (CDw32)	++	+	++	—	+	+	+	—
Human FcγRIII (CD16)	++	—	++	—	—	+	+	++

Table 1.2 Human immunoglobulins

Antibody	Approximate molecular weight (kDa)	Heavy chain	Approximate concentration in normal human serum (mg/ml)
IgG1	146	γ1	9
IgG2	146	γ2	3
IgG3	170	γ3	1
IgG4	146	γ4	0.5
IgM	970	μ	1.5
IgA1	160	α1	3
IgA2	160	α2	0.5
IgD	200	δ	0.03
IgE	200	ε	0.0001

1989). The role of the carbohydrate is not fully understood but it is believed to be important in maintaining the tertiary structure and disposition of the CH2 domains. IgG without carbohydrate has been shown to have an altered conformation in the lower hinge region and may result in disruption of the interaction sites (Lund *et al.*, 1990).

IgG represents approximately 70–75% of the total immunoglobulin in human serum, the remainder being molecules of the other classes mentioned above, IgM, IgA, IgD and IgE. Although the IgG structure is a good general model for antibody structure, these immunoglobulin classes differ in their structure, reflecting differences in their normal functions. The different classes of immunoglobulin use light chains of the same type (κ or λ) and thus differ predominantly in their heavy chains (Table 1.2).

IgM is the predominant antibody raised in primary responses to many antigens and represents approximately 10% of the immunoglobulin in human serum. IgM antibodies have a pentameric structure of the basic four chain unit such that a total of ten antigen-binding sites per molecule are present (Figure 1.6). The μ heavy chain does not have a hinge region but has an extra constant region domain, which is inserted between the CH1 and CH2 domains in the analogous IgG structure, such that the CH4 domain of IgM is analogous to the CH3 domain of IgG. There is also an extra tail of 19 amino acids on the heavy chain involved in polymer assembly. The heavy chains are held together by disulphide bonds between CH3 domains and there is also an extra polypeptide chain, the J chain, whose role is not fully understood although it may assist the process of assembly of the pentameric IgM molecule. The μ heavy chain is also more heavily glycosylated than the γ heavy chain, with sugar attached at five glycosylation sites. Although it is presented as a pentameric structure in Figure 1.6, this is not always the case and the occurrence of IgM molecules with different numbers of binding units is now well established. The production of hexamers and tetramers has been reported (Eskeland and Christensen, 1975) and in some cases hexamers may be a predominant structure (Cattaneo and Neuberger, 1987; Davis *et al.*, 1988). This multivalent structure provides the IgM molecule with a high binding avidity, to help compensate for the relatively low affinity of each binding site. Another consequence of the multivalent structure is the ability of one molecule of IgM to activate complement, which is thought to occur via binding to the IgM CH3 domain. Therefore IgM molecules are well adapted for their role in the primary immune response.

HOSPIRA EX. 1029
Page 18
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

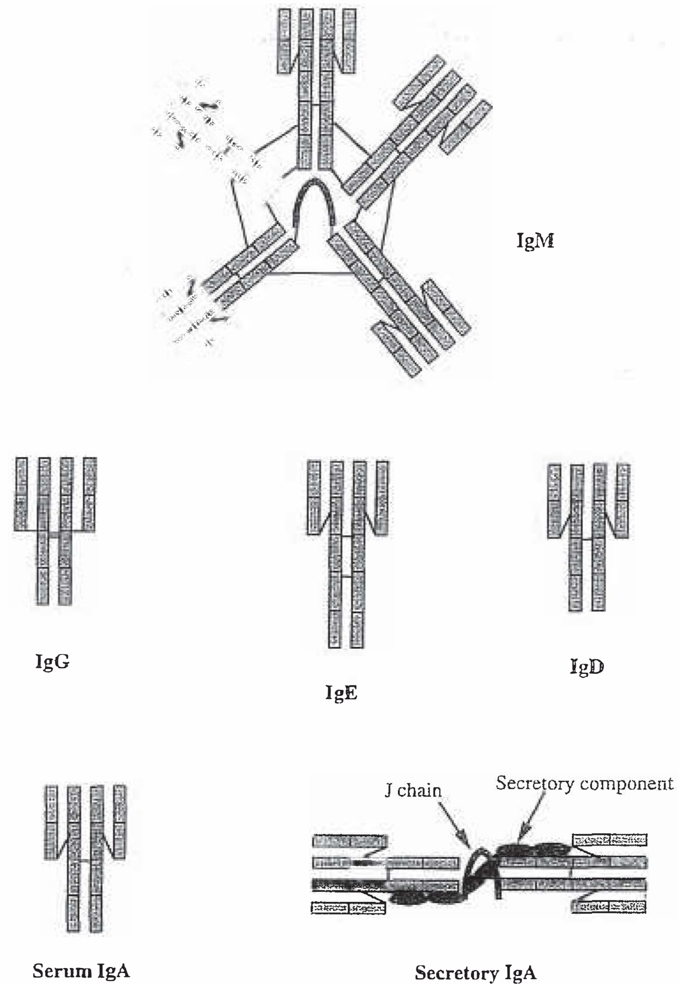


Figure 1.6 Organisation of protein chains in the immunoglobulin classes

There are two isotypes of IgA present in humans (IgA1 and IgA2) which together make up 15–20% of the total serum immunoglobulin. In addition, a form of IgA, termed secretory IgA or sIgA, is the predominant immunoglobulin present in external secretions such as saliva, milk, tears, genitourinary secretions and tracheobronchial secretions (Tomasi, 1992). The mucosal surfaces bathed in these secretions are a major site of exposure to the environment and thus secretory IgA is an important defence mechanism against invading organisms. In terms of quantity, sIgA is the major type of Ig produced, with estimates of 2g per day being produced in humans, more than other Ig forms. The IgA present in serum is largely monomeric and consists of two heavy chains and two light chains assembled in a similar manner to IgG. IgA1 is the predominant isotype, making up approximately 90% of the serum IgA. IgA heavy chains have three constant domains with IgA1 having a longer hinge region than IgA2. In addition, IgA heavy chains also

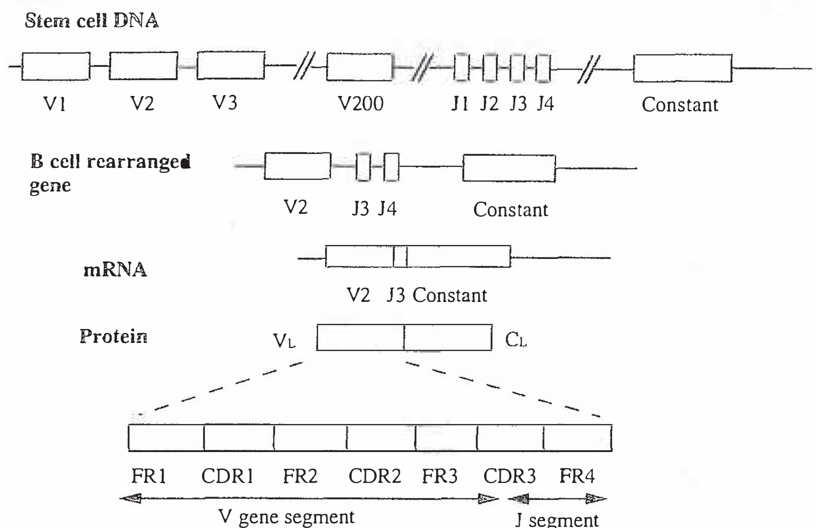
have a similar tail to IgM which allows polymerisation, and binding of the J chain to the α chain, through a C-terminal cysteine residue in a similar manner to IgM. sIgA exists predominantly in dimeric form with a molecular weight of approximately 385,000. sIgA also contains a secretory component which is an extra polypeptide chain of approximately 70 kDa, synthesised by epithelial cells and not the plasma cells. The secretory component is part of the receptor involved in transport of IgA into mucosal secretions which is cleaved during transport across endothelial surfaces to release the 70 kDa secretory component. This then becomes bound to the IgA by disulphide bonds (Fallgreen-Gebauer *et al.*, 1993). sIgA is more stable to proteolytic attack than the serum form and part of this increased stability may result from the association of the secretory component which protects the immunoglobulin from degradation (Tomasi, 1992).

Human IgD and IgE are relatively poorly understood although the overall structures are known (Figure 1.6). IgD has been known to exist in a membrane-bound form on B cells for some time, where along with membrane-bound IgM it plays an important role in initiating the antibody response as described above. In the circulation, IgD is present at low levels, less than 1% of the circulating immunoglobulin, and may also be involved in regulation of the immune response (Roes and Rajewsky, 1993). IgD has a structure similar to IgG except that the heavy chain is more heavily glycosylated and there is only one disulphide bond between the two heavy chains even though the hinge region is extended and similar in length to that of IgG3 which has 11 inter-heavy chain disulphide bonds. IgE is present at very low levels in normal human serum although levels are raised significantly in allergic diseases and parasitic infections. IgE is believed to have evolved for protection against parasitic diseases, though in industrialised countries its role in allergy and asthma is now more important (Sutton and Gould, 1993). IgE also contains two heavy chains and two light chains in an assembled IgG-like structure. The ε heavy chain, however, contains four constant domains and multiple glycosylation sites (Figure 1.6).

1.4 The organisation of antibody genes

Antibody heavy and light chains are encoded in the genome at separate genetic loci; the two light chain loci, κ and λ, and the heavy chain locus, IgH. In humans and mice these loci are found on different chromosomes. Within each locus there are exons which encode the constant region domains and clusters of exons which are used to form the unique variable domains by each B cell. There are three families of repeated gene segments which are used to form the variable region at the heavy chain locus, variable (V), diversity (D) and joining (J), while the light chains use only V and J gene segments. During the development of the B cell a series of recombination events takes place to form the variable domain exon. In the mouse, the light chain variable region is recombined from one V out of a choice of approximately 200, and one J out of a choice of 4. The heavy chain chooses one V out of a choice of approximately 100, one D out of approximately 50 and one of 4 J region gene segments. The variable region along with the signal sequence exon (to direct secretion of the antibody) and the promoter/enhancer (involved in transcription) is then recombined with the constant region exons for subsequent expression (Figure 1.7). In this way each B cell forms a unique antibody from a total of approximately 10^{10} possible combinations. This is a random process and requires antigen itself to select out B cells producing antibodies of useful specificity (Section 1.2). Each individual mouse probably has B cells expressing about 10^{8} specificities at one time. Further diversity in

HOSPIRA EX. 1029
Page 20
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374



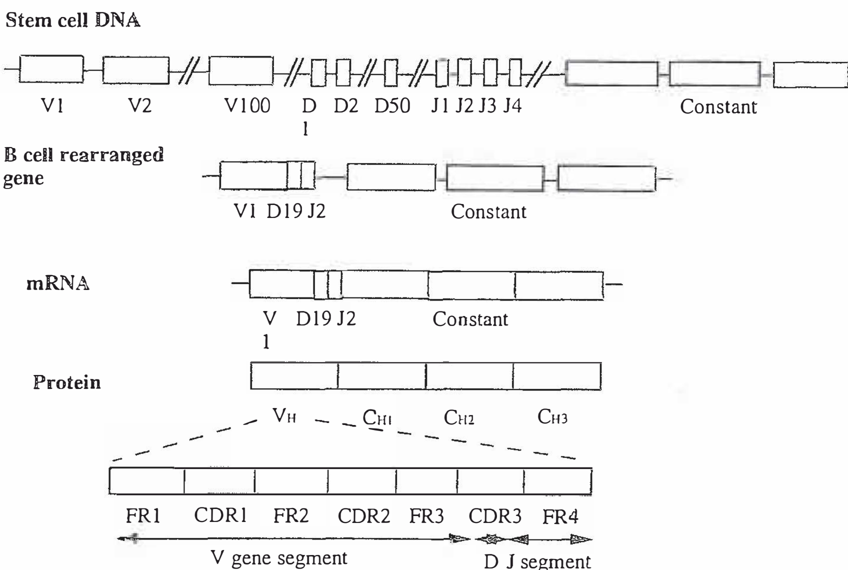


Figure 1.7 Gene organisation of IgG1, demonstrating an example of gene rearrangement to produce light and heavy chains

the antibody genes is generated because of imprecision in joining the V, D and J regions, as well as occasional incorporation of extra nucleotides at the junctions (Lieber, 1996).

The constant region exons next to the variable regions determine the isotype of the antibody produced. For the heavy chain this is initially μ, leading to the production of

HOSPIRA EX. 1029
Page 21
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

IgM. As the immune response progresses, B cells rearrange the genes such that other classes of immunoglobulin are produced: for example, switching the μ exons for γ1 exons to produce an IgG1. This phenomenon is known as class switching. Also antigen selects for B cells producing antibody of high binding affinity. This is achieved through hypermutation of the variable region sequences and selection of the cells producing antibody of the highest affinity. These cells then follow the same pathway as before, some differentiating into plasma cells and some into memory cells. This is the genetic basis of the process known as affinity maturation.

There is also some genetic variation in the constant region determined isotypes between individuals. These result from small variations in the constant region sequences of a particular isotype between populations, usually between different racial groups, and are known as allotypes. In humans, allotypes have been identified for IgG1, IgG2 and IgG3 as well as IgA, IgE and κ light chains.

1.5 Antigen-binding affinity and avidity

1.5.1 *Affinity*

A measure of the strength of the antibody:antigen interaction is essential in comparing MAbs to the same or different antigens. The strength of the antibody:antigen interaction is measured through the binding affinity. This can be considered as the sum of all the non-covalent interactions between antibody and antigen involved in the binding reaction. The antibody affinity is quantified through the association constant K_a.

For the binding reaction:

$$\text{Ab} + \text{Ag} \rightarrow \text{AbAg complex}$$

K_a is the equilibrium constant of this reaction, and can be determined from the equilibrium concentrations of the reactants and complex using the law of mass action:

$$K_a = \frac{[\text{AbAg complex}]}{[\text{Ab}]\,[\text{Ag}]}$$

The units of K_a are therefore M^{-1}.

Frequently the dissociation constant K_d of the reaction is used in preference to the association constant. The dissociation constant is the affinity constant of the reverse reaction, and is therefore given by:

$$K_d = \frac{[\text{Ab}]\,[\text{Ag}]}{[\text{AbAg complex}]}$$

K_d is also, of course, the reciprocal of the association constant: $K_d = 1/K_a$ and the units of K_d are M.

The popularity of K_d as a measure of antigen-binding strength stems from the determination of affinities through measuring the concentration of antibody required to complex with half of the antigen. At the 50% bound point the concentration of Ag = AbAg complex by definition. These therefore cancel each other out in the equation above, and K_d = concentration of antibody required to complex 50% of the antigen. High affinity antibodies will require a low concentration of antibody to achieve this, e.g. 10^{-8}–10^{-12} M.

In some circumstances the kinetic rate constants of the binding reaction, the binding on-rate k_{on} and the dissociation rate or off-rate k_{off}, may also be important. These can be

HOSPIRA EX. 1029
Page 22
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

considered measures of the rate of binding and dissociation whereas the overall strength of the reaction is given by K_a or K_d. The rate of the binding reaction is dependent on the concentrations of Ag and Ab and the rate constant k_{on}. Units for k_{on} are M^{-1} s^{-1}. The rate of dissociation is independent of the concentration of AbAg complex and depends only on the dissociation rate constant k_{off}. Units for k_{off} are s^{-1}. At equilibrium, by definition, the rate of binding is equal to the rate of dissociation, $k_{on} = k_{off}$.

$$k_{off}\,[\text{AbAg complex}] = k_{on}\,[\text{Ab}]\,[\text{Ag}]$$

Rearranging this equation gives:

$$\frac{k_{off}}{k_{on}} = \frac{[\text{Ab}]\,[\text{Ag}]}{[\text{AbAg complex}]}$$

Therefore the rate constants can be used to give K_a or K_d:

$$K_d = \frac{[\text{Ab}]\,[\text{Ag}]}{[\text{AbAg complex}]} = \frac{k_{off}}{k_{on}}$$

The values of k_{on} and k_{off} can be measured directly as initial rates using instrumentation such as the optical biosensor BIAcore device (see Chapter 3; Malmqvist, 1993). This allows rapid determination of K_d values without waiting for the reaction to come to equilibrium. Several methods for determination of affinity and kinetic parameters, either equilibrium-based or initial rate-based, are available and have been reviewed (Goldberg and Djavadi-Ohaniance, 1993; Malmqvist, 1993).

1.5.2 Avidity

The antibody affinity is a measure of the strength of binding of an individual antibody binding site to a single antigenic site. However, antibody molecules usually have more than one binding site (e.g. 2 for IgG, 10 for IgM), and many antigens contain more than one antigenic site (e.g. a cell surface or antigen immobilised on a solid support), and therefore multivalent binding may be possible. The strength with which a multivalent antibody binds antigen is termed avidity. The avidity of the antibody depends on the affinities of the individual antibody binding sites, but is greater than the binding affinity as all the antibody:antigen interactions must be broken simultaneously for the antibody to dissociate completely. Thus, although the affinities of the individual binding sites are the same, multivalency leads to a large effect on the dissociation rate resulting in high binding avidity. Avidity is also referred to as functional affinity.

1.6 Generation of monoclonal antibodies

1.6.1 Hybridoma technology

The techniques conventionally used for the production of MAbs are based on the original report by Kohler and Milstein (1975). This technology, known as hybridoma technology, has proved to be capable of producing rodent antibodies of predetermined specificity to a wide variety of different antigens, for example proteins, nucleic acids, carbohydrates and haptens. Since each individual B cell produces antibody of a single specificity the

HOSPIRA EX. 1029
Page 23
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

production of antibodies of a single specificity requires isolation of individual B cells. However, B cells are not normally capable of growth in culture, and thus the production of MAbs directly from B cells is not possible. Hybridoma technology allows the production of hybrid cell lines from B cells which secrete a single, monoclonal, antibody with one binding specificity which can potentially be produced in unlimited quantitities.

The general scheme for the production of MAbs by hybridoma technology is shown in Figure 1.8. Mice are immunised by injection of the antigen to which a MAb is required. Rats are also commonly used to generate MAbs, and in some cases (particularly when antibodies to mouse antigens are required) other species have been used such as hamsters (Houlden *et al.*, 1991), chickens (Nishinaka *et al.*, 1991) and rabbits (Speiker-Polet *et al.*, 1995). When an immune response has been raised, B cells are harvested from the rodent, usually from the spleen, and these are fused with myeloma cells by promoting fusion of the cell membranes with, for example, polyethylene glycol. Myeloma cells are derived from a mutant cell line of a B cell tumour, and cells are used which do not secrete any immunoglobulin, and which are essentially immortal and can be easily grown in culture. Fusion results in the production of immortalised hybrid cells or hybridomas. The remainder of the process is then one of isolation and propagation of hybridoma cells which have retained the ability of the B cell to produce antibody and the good growth characteristics of the myeloma cell. The process of hybridoma production can therefore be considered in three parts: immunisation, fusion and selection of the required hybridoma. Many protocols for immunisation and generation of hybridomas are available (e.g. Harlow and Lane, 1988; Donohoe *et al.*, 1995), and should be referred to for practical guidance.

Immunisation of the animal to raise an immune response is of key importance. Immunogens of different molecular types can be used and they do not need to be pure materials, as a hybridoma secreting a MAb of the required specificity can be selected out later. Different molecules vary greatly in their immunogenicity and thus require different immunisation protocols for an optimal response. Factors such as the form of the antigen, number and route of immunisations, carrier, adjuvant and the species and strain of animal used need to be considered. It is important to consider the conformation of the molecule which the MAb is desired to bind to. For example, will a protein be in its native conformation or denatured? MAbs of exquisite specificity can be generated, and therefore a desired isomer or molecular form of a particular antigen might be required.

There are two important exceptions to the range of immunogens which can be used. Antibodies will not normally be raised to 'self' antigens, and small antigens (less than approximately 1000 in molecular weight) will not raise a response unless conjugated to a high molecular weight carrier protein. For this purpose proteins are used, such as bovine serum albumin or keyhole limpet haemocyanin, which are very immunogenic in rodents, easily available and readily conjugated to small molecules. The generation of high affinity antibodies normally requires a T-cell-dependent B-cell response as described in Section 1.2, and thus both T and B cell epitopes are needed to allow the memory response and class switching to take place. T cells respond to proteolytically produced peptides of the antigen presented by MHC class II molecules and thus in general non-self proteins and glycoproteins elicit high affinity responses after multiple immunisations. Without protein, carbohydrates and other non-proteinaceous materials elicit only a T cell-independent response with little or no memory or class switching.

The type of antibody produced is also dependent on the number of immunisations. If IgM is required, only one immunisation is carried out as only a primary response is required, whereas IgG antibodies require multiple injections (usually three or four) at intervals of approximately 3–4 weeks to allow an effective secondary response. Adjuvants,

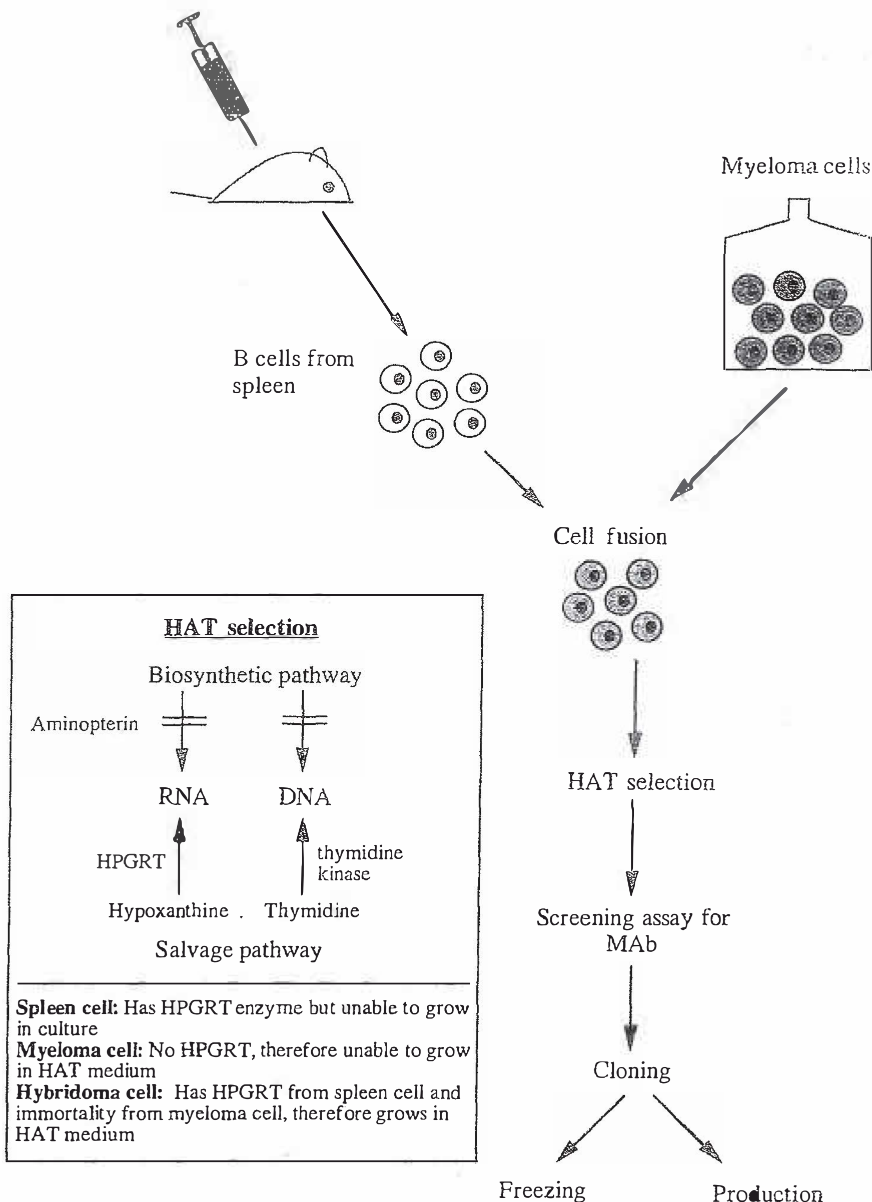


Figure 1.8 Production of monoclonal antibodies by hybridoma technology – see text for details

such as Freund's adjuvant, are widely used to aid the generation of an immune response to soluble antigens injected via the intraperitoneal, intradermal, intramuscular or subcutaneous routes which are usually used for primary immunisations (Harlow and Lane, 1988). Adjuvants are usually colloidal or oils, which are mixed with the soluble antigen to

produce an emulsion which is injected into the animal and provides a long-lasting local 'depot' of antigen which is slowly released and thus continually boosts the response. In addition, other components are sometimes included in the adjuvant mixture to activate the immune system non-specifically. For example, complete Freund's adjuvant contains killed mycobacteria for activation as well as oil and an emulsifying agent. Complete Freund's adjuvant is used for the first immunisation and subsequent booster immunisations are carried out in incomplete Freund's adjuvant (without the mycobacteria component) to maximise response to the antigen and avoid excessive inflammatory responses to mycobacteria. Insoluble antigens can often be used without adjuvant, as these form insoluble 'depots' on their own.

Genetic immunisation has recently been developed to allow immunisation with DNA encoding a protein of interest. The DNA is injected directly into mouse tissue, in a form which allows expression of protein from the DNA by the mouse tissue. An immune response is then generated against the protein which allows MAbs to be produced (Paddock *et al.*, 1994). This approach may be particularly useful when the protein is difficult to produce or purify sufficiently to allow direct immunisation. In addition, it may be possible to boost the immune response to poorly immunogenic proteins by producing fusion proteins with a highly immunogenic sequence.

To ensure that an immune response has been made to the antigen before harvesting B cells, blood samples can be obtained from the immunised animals and tested for the presence of specific antibodies. If positive, final boosts of soluble antigen are often made intravenously to increase the number of immune B cells present in the spleen prior to harvest. The spleen is the most common source of immune B cells, although cells can also be harvested from lymph nodes, particularly if soluble antigen is not available and a final intravenous boost cannot be given.

Several myeloma cell lines are available for fusion purposes from mice and rats. As these lines are derived from tumours of antibody-producing B cells, it is important to try to choose a line which does not produce any antibody of its own. If the resulting hybridoma produces two types of light and heavy chains these will probabably associate randomly resulting in a mixture of antibody molecules produced comprising the original myeloma antibody, the desired MAb and various mixed combinations of the two types of heavy and light chains. This mixing can be advantageous for the production of bispecific antibodies (see Chapter 2), but will reduce the efficiency of the initial isolation of a MAb and make purification of the desired MAb difficult. Another requirement of the myeloma cells is that they are deficient in the enzyme hypoxanthinephosphoribosyltransferase and will allow selection of the fused hybridoma cell over the parent myeloma cell (see below).

Fusion of the myeloma cells and the harvested B cells is usually accomplished using polyethylene glycol (PEG) as the agent to induce membrane fusion. Alternative methods are also available, for example the use of Sendai virus as used in early research or electrofusion. However, PEG fusion is achieved simply, cheaply and reliably and thus has become the most common method. Initially only the cell membranes fuse and two nuclei are present in the cell. Nuclear fusion takes place during mitosis and subsequent generations of the hybridoma cell often result in loss of some chromosomes. After fusion a mixture of hybridoma cells, spleen cells and myeloma cells are present and the next stage of the process is the selection of the hybridoma cells over the other cell types. Spleen cells will not grow in culture and thus growth of the cell mixture for a few days will readily remove residual spleen cells. Myeloma cells, on the other hand, will grow rapidly and would make isolation of any hybridoma cells very difficult. For this reason myeloma cells are used for the fusion which are deficient in the enzyme

hypoxanthinephosphoribosyltransferase (HPRT) and consequently are not able to use the salvage pathway for RNA synthesis. Selection is then achieved by using HAT medium (Figure 1.8). HAT medium contains hypoxanthine (H), aminopterin (A) and thymidine (T). Aminopterin is an effective inhibitor of RNA and DNA synthesis and thus will block growth of the myeloma cells. However, hybrid cells which have the HPRT enzyme from the spleen cells will be able to use the added hypoxanthine and thymidine to produce RNA via the salvage pathway and survive.

After selection in HAT medium, hybrid cells are grown on and tested to determine which colonies of cells produce antibodies of interest. Not all of the spleen cells which form hybrids are B cells, and thus there will be some hybrids which do not produce antibody at all, and also many cells will be present which produce antibodies which are not of interest. An important step is therefore the screening of clones of cells to identify those which produce the MAbs required. A large number of clones may need to be screened quickly to allow selection of those required for further culture. A suitable screening assay should therefore be rapid, able to screen many samples and capable of giving a result with the small amount of antibody produced by early clones of cells. There are many types of suitable assays to measure antigen-binding properties including ELISA (enzyme linked immunoadsorbent assay), radioimmunoassay, dot blots and western blots, immunohistochemistry and immunofluorescence. In addition, it may be desirable to screen for other properties such as isotype or specific functional properties such as complement activation or the ability to elicit ADCC. If possible, the assay which uses the antibody in the closest way to that required for the intended end use of the antibody is the most suitable, as this will ensure that the optimal properties of the antibody are selected.

Once suitable hybridomas have been identified, these must be cloned several times to ensure a stable, homogeneous colony of cells and to ensure that the antibody is indeed monoclonal. To do this requires the growth of a colony from a single cell. This is achieved by subculturing the cells by limiting dilution so that each well of the culture plate contains an average of less than one cell. Alternatively cloning can be carried out by plating out cells in semisolid agar or by single cell manipulation techniques. Individual clones of cells can than be screened for antibody production as before. Once cloned, the hybridoma cell is ready to be used for the production of large amounts of antibody, but it is also important to ensure that samples of cells are preserved by freezing in liquid nitrogen at each stage so that valuable hybridomas are not lost!

1.6.2 Human monoclonal antibodies

The generation of human MAbs is often desirable for clinical applications. This is because murine antibodies are recognised as foreign when administered to humans and therefore elicit an immune response directed against the administered MAb: a human anti-mouse antibody response or HAMA. This prevents repeat administration of MAb as the HAMA results in formation of immune complexes which are rapidly cleared, rendering the antibody ineffective. In addition, HAMA responses may result in adverse reactions by the patient such as allergic reactions, hepatic dysfunction and in some cases anaphylactic shock. Attempts to overcome this problem have driven much research both in reconstructing rodent antibodies by genetic engineering (see Chapter 2) and in attempts to generate human MAbs. Human antibodies may also be advantageous in that they are compatible with human effector mechanisms such as complement and ADCC which are often not fully activated by rodent antibodies.

HOSPIRA EX. 1029
Page 27
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 1.3 Problems in human MAb production using conventional techniques

Immunisation *in vivo* or *in vitro*
Source of human B lymphocytes
Immortalisation: EBV/choice of fusion partner cell line
Stability of human hybridoma/heterohybridoma cell lines
Selection of isotypes and high affinity MAbs
Scale up to produce large quantities of human MAbs

The production of human MAbs has not been straightforward and attempts have driven many technical innovations and improvements to antibody technology. Production of human MAbs by hybridoma techniques is usually difficult for several reasons (Table 1.3). For many antigens of interest, such as human disease markers, it is not appropriate to immunise humans directly with the antigen for ethical and moral reasons. Therefore it can be difficult to obtain suitably immune lymphocytes. The source of lymphocytes is also a major problem. It is usually not possible to harvest these from the spleen or lymph node and the most available and commonly used source is peripheral blood. B cells are comparatively poorly represented in peripheral blood and the majority of those present express surface IgM. Fusion therefore often results in cells which largely produce low affinity IgM antibodies. The choice of cell line to be used as a fusion partner is also problematic. Human myeloma lines have proved to be comparatively poor with respect to their growth characteristics and fusion efficiencies and usually secrete immunoglobulin of their own which complicates production and purification. Attempts to use murine cell lines, which do not produce any immunoglobulin of their own, as fusion partners have met with some success, although the hybrid cells produced can be unstable and may preferentially lose human chromosomes and thus the ability to secrete human antibody. More successful has been the development of mouse–human heteromyeloma cell lines as fusion partners. These are formed by fusing a human lymphoid tumour line with a mouse myeloma cell line and using the resulting hybrid cell line to fuse with the required B cells. The resulting fused cells are termed heterohybridomas or triomas and these often produce more antibody than cell lines produced with murine fusion partners alone and are relatively stable.

An alternative approach to immortalise human B cells has been the transformation of these cells with Epstein–Barr virus (EBV). EBV is a virus which preferentially infects B cells and activates them to divide and produce antibody. Some of the infected cells may be immortalised but this technique is relatively inefficient and cell lines are often unstable and lose the ability to produce antibody. Combinations of this approach with the use of heteromyeloma fusion are more efficient. For example, stimulating B cells with EBV followed by fusion with a mouse–human heteromyeloma has been used to produce several human MAbs (e.g. Kozbor *et al.*, 1982; Gustafsson and Hinkula, 1994).

To overcome the problem of being often unable to immunise humans *in vivo*, approaches to the *in vitro* immunisation of human B cells in culture have been developed (Borrebaeck, 1989; Koda and Glassy, 1990). Although developed primarily for use in human antibody production, *in vitro* immunisation was first used for the production of mouse antibody and may offer advantages for the production of rodent antibodies in some circumstances, for example when trying to raise antibodies to weakly antigenic epitopes which might be masked by dominant epitopes *in vivo*, or when trying to raise antibodies to hazardous antigens which it would not be possible to administer *in vivo*. *In*

HOSPIRA EX. 1029
Page 28
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

vitro immunisation is an attempt to achieve primary activation of B cells in culture and as such requires a complex *in vitro* protocol including co-cultivation with a variety of cytokines and mitogenic factors to stimulate B cells (Danielsson *et al.*, 1987; Darveau *et al.*, 1993). Suppressor cells also need to be eliminated from the population by cell separation or by treatment with agents such as leucyl leucine methyl ester which is cytotoxic to CD8+ T cells and natural killer cells (Borrebaeck *et al.*, 1988). The major drawback of *in vitro* immunisation approaches developed to date is that the antibodies produced are characteristic of a limited primary response and are therefore often of the IgM subclass with low to intermediate affinities for antigen.

Mice with a mutation resulting in severe combined immune deficiency which lack functional B and T cells (SCID mice) have been used in attempts to produce higher affinity human antibodies. Because of their immune deficiency, SCID mice can accept grafts of human lymphocytes isolated from peripheral blood which can be used to re-create a partial human immune system in the mouse (Mosier *et al.*, 1988). Cells can be transplanted from immunised donors or after *in vitro* immunisation and re-immunised in the mouse to generate secondary responses (Duchosal *et al.*, 1992; Carlsson *et al.*, 1992). Immune human B cells can then be harvested from the spleen or lymph nodes to allow antibody production.

In addition, it is possible to use SCID mice with human immune cells to generate primary responses using cells from donors not previously immunised (Sandhu *et al.*, 1994). This raises the prospect of producing a wide range of human antibodies in mice, and research towards developing suitable methods is under way (Walker and Gallagher, 1994). So far only a limited number of antigens have been investigated using this system and the limits to the ability of this system to produce high affinity antibodies to a range of different antigens will be a subject of intensive research over the next few years.

One approach which can successfully overcome the instability of human antibody-producing cell lines once they have been generated and the difficulties involved in their large-scale culture is gene rescue. In this approach the antibody genes are cloned from the cells and expressed in a stable alternative host cell such as a murine myeloma. For example, genes coding for human antibodies to tetanus toxoid and pseudomonal exotoxin A have been rescued from a mouse–human heterohybridoma and an EBV transformed B cell line respectively and re-expressed in a murine myeloma cell line (Gillies *et al.*, 1989b; Nakatani *et al.*, 1989). In both cases stable murine cell lines were obtained which secreted high levels of human antibody, similar to the yields of antibody produced by murine hybridoma cells. This technique also allows engineering of the human antibody, such as changing the isotype of the antibody if required. Engineering of antibodies is discussed in detail in Chapter 2.

1.6.3 Human MAbs from transgenic mice

An alternative approach to the generation of human antibodies is the genetic manipulation of mice to disable the production of mouse immunoglobulin and to introduce human immunoglobulin loci. The resulting transgenic mice are capable of producing human antibodies in reponse to challenge with antigen which can be isolated by conventional mouse hybridoma technology (Lonberg *et al.*, 1994; Green *et al.*, 1994). The human immunoglobulin loci are large and contain the V, D and J genes of the variable domains

HOSPIRA EX. 1029
Page 29
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

as well as the constant regions as described in Section 1.4. The transfer of the entire locus is technically extremely difficult and thus miniloci have been constructed containing a limited number of the heavy and light chain genes. These genes are introduced into mouse embryonic stem cells and implanted into a surrogate mother to develop. The resulting mouse strain therefore contains both human and mouse antibody genes. A separate mouse strain is produced from embryonic stem cells in which the mouse immunoglobulin heavy and κ light chain genes are disrupted and this is then bred with the strain containing mouse and human genes. Progeny containing only functional human immunoglobulin genes can then be isolated. These mice are capable of immune responses to administered antigen and although the entire diversity of V, D and J regions has not been reproduced, those present are utilised to produce diverse antibody molecules. In some instances somatic mutation has also been demonstrated, suggesting that an extensive diversity can be achieved, with both IgM and IgG antibodies being produced (Lonberg *et al.*, 1994). High avidity antibodies have been isolated; with a panel of human antibodies produced in this way to the human T cell marker CD4, K_a values of 10^9–10^{10} M^{-1} were produced (Fishwild *et al.*, 1996). Progress is also being made on the introduction of larger segments of the human Ig gene loci into mice, with transfer of large DNA segments or chromosome fragments containing the Ig loci now possible (Mendez *et al.*, 1997; Tomizuka *et al.*, 1997). This technology is very much in its infancy and, in the next few years, will no doubt develop to the point where a strain of mice can be produced which is capable of generating a range of useful human antibodies.

Attempts have been made to overcome many of the problems summarised in Table 1.3, and many human MAbs have been generated, but the technology is far from routine and relatively few high affinity antibodies have been produced. Recently an alternative technology for the production of antibodies which can bypass hybridoma technology has been developed, known as phage display. This can be used to produce human antibodies and is probably the method which will dominate human antibody production in the future.

1.6.4 Isolation of antibodies by phage display

An alternative approach to isolation of MAbs is to isolate the genes for the antibody directly and thus bypass hybridoma technology altogether. As the difference in antigen-binding specificities between antibodies lies entirely within the variable domains, it is only necessary to isolate genes for the variable regions which can then be joined to constant region genes by recombinant DNA techniques to allow expression of the required antibody molecule (see Chapter 2). Strategies for isolating antibody specificities using this approach have been developed over the past few years (Winter *et al.*, 1994; Burton and Barbas, 1994). These strategies attempt to mimic the key features of antibody generation by the immune system. To achieve this, repertoires of large numbers of antibody genes are isolated and expressed as antibody fragments displayed on the surface of bacteriophage. Ideally each phage displays a single antibody fragment. The phage with the appropriate antibody fragment displayed are then selected by binding to antigen attached to a solid support. Usually several rounds of selection are carried out to isolate the highest affinity antibody fragments. The selected phage can then be used to express soluble antibody fragment and to recover the antibody genes which are used to construct the desired form of antibody (Figure 1.9).

HOSPIRA EX. 1029
Page 30
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

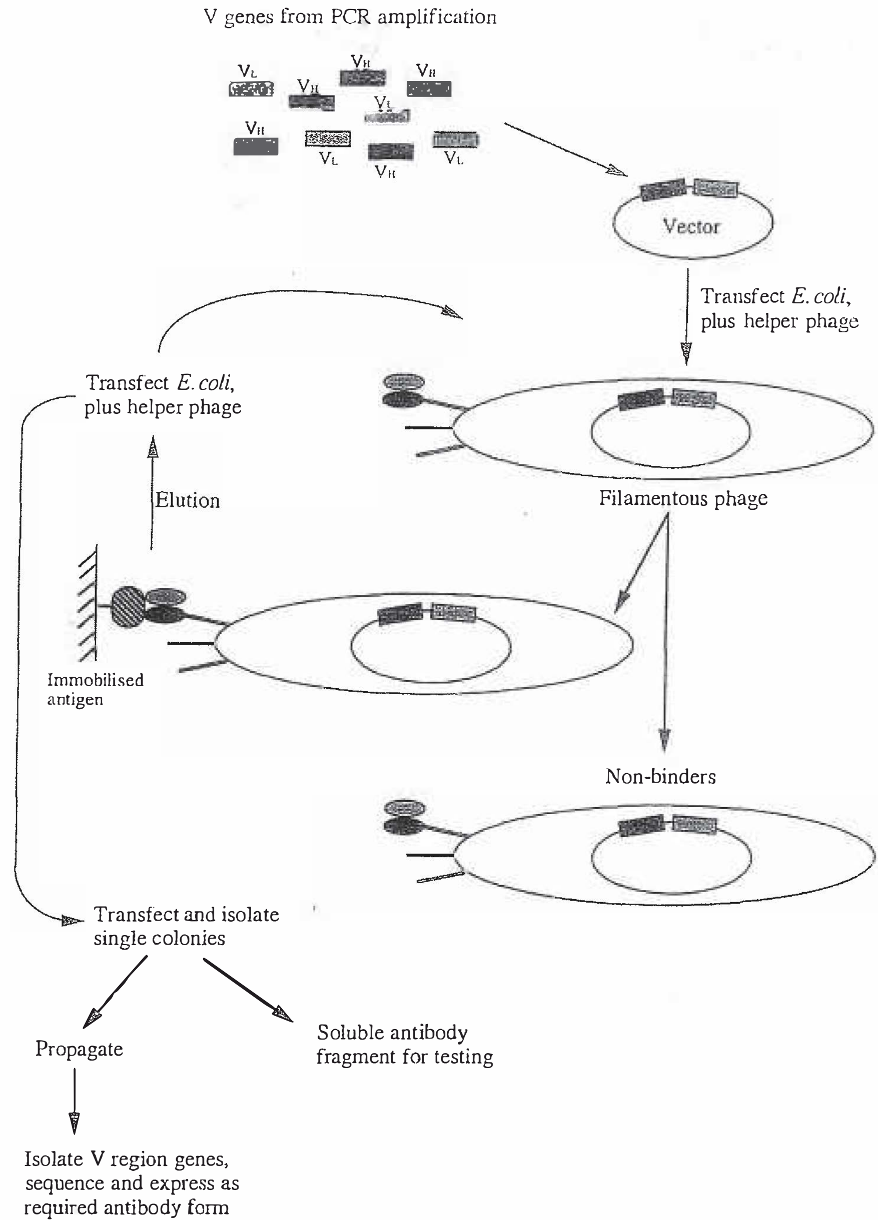


Figure 1.9 Production of monoclonal antibodies by phage display – see text for details

The first requirement is thus a repertoire of antibody genes. Several sources have been used. Genes can be isolated directly from B cells recovered from the spleen, bone marrow or peripheral blood of either immunised or non-immunised animals. Immunised animals contain larger numbers of genes encoding antibodies to the antigen of interest, and may

HOSPIRA EX. 1029
Page 31
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

also contain higher affinity antibody genes as a result of secondary immune responses. Therefore it is easier to produce high affinity antibodies from immunised animals. The technique is also applicable to non-immunised donors and has therefore attracted great interest as a technique for the production of human antibodies. As hybridoma technology is bypassed completely, phage display can also circumvent many of the other difficulties in producing human antibodies, such as immortalisation and instability of cell lines, discussed above (Table 1.3). The most common source of variable region genes is cDNA derived from mRNA and amplified by polymerase chain reaction (PCR) technology, although genes can also be recovered by PCR directly from genomic DNA. PCR technology amplifies DNA, producing many copies of the required sequences. Oligonucleotide primers are prepared for binding at the 5′ and 3′ ends of the target DNA on opposite strands which are then extended with a thermostable DNA polymerase. Several cycles of (a) DNA denaturation to single strands, (b) primer annealing and (c) extension by DNA polymerase are carried out in a thermal cycler that cycles between the three temperatures required for these processes. The amount of DNA increases exponentially with each cycle as each new DNA molecule can act as a template to produce new molecules. The flanking sequences of antibody variable region genes have been well characterised and sets of primers have been designed to allow both murine and human antibody genes to be amplified (Orlandi *et al*., 1989; Marks *et al*., 1991).

To allow selection of high affinity antibodies a large library of antibody genes is required in order to represent as much of the total repertoire of antibody genes as possible. The larger the library, the greater is the gene diversity and therefore the higher the chance of isolating high affinity specific antibodies. After immunisation of animals, or with humans who have been exposed to the required antigen, for example as a result of infection, the library constructed will have a large proportion of rearranged genes from immune B lymphocytes, and thus smaller numbers of antibody genes need to be screened to identify specific antibodies. Libraries from non-immunised individuals require screening of larger numbers but have the advantage that antibodies to different antigens can be isolated from a single library. Initial libraries were relatively small (10^7–10^9) and resulted in the isolation of weakly binding antibodies. Over the past few years rapid technological advances in library isolation have taken place, including the introduction of synthetic libraries in which random CDR sequences are introduced (Barbas, 1995). Library sizes of 10^{10} have now been achieved and allowed selection of high affinity, specific antibodies (Griffiths *et al*., 1994; Vaughan *et al*., 1996).

Two different types of antibody fragment have been widely used for display of antibody specificities on phage, Fab and single-chain Fv (scFv, see Chapter 2). Antibody fragments are used as these are efficiently expressed in bacterial systems (see Chapter 5) and they can be well presented on the surface of the phage. Phage display was first achieved using the phage λ (Huse *et al*., 1989), but subsequently filamentous phage such as M13, fd or f1 have been found to be more suitable systems (McCafferty *et al*., 1990; Clackson *et al*., 1991). These phages are single-stranded DNA phages which consist of a long single strand of DNA coated with approx. 2700 copies of the major coat protein pVIII and a low number of copies of four minor protein components, including the pIII protein which is normally present at 3–5 copies. The pIII protein is located at one end of the phage and is normally involved in infection of a bacterial cell to allow replication. Display at the phage surface is achieved by fusion of the antibody genes to one of the phage surface proteins. It was demonstrated by Smith (1985) that peptides could be presented on the surface of filamentous phage by fusion to the N-terminus of the pIII protein. This was extended to the fusion of antibody fragments resulting in their surface

HOSPIRA EX. 1029
Page 32
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

presentation in a form which could bind specifically to antigen (McCafferty *et al.*, 1990; Clackson *et al.*, 1991). Fusions to the pIII protein can be made by direct cloning into the phage genome, although this results in phage presenting an antibody fragment on each of its pIII proteins and results in phage of low infectivity due to preventing the normal function of the pIII protein. Also the presentation of multiple copies of the antibody fragment on each phage is not ideal as multivalent binding may then take place, where the low affinity binding of several copies may result in relatively high avidity interactions which may mask the binding of a single copy of a high affinity antibody fragment (Barbas *et al.*, 1991). A preferred system is therefore the use of phagemid vectors, plasmid vectors containing a viral origin of replication. Phagemids are co-introduced into the bacterial cell with helper phage which provide the genes for native phage proteins. Under appropriate conditions this results in a large number of phage particles containing a single antibody fragment (Garrard *et al.*, 1991). Several different vector systems have now been developed using either the whole or part of the pIII protein fused to either Fab or scFv fragments (Winter *et al.*, 1994; Burton and Barbas, 1994). The pVIII protein has also been used for phage display, although the large number of copies of this protein in the viral coat means that display of antibody fragments results in many antibody molecules on each phage, and thus this system has been relatively little used.

After assembly of the phage display library, selection must be carried out. This is done by binding to antigen which is attached to a solid phase. Antigen is often coated onto microtitre plates for this purpose or can be used in many other formats such as coated to beads or column matrices. Cells containing surface antigen can also be used or antigen in a form which can be readily captured such as biotinylated antigen, which can be captured on streptavidin-coated beads via the high affinity biotin–streptavidin interaction. Phage bound to the solid phase are then eluted either by competition with soluble antigen or by a change in pH or with a chaotropic agent to disrupt the antibody–antigen interaction in much the same way as used in immunoaffinity chromatography (see Chapter 3). Several rounds of selection are usually carried out to ensure isolation of specific binding phage.

After selection, the antibody genes are recovered and may be sequenced and manipulated as required. In some phage vector systems an amber codon is inserted between the pIII protein gene and that for the antibody fragment (Hoogenboom *et al.*, 1991). When grown in a supE supressor strain of *E. coli* the codon is read as glutamine and the antibody fragment is displayed on the phage surface, but when grown in a non-supressor strain the amber codon is read as a stop codon and soluble antibody fragment is secreted from the bacterial cell. This facilitates rapid analysis of the antigen-binding properties of the isolated antibody sequences without further genetic manipulation.

An interesting variation of phage display has been developed using phage which are only infective when carrying the desired antibody specificity (Spada and Pluckthun, 1997). Such selectively infective phage (SIP) are particularly useful in selecting antibody specificities from large libraries as they improve the specificity of the isolation of desired phage, reducing non-specifically binding phage from the system. Selective infectivity is conferred by using a truncated version of the pIII protein for display of the antibody fragment which is not sufficient to allow infectivity on its own. The pIII protein comprises three domains and the two N-terminal domains are required for phage infectivity. Antibody fragments can be displayed on the surface of phage through fusion to the C-terminal domain alone, resulting in non-infectious phage. Infectivity can then be restored through binding antigen fused to the N-terminal domains of pIII. Antigen–pIII fusions can be prepared either as fusion proteins or chemically, therefore allowing both protein and non-protein antigens to be used. As phage are only infective when antibody fragments

capable of binding antigen are present, background phage are reduced such that isolation of antibodies is simplified. Using this approach antibodies to a range of different antigens have been described (Spada and Pluckthun, 1997).

Although high affinity antibodies can be isolated directly from large libraries, it is often necessary to improve the antigen-binding affinity of antibodies isolated by phage display. Several strategies for this have been developed. This can be considered to be analogous to the process of affinity maturation undergone in the immune system to generate high affinity secondary response antibodies (see Section 1.2). One approach is to use mutagenesis strategies where sequences in the CDR regions are mutated either randomly or in a more directed fashion and antibodies with higher binding affinities selected by phage display (Yang *et al.*, 1995). This can be achieved either by several rounds of

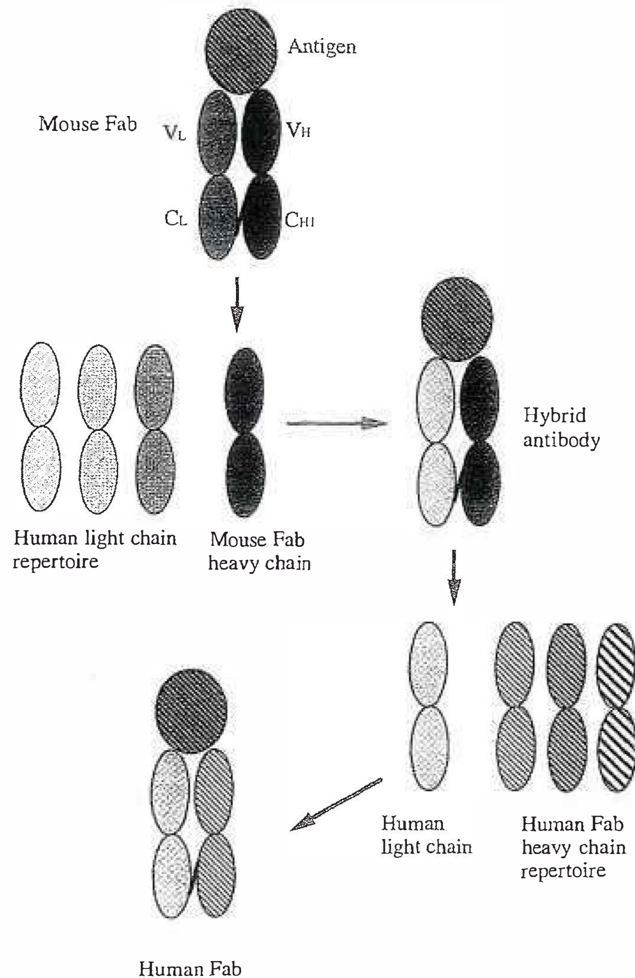


Figure 1.10 Epitope imprinted selection of human Fab from a mouse Fab to the same antigen (adapted from Jespers *et al.*, 1994)

sequential mutagenesis of CDR regions or by mutating them in parallel. To date sequential approaches have yielded the best results, with particular improvements coming from mutagenesis of CDR3 regions of the heavy and light chains. Antibodies to both HIV gp120 and the tumour-associated antigen p185^{HER2} have been improved to picomolar affinities by this approach (Yang *et al*., 1995; Schier *et al*., 1996). Residues outside the CDR regions may also have an impact on antigen-binding affinity (see Chapter 2) and mutagenesis strategies for these sequences may also need to be taken into account.

An alternative approach is to use chain shuffling for affinity maturation. In this technique one of the two antibody chains (e.g. VH) is used to recombine with a library of light chain genes and the higher affinity binding combination retrieved. The new antibody VL is then used to recombine with a library of heavy chain genes to find a higher affinity heavy chain. This can be repeated, combined with mutagenesis strategies or libraries of synthetic V gene sequences, to introduce further diversity and isolate higher affinity antibodies. In this way antibody affinities have been improved from K_d values of 10^{-7} M to 10^{-9} M (Marks *et al*., 1992).

An interesting technique for isolating human antibodies to antigens for which mouse antibodies already exist has been described based on chain shuffling (Jespers *et al*., 1994). This technique, known as epitope imprinted selection, starts with an existing mouse antibody heavy chain and selects light chains from a human light chain library which pair with the mouse heavy chain and bind to antigen. The light chain is then recombined with a library of human heavy chains and a human heavy chain is selected (Figure 1.10). This technique has been used to isolate a human antibody to TNFα with similar binding affinity to the original mouse antibody (Jespers *et al*., 1994).

The phage display approach offers several potential advantages over hybridoma technology. New antibody specificities can be isolated rapidly, with or without prior immunisation, and high affinity antibodies obtained. Human antibodies can be generated, including to self antigens which is difficult to achieve by other means (Griffiths *et al*., 1993) and antibodies to weak epitopes can be deliberately selected by masking immunologically dominant epitopes (Ditzel *et al*., 1995). However, it should not be seen as only a competing technology to hybridoma technology. It is possible to use both techniques to generate antibodies with different properties such that the most appropriate antibody for a specific application can be generated. In addition it is possible to use antibody chains isolated by hybridoma techniques in chain shuffling experiments with phage libraries and isolate novel high affinity antibody chains (Ames *et al*., 1995). Mutagenesis or chain shuffling techniques combined with phage display can also be used to increase the affinity or even change the epitope recognised by a pre-existing antibody, allowing manipulation of the antibody specificity (Jackson *et al*., 1995; Ohlin *et al*., 1996).

HOSPIRA EX. 1029
Page 35
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

2

Antibody Engineering: Design for Specific Applications

2.1 Introduction

Antibody molecules are very amenable to manipulation both chemically and by genetic engineering. As described in Chapter 1, the antibody molecule is made up of a series of domains which are capable of folding into their native structure independently. This has enabled many manipulations of the antibody molecule to be carried out without affecting the conformation of adjacent domains, and allows domains to be moved in position, or substituted with other molecules relatively easily. In addition, the beta sheet structure of the immunoglobulin fold allows some substitutions to be made within domains without loss of activity provided care is taken to ensure the integrity of the domain. In particular, as the antigen binding activity of the V domains is largely conferred by the CDR loops alone, it is possible to make changes in these loops, or switch them from one antibody to another, without disturbing the structure of the framework supporting them. This ease of genetic manipulation has led to the technology of antibody engineering which now allows the specific design of antibody molecules for particular applications.

Several factors must be considered in the design of antibody molecules, and these will be determined by the particular application for which the antibody is to be used. Some major issues to consider are listed in Table 2.1. A large range of modifications to the basic structure are possible, although the choice of one feature may limit the choices in other areas. Firstly, the antibody to be used must be of suitable specificity and affinity for the intended use, and subsequent manipulation should not usually result in alteration of the specificity, as this may lead to undesirable cross-reactivity with other, non-target, antigens. The affinity of the antibody for its antigen should not be drastically reduced, although in some instances this may be an advantage, for example in affinity purification applications. In some cases it has proved possible to improve affinity to a small extent and, in some therapeutic applications particularly, this may be advantageous. The valency of the antibody should also be considered and can be tailored for different applications. The normally divalent antibody can be made into monovalent antibody fragments or multiple binding sites linked together to increase the avidity of antigen binding. In addition, bispecific or even trispecific antibodies can be prepared with binding sites to more than one antigen. Antibody fragments of different sizes can be prepared which may have

HOSPIRA EX. 1029
Page 36
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 2.1 Issues in the design of antibodies for specific applications

Specificity and affinity of the binding site
Valency
Size
Requirement for effector function
Attachment of effector or reporter molecules
In vivo properties: immunogenicity
pharmacokinetics & metabolism
Cost of production

more appropriate properties *in vivo*, for example increased penetration into tissue or altered pharmacokinetics. The native effector or signalling functions of the antibody might be exploited for therapy, or specifically disabled if undesirable. Alternatively, a wide range of potentially therapeutic effector molecules, such as drugs, toxins, radionuclides or cytokines, can be attached to the antibody, as can reporter molecules for diagnostic uses. Specific properties can be designed into the molecule for *in vivo* use, such as appropriate pharmacokinetics and low immunogenicity to allow repeat usage. And of course, for industrial users the costs of producing the molecule required must be economic, and this can also be addressed by engineering. Forms of the antibody can be produced which are suitable for low-cost manufacturing and the use of high-yielding, low-cost expression systems can reduce costs further.

2.2 Isolation of variable region genes

At present it is not possible to design antibody variable domains capable of specific antigen binding *de novo*. Therefore a prerequisite for antibody engineering is the production of a suitable MAb and cloning of the variable region genes. With MAbs isolated by phage display, the gene is isolated directly, and variable region genes can be retrieved from hybridoma cells by several different methods. Early cloning of variable regions was carried out by isolating the rearranged genes from genomic clones (Seidman and Leder, 1978). Libraries of genomic clones constructed in a λ phage vector were size fractionated and screened with gene fragments or synthetic oligonucleotide probes to identify variable region heavy and light chain genes. This approach has the advantage that the Ig promoter and in some cases the Ig enhancer sequences can also be recovered as a single DNA fragment using suitable restriction sites which may be useful in subsequent expression studies (see Chapter 5). Another approach is to recover the genes from mRNA through cDNA cloning (e.g. Bothwell *et al*., 1981). As there is abundant mRNA for the antibody in most hybridoma lines, only small libraries of cDNA are required (approx. 5000–10 000 clones). The libraries are also screened with gene fragments or oligonucleotides to isolate the required genes. Care must be taken however, as hybridoma cell lines often contain aberrant variable region gene segments, derived from the fusion partner, some of which are transcribed. During both genomic and cDNA cloning these extra antibody sequences can be cloned inadvertently (Cabilly *et al*., 1984; Carroll *et al*., 1988; Neumaier *et al*., 1990), and thus all cloned sequences should be checked to ensure cloning of full length antibody variable regions for both heavy and light chains. In some cases the sequence of the antibody may be known or the N-terminal protein sequence can be determined, although in many cases identification of novel sequences is the goal. The extra

HOSPIRA EX. 1029
Page 37
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

light chains most commonly encountered as full length genes from mouse hybridomas are the κ138 or MOPC21 light chain (Cabilly and Riggs, 1985).

These cloning methodologies have now been largely replaced by cloning of variable region genes by PCR using the methods described in Section 1.7. This technique allows rapid isolation of variable region genes through PCR amplification, and can be carried out from very few cells (Liu *et al*., 1992). An excellent practical guide to isolation of variable region genes by PCR has been published (Adair, 1997). One problem using this technique is the introduction of errors into the sequence due to replication errors or cross-contamination. This is usually overcome by sequencing multiple independent clones. Also aberrant and extra gene sequences originating from the fusion partner may be identified as described above. These can be rapidly screened against with primers that anneal specifically to known sequences in these antibody chains. With this technique, as well as conventional cloning procedures, the best test of accuracy is a functional test of the antigen binding ability of the antibody expressed from the cloned sequence. This can be done rapidly by expressing the antibody either as murine antibody or directly as a chimeric antibody (see Section 2.3.1) in a transient expression system such as COS cells or CHO cells to produce small amounts of recombinant antibody for analysis (Whittle *et al*., 1987; see Chapter 5).

2.3 Overcoming immunogenicity

The application of MAbs for human therapy and diagnosis *in vivo* has been limited by many factors. One of the major problems encountered with the administration of murine MAbs to humans has been the generation of an immune response to the administered antibody protein known as human anti-mouse antibody response or HAMA. For antibody therapy in many types of diseases, repeat treatment will be required to achieve effective results. However, HAMA prevents repeat administration of antibody due to the formation of immune complexes which are rapidly cleared from the body, rendering repeat therapy ineffective. Also, in some cases, HAMA responses may result in adverse reactions such as allergic reactions or anaphylatic shock. Because of the problems in generating high-affinity human MAbs in many cases (discussed in Section 1.6), alternative solutions to this problem have been sought. These include the use of antibody engineering techniques to produce chimeric or fully humanised antibodies, chemical modification with polymers such as poly(ethylene glycol), the use of antibody fragments and immunosuppressant therapy.

2.3.1 Chimeric and humanised antibodies

One of the most successful approaches to overcoming immunogenicity has been the 'humanisation' of rodent MAbs by genetic engineering. A simple approach to making an antibody more human is the replacement of the constant domains of the antibody with constant domains of a human antibody (Morrison *et al*., 1984). The resulting chimeric antibody (Figure 2.1) contains only the variable regions of murine origin and would therefore be expected to be less immunogenic in people. Many chimeric antibodies have been prepared and shown to retain the full antigen binding ability of the parent murine antibody as well as taking on the constant region effector functions of the human antibody used.

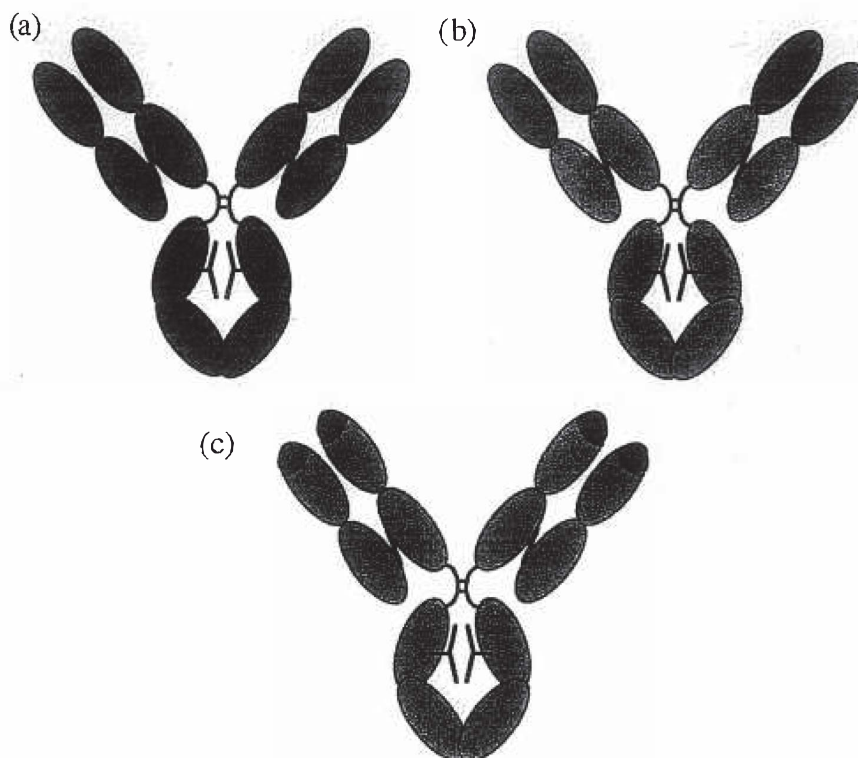


Figure 2.1 Humanisation of IgG: (a) parent murine antibody; (b) mouse:human chimeric antibody with the variable domains retained from the murine antibody and constant regions of human origin; (c) CDR-grafted antibody in which only the antigen-binding loops are retained from the murine antibody, the remainder of the sequence being of human origin

Construction of chimeric antibodies is relatively straightforward and requires simple ligation of the variable region genes 5′ to the constant region DNA and transfer into one of many available expression vectors. The constant region DNA can be in the form of genomic DNA (Whittle *et al.*, 1987) or cDNA (Liu *et al.*, 1987). If cloned by genomic cloning, a restriction site in the intron between the variable domain and constant domain can be used to assemble the required construct. As the intron sequences are removed during RNA splicing, alterations in the intron sequence are of little consequence. Alternatively cDNA clones require site-directed mutagenesis to introduce restriction sites at the V–C junctions to make the chimeric construct, taking care not to alter the required protein sequence. Also oligonucleotide linkers can be used to recreate variable region sequences from a convenient restriction site near the 3′ end of the V regions and introduce a splice donor site immediately after the coding sequence. A convenient restriction site is included to allow assembly of the chimeric gene. For PCR derived genes, primer design can allow incorporation of convenient sites for subsequent manipulation of the cDNA into an appropriate expression construct. PCR approaches using primers at the 5′ end of the coding region will not isolate the signal sequence, and thus this will need to be

added to allow secretion of the expressed antibody. Construction of an appropriate vector for expression of a chimeric antibody in mammalian cells requires assembly not just of the antibody heavy and light chain genes themselves but also of a suitable promoter and regulatory elements (see Chapter 5).

Chimeric antibodies have also been produced by homologous recombination in hybridoma cells (Yarnold and Fell, 1994). Homologous recombination is achieved by targeting a plasmid with the human constant region genes of choice to the immunoglobulin locus in the hybridoma cell, such that the murine constant regions are replaced with the targeted human sequences. This approach can be used to chimerise both heavy and light chains but at relatively low efficiency. The other drawbacks of this approach include the limited range of antibody engineering which can be achieved (for example, it is not possible to produce fully humanised antibodies by this technique) and the limitation of the expression level to that seen with the hybridoma, which may be significantly lower than expression levels achieved with many recombinant cell lines.

Transgenic mice have now been developed which can produce chimeric antibodies directly. A bacteriophage derived recombination sysytem known as Cre-*loxP* can be used to replace the mouse antibody constant domain gene segments in embryonic stem cells with human sequences (Zou *et al.*, 1994). Mice producing chimeric light chains have been produced as well as γl heavy chain recombinants and crossed together to produce offspring capable of producing γl antibodies with both chimeric heavy and light chains at similar levels to endogenous γl antibody in wild type mice. These mice are therefore theoretically capable of producing chimeric γl antibodies to any antigen to which the mice can respond. This technique has the advantage that chimeric constructs do not need to be made for each individual antibody, as it should be possible to recover chimeric antibodies directly by hybridoma or recombinant techniques; however, the disadvantages are similar to the use of recombination in cell lines, in that a relatively restricted amount of engineering is possible.

The HAMA response to murine MAbs is induced in a large proportion (50–100%) of patients receiving antibody (Khazaeli *et al.*, 1994). Table 2.2 summarises some of the data obtained from clinical trials with chimeric antibodies. Although care should be taken in interpreting the figures absolutely, as these are compiled from different investigators using different clinical protocols and different assays to measure the immune response, it is clear that the response to chimeric antibodies is reduced compared with HAMA responses to murine antibodies. The immune responses detected to chimeric antibodies have been directed toward the murine variable domains, and not to the human constant regions. In some cases responses to novel epitopes created at the interface of the murine variable and human constant regions have also been observed (Khazaeli *et al.*, 1991; Baker *et al.*, 1991). The chimeric antibodies vary in their extent of immunogenicity, although the reasons for this are not understood. It is possible that differences seen simply reflect the different immunogenicity of the antibody variable domains, and this has enabled predictions of the immunogenicity of individual chimeric antibodies in man to be made by testing immunogenicity in animals (Cartner *et al.*, 1993). The antibody responses observed towards the chimeric antibody variable domains include responses to both the framework regions and the antigen-binding loops themselves. Antibodies against the antigen-binding loops (the CDRs) are known as anti-idiotype antibodies, and can usually block antigen binding.

An alternative type of chimeric antibody produced from a monkey antibody variable region linked to human constant regions, termed a 'primatised' antibody, has also been produced (Newman *et al.*, 1992). Primate antibody sequences are more homologous to

Table 2.2 Immunogenicity of mouse:human chimeric antibodies in clinical studies

Antibody	Source	Indication	Dosing	Immune response	Reference
c17–1A (γ1)	Centocor	Colorectal carcinoma	1–3 doses, 4–40 mg	1/16, 6%	LoBuglio *et al.*, 1989 Meredith *et al.*, 1991
cB72.3 (γ4)	Celltech	Colorectal carcinoma	1–3 doses, 3–7 mg	16/24, 67%	Khazaeli *et al.*, 1991 Meredith *et al.*, 1992a
cB72.3 (γ4)	Celltech	Colorectal carcinoma	1–4 doses, 10–20 mg	3/6, 50%	Baker *et al.*, 1991
cAnti-CD4 (γ1)	Becton-Dickinson	Mycosis Fungoides	6 doses, 10–80 mg	2/7, 29%	Knox *et al.*, 1991
c14.18 (γ1)	Damon Biotech	Melanoma	1 dose, 5–100 mg	8/13, 62%	Saleh *et al.*, 1992
c14.18 (γ1)	Damon Biotech	Neuroblastoma	1–5 doses, 7–144 mg	3/5, 60%	Uttenreuther-Fischer *et al.*, 1995
cL6 (γ1)	InVitron/ Bristol Myers	Carcinoma (lung, breast or colon)	1–4 doses, 35–700 mg/m^2	4/18, 22%	Goodman *et al.*, 1993
NR-LU-13 (γ1)	NeoRx	Colorectal carcinoma	1 dose, 42 mg	6/8, 75%	Weiden *et al.*, 1993
C2B8	IDEC	B-cell lymphoma	1 dose, 10–500 mg/m^2	0/15, 0%	Maloney *et al.*, 1994
cAnti-CEA (γ4)	Ciba Geigy	Colorectal carcinoma	1 dose, 2–4 mg	2/9, 22%	Buchegger *et al.*, 1995
cMOv18 (γ1)	Centocor	Ovarian carcinoma	1 dose, 3 mg	0/24, 0%	Buist *et al.*, 1995

HOSPIRA EX. 1029
Page 41
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

human than murine sequences and thus it is possible that these may prove less immunogenic than mouse:human chimeric antibodies.

Humanisation of rodent antibodies can be taken further to produce fully humanised antibodies, in the form of reshaped, CDR-grafted or engineered human antibodies, in which much of the variable domain sequences are also replaced by human antibody sequence (Figure 2.1). In these approaches the antigen-binding loops are derived from the rodent antibody and much of the supporting framework is human. The relatively conserved structure of the human and rodent variable domain frameworks allow grafting of the hypervariable loops (CDRs) from one antibody onto another without loss of the overall conformation and structure of the loops and therefore allows antigen-binding ability to be retained. This should reduce any immune response against the variable domain framework. The remaining murine parts of the antibody, the CDRs, are also hypervariable in sequence in human antibodies and therefore more sequence variation may be permitted without generating immune responses.

The simplest form of CDR grafting involves the transfer of the CDR residues from the murine antibody to a human antibody variable domain framework. However, full antigen-binding activity is not achieved by this simple transfer, probably due to small conformational differences in the orientation of the antibody CDRs on the new framework structure. It has therefore proved necessary to introduce a small number of framework residue substitutions from the murine antibody to the humanised antibody, as well as the CDR residues, to restore full binding activity. Successful humanisation therefore requires several key choices to be made, including definition of the CDR residues, identification of human antibody framework sequences, and which if any framework residues to change to murine residues. Several different approaches to humanisation have been developed which largely reflect different approaches to making these choices. The first humanisation in this way was the heavy chain of the hapten binding antibody B1-8 (Jones *et al.*, 1986), and the humanisation of antibodies to complex protein antigens followed soon after (Verhoeyen *et al.*, 1988; Riechmann *et al.*, 1988a). Although difficulty in achieving full antigen binding activity was encountered early in the development of this technology, humanisation of murine antibodies is now relatively routine and many antibodies have been successfully humanised. A review of humanised antibodies, compiled in 1995, revealed over 100 then known examples in which humanisation of the variable domains had been successfully achieved (Adair and Bright, 1995).

The CDR residues are those residues most hypervariable in sequence between antibodies, and have been defined from a comparison of all known antibody sequences (Kabat *et al.*, 1991). All of the six antigen-binding loops are included within the six CDRs, which in several cases extend a short way beyond the structural loops. The choice of human framework is made from the known human antibody variable domain sequences. A human framework can be chosen for which a crystal structure is available, for example the heavy chains from the human antibodies KOL or NEW and the light chain from REI, so that the conformation of the loops can be modelled onto the existing structure and predictions made as to which framework residue substitutions may be required. Alternatively, the heavy and light chains from a single human antibody can be used, such as EU or LAY, or a consensus human antibody sequence. In many cases the human antibody of highest homology to the murine antibody to be humanised is used, as this requires fewer choices to be made with regard to possible framework substitutions. In addition, variable region sequences have been divided into a number of subgroups (Kabat *et al.*, 1991) which are useful in determining the choice of homologous framework.

HOSPIRA EX. 1029
Page 42
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

The remaining choices are which framework residues from the mouse sequence to transfer to the human framework. Some residues not in the CDRs may contribute to antigen binding directly or indirectly by affecting the conformation of the loops, by allowing stable packing of the domain structure or by interacting with the other variable domain at the heavy:light chain variable domain interface (Foote and Winter, 1992). The ideal way to determine which framework residues would be required to maintain an accurate conformation of the CDRs would be by analysis of an X-ray crystallographic structure of the binding site. However, the time taken to generate a crystal structure for each antibody to be humanised is usually prohibitive. Alternatively, these residues can be identified by generating a three-dimensional model of the humanised antibody variable regions and then evaluation of the role of particular residues (e.g. Queen *et al.*, 1989) or by alignment and analysis of the sequences to determine their likely structural location on the basis of known structures and thus enable estimation of the consequences of substitutions (e.g. Riechmann *et al.*, 1988a). In this way some unusual amino acids at particular positions in the individual human framework sequences can also be identified and changed. These approaches to humanisation have been alternatively termed CDR-grafting (Jones *et al.*, 1986), reshaping (Riechmann *et al.*, 1988a), hyperchimerisation (Junghans *et al.*, 1990) and civilisation (Kurrle *et al.*, 1990).

In some cases comparisons of different humanisation methods have been carried out with the same antibody. The antibody 0.5β recognises the gp120 envelope protein of HIV, and has been humanised using the human framework regions from REI for the light chain and from NEW for the heavy chain, both antibody chains for which structural information is available (Maeda *et al.*, 1991). Alternatively the heavy chain was humanised using the consensus sequence of human group I heavy chains with the same light chain. Although several variants were tested, the consensus sequence framework gave significantly more active antibody, probably as a result of its higher degree of homology with the 0.5β murine antibody (Maeda *et al.*, 1991). A similar result was observed with humanisation of the antibody CAMPATH-9 (Gorman *et al.*, 1991). In this case two heavy chain grafted variants were tested based on the framework sequences from NEW and KOL. Again the version using the antibody framework of highest homology was more active, in this case based on KOL. Other examples are the heavy chain of the anti-CD18 antibody 1B4 (Singer *et al.*, 1993), which was humanised using three different frameworks (NEW, GAL and JON), and the anti-interleukin 6 receptor antibody AUK12-20, which was humanised using two different framework sequences, HAX and consensus group I (Sato *et al.*, 1994). In each case the best retention of antigen-binding activity was achieved using the most homologous human antibody framework, be it a consensus sequence or from an individual human antibody.

A further approach to humanisation has been developed, known as veneering or resurfacing (Padlan, 1991). In this approach it is proposed that the immunogenicity of murine framework regions is determined by the surface residues alone and thus identification of the surface residues not involved with antigen binding and their substitution with 'human' residues will reduce immunogenicity. This approach has been successfully applied with retention of antigen-binding activity (Roguska *et al.*, 1994) and also combined with the 'traditional' CDR-grafting technique to humanise an antibody in an approach referred to as framework exchange (Benhar *et al.*, 1994).

Once designed, the humanised antibody variable domain can be assembled by several procedures. Site-directed mutagenesis of a single stranded DNA template (Riechmann *et al.*, 1988a), or by gene synthesis with alternating overlapping oligonucleotides, filling the gaps with DNA polymerase (Queen *et al.*, 1989), or by PCR procedures to gap fill

and amplify the DNA (Daugherty *et al.*, 1991). Of these the PCR approach is the most rapid and efficient although errors may be introduced, so several PCR assembled variable region genes may need to be sequenced to identify the correct product. The humanised variable region genes are then joined to constant region genes as described for chimeric antibodies.

For all humanisation approaches it is usually useful to produce several humanised variants of the antibody and produce small amounts of each for analysis of antigen binding or bioactivity. This can be achieved rapidly by transient expression of small amounts of antibody in mammalian cell systems such as COS or CHO cells (Whittle *et al.*, 1987; Baker *et al.*, 1994 – see Section 5.2). In theory it is only necessary to express the variable region genes as an Fv or Fab fragment to produce material for analysis (e.g. Benhar *et al.*, 1994). This can be achieved rapidly in *E. coli* expression systems; however, the analysis of the antigen-binding ability of these monovalent antigen-binding species is complicated by the possibility of formation of small amounts of dimeric or aggregated material which can greatly influence the outcome of the antigen-binding affinity measurement. For example, it has been shown that the presence of only 0.5% of $F(ab')_2$ in a monovalent Fab′ preparation can overestimate the antigen-binding measurement fivefold (Desplancq *et al.*, 1994). For this reason it is preferable to measure the antigen-binding ability of stable dimeric molecules such as IgG.

In some instances unexpected increases in antigen-binding affinity have been reported on humanisation (Co *et al.*, 1992; Carter *et al.*, 1992a, Baker *et al.*, 1994). This may be due to removal of a glycosylation site from the variable region (e.g. Co *et al.*, 1993), although in other cases the reason is unknown and may reflect subtle changes to the conformation of the binding site (e.g. Baker *et al.*, 1994). However, care must be taken to characterise the engineered antibody produced to ensure that properties of the antigen-binding site are not altered in an undesirable fashion, such as leading to a reduced specificity or some unexpected property. Subtle alterations in the recognition of the antigen by the engineered antibody are possible which may only be revealed by a detailed analysis (Kelley *et al.*, 1992).

Several humanised antibodies have now been examined in clinical studies and thus information on the success of humanisation in overcoming immunogenicity is beginning to emerge. Direct comparisons of immunogenicity of humanised antibodies with the parent murine antibody have not been carried out in patients, but several reports have appeared on the immunogenicity of humanised antibodies in monkeys compared to murine antibody (Hakimi *et al.*, 1991; Singer *et al.*, 1993; Stephens *et al.*, 1995). In each case, the immune response was much reduced compared to the parent murine antibodies, with an anti-idiotype response (i.e. directed to the CDRs) developed on repeat dosing. In the case reported in greatest detail (Stephens *et al.*, 1995), the CDRs appear less immunogenic when presented in the human framework since the anti-idiotype response to the humanised antibody was greatly reduced compared to the anti-idiotype component of the immune response to the murine antibody.

The immunogenicity of humanised antibodies in patients is also low, as summarised in Table 2.3. The earliest studies were carried out with the humanised antibody CAMPATH-1H. Two non-Hodgkin lymphoma patients treated with 1–20 mg doses of this antibody for up to 43 days showed no anti-CAMPATH-1H response during the course of treatment (Hale *et al.*, 1988). However, these results should not be over-interpreted, because such patients are somewhat immunocompromised prior to treatment and because the treatment itself is likely to be immunosuppressive. In subsequent studies CAMPATH-1H was administered to eight rheumatoid arthritis patients (Isaacs *et al.*, 1992) and four

Table 2.3 Immunogenicity of humanised antibodies in clinical studies

Antibody	Source	Indication	Dosing	Immune response	Reference
CAMPATH-1H	Univ. Cambridge/ Wellcome	Non-Hodgkin lymphoma		0/2	Hale *et al.*, 1988
CAMPATH-1H	Univ. Cambridge/ Wellcome	Rheumatoid arthritis	5 × 4 mg + 5 × 8 mg	0/8, 3/4 on retreatment	Isaacs *et al.*, 1992
CAMPATH-1H	Univ. Cambridge/ Wellcome	Systemic vasculitis	3 doses, 2–40 mg	1/4	Lockwood *et al.*, 1993
Humanised anti-Tac	PDL/Hoffman La Roche	Graft v. host disease	1–2 doses, 0.5–1.5 mg/kg	0/20	Anasetti *et al.*, 1994
Humanised M195	PDL/MSKCC	Myeloid leukemia	6 or 12 doses, 0.5–10 mg/m^2	0/13 (3 non-specific responses)	Caron *et al.*, 1994
CDP571	Celltech	Healthy volunteers	1 dose, 0.1–10 mg/kg	4/4 at 0.1 mg/kg 0/4 at 2, 5 and 10 mg/kg	Stephens *et al.*, 1995
hMN-14	Immunomedics	CEA producing cancer	1–3 doses, 1–20 mg	0/6 (without exposure to murine ab)	Sharkey *et al.*, 1995
rhuMAb HER2	Genentech	Breast cancer	250 mg + 10 doses 100 mg	0/46	Baselga *et al.*, 1996

HOSPIRA EX. 1029
Page 45
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

patients with systemic vasculitis (Lockwood *et al.*, 1993). In the rheumatoid arthritis study, 4–8 mg doses were given over 10 days. Clinical benefit was seen in seven of the patients, and anti-CAMPATH-1H antibodies were not detectable in any after one course of treatment. Of four patients given a second course of treatment, three showed a detectable anti-CAMPATH-1H response. In the study in systemic vasculitis, one out of four patients developed an anti-idiotype response after treatment. No data are yet available concerning the nature of this response, whether it interferes with efficacy, and if so, whether such interference can be overcome using larger doses. A study with a humanised anti-Tac antibody in patients with graft versus host disease noted no immune responses in any of the patients (Anasetti *et al.*, 1994); however, caution must also be exercised in the interpretation of this study due to the severe immunodeficiency of these patients. The humanised anti-CD33 antibody hM195 has been evaluated in 13 patients with myeloid leukemia at 0.5–10 mg/m^2 with 6 or 12 doses per patient without the onset of any immune response (Caron *et al.*, 1994). The immune response to the humanised anti-tumour necrosis factor antibody CDP571 has been examined after administration of a single dose of 0.1 to 10 mg/kg to fully immunocompetent human volunteers (Stephens *et al.*, 1995). Administration of the lower doses led to the development of a weak anti-idiotype response, predominantly of the IgM isotype, although at higher doses responses were very low or undetectable. The weak IgM anti-idiotype antibodies raised at low doses did not appear to result in rapid clearance of the circulating antibody or to neutralise the ability of the circulating CDP571 to bind TNF, suggesting that this type of response would not be problematic. This type of weak immune response is also seen with the administration of human MAbs (e.g. Steiss *et al.*, 1990) and may be indicative of the humanised antibody behaving in a similar manner to administered human antibody. In a study with a humanised anti-p185^{HER2} antibody, rhuMAb HER2, 46 patients were administered 250 mg of antibody followed by 10 weekly doses of 100 mg each (Baselga *et al.*, 1996). No immune responses were observed. The absence of immune responses after administration of these large doses of antibody is consistent with the results of the other studies. The humanised anti-tumour antibodies hMN-14 and Hu2PLAP have also been studied in humans, but in these cases the analysis of immune response data is difficult due to responses to chemical groups attached to the antibody (Kalofonos *et al.*, 1994) or the administration of murine antibody to the same patients (Sharkey *et al.*, 1995). Nevertheless, in patients receiving hMN-14 alone no immune response was detected (Sharkey *et al.*, 1995). These results taken together demonstrate that fully humanised antibodies have low immunogenicity, and should allow prolonged or repeat therapy in man.

2.3.2 Antibody fragments to reduce immunogenicity

The use of fragments of antibodies has also been advocated as a possible means to reduce the immunogenicity of rodent antibodies in man. Antibody fragments such as Fab, F(ab′)$_2$ and Fv can be produced from IgG by proteolytic digestion, or directly by recombinant means (see Section 2.4). Several comparative studies have shown that antibody fragments have reduced incidence of HAMA formation compared to IgG (e.g. Breitz *et al.*, 1992; Buist *et al.*, 1995), presumably as a result of the absence of the immunogenic rodent constant regions. However, there is still a sufficient immune response to murine Fab to prevent repeat dose therapy. To reduce responses, recombinant mouse:human chimeric and humanised antibody fragments have been produced. Similarly to studies with IgG, presentation of the murine variable region in a chimeric Fab fragment leads to less

immune reponse than when the murine fragment is used. For example, the Fab fragment of the antibody 7E3 which is directed against the platelet glycoprotein IIb/IIIa receptor has been evaluated in clinical studies as both a murine Fab and a mouse:human chimeric Fab. Immune responses to the chimeric Fab in a large phase III trial of approximately 1400 patients were seen in 5.8% of cases, whereas in similar patients treated with murine Fab, immune responses were detected in approximately 45% of patients (Knight *et al.*, 1995). This was seen even though most of the reponses towards the murine Fab were directed toward the variable region which is identical to that of the chimeric Fab. This result therefore suggests that the presentation of the variable region attached to a human constant region results in a significant advantage over murine antibody fragments.

2.3.3 Chemical modification to reduce immunogenicity

An alternative strategy which has been used to reduce the immunogenicity of many different proteins including MAbs is chemical modification. Addition of polymers such as poly(ethylene glycol) (PEG) and low molecular weight dextran have been shown to be able to reduce the immunogenicity of antibodies in animal model experiments (Kitamura *et al.*, 1991; Fagnani *et al.*, 1990).

PEG can be attached to antibody using a variety of different chemical linkages, usually by attachment to amine groups of lysine residues on the surface of the protein. Some of the methods which have been used for attachment of PEG to antibody are shown in Figure 2.2. Attachment via cyanuric chloride was the first method developed for PEG attachment to protein (Abuchowski *et al.*, 1977), but is relatively harsh and often leads to inactivation of the biological function of the protein. Attachment via *N*-hydroxysuccinimide esters is now preferred, as it is a more gentle procedure (Zalipsky and Lee, 1992). Other lysine-directed attachment methods such as tresyl chloride-activated PEG are now available and have been used for antibody fragments (Delgado *et al.*, 1996). A two-step method for attachment to lysine via thiol groups has also been described (Pedley *et al.*, 1994). In this method stable attachment is achieved under mild conditions by modification of lysine residues with 2-iminothiolane (Traut's reagent) to generate free thiol groups on the antibody, followed by reaction with a thiol-specific, PEG–maleimide reagent. This method has the advantage that the number of thiol groups introduced can be accurately measured and the consequent coupling of PEG to the antibody closely controlled.

PEG conjugation to antibodies results in reduced immunogenicity and also changes in other biological properties such as resistance to proteolysis and increased circulating half-life (see Section 2.7). The magnitude of the effect may be related to the degree of substitution with PEG achieved, i.e. the number of PEG molecules per antibody molecule. Attachment of large numbers of PEG molecules often leads to reduced ability to bind antigen, particularly if harsh attachment methods are used which may also damage other sites in the protein. Therefore, production of suitable conjugates is a compromise between attaching as much PEG as possible and retaining antigen-binding ability. For example, conjugates of a murine MAb prepared by Kitamura *et al.* (1991) showed reduced antigen-binding ability with 10 and 15 PEG molecules per antibody, although a conjugate with 5 PEG molecules attached was relatively unaffected. The conjugate with 10 PEG molecules attached did not elicit an immune response after multiple administrations to rabbits, although the unmodified antibody readily raised an immune response.

An alternative approach has been to use highly substituted IgG–PEG conjugates as tolerogens. A human IgG–PEG conjugate, with 20 PEG molecules per antibody, administered to mice was able to prevent 85–90% of the immune response to subsequently

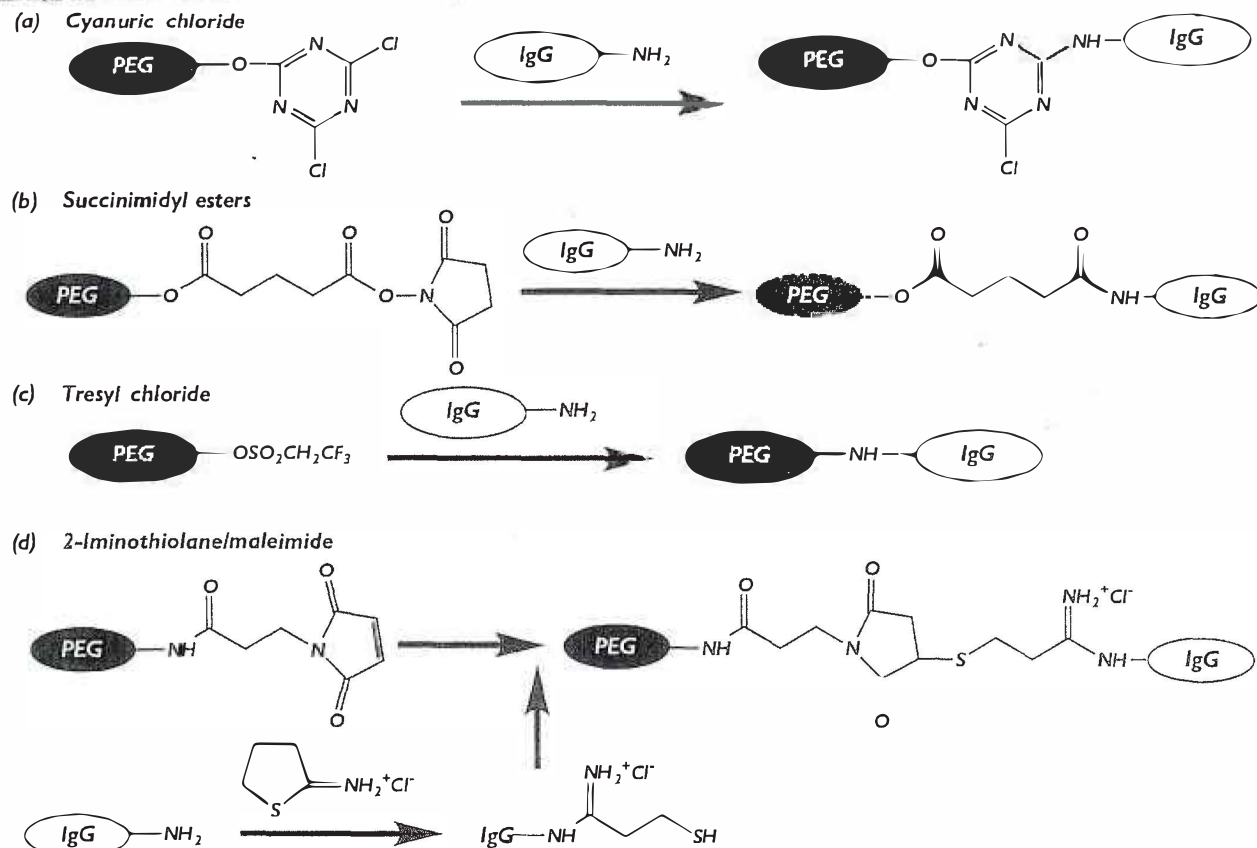


Figure 2.2 Examples of chemical methods for attachment of poly(ethylene glycol) to antibody: (a) attachment via cyanuric chloride-activated PEG; (b) attachment via succinimidyl active esters, e.g. succinimidyl glutarate-activated PEG; (c) attachment via tresyl chloride-activated PEG; (d) two-stage attachment via 2-iminothiolane and PEG–maleimide

HOSPIRA EX. 1029
Page 48
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

administered unmodified human IgG (Wilkinson *et al*., 1987). Subsequent studies have demonstrated that specific immunosuppression of HAMA can be achieved using this approach, such that reponses to the determinants of individual antibody protein chains are suppressed without affecting responses to other antigens (Bitoh *et al*., 1993).

The covalent attachment of low molecular weight dextrans has also been shown to reduce immunogenicity of IgG in a similar manner to PEG attachment. Conjugates with up to 17 dextran molecules per antibody have been prepared which retain >50% of their immunoreactivity (Fagnani *et al*., 1990). Tests of immunogenicity in animals reveal a significant reduction in immune response compared to the unmodified antibody.

2.3.4 *Immunosuppressive therapy*

A further approach to reduce the incidence of HAMA response has been to treat patients with immunosuppressive drugs. For example, cyclosporin A has been used to allow repeat dosing of a murine anti-carcinoembryonic antigen (CEA) antibody to patients with CEA producing tumours (Ledermann *et al*., 1988). A significant reduction in HAMA was observed which allowed up to four administrations of murine antibody to be given. However, cyclosporin A was effective for only a limited time and secondary responses were observed in some patients. Cyclosporin A and similar drugs are general immunosuppressants, and while their use may be possible in cancer and other severe diseases, their routine use for antibody therapy is unlikely.

2.4 Antibody fragments

Fragments of MAbs have been of interest for many years as small antigen targeting molecules. Antibody fragments are also useful 'building blocks' for the assembly of molecules designed to carry other agents to a desired antigen such as diagnostic reagents or therapeutic conjugates. For *in vivo* applications, fragments of antibodies have been of interest due to their altered pharmacokinetic behaviour which has been investigated for cancer therapy with cytotoxic agents, and for their rapid penetration into body tissues which may offer advantages for imaging and therapy techniques. Also, effector functions can be disabled by removal of the Fc portion, and cell internalisation may be disabled by making a monovalent binding fragment. Initially antibody fragments, notably $F(ab')_2$ and Fab fragments and occasionally functional Fv fragments, were produced by proteolysis of IgG molecules. More recently recombinant techniques have extended the range of antibody fragments available by allowing novel fragments to be produced, and have extended the range of potential applications by opening up the possibilities of economical large-scale manufacturing.

2.4.1 *Antibody fragments from proteolysis of IgG*

The production of Fab and $F(ab')_2$ fragments of antibodies by proteolysis of IgG has been established for many years (Figure 2.3). As described in Chapter 1, the area around the antibody hinge is more susceptible to proteolysis than the tightly folded domains and thus this is the point at which cleavage usually takes place. Proteolysis above the disulphide bonds in the hinge region with, for example, papain results in Fab fragments which are monovalent for antigen binding, whereas proteolysis below the hinge disulphide bonds,

HOSPIRA EX. 1029
Page 49
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

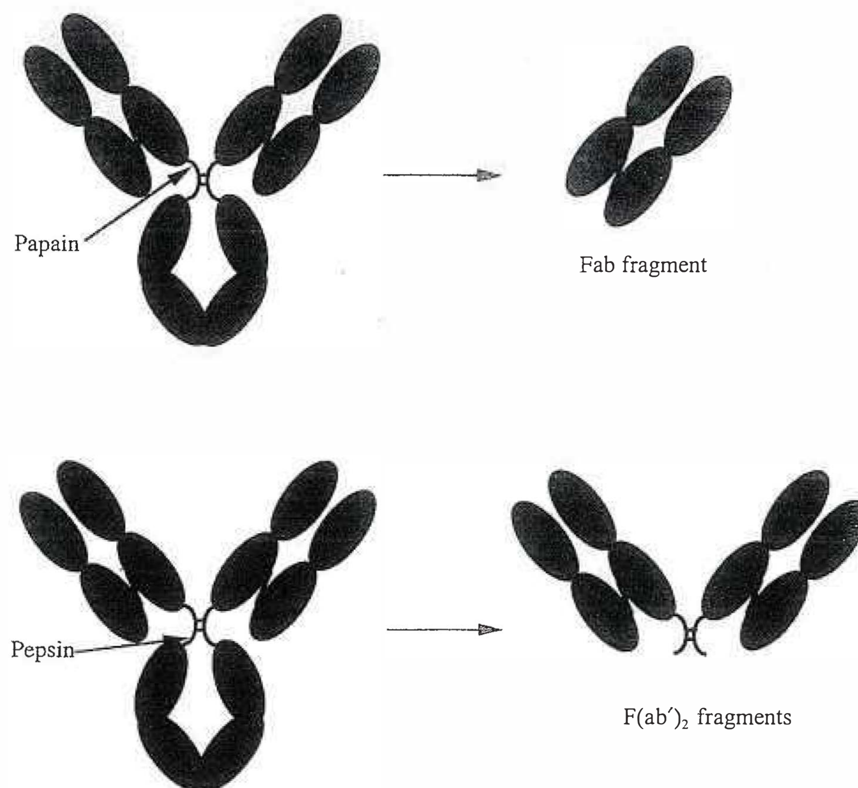


Figure 2.3 Diagram to represent the difference in cleavage point between the proteases pepsin and papain: papain cleaves IgG above the hinge region cysteines leading to the production of monovalent Fab fragments; pepsin cleaves IgG below the hinge region cysteines leading to the divalent $F(ab')_2$ fragment .

with enzymes such as pepsin, results in the divalent $F(ab')_2$ fragment. Fc fragment is also produced during the production of Fab fragment by papain digestion, although during the production of $F(ab')_2$ fragments with pepsin the Fc portion is substantially degraded. Typical enzymes used to produce Fab and $F(ab')_2$ fragments from murine IgG are given in Table 2.4. Fab′ fragments, which are Fab fragments with an attached hinge region, can be produced by selective reduction of the hinge disulphide bonds of $F(ab')_2$ fragments. The digestion of IgG is very subclass-dependent and different experimental conditions are required for the production of fragments from different antibody subclasses (Parham, 1986). Individual antibodies within a subclass can also vary in their susceptibility to digestion (e.g. Rea and Ultee, 1993). Therefore, to maximise the yields of active fragments achieved, it is usually necessary to carry out small-scale experiments to optimise the enzyme used, buffer and pH, incubation time and the ratio of enzyme to antibody.

The production of Fab fragments with the non-specific thiol protease papain or other related thiol proteases, such as ficin, is generally successful across a range of antibody types, but the production of active $F(ab')_2$ fragments is more variable. Murine monoclonal IgG1 $F(ab')_2$ can be produced in good yield with bromelain or ficin (Milenic *et al.*, 1989;

Table 2.4 Enzymes commonly used for the production of murine antibody fragments by proteolysis of monoclonal IgG

	Fab	$F(ab')_2$
IgG1	Papain	Bromelain, ficin, pepsin, papain
IgG2a	Papain	Pepsin
IgG2b	Papain, pepsin	Lysyl endopeptidase*
IgG3	Papain	Pepsin

*Based on one report, general applicability currently unknown.

Mariani *et al*., 1991) and in a few cases with appropriate buffer conditions with pepsin (Parham, 1986; Rea and Ultee, 1993). $F(ab')_2$ fragments can usually be generated from murine IgG2a and IgG3 antibodies with pepsin. However, the production of $F(ab')_2$ from murine IgG2b has not proved possible with pepsin or other enzymes, with rapid degradation to Fab/c and smaller fragments taking place (Parham, 1986), although one report suggests that careful digestion of a murine IgG2b with lysyl endopeptidase can allow $F(ab')_2$ formation (Yamaguchi *et al*., 1995). Conditions for $F(ab')_2$ production from the rat IgG subclasses have been described (Rousseaux *et al*., 1983), and human IgG1 and IgG4 $F(ab')_2$ can be produced readily with pepsin and bromelain respectively. It is also possible to produce fragments of IgM antibodies including Fab and $F(ab')_2$ with, for example, pepsin and trypsin (Parham, 1986), although as the individual binding site affinities of IgM antibodies are generally of low affinity (normally compensated for by the high avidity of the pentameric molecule) there has been relatively little use of these for other than experimental investigations. Similarly, murine IgA Fab, IgD Fab and IgE $F(ab')_2$ have been produced by proteolysis (Parham, 1986).

The production of other types of antibody fragment by proteolysis is more difficult. There has been much interest in the production of smaller antigen-binding fragments which may be more effective at penetrating tissues. The Fv fragment comprises only the paired heavy and light chain variable domains and is thus approximately half the size of a Fab fragment. Fv fragments were first prepared by pepsin digestion of the murine IgA myeloma protein, MOPC315 (Inbar *et al*., 1972). However, attempts to produce Fv fragments of other antibodies have proved largely unsuccessful, being restricted to a small number of cases of digestion of IgM (Lin and Putnam, 1978), IgA myeloma protein (Sharon and Givol, 1976), IgG containing λ light chain (Ornatowska and Glasel, 1991) and IgG2a with a deleted C_{H1} domain (Takahashi *et al*., 1991).

Although they are of great utility for experimental applications, the problems of expense, heterogeneity of product, difficulty of purification and limited transferability from one antibody to another have limited the applications of antibody fragments produced by proteolytic means. Also, the range of fragments which can be produced in this way is restricted, and thus much effort has recently been invested to investigate the potential of recombinant antibody fragments.

2.4.2 *Recombinant antibody fragments*

The direct expression of recombinant antibody fragments allows a range of antibody fragments to be produced using any monoclonal antibody specificity without the restrictions imposed by proteolysis of the native antibody structure. $F(ab')_2$, Fab′, Fab and Fv can all

be readily produced by expression of the appropriate gene segments in a suitable host, as can a range of novel fragment types. Antibody fragments can be expressed in mammalian cells and also in microbial systems, particularly *E. coli* (see Chapter 5). Two modes of expression are available for use with *E. coli*, intracellular expression and secretion to the periplasm. In common with other types of protein expressed in *E. coli*, intracellular expression of antibody fragments usually results in the accumulation of the expressed protein in a denatured form as an insoluble inclusion body which requires solubilisation and refolding to obtain active material (e.g. Bird *et al.*, 1988; Field *et al.*, 1989). This is often an expensive and low yielding procedure. Alternatively, attachment of a signal sequence to each protein chain can result in secretion of the antibody fragment to the periplasm, from which soluble active protein can be recovered (Skerra and Pluckthun, 1988; Better *et al.*, 1988). The amount of antibody fragment secreted varies between antibodies and between different types of antibody fragment. In general smaller antibody fragments are produced more efficiently than larger fragments, and therefore *E. coli* has become the most widely used expression host for Fv-based fragments, whereas with Fab fragments the most efficient expression system appears to depend on the particular antibody studied.

Fab-based fragments

Fab and Fab′ fragments are produced by co-expression of the light chain and Fd or Fd′ fragment of the heavy chain. These vary only in the presence of the hinge region in Fd′. Fab fragments are relatively stable due to extensive non-covalent interactions between the protein chains and also the presence of a disulphide bond anchoring the chains together. Assembly of expressed light chain and truncated heavy chain (Fd or Fd′) has generally been efficient. However, expression of Fd′ with light chain results in Fab′ production, with little or no $F(ab')_2$ produced. Expression in both mammalian cells and *E. coli* has been shown to result in high yields of Fab and Fab′ fragments and may result in more homogeneous molecules than those obtained by proteolysis.

For many applications the increased avidity of the bivalent molecule may be important and therefore attempts to produce bivalent antibody fragments have been made. Direct expression of $F(ab')_2$ can be achieved, but requires design of a hinge region containing multiple disulphide bonds. Hinge regions with single cysteine residues have resulted in very little $F(ab')_2$ production (Carter *et al.*, 1992a; King *et al.*, 1994) and hinge regions containing two cysteine residues results in only 10–30% $F(ab')_2$ formation (King *et al.*, 1992a; Better *et al.*, 1993). Engineering a hinge region with three potential disulphide bonds has resulted in the production of approx. 70% $F(ab')_2$ from Fab′ expressed in *E. coli* (Rodrigues *et al.*, 1993) and inclusion of five disulphide bonds in the hinge region has resulted in quantitative $F(ab')_2$ formation (King *et al.*, unpublished data). Alternatively, recombinant $F(ab')_2$ can be produced by *in vitro* re-oxidation of the hinge thiols of the expressed Fab′ (King *et al.*, 1992a) or chemically cross-linked $F(ab')_2$ can be produced by specifically cross-linking the hinge thiols (Carter *et al.*, 1992a; King *et al.*, 1994) (Figure 2.4). If chemical cross-linking is used a single hinge thiol is desirable to reduce side-reactions and allow high cross-linking yields. This is easily designed into the Fab′ construct to be expressed. Cross-linking is achieved using a cross-linker with two thiol reactive groups, usually maleimide groups, and can be used to generate $F(ab')_2$-like molecules with two Fab′s of the same antibody or using two different Fab′s to produce bispecific antibody fragments (Glennie *et al.*, 1987; see Section 2.5). Chemical cross-linking also allows the possibility of incorporating extra moeities into the cross-linking reagent and therefore the site-specific attachment of reagents away from the antigen-binding site (Figure 2.4).

HOSPIRA EX. 1029
Page 52
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

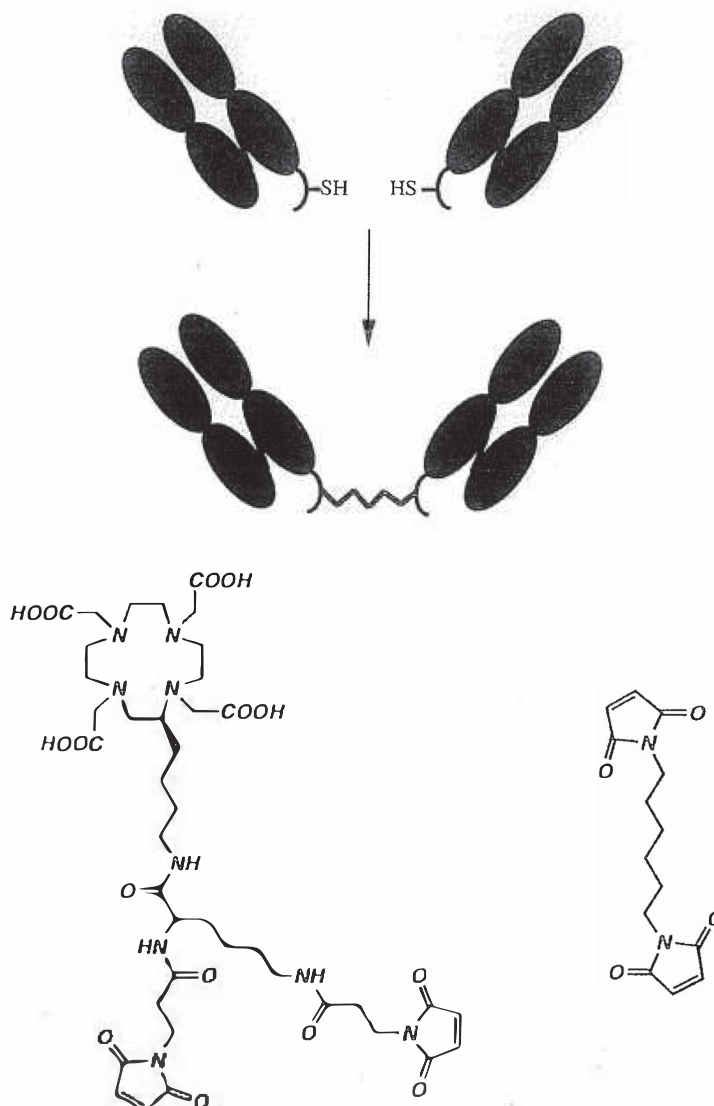


Figure 2.4 Chemical cross-linking of Fab′ fragments to form chemically cross-linked di-Fab, using either two Fab′s of the same specificity or two of different specificity, in which case a bispecific fragment is produced. Also shown are the structures of two linkers which can be used for this purpose. Hexane bis-maleimide is a simple linker which allows di-Fab formation through cross-linking cysteine thiol groups. Alternatively linkers can be used which contain sites for site-specific attachment. In this case a 12N4 macrocycle is incorporated for site-specific labelling with radioactive metals (King *et al.*, 1994).

For example, cross-linkers containing macrocyclic ligands for the stable attachment of radioisotopes have been used to allow formation of radiolabelled antibody fragments without the need for further derivatisation of the antibody (King *et al.*, 1994).

Fv-based fragments

The two chains of the Fv fragment are less stably associated than the Fd and light chain of the Fab fragment with no covalent bond and less non-covalent interaction, but nevertheless functional Fv fragments have been expressed for a number of different antibodies. Characterisation of Fv fragments has revealed that although the antigen-binding ability of the Fab fragment is retained, dissociation of the two domains takes place at low protein concentration or under physiological conditions. Thus strategies for stabilising Fv fragments have been of great interest. Two strategies have been investigated with a number of different antibodies: firstly, mutating a selected residue on each of the VH and VL chains to a cysteine to allow formation of a disulphide bond between the two domains; and secondly, the introduction of a peptide linker between the C-terminus of one domain and the N-terminus of the other, such that the Fv is produced as a single polypeptide chain known as a single-chain Fv (Figure 2.5).

The production of single-chain Fv's (scFv's) was first described in 1988 (Bird *et al.*, 1988; Huston *et al.*, 1988). Since then a large number of scFv's have been produced which retain antigen-binding characteristics, although in some cases antigen-binding affinity is reduced. Expression in *E. coli* has been the most frequently used production method, with both intracellular expression and secretion enabling high yields of scFv to be made. The production of scFv molecules requires the identification of a suitable peptide linker to span the 35–40 Å distance between the C-terminus of one domain and the N-terminus of the other and allow correct folding and assembly of the Fv structure. Several different types of linker have been used and shown to result in functional scFv. Linkers have been selected from searching existing protein structures for protein fragments of the appropriate length and conformation, or have been designed *de novo* based on simple, flexible structures with perhaps the most commonly used being the 15 amino acid sequence

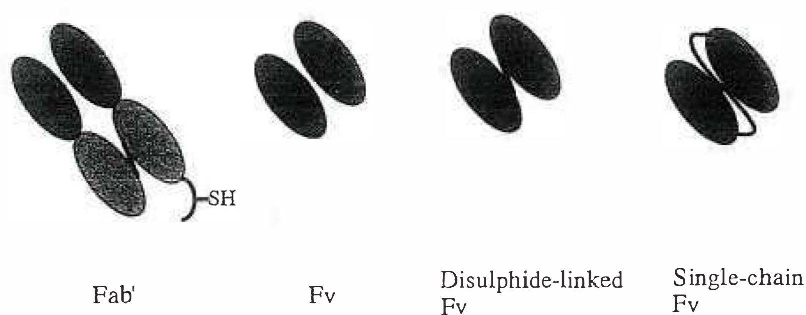


Figure 2.5 The organisation of domains in a Fab′ fragment compared to Fv, disulphide-linked Fv and single-chain Fv fragments: disulphide-linked Fvs are formed when a selected amino acid residue on both VL and VH is mutated to cysteine such that a disulphide bond can form between the variable domains; single-chain Fvs are formed as a result of the introduction of a peptide linker into the sequence, linking the C-terminus of one variable domain to the N-terminus of the second

HOSPIRA EX. 1029
Page 54
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

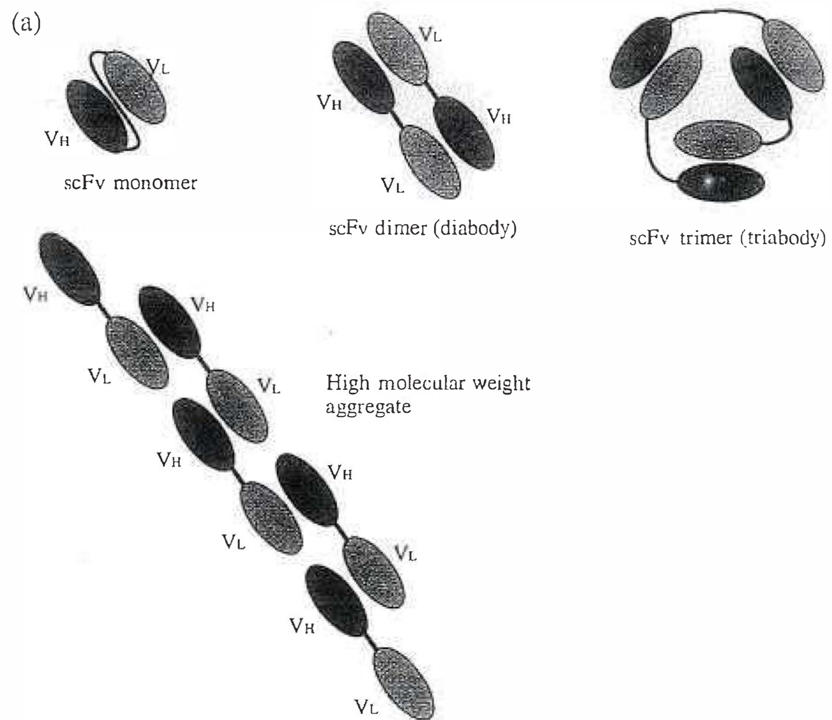
$(Gly_4Ser)_3$. Active single-chain Fv molecules have been produced in both of the two possible orientations, VH–linker–VL or VL–linker–VH; however, for some antibodies one particular orientation may be preferable as a free N-terminus of one domain, or C-terminus of the other, may be required to retain the native conformation and thus full antigen binding. For example, VL–linker–VH scFv's of the anti-tumour antibody B72.3 have been shown to be more active than VH–linker–VL with a range of different linker lengths (Desplancq *et al.*, 1994).

Many scFv's have been shown to be susceptible to aggregation with dimers and larger species formed in a concentration-dependent manner (reviewed by Raag and Whitlow, 1995). This is because the interactions between VH and VL are relatively weak and there appears to be an equilibrium set up between assembled and unfolded scFv in solution. This leads to domain interchange with neighbouring molecules taking place at high concentrations (Figure 2.6). This is particularly prevalent if strain is introduced into the conformation of the scFv molecule by the use of short linker peptides. Conversely, the use of longer peptide linkers relaxes any strain in the conformation and encourages monomer formation (Whitlow *et al.*, 1993). For example, the B72.3 scFv is in the form of mostly dimers and high molecular weight aggregates with linker lengths of 0, 5 and 10 amino acids but mostly monomeric with linkers of 15, 20, 25 and 30 amino acids (Desplancq *et al.*, 1994). In addition, the tendency towards dimer formation at high concentration decreases as the linker length increases (Figure 2.6). The potential of forming dimers with very short linkers, or no linker at all, can be exploited to produce stable dimeric structures which have been termed 'diabodies' (Holliger *et al.*, 1993). Such an approach can also be used to create molecules with two different binding specificities, bispecific diabodies, by fusing the VH of an antibody of one specificity to the VL of another and vice versa (see Section 2.5). The crystal structure of a diabody recognising a phospholipase enzyme has been solved and it has been demonstrated that the VH and VL domains on the two polypeptide chains associate as expected, although in this case there is also non-covalent association between two diabody molecules to form a tetrameric structure (Perisic *et al.*, 1994).

Fv's stabilised by disulphide linkages appear to be more stable structures than scFv's (Glockshuber *et al.*, 1990). The introduction of a disulphide bond between the VH and VL domains to form a disulphide-linked Fv requires the identification of residues in close proximity on each chain which are unlikely to affect directly the conformation of the binding site when mutated to cysteine, and will be capable of forming a disulphide bond without introducing strain into the structure of the Fv. Sites have been identified in both CDR regions and framework regions which appear to result in the formation of such disulphide bonds and allow the production of stabilised Fv fragments which retain antigen-binding characteristics (Glockshuber *et al.*, 1990; Reiter *et al.*, 1994). The use of sites in the framework region is likely to be of more general utility, and several different antibody Fv fragments have now been stabilised in this way (reviewed by Reiter and Pastan, 1996).

A different approach would be to use a single immunoglobulin domain for antigen binding. In a small number of cases a single-antigen binding domain may have antigen-binding activity. For example, VH domains with affinity for lysozyme in the 20 nM range have been isolated which have been termed single domain antibodies or dAbs (Ward *et al.*, 1989). Light chain domains may also have antigen-binding activity in some cases (Masat *et al.*, 1994). However, single domains have been difficult to express and are poorly soluble due to an exposed hydrophobic surface which normally associates with the other V domain, and thus such small antigen binding proteins have not been widely used.

(a)



(b)

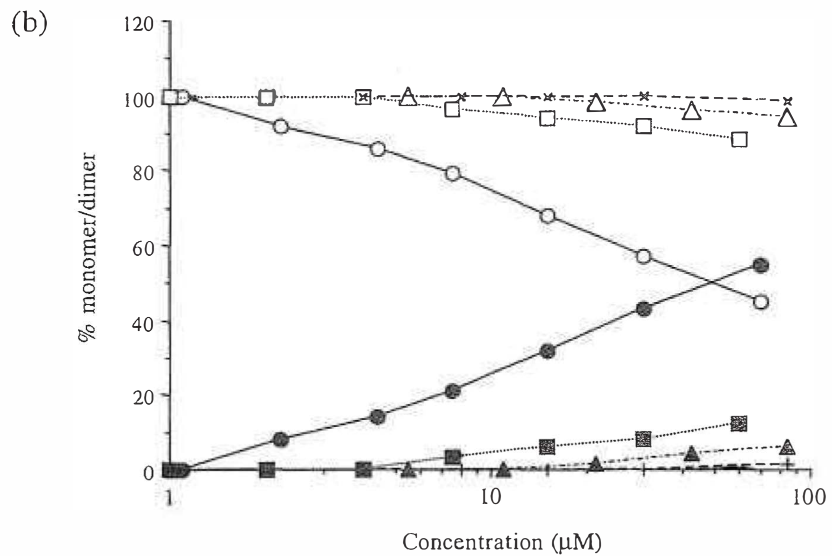


Figure 2.6 Aggregation of single-chain Fv: (a) representation of scFv monomer, dimer (diabody), trimer (triabody) and high-molecular weight aggregate; (b) extent of dimer formation with variation in linker length for scFv of the antibody B72.3, adapted from Desplancq *et al*. (1994). scFvs in the orientation VL–VH with linker lengths of 15, 20, 25 and 30 amino acids are shown. (○) Monomer, (●) dimer VL–15–VH; (□) monomer, (■) dimer VL–20–VH; (△) monomer, (▲) dimer VL–25–VH and (x) monomer, (+) dimer VL–30–VH.

Camels have been found to have naturally occurring antibodies without light chains, and thus attempts have been made to engineer the interface of the VH domain to mimic camel VH domains which may be more soluble (Davies and Riechmann, 1995). Although these attempts have been partially successful, in that antigen-binding specificities could be obtained from a library of such engineered VH domains, only low affinity binding has been achieved to date. This is probably a consequence of the reduction in available CDR regions for antigen binding from 6 to 3 compared to authentic Fv. In some cases it may be possible to increase the number of antigen contact residues, for example by engineering an extra antigen-binding loop which can be inserted into the framework 3 region of VH domains to increase binding affinity (Simon and Rajewsky, 1992).

Multivalent antibody fragments

As monovalent binding entities, Fv fragments suffer from relatively low avidity binding and therefore there has been much interest in producing multivalent Fv fragments which still retain a relatively small size. The production of 'diabodies' is one approach as described above, and some diabodies can be produced to high levels in the form of stable dimers (Zhu *et al*., 1996). However, the stability of such dimeric species varies from one antibody to another (Whitlow *et al*., 1994). In some cases, particularly with direct fusions of VH and VL, stable trimeric species are produced which have been termed 'triabodies' (Iliades *et al*., 1997). Some triabodies show improved avidity for antigen as expected from the increased number of binding sites, although this is not always the case. A crystal structure of a non-functional triabody produced from the variable domains of two anti-hapten antibodies has been solved and demonstrated a dramatic alteration in the conformation of the VH CDR3 antigen-binding loop (Pei *et al*., 1997).

Several alternative approaches have been examined in attempts to produce dimeric species (Figure 2.7). A peptide linker can be introduced between two scFv fragments to produce a single polypeptide chain di-scFv molecule. However, attempts to produce such dimers have often resulted in reduced antigen binding for the two binding sites, even when anti-hapten antibodies were used (Mallender and Voss, 1994). One of the most successful strategies to produce dimeric species has been to introduce a cysteine residue which can be used to introduce a disulphide bond or a chemical cross-link between two scFv fragments. Either this can be introduced directly at the C-terminus of an scFv, via a linker sequence, or a natural antibody hinge region can be attached to form an scFv′ fragment (Cumber *et al*., 1992; Adams *et al*., 1993; King *et al*., 1994). Dimeric scFv can then be formed in a similar manner to that described for Fab′ fragments above, and offers the same potential for site-specific attachment of radiolabels or other moieties (King *et al*., 1994).

An alternative approach has been the fusion of Fv fragments with peptides that naturally form dimeric structures. Amphipathic helices can be fused to the C-terminus of scFv fragments to allow spontaneous dimerisation (Pack and Pluckthun, 1992; Pack *et al*., 1993). These 'dimerisation domains' can be taken from, for example, a four-helix bundle structure or a leucine zipper from a naturally occurring protein. Leucine zippers are peptides of approximately 30 amino acids which naturally form amphipathic helices with leucine residues lining up on the hydrophobic face of the helix. The hydrophobic faces of two zipper peptides associate with each other through hydrophobic interactions. Therefore the association of these molecules occurs through non-covalent interactions, which require further stabilisation to form stable dimeric species. Stabilisation is achieved through engineering a disulphide bond to be formed between the two dimerisation domains when

HOSPIRA EX. 1029
Page 57

Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

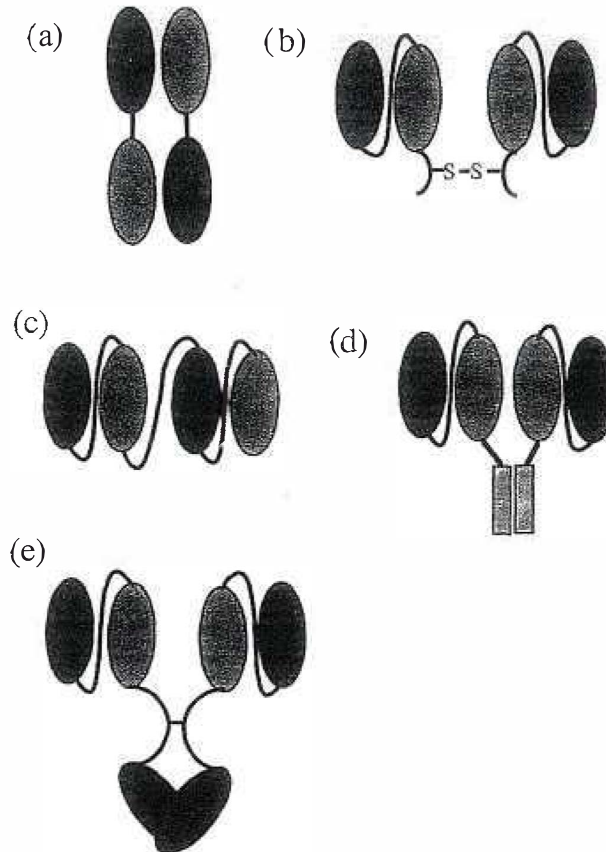


Figure 2.7 Strategies for formation of dimeric scFv species: (a) diabodies formed spontaneously from scFv with short peptide linkers; (b) formation of a disulphide bond or chemical cross-linking through attached hinge region peptides; (c) direct fusion of one scFv to another through an extra linking peptide; (d) the use of dimerisation domains such as leucine zippers; (e) minibody formed from the use of IgG CH3 domains and a hinge region peptide to drive, and stabilise, dimerisation

associated. This approach to dimer formation has the advantage that no *in vitro* manipulation is required after expression and purification of the molecule. However, for *in vivo* applications of Fv fragments, the added dimerisation domains may increase the immunogenicity of the molecule, thus limiting its usefulness. An alternative dimerisation domain is the IgG CH3 domain (Hu *et al*., 1996). As described in Chapter 1, there are extensive non-covalent interactions between IgG CH3 domains which help drive assembly of the native paired heavy chains in IgG. This interaction can also be used to promote dimerisation of scFv–CH3 fusion proteins which can be stabilised by inclusion of a hinge region containing cysteine residues to allow disulphide bond formation (Figure 2.7).

The use of self-assembling structures can be extended to produce higher avidity antigen-binding proteins such as tetrameric scFv's. A modified amphipathic helix can result in tetrameric fragments (Pack *et al*., 1995), as can fusions to the core region of streptavidin

HOSPIRA EX. 1029
Page 58
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

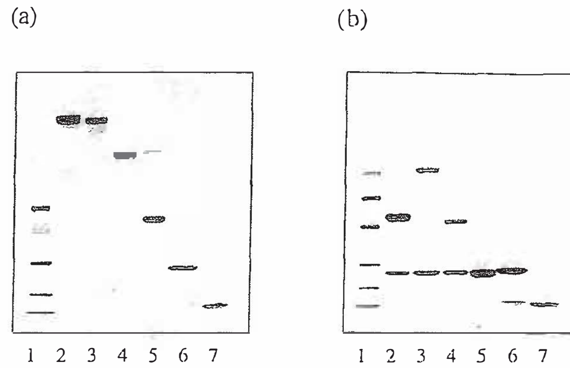


Figure 2.8 SDS-PAGE analysis of chemically cross-linked di-Fab and tri-Fab compared to other immunoglobulin forms: (a) (non-reducing conditions) lane 1, molecular weight markers; lane 2, IgG; lane 3, chemically cross-linked tri-Fab′; lane 4, chemically cross-linked di-Fab′; lane 5, Fab′ containing trace of $F(ab')_2$; lane 6, scFv; lane 7, Fv; (b) SDS-PAGE under reducing conditions, samples as for the non-reduced gel

which forms a natural tetrameric structure (Kipriyanov *et al.*, 1996). These fusion proteins can be difficult to express to high levels directly in soluble form, although procedures have been developed to enable recovery of active material from insoluble protein expressed in *E. coli* (Kipriyanov *et al.*, 1996). The tetramerisation domain of the human protein p53 has also been used to produce fusion proteins which assemble to tetrameric scFv's (Rheinnecker *et al.*, 1996). The use of human protein domains may have advantages for *in vivo* use as the human protein may be less immunogenic than entirely synthetic sequences or bacterial proteins such as streptavidin, although as a normally intracellular protein, p53 may not be the optimal choice for such reagents.

Trimeric and tetrameric antigen-binding proteins can also be produced by chemical cross-linking of Fab′ or scFv′ with tri- or tetra-maleimide cross-linking reagents (Schott *et al.*, 1993; King *et al.*, 1994), or by polyoxime chemistry (Werlen *et al.*, 1996). This approach can be easily used for a range of fragments from different antibodies, either recombinant or produced by proteolysis, and is perhaps the most attractive method for the production of multivalent fragments at present. Such cross-linked fragments can be produced in high yield and purified to homogeneity (Figure 2.8). Tri-scFv's and tri-Fab's produced in this way show increased avidity of binding to antigen and favourable *in vivo* properties (see Chapter 4).

2.5 Antibodies with multiple specificities

Bispecific antibodies have two different specific antigen-binding sites, one on each arm of the antibody molecule, and are potentially useful for a range of diagnostic or therapeutic applications. For example, a bispecific antibody can be prepared with one binding specificity to a target tissue antigen and a second specificity to peroxidase for use in immunohistochemistry (Milstein and Cuello, 1983). Alternatively, for therapy, a bispecific antibody could be used with one arm binding to a tumour cell antigen and one to a cell killing

HOSPIRA EX. 1029
Page 59
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

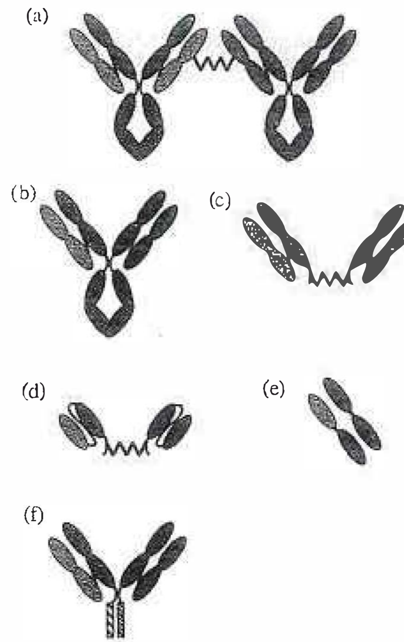


Figure 2.9 Approaches to bispecific antibody production: (a) chemical cross-linking of IgG, (b) production of IgG from a hybrid hybridoma, (c) chemical cross-linking of Fab's, (d) chemical cross-linking of scFvs, (e) bispecific diabody, (f) association through leucine zippers

agent such as a drug, toxin or effector cell (reviewed by Fanger and Guyre, 1991). There are now several approaches to the production of bispecific antibodies (Figure 2.9). All of these approaches have advantages and disadvantages with no one method being used predominantly at the present time.

Hybrid hybridoma cell lines, also known as quadromas, can be produced by the fusion of two antibody-secreting cells such that the resultant hybrid produces two individual heavy and light chains. This can be achieved using two hybridoma cells, or by direct fusion of a hybridoma cell and an immune spleen cell (triomas). Fusion of two existing hybridoma cell lines is usually preferable as the antibodies to be used can be characterised individually and thus the outcome is more predictable. The heavy and light chains associate with each other to produce IgG using the normal cellular mechanisms, but without any selectivity in the pairing of the chains ten combinations are possible, with the bispecific IgG making up only a small proportion of the total (Figure 2.10). Attempts to improve the proportion of bispecific produced have been made, for example, by using rat/mouse hybrid hybridomas wherein the light chains will only pair with the heavy chain of the same species (Lindhofer *et al.*, 1995). However, purification of the correct bispecific antibody from a mixture of very similar IgG molecules remains a considerable problem. If both antigens are available these can be used for sequential affinity purification although this is an expensive operation which is usually difficult to perform on a large scale. Alternatively, methods such as ion-exchange chromatography based on differences in charge of the individual IgG chains can be used (Allard *et al.*, 1992), or if the two

HOSPIRA EX. 1029
Page 60
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

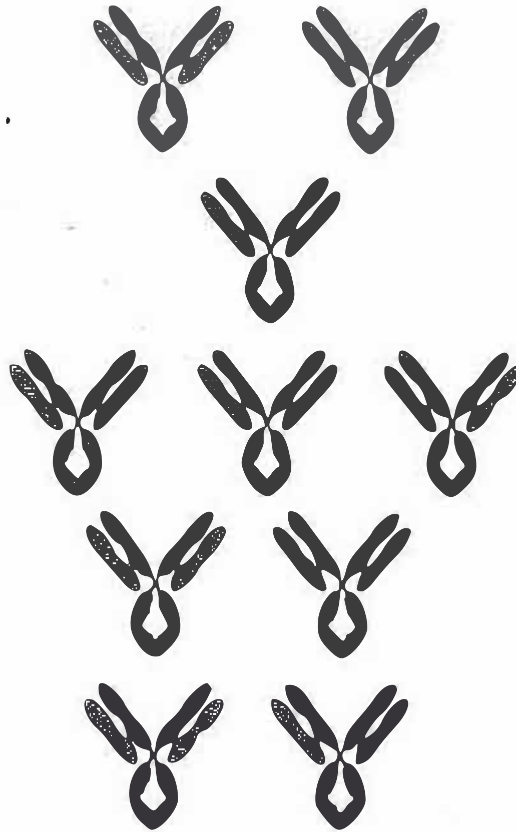


Figure 2.10 Combinations of heavy and light chains possible from a hybrid hybridoma

heavy chains are of different isotype, fractionation on protein A chromatography may be possible (Lindhofer *et al.*, 1995). A further problem with hybrid hybridomas is that they are usually unstable as a result of being polyploid due to carrying the chromosomes of each of the parent cells. Chromosome loss and loss of the ability to secrete bispecific antibody is a constant danger and maintenance of a productive cell line therefore requires frequent cloning to ensure that both sets of heavy and light chain genes are still present. An alternative method to overcome the instability problem is the introduction and expression of the genes for two heavy and light chains in the same cell by recombinant DNA techniques (Songsivilai *et al.*, 1989). This method also allows engineering of the antibody genes to introduce other desired features; for example, humanised antibody genes can be used.

A bispecific reagent can also be prepared simply by chemically cross-linking two IgG molecules (e.g. Karpovski *et al.*, 1984). This results in an undefined heteroaggregate of approx. 300 kDa, and one or both of the antigen-binding sites may very well be damaged during the non-specific chemistry required. This approach does have the advantage, however, that bivalent binding for both antigens may be retained in some cases resulting in high avidity binding to both antigens.

The production of bispecific IgGs by protein engineering techniques has also been investigated by designing specific protusions, 'knobs' on one CH3 domain and a corresponding 'hole' in the CH3 domain of the second antibody heavy chain (Ridgeway *et al.*, 1996). This is achieved by swapping a small amino acid at the interface with a large one to make the 'knob' and vice versa to produce a complementary 'hole'. Assembly of the two engineered heavy chains is thus favoured while assembly of the homodimers containing two 'knobs' or two 'holes' is unfavourable. Coexpression of the two heavy chains resulted in mostly heterodimer produced. However, pairing of the correct light chains may require further engineering of the interface between the heavy and light chains, possibly using further 'knobs' and 'holes'.

A more attractive alternative is to prepare Fab′ fragments from the two antibodies required and then to eross-link the two Fab′ fragments via the hinge cysteine residues to produce bispecific $F(ab')_2$. This can be achieved by use of a thiol reactive cross-linker (Figure 2.4). Methods have been devised for the preparation of bispecific molecules without any homodimers produced, and such bispecific reagents have been shown to maintain the full functionality of each binding site (Brennan *et al.*, 1985; Glennie *et al.*, 1987). Chemically cross-linked $F(ab')_2$ produced with bis-maleimide reagents, which therefore contain thioether bonds between the two Fab′s, are produced in higher yields and are more stable than disulphide-linked molecules (Glennie *et al.*, 1987). This approach can be used with Fab′ molecules produced by proteolysis or with recombinant Fab′ or other antibody fragments engineered to contain a free thiol group, such as scFv′. If Fab′ with multiple hinge thiols is used, or alternatively tri-functional linkers, it is also possible to produce bispecific tri-Fab with two antigen-binding arms to one of the antigens, allowing higher avidity binding (Tutt *et al.*, 1991a). This approach can be modified to allow the production of trispecific antibody fragments, which allows cross-linking of two independent antigens on an effector cell as well as target antigen (Tutt *et al.*, 1991b).

The formation of bispecific $F(ab')_2$ molecules can also be accomplished using 'dimerisation domains' (Kostelny *et al.*, 1992). Leucine zipper peptides have been taken from the transcription factor proteins Jun and Fos which preferentially associate with each other to form a heterodimer. These zipper peptides have been fused to the C-terminus of the Fd′ chains of two Fab′ fragments and expressed as fusion proteins. When co-expressed *in vivo* little heterodimer was produced, but when the individual Fab′-Jun and Fab′-Fos proteins were expressed and purified separately, they could be reduced and recombined *in vitro* to produce the bispecific molecule in high yield. The design of these molecules also incorporates hinge disulphides to stabilise the bispecific $F(ab')_2$ produced. The Jun and Fos peptides attached may increase the immunogenicity of the $F(ab')_2$, but further design could incorporate proteolytic cleavage sites between the Fab′ and the dimerisation domains to allow their subsequent removal once the disulphide bonds were formed.

Bispecific scFv molecules can also be produced by several means using techniques similar to those described for the formation of homodimers above (Section 2.4.2). Direct fusion of two scFv molecules to form single polypeptide molecules containing two different specificities has been attempted by several groups. Initial attempts to express such bispecific molecules in *E. coli* resulted in insoluble material, even if secretion to the periplasm was attempted (Gruber *et al.*, 1994; Mallender and Voss, 1994). Refolding of functional molecules was achieved but resulted in reduced antigen-binding activity of one or both antigen-binding sites. Subsequent expression of single-chain bispecific constructs in mammalian cells resulted in molecules in which both binding sites appeared to be functional (Mack *et al.*, 1995; Jost *et al.*, 1996).

Fusion of the VL of one antibody to the VH of a second antibody with a short linker and coexpression with the VL of antibody two fused to the VH of antibody one allows bispecific diabodies to be produced (Figure 2.9). Expression of high yields of bispecific diabodies in *E. coli* is possible, with yields of 935 mg/l reported after purification with approximately 75% as functional heterodimers (Zhu *et al.*, 1996). Engineering of the domain interfaces has also been investigated to improve assembly of dimeric molecules (Zhu *et al.*, 1997). Improvements in the proportion of functional heterodimer to 92% were reported by engineering the interface to contain 'knobs' and 'holes' as described for the pairing of two heavy chains above. Bispecific diabodies can also be combined with phage display technology to allow selection of diabodies with improved binding and association properties (McGuinness *et al.*, 1996). Although it is easier to produce in *E. coli*, non-specific association of diabodies has been demonstrated (Perisic *et al.*, 1994), and further work examining both the physical properties of these molecules and their *in vivo* characteristics is required.

Genetic fusions of anti-dansyl scFv's to the C-terminus of anti-dextran antibody, either as IgG or as $F(ab')_2$, has been described resulting in tetravalent bispecific molecules (Coloma and Morrison, 1997). Although produced at relatively low levels (0.5–5 mg/l), proteins capable of binding both antigens resulted. It was not clear however, whether the potential for bivalent binding by both specificities was achieved.

2.6 Engineering effector functions

For many applications a simple blocking or neutralising effect of antibody is not enough, and it is desirable to use the antibody to target a diagnostic or therapeutic effector mechanism. Therapeutics can utilise natural effector functions such as complement activation, phagocytosis or ADCC, or entirely novel functions can be introduced. For example, the antibody can be used to target radioisotopes, drugs or toxins to kill tumour cells. Novel functions can be introduced by chemical coupling to antibody molecules or in many cases by recombinant DNA techniques. Similarly, reagents useful as reporter molecules for diagnostic agents, such as enzymes or peptides, can be attached to antibodies either chemically or by means of recombinant DNA. Antibody engineering can thus be used to tailor the effector functions required for individual applications.

2.6.1 Engineering natural effector functions

Use of different human isotype constant regions for chimeric and humanised antibodies can confer different biological properties as expected. For example, Shaw *et al.* (1988) produced a set of mouse/human chimeric antibodies from the mouse MAb 17-1A which recognises a tumour-associated antigen. It was demonstrated that the chimeric molecules could activate the effector functions expected for each particular human IgG isotype. This has also been shown for several other sets of matched chimeric or humanised antibodies. Thus the choice of isotype for a recombinant antibody allows the selection of the desired effector function profile. For example, human IgG1 and IgG3 are the most active IgG isotypes in binding to Fc receptors and eliciting ADCC responses, whereas IgG2 or IgG4 can be chosen to minimise Fc binding and ADCC effects. The ability of human antibodies to mediate complement-dependent lysis is highly dependent on a number of poorly understood factors, including antigen access and density, antigen size and proximity to

the cell membrane. However, comparisons of sets of recombinant antibodies with identical variable regions have revealed that human IgG1 and IgG3 are also the most active isotypes for complement activation (see Table 1.1) and thus when an active isotype is required one of these two should be chosen. IgG3 molecules are difficult to purify and handle; their long hinge region results in susceptibility to proteolysis and aggregation, and therefore IgG1 is a common choice when an active antibody is required.

In some cases isotypes are chosen for chimeric and humanised antibodies to mimic the functional properties of the original rodent antibody. The effector functions of murine and human isotypes are compared in Table 1.1. Most mouse and rat antibodies are poorly effective at activation of human effector functions, although mouse IgG2a and rat IgG2b are more efficient than other isotypes. Active human isotypes can confer human effector functions to humanised mouse antibodies previously unable to mediate such effects (e.g. Junghans *et al.*, 1990; Caron *et al.*, 1992a), although this is not always the case due to restrictions of the antibody/antigen interaction mentioned above, and also due to the existence of specific cellular protective mechanisms. As more is understood of the molecular basis of Fc receptor binding and complement activation, specific mutations can be made to alter effector functions of a particular isotype. For example, in many *in vivo* situations antibodies are used as blocking or neutralising molecules and effector functions are undesirable as these could lead to unnecessary toxicity. In these cases human isotypes such as IgG2 or IgG4 have been used, although even these relatively neutral isotypes have low level functionality for both ADCC and complement lysis, which may be undesired. The recognition site for Fc receptor binding on the lower hinge/$C_{H}2$ domain has been mapped to residues Leu234–Ser329, and mutations in these residues have been shown to reduce Fc receptor binding (Lund *et al.*, 1991; Canfield and Morrison, 1991). The residue Leu235 in this motif has been changed to Glu in a humanised IgG4 and shown to remove the residual Fc receptor binding of this isotype (Alegre *et al.*, 1992). In engineering human antibodies for specific effects of this type, however, it must be remembered that mutations from the natural sequence may have unexpected effects on other functional properties or may increase the immunogenicity of the molecule, and therefore careful testing of the engineered molecule is required.

Other considerations must also be borne in mind when choosing an antibody isotype. The flexibility of the hinge region varies between the human IgG isotypes, and this may affect binding properties, particularly to cell surface antigens. For example, the isotype choice for a chimeric antibody against intercellular adhesion molecule 1 (ICAM-1) affected the antigen-binding properties of the antibody (Morelock *et al.*, 1994). In this case a chimeric IgG1 variant had equivalent antigen-binding ability to the murine antibody, yet less active molecules were produced with human IgG4 or IgG2 constant regions. The affinity of the individual binding sites was shown to be the same for all the chimeric antibodies, the difference being in the avidity due to differences in the ease of bivalent binding to this cell surface antigen. This correlated with the different flexibility of the hinge region in these antibodies, IgG1 being the most flexible with highest avidity and IgG2 the least flexible with the lowest avidity. Similarly, differences in antigen-binding properties between antibodies with identical variable regions have been identified in other antibody–antigen systems with both murine and human antibodies (Cooper *et al.*, 1993; McCloskey *et al.*, 1996).

Human IgG4 is a common isotype choice when an antibody with few effector functions is required. Natural human IgG4 contains a proportion of molecules in which the inter-heavy chain disulphide bonds do not form although the two halves of the molecule are still assembled through non-covalent interactions (King *et al.*, 1992a). During analysis by

HOSPIRA EX. 1029
Page 64
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

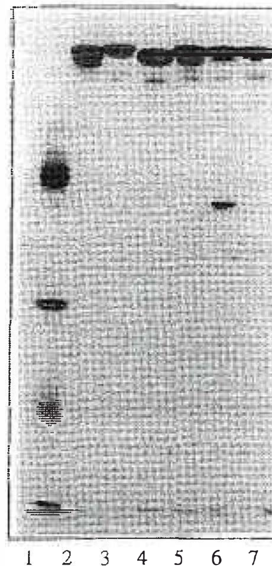


Figure 2.11 SDS-PAGE of an isotype series of the mouse:human chimeric antibody B72.3 under non-reducing conditions; the presence of a band of non-disulphide-linked molecules is observed at approx. 80 kDa for IgG4: lane 1, molecular weight markers; lane 2, murine B72.3; lane 3, chimeric IgG1; lane 4, chimeric IgG2; lane 5, chimeric IgG3; lane 6, chimeric IgG4; lane 7, chimeric IgG4P, Ser-Pro mutant (see text for details)

SDS-PAGE non-covalent interactions are broken such that two bands are seen under non-reducing conditions, one of disulphide bonded IgG at approx. 155 kDa and one representing IgG with no inter-heavy chain disulphide bond at approx. 80 kDa (Figure 2.11). This can lead to confusion during analysis of purified antibody as the 80 kDa band (which is present at 5–25% depending on the particular IgG4 antibody) may be viewed as an impurity, although it is present in all natural IgG4 antibodies studied as well as recombinant antibodies (King *et al.*, 1992a). The core hinge region of human IgG4 contains the sequence CPSC, compared to IgG1 which contains the sequence CPPC. The serine residue present in the IgG4 sequence leads to increased flexibility in this region and therefore a proportion of molecules form disulphide bonds within the same protein chain (an intrachain disulphide) rather than bridging to the other heavy chain in the IgG tetramer to form the interchain disulphide (Bloom *et al.*, 1997). Changing the serine residue to a proline to give the same core sequence as IgG1 allows complete formation of inter-heavy chain disulphides in the IgG4 molecule, hence removing the 80 kDa band seen on SDS-PAGE (Angal *et al.*, 1993). This altered isotype, termed IgG4P, has been used for several chimeric and engineered human antibodies to overcome the double-banded gel pattern (e.g. Owens and Robinson, 1995).

It is also possible to produce recombinant antibodies of other classes which are functional in Fc receptor binding. Mouse:human chimeric IgM, IgA1, IgA2 and IgD molecules have all been expressed and have shown functional binding to their respective receptors (Bruggemann *et al.*, 1987; Morton *et al.*, 1993; Shin *et al.*, 1992). The properties of these constant regions are therefore also available for incorporation into recombinant

Figure 2.12 Dimeric IgG formed through mutation of a CH3 domain residue to cysteine (see text for details)

antibodies if required. In some cases these isotypes may offer advantages, for example a chimeric IgA2 has been used to translocate across an epithelial cell layer via the polymeric Ig receptor for IgA and therefore target tumour cells by reaching an antigen more abundant on the apical cell surface (Terskikh *et al*., 1994).

Although the amino acids involved in the interaction sites for Fc receptor binding and complement activation are beginning to be identified, at present the structural basis by which effector functions are elicited is not fully understood. Mutations in recombinant antibody molecules are playing a large role in elucidating recognition sites for individual functional effects. When the molecular basis of these interactions is understood it may be possible to tailor antibodies to bind to a chosen set of receptors and so design their *in vivo* properties.

The role of carbohydrate in maintaining the conformation of the IgG CH2 domains must also be considered. Removal of carbohydrate by mutation of the normal attachment site for N-linked carbohydrate at position 297 in the CH2 domain results in severe reductions in the ability of antibody to mediate effector functions (Tao and Morrison, 1989). This is probably a consequence of altered conformation of the antibody molecule (Lund *et al*., 1990). Recent studies have shown that mutation of amino acids involved in structural contacts with antibody carbohydrate can influence effector functions as well as the profile of sugar residues attached to the antibody itself (Lund *et al*., 1996). Manipulation of antibody carbohydrate structure may, therefore, represent another route towards engineering antibody effector functions.

Another approach to alter effector functions is the production of polymeric IgG antibodies. As described in Chapter 1, the ability of IgG to elicit complement activation is dependent on multiple molecules attaching to antigen to allow Fc clustering. Pentameric and hexameric IgM molecules contain multiple attachment sites for C1q binding which are revealed by a conformational change upon antigen binding and thus IgM molecules in general are more active at complement activation than IgG. Simple chemical cross-linking can be used to produce dimeric or polymeric species with increased antigen-binding avidity (Tsai *et al*., 1995). However, antibody engineering allows more controlled and reproducible site-specific linking strategies to be considered. Two approaches to engineering IgG molecules for increased effector function via multimerisation have been investigated. Substitution of a serine residue near the C-terminus of the CH3 domain (Ser444) to cysteine allowed the production of IgG dimer of a chimeric human IgG1 (Figure 2.12). This mutation resulted in 50% of the molecules forming dimeric IgG, reminiscent of IgA dimers, which were approx. 200-fold more potent in complement-mediated lysis (Shopes, 1992). The same mutation introduced into a humanised IgG1, huG1-M195, also resulted in the formation of IgG dimers which were more potent at both complement activation and ADCC (Caron *et al*., 1992a). In addition, dimers of an antibody to an internalising antigen such as huG1-M195 internalise into cells more rapidly than the IgG, which may offer advantages for delivery of cytotoxic agents to cells *in vivo*

HOSPIRA EX. 1029
Page 66
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

(see Chapter 4). The second approach investigated has been the expression of IgG molecules with a tailpiece from the IgM μ chain which results in the production of IgM-like IgG polymers with up to six IgG units (Smith and Morrison, 1994; Smith *et al.*, 1995). All four human IgG isotypes have been shown to result in polymers using this approach, and as expected the ability of such multimeric molecules to mediate complement activation is increased, as is their ability to interact with the FcγRII receptor. Disadvantages include the heterogeneity of polymers produced, with species from monomers to hexamers present, activation of complement in the absence of antigen for some isotypes, and altered *in vivo* tissue distribution and clearance properties, with rapid loss of polymeric species from the circulation.

Another approach is the use of bispecific antibody fragments to redirect effector functions. Bispecific diabodies have been produced with one specificity for target antigen and the second for serum immunoglobulin (Holliger *et al.*, 1997). Such diabodies can retarget serum Ig to the target antigen and elicit a range of effector functions including complement activation, phagocytosis and ADCC. This potentially exciting strategy offers the potential to develop reagents capable of retargeting specific immunoglobulin types and thus their particular effector functions, or all immunoglobulins via a common epitope such as light chain constant domain (Holliger *et al.*, 1997). Similarly, more specific reagents may be produced through retargeting individual effector molecules. For example, a bispecific diabody with one specificity towards complement component C1q and the other against lysozyme as a model target antigen was capable of activating complement resulting in specific target cell lysis (Kontermann *et al.*, 1997).

2.6.2 *Attachment of diagnostic or therapeutic agents*

Chemical conjugates

Chemical conjugates of antibodies with drugs, radioisotopes, proteins or other molecules have been widely investigated, and a range of chemical approaches are available and widely used. Chemical conjugation is often the quickest route to the preparation of conjugates for many purposes, and in some instances conjugates with superior properties to those produced as fusion proteins may result (see below). Indeed, chemical conjugates of antibodies with enzymes or other reporter groups are a mainstay of the diagnostics industry (see Chapter 3). Chemical conjugation is, of course, the only option for the attachment of non-proteinaceous materials such as drugs to antibodies, and the ability to use a range of different linker chemistries allows variation in the properties of the reagents produced to be tested until the optimum is identified. The major issues in the production of such conjugates are the degree of substitution which can be achieved, the stability of the linkage and the biological activity of the resulting conjugate. Loss of some or all of the antigen-binding ability of the antibody is a common consequence of non-specific conjugation methods which may result from the modification of amino acids at or close to the CDR amino acid residues involved in antigen binding. This is more pronounced when antibody fragments are used, as there is more chance of randomly modifying a residue important for function simply due to the smaller number of amino acids present. Similarly, care must also be taken that the effector or reporter molecule attached is not damaged by the conjugation methodology. Therefore, choice of the conjugation method and control of the conjugation process to minimise such effects is a major

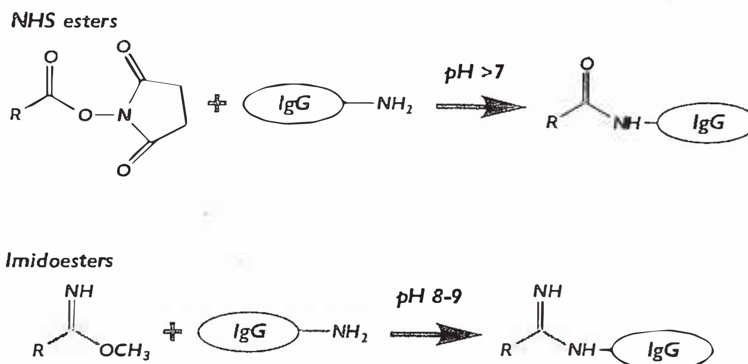
consideration. Other drawbacks of this approach include the heterogeneous nature of the conjugates produced which can lead to difficulties with batch-to-batch variation.

A variety of amino acid residues on the surface of antibody molecules can be used for chemical modification and coupling. Residues with useful functional groups for attachment include tyrosine (phenolic hydroxyl group), aspartic and glutamic acid (carboxyl group), lysine (amino group) and cysteine (thiol group). The tyrosine phenolic hydroxl group is reactive towards electrophilic agents, and its major role as a modification site is as a site for labelling of proteins with radioactive iodine. The carboxyl group of the acidic aspartate and glutamate residues can be used for coupling with carbodiimides such as EDC which react with both carboxyl and amine groups. However, such reagents are relatively non-specific and often lead to antibody aggregation and precipitation. The most useful and widely used sites for attachment to antibodies are the terminal amino group of surface lysine residues and the free thiol group of cysteine residues which can be generated by reduction of disulphide bonds in the hinge region. A large variety of reagents are available as linkers for attachment to these two groups under mild conditions which minimise damage to the antibody (Figure 2.13). Immunoconjugates which incorporate low molecular weight ligands are usually prepared using a derivative of the ligand with a suitable amino acid reactive group, whereas for protein conjugates a linker is required. Both homobifunctional linkers with two identical reactive groups and heterobifunctional linkers with reactive groups for different moieties are commercially available.

The most useful amine reactive groups include imidoesters and *N*-hydroxysuccinimide esters. Both of these groups react with the terminal amino group of lysine residues under relatively mild conditions (Figure 2.13), but many imidoesters require high pH (>9) for selective reactivity and can be reversible. Therefore *N*-hydroxysuccinimide esters have become the most popular reagents for linking to lysine residues due to their efficient coupling at physiological pH and the stability of the linkage. The amine groups at the N-terminus of the protein chains can also react with these linkers. Other lysine-reactive groups are not useful for protein–protein linkage but are commonly used for attachment of small chemical moieties such as drugs, or fluorescent reporter groups onto antibodies. For example, fluorescein and rhodamine isothiocyanates are commonly used for the attachment of these fluorescent groups to antibodies, and several amine-reactive groups have been used for polyethylene glycol attachment to antibodies (see Figure 2.2).

The cysteine thiol group is the most reactive amino acid side chain and is thus a useful site for specific conjugation. Thiol-reactive groups for antibody modification include maleimide, vinylsulphone, haloacetyl or pyridyl disulphide groups (Figure 2.13). Maleimides react selectively with cysteine residues at neutral pH, although there is reactivity with amine groups at higher pH values. A stable thioether bond is generated. Haloacetyl groups, usually containing iodoacetyl, are also useful reagents for introducing stable thioether bonds into immunoconjugates. These reagents are less selective than maleimides and at high concentrations can also react with histidine groups. Also, free iodine is generated under some conditions which may react with tyrosine, tryptophan or histidine residues causing unnecessary antibody damage. Pyridyl disulphides react with thiols to form a disulphide bond and are useful in situations when a less stable chemical linkage is required. A major advantage of coupling via pyridyl disulphides is the release of pyridine-2-thione which can be used to monitor the reaction spectrophotometrically at 343 nm. Antibody molecules do not normally contain a free thiol group as all of the available cysteine residues form disulphide bonds. Free thiols can be liberated by reduction of the hinge region of IgG, or of $F(ab')_2$ fragments to form Fab′ which is a method of site-specific labelling (see below). Alternatively, surface lysine residues can be modified to

(a)



(b)

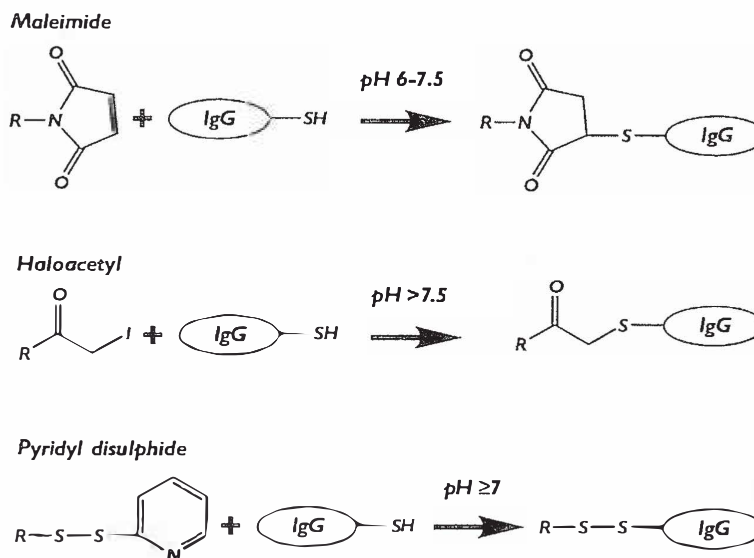


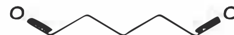
Figure 2.13 Examples of amine and thiol reactive linkers commonly used for chemical modification of antibodies: (a) lysine reactive linkers such as NHS esters and imidoesters; (b) thiol reactive linkers such as maleimide, haloacetyl and pyridyl disulphide

introduce a thiol group using reagents such as 2-iminothiolane, SATA or SPDP (Figure 2.14), which in turn can be used as an attachment point. One advantage of modifying lysine residues with thiol groups in this way is to achieve good control over the conjugation reaction, and thus minimise loss of biological properties of the conjugate. While

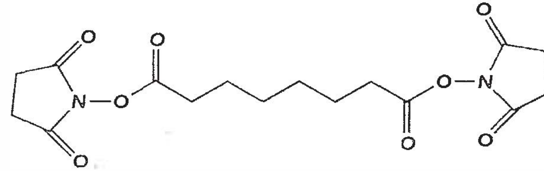
HOSPIRA EX. 1029
Page 69
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

(a) Homobifunctional

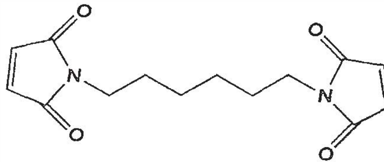
Glutaraldehyde



DSS

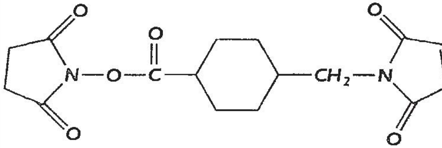


BMH

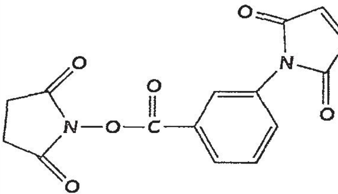


(b) Heterobifunctional

SMCC



MBS



SPDP

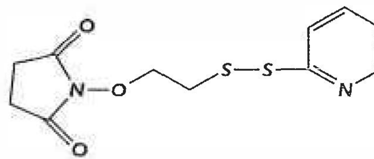


Figure 2.14 Some useful commercially available protein cross-linking reagents: (a) homobifunctional reagents glutaraldehyde, disuccinimidyl suberate (DSS) and bis-maleimidohexane (BMH); (b) heterobifunctional reagents, succinimydl 4-*N*-maleimidomethylcyclohexane-1-carboxylate (SMCC), maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) and *N*-succinimidyl-3-[2-pyridyldithio]propionate (SPDP)

HOSPIRA EX. 1029
Page 70
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

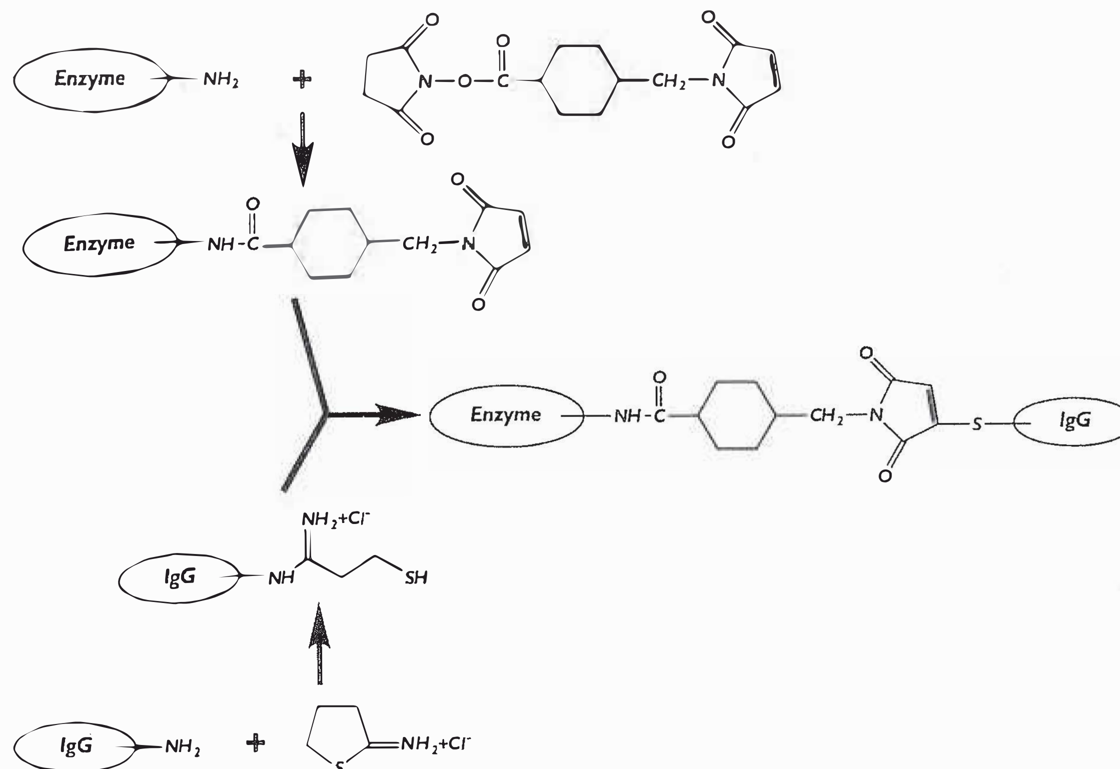


Figure 2.15 Example reaction scheme for attachment of an enzyme to antibody using SMCC attachment to enzyme and 2-iminothiolane activation of IgG

HOSPIRA EX. 1029
Page 71
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

titration of lysine groups to determine the level of substitution is an insensitive procedure, the presence of one thiol group per antibody can be easily measured using reagents such as dithionitrobenzoic acid (DTNB) or dithiodipyridine (DTDP).

Both homobifunctional and heterobifunctional reagents are readily available (Figure 2.14). Homobifunctional cross-linkers which are either lysine-reactive or thiol-reactive can be used for IgG conjugation, although in some cases their use can result in unacceptable aggregation. The use of heterobifunctional linkers often allows more control of the conjugation reaction. Heterobifunctional reagents with one thiol-reactive and one amine-reactive group are particularly useful for the production of stable conjugates with minimum aggregation, such as antibody–enzyme conjugates. A typical reaction strategy is shown in Figure 2.15. The introduction of such a stable linkage is desirable in many cases though for some applications, for example in the construction of immunotoxins (see Chapter 4), a reducible linkage has been shown to be useful. In these cases linkers such as SPDP can be used to form a disulphide linkage after reaction of SPDP with each reagent individually, or alternatively heterobifunctional reagents with a disulphide group between the two functional groups can be used. In a similar way, sites for attachment of other functionalities can be designed into the linkers used. For example, sites for radiolabelling either with iodine or by chelation of radioactive metals can be incorporated.

Site-specific attachment

As a consequence of the loss of antigen-binding avidity often seen on conjugation, several methods for attachment of reagents at specific sites away from the antigen-binding site have been investigated (Table 2.5). As mentioned above, the generation of free cysteines by selective reduction of the hinge region has been used for both IgG and antibody fragments, for example to attach fluorescent groups (Packard *et al*., 1986) or for site-specific radiolabelling (King *et al*., 1994). The Fc region carbohydrate also provides a natural specific attachment site for IgG molecules. Carbohydrate is usually modified by periodate oxidation to generate reactive aldehydes which can then be used to attach reactive amine containing compounds by Schiff base formation. As the aldehydes can react with amine groups, reactions are carried out at low pH so that the lysine residues are protonated and unreactive. Hydrazide groups are most suitable for attachment to the aldehydes generated since they are reactive at low pH to form a hydrazone linkage. The linkage can then be further stabilised by reduction with sodium cyanoborohydride to form a hydrazine linkage (Figure 2.16). Comparisons of conjugates made by attachment to

Table 2.5 Approaches to site-specific labelling of antibodies and antibody fragments

Strategy	Reference
Modification of hinge cysteine residues	Packard *et al*., 1986
Introduction of surface cysteines in constant region domains by protein engineering	Lyons *et al*., 1990
Modification of Fc carbohydrate	Rodwell *et al*., 1986
Introduction of glycosylation site to light chain by protein engineering	Leung *et al*., 1995
Introduction of extra lysine residues by protein engineering	Hemminki *et al*., 1995
Addition to C-terminus of antibody fragments by reverse proteolysis	Fisch *et al*., 1992

HOSPIRA EX. 1029
Page 72
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

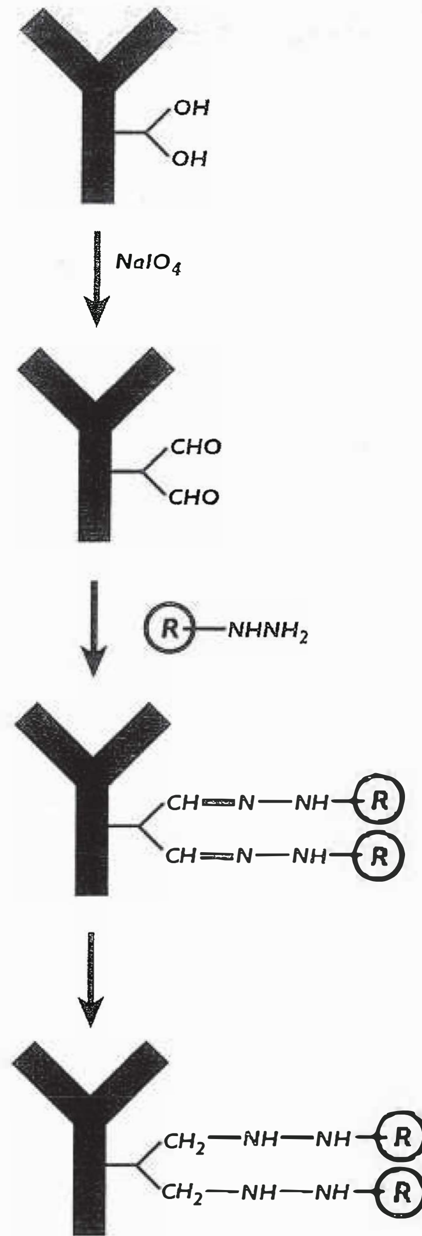


Figure 2.16 Reaction scheme for site-specific attachment to antibody carbohydrate via periodate oxidation

Fc carbohydrate with conventional conjugates with ligands randomly attached to lysine residues have demonstrated significantly improved antigen-binding properties (Rodwell *et al.*, 1986). The disadvantages of this approach are the relatively harsh conditions required, which can damage and aggregate some antibody molecules. Methionine residues present in some antibody variable regions may be particularly susceptible to oxidation by periodate, which can lead to loss of antigen-binding avidity. In some cases histidine or tryptophan residues might also be affected.

Antibody engineering can be used to introduce specific attachment sites into antibody molecules, and this can be incorporated as part of the design of an engineered molecule. Extra cysteine residues can be introduced onto the surface of antibody constant domains to provide a specific attachment site without the need to disrupt native disulphide bonds. Introduction of specific cysteine sites in the CH1 domain of the IgG heavy chain has been shown to result in sites to which ligands can be attached without any loss of antigen binding (Lyons *et al.*, 1990). Alternatively, extra carbohydrate sites can be engineered into the molecule to allow attachment via periodate oxidation. Some antibody light chains have an unusual natural glycosylation site, and thus the light chain has been used as a site to introduce a glycosylation site into antibodies which do not normally have carbohydrate attached to the light chain (Leung *et al.*, 1995). A third engineering strategy is to introduce extra lysine residues into the surface of the constant region domains (Hemminki *et al.*, 1995). Although this approach does not introduce a unique labelling site, lysine-reactive reagents are more likely to modify the antibody at the increased concentration of lysine residues in the constant region, resulting in the retention of more antigen-binding reactivity.

A more specialised approach is the use of reverse proteolysis to attach reagents specifically at the C-terminus of Fab′ heavy chains (Fisch *et al.*, 1992). After production of a $F(ab')_2$ fragment by the protease lysyl endopeptidase, experimental conditions can be altered such that the same protease working in reverse is capable of the specific attachment of carbohydrazide groups to the C-terminus of the $F(ab')_2$ heavy chains. These carbohydrazide groups could then be used as an attachment point for a radiolabelled chelator reacting via an aldehyde group to form a hydrazone linkage. A similar approach has also been used to attach an aldehyde-modified enzyme to the C-terminus of $F(ab')_2$ (Werlen *et al.*, 1994).

Fusion proteins

An attractive alternative to chemical conjugation for antibody–protein conjugates is the direct expression of fusion proteins. This approach can be viewed as another method of site-specific attachment, as the protein to be fused is attached away from the antigen-binding site and no further modification is necessary. The antibody or antibody fragment is fused to the protein at the gene level, usually via attachment of the sequence to either full length or a truncated heavy chain. Expression of functional fusion proteins was first shown by fusion of a Fab fragment to Staphylococcal nuclease (Neuberger *et al.*, 1984). Since then the production of active antibody and antibody fragment fusion proteins with a variety of different enzymes, cytokines, and other proteins has been achieved (Table 2.6). Fusion proteins overcome problems with loss of antigen-binding ability on chemical conjugation and are also well-defined, homogeneous reagents. Disadvantages are that the flexibility of using linkers with varying chemical stability is lost, and in some cases fusion proteins have proved difficult to express in a functional form.

HOSPIRA EX. 1029
Page 74
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 2.6 Some examples of antibody fusion proteins

Antibody form	Fusion	Reference
Enzyme fusions		
$F(ab')_2$	Staphylococcal nuclease	Neuberger *et al.*, 1984
scFv, dsFv	β-lactamase	Goshorn *et al.*, 1993 Rodrigues *et al.*, 1995
Fab′	β-glucuronidase	Bosslet *et al.*, 1992
scFv, $F(ab')_2$	Alkaline phosphatase	Wels *et al.*, 1992 Ducancel *et al.*, 1993
scFv	Urokinase	Holvoet *et al.*, 1991
Toxin fusions		
scFv, dsFv	Pseudomonas exotoxin	Brinkmann *et al.*, 1991 Reiter *et al.*, 1996
scFv, Fab, $F(ab')_2$	Gelonin	Better *et al.*, 1995
Fab′, scFv	Angiogenin	Rybak *et al.*, 1992 Newton *et al.*, 1996
scFv	Eosinophil-derived neurotoxin	Newton *et al.*, 1994
Cytokines and growth factors		
scFv, $F(ab')_2$	IL-2	Fell *et al.*, 1991 Dorai *et al.*, 1994
IgG, Fab	IL-2, GM-CSF TNFα, TNFβ	Gillies *et al.*, 1992 Gillies *et al.*, 1993
Fab, $F(ab')_2$, IgG	IGF-1, IGF-2	Shin *et al.*, 1994
Other proteins		
$F(ab')_2$	Aequorin	Casadei *et al.*, 1990
Fab, $F(ab')_2$, IgG	Avidin	Shin *et al.*, 1997
Fab	Biotin carboxyl carrier protein	Weiss *et al.*, 1994
Fab, $F(ab')_2$, IgG	Transferrin	Shin *et al.*, 1995
Fab	Staphylococcal enterotoxin A	Dohlstein *et al.*, 1994
scFv	Protein A fragment	Tai *et al.*, 1990
$F(ab')_2$	Metallothionein	Das *et al.*, 1992

The choice of antibody form for fusion purposes depends on the other properties of the molecule required, e.g. overall size, pharmacokinetics and valency. As shown in Table 2.6, fusion proteins with single-chain Fv, disulphide-linked Fv, Fab, $F(ab')_2$ and IgG have all been constructed. However, the choice of construct can have unexpected effects on the assembly and activity of the fusion protein. A series of fusions of interleukin-2 (IL-2) to the heavy chain of the chimeric anti-ganglioside antibody ch14.18 have been made with addition directly after the Fab such that the entire Fc is replaced by IL-2, after CH2 such that the CH3 domain is replaced with IL-2, and fusion to the end of the entire heavy chain (Gillies *et al.*, 1992). The Fab fusion was inactive in antigen binding and the fusion

HOSPIRA EX. 1029
Page 75
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

to CH2, although capable of binding antigen, did not assemble correctly to the divalent species even though a full-length hinge region was present. The fusion to the end of the entire heavy chain resulted in enhanced antigen binding of the antibody and the molecule was shown to be fully assembled. The IL-2 fused to the antibody remained active although the antibody effector functions were slightly reduced.

In some case spacer peptides have been included between the antibody and fused protein which may assist folding or activity of the fused protein. For example, a linker between an scFv toward the transferrin receptor and the RNAse angiogenin was shown to be required for optimal activity of both the scFv and the enzyme (Newton *et al.*, 1996). Also, spacers can be designed to allow proteolytic release of the fused protein when internalised into cells. Other choices in the nature of the construct also need to be made, as the fusion can be made at the C-terminus or N-terminus of the antibody chain and to heavy or light chain. In a study of antibody fragment fusions to the plant toxin gelonin, fusions to Fab, $F(ab')_2$ and scFv were made at either the N- or C-terminus (Better *et al.*, 1995). Of these immunotoxins, the divalent $F(ab')_2$ variants were the most potent, as might be expected due to divalent antigen binding and possibly more effective internalisation into target cells. scFv and Fab conjugates with gelonin fused at the N-terminus or C-terminus of the light or heavy chain were all approximately equipotent. Linkers were examined with sites for trypsin-like proteases or with sites for the lysosomal enzymes cathepsin B and D. These are expected to be cleaved intracellularly and may assist gelonin release and hence cytotoxicity. However, in this study there was little difference between the cytotoxicity of the constructs with and without the cleavable linkers, suggesting that the fusion proteins themselves may be active without the need for release of free gelonin.

The types of protein used in fusion proteins includes those used to induce dimerisation (Section 2.4.2), peptide or protein tags used to simplify antibody fragment purification (see Chapter 5), enzymes for both therapeutic and diagnostic purposes, cytokines and growth factors, and many others as shown in Table 2.6. Fusions can also be used to couple some non-proteinaceous materials to antibodies via specific binding proteins. Fusion of the metal-binding protein metallothionein to antibody fragments can be used to allow subsequent binding of the radionuclide ^{99m}Tc (Das *et al.*, 1992). A small peptide which can chelate ^{99m}Tc has also been used effectively (George *et al.*, 1995). Another approach to radiolabelling is to attach a peptide sequence which can be enzymically labelled with ^{32}P (Neri *et al.*, 1996). Non-proteinaceous materials which are naturally bound by other proteins can be attached by fusion of a suitable 'acceptor' sequence to the antibody fragment followed by expression in *E. coli*. In this way lipid-tagged scFv fragments have been produced by fusion of the amino terminal part of the *E. coli* major lipoprotein, which is sufficient for attachment of lipid during expression (Laukkanen *et al.*, 1993). Biotin can be attached via the biotin-carboxyl carrier protein (BCCP) subunit of *E. coli* acetyl-CoA carboxylase (Weiss *et al.*, 1994). In this case a Fab-BCCP fusion was expressed in *E. coli* and biotin was attached during secretion of the fusion protein.

2.7 Engineering pharmacokinetics and biodistribution

For therapeutic applications of MAbs, the pharmacokinetics and biodistribution of the agent to be used in humans is of great importance. The degree of binding to the target antigen *in vivo* is dependent not only on binding characteristics of the antibody such as affinity and avidity which can be measured *in vitro*, but also on a variety of other factors such as the accessibility of the antigen, the concentration, and the time of exposure of the

HOSPIRA EX. 1029
Page 76
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

antibody to the target antigen. Access to antigens in the vascular compartment is relatively straightforward when antibodies are injected intravenously, but access to other body tissues is usually more difficult. Such extravascular targets require the antibody to pass through the endothelial cell layer lining the blood capillaries to reach interstitial fluid compartments. The permeability of blood vessels to proteins varies according to the size of the protein, with smaller proteins able to reach interstitial spaces more quickly. Therefore, large proteins such as intact antibody molecules penetrate into tissues relatively slowly, while antibody fragments penetrate tissues more quickly. However, the amount of antibody to reach the target tissue is also dependent on the concentration in circulation and the rate of clearance from the blood, and therefore the study of antibody pharmacokinetics has an important role to play.

In both human and animal systems, antibody clearance from the blood follows a classical two-compartment kinetic model. Initially, a short distribution phase, or alpha phase, takes place during which the antibody is distributed throughout body tissues, i.e. equilibrates between intravascular and extravascular compartments. This is followed by an elimination or clearance phase (beta phase) in which the antibody is metabolised and excreted. The rates of these two phases are commonly calculated and quoted as half-life values ($t_{\frac{1}{2}}\alpha$ and $t_{\frac{1}{2}}\beta$). Alternatively, a single-compartment kinetic model may be used, in which case a single half-life can be calculated.

2.7.1 Pharmacokinetics of IgG

Human IgG molecules have a long circulating half-life in humans, with $t_{\frac{1}{2}}\alpha$ of 18–22 hours and $t_{\frac{1}{2}}\beta$ of 21–23 days for human IgG1, 2 or 4 (reviewed by Mariani and Strober, 1990). Human IgG3 has a shorter half-life with a $t_{\frac{1}{2}}\beta$ of approximately 9 days. This may be due to the extended hinge region of IgG3 which makes the molecule more susceptible to proteolytic cleavage. Human IgM is cleared more quickly, with a half-life of approximately 5 days. This very large protein remains largely in the vascular compartment and thus pharmacokinetic data are normally described using a one-compartment kinetic model. The pharmacokinetics of human IgG are unusual in that the half-life varies with concentration. At very high IgG levels, such as those seen in myeloma patients which can approach 100 mg per ml of serum, the half-life is decreased to similar values to IgM, while in patients with hypogammaglobulinemia with reduced plasma IgG levels a prolonged half-life is observed. It has been postulated that a receptor-mediated event is thus responsible for maintaining the long circulating half-life for IgG, through IgG binding, preventing degradation and resulting in recirculation to the plasma (Brambell *et al.*, 1964). This hypothesis has been supported by subsequent experimental investigations. It is possible to calculate the amount of IgG which is protected from degradation by this receptor-mediated process in humans at full saturation. This is approximately 150 mg per kg body weight per day (Mariani and Strober, 1990).

It has been known for some time that this receptor-mediated recycling is mediated by the Fc region of the antibody, as isolated Fc has a long half-life, whereas Fab and $F(ab')_2$ are cleared relatively rapidly. Also, co-administering Fc with IgG can result in reduced IgG half-life in a similar manner to increasing the IgG dose. Recently the amino acids in the Fc fragment important for maintaining IgG in circulation in mice have been elucidated by site-directed mutagenesis studies (Kim *et al.*, 1994; Ghetie *et al.*, 1996). The receptor binding site is located at the interface region between the CH2 and CH3 domains and overlaps with the binding site for Staphylococcal protein A, commonly used for purification of IgG (see Chapter 5). Thus protein A complexes are rapidly cleared due to

HOSPIRA EX. 1029
Page 77
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

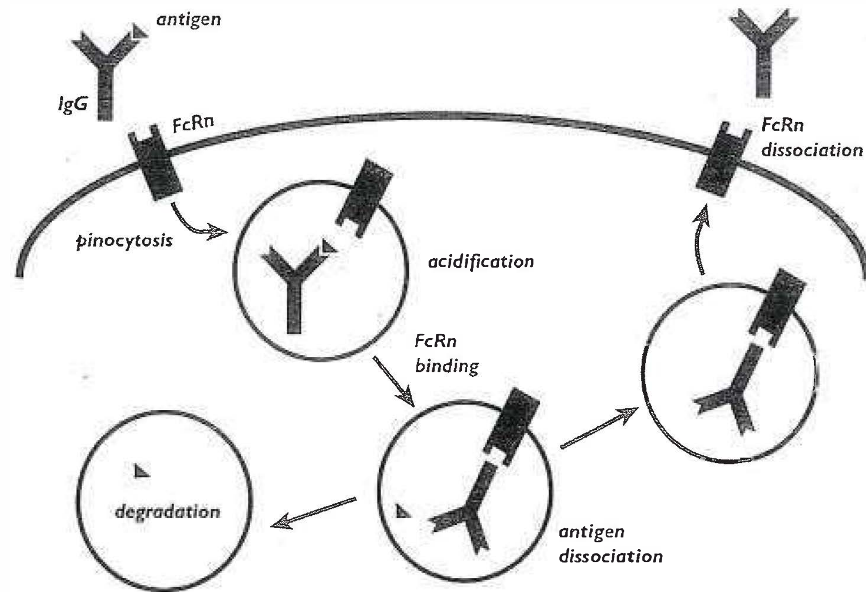


Figure 2.17 Mechanism of FcRn-mediated salvage of IgG from catabolic degradation: the low pH of the endosome results in tight binding of IgG to the FcRn receptor, leading to protection from degradation and return to the cell surface where the higher pH allows re-release of the IgG into circulation

competition with the receptor-mediated process, and isolated Fc fragments have the same $t_{\frac{1}{2}}\beta$ as the whole IgG molecule. The cellular receptor responsible for maintaining half-life has been identified as the neonatal Fc receptor, FcRn. This is the same receptor which is responsible for transfer of IgG from the mother to the neonate which provides immunity for the first few weeks after birth. Mice deficient in this receptor are unable to acquire IgG from the mother, have reduced plasma IgG levels and clear administered IgG or Fc with an abnormally short half-life (Ghetie *et al*., 1996). The FcRn-mediated pathway for maintaining high IgG levels is thought to operate through its pH dependence (Figure 2.17). IgG is strongly bound to FcRn at low pH and dissociates at higher pH. IgG is internalised into cells and salvaged from the endosome during acidification due to binding to FcRn at low pH. Low pH may also strip any bound antigen from the antibody which would then be degraded in the lysosome. Antibody is then recycled to the cell surface where the higher pH of the extracellular fluid promotes dissociation from FcRn and return of the IgG to the circulation.

Many murine MAbs have been examined in human studies in a variety of diagnostic and therapeutic situations. The half-lives of such murine antibodies vary both from antibody to antibody and from patient to patient, and depend on a number of factors including isotype and the presence of circulating antigen. In general, $t_{\frac{1}{2}}\beta$ values for murine antibodies in humans have been in the range of 15–48 hours on the first administration. Such studies are complicated by human immune responses to murine antibodies (HAMA, see Section 2.3) which result in rapid clearance of murine antibodies on repeat injection. There is now a growing collection of data on the pharmacokinetics of chimeric and humanised antibodies in man, some of which is summarised in Table 2.7. These data are

Table 2.7 Pharmacokinetics of some chimeric and humanised antibodies in humans

Antibody	Indication	$t_{\frac{1}{2}}\alpha$	$t_{\frac{1}{2}}\beta$	Reference
c17-1A (γ1)	Colorectal carcinoma	18 ± 2 hours	101 ± 16 hours	LoBuglio *et al.*, 1989 Meredith *et al.*, 1991
cB72.3 (γ4)	Colorectal carcinoma	18 ± 7 hours	224 ± 66 hours	Khazaeli *et al.*, 1991 Meredith *et al.*, 1992a
c14.18 (γ1)	Melanoma	24 ± 1 hours	181 ± 73 hours	Saleh *et al.*, 1992
cL6 (γ1)*	Carcinoma		54–109 hours	Goodman *et al.*, 1993
C2B8 (γ1)*	B-cell lymphoma		106 (38–252 hours)	Maloney *et al.*, 1994
cAnti-CEA (γ4)*	Colorectal carcinoma	7 (1.4–18 hours)	91 (30–292 hours)	Buchegger *et al.*, 1995
hu2PLAP (γ1)*	Carcinoma		73.1 ± 30.2 hours	Hird *et al.*, 1991
Humanised anti-Tac(γ1)*	Graft v. host disease		88† hours (44–360)	Anasetti *et al.*, 1994
Humanised M195 (γ1)*	Myeloid leukemia	0.3 ± 0.4 hours	38 ± 9 hours	Caron *et al.*, 1994
CDP571 (γ4)	Healthy volunteers	19.6 hours (1–49)	225† hours (87–537)	Stephens *et al.*, 1995
hMN-14 (γ1)*	CEA producing cancer	5.3 ± 6.2 hours	56 ± 32 hours	Sharkey *et al.*, 1995
rhuMAb HER2 (γ1)	Breast cancer		199 ± 120 hours	Baselga *et al.*, 1996
hCTM01 (γ4)*	Ovarian cancer	4.2 ± 6.1 hours	64.7 ± 20.2 hours	van Hof *et al.*, 1996

*In these cases circulating antigen, or large pools of readily accessible antigen, are known to be present which may reduce antibody half-life in some cases.
†Increase in $t_{\frac{1}{2}}$ with increasing dose.

HOSPIRA EX. 1029
Page 79
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 2.8 Approaches used to manipulate antibody pharmacokinetics

Use of particular isotypes
Clearing regimes: anti-antibody *in vivo*
avidin–biotin system
extracorporeal immunoadsorption
Chemical modification: galactosylation
Engineering antibody domain deletions
Antibody fragments

difficult to compare due to different antibodies being used in a variety of different clinical conditions with, in some cases, the presence of pools of circulating antigen, or because some of the antibodies used target readily available antigen on blood cells. However, it is apparent that the $t_{\frac{1}{2}}\beta$ values for chimeric and humanised antibodies are consistently higher than those for murine antibodies. In some instances the same antibody has been administered to man in both murine and chimeric forms. In all of these cases the antibody half-life is significantly longer for the chimeric antibody, for example the $t_{\frac{1}{2}}\beta$ of 17-1A was increased from 16 hours for the murine antibody to 101 hours for the chimeric IgG1 version, and that of B72.3 from 24–48 hours for the murine antibody to 224 hours for the chimeric IgG4 (LoBuglio *et al*., 1989; Khazaeli *et al*., 1991). In the one report to date of the pharmacokinetics of a humanised antibody in healthy volunteers, the anti-TNF antibody CDP571 had a half-life which approached the 21 day half-life of normal human IgG4 (Stephens *et al*., 1995), suggesting that in healthy subjects at least, the normal mechanisms for regulation of IgG half-life described above also apply to humanised antibodies.

The long half-life of humanised antibodies is ideal for some applications, for example in providing protection against infection, or providing long-term neutralisation of a cytokine. However, antibodies have also been widely investigated for their ability to deliver diagnostic or therapeutic agents such as drugs, radionuclides or protein toxins to sites such as tumour cells in attempts to detect and/or destroy them. In these cases a long circulating half-life can result in unacceptable toxicity. For example, a circulating antibody–radionuclide conjugate will irradiate other tissues and limit the amount of radioactivity that can be delivered to a tumour. The ideal molecule would target the toxic agent to the tumour cells and clear rapidly from the rest of the body. Therefore there has been much interest both in strategies to remove antibody from circulation more rapidly and in designing molecules with shorter *in vivo* half-lives. Some approaches to this problem are summarised in Table 2.8.

The choice of a particular isotype for an engineered antibody can influence the pharmacokinetic properties of the antibody. The γ1 and γ4 isotypes of chimeric B72.3 have been compared directly for their pharmacokinetics in cynomolgous monkeys (Hutzell *et al*., 1991). The $t_{\frac{1}{2}}\beta$ was 90 hours for the γ1 and 262 hours for the γ4 isotype. Although direct comparisons have not been done in humans, the same trend has been observed (see Table 2.7), with γ1 antibodies usually clearing faster than γ4. However, the clearance of γ1 antibodies is still slow, and other strategies are required to reduce the half-life further.

2.7.2 *Pharmacokinetics of antibody fragments*

As the catabolic site for IgG lies within the Fc domain, there is no specific mechanism for maintaining the serum level of $F(ab')_2$ or smaller antigen-binding fragments and these are

HOSPIRA EX. 1029
Page 80
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

cleared from circulation more rapidly than IgG. One of the most useful approaches to developing molecules with short *in vivo* half-life has thus been the use of antibody fragments. Small antibody fragments are filtered through the kidney, from the glomerular capillaries to the renal tubules, and thus rapidly excreted. The nominal molecular weight cut-off for kidney filtration is believed to be approx. 60–70 kDa, and thus Fab and Fv fragments are rapidly cleared through this route. During this process antibody fragments are often re-absorbed by the kidney tubules and metabolised. As well as shorter $t_{\frac{1}{2}}\beta$ values, antibody fragments also distribute to, and penetrate, tissues more rapidly, which is reflected in a shorter α phase. For example, the ability to penetrate into tumour tissue has been shown to be size-related, with smaller fragments demonstrating faster penetration (Yokota *et al.*, 1992).

There have been many studies comparing the pharmacokinetics of IgG with $F(ab')_2$ and Fab fragments in animal models. In the majority of studies Fab clears faster than $F(ab')_2$, which is considerably faster than IgG. For example, analysis of the murine antibody MOPC21 revealed the residence time in the body for IgG, $F(ab')_2$ and Fab to be 8.5, 0.5 and 0.2 days respectively (Covell *et al.*, 1986). A similar pattern was observed with B72.3 Fab, $F(ab')_2$ and IgG labelled with either of two different radioisotopes (Brown *et al.*, 1987). In several human studies $F(ab')_2$ has been shown to be cleared more rapidly than IgG. For example, Buist *et al.* (1993) found a plasma elimination half-life of 70 hours for chimeric MOv18 and 20 hours for $F(ab')_2$. However, this is not the case for all studies. In a trial of ^{131}I-radiolabelled anti-CEA antibody a similar half-life was observed for IgG and $F(ab')_2$ (Lane *et al.*, 1994).

$F(ab')_2$ molecules from different IgG isotypes have different pharmacokinetic properties. A comparison of chimeric $F(ab')_2$ of the human IgG isotypes $\gamma 1$, 2 and 4 revealed that the $\gamma 2$ version was retained in circulation longer than the other isotypes (Buchegger *et al.*, 1992). This pattern may reflect the number of disulphide bonds in the hinge region, 4 for $\gamma 2$, 2 for $\gamma 1$ and $\gamma 4$, as $F(ab')_2$ with more disulphide bonds between the two heavy chains may take longer to be metabolised, either reductively or proteolytically, *in vivo*. As $F(ab')_2$ is above the molecular weight cut-off for rapid kidney clearance, metabolism to smaller fragments probably plays an important role in $F(ab')_2$ pharmacokinetics. Recombinant $F(ab')_2$ of the same isotype with different numbers of disulphide bonds inserted in the hinge region also demonstrated that more disulphide bonds results in a longer half-life (Rodrigues *et al.*, 1993). Similarly, $F(ab')_2$ with a chemically cross-linked hinge region has been shown to have a longer residence time in the blood than $F(ab')_2$ linked by a single disulphide bond (Rodrigues *et al.*, 1993; King *et al.*, 1994).

Fv and scFv fragments have also been examined in experimental systems and shown to clear very rapidly (Milenic *et al.*, 1991; King *et al.*, 1992b). A comparison of scFv with other forms of the antibody CC49 showed very rapid plasma and whole body clearance for the scFv in both mouse and monkey studies, with 80% of scFv lost from the plasma in 15 minutes in mice and 95% removed from the plasma of monkeys by 30 minutes (Milenic *et al.*, 1991). Calculation of $t_{\frac{1}{2}}\alpha$ and $t_{\frac{1}{2}}\beta$ values can be misleading due to not taking account of the duration of each phase which may differ between antibody fragment forms. However, results for comparisons of some fragment forms of two antibodies, one murine and one chimeric, are demonstrated in Table 2.9. Differences in overall values probably reflect differences in the method of calculation used in each case, but it is easily seen that the overall trends are maintained between fragments. The pharmacokinetics of divalent scFv fragments prepared by a number of techniques including disulphide linkage, chemical cross-linking and the use of dimerisation domains have also been examined (Adams *et al.*, 1993; King *et al.*, 1994; Haunschild *et al.*, 1995). In

HOSPIRA EX. 1029
Page 81
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 2.9 Pharmacokinetics of antibody fragments in mice: data for CC49 from Milenic *et al.* (1991) and for cB72.3 from King *et al.* (1994); all values in hours

	CC49		cB72.3	
	$t_{\frac{1}{2}}\alpha$	$t_{\frac{1}{2}}\beta$	$t_{\frac{1}{2}}\alpha$	$t_{\frac{1}{2}}\beta$
IgG	0.65	113	11.1	56.4
$F(ab')_2$	0.43	12	0.87	26.1
Cross-linked $F(ab')_2$			3.14	21.2
Fab′	0.15	1.5		
scFv	0.06	1.5	0.23	4.0

all cases the half-life of the dimeric species is increased only slightly, the di-scFv still being rapidly removed from circulation. The preparation of tri-scFv results in a further small increase in half-life, though these multivalent molecules are still cleared rapidly (King *et al.*, 1994).

Further control of half-life of antibody fragments can be exerted by PEGylation as described in Section 2.3.3. PEGylation of many different proteins, including antibody fragments, has resulted in conjugates with increased half-life. Comparison of the *in vivo* properties of IgG, $F(ab')_2$ and Fab′ fragments with and without PEG attached revealed that PEGylated IgG was relatively unaffected, whereas the half-life of $F(ab')_2$ and Fab′ was increased by PEGylation (Pedley *et al.*, 1994). Application of this technology to smaller Fv-based fragments allows the preparation of a series of molecules with a range of different half-lives which can then be selected for a particular application.

Selective engineering of the IgG molecule can also be carried out to affect half-life. Removal of antibody carbohydrate in the CH2 domain can be achieved by substitution of the Asn residue at position 297 which is normally glycosylated. Such aglycosyl antibodies may have a reduced half-life, although this appears to be dependent on isotype. An aglycosylated mouse:human chimeric IgG4 and chimeric IgG3 antibody cleared faster than the native IgG, whereas aglycosyl chimeric IgG1 was relatively unaffected (Tao and Morrison, 1989; Rhind *et al.*, 1990). Aglycosyl mouse IgG2b also clears more rapidly than the glycosylated antibody (Wawrzynczak *et al.*, 1992). This may reflect differences in the role of the antibody carbohydrate in maintaining the conformation of the two CH2 domains, which in turn would affect FcRn interaction. Alternatively, differing conformational effects between isotypes may result in increased susceptibility to proteolysis for those isotypes which clear rapidly in aglycosyl form. More subtle variation in the carbohydrate structure can also affect half-life. A chimeric IgG1 antibody produced in a mutant CHO cell line, which is incapable of processing a high-mannose intermediate through the terminal steps of the glycosylation pathway, has a shorter half-life than the same antibody produced in a wild-type CHO line (Wright and Morrison, 1994). This is probably a result of enhanced clearance through mannose receptors in the liver, as proteolytic stability of the antibody was unaffected.

The serum persistence of Fc has been increased by introducing mutations into the FcRn binding region (Ghetie *et al.*, 1997b). Random mutagenesis of the sequence around the FcRn binding site, followed by selection using phage display, allowed variants with higher affinity for FcRn to be isolated with serum half-life increased from 120 to 150 hours. Specific engineering to disrupt FcRn binding in an IgG has not yet been reported, although mutations in the CH2 and CH3 domains which have this effect in Fc fragments

have been described (Kim *et al.*, 1994). Mutation of these key residues may allow the production of antibodies with a range of serum half-lives. Removal of the entire CH2 domain of the IgG heavy chain has been reported (Mueller *et al.*, 1990). The resulting truncated heavy chain still allows assembly of a tetrameric structure with full antigen-binding ability. The pharmacokinetics of such CH2 deleted variants are similar to those of stabilised $F(ab')_2$ fragments with a $t_{\frac{1}{2}}\alpha$ of 1.5 hours and a $t_{\frac{1}{2}}\beta$ of 12 hours reported in mice (Mueller *et al.*, 1990). This again demonstrates disruption of the FcRn receptor binding site.

As described in Section 2.5, bispecific diabodies can be produced with one specificity for target antigen and one for serum immunoglobulin. This results in not only the ability to retarget effector functions, but also a significant increase in half-life compared to a control diabody (Holliger *et al.*, 1997). A β-phase half-life of 10 hours was reported for serum Ig targeted diabody, compared to less than 1 hour for the control diabody. This was still significantly less than the IgG alone, possibly as a result of dissociation of the diabody from the IgG during the low pH values experienced during FcRn-mediated recycling (Figure 2.17).

2.7.3 Clearance

A further approach to remove antibody from circulation has been to allow antibody to bind to its target for a period of time and then attempt to remove the residual antibody. Such 'clearance' strategies have been investigated particularly with radiolabelled anti-tumour antibodies. Clearance can be achieved using a second antibody with specificity for the anti-tumour antibody. After the desired time interval, injection of the second antibody results in binding to the radiolabelled antitumour antibody to form immune complexes. These are then rapidly removed by clearance through the liver and to a lesser extent other clearance organs. Initial work used liposomally entrapped second antibody to achieve this, although equivalent results were later generated with second antibody alone (Begent *et al.*, 1982; Pedley *et al.*, 1989). Effective clearance of the radiolabelled antibody has been observed using such systems with enhanced tumour : blood ratios of radioactivity allowing improved imaging of tumours, and resulting in less toxicity associated with blood-borne activity (Blumenthal *et al.*, 1989). A variation of this approach uses the high-affinity interaction between avidin and biotin to remove antibody from circulation. The initial radiolabelled antibody is conjugated to biotin before use and then can be cleared through administration of avidin or streptavidin (Paganelli *et al.*, 1991). A comparison of these two approaches found that superior clearance could be achieved with second antibody, with a larger and faster reduction in blood levels achieved (Marshall *et al.*, 1994).

Problems in using this approach include the relative complexity and expense of preparing and using a clearing agent. Also, the clearance system may result in the sudden deposition of large amounts of immune complex and consequently radioactivity into the liver, or other clearance organ, which may result in toxic effects. This can be overcome using a related method known as extracorporeal immunoadsorption. In this procedure blood is pumped outside the body through an extracorporeal shunt in which the radiolabelled antibody is removed by binding to an affinity column before being returned to the patient (Johnson *et al.*, 1991b). The affinity column can be prepared from either antigen or second antibody attached to a solid support. Clinical trials with this approach have proved that most of the circulating activity can be removed from the patient efficiently,

although the complexity and expense of such a procedure has meant that it has not been widely used.

2.7.4 Chemical modification

Chemical modification of antibody has also been used to alter half-life of antibody. Attachment of galactose, galactosylation, allows recognition of the conjugate by the asialoglycoprotein receptor in the liver resulting in very rapid clearance. This procedure was developed for use with antibodies administered locally, for example intra-peritoneally for ovarian cancer, such that when antibody leaked out from the peritoneal cavity to the blood, the circulating activity was rapidly removed (Mattes, 1987). However, penetration of antibody from the peritoneal cavity to ovarian tumours is not very effective and hence galactosylated antibody has also been evaluated for intravenous cancer therapy (Ong *et al.*, 1991). In this case it was necessary to inhibit the action of the asialoglycoprotein receptor for 2–3 days by injection of large amounts of asialo-bovine submaxillary mucin which initially saturates the receptor. Inhibition for 2–3 days allowed the galactosylated antibody to bind to experimental tumours in nude mice, after which time the inhibitor was cleared and antibody removed from the circulation. In common with the *in vivo* clearance mechanisms described above, this procedure can result in the rapid deposition of antibody in the liver, and thus is restricted to use with therapeutic agents which will not be toxic to liver cells. Radioiodinated antibody appears to be rapidly metabolised by the liver with the excretion of radio-iodine, and thus may be useful for therapy with such galactosylated conjugates (Ong *et al.*, 1991). But perhaps the most useful application of galactosylated antibody is as a second antibody clearing agent. Use of galactosylated second antibody increases the rate of removal of the first antibody from the circulation and has been applied to the removal of antibody–enzyme conjugates from circulation prior to prodrug therapy (Sharma *et al.*, 1990; see Chapter 4).

2.7.5 Fc region to extend half-life

The finding that antibody half-life is dependent on the Fc region as described above, and that isolated Fc regions have the same extended $t_{\frac{1}{2}}\beta$ as IgG molecules, has led to the use of the Fc region to extend the half-life of other proteins. CD4 is a receptor for the HIV virus, and has been of interest for AIDS therapy. However, soluble CD4 has a very short plasma half-life and was therefore difficult to examine *in vivo*. Fusion proteins between CD4 and IgG Fc, termed immunoadhesins, have been prepared which use the IgG Fc region to increase dramatically the half-life of the soluble CD4 protein (Capon *et al.*, 1989). Since then a similar approach has been used to produce Fc fusion proteins with a range of different proteins in attempts to improve half-life (Charnow and Ashkenazi, 1996). An additional property of many immunoadhesins is the retention of some Fc effector functions, and a range of applications are open to such molecules both as research reagents and as potential human therapeutics. The CH2 domain alone can also be used to extend half-life, though the increase in $t_{\frac{1}{2}}\beta$ is less than can be achieved with whole Fc fusions. However, the use of this single domain allows the design of molecules with an internal CH2 domain. For example, a CD4–CH2–PE40 toxin molecule has been produced with extended plasma half-life (Batra *et al.*, 1993).

HOSPIRA EX. 1029
Page 84
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

3

Monoclonal Antibodies in Research and Diagnostic Applications

3.1 Introduction

Antibodies have proved to be invaluable reagents for the detection and quantitation of many types of substances both *in vitro* and *in vivo* and have, therefore, found wide application in both the research laboratory and the diagnostics industry. Many of the techniques used in these two situations are similar, and thus it is helpful to review them together. In a chapter of this length it is impossible to cover all the ways that MAbs have been used in these areas; thus the emphasis in this chapter is on applications which are particularly widely used and those which are likely to benefit most from the ability to design engineered or modified antibodies.

3.2 Immunoassays in diagnostics and research

Immunoassays are carried out to detect and quantitate the presence of a particular antigen or antibody in a test fluid. The development of immunoassays began with polyclonal antisera and thus precedes the introduction of MAbs. However, today MAbs are widely used, and many assays routinely used in, for example, hospital clinical biochemistry laboratories which rely on them. In fact MAbs have found widespread use in laboratories of all types, in applications from drug discovery to detecting drugs of abuse in athletes and horses. In contrast to polyclonal antisera, production of MAbs allows a potentially unlimited supply of identical reagent which can be selected from a number of different clones to have the optimal characteristics for the intended assay. For some assay formats such as radioimmunoassay, however, polyclonal antisera remain the best reagents, as they are frequently of higher apparent affinity than MAbs. Therefore, it is useful to examine the types of immunoassays to appreciate the role of monoclonal antibody reagents. Some of the properties of MAbs of interest for immunoassay development are given in Table 3.1.

HOSPIRA EX. 1029
Page 85
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 3.1 Properties of monoclonal antibodies important for immunoassay design

Unlimited quantity of identical antibody
Antibodies to distinct epitopes can be selected from panels of clones
Easily purified to reduce background binding
Readily digested to produce fragments with equivalent specificity
Can be produced to impure antigen
Often of lower apparent affinity than polyclonal antisera

3.2.1 *Radioimmunoassay*

Radioimmunoassay (RIA) methodology was first developed in the 1960s and represented the first sensitive method for immunoassay (Yallow and Berson, 1960). RIAs are competitive assays based on the competition of radiolabelled antigen with antigen present in the sample to be assayed for binding to antibody (Figure 3.1). The antibody complex is then separated from the free antigen and the amount of bound material determined by counting the radioactivity. Separation can be achieved by a number of methods, although it is most convenient to use antibody immobilised onto a solid phase directly, or to

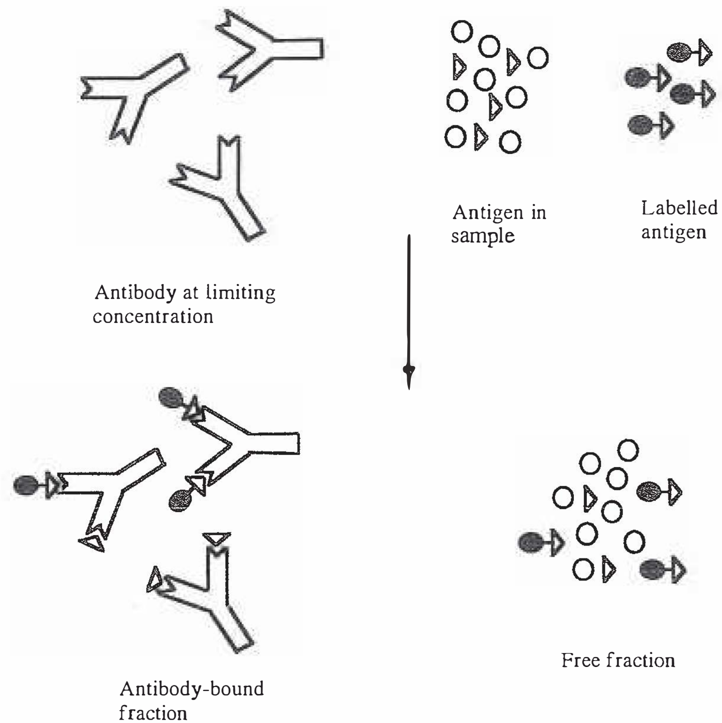


Figure 3.1 Format of radioimmunoassay: antibody at limiting concentration is added to sample containing antigen and radiolabelled antigen; the antibody-bound fraction is then separated and either free or antibody-bound counts (or both) are measured

HOSPIRA EX. 1029
Page 86
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

remove the antibody–antigen complex by a second immobilised antibody which recognises the first antibody used. Antibody can be immobilised to many types of solid supports, e.g. microparticles such as cellulose, sepharose and paramagnetic particles, or to large solid phase plastic surfaces such as beads, tubes or microwells. The RIA assay format requires excess antigen over antibody and for high sensitivity small amounts of high affinity antibody are required. Polyclonal antisera often have very high apparent affinity or avidity, probably due to a small proportion of the total antibody present having high affinity and to cooperativity between antibody molecules. The mixture of antibodies present in a polyclonal antiserum may allow attachment of several antibody molecules to the antigen at different sites and, as antibody molecules are bivalent, this can lead to the formation of multimeric complexes, which lead to a low off-rate and higher apparent affinity. Therefore, polyclonal sera have remained the ideal reagents for RIA, and although MAbs can be used their performance is often not optimal. However, RIA techniques are relatively long and tedious to carry out and in many cases have been superseded by MAb techniques based on other assay formats.

3.2.2 *Immunoradiometric assay*

The immunoradiometric assay (IRMA) uses an excess of labelled antibody rather than antigen to quantitate the antigen present. This means that sensitivity is not dependent on antibody affinity. The IRMA was initially developed as a single-site assay in which excess labelled antibody was added to the sample containing antigen to form immune complexes (Miles and Hales, 1968). The remaining antibody was then removed by incubation with solid-phase antigen and after separation of the solid and liquid phases, counting of either phase could be carried out to determine the amount of antigen present (Figure 3.2). This form of IRMA is not widely used due to the requirement for large amounts of both labelled antibody and immobilised antigen. A variation of the IRMA, the two-site IRMA, has become a much more useful assay format. In this assay antigens are quantitated using two antibodies which bind to different epitopes (Woodhead *et al.*, 1974). Antibody is immobilised onto a solid phase and incubated with sample containing antigen. After binding of the antigen to the solid-phase antibody, excess labelled second antibody is added which binds to a second epitope on the antigen. The excess labelled antibody is then washed away and the amount of bound antibody quantitated by counting (Figure 3.3). Requirements for this type of assay are an antigen which is large enough to contain two epitopes, and supplies of two different specific antibodies. This type of assay format is very difficult to achieve with polyclonals, and requires extensive purification of the antisera to isolate preparations binding to individual epitopes. MAbs are ideally suited, however, as different clones can be tested until two non-competing antibodies are identified. These assays are much more rapid, sensitive and robust than competitive binding assays such as RIA and are now widely used.

Iodine 125 (^{125}I), a high-energy gamma emitter, is usually used as the isotope for antibody labelling in IRMA applications. It allows the development of sensitive assays due to the ability to achieve high specific activity labelled antibody preparations. ^{125}I is attached to antibodies via two main methodologies, either by attachment to tyrosine residues or by attachment to lysine residues. Attachment to tyrosine is achieved by oxidation of sodium iodide ($Na^{125}I$) to form molecular iodine which reacts rapidly with tyrosine side chains (Figure 3.4). The reaction is then stopped by the addition of a reducing agent (e.g. sodium metabisulphite) to stop generation of reactive iodine, or by

HOSPIRA EX. 1029
Page 87
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

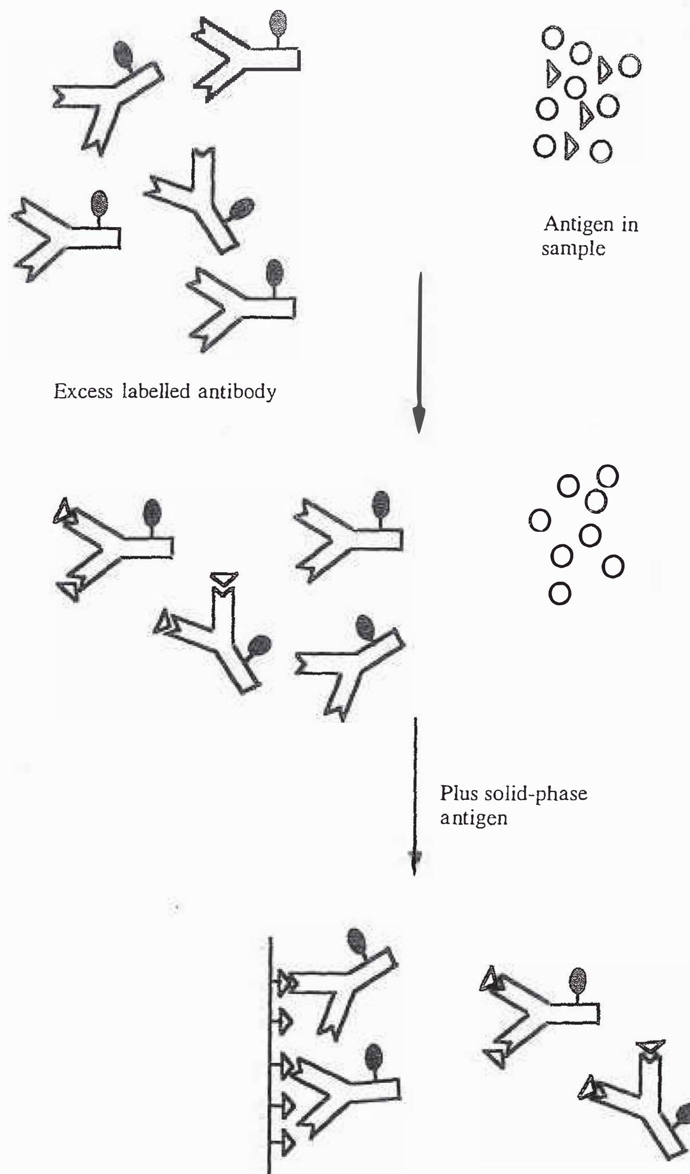


Figure 3.2 Format of immunoradiometric assay: excess labelled antibody binds antigen in the sample and is then added to solid-phase antigen; determination of bound or free counts allows quantitation of the amount of antigen present in the sample

adding excess free tyrosine to mop up all of the reactive iodine. High specific activities can be generated using this methodology, although care must be taken that oxidative damage to the antibody is minimised. Commonly used oxidants are chloramine T or solid-phase reagents such as iodogen coated onto a plastic bead or tube. The use of solid-phase

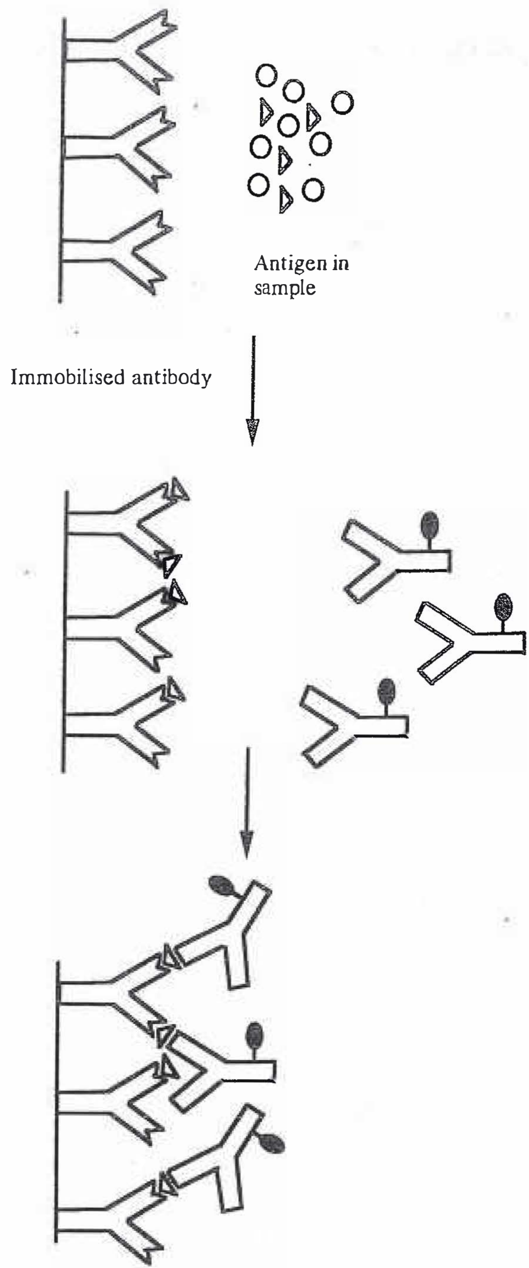


Figure 3.3 Format of two-site immunoradiometric assay: immobilised antibody captures antigen in the sample; a second antibody reactive with a second epitope on the antigen is then used in labelled form to bind to antigen and reveal the presence of antigen

HOSPIRA EX. 1029
Page 89
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

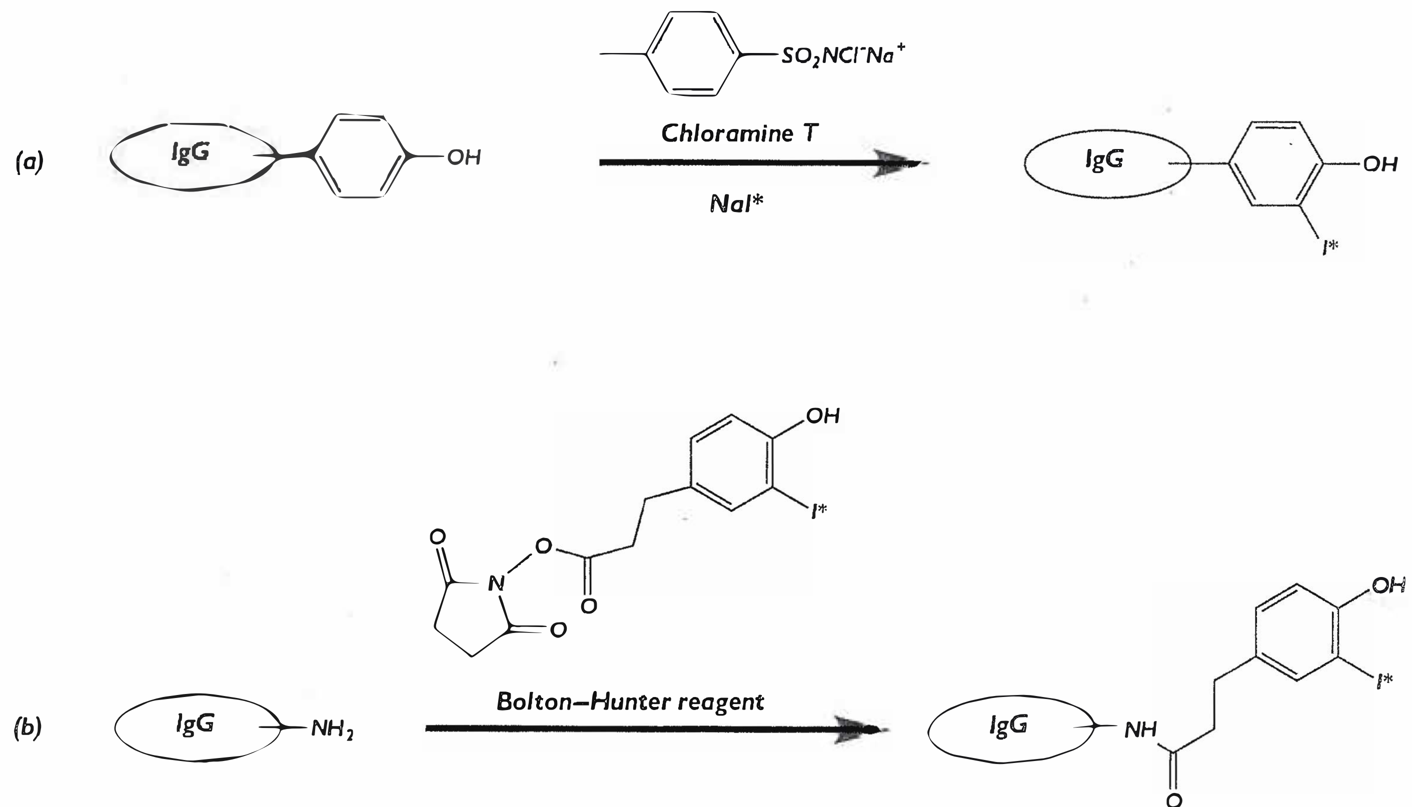


Figure 3.4 Radioiodination of MAb by (a) the use of chloramine T and sodium iodide and (b) Bolton–Hunter reagent (Bolton and Hunter, 1973)

HOSPIRA EX. 1029
Page 90
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

reagents allows rapid removal of oxidant from the system and thus can result in less damage to the antibody. Attachment to lysine residues is accomplished via indirect labelling using amino reactive compounds pre-labelled with ^{125}I. This approach was first described using the ^{125}I-labelled *N*-hydroxysuccinimide ester of 3-(4-hydroxyphenyl) propanoic acid (Bolton and Hunter, 1973), subsequently known as Bolton–Hunter reagent. This and other similar reagents are commercially available in radiolabelled form and allow simple labelling of antibodies under mild conditions (Figure 3.4). Specific activities achieved with Bolton–Hunter reagent are usually lower than with oxidative methods as the incorporation efficiency is lower, but for antibodies sensitive to oxidative damage and in cases where relatively low specific activity is required, this method is particularly useful.

The limitation in sensitivity of the two-site IRMA is the specific activity of the labelled antibody, the affinity of the first antibody and the level of non-specific binding. MAbs can be readily purified, and antigen-binding fragments such as $F(ab')_2$ easily produced, which can reduce levels of non-specific binding to surfaces. Low specific activity is often due to antibody damage during radiolabelling. The availability of large amounts of MAb allows the screening of several radiolabelling methods and the development of a suitable method to minimise such damage.

3.2.3 Non-isotopic immunoassays

A major disadvantage with the use of the two-site IRMA is the need to use radiolabelled antibody. ^{125}I has a half-life of 60 days and thus new labelled preparations need to be continually produced. Also, the use of radiolabelled materials requires stringent precautions in the laboratory to prevent radioactive contamination. Several alternative labels have been investigated, which are replacing radioisotopes. These include enzymes, fluorescent and chemiluminescent labels, and indirect methods (Table 3.2).

Suitable enzymes can be attached to antibodies without loss of activity, and can convert substrate to a coloured product which is then quantitated spectrophotometrically. Enzyme detection has become the standard method for most laboratory immunoassays and two-site (or sandwich) ELISAs (enzyme-linked immunosorbent assays) are very

Table 3.2 Commonly used non-isotopic labels for immunoassays

Enzymes	Horseradish peroxidase Alkaline phosphatase Urease β-galactosidase Xanthine oxidase
Fluorescent labels	Fluorescein Phycoerythrin Europium chelates and other lanthanide chelates
Chemiluminescent	Luminol/isoluminol Acridinium esters
Indirect methods	Biotin Streptavidin/avidin Protein A, protein G

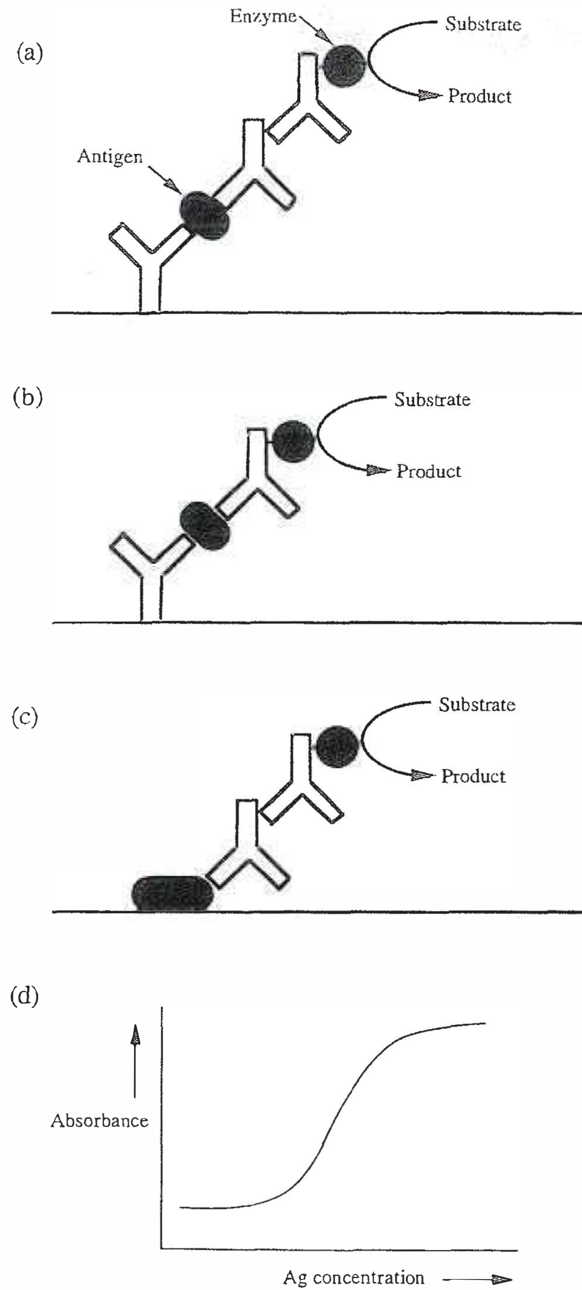


Figure 3.5 Common ELISA formats: (a) two-site ELISA for antigen quantitation using enzyme conjugated anti-antibody to detect; (b) two-site ELISA for antigen quantitation using directly conjugated second antibody; (c) determination of antigen-specific antibody; (d) expected form of standard curve for such direct binding ELISA formats

widely used. These are similar in format to immunoradiometric assays, although often an extra antibody layer is included to help amplify the signal generated by the enzyme (Figure 3.5). The use of such formats on microtitre plates allows assays to be carried out simply and quickly. Commonly used enzymes such as alkaline phosphatase and horseradish peroxidase can convert substrate to product with a high turnover number to allow the generation of a large amount of coloured product per enzyme molecule bound, resulting in amplification of the signal.

ELISAs are commonly used for measuring antibody as well as antigen. In this case ELISAs with immobilised antigen can be used to measure the amount of antigen-specific antibody by detecting with an anti-antibody reagent after binding. Alternatively, the amount of total antibody in a sample can be quantitated in a two-site ELISA using, for example, immobilised antibody to the light chain and detecting with enzyme conjugated to antibody directed to the heavy chain or vice versa. ELISAs can also be used in a number of competitive formats, for example in the detection of soluble antigens (Figure 3.6), and can also be carried out with whole cells as the antigen (Feit *et al*., 1983). ELISAs are usually carried out on plastic microtitre plates on which the 'capture' reagent is immobilised. These plates are usually made of polystyrene which is optically clear and are commonly used in the standard 96-well format. Such plates are particularly advantageous as they allow dilutions of standards and samples to be run under identical conditions on the same plate, which is ideal for an accurate result. Antibody or antigen is normally immobilised to the plate by passive adsorption through hydrophobic interactions, although it has been suggested that covalent binding may improve assay performance (Douglas and Monteith, 1994).

Antibody–enzyme conjugates are often produced by chemical coupling (see Section 2.6.2). This is usually a satisfactory procedure although there are often variations from batch to batch and in some cases it can lead to loss of some of the antigen-binding activity of the antibody or to loss of enzyme activity. Site-specific conjugation to Fc region carbohydrate has been investigated, and alkaline phosphatase conjugates prepared in this way (Section 2.6.2) have been shown to retain full binding activity (Husain and Bienarz, 1994). Recently, attempts have been made to generate suitable reagents by expression of antibody–enzyme fusion proteins. Alkaline phosphatase fusions to both scFv and $F(ab')_2$ fragments have been prepared, expressed in *E. coli* and shown to be fully functional for both antigen binding and enzyme activity (Wels *et al*., 1992; Ducancel *et al*., 1993). For application in immunoassays a general reagent consisting of anti-human IgG scFv or $F(ab')_2$ fused to alkaline phosphatase has been produced and shown to be capable of detecting human IgG to hepatitis B antigen in an ELISA (Carrier *et al*., 1995). An additional advantage of this approach is that the use of antibody fragments has been shown to reduce background binding in ELISAs compared to intact IgG molecules. The ease of recombinant techniques for the direct expression of antibody fragment–enzyme fusion proteins suggests that this route of production may become increasingly important in immunoassay development.

3.2.4 *Improving sensitivity*

Several approaches have been investigated to improve the sensitivity of immunoassays, to develop ultrasensitive assays, by manipulation of the detection system (Kricka, 1993). Increases in enzyme amplification of the signal generated have been described in which the product of the antibody–enzyme conjugate reaction is used to set up a cycle in which

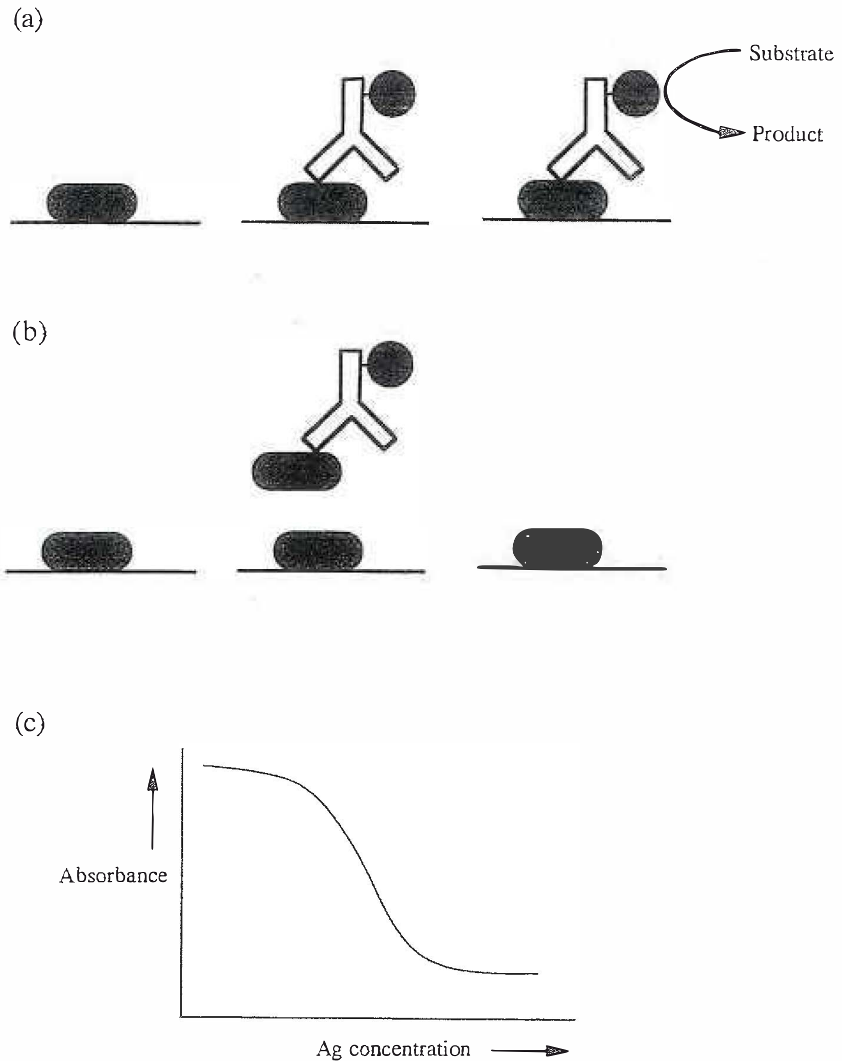


Figure 3.6 Competitive ELISA format assay: (a) in the absence of antigen in the sample, conjugated antibody binds to immobilised antigen and gives rise to the production of coloured product; (b) in the presence of antigen in the sample, conjugated antibody is prevented from binding to immobilised antigen leading to the absence of signal; (c) expected form of standard curve from such a competitive ELISA

a second enzyme system generates large amounts of coloured product as shown in Figure 3.7 (Stanley *et al.*, 1985). A high degree of signal amplification results as each NAD molecule generated can be responsible for the production of many hundred formazan molecules, and this has led to the development of several immunoassays with reported sensitivities over 100-fold higher than conventional alkaline phosphatase substrates such

HOSPIRA EX. 1029
Page 94
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

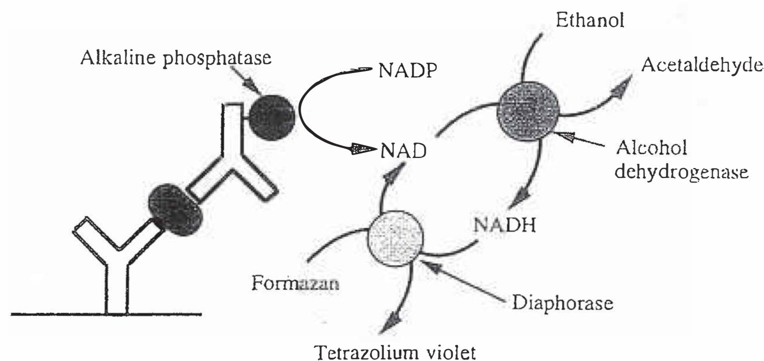


Figure 3.7 Example of an amplification mechanism for enhancing signal in ELISA

as *p*-nitrophenyl phosphate (Clark and Price, 1986). In many cases such highly sensitive assays may have problems with the level of non-specific background and thus careful optimisation of the assay is usually required.

Enzyme labels can also be made more sensitive by the use of substrates, resulting in the generation of fluorescent or chemiluminescent substances, which can be detected with greater sensitivity than colorimetric reactions. Substrates resulting in both fluorescent and chemiluminescent assays are available for the most commonly used enzymes, horseradish peroxidase and alkaline phosphatase (e.g. Albrecht *et al.*, 1994; Akhavan-Tafti *et al.*, 1995). Enzyme amplification cycles have also been combined with fluorescent readouts to allow determination of very small amounts of alkaline phosphatase, down to one thousandth of an attomole (one zeptomole or 350 molecules) when applied to an immunoassay of proinsulin (Cook and Self, 1993).

Fluorescent labels can also be directly attached to antibody and used as a detection system for immunoassays. The major problem with the use of fluorescent labels, however, is the background level of fluorescence generated by many biological substances and by plastics used for immobilisation. Hence conventional fluorescent compounds used in immunocytochemistry (see Section 3.4) such as fluorescein are of relatively little use. This can be overcome by the use of time-resolved fluorescent techniques using lanthanide chelates as the fluorescent reagents (Diamandis, 1988). In this technique pulses of light are used to excite the fluorescent material present and then the emitted light is measured after a short time interval of a few hundred microseconds. As background fluorescence is due to very rapid events, with fluorescence lifetimes of 100 nanoseconds or less, the interference can be removed by using fluorescent compounds which emit light for longer periods (1000 microseconds or more) and measuring emitted light after the background fluorescence has died away. Measurement of light emitted by such systems requires instruments which can pulse and measure fluorescence many times per second to build up a strong signal, and several such instruments have been commercialised. Chelates of lanthanides such as europium or terbium are suitable reagents as they have ideal properties of long fluorescence lifetimes, large Stokes shifts (the difference between the wavelength of light used for excitation and that emitted) and narrow emission wavelength bands. Many sensitive assays have been developed using this technology and they are widely used in clinical diagnostics. Attempts have also been made to produce recombinant molecules capable of binding lanthanides directly. A fusion protein between a

single-chain Fv and an engineered lanthanide binding protein has been produced and shown to bind terbium (MacKenzie *et al.*, 1995).

Chemiluminescent reactions have also been explored as a sensitive means of detection. Chemiluminescence results from a reaction in which the product is in an electronically excited state. Electrons in the excited state then relax to their ground state with the emission of light. Most useful chemiluminescent labels for direct attachment to antibodies have been the acridinium esters, which are capable of coupling to antibodies or antigens without a large loss in their quantum yield (Weeks *et al.*, 1983). They are usually coupled to antibody or antigen as *N*-hydroxysuccinimide esters which allows high coupling efficiencies, and chemiluminescence is initiated simply by the addition of hydrogen peroxide. An alternative luminescent reagent for attachment to antibody is the calcium-activated photoprotein aequorin, initially isolated from the jellyfish *Aequorea victoria*. Aequorin conjugated antibody has been used for the development of several sensitive immunoassays for hormones such as thyrotrophin, chorionic gonadotrophin, lutrophin and follitrophin (Rigl *et al.*, 1995). As a protein, aequorin can also be attached to antibody by recombinant means, and the construction and expression of a functional Fab′–aequorin fusion protein has been described (Casadei *et al.*, 1990).

The use of an antibody-linked peroxidase system to generate a chemiluminescent acridinium ester has been reported to result in longer duration of light than that generated from directly conjugated acridinium esters (Akhavan-Tafti *et al.*, 1995). Peroxidase also catalyses the oxidation of luminol by hydrogen peroxide which can be used to generate a strong signal in the presence of a suitable enhancer such as 4-iodophenol, 1,6-dibromo-2-naphthol or 6-hydroxybenzothiazole (Thorpe and Kricka, 1986). Such enhanced chemiluminescent assays have been shown to be useful in many situations, for example in the measurement of thyroxine (Christofides and Sheehan, 1995). Other enzymes can also be used, and xanthine oxidase has been shown to be useful in a luminol-based immunoassay system for interleukin-5 (Rongen *et al.*, 1997). Several substrates for alkaline phosphatase conjugates have been developed which give rise to chemiluminescent compounds after enzyme action (Bronstein *et al.*, 1989). The use of the substrate adamantyl 1,2-dioxetane aryl phosphate (AMPPD) has been shown to result in particularly low detection levels, down to one zeptomole, when applied to immunoassay of thyrotrophin (Bronstein *et al.*, 1989).

A simple method to increase sensitivity of immunoassays might be simply to put more of the label on to each detecting antibody molecule. This is limited by loss of immunoreactivity of the antibody when too many labelling molecules are attached. One way to increase this is to attach a carrier molecule, which can be loaded up with labels. Poly-lysine has been used in this way for the attachment of multiple fluorescent groups (Exley and Ekeke, 1981). However, the most useful strategy to date has been the use of the biotin:avidin or biotin:streptavidin interaction. Each streptavidin or avidin molecule can bind four biotin groups with high affinity. The detecting antibody can be labelled with several biotin molecules until the limit for retention of antibody-binding activity is reached. When bound in an assay, biotinylated antibody can then be revealed with streptavidin that is labelled with multiple numbers of signal generating molecules such as enzymes or fluorescent or chemiluminescent reagents, hence leading to a considerable amplification in signal. For example, thyroglobulin molecules labelled with up to 480 europium chelate groups can be attached to streptavidin and used to detect biotinylated antibody resulting in an amplification of 4500- to 6750-fold (Diamandis, 1991). Biotinylated antibody can also be produced by recombinant means by use of Fab fusion proteins to the biotin–carboxyl carrier protein (BCCP) subunit of *E. coli* acetyl-CoA carboxylase (Weiss

HOSPIRA EX. 1029
Page 96
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

et al., 1994). However, production of fully biotinylated material is difficult due to the requirement for sufficient biotin ligase enzyme to allow *in vivo* incorporation of biotin during expression of the Fab–BCCP fusion protein. Only approximately one sixth of the fusion protein produced was able to react with streptavidin (Weiss *et al.*, 1994).

Another approach to amplify signal is to attach the detecting antibody to a liposome, into which have been entrapped many detectable molecules, such as enzymes, fluorescent or chemiluminescent reagents. Liposomes are closed spherical structures in which a phospholipid bilayer encloses an aqueous compartment in which it is possible to entrap 10^3–10^6 water-soluble molecules in a stable manner. When used in an immunoassay, the liposome can be lysed at the end of the assay and the contents quantitated. Immunoliposomes can be produced by coupling antibody to lipid via a heterobifunctional cross-linker, either randomly or site-specifically via antibody carbohydrate (Torchilin, 1994; Ansell *et al.*, 1996). However, immunoliposomes often show a lower association rate than free antibody and may also increase non-specific binding levels. Nevertheless, direct comparisons of immunoassays for thyrotrophin with antibody conventionally labelled with an acridinium ester (10 labels per antibody) with an immunoliposome containing approx. 250 000 labels per liposome resulted in an improvement in signal to background ratio of approx. 3-fold for the immunoliposome assay (Law *et al.*, 1989). Lipid-tagged antibodies have also been produced by expression of scFv fragments fused to part of the *E. coli* major lipoprotein which is tagged with lipid when expressed in *E. coli*. The resulting lipid-tagged antibody fragments can be incorporated into liposomes with high efficiency and have been used to generate europium chelate loaded liposomes for a use in fluoroimmunoassay (Laukkanen *et al.*, 1995). A higher signal was generated using liposomally entrapped europium chelate compared to conventionally labelled scFv, resulting in a more sensitive assay.

The polymerase chain reaction (PCR – see Chapter 1) has also been adapted for use as a sensitive detection system for immunoassay, termed immuno-PCR (Sano *et al.*, 1992). Antibody is coupled to DNA through the avidin–biotin system. In the first system described, streptavidin is first bound to immobilised antibody through a protein A–streptavidin conjugate. This is then used to capture biotinylated DNA which is then detected and amplified through PCR with the resulting products analysed by electrophoresis. The resulting assay has been shown to be extremely sensitive, capable of detecting as few as 600 molecules in an immunoassay of bovine serum albumin (Sano *et al.*, 1992). PCR has also been applied to the simultaneous determination of multiple analytes (Hendrickson *et al.*, 1995). In this case human thyroid stimulating hormone, human chorionic gonadotropin and *E. coli* β-galactosidase were measured by a combination of three specific MAbs, each conjugated to a unique oligonucleotide which were designed to result in different size PCR products from the same primer sequence. The different size products could be resolved by electrophoresis, resulting in an assay which was 2–3 orders of magnitude more sensitive than a comparable ELISA.

3.2.5 Assay formats

The majority of immunoassay formats rely on the separation of bound and free antibody before detection. Homogeneous assays in which there is no need to separate bound and free antibody have been investigated in attempts to produce simplified assays. Several formats have been successful as competitive assays which are easy to use and automate,

HOSPIRA EX. 1029
Page 97
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

although sensitivity is often relatively low. The enzyme multiplied immunoassay technique, known as EMIT, uses glucose-6-phosphate dehydrogenase–antigen conjugates which catalyse the conversion of substrate to coloured product. When bound by antibody, enzyme activity is inhibited and therefore the amount of coloured product is proportional to the amount of competing antigen present (Armbruster *et al*., 1993). Later versions of this system use a recombinant form of the enzyme which is more suitable for automation (Vogl *et al*., 1996). A similar system, cloned enzyme donor immunoassay, known as CEDIA, also operates as a competitive assay. In this system two inactive fragments of β-galactosidase, enzyme donor and enzyme acceptor, are produced by recombinant means which when mixed together associate to produce active enzyme. When the enzyme donor is conjugated to antigen, antibody binding prevents association and formation of the active enzyme. Any free antigen competes for binding to the antibody, hence allowing enzyme activity to be generated by association of the antigen–enzyme donor with the acceptor (Henderson *et al*., 1986). Such homogeneous systems have been developed for immunoassay of many small molecular weight substances such as cortisol, ferritin, digoxin, folate, vitamin B12 and drugs of abuse (Van der Weide *et al*., 1992; Armbruster *et al*., 1995). Other homogeneous assay formats have also been developed with fluorescent endpoints, including fluorescence polarisation (Colbert *et al*., 1985), fluorescence energy transfer (Calvin *et al*., 1986) and fluorescence quenching (Barnard *et al*., 1989). Many other formats have been investigated, including some based on light scattering which may be simple to automate (Armbruster *et al*., 1993). Overall the advantages of homogeneous assays are in speed and convenience. However, the sensitivity of homogeneous immunoassays remains limited, largely because the use of a separation step in other assay formats allows washing, which reduces or removes interfering substances and non-specific binding events.

For most large diagnostic laboratories there is now a requirement for a high degree of automation in performing immunoassays, and many automated assays are in use and continue to be developed. Both heterogeneous and homogeneous assay formats have been automated using a wide variety of detection systems, including enzyme reactions, fluorescence, chemiluminescence and light-scattering techniques (Chan, 1996). Attempts are being made to develop all-in-one formats suitable for automation (Lovgren *et al*., 1996). Whether any of these systems will eventually dominate the future market for automated assays or whether the current range of immunoassay techniques will continue to be extended remains to be seen.

While large diagnostics centres require specialised instrumentation, there has also been much interest in developing 'point of need' assays which can be performed simply in the clinic, doctor's office or home to give a simple + or – readout. Applications include home pregnancy testing (measurement of chorionic gonadotrophin, hCG) and fertility testing (lutrophin measurement). Several useful formats have been developed and commercialised based on immunochromatographic test strips and membranes. For example, the hCG test strip, known as Clearblue One Step (Unipath) has been developed for home use. In this test coloured submicron latex particles are used to indicate a result by formation of a blue line in the large window and the small window (positive) or the small window alone (negative). A sample of urine is captured on a porous applicator which is then held in contact with the test membrane which has three zones of antibody (Davidson, 1992). Urine is used as the mobile phase and as it passes along the membrane it reaches the first zone consisting of blue microparticles with a Mab to the alpha subunit of hCG attached. The urine picks up the particles and carries them to the second zone, under the large window, which has a MAb to the beta subunit of hCG immobilised on the membrane.

HOSPIRA EX. 1029
Page 98
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

If hCG is present the coloured particles will bind resulting in a blue line under the large window. The urine continues to migrate along the membrane and reaches the third zone, under the small window, when excess or unreacted particles will be trapped resulting in a blue line whether hCG is present or not, allowing a control on the assay format. This assay can be carried out in three minutes with an overall performance (sensitivity and specificity) of 99% (Davidson, 1992). Many variants of such tests are now available which have successfully extended the applications of MAbs in the home for such applications as pregnancy testing (hCG assay), ovulation prediction (luteinising hormone, follicle stimulating hormone) and contraception (luteinising hormone and oestrone-3-glucuronide). Similar tests are under development or in use for applications such as identification of infectious diseases, drugs of abuse screening and monitoring disease markers.

3.2.6 Advantages of monoclonal antibodies in immunoassay

Although many immunoassays are still carried out with polyclonal antibody reagents, MAbs have clearly demonstrated an important role as described above, and have allowed a whole new generation of assays to be developed of importance in the research laboratory, the diagnostics industry and even the home. In many cases the combination of monoclonal and polyclonal reagents may allow powerful assays to be developed, and the design of a particular assay can take account of the properties of the individual reagents available.

By selection of an appropriate MAb, the interference of closely related substances in an assay can often be removed so that specificity is increased. For example, a MAb has been raised to the C-terminal part of human brain acetylcholinesterase which can distinguish between brain acetylcholinesterase and that present in red blood cells (Boschetti *et al*., 1996). Brain acetylcholinesterase leaks into the amniotic fluid in prenatal neural tube defects as well as neurological disorders. Samples of amniotic fluid from amniocentesis are often contaminated with blood and thus an assay which can distinguish between the two forms could be important in allowing accurate diagnosis of such serious conditions, reducing any false positive results. Closely related compounds may also be problematic in therapeutic drug measurements. Monoclonal reagents have shown superior specificity in the measurement of digoxin (Datta *et al*., 1996), and can be used to discriminate between a drug itself and its metabolites, as shown in the case of cyclosporin monitoring to allow adjustment of individual dosing regimens (Quesniaux, 1991). Another common form of interference in immunoassays is the presence of autoantibodies to the sample antigen. The use of MAbs in such cases also allows reduced interference, as demonstrated in the case of an IRMA for the measurement of serum thyroglobulin (Marquet *et al*., 1996).

Assay sensitivity may also be increased with MAbs. Simple substitution of a polyclonal capture antibody with a monoclonal has resulted in increased sensitivity in a fluorescent assay for salmon calcitonin (Rong *et al*., 1997). Similarly, sensitivity for the human cancer marker, prostate specific antigen, has been increased in another immunofluorometric assay which may be of utility in the diagnosis and staging of prostate and breast cancers (Ferguson *et al*., 1996).

One problem with the use of MAbs has been in the detection of small molecular weight materials, such as many drugs, which are often not large enough to contain two distinct epitopes for use in two-site assays. Thus the measurement of such substances has relied largely on competitive techniques, using detection either with radioisotopes

HOSPIRA EX. 1029
Page 99
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

(RIA) or with non-isotopic techniques such as those described above. Non-competitive approaches are now being developed. Two-site ELISAs have been developed based on the interaction of a small molecular weight analyte, such as digoxin, with an antibody which can then be detected by an antibody which recognises the new epitope formed by the bound analyte in the immune complex (Self *et al*., 1994). Alternatively, assays have been developed using one second antibody which sees the antibody–analyte immune complex and one which recognises the primary antibody only when the analyte is not bound (Mares *et al*., 1995).

MAbs have found applications in the measurement of a wide range of substances including hormones, metabolites, disease markers, therapeutic drugs, drugs of abuse and food and environmental contaminants such as mycotoxins, microorganisms, herbicides and pesticides. Antibody engineering technology is also beginning to have an impact on the design of immunoassays. Direct expression of fusion proteins may result in improved, homogeneous reagents in both enzyme-based and fluorescent assays. Antibody specificities can be directly expressed as fragments which may exhibit fewer problems of non-specific binding, and the power of phage selection of antibodies (see Chapter 1) allows rapid selection of useful binding specificities. Phage libraries may also allow the generation of useful antibodies which are difficult or impossible to obtain by other means, such as for antigens which are highly conserved between species, and it has been demonstrated that phage-bound antibody fragments may even be used directly in immunoassays (Navarra-Teulon *et al*., 1995). The applications of bispecific antibodies are also likely to increase as their production becomes simpler (see Section 2.5). For example, bispecific antibodies can be produced with specificities for two epitopes on the same target which may offer advantages in specificity and affinity of binding and extend applications in immunoassay development (Cook and Wood, 1994).

Bispecific antibodies are also finding application in simple immunoassay formats. Rapid diagnosis of pulmonary embolism (PE) in the clinic may be crucial to further treatment and it has been suggested that high levels of the fibrin degradation product, D-dimer, is associated with PE (Bridey *et al*., 1989). A rapid assay has been developed as an exclusionary test for PE which can allow exclusion of patients from potentially hazardous anti-coagulant therapy (Ginsberg *et al*., 1995). This assay uses a bispecific antibody in which one arm recognises D-dimer and one red blood cells. The antibody causes agglutination of the patient's own red blood cells in the test tube, hence allowing a simple positive or negative test to be performed within a few minutes of taking a blood sample.

Entirely novel assay formats are also possible based on recombinant antibody fragment production. It has been shown that the interaction between the two variable domains of an antibody (VH and VL) is considerably strengthened in the presence of antigen (King *et al*., 1993). This interaction has been used to develop an open sandwich ELISA method for the detection of antigen in which VL immobilised on an ELISA plate is used to bind antigen in the presence of phage displayed VH. The resulting complex is then detected by use of a peroxidase labelled anti-phage antibody (Ueda *et al*., 1996). If such antigen-promoted association of VH and VL is generally applicable then an exciting new class of immunoassays could result for both large and small antigens.

3.3 Immunosensors

An alternative to conventional immunoassays is the use of a biosensor based on antibody:antigen interaction, termed an immunosensor. Immunosensors are useful analytical

HOSPIRA EX. 1029
Page 100
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

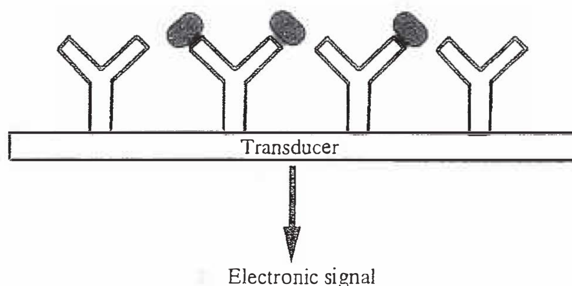


Figure 3.8 Diagram of a general immunosensor: antigen binding is detected by generation of signal which is converted to electronic format by a transducer

tools for monitoring antibody:antigen reactions in real time, often without the need for labelling either of the components. As such there has been great interest in developing immunosensors for analytical applications in the clinic, as well as for environmental monitoring and for food analysis. Immunosensors have been produced which are capable of detecting medical diagnostic markers such as hormones, drugs (therapeutic and abused), microorganisms and environmental pollutants such as pesticides. Under appropriate conditions immunosensors can also be used to analyse the kinetics of antibody:antigen binding.

An immunosensor is a solid-state device in which the antibody:antigen reaction is detected via a transducer which provides a signal that the binding reaction has taken place (Figure 3.8). Three main types of transducer have been used in biosensor technology; these exploit changes in mass (piezoelectric or acoustic wave), electrochemical properties (potentiometric, conductimetric or amperometric) or optical properties (fluorescent, reflective, luminescent, surface plasmon resonance or waveguide properties).

3.3.1 *Mass-detecting immunosensors*

Piezoelectric materials, such as quartz crystals, can be made to resonate by application of an alternating electric field with the frequency of the resulting oscillation being determined by the mass of the crystal. In piezoelectric biosensors, quartz crystals are coated with antibody. Binding of antigen results in a change in mass which is monitored through the change in frequency of the crystal oscillation. One problem with the use of such immunosensors is non-specific binding of sample components to the crystal, but sensitive devices have been produced for such applications as insulin measurement (Raman-Suri *et al.*, 1995), metamphetamine detection in human urine (Miura *et al.*, 1993), herbicide detection in drinking water (Yokoyama *et al.*, 1995) and detection of viruses and bacteria (Konig and Gratzel, 1993).

A further variant of the piezoelectric sensor uses surface acoustic waves to monitor binding to the crystal surface. In this application the oscillation of the crystal is at higher frequency and an acoustic wave is generated by application of an alternating voltage across interlaced electrodes, known as an interdigital transducer. A second interdigital transducer detects the acoustic signal a few millimetres away. Binding of sample to the crystal slows the acoustic wave, with the change in velocity being proportional to the analyte concentration. Such devices have the potential for higher sensitivity but may suffer more interference from factors such as temperature, pressure and conductivity, all of which may affect the properties of the acoustic wave. An acoustic wave sensor for

HOSPIRA EX. 1029
Page 101
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

measurement of the pesticide atrazine enabled sensitive detection in a competitive assay, with the ability to re-use the device 48 times with only a 30% loss in response (Tom-Moy *et al.*, 1995).

3.3.2 Electrochemical immunosensors

Potentiometric immunosensors are based on the change in potential that results when antibody is immobilised on an electrode and its antigen binds to it. Antibodies, like all proteins, are polyelectrolytes and in many cases binding of antigen will alter the charge. Therefore the potential difference between an electrode with immobilised antibody and a reference electrode will depend on the concentration of antigen present. However, direct detection results in only small changes in potential (1–5 mV) and low signal : noise ratios because the the charge density on the antibody is low compared with background ions. Also, signal is dependent on variables such as pH and ionic strength of the sample and sensitive to interference from other ions. Attempts to improve such sensors have included the development of antibody field-effect transistor devices (immunoFET). ImmunoFET operates through measurement of conductivity through a channel region between source and drain electrodes. The conductivity is controlled by the strength of electric field generated by the gate, providing an amplification effect. Antibody is immobilised on a membrane which must be thin enough to allow redistribution of the small charge changes which occur on antigen binding. However, reliable immunoFET sensors have not been developed due, at least in part, to problems in manufacturing suitable membranes.

Conductimetric sensors monitor variations in the conductivity between two electrodes by measuring variations in the current across the electrodes. Many reactions lead to variations in ion concentration through either the consumption or production of ionic species, and thus such sensors have been developed with many enzyme systems. Application of conductimetric sensor technology to immunosensors has been limited to date.

Amperometric sensors measure the current generated when electroactive species are reduced or oxidised at the electrode. Antibodies are not directly electroactive and thus enzyme labels are used to generate electroactive species which can then be measured. Most often such sensors use oxygen or hydrogen peroxide electrodes, with the current produced being directly proportional to the amount of oxygen or hydrogen peroxide reduced or oxidised. Catalase was the first label used to construct an immunosensor in this way. MAb to human chorionic gonadotropin (hCG) was immobilised on an oxygen electrode to which hCG and catalase-labelled hCG could compete for binding (Aizawa *et al.*, 1979). Subsequently alternative labels, such as alkaline phosphatase, have been used in the detection of several antigens (Treloar *et al.*, 1994). Substrates for alkaline phosphatase which can be used to generate electroactive compounds include *p*-aminophenol phosphate or *N*-ferrocenyl-4-aminophenol phosphate. Alternative labels have also been developed for such applications as measurement of apolipoprotein E in serum (Meusel *et al.*, 1995) and detection of herbicides such as 2,4-dichlorophenoxyacetic acid using a disposable immunosensor (Kalab and Skladal, 1995). The development of amperometric immunosensors has also been extended into *in vivo* use for the measurement of corticosteroids (Cook, 1997). This system used an immunosenor with antibodies immobilised on the electrode surface surrounded by a dialysis membrane which allows corticosteroids to equilibrate across. When the corticosteroid to be measured has equilibrated, a solution of corticosteroid conjugated to horseradish peroxidase is introduced into the internal cavity of the electrode, which then competes for antibody binding. The electrode surface

can then be washed and peroxidase substrate added which is detected amperometrically. The probe can be regenerated with 1 mM HCl, allowing re-use every three minutes. The small size of this probe allowed implantation into the circulatory system of animals for monitoring hormone levels in real time, with the probe viable for several hundred measurements over 48 hours. The development of such systems may allow real-time, *in vivo* monitoring of a range of substances in the future.

3.3.3 *Optical immunosensors*

Optical immunosensors can be used with or without labels. Some sensors use labels such as enzymes or fluorescent or luminescent reagents to generate the optical signal, whereas in other cases binding is detected through the use of techniques such as total internal reflection, surface plasmon resonance or dielectric waveguides.

Labelled antibody immunosensors based on fluorescence or luminescence are attractive due to the relatively simple instrument design possible. Also, the use of optical fibre technology has enabled many devices to be miniaturised. These essentially operate via the generation or quenching of light at the surface of the optical fibre. Chemiluminescent assays have been developed based on the generation of light from the peroxidase catalysed oxidation of luminol, and fluorescent devices have been developed using different fluorescent labels, for applications such as the measurement of the cancer marker, prostate specific antigen in whole blood (Daniels *et al.*, 1995).

The most widely used optical immunosensors do not require labelled reagents. Surface plasmon resonance (SPR) uses light directed towards a layer of low refractive index from one of higher refractive index at an angle such that total internal reflection occurs. A metal film coated with a dextran layer is used to immobilise the antibody (or antigen). When light hits the metal film, surface plasmons in the film are excited and an evanescent wave is generated, a process known as SPR, which results in a decrease in the intensity of reflected light (Figure 3.9). When antigen binds to the immobilised antibody, the resulting change in refractive index causes a change in the angle at which the drop in reflected light intensity occurs. Continuous monitoring of the angle and intensity of reflected light therefore allows real-time analysis of binding events. A commercial system based on this principle, known as the BIAcore™, is now widely used for the analysis of biological interactions (Malmqvist, 1993). In this instrument SPR technology has been combined with a microfluidics system such that continuous monitoring is possible (Figure 3.9). This not only enables quantitative measurements to be made but also enables detailed kinetic analysis of binding interactions to be performed in real time. This instrument has therefore found many applications in the analysis of antibody: antigen interactions. BIAcore™ analysis is now used to determine the binding affinities of MAbs, and is widely used to monitor the outcome of antibody engineering experiments, for example in the selection of phage displayed antibodies, in the characterisation of antibody fragments and in epitope mapping (Malmborg and Borrebaeck, 1995).

Another commercially available type of optical biosensor, the IAsys™, is based on a combination of SPR technology with waveguide technology. This device has found applications in the area of bioprocess monitoring. The production of an Fv fragment could be monitored through fermentation and purification processes (Holwill *et al.*, 1996). Measurements were performed in five seconds, with an overall assay time of two minutes. This constant monitoring of the process allowed in-process decisions to be made to improve the process efficiency. A comparative study which used both BIAcore™ and the IAsys™

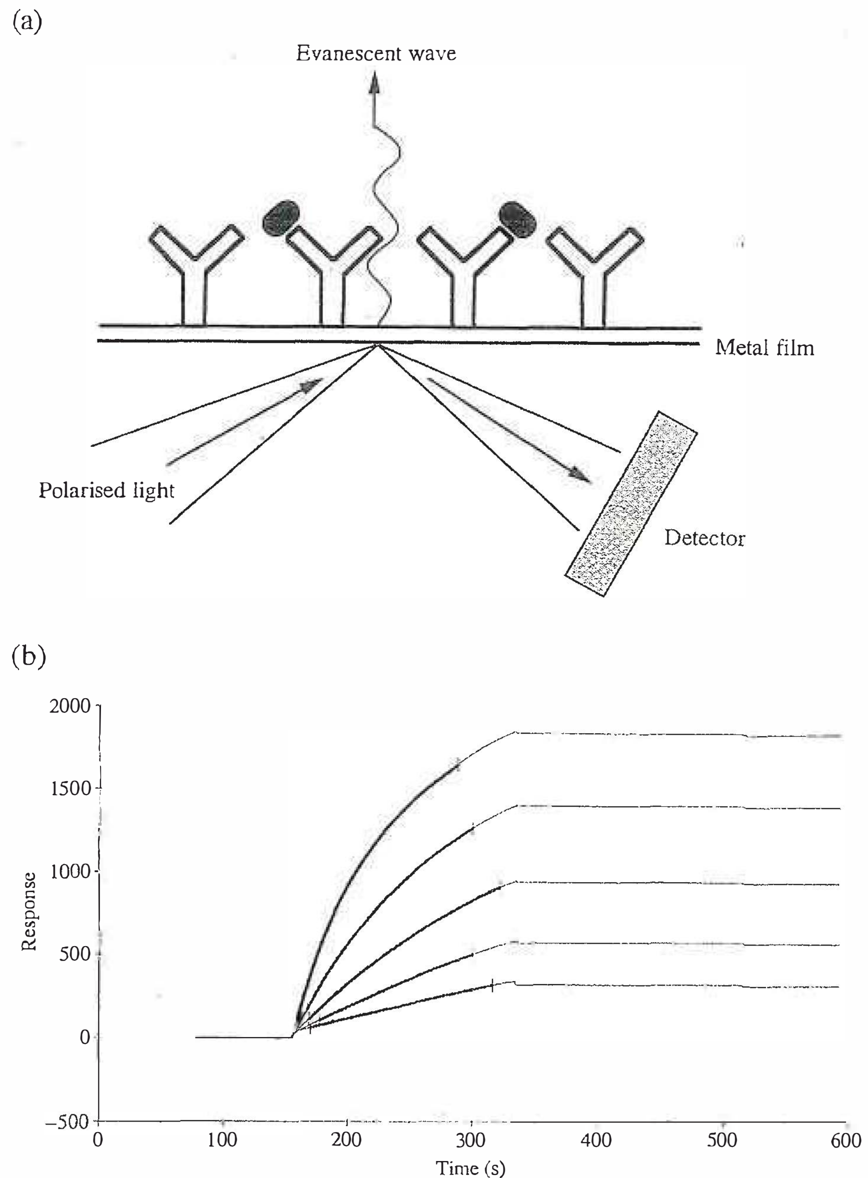


Figure 3.9 (a) Schematic diagram of BIAcore optical biosensor. Polarised light is reflected in the gold film of the sensor chip and detected on a diode array. Surface plasmon resonance is observed as a decrease in light intensity for a specific angle of incidence. The angle changes as the refractive index in the vicinity of the surface changes due to binding of large molecules on the immobilised ligand on the sensor chip. (b) Example of BIAcore sensorgrams for the binding, and slow dissociation, of IgG to an antigen-coated chip at five different concentrations. Measurement of inital on-rate and off-rate can be made to allow estimation of binding affinity.

HOSPIRA EX. 1029
Page 104
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

system to monitor the kinetics of binding of lysozyme to anti-lysozyme antibody found good agreement between results obtained from the two systems (Yeung *et al.*, 1995).

3.4 Immunocytochemistry

Immunocytochemistry allows the detection and location of antigens within cells and tissues by the application of labelled antibody (either directly labelled or via a labelled second antibody reagent) followed by microscopy. Suitable labels to allow both light microscopy and electron microscopy applications are available. In the research laboratory immunocytochemistry is used to visualise antigens and their subcellular locations in tissues. Double-labelling techniques, in which antibodies to two different antigens are applied with different labels, allow comparison of the relative distributions of two antigens in the same tissue samples. This is compared with conventional histological stains and markers to build up a detailed picture of antigenic distribution. Immunocytochemistry is also widely used in diagnostic pathology, particularly in the identification of types of tumour cells, infectious organisms or inflammatory cells.

Detailed methodologies for immunocytochemistry procedures for light and electron microscopy have been described (Beesley, 1993; Polak and Priestley, 1992), and therefore the technique is summarised only briefly here. Immunocytochemistry requires firstly tissue preparation followed by antibody binding and then detection. Two major types of tissue sections are used in immunocytochemical studies: fixed and frozen. Frozen sections are more difficult to prepare but the technique is relatively gentle and leaves more of the immunological features of the tissue intact. Fixed tissue sections are prepared, often in formalin-based fixative, and embedded in paraffin prior to cutting slices. Such fixed sections are simple to prepare, retain cellular morphology and can be stored for long periods but the process of preparation is more likely to destroy antigenic epitopes within the tissue. Nevertheless such fixed sections are widely used in diagnostic pathology due to their ease of preparation, and the existence of extensive slide libraries which can be used for comparative purposes. Cell suspensions from cell culture experiments, and cell smears from, for example, needle aspirates or tissue scrapings, can also be used for immunocytochemistry after suitable fixing procedures. Such slides obviously do not preserve the morphology of the intact tissue but are useful in many subcellular studies, or in the identification of infectious organisms in tissue samples.

MAbs offer improved specificity compared to many polyclonal reagents also used in immunocytochemistry. However, the loss of a MAb epitope on tissue fixing is more likely than the loss of all the epitopes recognised by a polyclonal antiserum. Therefore, polyclonal reagents may be preferred for the detection of some antigens, particularly when using fixed tissue sections. Non-specific binding is generally reduced by use of monoclonal reagents. In some tissues non-specific binding via Fc receptors may also be a significant problem which can be overcome by use of antibody fragments such as Fab and $F(ab')_2$. In addition, antibody fragments may ease the penetration of the reagent into tissues. Smaller fragments such as engineered Fv fragments may also be beneficial and have also been developed for use in immunocytochemistry applications (Kleymann *et al.*, 1995).

Antibodies can be directly labelled for immunocytochemistry, but if several antigens are to be detected, this requires preparation of several individual labelled reagents. The use of labelled second antibody allows the detection of several antibodies with a single reagent. For example, sections can be probed with mouse MAbs and then detected with

HOSPIRA EX. 1029
Page 105
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 3.3 Examples of antibody labels for immunocytochemistry

Enzymes	Substrates	Colour
Horseradish peroxidase	Diaminobenzidine	Brown
	Tetramethylbenzidine	Blue
	Aminoethylcarbazole	Red
	4-chloro-1-naphthol	Blue-black
Alkaline phosphatase	Naphthol AS phosphate + fast red, blue or violet	Red, blue or violet
	Bromochloroindolyl phosphate + nitroblue tetrazolium	Blue
Fluorescent labels	Fluorescein	Green
	Rhodamine	Red
	Phycoerythrin	Orange
	Texas Red	Red
	Cyanin 3 or 5	Red or orange
Metal labels	Colloidal gold 1–40 nm	Electron-dense
	Gold + silver enhancement	Dark brown or black

a labelled anti-mouse Ig reagent. Second antibody methods also allow some amplification on detection, and avoid the problem of developing labelling methods to minimise loss of antigen-binding properties with many different antibodies. Alternatively, biotinylated antibodies which can be detected with a labelled streptavidin conjugate are widely used.

The major types of label used in immunocytochemistry are enzymes used to generate insoluble coloured products, fluorescent labels and colloidal gold particles (Table 3.3). Both fluorescent and enzyme substrate labels are available in different colours such that two or more antigens may be visualised simultaneously. For example, fluorescein and phycoerythrin can be used to detect two different antigens under a fluorescent microscope with differential colour fluorescence, green and orange respectively. Horseradish peroxidase is the most widely used enzyme label for animal tissues, though not for plant tissues due to the presence of endogenous peroxidase activity which can lead to problems with non-specific staining. Conversely plant tissues can be stained with alkaline phosphatase conjugates, although endogenous activity makes this technique unsuitable for many animal tissues. In practice, however, both enzymes are suitable for most types of tissue, as endogenous activity is easily inhibited. Colloidal gold is suitable as a label for use in both light and electron microscopy. In light microscopy, the signal from gold-labelled antibodies is seen as a pinkish colour. This is usually intensified by the use of silver enhancement in which the gold catalyses the reduction of silver ions to metallic silver which results in a stable signal. Under the electron microscope, colloidal gold is seen as dense black round particles that can easily be distinguished from cellular structures. Different size particles are available, for example, 5 nm and 30 nm, which can be used to allow the detection and relative quantification of two antigens simultaneously.

Many of the problems observed with immunocytochemistry are similar to those seen with immunoassay development. Non-specific binding is also a problem which can be overcome in many cases by purification of suitable reagents, or the use of antibody fragments or alternative labelling techniques.

HOSPIRA EX. 1029
Page 106
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

One of the major uses of immunocytochemistry is the identification and classification of human tumour types (Giovagnoli and Vecchione, 1996). Information gained in this way can be of direct benefit in patient care. MAbs can allow the identification of the primary site of metastatic carcinoma, give information on the malignancy of the tumour and in some instances be an aid to determining prognosis. For example, the breast cancer marker p185^{HER2} has been associated with increased probability of relapse and poor outcome (Slamon *et al.*, 1987). Immunocytochemistry to detect expression of this marker in breast cancer tissue is thus useful in determining likely patient prognosis. Similarly, applications of MAbs in the detection and identification of microorganisms are important clinically such as in the demonstration of human cytomegalovirus infection (Jahn and Plachter, 1993). There is also a role for immunocytochemistry in other disease states in the detection of cellular infiltrates and the determination of their activation state, the localisation of adhesion molecules and identification of locally produced cytokines. In pulmonary diseases such as allergic asthma and sarcoidosis, the analysis of cytokines from cells present in bronchoalveolar lavage fluid using immunocytochemistry can give an insight into the disease process (Krouwels *et al.*, 1997).

3.5 Flow cytometry and cell sorting (FACS)

Flow cytometry and fluorescence activated cell sorting techniques (FACS) are widely used in cell biology research for both analytical and preparative purposes. Fluorescently labelled antibodies capable of binding to the cells of interest are used to tag these cells, such that the cells become fluorescent. FACS equipment resolves mixtures of cells based on this fluorescence. Within the instrument a stream of single cells passes through a laser light beam and fluorescent cells can be individually detected. The distribution of antigens within the population of cells can therefore be readily studied. Preparative isolation of populations of cells becomes possible as the instrument can selectively impart an electrostatic charge to labelled cells and not others. The cells then pass through an electric field when the cells carrying a charge are deflected into a different container from the uncharged cells. As such this technique can be considered as an immunopurification technique for whole cells, which has been used for both research and clinical applications. Applications include the separation of cell populations for functional studies and the isolation of rare transfected cells to simplify cloning.

FACS techniques can also be performed with two different fluorescent labels, allowing the simultaneous detection of two cellular antigens. Such analyses are widely used in characterising populations of cells, for example in the characterisation of hematopoietic stem cells used for autologous transplantation, using antibodies to CD34 and CD19 (Fritsch *et al.*, 1995). The use of FACS for preparing a purified population of CD34 positive bone marrow cells for use in transplantation studies has also been described (Rondelli *et al.*, 1996). The application of flow cytometry to detect cellular antigens is not restricted to cell surface markers: intracellular substances can also be detected. The use of flow cytometry in the detection of intracellular interleukin-4 and -5 and interferon-γ has been compared with immunocytochemistry (Krouwels *et al.*, 1997). In this comparison flow cytometry was shown to be more suitable for use with double staining techniques for detection of more than one antigen and allowed isolation of defined cell populations. However, the detection of these cytokines was apparently less sensitive than with immunocytochemistry.

3.6 Western blotting (immunoblotting)

The technique of western blotting, also known as immunoblotting, is used to detect protein antigens after polyacrylamide gel electrophoresis (PAGE). PAGE is used to separate proteins in an electric current, and in its most widely used form is carried out in the presence of the detergent sodium dododecyl sulphate (SDS). SDS denatures proteins and binds to them such that a uniform negative charge is imparted to each protein. Therefore, when proteins are run through a gel using an electric current in the presence of SDS (SDS-PAGE) they can be separated on the basis of molecular weight. The range of molecular weights which can be separated depends on the pore size in the gel used and this is normally controlled by the concentration of acrylamide and cross-linker used in formation of the polyacrylamide gel. Proteins separated on SDS-PAGE are then visualised using a general protein stain such as coomassie blue or, more sensitively, silver stain. However, general protein stains stain every protein, and it is often desirable to be able to identify specific proteins. This can be achieved by western blotting. After SDS-PAGE the proteins present in the gel are transferred laterally (blotted) onto a membrane of nitrocellulose or an alternative polymer such as polyvinylidene difluoride (PVDF). The protein (or proteins) of interest can then be detected using the desired antibody which is either conjugated to a suitable label or detected by a secondary labelled antibody reagent. Labels commonly used include the radioisotope iodine-125 followed by exposure to X-ray film, colloidal gold with silver enhancement (see Section 3.4), and the enzymes horseradish peroxidase and alkaline phosphatase either with insoluble coloured substrates or, in the case of peroxidase, to generate a chemiluminescent readout by oxidation of luminol in the presence of hydrogen peroxide. Chemiluminescent detection by HRP-conjugated antibodies is usually enhanced with chemical enhancers and is a particularly sensitive detection method. An additional advantage is that after detection the signal can be removed by stripping off the bound antibody with protein denaturants as first developed for use with radiolabelled antibodies (Kaufmann *et al.*, 1987). This allows the blot to be reprobed with another antibody and re-detected, thus allowing several different antigens to be examined sequentially on the same blot. Western blotting is not confined to SDS-PAGE but can also be used to identify proteins separated by electrophoresis under non-denaturing conditions or by isoelectric focusing.

An example of a western blot is shown in Figure 3.10. Both polyclonal and monoclonal antibodies are used in western blotting. MAbs offer excellent specificity, but as detection is carried out on denatured proteins, reactivity of the MAb with the native conformation of the protein does not necessarily allow the antibody to be used in western blotting. This is because the antibody may recognise an epitope not made up of a stretch of linear polypeptide sequence but of parts of the protein which are spatially close in the protein's native conformation due to folding. Polyclonal antisera which recognise many different epitopes are more likely to contain antibodies which recognise some linear polypeptide sequences, and thus are often more successful in western blotting applications. However, screening can be carried out to identify those MAbs which are suitable for blotting.

The applications of western blotting in the research laboratory are wide and include the detection and analysis of natural proteins of low abundance, the analysis of recombinant protein expression and the detection of contaminant proteins. The combination of immunological reactivity with size analysis gained from western blotting allows analysis of protein processing and post-translational modifications. The use of MAbs allows precise information to be gained which would not be possible otherwise. This is demonstrated by a recent study to determine the role of matrix metalloprotease enzymes in human arthritis

HOSPIRA EX. 1029
Page 108
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

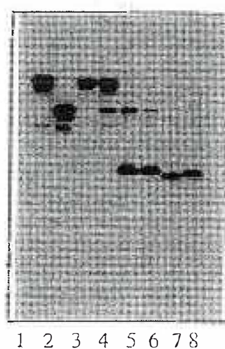


Figure 3.10 Example of a western blot. SDS-PAGE was carried out using a 4–20% polyacrylamide gel under non-reducing conditions. The blotted gel was probed with a murine monoclonal antibody to IgG Fd (Fab heavy chain) followed by a polyclonal rabbit anti-mouse IgG–horseradish peroxidase conjugate. The blot was then developed using enhanced chemiluminescence. Lane 1, recombinant human IgG4 (hIgG4); lane 2, $F(ab')_2$ derived from hIgG4; lane 3, hIgG1; lane 4, hIgG4 and $F(ab')_2$ mixture; lane 5, γ1 hFab' and $F(ab')_2$ expressed in mammalian cells; lane 6, γ1 hFab' and $F(ab')_2$ expressed in *E. coli*; lane 7, γ4 hFab'(a) expressed in mammalian cells; lane 8, γ4 hFab'(b) expressed in *E. coli*.

(Fosang *et al.*, 1996). Aggrecan is the major proteoglycan in cartilage and the molecule that allows the tissue to bear weight by deforming under compression. A MAb recognising the cleaved products of metalloprotease action on aggrecan allowed the detection of cleaved products in synovial fluid by western blot which could be quantitated with an ELISA using the same antibody. This analysis allows the protease activity involved to be characterised, leading to further insight into the degradative mechanisms involved in the arthritic process.

Western blotting can also be used in routine diagnostic procedures, for example in the confirmation of HIV infection (Gurtler, 1996). In this case anti-HIV antibodies are detected initially by ELISA tests, and reactive results are confirmed by western blotting which can allow differentiation of HIV types and subtypes. Western blotting has also been used to determine alcohol abuse by measurement of carbohydrate-deficient transferrins (Anton and Bean, 1994). Carbohydrate-deficient transferrins are produced in greater quantities than usual during periods of heavy alcohol intake due to decreased glycosylation of transferrin before release into the blood. Western blotting of serum samples allows separation and detection of the carbohydrate-deficient transferrin from the bulk of normal transferrin. In this case isoelectric focusing is used as the separation technique, where the difference in charge of the carbohydrate-deficient transferrin provides the basis for good separation from a large amount of normal transferrin.

Recombinant fusion proteins are also beginning to play a role in western blotting applications. Fusions of an scFv recognising the plant protein phytochrome have been made to both alkaline phosphatase and the IgG binding protein staphylococcal protein A (Gandecha *et al.*, 1994). Western blotting of extracts from oat seedlings containing phytochrome demonstrated that both of these fusion proteins could be successfully used for western blotting. The alkaline phosphatase conjugate was visualised directly using the chromogenic substrates BCIP/NBT (bromochloroindolyl phosphate + nitroblue

HOSPIRA EX. 1029
Page 109
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

tetrazolium), whereas the protein A conjugate was used in a two-step method, detected by an IgG–alkaline phosphatase conjugate. The fusion proteins were expressed in *E. coli*, and detection on the western blots was possible using the fusion proteins directly from *E. coli* lysates without the need to purify the reagents. The functionality of such conjugates suggests that the role of recombinant fusion proteins in the immunoassay field in general will increase and allow the use of simple single-step procedures, which may be advantageous in reducing non-specific binding problems.

3.7 Immunopurification

Immunopurification uses the specificity of the antibody to bind to a substance of interest and isolate it in a purified form. Antibody is usually coupled to a solid phase, such as beaded agarose, and packed into a chromatography column for use in the procedure known as immunoaffinity chromatography. This is a type of affinity chromatography in which antibodies are used as the binding moiety to retain the protein, or other substance of interest, while contaminating material flows through the column. The bound protein is then eluted by a change in buffer conditions to those which promote dissociation of the antibody–antigen complex (Figure 3.11). Immunoaffinity chromatography is used for the simple, rapid purification of proteins in the research laboratory both for preparation of the protein itself and as a quantitative measure, i.e. assay. In addition, immunoaffinity chromatography is used commercially for the purification of a number of pharmaceutical proteins, particularly high value proteins which are difficult to purify by other means. Because the interaction between antibody and antigen is very specific, a high degree of purification is achieved in a single step. Therefore, the use of immunoaffinity chromatography negates the requirement to develop protein-specific, multi-step purification protocols and allows rapid isolation of protein for research purposes.

Elution from the immunoaffinity chromatography column is achieved by disruption of the antibody–antigen interaction using mild denaturation such that both antigen and antibody are not irreversibly denatured. Commonly used eluents include extremes of pH and chaotropic agents such as thiocyanate, which often allow active material to be obtained. However, if the affinity of the antibody for antigen is very high it may be difficult to elute bound antigen without irreversible denaturation. Relatively low affinity MAbs are therefore ideal for successful immunopurification. Antibodies with affinity constants in the range 10^{-4}–10^{-8} M have been suggested to be most suitable (Phillips, 1989). However, the best way to select an antibody for immunopurification is by testing a panel of antibodies, to see which allow elution of the antigen under mild conditions. This can be achieved conveniently using ELISA or BIAcore format assays in which several eluent solutions are also screened, or antibody coupled to solid-phase beads can be used in small-scale experiments. When suitable antibodies have been identified, the antibody is coupled to a solid phase, usually a cross-linked beaded agarose material such as Sepharose, suitable for use in immunoaffinity chromatography. Many such matrices are commercially available in pre-activated form, which allows simple attachment of the antibody through amine groups with matrices derivatised with linkers such as *N*-hydroxysuccinimide or cyanogen bromide (see Section 2.6.2). Matrices are also available which allow site-specific attachment of antibodies, such that more of the antigen-binding ability is retained.

Random coupling through lysine residues often results in only 10–15% of the antibody retaining its antigen-binding activity due to a combination of chemical modification of

HOSPIRA EX. 1029
Page 110
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

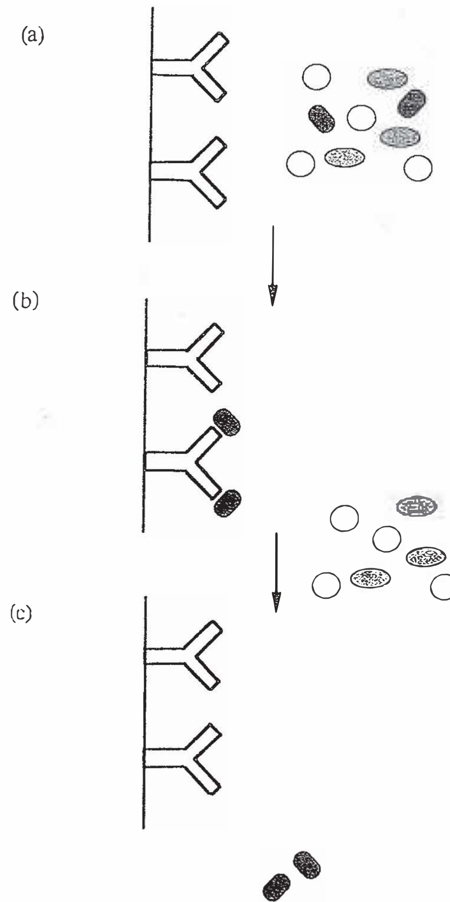


Figure 3.11 Immunoaffinity chromatography: immobilised antibody is used to selectively bind the ligand of interest (a); after washing to remove non-bound material (b), bound ligand is eluted in purified form (c), regenerating the immobilised antibody for re-use

important amino acid residues in the binding site of the antibody, and lack of orientation of antibody on the solid phase, such that many binding sites may be occluded (Fowell and Chase, 1986). Similar methodology is used for site-specific attachment as for site-specific protein–protein conjugates (see Section 2.6.2). Site-specific attachment can be achieved through attachment to Fc carbohydrate by periodate oxidation followed by reaction with hydrazide activated matrix (O'Shanessy, 1990). By attaching the antibody via the Fc this method orients the antibody such that the antibody-binding sites are less likely to be occluded by the matrix as well as avoiding modification of important amino acids in the CDR regions. Similarly, Fc region binding proteins have been used to orient the antibody correctly such that the antigen-binding site is still available. Staphylococcal protein A and Streptococcal protein G are widely used in antibody purification as they bind IgG specifically through Fc region binding sites between the CH2 and CH3 domains (see Chapter 5). Protein A and protein G linked to solid phases are therefore widely available reagents.

Binding of antibody for immunopurification to the protein A matrix followed by chemical cross-linking can therefore be used to immobilise antibody in oriented fashion, retaining a larger proportion of active antigen-binding sites (Sisson and Castor, 1990). Fab′ fragments can also be used for site-specific attachment to matrices through the hinge region free thiol groups. Maleimide or iodoacetyl activated matrices can be used to generate Fab′ solid phases in which most of the antigen-binding ability of the Fab′ is retained (Prisyazhnoy *et al*., 1988). Immobilised Fab′ fragments may have additional advantages in that the lower avidity of the monovalent Fab′ fragment for antigen may allow elution of bound antigen under less harsh conditions than for intact IgG, particularly when low immobilised density on the solid phase is used.

Immunoaffinity chromatography is widely used for the purification of both natural and recombinant proteins for research purposes, particularly when the source of the protein contains only small amounts among many other contaminating proteins. With recombinant proteins it is not always necessary to have a MAb to the protein, as specific 'tags' can be added to the gene before expression such that the resulting fusion protein can be easily purified. The commercially available FLAG system is one such 'tag' (Hopp *et al*., 1988). The FLAG epitope consists of the 8 amino acid sequence Asp-Tyr-Lys-$(Asp)_4$-Lys, and vectors are available which allow fusion of the gene for this sequence to the recombinant gene of interest. The resulting fusion protein can then be purified using an immobilised MAb which recognises a calcium complex of the FLAG peptide. The MAb used is conformation-specific and in the absence of calcium the antibody dissociates from the peptide tag. This system therefore allows for very mild elution of the tagged recombinant protein using EDTA to remove calcium or, alternatively, a competitive peptide can be used (Hopp *et al*., 1996). The FLAG system has proved of wide applicability and has been used to purify a wide range of protein targets including interleukin-3 (Park *et al*., 1989), β2-adrenergic receptors (Guan *et al*., 1992), tumor necrosis factor (Su *et al*., 1992), transcription factors (Chiang and Roeder, 1993), antibody fragments (Knappik and Pluckthun, 1994) and interleukin-5 receptors (Brown *et al*., 1995).

Immunoaffinity chromatography is also increasingly used as a form of immunoassay. Immobilised antibody is used to capture the analyte of interest, which is then eluted and quantitated by UV absorbance or other means. The development of useful assays has been facilitated by the development of new solid-phase materials which can be operated at high flow rates allowing very fast assays to be performed and automated (Afeyan *et al*., 1992). Assays are not restricted to proteins, and for other analytes immunoaffinity chromatography is often coupled with detection techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC) or mass spectrometry (MS). For example, the β2-agonists salbutamol and clenbuterol have been determined in tissue samples (Pou *et al*., 1994), and both aflatoxin contamination in cheeses and cannabis metabolites in saliva have been determined by combining immunoaffinity chromatography with HPLC (Dragacci *et al*., 1995; Kircher and Parlar, 1996).

The high affinity of the antibody:antigen interaction also allows immunoaffinity chromatography to be used for the specific removal of trace contaminants which may be present at low levels. This includes the removal of protein contaminants from pharmaceutical proteins for *in vivo* use, and applications such as the removal of trace bacterial contamination from foodstuffs. Molloy *et al*. (1995) have demonstrated that an immobilised scFv which recognises a lipoprotein surface component of *Pseudomonas aeruginosa* can remove cells of this bacterium from mixed cultures and from milk. This study demonstrated that immobilised antibody fragments could be effective and potentially

HOSPIRA EX. 1029
Page 112
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

economic in this type of application and suggests that scale-up of this technology may be possible to allow the use of immunoaffinity columns for removal of toxic materials from the environment.

The use of immunoaffinity columns for specific removal of materials may also find clinical application. Several groups have attempted to develop systems in which plasma is removed from the patient, passed through an immunoaffinity column and then returned to the patient. Sato *et al.* (1989) have suggested that specific removal of IgE is useful in the treatment of patients with allergy or other conditions in which the level of IgE is abnormally high. Another application is the use of immunoaffinity techniques for the removal of radiolabelled antibodies from the blood following radioimmunotherapy of tumours (Norrgren *et al.*, 1991). Radioimmunotherapy is described in Section 4.2.5. Briefly, antibody to a tumour-associated antigen is used to target a radioisotope to tumour cells in an attempt to kill them. However, to deliver a toxic dose to the tumour requires injection of a large amount of radiolabelled antibody which is often toxic to normal tissues such as bone marrow. Therefore attempts have been made to allow antibody to localise to tumour cells and then remove excess antibody from the blood using immunoaffinity techniques, hence reducing normal tissue toxicity.

Many recombinant proteins are now used as pharmaceuticals. Immunopurification would be an attractive method for purification of many of these pharmaceutical proteins. However, the production of MAbs in large amounts is relatively expensive compared to other chromatographic ligands, and therefore the use of immunopurification techniques in the production of proteins for pharmaceutical use has been limited. Also, when intended for use in the manufacture of a pharmaceutical protein the MAb must be produced to a standard which is as high as that demanded of the pharmaceutical protein itself. Nevertheless, immunopurification has been used for some high-value proteins which are difficult to prepare by other means, for example interferons, factor VII, factor VIII and factor IX (Bailon and Roy, 1990; Kim *et al.*, 1992).

Antibody fragments which can be produced in bacterial expression systems may be manufactured much more economically than intact IgG (see Chapter 5) and thus there has been interest in the development of immunoaffinity columns using Fv and scFv fragments. Engineered antibody fragments can also be produced with sites for specific attachment to the solid phase designed such that minimal antigen binding activity is lost on immobilisation. For example a hinge region containing a single thiol group can be engineered onto the C-terminus of an scFv fragment to allow site-specific conjugation (King *et al.*, 1994). Both Fv and scFv fragments have been immobilised and used for immunoaffinity chromatography (Berry *et al.*, 1991; Spitznagel and Clark, 1993; Molloy *et al.*, 1995). scFv may be a better reagent to use as the covalent linkage between VH and VL results in less leakage from the column during elution (Berry and Pierce, 1993). In a comparison of IgG, Fab′ and Fv fragments it was found that immobilisation of Fv resulted in the highest binding capacity, presumably due to the small size of the fragment (Spitznagel and Clark, 1993). However, antibody fragments with exposed hydrophobic patches on the surface of the protein must not be used, as these may result in high levels of non-specific binding to the column. Use of a single VH domain which could bind antigen (dAb) was not successful as an immunopurification reagent for this reason (Berry and Davies, 1992).

The development of these low-cost affinity materials will no doubt open up a range of applications for immunopurification which have been limited to date by cost. This could include the design of reagents for the simple purification of pharmaceutical proteins, and

HOSPIRA EX. 1029
Page 113
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

applications such as removal of environmental contaminants. A further example of this is the generation of an scFv to the herbicide paraquat specifically for use as an immunopurification reagent (Graham *et al*., 1995). The use of phage technology will allow the selection of the most suitable antibody for a particular application, which can then be reconstructed in the most suitable format for immobilisation.

3.8 Antibodies in structural biology

MAb fragments have also found a role in the determination of protein structures by X-ray crystallography. Many proteins of biological interest are unable to form the high-quality crystals required for structural determination due to heterogeneity, insolubility, flexibility or polydispersity in solution. Antibody fragments such as Fab and Fv are soluble, bind tightly to antigen and can thus effectively transform aggregated protein into a soluble monodisperse sample suitable for crystallisation (Kovari *et al*., 1995). The HIV capsid protein p24 was able to be crystallised only as a Fab–p24 complex which prevented oligomerisation of the p24 protein (Prongay *et al*., 1990). In the case of HIV reverse transcriptase, a Fab fragment was used to decrease the mobility of a region of the protein allowing rapid determination of the structure (Jacobo-Molina *et al*., 1993). Fv fragments are also particularly useful and have been used to allow determination of the membrane protein bacterial cytochrome c oxidase by enlargement of the polar surface to allow formation of crystal lattice (Ostermeier *et al*., 1995). It is usually necessary to screen a large number of antibody fragments to find a suitable one for crystallisation which not only stabilises the protein of interest but also allows suitable crystal contacts to form. Ideal antibody fragments will not alter the conformation of the protein of interest, although it should be remembered that it is often the case that the conformation of the antibody itself may change on binding antigen (see Chapter 1). Selection of appropriate antibody fragments from phage display may allow rapid identification of suitable reagents.

3.9 *In vivo* diagnostics

The use of antibodies for imaging disease has been developed to the point where there are now several products marketed for clinical use (Table 3.4) and many more under development. Antibodies to suitable antigens can be used to confirm the presence and extent of disease in the body, identify its location and monitor the progress of any therapy. To achieve this the antibody is labelled with a suitable radioisotope, usually a short half-life gamma emitter, and the emissions from the isotope detected with a gamma camera. Antibodies can also be labelled with positron emitting isotopes for use in positron emission tomography scanning. This technique is variously known as radioimmunodetection (RAID) or radioimmunoscintigraphy (RIS). Advances in instrumentation, developing such techniques as single photon emission computerised tomography (SPECT), have contributed to the popularity of development of RIS by allowing improved accuracy and detection of lesions not seen using other techniques. An alternative form of radioimmunodetection is used to aid surgical procedures, known as radioimmunoguided surgery (RIGS). In this technique radiolabelled antibody localised to the site of disease is detected by a hand-held monitor to allow simple identification and resection of diseased tissue during surgery.

HOSPIRA EX. 1029
Page 114
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 3.4 Some commercially marketed monoclonal antibody imaging agents

Imaging agent	Antibody	Isotope	Disease	Company
OncoScint CR/OV	B72.3 IgG (anti-TAG72)	^{111}In	Colorectal and ovarian cancer	Cytogen
CEA-Scan	Immu-4 Fab′ (anti-CEA)	^{99m}Tc	Colorectal cancer	Immunomedics
LeukoScan	anti-NCA-90 Fab′	^{99m}Tc	Infection/inflammation	Immunomedics
Myoscint	anti-myosin Fab	^{111}In	Heart disease	Centocor
ProstaScint	7E11-C5.3 IgG	^{111}In	Prostate cancer	Cytogen
Verluma	NR-LU-10 Fab′	^{99m}Tc	Small-cell lung cancer	NeoRx

3.9.1 *Radioimmunodetection of human tumours*

A major impetus to the development of RAID has been the desire to develop techniques to allow detection of human tumours and determine the extent and location of disease, particularly monitoring metastatic spread. In many cases accurate, early diagnosis using RAID can be a contributor to effective treatment, with the major use of RAID being to monitor disease in patients following treatment by surgery, radiotherapy or chemotherapy (Larson, 1995). Many tumour-associated antigens are now known and characterised, and antibodies to these allow targeting to different tumour types (Table 3.5). However, obtaining high-quality tumour images from radiolabelled antibodies is not straightforward and the immunoconjugate to be used requires careful optimisation of not only the antibody specificity and the radioisotope, but also the form of the antibody used and the method of attachment of the isotope to the antibody. Some of the many factors affecting RAID are given in Table 3.6.

Tumour-associated antigens

Useful tumour-associated antigens are absent on normal tissues and present at high levels on tumour cells, preferably homogeneously on all cells of the tumour. Antigen should also not be shed from the tumour into the blood. In reality there are no perfect, tumour-specific antigens and the choice of a suitable target requires compromise over one or more of these characteristics. Nevertheless, high-quality tumour imaging can be achieved through optimisation of the immunoconjugate and the use of alternative strategies to overcome the individual problems of particular targets. For example, antibodies to antigens which are present on normal tissues, but inaccessible to the administered antibody, have been used successfully in imaging studies, and the presence of circulating antigen can often be overcome through increasing the dose of antibody administered to saturate circulating antigen, or the use of an unlabelled antibody pre-dose (van Hof *et al.*, 1996).

Table 3.5 Some commonly used tumour-associated antigens, with examples of antibodies raised against them

Antigen	Tumour type	Representative antibody
Tumour-associated glycoprotein72 (TAG72), 72 kDa glycoprotein	Pancarcinoma	B72.3, CC49
Carcinoembryonic antigen (CEA), 180 kDa glycoprotein	Pancarcinoma	NP-4, A5B7
Polymorphic epithelial mucin (PEM), >100 kDa glycoprotein	Ovarian, breast, lung	HMFG1
Epithelial membrane antigen (EMA), 40 kDa glycoprotein	Colorectal (and other epithelial tumours)	17-1A
Epidermal growth factor receptor (EGFR), 175 kDa glycoprotein	Breast, lung	425
$p185^{HER2}$/ c-*erb*-B2 (185 kDa glycoprotein)	Breast, lung	4D5
Prostate-specific membrane antigen (PSMA), 100 kDa glycoprotein	Prostate	7E11-C5.3
CD33 67 kDa glycoprotein	Myeloid leukemia	P67.6, M195
CD20 35 kDa glycoprotein	Lymphoma	C2B8
GD2 ganglioside	Melanoma, neuroblastoma	14-18

The homogeneity of tumour-associated antigen expression is less important for RAID than for targeted therapy (see Chapter 4), as good imaging can be achieved as long as enough labelled antibody can reach the tumour site to generate a high tumour : backgound ratio. Hence tumours with as few as 15% of the cells expressing the relevant antigen have

Table 3.6 Factors affecting radioimmunodetection of cancer

Tumour-associated antigen	Specificity (presence in normal tissues) Antigen density and homogeneity in tumour Presence of shed antigen in blood
Antibody	Specificity Affinity and avidity Molecular size – penetration into tumour – pharmacokinetics Internalisation Immunogenicity
Radioisotope	Method of attachment to antibody (stability of conjugate and retention of antigen binding) Half-life Suitability of emission energy for imaging
Tumour	Location in the body Size Vascularisation and vascular permeability

been successfully imaged (Doerr *et al.*, 1991). When developing an antibody for RAID it is desirable that it can be used for several different types of tumour. Such pan-reactive antibodies have been identified using widely distributed tumour-associated antigens such as carcinoembryonic antigen (CEA) which is present on cells of colorectal, breast, ovarian and lung tumours, and polymorphic epithelial mucin which is present on ovarian, lung and breast tumour cells (Table 3.5).

Form of antibody

The generation of a high tumour : background ratio is also the key to the selection of the best form of antibody to use for tumour imaging. Intact antibody circulates in the blood for long periods and builds up relatively high levels of activity at the tumour site. However, the long residence time of labelled antibody in the blood means that relatively poor tumour : blood ratios are generated at early time points, and tumour imaging is only possible days later when sufficient antibody has cleared from the blood. Antibody fragments clear much more rapidly (see Section 2.7) and although lower levels of activity are accumulated at the tumour site, higher tumour : blood ratios are generated at early time points, allowing imaging to take place on the same day. Many studies have been carried out with $F(ab')_2$ and Fab′ fragments generated by digestion from IgG and, more recently, the generation of recombinant fragments of anti-tumour antibodies has allowed smaller fragments such as Fv and scFv to be tested. Studies of Fab′ and $F(ab')_2$ compared to IgG demonstrate that faster imaging is possible with antibody fragments leading to the detection of small tumour deposits with higher sensitivity (Lane *et al.*, 1994; Behr *et al.*, 1995). In comparative studies of IgG, $F(ab')_2$, Fab′, Fv and scFv, higher ratios were generated with smaller fragments, the best being the Fv and scFv fragments (Milenic *et al.*, 1991; King *et al.*, 1992b). There is also strong evidence that smaller fragments penetrate further into the tumour mass, again with scFv more effective than Fab′ and $F(ab')_2$ (Yokota *et al.*, 1992). Clinical imaging with scFv has also been found to be very effective. Using an anti-CEA scFv derived from phage display, Begent *et al.* (1996) have demonstrated that high-quality tumour images could be obtained with improved sensitivity over conventional imaging using X-ray computerised tomography (CT). Several liver metastases of colorectal tumours not seen at all by conventional CT were detected using scFv imaging. However, good tumour imaging is also dependent on the biology of the system under investigation. Hence although for most tumours antibody fragments are preferable, for some tumours which are less accessible or less well vascularised, early imaging with antibody fragments may be difficult and better results may be observed by imaging later with an intact IgG (Behr *et al.*, 1995).

Further attempts to optimise the form of antibody for RAID have led to the testing of divalent and trivalent Fab′ and scFv fragments. Fab′ and scFv fragments are monovalent with respect to antigen binding which leads to low avidity of binding (see Chapter 1). Production of multivalent forms of scFv may enable the benefits of small size to be retained while enabling better binding ability leading to increased tumour uptake. scFv′s expressed with a hinge region attached have been used to prepare di-scFv′s which have shown increased tumour uptake leading to superior tumour : blood ratios (Adams *et al.*, 1993; King *et al.*, 1994). Tri-scFv′s have improved ratios further due to increased antigen-binding ability with only a small increase in blood retention (King *et al.*, 1994). Cross-linked Fab′ fragments also show improved tumour targeting which may lead to improved agents for both RAID and targeted therapy (see Chapter 4).

HOSPIRA EX. 1029
Page 117
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

Table 3.7 Commonly used radioisotopes for development of RAID

Radionuclide	Emission	Half-life (hours)	Energy (keV)	Comments
^{131}I	$\gamma + \beta$	193	364	Reactor produced, also used as therapeutic isotope (β emission)
^{123}I	γ	13	159	Cyclotron produced
^{111}In	γ	68	171	Cyclotron produced, matched to ^{90}Y for therapy, used clinically
^{99m}Tc	γ	6	141	Generator produced, most suited to available cameras, readily available and widely used clinically
^{67}Ga	γ	80	184	Cyclotron produced, in clinical use as citrate
^{64}Cu	$\beta+$	13	511	For use in PET scanning

Radioisotopes

Suitable radioisotopes for RAID are usually short half-life gamma emitters, although positron emitting isotopes are also under development for positron emission tomography (PET) applications (Table 3.7). For use in RAID, radioisotopes must be of sufficient energy to reach the detector outside the body whilst having a low linear energy transfer (LET, the energy deposited by the isotope over its pathway) resulting in the minimum damage to cells within the body. Optimal tumour imaging requires a good match between the energy of the gamma emission and the gamma camera detector. Most current gamma cameras are best used with gamma emissions in the range of 100–200 keV. Isotopes also need to be available in carrier-free form and must have suitable chemical properties to allow attachment to antibody. They must not have hazardous daughter isotopes, and should be of suitable half-life to allow preparation of the radiolabelled antibody, localisation and clearance of blood activity. As such the half-life of the isotope used needs to be matched to the biological half-life of the form of antibody used. For example, indium-111 is well suited to RAID with intact IgG, while technetium-99m is well suited to rapidly clearing antibody fragments such as Fab′ and scFv.

Iodine isotopes have been widely investigated due to their ready availability and well-developed methods for radioiodination of antibodies at either tyrosine or lysine residues (see Section 3.2.2). ^{131}I is not an ideal isotope for RAID, and is used primarily in experimental studies of tumour targeting for therapy. However, the ability to image tumours using the same labelled immunoconjugate used for therapy is valuable as it allows tumour dose estimates to be determined (DeNardo *et al.*, 1996a). ^{123}I is a potentially useful diagnostic nuclide although it is relatively poorly available and expensive. Its short half-life means it is best suited to imaging with antibody fragments and has been used successfully to image colorectal tumours with anti-CEA $F(ab')_2$, Fab and scFv (Goldenberg *et al.*, 1990; Begent *et al.*, 1996). One problem common to all radioiodinated antibodies is their relative instability *in vivo*. Antibodies are deiodinated *in vivo* which leads to iodine leaking out from the tumour and often to the accumulation of free iodine in the thyroid and stomach. The accumulation of free iodine can be blocked by pre-treatment

with cold iodine, although deiodination cannot be prevented. Internalising antibodies may also be rapidly metabolised leading to the expulsion from the cell of small molecular weight iodinated metabolites (Press *et al*., 1996).

Technetium-99m is probably the isotope of choice for most RAID applications. It is cheap, very readily available from generators in all hospital nuclear medicine departments, leads to low radiation exposure to the patient and has ideal physical properties for detection by gamma cameras. ^{99m}Tc can be attached to antibody either directly or via a chelating agent. ^{99m}Tc is prepared from a generator in the +7 state as pertechnetate and requires reduction, usually achieved with Sn^{2+} or ascorbate, to the +5 state for antibody labelling. Thiol groups are particularly good ligands for binding ^{99m}Tc and direct labelling of IgG is achieved through reduction of disulphide bonds in the antibody molecule, presumably in the hinge region, which are then used to bind ^{99m}Tc. Sn^{2+} can be used to reduce both pertechnetate and the antibody itself, and often an 'intermediate chelator' is used such as glucarate. Several kit formulations have been developed to allow ^{99m}Tc labelling via such direct labelling methods (e.g. Pak *et al*., 1992, Alauddin *et al*., 1992). Similarly, Fab′ fragments can be labelled via free thiol groups in the hinge region, and scFv fragments have been engineered specifically to contain C-terminal cysteine residues to allow ^{99m}Tc labelling (George *et al*., 1995; Verhaar *et al*., 1996).

Bifunctional chelating agents have been developed for radiolabelling with different metallic radionuclides. These are termed bifunctional due to having a reactive group for antibody attachment and a chelating group for binding the radiometal. Thus the bifunctional reagent can be used to form an antibody–chelator conjugate which can then bind radiometal under mild conditions. Alternatively, the chelator can be pre-labelled with radiometal before attachment to the antibody, although this method has the disadvantage that more handling of radiolabelled materials is required and manipulations need to be carried out rapidly to avoid extensive radioactive decay. Several chelators for ^{99m}Tc labelling have been developed, many of which use thiol ligands. Diamide dimercaptide ligands, also known as N_2S_2 ligands, form a stable tetradentate complex with technetium (Figure 3.12), although problems with non-specific binding of technetium to antibody protein have resulted in a pre-labelling method being most successful (Fritzberg *et al*., 1988). Nevertheless methods have been developed to reduce this to a kit form for simple radiolabelling of antibody fragments (Kasina *et al*., 1991). N_3S ligands (Figure 3.12), alternative N_2S_2 chelates based on bis-aminoethanethiols, and other chelating groups such as hydrazino nicotinamides have also been developed which allow formation of the antibody–chelator conjugate before labelling (Weber *et al*., 1990; Eisenhut *et al*., 1996; Ultee *et al*., 1997). Macrocyclic ligands based on cyclam which form very stable technetium complexes have also been examined (Morphy *et al*., 1988). Recombinant proteins with specific groups capable of binding technetium are also under development, for example fusion of the metal-binding protein metallothionein to antibody fragments can be used to allow subsequent binding of ^{99m}Tc (Das *et al*., 1992).

Comparisons of direct labelling with chelation have demonstrated that the use of defined chelation gives a more stable complex and results in higher retention of technetium in tumour tissue and less non-specific uptake in normal tissues, resulting in improved tumour images (Hnatowich *et al*., 1993; Ultee *et al*., 1997). Nevertheless, clinical imaging of tumours has been successful with both types of procedure (Behr *et al*., 1995; Eary *et al*., 1989), and commercially available preparations include technetium-based tumour imaging agents using Fab′ fragments labelled with both methodologies. Immunomedics' CEA-scan™ for imaging colorectal tumours uses a direct labelling procedure, whereas Verluma™ developed by NeoRx for imaging lung tumours is based on chelation methodology.

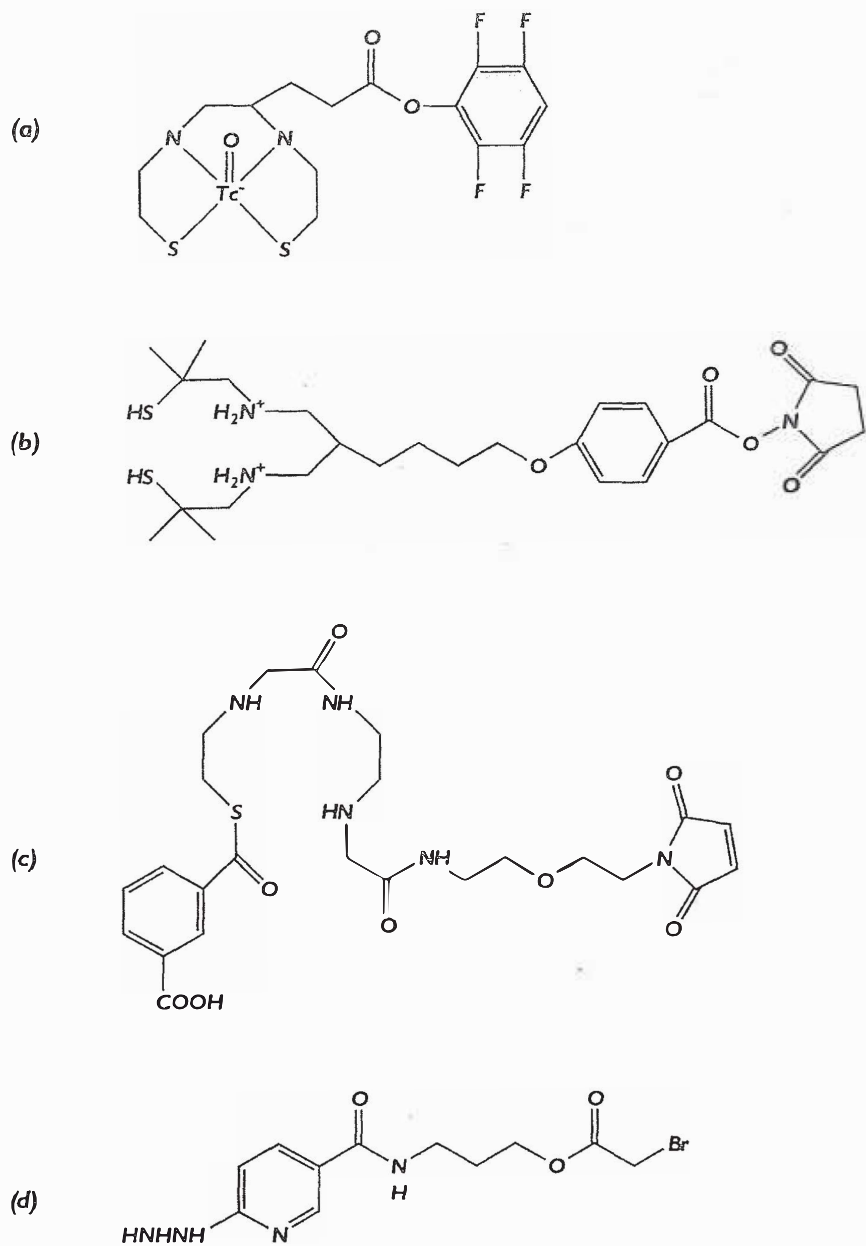


Figure 3.12 Examples of ligands for attachment to MAb which can be used to chelate technetium-99m: (a) active ester of ^{99m}Tc-4,5-bis-(thioacetamide)pentanoate for attachment to amine groups (Fritzberg *et al.*, 1988); (b) 6-(4′-(4″-carboxyphenoxy)butyl) -2-10-dimercapto-2,10-dimethyl-4,8-diazaundecane for attachment to amine groups (Eisenhut *et al.*, 1996); (c) *N*-(5-maleimido-3-oxapentyl)[2-[(3-carboxybenzoyl)-thio]acetyl]glycylglycylglycinamide (N_3S ligand) for attachment to thiol groups (Weber *et al.*, 1990); (d) bromoacetyl hydrazinonicotinamide hydrobromide for attachment to thiol groups (Ultee *et al.*, 1977)

HOSPIRA EX. 1029
Page 120
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

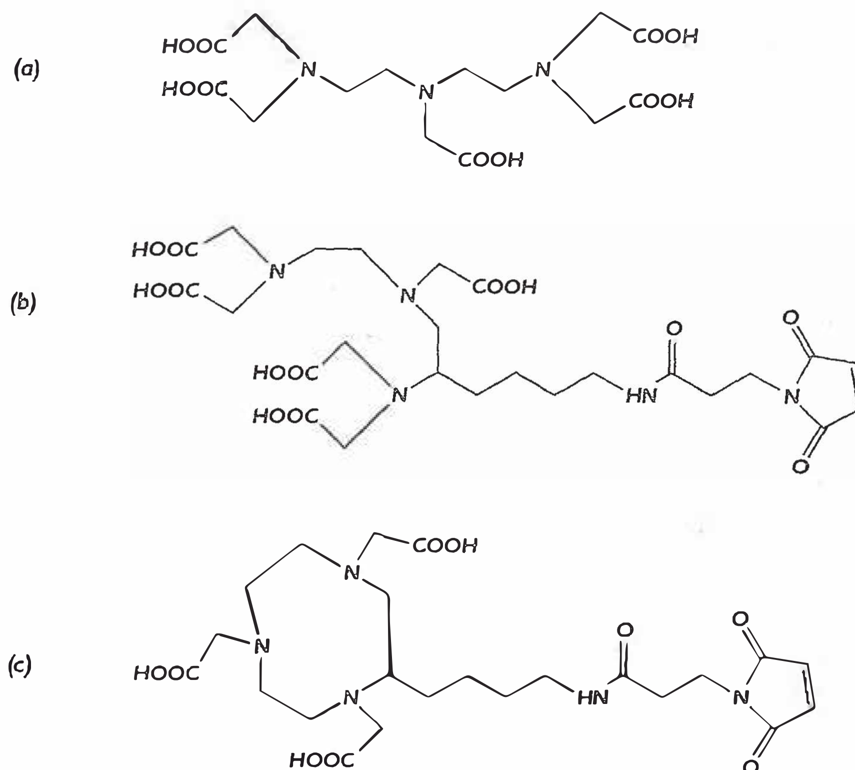


Figure 3.13 Examples of ligands for attachment of indium-111 to MAb: (a) DTPA; (b) derivatised DTPA (Harrison *et al.*, 1991); (c) 9N3 macrocycle (Turner *et al.*, 1994)

Indium-111 is also widely used for RAID applications and requires the use of a bifunctional chelator to allow labelling. Early studies used the cyclic anhydride of diethylenetriaminepentaacetic acid (DTPA) for attachment to antibody and subsequent chelation of ^{111}In (Hnatowich *et al.*, 1983). Although efficient radiolabelling can be achieved, the stability of the ^{111}In–DTPA complex is relatively low as one of the chelation arms is used for attachment to the antibody. *In vivo* dissociation of the complex can take place, leading to the formation of ^{111}In–transferrin which is subsequently deposited in the liver (Schumacher *et al.*, 1990). Subsequently, new derivatives of DTPA were produced such that a separate antibody attachment site could be used, preserving all eight coordination sites for ^{111}In (Figure 3.13), and resulting in more stable immunoconjugates (Brechbiel *et al.*, 1986). However, the most stable complexes of ^{111}In known are formed with macrocyclic ligands, and maleimide derivatives for attachment to antibodies have been developed which retain all of their stability toward binding ^{111}In (Figure 3.13; Craig *et al.*, 1989). Comparative biodistributions of the 9N3 macrocyclic ligand with DTPA and a bifunctional DTPA derivative revealed that the macrocyclic ligand resulted in improved tumour localisation with higher levels of activity in tumour and less in normal tissues (Turner *et al.*, 1994). The macrocyclic ligand DOTA (12N4), best known for labelling with ^{90}Y for radioimmunotherapy (see Section 4.2.5), can also be used to form a stable

HOSPIRA EX. 1029
Page 121
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

complex with ^{111}In. Comparative biodistributions demonstrate little difference between the 9N3 and 12N4 macrocycles, although labelling of 12N4 with ^{111}In is more difficult (Turner *et al*., 1994). Labelling procedures for 9N3 macrocyle immunoconjugates have been developed to allow reproducible ^{111}In incorporation efficiencies of >95% (Haines, A.M.R. and King, D.J., unpublished data), and clinical studies with such conjugates show good tumour imaging characteristics (van Hof *et al*., 1996).

Fewer studies have been performed with alternative radionuclides, although gallium-67 is a potentially useful reagent which forms stable complexes with macrocyclic ligands (Craig *et al*., 1989). Positron emission tomography (PET) imaging can be used with suitable radionuclides such as copper-64, bromine-76 and zirconium-89, allowing high resolution imaging to be carried out. Suitable macrocyclic ligands for stable attachment of ^{64}Cu have also been developed (Moi *et al*., 1985). However, the application of antibodies to PET has been slow to develop due at least partly to the expensive equipment required for imaging studies to be performed. Experimental PET imaging studies have demonstrated good tumour imaging in animal models with a variety of isotopes (Anderson *et al*., 1992; Lovquist *et al*., 1997; Meijs *et al*., 1997). In clinical studies of colorectal carcinoma, ^{64}Cu-labelled antibody demonstrated impressive radioimmunodetection of small tumours in the abdomen and pelvis (Philpott *et al*., 1995).

Two- and three-step targeting approaches

An alternative approach to improving tumour : blood ratios for RIS is the use of two-step targeting strategies. The simplest form of two-step tumour imaging is to use a second antibody reagent to clear blood background activity, hence improving the signal : noise ratio and the quality of the image. Immune complexes formed by a second antibody are rapidly removed from the circulation by the reticuloendothelial system, particularly in the liver. This can be viewed as an alternative to the use of rapidly clearing antibody fragments. Antibodies to the antibody itself or the radiolabelled chelator can be used (Reardan *et al*., 1985; Pedley *et al*., 1989). The use of antibodies to the chelator ensures that any radiolabelled metabolites in the circulation are also rapidly removed. Biotinylated antibodies have also been investigated and shown to be rapidly cleared by the administration of streptavidin (Marshall *et al*., 1994).

In alternative approaches the administration of antibody and radiolabel are separated. Antibody is allowed to localise to tumour and sufficient time is allowed for antibody clearance from the blood and non-target tissues. Radioisotope is then injected separately in a form which can be readily captured by the tumour-bound antibody (Figure 3.14). Bispecific antibodies have been developed with specificity for tumour and a radiometal chelator such as DTPA (Goodwin *et al*., 1988; LeDoussal *et al*., 1989). After localisation of the bispecific antibody, and clearance from the blood, radiolabelled metal chelate is added which is then bound by the antibody localised at the tumour site and rapidly cleared from the rest of the body through renal excretion. Early studies revealed that divalent metal chelators which were able to bind two antibody binding sites simultaneously resulted in enhanced affinity for the tumour site and improved imaging (Goodwin *et al*., 1988; LeDoussal *et al*., 1989). A successful strategy may be the use of a di-Fab or tri-Fab construct, with one or two Fab arms binding to the tumour antigen and one available for binding the radiolabelled chelator. Bivalent chelator may then cross-link two antibodies at the tumour surface, resulting in its increased affinity. Such bispecific antibodies have been successfully used to image medullary thyroid carcinoma, colorectal

HOSPIRA EX. 1029
Page 122
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

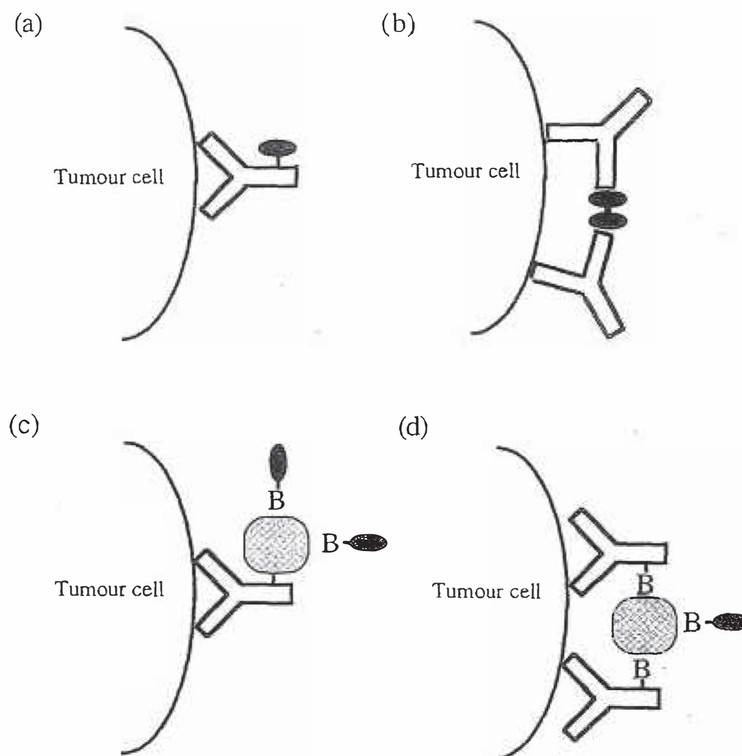


Figure 3.14 Strategies for tumour imaging: (a) one-step, directly labelled antibody; (b) two-step, bispecific anti-tumour antibody and anti-labelled hapten; (c) two-step, anti-tumour antibody–avidin conjugate and labelled biotin; (d) three-step, anti-tumour antibody–biotin conjugate, avidin, then labelled biotin (see text for details)

tumours and non-small cell lung tumours in patients (Peltier *et al.*, 1993; LeDoussal *et al.*, 1993; Vuillez *et al.*, 1997).

Alternative strategies have also been developed using the high affinity of the avidin:biotin system to capture radiolabelled small molecules from the blood as a two- or three-step imaging system. In the two-step version, antibody–avidin conjugate is injected and allowed to localise to tumour and clear from the blood. This is followed by injection of a low molecular weight biotinylated radiolabelled ligand which is captured by the tumour-bound avidin complex (Hnatowich *et al.*, 1987). Further development of this system has resulted in a three-step system in which biotinylated MAbs are injected and allowed to bind to the tumour site. This is followed by injection of avidin (or streptavidin) which binds to the tumour-bound antibody and has the additional advantage of forming a complex with antibody remaining in the blood which is rapidly cleared. Radiolabelled biotin is then added which is bound at the tumour site and cleared from the rest of the body (Paganelli *et al.*, 1991). Such three-step targeting allows the use of cocktails of biotinylated antibodies if desired which improves sensitivity through simultaneous detection of several tumour antigens, and has been used to detect small tumour deposits in patients, not visible by other techniques (Magnani *et al.*, 1996). The disadvantages are

HOSPIRA EX. 1029
Page 123
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

their relative complexity, and the immunogenicity of some of the components used, such as streptavidin. Whether such methods will find long-term application remains to be seen.

3.9.2 Radioimmunoguided surgery

Radioimmunoguided surgery (RIGS) may prove to be a useful aid to surgery for the removal of tumour tissue. Patients are pre-administered radiolabelled antibody, and a hand-held gamma detecting probe is used intraoperatively to locate occult tumour which may otherwise escape detection. Some studies have suggested that the sensitivity obtained with RIGS may be better than with radioimmunoscintigraphy (Hinkle *et al*., 1991), and RIGS has been shown to improve assessment of tumour spread and improve surgical intervention (Arnold *et al*., 1992). For example, in patients with primary colorectal tumours RIGS with the anti-TAG72 antibody CC49 allowed detection of 86% of tumours, whereas in patients undergoing second-look surgery for recurrent disease 97% of tumour deposits were detected (Arnold *et al*., 1992), the sensitivity of detection apparently being linked to antigen expression. Most studies have used antibody labelled with the weak gamma emitter ^{125}I, although ^{99m}Tc-labelled antibody has also been successfully tested (Ind *et al*., 1994). Many of the parameters involved in effective RIGS are the same as those described above. For example, improvements are offered by the use of rapidly clearing scFv fragments allowing RIGS to be performed more rapidly after administration of the labelled targeting molecule (Nieroda *et al*., 1995). Additionally, limitations due to antigen expression can be addressed by appropriate selection of antibodies or, in the case of CC49, by up-regulation of TAG72 antigen expression with γ-interferon treatment (Nieroda *et al*., 1995).

3.9.3 Non-tumour radioimmunodetection

MAb-based radioimmunodetection has also been applied in several other disease situations, where precise imaging may offer useful clinical information. These include detection of myocardial necrosis, imaging of blood clots and the detection of infection and inflammation.

Myocardial necrosis takes place in a range of ischaemic, inflammatory and toxic heart diseases and results in breakdown of cell membranes, exposing cardiac myosin which is then accessible to a MAb administered systemically. Fab to the heavy chain of cardiac myosin labelled with ^{111}In has been used to image damage as a result of myocardial infarction (Khaw *et al*., 1987), myocarditis and cardiac transplant rejection (Carrio *et al*., 1988). It may also be beneficial in the identification of cardiac involvement in the autoimmune disease systemic lupus erythematosus (Morguet *et al*., 1995), and in the assessment of cardiac toxicity following cancer chemotherapy with doxorubicin (Carrio *et al*., 1995). Localisation of labelled antibody to the site of damage is usually very rapid, even when blood flow is reduced as a result of that damage, and the quality of the images obtained is dependent on rapid clearance of blood activity. Therefore most studies have used Fab fragments with rapid clearance times, usually labelled with ^{111}In via the chelator DTPA. However, with ^{111}In-Fab it normally takes 24–48 hours for blood pool activity in the heart to decrease sufficiently to allow good interpretation of images, which has limited the use of imaging in conditions such as myocardial infarction where rapid imaging is required. New agents are now being designed to allow more rapid imaging. In an

HOSPIRA EX. 1029
Page 124
Celltrion, Inc. 1195
Celltrion v. Genentech
IPR2017-01374

animal model system, an anti-myosin scFv has been shown to retain the same accumulation at sites of infarction as the Fab fragment, although clearing more rapidly from the blood (Nedelman *et al.*, 1993). In addition the scFv was labelled with ^{99m}Tc via a bifunctional chelator, which allows earlier imaging than with ^{111}In as discussed above (Section 3.9.1). Another approach has been to chemically modify anti-myosin Fab′ so as to impart a negative charge (Khaw *et al.*, 1991). Positively charged Fab′ may interact non-specifically through ionic interactions with negatively charged cell surfaces. Chemical modification of the Fab through attachment of a negatively charged polymer decreased background activity and allowed earlier imaging.

There has been much interest in the development of anti-fibrin antibodies for imaging blood clots, in both deep vein thrombosis and arterial thrombosis. Anti-fibrin antibodies have been identified which bind to fibrin but not circulating fibrinogen and thus can be used to visualise thrombosis. Both ^{111}In and ^{99m}Tc have been used in clinical studies, with high specificity and sensitivity of thrombus detection in deep vein thrombosis (DeFaucal *et al.*, 1991; Schiable *et al.*, 1992). Results in imaging arterial disease have been less impressive and ^{99m}Tc-antifibrin Fab′ was less effective in thrombus detection than labelled platelets (Stratton *et al.*, 1994). Venous thrombi comprise mostly thrombin and red blood cells whereas acute arterial thrombi contain more platelets with smaller amounts of fibrin and red blood cells. Hence a smaller antigen pool is present in arterial disease, which may be responsible for poorer imaging results. Anti-platelet MAbs have also been investigated as agents for detection of thrombi (Oster *et al.*, 1985), and may be potentially useful in the detection of active arterial thrombi, although further clinical evaluation is required. Again attempts to use more rapidly targeting and clearing molecules have been made. An scFv has been expressed with specificity for fibrin fragment D-dimer (Laroche *et al.*, 1991), and a peptide designed from an antibody CDR loop to mimic an anti-platelet antibody binding site has been produced (Knight *et al.*, 1994). Although some specificity was seen for ^{99m}Tc-labelled peptide, the levels accumulated at the thrombus were too low for useful imaging.

RAID is also under development in several other disease states to enable localisation of disease and to monitor therapy. Antibodies specific for polymorphonuclear leukocyte (PMN) surface antigens have been developed for imaging inflammatory processes at sites of infection. A ^{99m}Tc-Fab′ to the cell surface antigen NCA-90 has shown rapid targeting in clinical studies detecting soft-tissue infections and osteomyelitis, and is the basis of the agent LeukoScan™ (Becker *et al.*, 1994). Similarly, ^{99m}Tc-labelled anti-CD15 antibody has been used to image inflammatory disease, labelled either directly or via a DTPA-based chelator with no significant difference in image quality (Thakur *et al.*, 1996). Labelled polyclonal human IgG is also capable of imaging sites of inflammation, probably due to increased vascular permeability at the site or to binding to Fc receptors up-regulated at the site (Rubin *et al.*, 1989). The use of specific reagents may, however, increase confidence in interpretation of the scans as non-specific reagents may lead to scans which are difficult to interpret with often low or diffuse uptake (Thakur *et al.*, 1991). An interesting follow-up to non-specific imaging uses a two-step strategy (Kranenborg *et al.*, 1997). An unlabelled, monoclonal anti-DTPA antibody is used to localise to the site of infection non-specifically. After localisation and clearance from the blood has taken place, DTPA labelled with ^{99m}Tc is administered which binds to the sequestered antibody with the remainder rapidly cleared from the circulation, leading to low background levels and clearer imaging.

An alternative to imaging via antibodies to PMNs is to image the inflamed endothelium. Certain adhesion molecules are up-regulated during inflammation as part of the mechanism