

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO			
RAD:	11-133166- -8	FECHA:	2014-02-25 12:34:17
DEP:	2020 DIRECCION DE NUEVAS CREACIONES	EVE:	379 FASE NACIONAL INCLUIDO CAPITULO II
TRA:	11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS:	6
ACT:	519 PRESENTACION		

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
JERONIMO CASTILLO BONILLA

Asunto: Radicación: 11-133166- -8
 Trámite: 11
 Evento: 379
 Actuación: 519
 Oficio: 2406
 Folios: 6

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I		Capítulo II	X
No. Solicitud Internacional	PCT/US2010/027242				
Fecha de Presentación Internacional	12/03/2010				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 11-133166-00000-0000	7/Octubre/2011
Reivindicaciones:	Radicado No. 11-133166-00000-0000	7/Octubre/2011
	Radicado No. 11-133166-00007-0000	29/Mayo/2013
Dibujos:	Radicado No. 11-133166-00000-0000	7/Octubre/2011
Respuesta del solicitante:	Radicado No. 11-133166-00007-0000	29/Mayo/2013
Prioridad:	US 61/160,199	13/Marzo/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones aceptadas

Se acepta el nuevo capítulo reivindicatorio (Radicado No. 11-133166-00007-0000) porque no se amplía el objeto de la solicitud.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso, se tiene en cuenta la totalidad del nuevo capítulo reivindicatorio (Radicado No. 11-133166-00007-0000) para el examen de patentabilidad.

Título de la solicitud: “Células útiles para ensayos de actividad del serotipo A de la toxina botulínica basado en inmunoensayos”.

El problema planteado en esta solicitud consiste en establecer una línea celular para un ensayo de actividad de toxina botulínica.

Para resolver este problema técnico la solicitud en estudio proporciona una línea celular clonal que comprende células susceptibles de intoxicación por neurotoxina botulínica A (BoNT/A).

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N 5/079; C12Q 1/37; G01N 33/50.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 – 4 carecen de claridad y concisión porque definen la línea celular por el nivel de expresión de un grupo de genes, con respecto a los niveles de expresión en células de la línea celular SiMa original DSMZ ACC 164. Debido a que la obtención de este tipo de línea celular clonal se logra luego del análisis de miles de colonias aisladas, la invención no se encuentra divulgada de manera suficientemente completa para que la persona del oficio normalmente versada en la materia pueda ejecutarla. De esta forma, es necesario presentar el Certificado de Depósito para la línea celular clonal a la que se hace referencia en las reivindicaciones 1 - 4.

De otra parte, este Despacho encuentra que si bien la solicitud describe los genes sobre los cuales la línea celular clonal presenta niveles de expresión aumentados, la tabla 13 de la presente solicitud muestra que el listado de genes que son expresados diferencialmente 4 o más veces en líneas celulares clonales BB10 y H1 sensibles a BoNT/A se hace con respecto a la línea 2D6 sensible a BoNT/A, y no frente a genes en células de la línea celular SiMa original DSMZ ACC 164, como reclama la reivindicación 1 de la solicitud. Igualmente, la tabla 13 hace mención a otros genes como ATAD2, CDC45L, CNTN1, Cyclin A, entre otros, que no son mencionados en la reivindicación 1 de la solicitud, de manera que no es claro que solamente un nivel de expresión genética aumentada de los genes seleccionados del grupo ADAMTS9, ASCLI, BASPI, DOK5, DPYD, GNB4, GNGII, GTSFI, MAOA, MINA, MSN, PEG3, PLK2, PRSS12, RNF182, SLC44A5, 15

SPARC, TFPI2 y TPTE compartan la actividad de la línea celular clonal reclamada.

Se sugiere a la solicitante complementar el término “nivel de expresión genética” con “nivel de expresión de ARNm”, debido a que es el parámetro que se utilizó para determinar los niveles aumentados de expresión genética (Fl. 748).

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: marzo 13 de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	EP1718756	“Botulinum toxin screening assays”	8/Noviembre/2006
D3	G. Moldenhauer. Handbook of Therapeutic Antibodies. Pág. 33	Selection Strategies I: Monoclonal antibodies	26/Marzo/2007

El documento D1¹ divulga una línea celular SiMa que comprende células susceptibles de intoxicación por una BoNT/A con número de depósito DSMZ ACC 164 (Resumen y Párr. 78).

El documento D3² enseña un método para la producción de una línea celular clonal (Pág. 33).

7. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en una línea celular clonal establecida que comprende células susceptibles de intoxicación por una BoNT/A, donde las células presentan una diferencia de 1.5 veces en los niveles de expresión genética de tres o más genes, en comparación con los niveles de expresión de estos genes en células de la línea celular SiMa original DSMZ ACC 164 y donde los niveles de expresión genética se seleccionan del grupo de ADAMTS9, ASCLI, BASPI, DOK5, DPYD, GNB4, GNGII, GTSFI, MAOA, MINA, MSN, PEG3, PLK2, PRSS12, RNF182, SLC44A5, 15 SPARC, TFPI2 y TPTE y donde la línea celular clonal no ha sido transfectada con exógeno FGFR3 y comprende células susceptibles de intoxicación por BoNT/A en alrededor de 100 pM o menos de una BoNT/A.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D3
Una línea celular clonal establecida que comprende células susceptibles de intoxicación por una BoNT/A, donde las células presentan una diferencia de 1.5 veces en los niveles de expresión genética	Una línea celular SiMa que comprende células susceptibles de	Un método para la producción de una línea celular clonal.

¹http://worldwide.espacenet.com/publicationDetails/biblio?CC=EP&NR=1718756A2&KC=A2&FT=D&ND=&date=20061108&DB=&locale=en_EP
² <http://onlinelibrary.wiley.com/book/10.1002/9783527619740>

de tres o más genes, en comparación con los niveles de expresión de estos genes en células de la línea celular SiMa original DSMZ ACC 164 y donde los niveles de expresión genética se seleccionan del grupo de ADAMTS9, ASCLI, BASPI, DOK5, DPYD, GNB4, GNGII, GTSFI, MAOA, MINA, MSN, PEG3, PLK2, PRSS12, RNF182, SLC44A5, 15 SPARC, TFPI2 y TPTE y donde la línea celular clonal no ha sido transfectada con exógeno FGFR3 y comprende células susceptibles de intoxicación por BoNT/A en alrededor de 100 pM o menos de una BoNT/A.	intoxicación por una BoNT/A con número de depósito DSMZ ACC 164. (Resumen y Párr. 78)	(Pág. 33)
--	---	------------------

Como se dijo anteriormente, el documento D1 es considerado el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una línea celular SiMa que comprende células susceptibles de intoxicación por una BoNT/A con número de depósito DSMZ ACC 164 (Resumen y Párr. 78).

La diferencia entre la invención, definida en la reivindicación 1, y la línea celular divulgada en el documento D1 consiste en que la línea celular es una línea celular clonal con niveles aumentados en la expresión de genes seleccionados de ADAMTS9, ASCLI, BASPI, DOK5, DPYD, GNB4, GNGII, GTSFI, MAOA, MINA, MSN, PEG3, PLK2, PRSS12, RNF182, SLC44A5, 15 SPARC, TFPI2 y TPTE.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la línea celular clonal reclamada en esta solicitud porque además de los genes seleccionados de ADAMTS9, ASCLI, BASPI, DOK5, DPYD, GNB4, GNGII, GTSFI, MAOA, MINA, MSN, PEG3, PLK2, PRSS12, RNF182, SLC44A5, 15 SPARC, TFPI2 y TPTE, existen otros genes involucrados en rutas alternativas, cuya expresión aumentada se encuentra asociada a la susceptibilidad de intoxicación por BoNT/A, de acuerdo a la descripción (Fl. 762, Tabla 13).

El problema técnico objetivo de esta solicitud consiste en la necesidad de una línea celular clonal alternativa a la que se puede obtener, por medio de técnicas rutinarias, de la línea celular conocida en el documento D1.

Teniendo en cuenta que el documento D1 ya divulgaba una línea celular SiMa que comprende células susceptibles de intoxicación por una BoNT/A con número de depósito DSMZ ACC 164 (Resumen y Párr. 78) y que el documento D3 enseña que la obtención de líneas celulares clonales de una población heterogénea de células es una actividad rutinaria en el campo de la biología celular y molecular, utilizando, por ejemplo, la metodología de dilución limitante (Pág. 33), la persona normalmente versada en la materia, utilizaría esta técnica para aislar células independientes, caracterizar su perfil de expresión genética, y establecer la línea de origen clonal como la de la reivindicación 1 de la solicitud estudiada. Lo anterior considerando que la línea celular SiMa es derivada de tejido tumoral de neuroblastoma, la persona normalmente versada en la materia se vería motivada a aislar e investigar esta población heterogénea de células para el establecimiento de una línea celular clonal, lo cual es considerado obvio y por lo tanto, sin altura inventiva.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y en las reivindicaciones 2 – 4 es obvia y no tiene nivel inventivo.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	EP1718756	1 –4	Nivel inventivo
D3	G. Moldenhauer. Handbook of Therapeutic Antibodies. Pág. 33	1 – 4	Nivel inventivo

9. Respuesta a los argumentos del señor Solicitante

En referencia al argumento del solicitante, según el cual, con la información detallada en la solicitud, una persona del oficio normalmente versada en la materia no tendría problema en identificar las líneas celulares clonales con susceptibilidad a la BoNT/A y con buena viabilidad, este Despacho encuentra que si bien la solicitud describe los genes sobre los cuales la línea celular clonal presenta niveles de expresión aumentados, la tabla 13 de la presente solicitud muestra que el listado de genes que son expresados diferencialmente 4 o más veces en líneas celulares clonales BB10 y H1 sensibles a BoNT/A se hace con respecto a la línea 2D6 sensible a BoNT/A, y no frente a genes en células de la línea celular SiMa original DSMZ ACC 164, como reclama la reivindicación 1 de la solicitud. De esta forma, la invención, al no estar caracterizada de manera tal que pueda ser ejecutada por una persona del oficio normalmente versada en la materia, debe definirse con el respectivo depósito de material biológico, tanto de la línea celular clonal reclamada, como de la línea celular clonal sobre la que se tiene evidencia descriptiva que existe unos niveles superiores de expresión genética.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-03-04

Elaboró: Moncaleano Prado Juan David

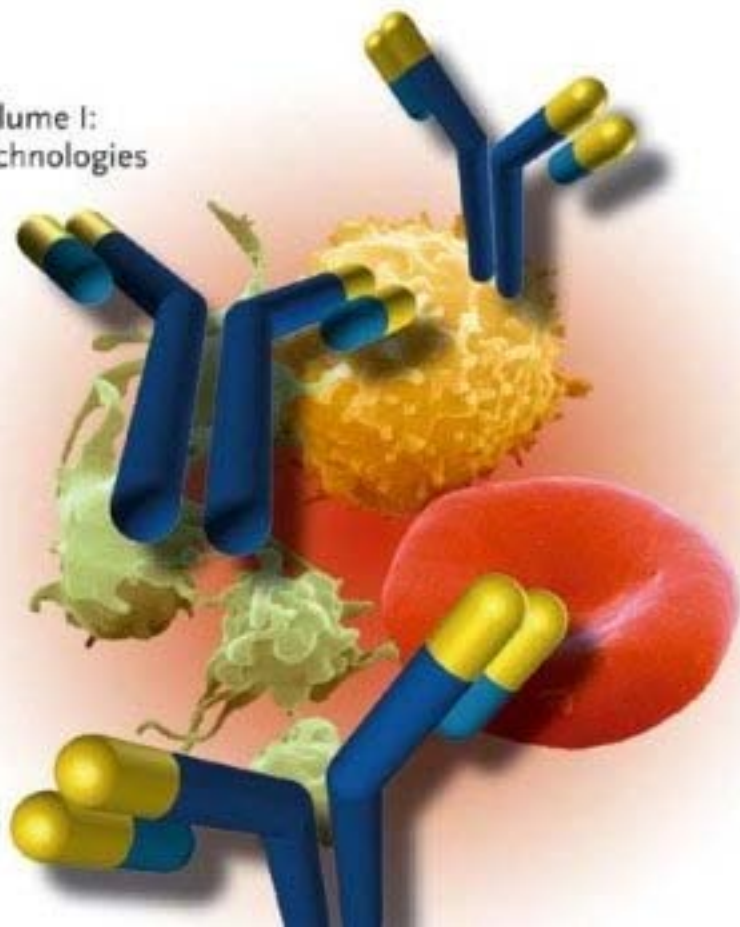
Revisó: Consuelo Leguizamon Leguizamon

Edited by Stefan Dübel

 WILEY-VCH

Handbook of Therapeutic Antibodies

Volume I:
Technologies



**Handbook of
Therapeutic Antibodies**

*Edited by
Stefan Dübel*

1807–2007 Knowledge for Generations

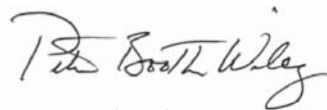
Each generation has its unique needs and aspirations. When Charles Wiley first opened his small printing shop in lower Manhattan in 1807, it was a generation of boundless potential searching for an identity. And we were there, helping to define a new American literary tradition. Over half a century later, in the midst of the Second Industrial Revolution, it was a generation focused on building the future. Once again, we were there, supplying the critical scientific, technical, and engineering knowledge that helped frame the world. Throughout the 20th Century, and into the new millennium, nations began to reach out beyond their own borders and a new international community was born. Wiley was there, expanding its operations around the world to enable a global exchange of ideas, opinions, and know-how.

For 200 years, Wiley has been an integral part of each generation's journey, enabling the flow of information and understanding necessary to meet their needs and fulfill their aspirations. Today, bold new technologies are changing the way we live and learn. Wiley will be there, providing you the must-have knowledge you need to imagine new worlds, new possibilities, and new opportunities.

Generations come and go, but you can always count on Wiley to provide you the knowledge you need, when and where you need it!



William J. Pesce
President and Chief Executive Officer



Peter Booth Wiley
Chairman of the Board

Handbook of Therapeutic Antibodies

Volume I

Edited by
Stefan Dübel



WILEY-VCH Verlag GmbH & Co. KGaA

The Editor

Prof. Dr. Stefan Dübel

Technical University of Braunschweig
Institute of Biochemistry and Biotechnology
Spielmannstr. 7
38106 Braunschweig
Germany

■ All books published by Wiley-VCH are carefully produced. Nevertheless, authors, editors, and publisher do not warrant the information contained in these books, including this book, to be free of errors. Readers are advised to keep in mind that statements, data, drug dosages, illustrations, procedural details or other items may inadvertently be inaccurate.

Library of Congress Card No.:

applied for

British Library Cataloguing-in-Publication Data

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

Bibliographic information published by the Deutsche Nationalbibliothek

Die Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at <<http://dnb.d-nb.de>>.

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

All rights reserved (including those of translation into other languages). No part of this book may be reproduced in any form – by photoprinting, microfilm, or any other means – nor transmitted or translated into a machine language without written permission from the publishers. Registered names, trademarks, etc. used in this book, even when not specifically marked as such, are not to be considered unprotected by law.

Cover Design: Schulz Grafik-Design, Fussgönheim

Wiley Bicentennial Logo: Richard J. Pacifico

Composition SNP Best-set Typesetter Ltd.,
Hong Kong

Printing betz-druck GmbH, Darmstadt

Bookbinding Litges & Dopf GmbH, Heppenheim

Printed in the Federal Republic of Germany
Printed on acid-free paper

ISBN 978-3-527-31453-9

Dedication

To Inge, Hans, Ulrike and Tasso Dübel – the four best things in my life

Stefan Dübel

Contents

Volume I

Overview of Therapeutic Antibodies XIX

A Greeting by the Editor XXI

Foreword XXIII

List of Authors XXV

Introduction

- 1 Therapeutic Antibodies – From Past to Future 3**
Stefan Dübel
- 1.1 An Exciting Start – and a Long Trek 3
- 1.2 The Gold Rush 8
- 1.3 Success and Disappointment 9
- 1.4 The Gleaming Horizon 14
- Further Reading 15
- References 15

Part I Selecting and Shaping the Antibody Molecule

- 2 Selection Strategies I: Monoclonal Antibodies 19**
Gerhard Moldenhauer
- 2.1 Introduction 19
- 2.2 Historical Remarks 20
- 2.3 Antibody Structure and Function 21
- 2.3.1 Membrane-bound and Secreted Forms of Antibodies 21
- 2.3.2 Monoclonal Antibodies 23
- 2.4 Production of Monoclonal Antibodies 24
- 2.4.1 Immunization 24
- 2.4.2 Myeloma Cell Lines 25
- 2.4.3 Cell Fusion 25

2.4.4	Drug Selection of Hybridomas	28
2.4.5	Screening Hybridoma Cultures for Specific Antibody	29
2.4.5.1	Enzyme-linked Immunosorbent Assay (ELISA)	29
2.4.5.2	Flow Cytometry	31
2.4.5.3	Immunohistology and Immunocytology	31
2.4.5.4	Cytotoxicity Assays	32
2.4.5.5	Screening for Function	32
2.4.6	Cloning	33
2.4.7	Expansion and Freezing of Hybridoma Clones	33
2.5	Purification and Modification of Monoclonal Antibodies	34
2.5.1	Mass Culture and Purification of Monoclonal Antibody	34
2.5.2	Fragmentation of Monoclonal IgG Antibodies	34
2.5.3	Labeling of Monoclonal Antibodies	35
2.6	Monoclonal Antibodies for Tumor Therapy	35
2.6.1	Leukocyte Differentiation Antigens	35
2.6.2	Epithelial Differentiation Antigens	37
2.6.3	Mechanisms of Action of Monoclonal Antibodies	38
2.6.4	Human Monoclonal Antibodies	39
2.7	Outlook	40
	References	40

3 Selection Strategies II: Antibody Phage Display 45

Michael Hust, Lars Toleikis and Stefan Dübel

3.1	Introduction	45
3.2	The Phage Display System	48
3.3	Selection and Evaluation of Binders	50
3.4	Phage Display Vectors	52
3.5	Phage Display Libraries	57
3.6	Generation of Phage Display Libraries	61
	References	62

4 Selection Strategies III: Transgenic Mice 69

Marianne Brüggemann, Jennifer A. Smith, Michael J. Osborn, and Xiangang Zou

4.1	Introduction	69
4.2	Human Ig Genes and Loci	69
4.2.1	Minigene Constructs	71
4.2.2	Yeast Artificial Chromosomes (YACs)	74
4.2.3	Chromosome Fragments	76
4.3	Transgenic Ig Strains	77
4.3.1	Stability of the Transloci	78
4.3.2	Silenced Endogenous Loci	79
4.3.3	Immune Responses and Affinity of Human Ig	79
4.3.4	Ig Replacement	83

4.4	Complementary Strategies	84
4.4.1	H-chain-only Ig	84
4.4.2	<i>In vivo</i> Mutation	86
4.5	Outlook	86
	References	87
5	Bioinformatics Tools for Antibody Engineering	95
	<i>Andrew C.R. Martin and James Allen</i>	
5.1	Introduction	95
5.1.1	Brief Review of Antibody Structure	95
5.1.2	Conventions Used in this Chapter	96
5.2	Numbering Schemes for Antibodies	96
5.2.1	The Kabat Numbering Scheme	97
5.2.1.1	The Chothia Numbering Scheme	98
5.2.2	The IMGT Numbering Scheme	100
5.2.3	Honegger and Plückthun Numbering Scheme	100
5.3	Definition of the CDRs and Related Regions	100
5.4	Antibody Sequence Data	102
5.4.1	Antibody Sequence Databanks	102
5.4.2	Germline Sequence Databases	103
5.4.3	Web Resources for Sequence Analysis	104
5.4.3.1	Kabat Data	104
5.4.3.2	IMGT Data	105
5.5	Antibody Structure Data	105
5.6	Sequence Families	106
5.6.1	Families and Subgroups	106
5.6.2	Human Family Chronology	107
5.6.2.1	Human Heavy Chain Variable Genes (V_H)	107
5.6.2.2	Human Light Chain Variable Genes (V_K and V_L)	107
5.6.3	Mouse Family Chronology	108
5.6.3.1	Mouse Heavy Chain Variable Genes (V_H)	108
5.6.3.2	Mouse Light Chain Variable Genes (V_K and V_L)	108
5.6.4	Correspondence Between Human and Mouse Families	109
5.6.4.1	Heavy Chain Variable Genes (V_H)	109
5.6.4.2	Light Chain Variable Genes (V_K and V_L)	109
5.6.5	Tools for Assigning Subgroups	110
5.7	Screening new antibody sequences	111
5.8	Antibody Structure Prediction	111
5.8.1	Build the framework	112
5.8.2	Build the CDRs	112
5.8.3	Automated Modeling Tools	112
5.9	Summary	113
	References	113
	Websites	116
	Note added in proof	117

6 Molecular Engineering I: Humanization 119

José W. Saldanha

- 6.1 Introduction 119
- 6.2 History of Humanization 120
- 6.3 CDR Grafting 120
- 6.4 The Design Cycle 122
 - 6.4.1 Analysis of the Source (Donor) Sequence 123
 - 6.4.1.1 Complementarity Determining Regions (CDRs) 123
 - 6.4.1.2 Canonical Residues 123
 - 6.4.1.3 Interface Packing Residues 124
 - 6.4.1.4 Rare Framework Residues 124
 - 6.4.1.5 N- or O-Glycosylation Sites 125
 - 6.4.2 Three-Dimensional Computer Modeling of the Antibody Structure 126
 - 6.4.3 Choice of Human Framework Sequences 128
 - 6.4.3.1 Fixed Frameworks or Best Fit? 128
 - 6.4.3.2 V_L/V_H Frameworks from the Same or Different Clone? 130
 - 6.4.3.3 Human Subgroup Consensus or Expressed Framework? 131
 - 6.4.3.4 Germline Frameworks 131
 - 6.4.3.5 Database Search 131
 - 6.4.4 Identify Putative Backmutations 132
- 6.5 Other Approaches to Antibody Humanization 134
 - 6.5.1 Resurfacing/Veneering 134
 - 6.5.2 SDR Transfer 135
 - 6.5.3 DeImmunization Technology 135
 - 6.5.4 Phage Libraries 136
- References 137

7 Molecular Engineering II: Antibody Affinity 145

Lorin Roskos, Scott Klakamp, Meina Liang, Rosalin Arends, and Larry Green

- 7.1 Introduction 145
- 7.2 Affinity Maturation 145
 - 7.2.1 Maturation *In Vivo* 145
 - 7.2.2 Maturation *In Vitro* 147
- 7.3 Effect of Affinity on Antigen Binding and Antibody Potency 148
 - 7.3.1 Binding and Potency *In Vitro* 150
 - 7.3.2 Binding and Potency *In Vivo* 152
- 7.4 High-Throughput Selection of Hybridomas Secreting High-Affinity Antibodies 154
 - 7.4.1 Soluble Antigens 154
 - 7.4.2 Cell Surface Antigens 157
- 7.5 Kinetic and Equilibrium Determinations of Antibody Affinity 158

7.5.1	Biacore Technology	158
7.5.2	KinExA Technology	163
7.5.3	Cell-based K_D Titrations	166
7.6	Conclusions	167
	References	168
8	Molecular Engineering III: Fc Engineering	171
	<i>Matthias Peipp, Thomas Beyer, Michael Dechant, and Thomas Valerius</i>	
8.1	Mechanisms of Action of Monoclonal Antibodies	171
8.1.1	Introduction	171
8.1.2	Preclinical Evidence	172
8.1.3	Clinical Evidence	173
8.2	Modifying Effector Functions	175
8.2.1	Antibody Isotype	175
8.2.1.1	IgG Antibodies	175
8.2.1.2	IgA Antibodies	177
8.2.2	Altered Fc Receptor Binding	179
8.2.2.1	Introduction	179
8.2.2.2	Glyco-Engineered Antibodies	180
8.2.2.3	Protein-Engineered Antibodies	183
8.2.3	Altered Complement Activation	188
8.3	Modifying the Pharmacokinetics of Antibodies	189
8.3.1	Introduction	189
8.3.2	Modifying Binding to FcRn	189
8.4	Summary and Conclusions	190
	References	190
Part II	The Way into the Clinic	197
9	Production and Downstream Processing	199
	<i>Klaus Bergemann, Christian Eckermann, Patrick Garidel, Stefanos Grammatikos, Alexander Jacobi, Hitto Kaufmann, Ralph Kempken, and Sandra Pisch-Heberle</i>	
9.1	Introduction	199
9.2	Upstream Processing	200
9.2.1	Expression System	200
9.2.2	Cell Culture Media	203
9.2.3	Cell Culture Process Design	204
9.2.4	Cell Culture Process Optimization	206
9.2.5	Scale-up, Economy of Scale	207
9.2.6	Harvest	208
9.3	Downstream Processing	210
9.3.1	Platform Technologies for Downstream Processing of Monoclonal Antibodies	210
9.3.2	Primary Recovery	211

9.3.2.1	Ultra-/Diafiltration (UF/DF)	211
9.3.2.2	Affinity Chromatography	212
9.3.3	Virus Clearance	212
9.3.4	Purification and Polishing	213
9.3.4.1	Hydrophobic Interaction Chromatography	213
9.3.4.2	Ion Exchange Chromatography	214
9.3.5	Final Formulation	215
9.3.6	Integrated Downstream Process Development	216
9.3.7	Future Perspectives	217
9.4	Formulation Development	217
9.4.1	Challenges during Early Formulation Development Phase of Biopharmaceuticals	217
9.4.2	Strategies and Analytical Tools for Rapid and Economic Formulation Development	218
9.4.3	Stabilization of Liquid Protein Formulations by Excipients	223
9.5	Protein Characterization and Quality Control Testing	224
9.5.1	Protein Characterization	224
9.5.1.1	Protein Variants	224
9.5.1.2	Overall Structural Confirmation (Higher Order Structure)	227
9.5.1.3	Relationship Between Physicochemical/Structural Properties and Biological Activity	227
9.5.2	Quality Control Testing	228
9.5.3	Stability Testing	231
9.6	Overall Development Strategy and Outlook	232
9.7	Outlook	233
	References	234

10 Pharmaceutical Formulation and Clinical Application 239

Gabriele Reich

10.1	Introduction	239
10.2	Clinical Application	240
10.2.1	Therapeutic Areas of Antibody Drugs	240
10.2.2	Antibody-Mediated Drug Delivery	241
10.2.3	PEGylated Antibodies and Antibody Fragments	243
10.2.4	Routes of Administration	244
10.3	Pharmaceutical Product Development	245
10.4	Stability Issues	246
10.4.1	Degradation Pathways	246
10.4.1.1	Chemical Degradation	247
10.4.1.2	Physical Degradation	248
10.4.2	Design of Stability Studies	249
10.4.2.1	Regulatory Aspects	249
10.4.2.2	Analytical Tools	250
10.4.2.3	Practical Approach	251
10.5	Formulation and Manufacturing of Parenteral Delivery Systems	252

10.5.1	Ready-to-Use Solutions and Concentrates	252
10.5.1.1	Appropriate Excipients	253
10.5.2	Freeze-Dried Powders	255
10.5.2.1	Appropriate Excipients	257
10.5.3	Crystalline Suspensions	258
10.5.4	Carrier-based Systems	259
10.6	Formulation and Manufacturing of Local Delivery Systems	260
10.6.1	Inhalation Powders	260
10.6.1.1	Spray Drying	260
10.6.1.2	Spray Freeze-drying	261
10.6.2	Various Dosage Forms	262
10.7	Outlook	262
	References	263
11	Immunogenicity of Antibody Therapeutics	267
	<i>Huub Schellekens, Daan Crommelin, and Wim Jiskoot</i>	
11.1	Introduction	267
11.2	Assays for Antibodies Induced by Monoclonal Antibodies	268
11.3	Mechanisms of Antibody Induction	269
11.4	Factors Influencing the Immunogenicity	270
11.5	Consequences of the Immunogenicity of Monoclonal Antibodies	272
11.6	Prediction of the Anti-mAb Response	273
11.7	Reduction of Immunogenicity of Monoclonal Antibodies	274
11.8	Conclusion	275
	References	275
12	Regulatory Considerations	277
	<i>Marjorie A. Shapiro, Patrick G. Swann, and Melanie Hartsough</i>	
12.1	Introduction	277
12.2	Regulatory Authority	280
12.3	Chemistry, Manufacturing, and Controls Considerations	282
12.3.1	Cell Line Qualification	282
12.3.2	Quality Control Testing	283
12.3.3	Transmissible Spongiform Encephalopathy (TSE)	285
12.3.4	Product Stability	286
12.3.5	Reference Standard	287
12.3.6	Virus Clearance and Inactivation Studies	287
12.3.7	Abbreviated Product Safety Testing for Feasibility Trials in Serious or Immediately Life-Threatening Conditions	288
12.3.8	Comparability	288
12.4	Considerations for Preclinical Testing	289
12.4.1	Tissue Cross-Reactivity	290
12.4.2	Relevant Species	291

12.4.3	Pharmacodynamic and Pharmacokinetic Studies	291
12.4.4	Toxicology	292
12.4.5	Immunogenicity	295
12.4.6	Comparability	296
12.5	Conclusions	297
	References	297
13	Intellectual Property Issues	301
	<i>Michael Braunagel and Rathin C. Das</i>	
13.1	Introduction	301
13.2	Why Intellectual Property Rights are Important	301
13.3	Recombinant Antibody Technologies	303
13.4	Antibody Humanization	304
13.5	Human Antibody Technology	304
13.6	Antibody Production	311
13.6.1	Genesis of New Cabilly	311
13.6.2	Xoma Patents	313
13.7	Litigations and Cross-licensing	314
13.8	Other Cross-licensing	316
13.9	Litigation between CAT and Abbott	316
13.10	Importation of Data	317
13.11	The Single-Chain Antibody Technology	318
13.12	US Patent Issued on Polyclonal Antibody Libraries	320
13.13	Conclusion	321

Volume II

Overview of Therapeutic Antibodies XV

A Greeting by the Editor XVII

Foreword XIX

List of Authors XXI

Part III Beyond IgG – Modified Antibodies

1 Immunoscintigraphy and Radioimmunotherapy 325

Jason L. J. Dearling and Alexandra Huhlov

2 Bispecific Antibodies 345

Dafne Müller and Roland E. Kontermann

3 Immunotoxins and Beyond: Targeted RNases 379

Susanna M. Rybak and Dianne L. Newton

Part IV Emerging Concepts

- 4 Automation of Selection and Engineering** 413
Zoltán Konthur
- 5 Emerging Technologies for Antibody Selection** 431
Mingyue He and Michael J. Taussig
- 6 Emerging Alternative Production Systems** 445
Thomas Jostock
- 7 Non-Antibody Scaffolds** 467
Markus Fiedler and Arne Skerra
- 8 Emerging Therapeutic Concepts I: ADEPT** 501
Surinder K. Sharma, Kerry A. Chester, and Kenneth D. Bagshawe
- 9 Emerging Therapeutic Concepts II: Nanotechnology** 515
Dimitar S. Dimitrov, Igor A. Sidorov, Yang Feng, Ponraj Prabakaran, Michaela A.E. Arndt, Jürgen Krauss, and Susanna M. Rybak
- 10 Emerging Therapeutic Concepts III: Chimeric Immunoglobulin T Cell Receptors, T-Bodies** 533
Thomas Schirrmann and Gabriele Pecher
- 11 Emerging Therapeutic Concepts IV: Anti-idiotypic Antibodies** 573
Peter Fischer and Martina M. Uttenreuther-Fischer

Part V Ongoing Clinical Studies

- 12 Antibodies in Phase I/II/III: Cancer Therapy** 593
P. Markus Deckert
- 13 Antibodies in Phase I/II/III: Targeting TNF** 673
Martin H. Holtmann and Markus F. Neurath

Volume III

- Overview of Therapeutic Antibodies** XXIII
- A Greeting by the Editor** XXV
- Foreword** XXVII
- List of Authors** XXIX

Volume III Approved Therapeutics

- 1 **Adalimumab (Humira)** 697
Hartmut Kupper, Jochen Salfeld, Daniel Tracey, and Joachim R. Kalden
- 2 **Alemtuzumab (MabCampath)** 733
Thomas Elter, Andreas Engert, and Michael Hallek
- 3 **Bevacizumab (Avastin)** 779
Eduardo Díaz-Rubio, Edith A. Perez, and Guiseppe Giaccone
- 4 **Cetuximab (Erbix, C-225)** 813
Norbert Schleucher and Udo Vanhoefer
- 5 **Efalizumab (Raptiva)** 827
Karlheinz Schmitt-Rau and Sigbert Jahn
- 6 ^{99m}Tc-Fanolesomab (NeutroSpec) 851
Christopher J. Palestro, Josephine N. Rini, and Charito Love
- 7 **Gemtuzumab Ozogamicin (Mylotarg)** 869
Matthias Peipp and Martin Gramatzki
- 8 **Infliximab (Remicade)** 885
Maria Wiekowski and Christian Antoni
- 9 **Muromonab-CD3 (Orthoclone OKT3)** 905
Harald Becker
- 10 **Natalizumab (Tysabri)** 941
Sebastian Schimrigk and Ralf Gold
- 11 **Omalizumab (Xolair)**
Anti-Immunoglobulin E Treatment in Allergic Diseases 951
Claus Kroegel and Martin Foerster
- 12 **Palivizumab (Synagis)** 999
Alexander C. Schmidt
- 13 **Rituximab (Rituxan)** 1033
Michael Wenger
- 14 **Trastuzumab (Herceptin)**
A Treatment for HER2-Positive Breast Cancer 1109
Paul Ellis

15	Abciximab, Arcitumomab, Basiliximab, Capromab, Cotara, Daclizumab, Edrecolomab, Ibritumomab, Igovomab, Nofetumomab, Satumomab, Sulesomab, Tositumomab, and Votumumab	1131
	<i>Christian Menzel and Stefan Dübel</i>	

Index	1149
--------------	-------------

Overview of Therapeutic Antibodies

Trade name	FDA name	Chapter	Page
Avastin	bevacizumab	3	781
Bexxar	tositumomab	15	1145
CEA-Scan	arcitumomab	15	1134
Cotara	–	15	1137
Erbitux	cetuximab	4	815
Herceptin	trastuzumab	14	1111
HumaSpect-Tc	votumumab	15	1147
Humira	adalimumab	1	699
Indimacis-125	igovomab	15	1142
LeukoScan	sulesomab	15	1144
Leukosite	alemtuzumab	2	735
MabCampath	alemtuzumab	2	735
Mylotarg	gemtuzumab	7	871
Neutrospec	fanolesomab	6	853
OncoScint	satumomab	15	1143
Oncorad	satumomab	15	1143
Orthoclone	muromonab	9	907
Panorex	edrecolomab	15	1139
ProstaScint	capromab	15	1135
Raptiva	efalizumab	5	829
Remicade	infliximab	8	887
ReoPro	abciximab	15	1133
Rituxan	rituximab	13	1035
Simulect	basiliximab	15	1137
Synagis	palivizumab	12	1001
Tysabri	natalizumab	10	943
Verluma	nofetumomab	15	1142
Xolair	omalizumab	11	953
Zenapax	daclizumab	15	1138
Zevalin	imbricitumomab	15	1141

A Greeting by the Editor

Today, therapeutic antibodies are essential assets for physicians fighting cancer, inflammation, and infections. These new therapeutic tools are a result of an immense explosion of research sparked by novel methods in gene technology which became available between 1985 and 1995.

This handbook endeavors to present the fascinating story of the tremendous achievements that have been made in strengthening humanity's weapons arsenal against widespread diseases. This story not only includes the scientific and clinical basics, but covers the entire chain of therapeutic antibody production – from downstream processing to Food and Drug Administration approval, galenics – and even critical intellectual property issues.

A significant part is devoted to emerging developments of all aspects of this process, including an article showing that antibodies may only be the first generation of clinically used targeting molecules, making the IgG obsolete in future developments, and novel ideas for alternative therapeutic paradigms.

Finally, approved antibody therapeutics are presented in detail in separate chapters, allowing the clinicians to quickly gain a comprehensive understanding of individual therapeutics.

In such a fast-developing area, it is difficult to keep pace with the rapidly growing information. For example, a PubMed search with “Herceptin” yields more than 1500 citations. Consequently, we have tried to extract the essentials from this vast resource, offering a comprehensive basis of knowledge on all relevant aspects of antibody therapeutics for the researcher, the company expert, and the bedside clinician.

At this point, I express my deep gratitude to all the colleagues who wrote for these books. Without their enthusiasm this project would never have materialized. I would also like to thank Dr Pauly from the publisher's office, who paved the way for this three-volume endeavor, and the biologist Ulrike Dübel – my wife. Both played essential roles in keeping the project on track throughout the organizational labyrinth of its production. The hard work and continuous suggestions of all of these colleagues were crucial in allowing the idea of a comprehensive handbook on therapeutic antibodies to finally become a reality.

Braunschweig, December 2006

Stefan Dübel

Foreword

The most characterized class of proteins are the antibodies. After more than a century of intense analysis, antibodies continue to amaze and inspire. This *Handbook of Therapeutic Antibodies* is not just an assembly of articles but rather a state-of-the-art comprehensive compendium, which will appeal to all those interested in antibodies, whether from academia, industry, or the clinic. It is an unrivaled resource which shows how mature the antibody field has become and how precisely the antibody molecule can be manipulated and utilized.

From humble beginnings when the classic monoclonal antibody paper by Kohler and Milstein ended with the line, “such cultures could be valuable for medical and industrial use” to the current Handbook you hold in your hand, the field is still in its relative infancy. As information obtained from clinical studies becomes better understood then further applications will become more streamlined and predictable. This Handbook will go a long way to achieving that goal. With the application of reproducible recombinant DNA methods the antibody molecule has become as plastic and varied as provided by nature. This then takes the focus away from the antibody, which can be easily manipulated, to what the antibody recognizes. Since any type, style, shape, affinity, and form of antibody can be generated, then what the antibody recognizes now becomes important.

All antibodies have one focus, namely, its antigen or more precisely, its epitope. In the realm of antibody applications antigen means “target.” The generation of any sort of antibody and/or fragment is now a relatively simple procedure so the focus of this work has shifted to the target, and rightly so. Once a target has been identified then any type of antibody can be generated to that molecule. Many of the currently US Food and Drug Administration (FDA) approved antibodies were obtained in this manner. If the target is unknown then the focus is on the specificity of the antibody and ultimately the antigen it recognizes.

As the field continues to mature the applications of antibodies will essentially mimic as much of the natural human immune response as possible. In this respect immunotherapy may become immunomanipulation, where the immune system is being manipulated by antibodies. With the success of antibody monotherapy the next phase of clinical applications is the use of antibodies with standard chemotherapy, and preliminary studies suggest the combination of

these two modalities is showing a benefit to the patient. When enough antibodies become available then cocktails of antibodies will be formulated for medical use. Since the natural antibody response is an oligoclonal response then cocktails of antibodies can be created by use of various *in vitro* methods to duplicate this in a therapeutic setting. In essence, this will be oligotherapy with a few antibodies. After all, this is what nature does and duplicating this natural immune response may be effective immunotherapy.

And all of this brings us back full circle to where it all starts and ends, the antibody molecule. No matter what version, isotype, form, or combination used the antibody molecule must first be made and shown to be biologically active. Currently, many of the steps and procedures to generate antibodies can be obtained in kit form and therefore are highly reproducible, making the creation of antibodies a straightforward process. Once the antibody molecule has been generated it must be produced in large scale for clinical and industrial applications. More often than not this means inserting the antibody genes into an expression system compatible with the end use of the antibody (or fragment). Since many of the steps in generating clinically useful antibodies are labor intensive and costly, care must be used to select antibodies with the specificity and activity of interest before they are mass produced. For commercial applications the FDA will be involved so their guidelines must be followed.

Stating the obvious, it would have been nice to have this Handbook series in the late 1970s when I entered the antibody field. It certainly would have made the work a lot easier! And here it is, about 30 years later, and the generation of antibodies has become “handbook easy.” In this respect I am envious of those starting out in this field. The recipes are now readily available so the real challenge now is not in making antibodies but rather in the applications of antibodies. It is hoped that this Handbook will provide a bright beacon where others may easily follow and generate antibodies which will improve our health. The immune system works and works well; those using this Handbook will continue to amaze and inspire.

Mark Glassy

Chairman & Professor, The Rajko Medenica Research Foundation, San Diego,
CA, USA

Chief Executive Officer, Shantha West, Inc., San Diego, CA, USA

December 2006

List of Authors

James Allen

University College London
Department of Biochemistry and
Molecular Biology
Gower Street
Darwin Building
London WC1E 6BT
UK

Christian Antoni

Schering-Plough
Clinical Research Allergy/
Respiratory/Immunology
2015 Galloping Hill Rd
Kenilworth, NJ 07033
USA

Rosalin Arends

Pfizer Inc.
MS 8220-3323
Eastern Point Road
Groten, CT 06339
USA

Michaela A.E. Arndt

Department of Medical Oncology
and Cancer Research
University of Essen
Hufelandstr. 55
45122 Essen
Germany

Kenneth D. Bagshawe

Department of Oncology
Charing Cross Campus
Imperial College London
Fulham Palace Road
London W6 8RF
UK

Harald Becker

Wetzbach 26 D
64673 Zwingenberg
Germany

Klaus Bergemann

Boehringer Ingelheim Pharma GmbH
& Co. KG
BioPharmaceuticals
Birkendorfer Str. 65
88397 Biberach a.d. Riss
Germany

Thomas Beyer

University Schleswig-Holstein
Campus Kiel
Division of Nephrology
Schittenhelmstr. 12
24105 Kiel
Germany

Michael Braunagel

Affitech AS
Gaustadalléen 21
0349 Oslo
Norway

Marianne Brüggemann

The Babraham Institute
Protein Technologies Laboratory
Babraham
Cambridge CB22 3AT
UK

Kerry A. Chester

CR UK Targeting & Imaging
Group
Department of Oncology
Hampstead Campus
UCL, Rowland Hill Street
London NW3 2PF
UK

Daan J.A. Crommelin

Utrecht University
Utrecht Institute for
Pharmaceutical Sciences (UIPS)
Sorbonnelaan 16
3584 CA Utrecht
The Netherlands

Rathin C. Das

Affitech USA, Inc.
1945 Arsol Grande
Walnut Creek, CA 94595
USA

Jason L.J. Dearling

Royal Free and University College
Medical School
University College London
Cancer Research UK Targeting &
Imaging Group
Department of Oncology
Rowland Hill Street
Hampstead Campus
London NW3 2PF
UK

Michael Dechant

University Schleswig-Holstein
Campus Kiel
Division of Nephrology
Schittenhelmstr. 12
24105 Kiel
Germany

Peter Markus Deckert

Charité Universitätsmedizin Berlin
Medical Clinic III – Haematology,
Oncology and Transfusion Medicine
Campus Benjamin Franklin
Hindenburgdamm 30
12200 Berlin
Germany

Eduardo Díaz-Rubio

Hospital Clínico San Carlos
Medical Oncology Department
28040 Madrid
Spain

Dimitar S. Dimitrov

Protein Interactions Group
Center for Cancer Research
Nanobiology Program
CCR, NCI-Frederick, NIH
Frederick, MD 21702
USA

Stefan Dübel

Technical University of Braunschweig
Institute of Biochemistry and
Biotechnology
Spielmannstr. 7
38106 Braunschweig
Germany

Christian Eckermann

Boehringer Ingelheim Pharma GmbH
& Co. KG
BioPharmaceuticals
Birkendorfer Str. 65
88397 Biberach a.d. Riss
Germany

Paul Ellis

Department of Medical Oncology
Guy's Hospital
Thomas Guy House
St. Thomas Street
London SE1 9RT
UK

Thomas Elter

Department of Hematology and
Oncology
University of Cologne
Kerpener Str. 62
50937 Köln
Germany

Andreas Engert

Department of Hematology and
Oncology
University of Cologne
Kerpener Str. 62
50937 Köln
Germany

Yang Feng

Protein Interactions Group
Center for Cancer Research
Nanobiology Program
CCR, NCI-Frederick, NIH
Frederick, MD 21702
USA

Markus Fiedler

Scil Proteins GmbH
Affilin Discovery
Heinrich-Damerow-Str. 1
06120 Halle an der Saale
Germany

Peter Fischer

Boehringer Ingelheim Pharma
GmbH & Co. KG
Department of R&D Licensing &
Information Management
Birkendorfer Str. 65, K41-00-01
88397 Biberach a.d. Riss
Germany

Martin Foerster

Friedrich-Schiller-University
Department of Pneumology and
Allergy
Medical Clinics I
Erlanger Allee 101
07740 Jena
Germany

Patrick Garidel

Boehringer Ingelheim Pharma GmbH
& Co. KG
BioPharmaceuticals
Birkendorfer Str. 65
88397 Biberach a.d. Riss
Germany

Guiseppe Giaccone

Vrije Universiteit Medical Center
Department of Medical Oncology
De Boelelaan 1117
1081 HV Amsterdam
The Netherlands

Ralf Gold

Department of Neurology
St. Josef Hospital
Ruhr University Bochum
Gudrunstr. 56
44791 Bochum
Germany

Martin Gramatzki

University of Schleswig-Holstein
Campus Kiel
Division of Stem Cell Transplantation
and Immunotherapy
Schittenhelmstr. 12
24105 Kiel
Germany

Stefanos Grammatikos

Boehringer Ingelheim Pharma
GmbH & Co. KG
BioPharmaceuticals
Birkendorfer Str. 65
88397 Biberach a.d. Riss
Germany

Larry Green

Abgenix, Inc.
6701 Kaiser Drive
Fremont, CA 94555
USA

Michael Hallek

Department of Hematology and
Oncology
University of Cologne
Kerpener Str. 62
50937 Köln
Germany

Melanie Hartsough

Center for Drug Evaluation and
Research
Food and Drug Administration
Division of Biological Oncology
Products
10903 New Hampshire Ave.
Silver Spring, MD 20993
USA

Mingyue He

The Babraham Institute
Technology Research Group
Cambridge CB2 4AT
UK

Martin H. Holtmann

Johannes-Gutenberg-University
1st Department of Medicine
Rangenbeckstr. 1
55131 Mainz
Germany

Alexandra Huhlov

Royal Free and University College
Medical School
University College London
Cancer Research UK Targeting &
Imaging Group
Department of Oncology
Rowland Hill Street
Hampstead Campus
London NW3 2PF
UK

Michael Hust

Technical University of Braunschweig
Institute of Biochemistry and
Biotechnology
Spielmannstr. 7
38106 Braunschweig
Germany

Alexander Jacobi

Boehringer Ingelheim Pharma GmbH
& Co. KG
BioPharmaceuticals
Birkendorfer Str. 65
88397 Biberach a. d. Riss
Germany

Sigbert Jahn

Serono GmbH
Freisinger Str. 5
85716 Unterschleissheim
Germany

Wim Jiskoot

Gorlaeus Laboratories
Leiden/Amsterdam Center for Drug
Research (LACDR)
Division of Drug Delivery Technology
P.O. Box 9502
2300 RA Leiden
The Netherlands

Thomas Jostock

Novartis Pharma AG
 Biotechnology Development
 Cell and Process R & D
 CH-4002 Basel
 Switzerland

Joachim R. Kalden

University of Erlangen-Nürnberg
 Medical Clinic III
 Rheumatology, Immunology &
 Oncology
 Krankenhausstrasse 12
 91052 Erlangen
 Germany

Hitto Kaufmann

Boehringer Ingelheim Pharma
 GmbH & Co. KG
 BioPharmaceuticals
 Birkendorfer Str. 65
 88397 Biberach a.d. Riss
 Germany

Ralph Kempken

Boehringer Ingelheim Pharma
 GmbH & Co. KG
 BioPharmaceuticals
 Birkendorfer Str. 65
 88397 Biberach a.d. Riss
 Germany

Scott Klakamp

AstraZeneca Pharmaceuticals LP
 24500 Clawiter Road
 Hayward, CA 94545
 USA

Roland E. Kontermann

University Stuttgart
 Institute for Cell Biology and
 Immunology
 Allmandring 31
 70569 Stuttgart
 Germany

Zoltán Konthur

Max Planck Institute for Molecular
 Genetics
 Department of Vertebrate Genomics
 Ihnestr. 63–73
 14195 Berlin
 Germany

Jürgen Krauss

Department of Medical Oncology and
 Cancer Research
 University of Essen
 Hufelandstr. 55
 45122 Essen
 Germany

Claus Kroegel

Friedrich-Schiller-University
 Department of Pneumology and
 Allergy
 Medical Clinics I
 Erlanger Allee 101
 07740 Jena
 Germany

Hartmut Kupper

Abbott GmbH & Co. KG
 Knollstr. 50
 67061 Ludwigshafen
 Germany

Meina Liang

AstraZeneca Pharmaceuticals LP
 24500 Clawiter Road
 Hayward, CA 94545
 USA

Charito Love

Long Island Jewish Medical Center
 Division of Nuclear Medicine
 New Hyde Park, New York
 USA

Andrew C.R. Martin

University College London
Department of Biochemistry and
Molecular Biology
Darwin Building
Gower Street
London WC1E 6BT
UK

Christian Menzel

Technical University of
Braunschweig
Institute of Biochemistry and
Biotechnology
Spielmannstr. 7
38106 Braunschweig
Germany

Gerhard Moldenhauer

German Cancer Research Center
Department of Molecular
Immunology
Tumor Immunology Program
Im Neuenheimer Feld 280
69120 Heidelberg
Germany

Dafne Müller

University Stuttgart
Institute for Cell Biology and
Immunology
Allmandring 31
70569 Stuttgart
Germany

Markus F. Neurath

Johannes-Gutenberg-University
1st Department of Medicine
Langenbeckstr. 1
55131 Mainz
Germany

Dianne L. Newton

SAIC Frederick, Inc.
Developmental Therapeutics Program
National Cancer Institute at Frederick
Frederick, MD 21702
USA

Michael J. Osborn

The Babraham Institute
Protein Technologies Laboratory
Babraham
Cambridge CB22 3AT
UK

Christopher J. Palestro

Albert Einstein College of Medicine
Bronx, New York
USA
and:
Long Island Jewish Medical Center
New Hyde Park, New York
USA

Gabriele Pecher

Humboldt University Berlin
Medical Clinic for Oncology and
Hematology
Charité Campus Mitte
Charitéplatz 1
10117 Berlin
Germany

Matthias Peipp

University Schleswig-Holstein
Campus Kiel
Division of Stem Cell Transplantation
and Immunotherapy
Schittenhelmstr. 12
24105 Kiel
Germany

Edith A. Perez

Mayo Clinic Jacksonville
Division of Hematology/
Oncology
4500 San Pablo Road
Jacksonville, FL 32224
USA

Sandra Pisch-Heberle

Boehringer Ingelheim Pharma
GmbH & Co. KG
BioPharmaceuticals
Birkendorfer Str. 65
88397 Biberach a.d. Riss
Germany

Ponraj Prabakaran

Protein Interactions Group
Center for Cancer Research
Nanobiology Program
CCR, NCI-Frederick
Frederick, MD 21702
USA

Gabriele Reich

Ruprecht-Karls-University
Institute of Pharmacy and
Molecular Biotechnology
(IPMB)
Department of Pharmaceutical
Technology and Pharmacology
Im Neuenheimer Feld 366
69120 Heidelberg
Germany

Josephine N. Rini

Albert Einstein College of
Medicine
Bronx, New York
USA
And:
Long Island Jewish Medical
Center
Division of Nuclear Medicine
New Hyde Park, New York
USA

Lorin Roskos

AstraZeneca Pharmaceuticals LP
24500 Clawiter Road
Hayward, CA 94545
USA

Susanna M. Rybak

Bionamomics LLC
411 Walnut Street, #3036
Green Cove Springs, FL 32043
USA

José W. Saldanha

National Institute for Medical
Research
Division of Mathematical Biology
The Ridgeway
Mill Hill
London NW7 1AA
UK

Jochen Salfeld

Abbott Bioresearch Center
100 Research Drive
Worcester, MA 01605
USA

Huub Schellekens

Utrecht University
Department of Pharmaceutics Sciences
Department of Innovation Studies
Sorbonnelaan 16
3584 CA Utrecht
The Netherlands

Sebastian Schimrigk

Ruhr University Bochum
St. Josef Hospital
Department of Neurology
Gudrunstr. 56
44791 Bochum
Germany

Thomas Schirrmann

Technical University
Braunschweig
Institute of Biochemistry and
Biotechnology
Department of Biotechnology
Spielmannstr. 7
38106 Braunschweig
Germany

Norbert Schleucher

Hematology and Medical Oncology
Marienkrankenhaus Hamburg
Alfredstr. 9
22087 Hamburg
Germany

Alexander C. Schmidt

National Institutes of Health
National Institute of Allergy and
Infectious Diseases
Laboratory of Infectious Diseases
50 South Drive, Room 6130
Bethesda, MD 20892
USA

And:

Charité Medical Center at Free
University and Humboldt
University Berlin
Center for Perinatal Medicine
and Pediatrics
Schumannstr 20/21
13353 Berlin
Germany

Karlheinz Schmitt-Rau

Serono GmbH
Freisinger Str. 5
85716 Unterschleissheim
Germany

Marjorie A. Shapiro

Center for Drugs Evaluation and
Research
Food and Drug Administration
Division of Monoclonal Antibodies
HFD-123
5600 Fishers Lane
Rockville, MD 20872
USA

Surinder K Sharma

CR UK Targeting & Imaging Group
Department of Oncology
Hampstead Campus
UCL, Rowland Hill Street
London NW3 2PF
UK

Igor A. Sidorov

Center for Cancer Research
Nanobiology Program
CCR, NCI-Frederick, NIH
Protein Interactions Group
P.O. Box B, Miller Drive
Frederick, MD 21702-1201
USA

Arne Skerra

Technical University Munich
Chair for Biological Chemistry
An der Saatzeit 5
85350 Freising-Weihenstephan
Germany

Jennifer A. Smith

The Babraham Institute
Protein Technologies Laboratory
Babraham
Cambridge CB22 3AT
UK

Patrick G. Swann

Center for Drug Evaluation and
Research
Food and Drug Administration
Division of Monoclonal
Antibodies
HFD-123
5600 Fishers Lane
Rockville, MD 20872
USA

Michael J. Taussig

The Babraham Institute
Technology Research Group
Cambridge CB2 4AT
UK

Lars Toleikis

RZPD, Deutsches
Ressourcenzentrum für
Genomforschung GmbH
Im Neuenheimer Feld 515
69120 Heidelberg
Germany

Daniel Tracey

Abbott Bioresearch Center
100 Research Drive
Worcester, MA 01605
USA

Martina M. Uttenreuther-Fischer

Boehringer Ingelheim Pharma
GmbH & Co. KG
Department of Medicine
Clinical Research Oncology
Birkendorfer Str. 65
88397 Biberach a.d. Riss

Thomas Valerius

University Schleswig-Holstein
Campus Kiel
Division of Nephrology
Schittenhelmstr. 12
24105 Kiel
Germany

Udo Vanhoefer

Department of Medicine
Hematology and Medical Oncology,
Gastroenterology and Infectious
Diseases
Marienkrankenhaus Hamburg
Alfredstr. 9
22087 Hamburg
Germany

Michael Wenger

International Medical Leader
F. Hoffmann-La Roche Ltd.
Bldg. 74/4W
CH-4070 Basel
Switzerland

Maria Wiekowski

Schering-Plough
Clinical Research Allergy/Respiratory/
Immunology
2015 Galloping Hill Road
Kenilworth, NJ 07033
USA

Xiangang Zou

The Babraham Institute
Protein Technologies Laboratory
Cambridge CB22 3AT
UK

Contents

Volume II

Overview of Therapeutic Antibodies XV

A Greeting by the Editor XVII

Foreword XIX

List of Authors XXI

Part III Beyond IgG – Modified Antibodies

1 Immunoscintigraphy and Radioimmunotherapy 325

Jason L. J. Dearling and Alexandra Huhlov

1.1 Introduction 325

1.2 Solid Tumors as Targets for Antibody-Based Therapeutics 326

1.3 Antigen 326

1.4 Antibodies as Vehicles for Radionuclide Delivery 327

1.5 Radioisotope 334

1.6 Improving Radioimmunoscintigraphy and Therapy 337

1.7 Summary 338

References 339

2 Bispecific Antibodies 345

Dafne Müller and Roland E. Kontermann

2.1 Introduction 345

2.2 The Generation of Bispecific Antibodies 346

2.3 Bispecific Antibodies and Retargeting of Effector Cells 354

2.4 Bispecific Antibodies and Retargeting of Effector Molecules 361

2.5 Bispecific Antibodies as Agonists or Antagonists 364

2.6 Bispecific Antibodies and Somatic Gene Therapy 366

2.7 Outlook 367

References 368

3 Immunotoxins and Beyond: Targeted RNases 379*Susanna M. Rybak and Dianne L. Newton*

- 3.1 Introduction 379
- 3.2 Immunotoxins 382
- 3.3 Targeted RNases 389
- 3.4 Targeted Onconase 394
- 3.5 Outlook 399
- References 400

Part IV Emerging Concepts**4 Automation of Selection and Engineering 413***Zoltán Konthur*

- 4.1 Introduction 413
- 4.2 General Considerations for the Automation of Antibody Generation 415
- 4.3 Development of an Antibody Generation Pipeline 417
- 4.4 Conclusion 427
- References 428

5 Emerging Technologies for Antibody Selection 431*Mingyue He and Michael J. Taussig*

- 5.1 Introduction 431
- 5.2 Display Technologies 432
- 5.3 Antibody Libraries 433
- 5.4 Antibody Selection and Maturation *In Vitro* 435
- 5.5 Linking Antibodies to mRNA: Ribosome and mRNA Display 436
- 5.6 Advantages of Ribosome Display 436
- 5.7 Ribosome Display Systems 437
- 5.8 Antibody Generation by Ribosome Display 440
- 5.9 Summary 440
- References 441

6 Emerging Alternative Production Systems 445*Thomas Jostock*

- 6.1 Introduction 445
- 6.2 Production Systems 446
- 6.3 Outlook 458
- References 460

7 Non-Antibody Scaffolds 467*Markus Fiedler and Arne Skerra*

- 7.1 Introduction 467
- 7.2 Motivation for Therapeutic Use of Alternative Binding Proteins 467

7.3	Single-Domain Immunoglobulins	473
7.4	Scaffold Proteins Presenting a Contiguous Hypervariable Loop Region	480
7.5	Scaffold Proteins for Display of Individual Extended Loops	484
7.6	Scaffold Proteins Providing a Rigid Secondary Structure Interface	488
7.7	Conclusions and Outlook: Therapeutic Potential and Ongoing Developments	491
	References	492
8	Emerging Therapeutic Concepts I: ADEPT	501
	<i>Surinder K. Sharma, Kerry A. Chester, and Kenneth D. Bagshawe</i>	
8.1	Introduction and Basic Principles of ADEPT	501
8.2	Preclinical Studies	503
8.3	Clinical Studies	505
8.4	Immunogenicity	506
8.5	Important Considerations/Outlook	508
	References	509
9	Emerging Therapeutic Concepts II: Nanotechnology	515
	<i>Dimitar S. Dimitrov, Igor A. Sidorov, Yang Feng, Ponraj Prabakaran, Michaela A.E. Arndt, Jürgen Krauss, and Susanna M. Rybak</i>	
9.1	Introduction	515
9.2	Nanoliposomes, Gold Nanoshells, Quantum Dots, and Other Nanoparticles with Biomedical Potential	516
9.3	Conjugation of Antibodies to Nanoparticles	518
9.4	Nanoparticle–Antibody Conjugates for the Treatment of Cancer and Other Diseases	520
9.5	Comparison Between Nanoparticle–Antibody Conjugates and Fusion Proteins for Cancer Diagnosis and Treatment	522
9.6	Conclusions	526
9.7	Summary	526
	References	527
	Appendix	529
10	Emerging Therapeutic Concepts III: Chimeric Immunoglobulin T Cell Receptors, T-Bodies	533
	<i>Thomas Schirrmann and Gabriele Pecher</i>	
10.1	Introduction	533
10.2	Chimeric Immunoglobulin T-Cell Receptors – “T-Bodies”	534
10.3	Preclinical Studies	543
10.4	Therapeutic Considerations	552

10.5	Perspectives	558
10.6	Conclusions	561
	References	561

11 Emerging Therapeutic Concepts IV: Anti-idiotypic Antibodies 573

Peter Fischer and Martina M. Uttenreuther-Fischer

11.1	Introduction	573
11.2	Definition of Anti-idiotypic Antibodies	573
11.3	Anti-idiotypic Antibodies as Autoantigens	575
11.4	Anti-idiotypes as a Tool for Generating Specific Antibodies	575
11.5	Intravenous Immunoglobulin Preparations (IVIG)	575
11.6	Anti-idiotypic Antibodies as Possible Superantigens?	577
11.7	Anti-idiotypic Antibodies in Cancer Therapy	578
11.8	Ab2 β Vaccine Trials Mimicking GD ₂	580
11.9	Molecular Characterization of the Anti-idiotypic Immune Response of a Relapse-free Cancer Patient	581
11.10	Conclusion	583
	References	583

Part V Ongoing Clinical Studies

12 Antibodies in Phase I/II/III: Cancer Therapy 593

P. Markus Deckert

12.1	Introduction	593
12.2	Novel Antibody Constructs	595
12.3	Specific Targeting and Effector Mechanisms	606
12.4	Antigens Without Known Effector Function	627
12.5	Disease-specific Concepts of Unknown Antigen Function and Structure	656
12.6	Summary	656
	References	659

13 Antibodies in Phase I/II/III: Targeting TNF 673

Martin H. Holtmann and Markus F. Neurath

13.1	Introduction	673
13.2	Inflammatory Bowel Disease	674
13.3	Pathophysiologic Role of T Cells	675
13.4	Tumor Necrosis Factor- α	675
13.5	TNF Receptors and Signaling	676
13.6	Anti-TNF Antibodies and Fusion Proteins in Clinical Testing	679
13.7	Mechanisms of Action	683
13.8	Other Anti-TNF Biologicals	683
13.9	Other Cytokine-based and Anti-CD4 ⁺ T-Cell Approaches	684
13.10	Perspective	685
	References	686

Volume I

Overview of Therapeutic Antibodies XIX

A Greeting by the Editor XXI

Foreword XXIII

List of Authors XXV

Introduction

1 Therapeutic Antibodies – From Past to Future 3

Stefan Dübel

Part I Selecting and Shaping the Antibody Molecule

2 Selection Strategies I: Monoclonal Antibodies 19

Gerhard Moldenhauer

3 Selection Strategies II: Antibody Phage Display 45

Michael Hust, Lars Toleikis and Stefan Dübel

4 Selection Strategies III: Transgenic Mice 69

*Marianne Brüggemann, Jennifer A. Smith, Michael J. Osborn,
and Xiangang Zou*

5 Bioinformatics Tools for Antibody Engineering 95

Andrew C.R. Martin and James Allen

6 Molecular Engineering I: Humanization 119

José W. Saldanha

7 Molecular Engineering II: Antibody Affinity 145

*Lorin Roskos, Scott Klakamp, Meina Liang, Rosalin Arends,
and Larry Green*

8 Molecular Engineering III: Fc Engineering 171

Matthias Peipp, Thomas Beyer, Michael Dechant, and Thomas Valerius

Part II The Way into the Clinic 197

9 Production and Downstream Processing 199

*Klaus Bergemann, Christian Eckermann, Patrick Garidel,
Stefanos Grammatikos, Alexander Jacobi, Hitto Kaufmann,
Ralph Kempken, and Sandra Pisch-Heberle*

- 10 Pharmaceutical Formulation and Clinical Application** 239
Gabriele Reich
- 11 Immunogenicity of Antibody Therapeutics** 267
Huub Schellekens, Daan Crommelin, and Wim Jiskoot
- 12 Regulatory Considerations** 277
Marjorie A. Shapiro, Patrick G. Swann, and Melanie Hartsough
- 13 Intellectual Property Issues** 301
Michael Braunagel and Rathin C. Das

Volume III

Overview of Therapeutic Antibodies XXIII

A Greeting by the Editor XXV

Foreword XXVII

List of Authors XXIX

Volume III Approved Therapeutics

- 1 Adalimumab (Humira)** 697
Hartmut Kupper, Jochen Salfeld, Daniel Tracey, and Joachim R. Kalden
- 2 Alemtuzumab (MabCampath)** 733
Thomas Elter, Andreas Engert, and Michael Hallek
- 3 Bevacizumab (Avastin)** 779
Eduardo Díaz-Rubio, Edith A. Perez, and Guiseppe Giaccone
- 4 Cetuximab (Erbix, C-225)** 813
Norbert Schleucher and Udo Vanhoefer
- 5 Efalizumab (Raptiva)** 827
Karlheinz Schmitt-Rau and Sigbert Jahn
- 6 ^{99m}Tc-Fanolesomab (NeutroSpec)** 851
Christopher J. Palestro, Josephine N. Rini, and Charito Love
- 7 Gemtuzumab Ozogamicin (Mylotarg)** 869
Matthias Peipp and Martin Gramatzki

- 8 Infliximab (Remicade)** 885
Maria Wiekowski and Christian Antoni
- 9 Muromonab-CD3 (Orthoclone OKT3)** 905
Harald Becker
- 10 Natalizumab (Tysabri)** 941
Sebastian Schimrigk and Ralf Gold
- 11 Omalizumab (Xolair)**
Anti-Immunoglobulin E Treatment in Allergic Diseases 951
Claus Kroegel and Martin Foerster
- 12 Palivizumab (Synagis)** 999
Alexander C. Schmidt
- 13 Rituximab (Rituxan)** 1033
Michael Wenger
- 14 Trastuzumab (Herceptin)**
A Treatment for HER2-Positive Breast Cancer 1109
Paul Ellis
- 15 Abciximab, Arcitumomab, Basiliximab, Capromab, Cotara, Daclizumab, Edrecolomab, Ibritumomab, Igovomab, Nofetumomab, Satumomab, Sulesomab, Tositumomab, and Votumumab** 1131
Christian Menzel and Stefan Dübel
- Index** 1149

Contents

Volume III

Overview of Therapeutic Antibodies XXIII

A Greeting by the Editor XXV

Foreword XXVII

List of Authors XXIX

Volume III Approved Therapeutics

1	Adalimumab (Humira)	697
	<i>Hartmut Kupper, Jochen Salfeld, Daniel Tracey, and Joachim R. Kalden</i>	
1.1	Introduction	697
1.2	Pharmacology	698
1.3	Pharmacokinetics	700
1.4	Adalimumab Comparisons with Infliximab and Etanercept	701
1.5	Indications	703
1.5.1	Rheumatoid Arthritis	703
1.5.2	Psoriatic Arthritis	703
1.5.3	Ankylosing Spondylitis	704
1.5.4	Dosing and Administration	705
1.6	Clinical Experience	705
1.6.1	Studies in Rheumatoid Arthritis	705
1.6.1.1	Adalimumab in Combination with MTX	705
1.6.1.2	Adalimumab with Traditional DMARDs	708
1.6.1.2.1	Additional Experience: Adalimumab with Traditional DMARDs	709
1.6.1.3	Adalimumab Monotherapy	709
1.6.1.4	Pivotal Study in Early Rheumatoid Arthritis	710
1.6.1.5	Adalimumab in Patients Previously Treated with other Anti-TNF Agents	712

1.6.2	Pivotal Studies in Psoriatic Arthritis	713
1.6.3	Ankylosing Spondylitis	716
1.7	Review of Adalimumab Safety	717
1.7.1	Safety in Specific Indications	717
1.7.1.1	Long-Term Safety in Rheumatoid Arthritis	717
1.7.1.2	Safety Profile in Psoriatic Arthritis and Ankylosing Spondylitis	719
1.7.2	Immune System Function	719
1.7.3	Infections	719
1.7.3.1	Tuberculosis	720
1.7.3.2	Opportunistic Infections	720
1.7.4	Other Conditions	720
1.7.4.1	Lymphoma	720
1.7.4.2	Demyelinating Conditions	720
1.7.4.3	Autoantibodies and Autoimmune Diseases	721
1.8	Special Conditions and Populations	721
1.8.1	Pregnancy and Lactation	721
1.8.2	Elderly	721
1.8.3	Children and Adolescents	721
1.8.4	Impaired Renal and/or Hepatic Function	722
1.8.5	Congestive Heart Failure	722
1.9	Storage and Administration	722
1.10	Outlook and New Indications	723
1.10.1	Psoriasis	723
1.10.2	Crohn's Disease	723
1.10.3	Juvenile Rheumatoid Arthritis	726
1.11	Conclusions	727
	References	727
2	Alemtuzumab (MabCampath)	733
	<i>Thomas Elter, Andreas Engert, and Michael Hallek</i>	
2.1	Introduction	733
2.2	Basic Principles	734
2.2.1	Antibody Features and Production	734
2.2.1.1	Features of Alemtuzumab (Campath-1H)	734
2.2.1.2	Antibody Production	734
2.3	Mechanism of Action	734
2.3.1	Molecular Target and Target Expression	734
2.3.2	Mechanism of Cell Lysis	737
2.3.3	Immunogenicity and Antiglobulin Response	738
2.4	Clinical Studies with Alemtuzumab	738
2.4.1	Pharmacokinetic Studies	738
2.4.2	Chronic Lymphocytic Leukemia (CLL)	740
2.4.2.1	Relapsed/Refractory CLL	740
2.4.2.2	Minimal Residual Disease in CLL	746

2.4.2.3	Treatment-Naïve CLL	748
2.4.2.4	Chemoimmunotherapy Combinations	749
2.4.2.5	Immunotherapy Combination	753
2.4.2.6	Safety with Alemtuzumab in CLL	753
2.4.2.6.1	Infusion-related Adverse Events	754
2.4.2.6.2	Hematologic Toxicities	755
2.4.2.6.3	Immunosuppression and Infectious Events	756
2.4.3	T-Cell Leukemias and Lymphomas	758
2.4.3.1	T-Cell Lymphomas (Cutaneous/Peripheral T-Cell Lymphoma)	758
2.4.3.2	T-Cell Prolymphocytic Leukemia (T-PLL)	761
2.4.3.3	Adult T-Cell Leukemia	762
2.4.4	Non-Hodgkin's Lymphoma (NHL)	763
2.4.5	Stem Cell Transplantation (SCT)	765
2.4.5.1	Donor T-Cell Depletion (Prevention of GvHD) and Prevention of Graft Rejection	765
2.4.5.2	Reduced-Intensity/Non-Myeloablative Conditioning	766
2.4.5.3	Safety	768
2.5	The Future Outlook	769
2.5.1	Other Hematologic Malignancies (Multiple Myeloma, Acute Leukemias)	769
2.5.2	Prevention of GvHD in Solid Organ Transplantation	769
2.5.3	Applications in Autoimmune Disease	772
	References	772
3	Bevacizumab (Avastin)	779
	<i>Eduardo Díaz-Rubio, Edith A. Perez, and Guiseppe Giaccone</i>	
3.1	Introduction	779
3.1.1	Angiogenesis is Vital for Tumor Development	779
3.1.2	Vascular Endothelial Growth Factor: A Key Angiogenic Factor	780
3.1.3	Targeting Angiogenesis and VEGF: A Rational Treatment Option	781
3.2	The Development of Bevacizumab	783
3.2.1	Origin and Genetic Engineering	783
3.2.2	Mode of Action	784
3.2.3	Preclinical Activity of Bevacizumab	784
3.3	Clinical Trials of Bevacizumab in Key Tumor Types	785
3.3.1	Colorectal Cancer	786
3.3.1.1	Efficacy of Bevacizumab in CRC	786
3.3.1.2	Safety and Management of Bevacizumab in CRC	790
3.3.1.3	Future Use of Bevacizumab in CRC	792
3.3.2	Non-Small Cell Lung Cancer	795
3.3.2.1	Efficacy of Bevacizumab in NSCLC	795
3.3.2.2	Safety and Management of Bevacizumab in NSCLC	798
3.3.2.3	Future Use of Bevacizumab in NSCLC	799

3.3.3	Breast Cancer	799
3.3.3.1	Efficacy of Bevacizumab in Breast Cancer	799
3.3.3.2	Safety and Management of Bevacizumab in Breast Cancer	802
3.3.3.3	Future Use of Bevacizumab in Breast Cancer	802
3.4	The Potential of Bevacizumab	803
3.5	Conclusions	805
	References	806
4	Cetuximab (Erbix, C-225)	813
	<i>Norbert Schleucher and Udo Vanhoefer</i>	
4.1	Introduction	813
4.2	Preclinical Activity	815
4.3	Clinical Data I: Outcome of Monotherapy	816
4.4	Clinical Data II: Outcome of Combination Therapy	817
4.4.1	Colorectal Cancer	817
4.4.2	Head and Neck Cancer	819
4.4.3	Non-Small Cell Lung Cancer (NSCLC)	819
4.4.4	Other Tumors	820
4.5	Clinical Data III: Cetuximab and Radiotherapy/Chemo-Radiotherapy	820
4.5.1	The PARC Study	820
4.5.1.1	Dosing Schedule	821
4.5.1.2	Determinants of Cetuximab Efficacy	821
4.5.1.3	Treatment of Skin Toxicity	821
	References	822
5	Efalizumab (Raptiva)	827
	<i>Karlheinz Schmitt-Rau and Sigbert Jahn</i>	
5.1	Introduction	827
5.2	Development and Characterization of the Antibody	828
5.3	Efalizumab in the Treatment of Psoriasis	829
5.3.1	Psoriasis: Prevalence, Characteristics, and Therapeutic Options	829
5.3.2	Pathogenesis of Psoriasis	831
5.3.2.1	T-Cell Activation	831
5.3.2.2	T-Cell Migration and Extravasation	832
5.3.2.3	T-Cell Reactivation	832
5.3.3	Efalizumab: The Mechanism of Action	833
5.4	Pharmacology and Toxicology of Efalizumab	834
5.4.1	Preclinical Studies	834
5.4.2	Pharmacodynamics	834
5.4.3	Pharmacokinetics	835
5.4.3.1	Absorption	835
5.4.3.2	Distribution	836
5.4.3.3	Biotransformation	836

5.4.3.4	Elimination	836
5.5	Clinical Development of Efalizumab	837
5.5.1	Clinical Efficacy	837
5.5.1.1	Randomized, Placebo-Controlled, Double-Blind Studies	837
5.5.1.2	Extended Treatment and Re-Treatment	838
5.5.1.3	Long-Term Efficacy	840
5.5.2	Safety and Tolerability	841
5.5.3	Health-Related Quality of Life (HRQoL)	844
5.6	Practical Considerations for Therapy with Efalizumab	845
5.6.1	Managing Patients during Long-Term Efalizumab Treatment	845
5.6.2	Other Concerns	846
5.7	Summary	847
	References	847
6	^{99m}Tc-Fanolesomab (NeutroSpec)	851
	<i>Christopher J. Palestro, Josephine N. Rini, and Charito Love</i>	
6.1	Introduction	851
6.2	The Agent	851
6.3	Pharmacokinetics/Dosimetry	852
6.4	Biodistribution	852
6.5	Technique	854
6.6	Indications	856
6.6.1	Appendicitis	856
6.6.2	Osteomyelitis	859
6.6.3	Other Infections	861
6.7	Adverse Side Effects and Safety	863
6.8	Summary	865
	References	865
7	Gemtuzumab Ozogamicin (Mylotarg)	869
	<i>Matthias Peipp and Martin Gramatzki</i>	
7.1	Introduction	869
7.2	CD33 as a Target Antigen in Acute Leukemia Therapy	870
7.3	Gemtuzumab Ozogamicin	871
7.3.1	The IgG4 Moiety	872
7.3.2	Calicheamicin	872
7.3.3	The Design of Antibody-Calicheamicin Conjugates: Humanization and Choice of Linker	873
7.4	Mechanisms of Action	874
7.5	Potential Mechanisms of Resistance	874
7.6	Clinical Trials: The Data of GO	875
7.7	Summary and Conclusions	877
	References	878

8	Infliximab (Remicade)	885
	<i>Maria Wiekowski and Christian Antoni</i>	
8.1	Antibody Characteristics	885
8.2	Preclinical Characterization	886
8.3	Pharmacokinetics	886
8.4	Clinical Response	887
8.4.1	Therapeutic Indications	887
8.4.1.1	Crohn's Disease	888
8.4.1.2	Rheumatoid Arthritis	889
8.4.1.3	Ankylosing Spondylitis	890
8.4.1.4	Psoriatic Arthritis	891
8.4.1.5	Psoriasis	892
8.4.1.6	Ulcerative Colitis	893
8.5	Safety	894
8.5.1	Serious Infections	894
8.5.1.1	Tuberculosis	894
8.5.2	Antibody Formation against Infliximab	895
8.5.3	Infusion Reactions/Delayed Hypersensitivity Reactions	896
8.5.4	Auto-Antibody Formation	896
8.5.5	Neurological Disorders/Demyelinating Disease	896
8.5.6	Malignancies/Lymphoma	897
8.5.7	Congestive Heart Failure	897
8.5.8	Other Adverse Events	898
8.5.8.1	Hepatic Events	898
8.5.8.2	Pregnancy Outcome	898
8.6	Summary	898
	References	899
9	Muromonab-CD3 (Orthoclone OKT3)	905
	<i>Harald Becker</i>	
9.1	Introduction	905
9.2	Production of the Monoclonal Antibody	906
9.3	The Pharmacology of Muromonab-CD3	908
9.3.1	Pharmacokinetic Properties of Muromonab-CD3	909
9.3.2	Pharmacodynamics of Muromonab-CD3	910
9.3.3	Activation of Human T Cells	911
9.3.4	Immunogenicity	912
9.3.5	Interactions	913
9.4	Therapeutic Use	914
9.4.1	Renal and or Renal-Pancreas Transplant Recipients	916
9.4.2	Liver Transplant Recipients	919
9.4.3	Cardiac Transplant Recipients	919
9.5	Cytokine Release Syndrome	924
9.5.1	The Pathophysiology of the Cytokine Release Syndrome	924
9.5.2	Symptoms of the Cytokine Release Syndrome	926

9.5.3	Muromonab-CD3 and the Cytokine Release Syndrome	927
9.5.4	Management of the Cytokine Release Syndrome	929
9.5.4.1	Methylprednisolone	929
9.5.4.2	Pentoxifylline	931
9.5.4.3	Indomethacin	932
9.5.4.4	Recombinant Human Soluble Tumor Necrosis Factor Receptor (TNFR:Fc)	932
9.5.4.5	Anti-TNF Monoclonal Antibodies	933
9.6	The Consequences of Immunosuppression	933
9.6.1	Infections	933
9.6.2	Neoplasia	934
	References	935

10 Natalizumab (Tysabri) 941 *Sebastian Schimrigk and Ralf Gold*

10.1	Introduction	941
10.2	Basic Principles	942
10.3	Mode of Action	943
10.3.1	Pharmacodynamic Profile	944
10.3.2	Pharmacokinetics	944
10.4	Technology	945
10.5	Clinical Findings	945
10.5.1	Phase I Clinical Trials	945
10.5.2	Phase II Clinical Trials	945
10.5.3	Phase III Clinical Trials	946
10.5.4	Adverse Side Effects	947
10.5.5	Neutralizing Antibodies	947
10.6	Indications for Tysabri	947
10.6.1	Clinical Applications	948
10.6.1.1	Preparation and Administration of Natalizumab	948
10.7	Outlook	949
	References	949

11 Omalizumab (Xolair) Anti-Immunoglobulin E Treatment in Allergic Diseases 951 *Claus Kroegel and Martin Foerster*

11.1	Introduction	952
11.2	The Biology of the IgE Molecule	953
11.2.1	IgE Distribution and Blood Concentration	956
11.2.2	IgE Synthesis and Regulation	956
11.3	IgE Receptors	957
11.3.1	FcεRI (High-Affinity IgE Receptor)	957
11.3.2	FcεRII (Low-Affinity IgE Receptor, CD23)	959
11.4	Cell Distribution of IgE	962

11.4.1	Effector Cell-Associated IgE	962
11.4.2	Antigen-Presenting Cell-Associated IgE	962
11.5	Physiologic and Pathophysiologic Significance of IgE	963
11.6	The Concept of Anti-IgE-Based Treatment	964
11.7	Construction of the Monoclonal Anti-IgE Molecule	964
11.7.1	Antibody Generation	964
11.7.2	Complex Formation and Tissue Distribution	965
11.7.3	Preclinical Results	967
11.7.4	Clinical Studies	969
11.8	Anti-Inflammatory Effects of Omalizumab	973
11.8.1	Effects on Serum Free IgE Levels	974
11.8.2	Effect on Cytokines	974
11.8.3	Effects on FcεRI Cell Expression	975
11.8.4	Effect on Dendritic Cell APCs	975
11.8.5	Effect on Eosinophils	976
11.8.6	Effects on B Cells	977
11.9	Pharmacological Properties of Omalizumab	977
11.9.1	Pharmacodynamics	977
11.9.2	Pharmacokinetics	978
11.10	Adverse Effects	978
11.10.1	Systemic Side Effects	978
11.10.2	Local Reactions	979
11.10.3	Serious Adverse Effects	979
11.10.4	Immune Complex Diseases.	979
11.10.5	Long-Term Adverse Effects	980
11.11	Indications	980
11.12	Contraindications	981
11.13	Preparation for Use	982
11.14	Administration	982
11.15	Clinical Dosing	983
11.16	Dosing Adjustments	984
11.17	Precautions and Contraindications	984
11.17.1	Drug Interactions	984
11.17.2	Pregnancy and Lactation	984
11.18	Monitoring of Therapy	985
11.19	Cost	985
11.20	Response to Treatment	986
11.20.1	Onset of Action of Anti-Immunoglobulin E Effect	986
11.20.2	Duration of Treatment	986
11.21	Non-Approved Diseases	987
11.21.1	Allergic Rhinitis	987
11.21.2	Other Clinical Applications	987
11.22	Areas of Uncertainty	989
11.23	Outlook	990
	References	991

12	Palivizumab (Synagis)	999
	<i>Alexander C. Schmidt</i>	
12.1	Nature, Role in Disease, and Biology of the Target	999
12.1.1	Respiratory Syncytial Virus (RSV)-Induced Disease and RSV Epidemiology	999
12.1.2	The Target of the Antibody: The RSV Virion	1000
12.1.3	Correlates of Protection from Disease	1001
12.2	Origin, Engineering, and Humanization of the Antibody	1003
12.3	Mechanism of Action and Preclinical Results	1006
12.4	Production, Downstream Processing, and Galenics of the Antibody	1007
12.4.1	Production	1007
12.4.2	Downstream Processing	1008
12.4.3	Formulations	1008
12.4.4	Specifications	1009
12.5	Summary of Results from Clinical Studies	1010
12.5.1	Phase III Trials	1010
12.5.1.1	Palivizumab in Premature Infants and Children with BPD	1010
12.5.1.2	Palivizumab in Children with Significant CHD	1012
12.6	Indications and Usage	1014
12.7	Clinical Reports after Approval	1014
12.8	Is Protective Efficacy a Function of Palivizumab Serum Concentration?	1021
12.9	Post-Marketing Experience with Regard to Adverse Events	1023
12.10	Ongoing Clinical Studies and Outlook	1024
12.11	Summary	1025
	References	1026
13	Rituximab (Rituxan)	1033
	<i>Michael Wenger</i>	
13.1	Introduction	1033
13.1.1	Production, Design, and Structure of Rituximab	1033
13.1.2	CD20 as a Therapeutic Target	1034
13.1.3	Mode of Action	1035
13.1.4	Preclinical Studies	1037
13.1.5	Pharmacokinetic Studies	1038
13.2	Rituximab Clinical Data in NHL and CLL	1039
13.2.1	Overview of NHL and CLL	1039
13.2.2	Rituximab plus Chemotherapy Induction Therapy in Indolent NHL	1041
13.2.2.1	Rituximab plus Chemotherapy in Previously Untreated Indolent NHL	1041
13.2.2.1.1	Concurrent Rituximab and Chemotherapy: Phase II Studies	1042
13.2.2.1.2	Chemotherapy and Sequential Rituximab: Phase II Studies	1046

13.2.2.1.3	Chemotherapy plus Rituximab: Phase III Studies	1047
13.2.2.2	Rituximab plus Chemotherapy in Relapsed/Refractory Indolent NHL	1049
13.2.2.2.1	Rituximab and Chemotherapy: Phase II Trials	1050
13.2.2.2.2	Rituximab and Chemotherapy: Phase III Trials	1050
13.2.2.3	Meta-Analysis of Rituximab and Chemotherapy in Indolent NHL	1053
13.2.3	Induction Therapy with Rituximab plus Immune System Modulators in Indolent NHL	1053
13.2.3.1	Rituximab plus Immune System Modulators	1054
13.2.3.2	Rituximab plus Immune Modulators in Relapsed/Refractory Indolent NHL	1054
13.2.4	Induction with Rituximab Monotherapy in Indolent NHL	1055
13.2.4.1	Rituximab Monotherapy in Previously Untreated Indolent NHL	1055
13.2.4.2	Rituximab Monotherapy in Relapsed Indolent NHL	1055
13.2.5	Rituximab in Other Subtypes of Indolent Lymphoma	1056
13.2.5.1	Rituximab in Marginal Zone Lymphoma	1056
13.2.5.2	Rituximab in Small Lymphocytic Lymphoma	1057
13.2.5.3	Rituximab in Waldenström's Macroglobulinemia	1057
13.2.6	Rituximab Maintenance Therapy	1058
13.2.6.1	Rituximab Maintenance Therapy Following Monotherapy Induction	1058
13.2.6.2	Rituximab Maintenance Therapy Following Chemotherapy Induction	1061
13.2.6.3	Rituximab Maintenance Therapy Following Rituximab Chemotherapy Induction	1062
13.2.7	Rituximab Retreatment	1062
13.2.8	Rituximab in Aggressive NHL	1063
13.2.8.1	Rituximab plus Chemotherapy in Previously Untreated Aggressive NHL: Phase II Studies	1064
13.2.8.2	Rituximab plus Chemotherapy in Previously Untreated DLBCL: Phase III Studies and Population Analysis	1068
13.2.8.2.1	The GELA LNH98-5 Trial	1068
13.2.8.2.2	The Intergroup E4494 Trial	1069
13.2.8.2.3	The MInT Trial	1070
13.2.8.2.4	BCCA Population Analysis	1071
13.2.8.3	Rituximab plus Dose-Densified Chemotherapy in Previously Untreated Aggressive NHL: Phase III Studies	1072
13.2.8.4	Rituximab plus Chemotherapy in Relapsed Aggressive NHL	1072
13.2.8.5	Rituximab Monotherapy in Aggressive NHL	1073
13.2.8.6	Rituximab in Other Subtypes of Aggressive B-Cell NHL	1073
13.2.8.6.1	Rituximab in PMBCL	1073

13.2.8.6.2	Rituximab in Burkitt's and Burkitt-Like Lymphoma or Lymphoblastic Lymphoma/Leukemia	1074
13.2.9	Rituximab in MCL	1074
13.2.9.1	Rituximab plus Chemotherapy in Previously Untreated MCL	1074
13.2.9.2	Rituximab plus Chemotherapy in Relapsed MCL	1076
13.2.9.3	Rituximab Monotherapy in MCL	1077
13.2.10	Rituximab in CLL	1077
13.2.10.1	Rituximab plus Chemotherapy in Previously Untreated CLL	1077
13.2.10.2	Rituximab plus Chemotherapy in Relapsed CLL	1078
13.2.10.3	Rituximab with Immune System Modulators in CLL	1081
13.2.10.4	Rituximab Monotherapy in CLL	1082
13.2.11	Rituximab in the Transplant Setting	1082
13.2.12	Rituximab in Other Malignancies of B-Cell Origin	1083
13.2.12.1	Rituximab in PTLD	1084
13.2.12.2	Rituximab in HIV-Associated Lymphoma	1084
13.2.12.3	Rituximab in PCNSL	1084
13.2.12.4	Rituximab in HCL	1085
13.2.12.5	Rituximab in HD	1085
13.3	Rituximab in Autoimmune Disorders	1085
13.3.1	Rituximab in RA	1086
13.3.2	Rituximab in SLE	1087
13.3.3	Rituximab in Autoimmune Cytopenias and Hemophilia	1088
13.3.4	Rituximab in Chronic Graft-versus-Host Disease (GvHD)	1088
13.3.5	Rituximab in Other Autoimmune Disorders	1088
13.4	Summary and Conclusions	1089
	References	1091

14 Trastuzumab (Herceptin)

	A Treatment for HER2-Positive Breast Cancer	1109
	<i>Paul Ellis</i>	
14.1	Introduction	1109
14.2	Metastatic Breast Cancer	1111
14.2.1	Trastuzumab Monotherapy	1111
14.2.2	Trastuzumab in Combination with Taxanes	1112
14.2.3	Trastuzumab in Combination with other Standard Chemotherapy	1114
14.2.4	Trastuzumab in Triple Combination	1115
14.2.5	Trastuzumab in Combination with Hormonal Therapies	1115
14.3	Early Breast Cancer	1115
14.4	Trastuzumab Treatment in other Tumor Types	1119
14.5	Safety	1120
14.5.1	Cardiac Adverse Events	1121
14.5.2	Infusion-related Reactions	1124
14.5.3	Age Considerations	1125

14.5.4	Patient Considerations	1125
14.6	Dosing/Scheduling	1126
14.7	Conclusions	1126
	References	1126
15	Abciximab, Arcitumomab, Basiliximab, Capromab, Cotara, Daclizumab, Edrecolomab, Ibritumomab, Igovomab, Nofetumomab, Satumomab, Sulesomab, Tositumomab, and Votumumab	1131
	<i>Christian Menzel and Stefan Dübel</i>	
15.1	Abciximab (Reopro)	1131
15.2	Arcitumomab (CEA-Scan)	1132
15.3	Basiliximab (Simulect)	1133
15.4	Capromab Pendetide (ProstaScint)	1135
15.5	¹³¹ I-chTNT-1/B (Cotara)	1136
15.6	Daclizumab (Zenapax)	1137
15.7	Edrecolomab (Panorex 17-1A)	1138
15.8	Gemtuzumab Ozogamicin (Mylotarg)	1139
15.9	Ibritumomab (Ibritumomab Tiuxetan, Zevalin)	1140
15.10	Igovomab (Indimacis-125)	1141
15.11	Nofetumomab (Verluma)	1142
15.12	Satumomab (OncoScint/Oncorad: B72.3n)	1143
15.13	Sulesomab (LeukoScan, MN-3)	1144
15.14	Tositumomab; Iodine ¹³¹ I Tositumomab (Bexxar)	1145
15.15	Votumumab (Humaspect)	1146
	Index	1149

Volume I

Overview of Therapeutic Antibodies XIX

A Greeting by the Editor XXI

Foreword XXIII

List of Authors XXV

Introduction

1 Therapeutic Antibodies – From Past to Future 3

Stefan Dübel

Part I Selecting and Shaping the Antibody Molecule

2 Selection Strategies I: Monoclonal Antibodies 19

Gerhard Moldenhauer

- 3 Selection Strategies II: Antibody Phage Display 45**
Michael Hust, Lars Toleikis and Stefan Dübel
- 4 Selection Strategies III: Transgenic Mice 69**
Marianne Brüggemann, Jennifer A. Smith, Michael J. Osborn, and Xiangang Zou
- 5 Bioinformatics Tools for Antibody Engineering 95**
Andrew C.R. Martin and James Allen
- 6 Molecular Engineering I: Humanization 119**
José W. Saldanha
- 7 Molecular Engineering II: Antibody Affinity 145**
Lorin Roskos, Scott Klakamp, Meina Liang, Rosalin Arends, and Larry Green
- 8 Molecular Engineering III: Fc Engineering 171**
Matthias Peipp, Thomas Beyer, Michael Dechant, and Thomas Valerius

Part II The Way into the Clinic 197

- 9 Production and Downstream Processing 199**
Klaus Bergemann, Christian Eckermann, Patrick Garidel, Stefanos Grammatikos, Alexander Jacobi, Hitto Kaufmann, Ralph Kempken, and Sandra Pisch-Heberle
- 10 Pharmaceutical Formulation and Clinical Application 239**
Gabriele Reich
- 11 Immunogenicity of Antibody Therapeutics 267**
Huub Schellekens, Daan Crommelin, and Wim Jiskoot
- 12 Regulatory Considerations 277**
Marjorie A. Shapiro, Patrick G. Swann, and Melanie Hartsough
- 13 Intellectual Property Issues 301**
Michael Braunagel and Rathin C. Das

Volume II

Overview of Therapeutic Antibodies XV

A Greeting by the Editor XVII

Foreword XIX

List of Authors XXI

Part III Beyond IgG – Modified Antibodies

- 1 Immunoscintigraphy and Radioimmunotherapy 325**
Jason L. J. Dearling and Alexandra Huhlov

- 2 Bispecific Antibodies 345**
Dafne Müller and Roland E. Kontermann

- 3 Immunotoxins and Beyond: Targeted RNases 379**
Susanna M. Rybak and Dianne L. Newton

Part IV Emerging Concepts

- 4 Automation of Selection and Engineering 413**
Zoltán Konthur

- 5 Emerging Technologies for Antibody Selection 431**
Mingyue He and Michael J. Taussig

- 6 Emerging Alternative Production Systems 445**
Thomas Jostock

- 7 Non-Antibody Scaffolds 467**
Markus Fiedler and Arne Skerra

- 8 Emerging Therapeutic Concepts I: ADEPT 501**
Surinder K. Sharma, Kerry A. Chester, and Kenneth D. Bagshawe

- 9 Emerging Therapeutic Concepts II: Nanotechnology 515**
Dimiter S. Dimitrov, Igor A. Sidorov, Yang Feng, Ponraj Prabakaran, Michaela A.E. Arndt, Jürgen Krauss, and Susanna M. Rybak

- 10 Emerging Therapeutic Concepts III: Chimeric Immunoglobulin
 T Cell Receptors, T-Bodies 533**
Thomas Schirrmann and Gabriele Pecher

- 11 Emerging Therapeutic Concepts IV: Anti-idiotypic Antibodies 573**
Peter Fischer and Martina M. Uttenreuther-Fischer

Part V Ongoing Clinical Studies

12 Antibodies in Phase I/II/III: Cancer Therapy 593

P. Markus Deckert

13 Antibodies in Phase I/II/III: Targeting TNF 673

Martin H. Holtmann and Markus F. Neurath

Introduction

1

Therapeutic Antibodies – From Past to Future*Stefan Dübel*

1.1

An Exciting Start – and a Long Trek

In the late nineteenth century, the German army doctor Emil von Behring (1854–1979), later first Nobel Laureate for Medicine, pioneered the therapeutic application of antibodies. He used blood serum for the treatment of tetanus and diphtheria (“Blutserumtherapie”). When his data were published in 1890 (Behring and Kitasato 1890), very little was known about the factors or mechanisms involved in immune defense. Despite this, his smart conclusion was that a human body needs some defense mechanism to fight foreign toxic substances and that these substances should be present in the blood – and therefore can be prepared from serum and used for therapy against the toxins or infections. It worked, and the success allowed him to found the first “biotech” company devoted to antibody-based therapy in 1904 – using his Nobel Prize money as “venture capital.” The company is still active in the business today as part of ChironBehring.

In 1908, Paul Ehrlich, the father of hematology (Ehrlich, 1880) and the first consistent concept of immunology (“lateral chain theory,” Fig. 1.1d, Ehrlich, 1908), got the second Nobel Prize related to antibody therapeutics for his groundbreaking work on serum, “particularly to the valency determination of sera preparations.” Ehrlich laid the foundations of antibody generation by performing systematic research on immunization schedules and their efficiency, and he was the first to describe different immunoglobulin subclasses. He also coined the phrases “passive vaccination” and “active vaccination.” His lateral chain theory (“Seitenketten,” sometimes misleadingly translated to “side chain theory”) postulated chemical receptors produced by blood cells that fitted intruding toxins (antigens). Through these chemical receptors, cells combine with antigens and the receptors are eventually released as circulating antitoxins (antibodies). Without any knowledge of molecular structure or biochemical binding mechanisms, Paul Ehrlich anticipated much of today’s knowledge on immunoglobulin generation and antibody–antigen interaction, even class switching (Fig. 1.1d).

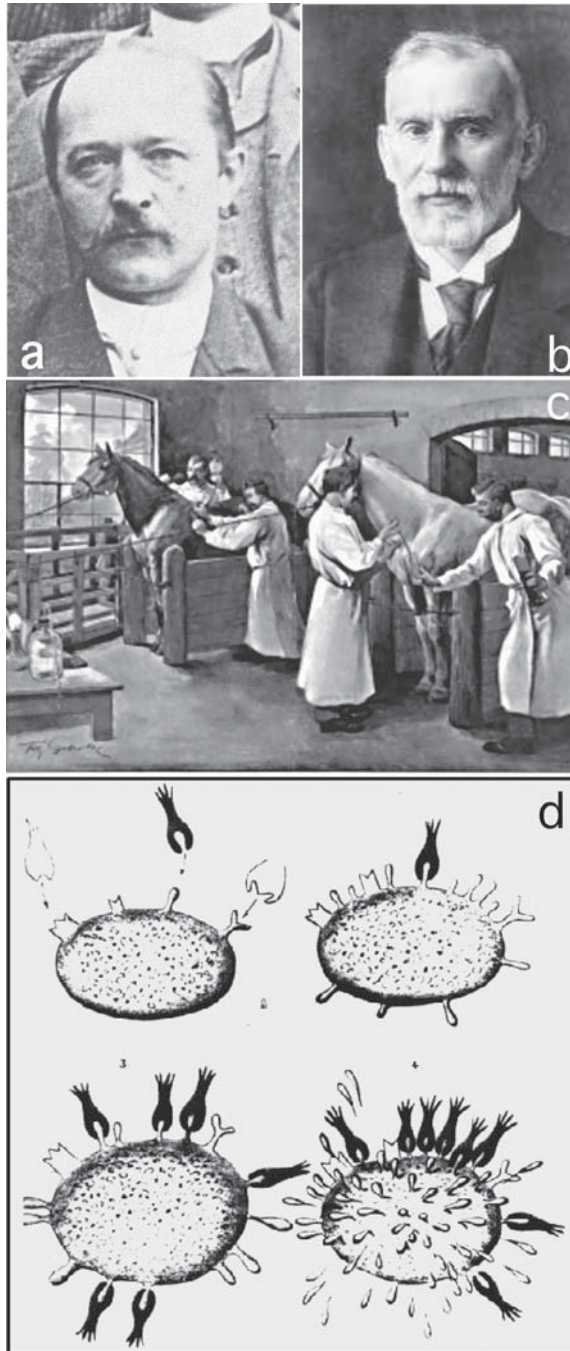


Fig. 1.1 We have come a long way since the first methods for the generation of antibody based therapeutics were established (c), pioneered by (a) Emil von Behring and (b) Paul Ehrlich in the last decade of the nineteenth century. (d) Drawing from Paul Ehrlich on the lateral chain theory (lateral chains = antibodies). He anticipated principles confirmed on a molecular basis

many decades later, like the binding of antigens by different specific antibodies (the “lock and key” principle), the differentiation and maturation of B cells and the class switch, allowing the initially cell-bound antibodies to be released in large amounts. Photos: Deutsches Historisches Museum, Berlin.

Passive and active vaccines were developed in rapid succession at the beginning of the twentieth century, and were successful in saving many lives. Snake and insect bites could be treated specifically, and beneficial effects were even observed with human serum immunoglobulin G (IgG) preparations without prior specific immunization (e.g. protection against hepatitis A).

The enormous success of all these blood products for the prevention and treatment of infections and intoxications, however, could not be expanded to other substantial disease areas, in particular cancer and autoimmunity. Here, the understanding of molecular processes in their etiology or at least the ability to identify molecules strongly correlated to their onset stimulated the desire to produce antibodies targeting these for therapeutic intervention. Unlike a snake bite, however, cancer and autoimmune diseases are chronic, and it was rapidly understood from animal models that antibodies have to be applied to the patient more than once. Immunologists knew very well at that time that repeated application of antigen during antiserum preparation is a good strategy to “booster” the immune response. When using animal serum antibodies to treat chronically ill humans, this must induce an immune response to the therapeutic agent. Further, a drug containing solely an IgG of defined specificity would be tremendously helpful to limit side effects and reach sufficient concentrations at the target site. These prerequisites could by no means be met by the well-established methods of serum antibody preparation.

Much has been learned about the antibody structure (Fig. 1.2) and its function since then (see Chapter 2, Vol I). Hopes were high when Cesar Milstein and Georg Köhler demonstrated that monoclonal antibodies could be produced in mouse cell culture (see Chapter 2, Vol I). However, the excitement of the late 1970s cooled rapidly when almost all first-generation monoclonal therapeutics failed during clinical evaluation. Only one of these products made it through to US Food and Drug Administration (FDA) approval in 1984 – Orthoclone (see Chapter 9, Vol III). However, this was a special case as the typical patient receiving this CD3 antibody was already immune suppressed by disease, a setup not commonly present in cancers or autoimmune diseases. Even more important, it was realized that simple binding to a target (inducing its neutralization) is in most cases not sufficient to make an antibody a good therapeutic agent to treat cancer or immune diseases. Effector functions, like complement activation or cellular responses triggered by Fc receptor binding, are obviously needed, but are not fully provided by the mouse monoclonals.

As a consequence, huge efforts were undertaken to exchange the antibody’s effector domains (constant regions) for human ones – thereby also removing the most immunogenic parts from the mouse IgG. By using the then available methods of molecular cloning and recombinant expression, most mouse antibodies were humanized prior to clinical testing. Various methods (chimerization and CDR-exchange-based humanization being the most widely used) were employed (see Chapter 6, Vol I). When tinkering with the antibody sequence, most candidate antibodies were also affinity matured to typical nanomolar and subnanomolar affinities (see Chapter 7, Vol I). The technology for humanization

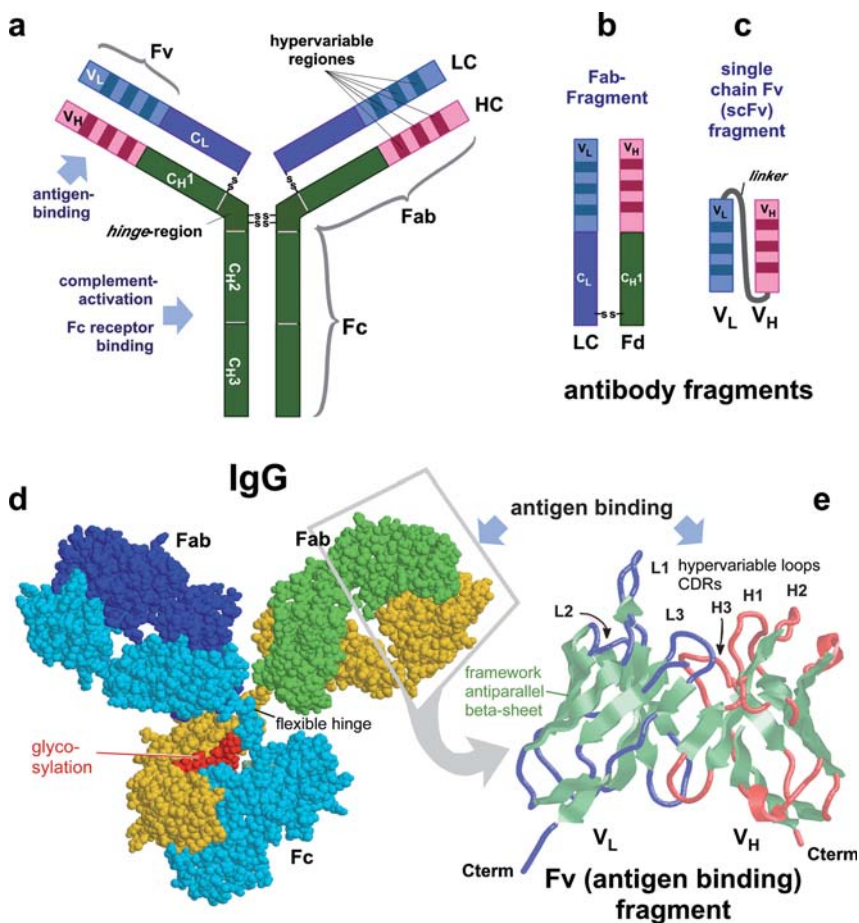


Fig. 1.2 Introduction to antibody structure. (a, d) IgG is a heterotetrameric protein assembled from two identical heavy and light chains (HC, LC), assembled by disulfide bonds. (b) Fab fragments contain the antigen-binding region, and can be generated by proteolysis or recombinant production. (c) In single-chain Fv fragments (scFv), the two antigen-contacting domains (variable regions of the heavy and light chains, V_H , V_L) are connected by an oligopeptide linker to form a single polypeptide. They can be produced in *E. coli* and are typically employed for the selection of human antibodies by phage display and other display systems. (d) Space fill model based on X-ray

crystallographic data of an antibody. The typical Y shape is only one of the many conformations the Fab fragments of an antibody can assume relative to each other. T-shaped structures can be assumed, and the hinge region to the Fc part can also bend significantly relative to the Fab fragments. (e) Alpha carbon backbone of an Fv fragment, the antigen-binding fragment of an antibody located at the two tips of the Y- or T-shaped complex, emphasizing the typical antiparallel beta sheet framework structure which holds together the hypervariable loops (L1–3, H1–3) composing the antigen-binding surface (CDRs).

and affinity maturation became available from the mid-1980s. Taking into account that drug development, testing and approval needs about 10 years to reach the market, a growing number of therapeutic antibodies were approved starting from the mid-1990s. These “first generation” recombinant antibodies – cloned from rodent hybridoma cells and improved by gene engineering – dominate the current list of approved antibody therapeutics.

Early in the 1990s, two novel enabling technologies were developed that revolutionized the generation of therapeutic antibodies, as for the first time they provided a robust and reliable method to prepare specific antibodies of human origin. Phage display (see Chapter 3, Vol I) and transgenic mice (see Chapter 4, Vol I) allowed the production of antibodies that are genetically 100% identical to human immunoglobulins. Recently, even transgenic rabbits producing human antibodies have been generated (Buelow et al. 2006). Many other approaches for the *in vitro* selection of antibodies, such as yeast display or ribosomal display, followed and are still in various stages of maturation. These are all based on the selection of antibodies from a large antibody gene repertoire in a heterologous expression system (Fig. 1.3).

The experience with other recombinant human protein drugs (e.g. insulin) raised great hopes that these human antibodies are completely stealthy in respect of an anti-drug immune reaction. However, it was soon realized that immunoge-

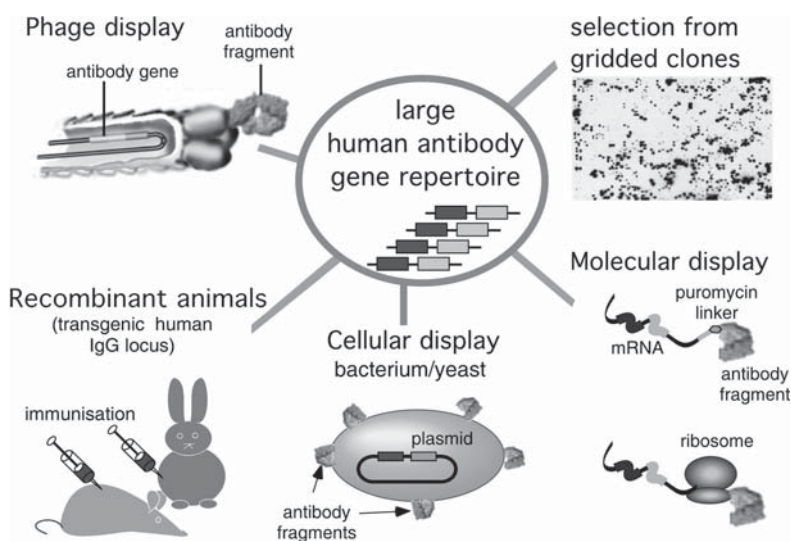


Fig. 1.3 Systems employed for the generation of human antibodies. They all include a heterologous expression of a repertoire of human immunoglobulin genes. Selection is achieved either *in vivo* by immunization (in case of the recombinant animals) or *in vitro* by binding to the antigen, allowing clonal selection of the gene host.

nicity was not switched off completely in most cases – Fc-glycosylation patterns resulting from recombinant production can be very different from endogenous human IgG, and a variable human antibody region with its “lottery”-derived CDRs (complementarity determining regions) generated outside of the context of the human immune system can be quite immunogenic. However, these problems were in most cases minor compared with the effects observed previously with animal-produced immunoglobulins. Today, the first antibody with a completely “human” sequence origin reached the pharmacy shelves: Humira (see Chapter 1, Vol III). It was genetically assembled entirely *in vitro*, with an antigen-binding region selected from an *E. coli*-hosted gene repertoire by phage display (see Chapter 3, Vol I). Many more antibodies derived from human gene repertoires, selected by phage display or generated in transgenic mice carrying the human immunoglobulin locus, have entered clinical testing. The *in vitro* technologies, like phage display, offer an additional advantage when antibody generation in animals is difficult, for example due to the high homology (resulting in low immunogenicity) of the human antigen used for immunization to a mouse protein, or in case of highly toxic or deadly pathogenic antigens. A few animal or even human serum-derived antibody products are still available (Rohrbach et al. 2003) but recombinant human or humanized products vastly dominate the current clinical studies.

Approval time was also getting increasingly short for antibodies when compared with a typical small molecule drug, and success rates of clinical studies improved, mainly due to the more predictable pharmacokinetics and lower risk of toxicity and other side effects when using molecules almost identical to the IgG in our veins – of course with the usual unavoidable exceptions, usually due to effects caused by the antigen binding itself (e.g. seen with TGN1412).

It is a surprising side note of history that in contrast to so many other biomedical achievements, almost all of the enabling antibody technologies (polyclonal sera, monoclonal antibodies, production of functional antibodies in *E. coli* and phage display, humanization, among others) were pioneered in Europe, mainly in the UK and Germany.

1.2

The Gold Rush

A gold rush for new therapeutic agents started when it was realized that all enabling technologies are in place to develop and produce monospecific, nearly human antibodies that are only mildly immunogenic but provide high-affinity target binding and human effector functions, long serum half-life and other pharmacologic advances. Many promising new concepts for the treatment of a huge variety of different diseases were envisaged. In fact, there are only few theoretical restrictions for antibody treatments. First comes the necessity to find a molecular target (antigen) accessible from the bloodstream (i.e. typically a target

at the cell surface that is located solely or in a higher concentration on the cell compartment to be effected). Second, the antibody in most cases needs to activate some immune reaction at the binding site (e.g. to kill a tumor cell). Exceptions are antibodies that act by neutralization of an infectious agent or an overexpressed factor, which can be achieved by simple sterical inhibition of the binding of the agent to its natural receptor. Affinity is no practical limitation anymore, as with existing methods antibodies usually can be engineered to provide affinities better than those needed for a maximal therapeutic effect (see Chapter 7, Vol I). Specificity is always an issue, of course, as no antibody is a priori unsusceptible to a cross-reaction, but many strategies have been developed to tackle this problem. Most simply, large numbers of different human antibody clones can now easily be evaluated in parallel using high-throughput assays (see Chapter 4, Vol II).

Some commentators are pessimistic about the shortage of manufacturing capacity for antibodies, and their high cost of production in comparison with a small molecule drug. They have calculated that health systems could not afford all of these new, expensive drugs even if they were made available. However, a scale-up of capacity and novel alternative production systems (e.g. microbial, eukaryotic or plant-derived) may allow much cheaper production of antibodies for many applications, and may even allow the “expensive” antibodies to enter new, low-margin therapeutic markets (see Chapter 6, Vol II). An example already paving the way in this direction is a plant-produced anti-carries antibody which is in phase II clinical testing today.

1.3

Success and Disappointment

All these exciting opportunities offered by the new technologies have resulted in an explosion in the number of clinical studies and approvals (Fig. 1.4). After the slow start in the 1980s and no substantial increase between 1985 and 2000 (Reichert 2001), the number of clinical studies has changed dramatically. Today, more than 400 studies are ongoing, targeting a broad range of diseases, from various cancers to infection and autoimmunity (Tables 1.1 and 1.2). Cancer therapeutics clearly dominate the field, but infection/immunity applications are catching up considering the low number of already approved antibodies, with about a third of the ongoing studies now. The nature of the antigens is just as diverse, with the approved antibodies targeting both cell surface markers (e.g. CD11a, CD20, CD25, CD33, CD52, EGFr, Her2) and soluble molecules (e.g. TNF- α , RSV, VEGF), with three different molecular formats: IgG, Fab fragments, and radioconjugates. A range of new targets and conjugates will follow. This is perfectly illustrated by the antibody variants made to block the tumor necrosis factor (TNF)/TNFR interaction to downregulate overshooting inflammations (Fig. 1.5). Starting with a clinically failed mouse hybridoma antibody, the next steps were chimeric and then humanized antibodies. Finally, a completely human antibody

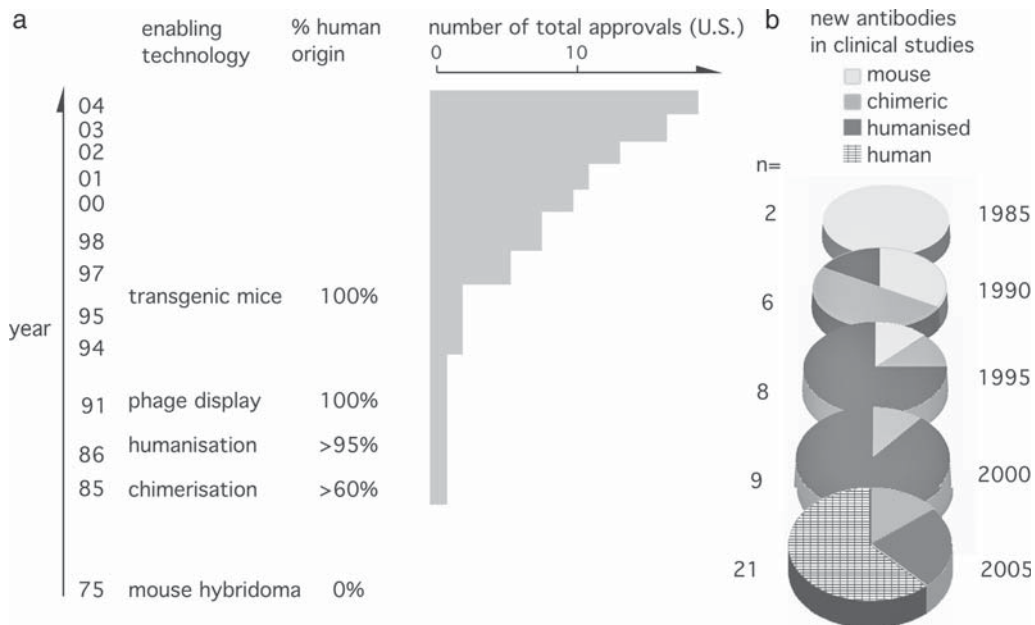


Fig. 1.4 Development history of therapeutic antibodies and the technologies for their generation. Data assembled from Reichert (2001), Reichert et al. (2005), Fabrizi (2005) and public domain sources of the companies.

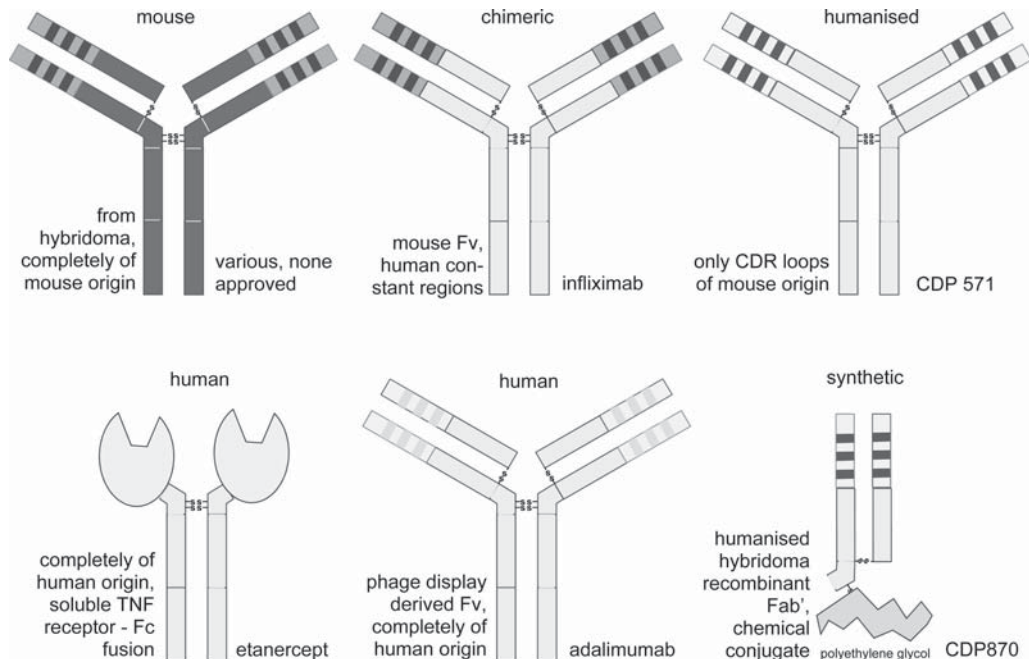


Fig. 1.5 The development of recombinant antibody variant TNF antagonists. Inspired by Rutgeerts et al. (2004).

Table 1.1 Clinical studies with therapeutic antibodies 2005.

<i>Disease</i>	<i>Clinical phase</i>			<i>Total</i>
	<i>I</i>	<i>II including I/II</i>	<i>III including II/III</i>	
Cancer				283
Systemic treatment	120	118	18	256
Ex-vivo purging				27
Infection and immunity	40	56	24	120
Total	160	174	42	403

Assembled from Bayes et al. (2005), Reichert et al. (2005), data presented on <http://www.clinicaltrials.gov>, <http://www.centerwatch.com/>, company homepages, other public domain sources and the Monoclonal Antibody Index.

and a human soluble receptor–Fc fusion were the next clinically tested and approved iterations of the idea. Now, a Fab fragment of human origin fused to PEG (polyethylene glycol) to improve its pharmacokinetics marks the sixth generation of TNF antagonist antibody constructs no longer resembling a molecule present in nature. Recombinant antibody technology may even reach its limits here, and a seventh-generation TNF antagonist could well be a novel synthetic binder of nonantibody structure (see Chapter 7, Vol II). A very good introduction to the clinical success story of recombinant antibodies is given by the review series by Reichert and colleagues (Reichert 2001, 2002; Reichert et al. 2005).

In February 2005, marketing of the therapeutic antibody natalizumab (marketed as Tysabri), a promising drug for the treatment of multiple sclerosis, was voluntarily suspended after only a few months of approval, based on reports of serious adverse events that have occurred in patients treated in combination with interferon beta-1a in clinical trials (Kleinschmidt-DeMasters and Tyler 2005). It seems, however, that this suspension will be released at time of print due to the reported positive effect of this drug. In December 2005, a voluntary market suspension due to serious and life-threatening cardiopulmonary events following the administration of NeutroSpec (technetium (^{99m}Tc) fanolesomab) was published. In March 2006, dramatic adverse effects not predicted from the animal studies were observed in clinical phase I for the anti-CD28 antibody TGN1412. These unfortunate stories of agents, some of which have even passed clinical studies for their approval, although far from final conclusion, as seen by the lift of the clinical hold for natalizumab after one year of evaluation, show that despite of all the theoretical advantages, there are still risks. These mainly originate from our still very incomplete understanding of the molecular and immunological processes, in particular in combination therapies. Here, great hopes are put on the

Table 1.2

<i>Drug name*</i>	<i>FDA name ** (original name)</i>	<i>Antigen</i>	<i>Ab origin ***</i>	<i>Tragetd diseases</i>	<i>Used for</i>
Avastin	bevacizumab (rhvMAb-VEGF)	VEGF	humanised	colon, breast, kidney, lung cancer, myeloma, mesothelioma	combin. therapy
Bexxar	tositumomab (B1)	CD20 (B cells)	murine	lymphoma (NHL)	therapy
CEA-Scan	arcitumomab (IMMU-4)	CEA	Fab' fragment, 111In conjug.	colorectal, lung, breast cancer	imaging
Cotara	– (TNT-1)	histone H1, DNA	chimeric	lung, uterine cancer, glioma, sarcoma	therapy (China)
Erbitux	cetuximab (C225)	EGFR	chimeric	breast, head and neck, pancreatic, colorectal, lung cancer, melanoma	therapy
Herceptin	trastuzumab (rhvMAb-4D5-8)	pl85/HER-2	humanised	breast, lung, ovarian, pancreatic cancer	therapy
HumaSpect-Tc	votumumab (88BV59H21-2V67-66)	CTAA 16.88	human BV-transformed cells 99mTc conjug.	Colorectal, breast, prostatic, lung, ovarian, pancreatic cancer	imaging
Humira	adalimumab (D2E7)	TNF-alpha	phage display, human library	rheumatoid arthritis, Crohn's disease	therapy
Indimacis-125	igovomab (OC125)	CA125	F(ab')2 fragment	Gastrointestinal, ovarian cancer	imaging
LeukoScan	sulesomab (MN-3)	NCA-90 (granulocytes)	Fab' fragment, 99Tcm conjug.	inflammation, infection, Crohn's disesase, Meckel's diverticulum, IBD	imaging
Leukosite	alentuzumab (CAMPATH-1H)	CD52	humanised from rat	leukemia (CLL), lymphoma	therapy
Mylotarg	gemtuzumab ozogamicin (hP67.6)	CD33	humanised, conjugated to calicheamicin (cytostatic)	leukemia (AML)	therapy
Myoscint	imciromab pentetate R11D10	myosin heavy chain	murine Fab, 111In conjug.	myocardial infarction	imaging
Neutrospec	fanolesomab (RB5)	CD15 granulocytes	murine IgM, 99Tcm conjug.	Inflammation, infection	imaging
OncoScint	satumomab (B72.3n)	TAG-72 (CA72-4) / sialyl Tn	murine, 111In conjugate	breast, pancreatic, colorectal, ovarian, lung, endometrial cancer	imaging
Oncorad				mesothelioma	

Orthoclone			CD3 (T cells)	murine	transplantation, rheumatoid arthritis	therapy
Panorex	muromonab (OKT3) edrecolomab (17-1A)		Ep-CAM	humanised	colon cancer	therapy (Germany) imaging
ProstaScint	capromab (7E11-C5.3)		PSA, PSMA	Murine, glycyI-tyrosyl-I-lysyl-DTPA konjugated	prostatic cancer	
Raptiva	efalizumab (hu-1124)		CD2a	humanised	psoriasis, rheumatoid arthritis transplantation, autoimmunity	therapy
Remicade	infliximab (cA2)		TNF-alpha	chimeric	Crohn's disease, psoriasis Rheumatoid arthritis, asthma, Ankylosing spondylitis Wegener's granulomatosis,	therapy
ReoPro	abciximab (7E3)		gp IIb/IIIa Receptor complex (platelets)	chimeric Fab	Restenosis, heart ischemic complication	therapy
Rituxan	rituximab		CD20 (B cells)	chimeric	Non-hodgkin Lymphoma Rheumatoid arthritis,	therapy
Simulect	basiliximab (CHB-201)		CD25	chimeric	transplantation	therapy
Synagis	palivizumab (1129)		F protein of RS virus	humanised	RSV infection in infants	therapy
Tysabri	natalizumab (HP2/1)		Integrin $\alpha 4\text{-}\beta$ (T cells)	humanised	multiple sclerosis	therapy
Verluma	nofetumomab merpentan NR-LU-10		ca. 40kDa glycoprotein	murine Fab, 99mTc conjug.	lung, colon, pancreatic, breast, ovarian, renal, gastric cancer	imaging
Xolair	omalizumab (rhuMAB-E25)		human IgE Fc	humanised	asthma, autoimmunity, peanut allergy	therapy
Zenapax	daclizumab (anti-Tac-H)		CD25	humanised	transplantation, asthma, autoimmunity, inflammation, multiple sclerosis	therapy
Zevalin	imbricitumomab (C2B8)		CD20	chimeric	lymphoma (NHL), autoimmunity, transplantation, rheumatoid arthritis	therapy

Table 2 Approved Antibody Therapeutics. Please note that a listing does not indicate that the antibodies are still available, approved or in use at date of print! Quite a number of products, including most of the radiolabelled mouse antibodies, have been withdrawn from the market or suspended. Some were approved by other bodies than the U.S. FDA (e.g. Panorex or Cotara).

* Trade names are Copyright of distributing companies.

** please see addendum for nomenclature.

*** chimeric / humanised indicates murine origin of V regions /CDRs, respectively, if not stated otherwise.

intense research going on worldwide into “-omics” and systems biology, which is intended to lead to a mathematical interaction model for all involved factors. Only then, and with quite a number of years to go, will there be a chance to better predict adverse effects of novel drugs and combination therapies on a truly rational basis.

1.4

The Gleaming Horizon

Despite all of the recent success stories of recombinant human-like IgGs, they do not mark the end of the development, but just the start. As we understand more and more of the complex molecular interactions between immune cells or in cancer tissues, and in expectation of a significant speed-up of knowledge gain from the “-omics” and systems biology approaches, we can endeavor to expand the design limits of an antibody drug. All approved drugs are based on IgG molecules close to the naive structure of the antibodies in our bloodstream (a few on the IgG–fragment Fab), sometimes conjugated to an effector or label. But this can only be the first step of engineering applied to this fascinating molecule. For example, by engineering the Fc glycosylation, dramatic improvements in efficiency can be obtained (Jefferis 2005).

Further, we should learn from nature by looking at the modular design it has used to create the highest diversity group of proteins from repeats of slightly changed domains with a single common basic structure (immunoglobulin fold). We can be inspired to utilize this modular approach for completely novel molecular designs. This has already successfully been attempted since the early 1990s, and led to a plethora of novel molecular designs. It allowed the creation of bispecific antibodies (see Chapter 2, Vol II), the adjustment of the size for optimal pharmacokinetics (Hu et al. 1996), and the addition of functions that nature does not provide with an IgG at all (see Chapters 1 to 3, Vol II). Unfortunately, clinical results with these new designs are sparse and frequently disappointing, although this may simply reflect the fact that the molecular design of today is still rather a result of trial and error than of an understanding of the underlying mechanisms, or is even dictated mainly by the developer’s patent portfolio. Nevertheless, this will change as well; an example is immunotoxins, which failed in many clinical studies for more than three decades, before novel concepts and acknowledgement of the vast body of knowledge collected throughout this time brought optimization and new ideas (see Chapter 3, Vol II). Fascinating concepts are under evaluation in hundreds of labs. There are so many ideas and so many parameters affecting therapeutic efficiency to be learned about that the development of antibody therapeutics will not reach saturation for any foreseeable time. Furthermore, major technology patents which have blocked some developments in the past will expire in the foreseeable future (see Chapter 13). Given that only the first generation of antibody drugs just has been approved in significant numbers, given the availability of recombinant human antibodies and the number

of targets in the developer's pipelines plus the advent of completely novel therapeutic strategies using antibody fusion proteins, therapeutic antibodies look forward to a golden future.

Further Reading

- Benny, K.C. (ed.) (2003) *Antibody Engineering: Methods and Protocols (Methods in Molecular Biology)*. Totowa, NJ: Humana Press (collection of relevant protocols).
- Breitling, F., Dübel, S. (1999) *Recombinant Antibodies*. New York: John Wiley and Sons (introduction to basic principles and applications).
- Brekke, O.H., Sandlie, I. (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Discov* 2: 52–62.
- Carter, P.J. (2006) Potent antibody therapeutics by design. *Nat Rev Immunol* 6: 343–357.
- Chowdhury, P.S., Wu, H. (2005) Tailor-made antibody therapeutics. *Methods* 36: 11–24.
- Gavilondo, J.V., Larrick, J.W. (2000) Antibody engineering at the millennium. *Biotechniques* 29: 128–132.
- Groner, B., Hartmann, C., Wels, W. (2004) Therapeutic antibodies. *Curr Mol Med* 4: 539–547.
- Haurum, J., Bregenholt, S. (2005) Recombinant polyclonal antibodies: therapeutic antibody technologies come full circle. *IDrugs* 8: 404–409.
- Hudson, P., Souriau, C. (2003) Engineered antibodies. *Nature Med* 9: 129–134.
- Huston, J.S., George, A.J. (2001) Engineered antibodies take center stage. *Hum Antibodies* 10: 127–142.
- Kontermann, R., Dübel, S. (ed.) (2001) *Antibody Engineering*. Heidelberg, New York: Springer (collection of relevant protocols).
- Monoclonal Antibodies and Therapies*. Supplement of *Nat Biotechnol*, November 2004 (collection of reviews).
- Monoclonal Antibody Index (MAI)*, Gallart Biotech SL, 2005 (collection of data on therapeutic antibodies, updated regularly).
- Presta, L.G. (2002) Engineering antibodies for therapy. *Curr Pharm Biotechnol* 3: 237–256.
- Subramanian, G. (ed.) (2004) *Antibodies: Volume 2: Novel Technologies and Therapeutic Use*. Heidelberg, New York: Springer.
- Urch, C.E., George, A.J.T. (eds) (2000) *Diagnostic and Therapeutic Antibodies (Methods in Molecular Medicine)*. Totowa, NJ: Humana Press.

References

- Bayes, M., Rabasseda, X., Prous, J.R. (2005) Gateways to clinical trials. *Methods Find Exp Clin Pharmacol* 27: 569–612.
- Behring, E., Kitasato, S. (1890) Über das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren. *Dt Med Wschr* 16: 1113–1114.
- Buelow, R., Puels, J., Hansen-Wester, I., Thorey, I., Schueler, N., Siewe, B., Niersbach, H., van Schooten, W., Platzer, J. (2006) Expression of a human antibody repertoire in transgenic rabbits. *Human Antibodies* 15: 19–23.
- Ehrlich, P. (1880) Methodologische Beiträge zur Physiologie und Pathologie der verschiedenen Formen der Leukocyten. *Z Klin Med* 1: 553–560.
- Ehrlich, P. (1908) Partial cell functions. Nobel Lecture, December 11: 1908.
- Hu S.Z., Shively, L.E., Raubitschek, A., Sherman, M., Williams, L.E., Wong, J.Y.C., Shively J.E., Wu A.M. (1996) Minibody: a novel engineered anti-carcinoembryonic

- antigen antibody fragment (single-chain Fv-C_H3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res* 56: 3055–3061.
- Jefferis, R. (2005) Glycosylation of recombinant antibody therapeutics. *Biotechnol Prog* 21: 11–16.
- Kleinschmidt-DeMasters, B.K., Tyler, K.L. (2005) Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med* 353: 369–374.
- Rutgeerts, P.J., Targan, S.R., Hanauer, S.B., Sandborn, W.J. (2004) Challenges in Crohn's disease: the role for current and future TNF antagonists. www.medscape.com/viewprogram/3261_pnt
- Reichert, J.M. (2001) Monoclonal antibodies in the clinic. *Nat Biotechnol* 19: 819–822.
- Reichert, J.M. (2002) Therapeutic monoclonal antibodies: trends in development and approval in the US. *Curr Opin Mol Ther* 4: 110–118.
- Reichert, J.M., Rosenzweig, C.J., Faben, L. B., Dewitz, M.C. (2005) Monoclonal antibody successes in the clinic. *Nat Biotechnol* 23: 1073–1078.
- Rohrbach, P., Broders, O., Toleikis, L., Dübel, S. (2003) Therapeutic antibodies and antibody fusion proteins. *Biotechnol/Genet Eng Rev* 20: 129–155.

Part I

Selecting and Shaping the Antibody Molecule

2

Selection Strategies I: Monoclonal Antibodies

Gerhard Moldenhauer

2.1

Introduction

Since the late nineteenth century and the days of Paul Ehrlich, who considered antibodies as “magic bullets” (Ehrlich, 1900), immunologists have been attracted by the idea of destroying tumor cells with antibody molecules alone or conjugates made up of such molecules. Emil von Behring and Shibasaburo Kitasato were the first to demonstrate the efficacy of a heterologous polyclonal antiserum directed against the exotoxin produced by *Corynebacterium diphtheria* (“Diphtherie-Heilserum”), thereby saving the lives of many children (von Behring and Kitasato 1890). Although similar attempts were subsequently made to employ antisera for tumor treatment, the outcomes were less successful. With the advent of monoclonal antibody (mAb) technology 30 years ago, there was renewed enthusiasm for the development of a modern immunotherapy for cancer. This promise was fulfilled, however, only in some rare cases of non-Hodgkin’s lymphomas (Levy and Miller 1990). The application of mouse mAbs for therapy has raised several problems. One major drawback was, for instance, that mouse antibodies are not usually able to activate human immunological effector functions as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). The formation of a human anti-mouse antibody (HAMA) response after repeated injection constitutes another reason for the low response rates observed (Khazaeli et al. 1994; DeNardo et al. 2003).

A breakthrough was achieved in 1997 with the approval of the chimeric (mouse/human) rituximab antibody for the treatment of relapsed/refractory low-grade non-Hodgkin’s lymphomas by the US Food and Drug Administration (FDA) (McLaughlin et al. 1998). During the past decade molecular biology has provided the means to create chimeric, humanized or fully human antibodies for therapy. To date, eight antibody-based cancer therapeutics have been approved and are on the market. They comprise unmodified antibodies as well as conjugates with toxins or radionuclides. Thus, antibody engineering finally has led to a renaissance of antibody-guided tumor therapy. The new reagents can interact with

human effector molecules and have been shown to synergize with or even substitute for conventional chemotherapeutic regimens (reviewed by Glennie and van de Winkel 2003; Brekke and Sandlie 2003; Ross et al. 2004; Stern and Herrmann 2005; Adams and Weiner 2005).

In this chapter I describe the principles of mAb creation by somatic cell hybridization, give an overview on frequently used screening procedures and modifications of the antibody molecule and finally outline the mechanisms by which antibodies exert their effector functions. For the sake of clarity, I have restricted my viewpoint mainly to the field of cancer research and oncology/hematology.

2.2

Historical Remarks

The immune system is capable of generating about 10^{11} to 10^{12} different antibody molecules. An individual B lymphocyte, however, synthesizes only the one distinct antibody it is genetically programmed for. How this enormous antibody repertoire is generated was a central question of immunology over the past 50 years. Starting from the natural clonal selection theory of antibody formation (Jerne 1955) it became obvious that two mechanisms affecting the immunoglobulin genes are of paramount importance: rearrangement of gene segments and somatic hypermutation. To study somatic mutations of antibody genes in more detail, an antibody-secreting cell line recognizing the same antigen was urgently needed. This was the aim of experiments performed by Georges Köhler and César Milstein, leading to the discovery of hybridoma technology for the production of mAbs (Köhler and Milstein 1975). One of the major methodological advances in biology and medicine, this work was honored by a Nobel Price in 1984, was achieved by answering an academic question (Milstein 1999). Recently, the immunologist Klaus Eichmann has published an interesting book on the scientific and historical background leading to the invention of hybridoma technology (Eichmann 2005).

Hybridoma production basically relies on the fusion of immunized lymphocytes from an experimental animal with immortal myeloma cells. The resulting cell hybrid contains the genetic material of both parents. From the tumor cell the hybrid acquires the capacity for indefinite growth while the B lymphocyte confers the ability to synthesize a specific antibody. After stabilization by repeated cell cloning, hybridomas produce fairly large quantities of identical mAb for years (Fig. 2.1). The early development of the method is documented in a couple of scientific anthologies (Melchers et al. 1978; Kennett et al. 1980; Hämmerling et al. 1981). Importantly, the first patient suffering from non-Hodgkin's lymphoma was treated in 1979 with a mouse mAb at the Dana Farber Cancer Center in Boston (Nadler et al. 1980). Taken together, mAbs have led to a revolution in basic sciences, medicine and industry during the past 30 years.

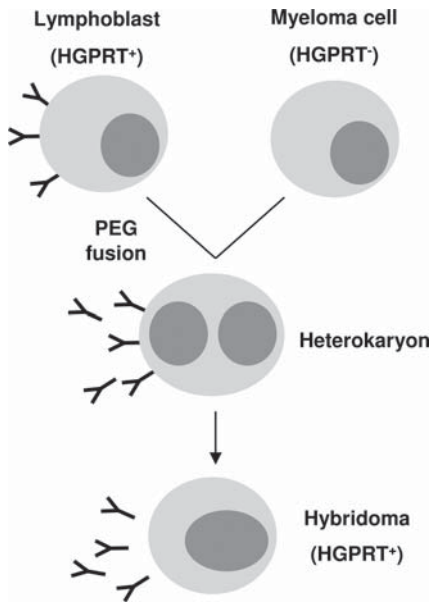


Fig. 2.1 Principle of hybridoma production by cell fusion. Both parents confer their key functions to the hybrid: production of an individual antibody (lymphoblast) and indefinite growth (myeloma cell). Introduction of a selectable marker (HGPRT) allows only hybridoma cells to proliferate.

2.3

Antibody Structure and Function

2.3.1

Membrane-bound and Secreted Forms of Antibodies

The basic structure of an IgG antibody has been elucidated as a symmetric monomer consisting of two identical heavy chains and two identical light chains, connected via disulfide bonds (Cohen and Porter 1964; Edelman et al. 1969). Five classes of immunoglobulins can be distinguished according to their distinct heavy chains: IgG, IgM, IgD, IgA, and IgE. Furthermore, each antibody contains one type of light chain, kappa or lambda. Both, heavy and light chains harbor a variable region of 110 amino acids at the N-terminus with three hypervariable segments called complementarity determining regions (CDRs). The hypervariable loops form the two antigen binding (or antigen-combining) sites of an IgG molecule and determine its specificity. In contrast, the constant part of the immunoglobulin (named the Fc portion) is responsible for secondary effects like activation of the complement system or binding to cellular Fc receptors (Fig. 2.2).

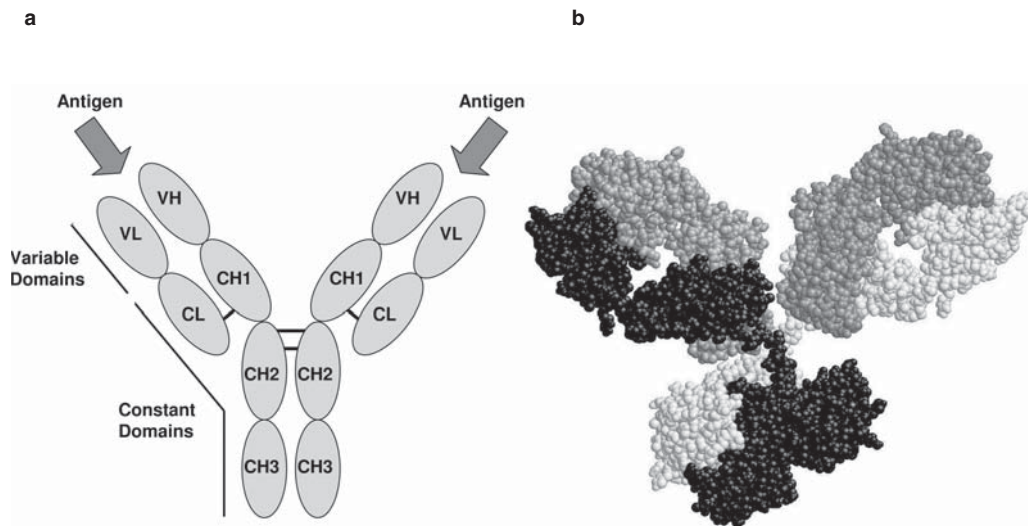


Fig. 2.2 Structure of an IgG molecule. The Y-shaped simplified representation in (a) shows the functional domains as well as the two antigen-binding sites. A more realistic space-fill model is depicted in (b) (courtesy of Dr C.W. von der Lieth, DKFZ Heidelberg).

Importantly, immunoglobulins display a dual function since they are exposed on the surface membrane of B lymphocyte as antigen receptors and are also secreted by plasma cells. They circulate in the blood and other body fluids and are able to bind, neutralize and eliminate foreign antigens, such as viruses, bacteria or toxins. Antibodies represent the effector molecules of the humoral immune system. Transmembrane and secreted forms of an antibody are generated by differential splicing of a primary transcript RNA. In the course of an ongoing immune response specific B lymphocytes undergo isotype switching, that is the transition of the IgM^+ and IgD^+ phenotype to IgG surface and secreted immunoglobulin. In addition, point mutations of the rearranged heavy and light chain variable genes occur in response to T lymphocyte signaling, giving rise to somatic hypermutation necessary to increase antibody affinity. These processes take place in the germinal centers of secondary lymphoid tissues such as lymph node and spleen. The enzyme activation-induced cytidine deaminase (AID) has been identified as a key player for both class switching and affinity maturation (Barreto et al. 2005). Detailed information on how an effective antibody response to antigen develops at a molecular level can be looked up in one of several excellent textbooks of immunobiology (Roitt et al. 2001; Abbas and Lichtman 2003; Janeway et al. 2005).

Immunoglobulin G (IgG, 150kDa) is the most abundant immunoglobulin in serum, accounting for up to 80% of all secreted antibodies. There are four dif-

ferent IgG isotypes in humans (IgG1, IgG2, IgG3, and IgG4) and mouse (IgG1, IgG2a, IgG2b, and IgG3). Human IgG1 and IgG3 antibodies are potent activators of the complement system and also bind with high affinity to Fc receptors on phagocytic cells, resulting in ADCC. Immunoglobulin M (IgM, 900kDa) accounts for approximately 10% of serum antibodies. It is expressed as monomer on B lymphocyte as antigen receptor, whereas the secreted form consists of a pentamer hold together by a J (joining) chain. Immunoglobulin D (IgD, 180kDa) is only found in fairly low amounts in the serum but is, together with IgM, the major membrane-bound form expressed on mature B cells. Immunoglobulin A (IgA, 160kDa) constitutes about 10–15% of serum antibodies. It represents the major antibody class, being secreted into tears, saliva, and mucus of the bronchial, genitourinary, and digestive tracts. While the prevalent form in the serum is a monomer, the secretory IgA usually consists of dimers covalently connected via J (joining) chains together with an additional polypeptide called secretory component. Immunoglobulin E (IgE, 180kDa) is found in the serum only in trace amounts. IgE antibodies are responsible for immediate hypersensitivity and cause the symptoms of hay fever, asthma and anaphylactic shock. Mast cells and basophils bind IgE via Fc receptors and subsequent contact with an allergen will cause degranulation and release of histamine and other mediators.

2.3.2

Monoclonal Antibodies

The key feature of an mAb is its unique specificity. It recognizes only one particular antigenic determinant (called epitope) on a given molecule – that means it is monospecific. All antibodies secreted by an individual hybridoma represent identical immunoglobulin molecules that display identical binding strengths to its antigen (referred to as affinity) and have identical physicochemical properties (isotype, stability). This homogeneity will give rise to the same immunological effector functions. In principle, mAbs can be produced in unlimited quantities, the hybridoma cell itself survives after cryopreservation at least for decades.

When compared with polyclonal antisera (for instance from rabbit) the affinity of mAbs might sometimes be inferior. Because mAbs consist of homogeneous molecules of the same isotype they may not elicit certain biological responses. The majority of monoclonals are directed against conformational epitopes of an antigen and may lose reactivity when tested on denatured samples, for instances by Western blotting or by immunohistology on paraffin sections. In common with polyclonal reagents, mAbs may show unexpected cross-reactivity with antigens being expressed in unrelated tissues.

Being glycoproteins, antibodies are potent immunogens when injected into another species. There are three different types of antigenic determinants against which an immune response can be induced: anti-isotype, anti-allotype, and anti-idiotypic. If a human being is injected several times with a mouse mAb, it is very likely a HAMA response will develop (Khazaeli et al. 1994; DeNardo et al. 2003).

2.4

Production of Monoclonal Antibodies

2.4.1

Immunization

The aim of an immunization is to elicit a strong immune response against a certain antigen.

For mAb production, most commonly mice and rats and less frequently hamsters and rabbits are immunized with antigen by distinct routes of administration. Antigen may consist of cellular components, purified proteins, peptides, carbohydrates, lipids, or nucleic acids and for each of these specific immunization protocols are available (Harlow and Lane 1988; Goding 1996; Coligan et al. 2004). The purity of the antigen used for immunization plays a major role in the outcome of antibody response. If rather impure preparations are used, problems may arise from the possible immunodominance of contaminants. This might occur when complete cells are employed as immunogen; on the other hand intact cells are highly immunogenic. Molecular biology allows the expression of fusion proteins in eukaryotic cells, a method that has largely improved the preparation of immunizing agents.

Especially for mounting an immune response against soluble antigens, the use of a strong adjuvant is highly recommended. Adjuvants are nonspecific stimulators of the immune system, the most famous representative being Freund's complete adjuvant (Freund 1956). This consists of mineral oil and inactivated *Mycobacterium tuberculosis* particles. When mixed with immunogen, a water-in-oil emulsion is prepared that allows the release of antigen over a long period of time. The mycobacteria give rise to an inflammatory response with the production of numerous cytokines. In general, for the first immunization it is appropriate to use a strong adjuvant (complete Freund's or *Bordetella pertussis*), whereas the second injection and following challenges can be given with incomplete Freund's (mineral oil only) or without any adjuvant. By repeated immunization the response of the animal is shifted against high-affinity antibodies of IgG isotype. Since previously activated lymphoblasts show preferential fusion with myeloma cells, the final booster immunization should be given 3 days prior to fusion to maximize the yield of hybrids.

If the amount of chosen protein antigen is short or if the antigen is not available at all, intrasplenic immunization with minute amounts of antigen (Spitz et al. 1984; Grohmann et al. 1991) or DNA immunization (Barry et al. 1994) might offer alternative approaches. During the past years a couple of useful antibodies were produced by immunizing with synthetic peptides coupled to immunogenic carriers like keyhole limpet hemacyanin (KLH) or bovine serum albumin (BSA). For this, only the amino acid sequence of the protein has to be known, which can be found in several databases. Coupling of peptides carrying an N- or C-terminal cysteine residue to the carrier is achieved by *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester or another heterobifunctional crosslinker (Green et al. 1982). One

drawback of the method is that such anti-peptide antibodies often exclusively react with the denatured but not with the native protein. *In vitro* immunization, invented to prime naive lymphocytes in cell culture, did not fulfill the expectations because in most instances solely an IgM response was induced (Borrebaeck 1983).

2.4.2

Myeloma Cell Lines

Multiple myeloma or plasmacytoma represents a malignancy of plasma cells in which large numbers of antibody-secreting cells residing in bone marrow are produced. They secrete monoclonal immunoglobulin, the specificity of which is usually not known, therefore they are regarded as “antibody without antigen.” All available mouse myeloma lines for fusion are derived from the MOPC-21 tumor that has been induced in BALB/c mice by mineral oil injection into the peritoneal cavity (mineral oil-induced plasmacytoma) and was then adapted to growth in tissue culture. While the very first hybridomas were made with myeloma fusion partners that endogenously secreted complete antibody, later on loss variants were selected producing solely kappa light chains (e.g. P3-NS1-Ag4-1; Köhler et al. 1976) or no immunoglobulin. Such nonproducer lines are mostly used for cell fusion these days, prominent examples being X63-Ag8.653 (Kearney et al. 1979), Sp2/0-Ag-14 (Köhler and Milstein 1976) and F0 (Fazekas de St. Groth and Scheidegger 1980).

Similarly, for the production of mAbs against mouse antigens some rat myeloma lines have been established from the LOU/C strain. Frequently used are Y3-Ag1.2.3 (secreting kappa light chains; Galfrè et al. 1979) and line IR983F (non-producer; Bazin et al. 1990). Since rats are not that much easier to handle than mice and since rat hybridomas are sometimes dependent on growth factors, making cell culture more complicated, interspecies hybrids have been constructed. For this, immune rat spleen cells were fused with a murine nonsecretor myeloma cell line. These rat/mouse hybrids turned out to be stable and secreted amounts of mAbs comparable to mouse/mouse hybridomas (Ledbetter and Herzenberg 1979). Mouse interspecies hybridomas have also been created with hamster and rabbit lymphoblasts to obtain respective mAbs (Sanchez-Madrid et al. 1983; Raybould and Takahashi 1988). A more advanced method for the production of mouse antibodies against mouse antigens is the use of knockout mice for immunization. Since they lack expression of the target antigen they are not tolerant and are able to mount a normal immune response.

2.4.3

Cell Fusion

In early experiments cell fusion was facilitated by means of agglutinating viruses like Sendai. The introduction of polyethylene glycol (PEG) as a fusing agent (Pontecorvo 1975) has simplified the procedure drastically and is used throughout

the field today. PEG renders the membrane of cells to be fused gluey, so that they stick together. Subsequently plasma membrane fusion occurs, giving rise to a cell with two (or more) nuclei called a heterokaryon. During cell division the nuclear membranes are degraded and the chromosomes are distributed into the daughter cell. These hybrid cells contain only one nucleus but the genetic material of both parents and are named synkaryons. The double set of chromosomes in hybrids causes genetic instability during further mitoses, leading to improper segregation or loss of chromosomes. If chromosomes coding for immunoglobulin heavy or light chain genes (in the mouse chromosomes 6, 12, and 16) are affected, antibody secretion of this hybrid will ultimately stop. To prevent overgrowth of early hybridoma culture by nonproducing variants, immediate cloning of cultures is mandatory. Following many cell divisions the hybrid line is stabilized in its chromosomal inventory. However, prior to mass production of hybridoma cells and in case the antibody titer of culture supernatant declines recloning should be performed (Fig. 2.3).

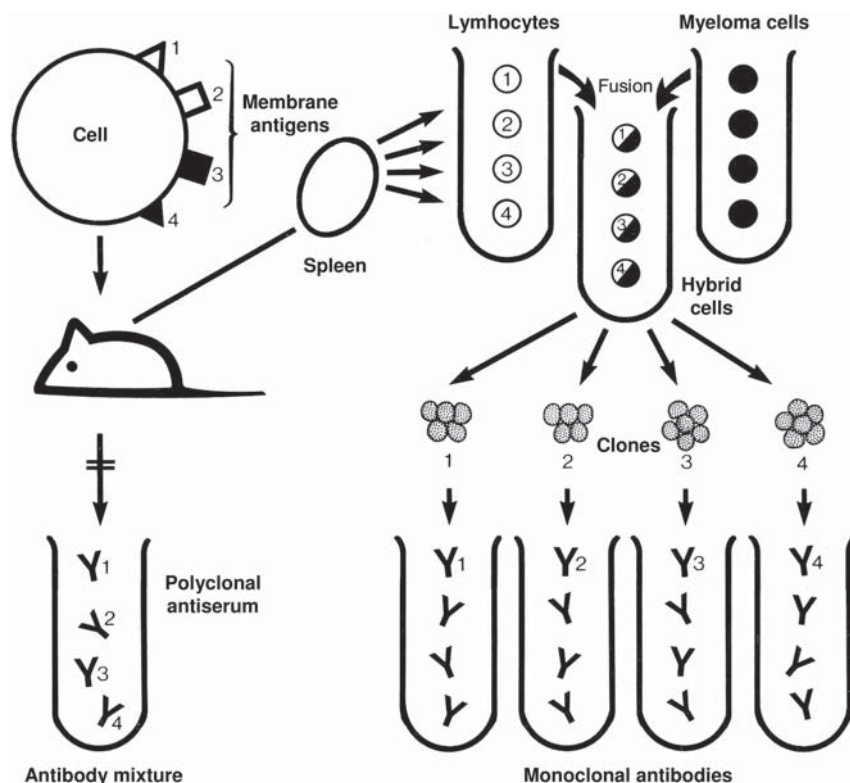


Fig. 2.3 Generation of monoclonal antibodies. Spleen cells from an immunized mouse are fused with myeloma cells to obtain hybridomas. After reduction to individual cell clones they secrete monoclonal antibodies of defined specificity.

Factors critically influencing the outcome of a fusion experiment are the choice of fetal calf serum (FCS) and the health status of the immunized mice. Depending on the content of the growth-promoting constituents, some FCS batches evolve better suited for hybridoma growth than others and it is worth testing for an optimal one. Bacterial and viral infections of rodents can cause severe immune suppression and will lead to low yields of hybridomas. Some protocols recommend the use of feeder cells as a source of growth factors as well as lysis of erythrocytes present in spleen cell preparations from immunized animal prior to fusion. In addition, repeated medium changes to remove potentially harmful substances derived from dying cells have been suggested. In my experience a simplified method works best, leaving the fused cells as untouched as possible. If a spleen from an immunized mouse (containing approximately $1\text{--}1.2 \times 10^8$ lymphocytes) is fused with an equal number of myeloma cells and the fusion mixture is distributed into 15–20 96-well microtiter plates (1440–1920 individual cultures), cell density is high enough to support hybridoma growth even without feeder cells and medium exchange (Fig. 2.4).

As an alternative to the PEG procedure described, electrically induced cell fusion has been developed. It is based on the delivery of high-voltage electrical field pulses to physically fuse lymphoblasts and myeloma cells (Ohnishi et al. 1987; Schmitt and Zimmermann 1989). More recently, a method was published

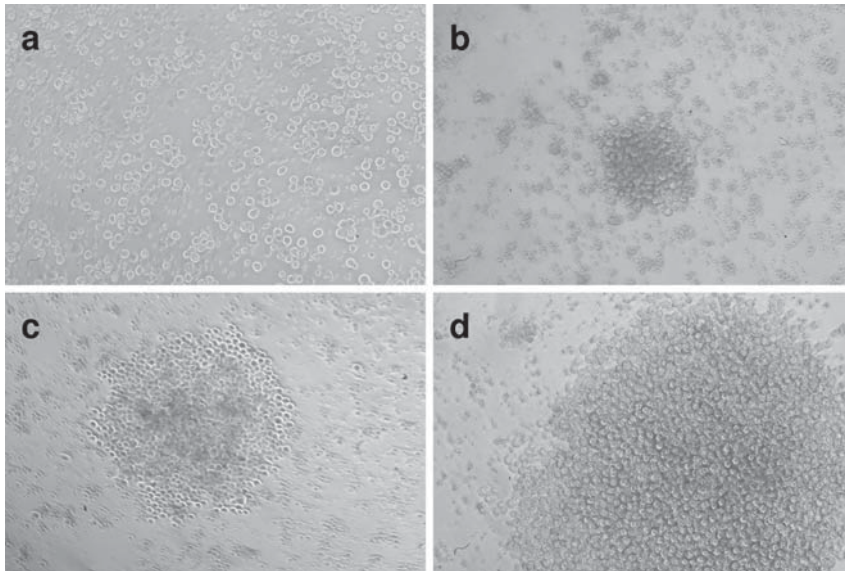


Fig. 2.4 Time course of hybridoma development visualized by phase-contrast light microscopy. Fusion mixture is plated on day 0 (a) and early hybridomas became visible after 4–6 days (b). At day 6–10 hybridomas grow vigorously (c) and are ready for screening at day 10–14 (d).

allowing the production of mAbs without hybridomas by using transgenic mice harboring a mutant temperature-sensitive simian virus-40 large tumor antigen (Pasqualini and Arap 2004).

2.4.4

Drug Selection of Hybridomas

During somatic cell hybridization only a small number of cells will actually fuse and only a minor proportion of them will develop into hybridomas (in the range of 1 in 10^5). Consequently, the culture is rapidly overgrown by myeloma cells or myeloma–myeloma fusions that also occur. This makes the introduction of a drug selection system indispensable. To accomplish this, enzyme-deficient myeloma lines are employed for cell fusion. In the system described by Littlefield (1964) the myeloma lacks the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). If the main biosynthetic pathway for purine and pyrimidine nucleotides required for both DNA and RNA synthesis is blocked by folic acid antagonists like aminopterin, cells can survive using an alternative “salvage pathway” that requires the enzymes HGPRT and thymidine kinase (Fig. 2.5). If the fusion mixture is cultured in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) only hybrids can actively grow. Normal spleen cells from the immunized mouse will die spontaneously *in vitro*. HGPRT-deficient myeloma cells undergo cell death in the presence of aminopterin since they cannot use the

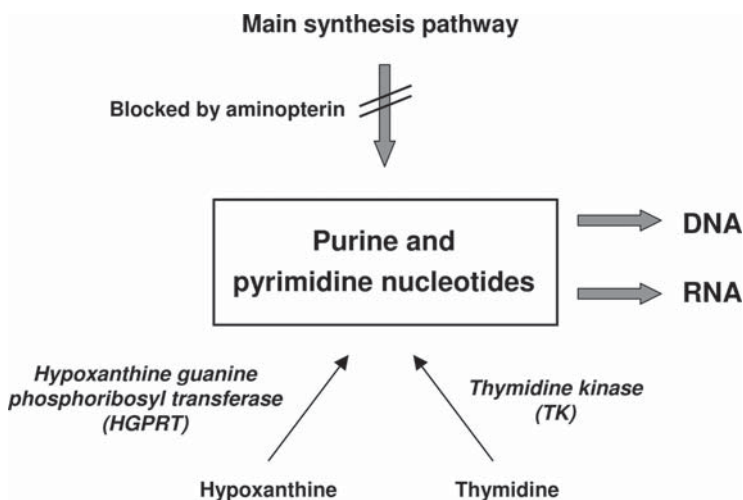


Fig. 2.5 Chemical selection of hybridomas. While the main pathway for synthesis of purine and pyrimidine nucleotides is blocked by the folic acid antagonist aminopterin, cells are forced to use the salvage pathway that requires the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK).

salvage pathway. Hybridomas having acquired HGPRT from immune lymphoblast represent the only cell type that continues to proliferate.

HGPRT-negative myeloma mutants can be selected by culturing the cells in the presence of toxic purine analogs like 8-azaguanine or 6-thioguanine. Cells harboring the enzyme are killed after incorporation of the toxic nucleotide. Spontaneously arising mutants can be simply established because the enzyme is encoded on the X chromosome and only one gene locus has to be targeted.

2.4.5

Screening Hybridoma Cultures for Specific Antibody

Establishment of a reliable, sensitive, and fast screening assay for the detection of desired mAb is the most important prerequisite for successful hybridoma production. It is not recommended to start a fusion experiment before an appropriate screening assay has been set up. There are numerous different test types available for the initial screening that all are based on the measurement of antigen–antibody binding. Taking blood from the immunized animal for instance by puncture of the retro-orbital venous plexus will provide a serum sample that is very useful to establish a sophisticated assay system for screening.

During the decade following invention of hybridoma technology many laboratories have successfully applied solid-phase and cellular radioimmunoassay using ^{125}I -iodine-labeled second step antibodies or protein A (Bjork et al. 1972). This method as well as laborious rosette techniques employing sheep red blood cells as indicator for antibody binding are only of historical interest today. The most frequently performed tests for early hybridoma screening are discussed below. Of course much more tailor-made assays are now established to identify mAbs with special features, such as antibodies reacting with sugar or glycolipid epitopes, antibodies working in Western blotting or neutralizing antibodies against a bacterial toxin or a virus.

2.4.5.1 Enzyme-linked Immunosorbent Assay (ELISA)

Solid-phase ELISA (Engvall and Perlman 1971), where the antigen is immobilized on the well of a microtiter plate, represents a universal test system that can easily be customized and allows rapid analysis of many samples in parallel. Special ELISA plates (the surfaces of which are specifically treated) are coated first with (semi-) purified antigen or peptide that attaches to the plastic surface by adhesive forces. To prevent nonspecific binding plates are then blocked by incubation with a gelatin or bovine albumin solution. Plates can thus be stored in the cold for month. Hybridoma supernatants are allowed to react with immobilized antigen for a while. If the supernatant contains specific antibody it will be strongly retained by its corresponding antigen whereas all other contaminating proteins are subsequently washed away. Next, bound mAb is detected by an enzyme-labeled second step reagent, usually anti-mouse immunoglobulin linked to horseradish peroxidase or alkaline phosphatase, that attaches to the already formed

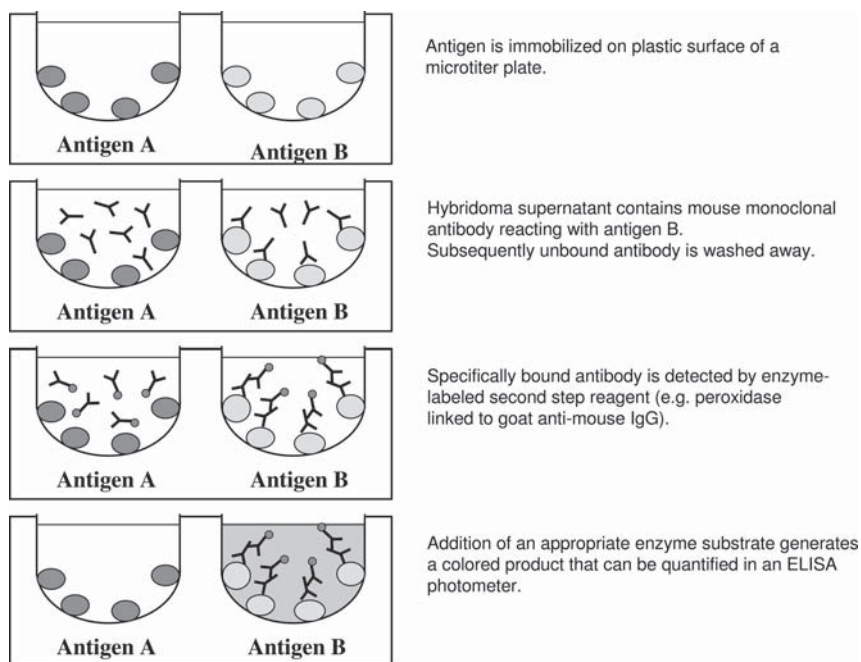


Fig. 2.6 Solid-phase ELISA for antibody screening.

antigen–antibody complex. Finally, the bound conjugate is visualized by a substrate reaction in which a colorless substrate is enzymatically converted into a dye. The reaction can be quantified by an ELISA photometer measuring the appropriate optical density in each well of the plate (Fig. 2.6).

ELISA is also the method of choice to determine the heavy and light chain subclasses of hybridoma antibodies. For this purpose, isotype-specific, enzyme-labeled secondary antibodies are supplied by commercial sources. Isotype-specific enzyme immunoassay was also engaged to select for isotype switch variants of hybridomas. The idea behind it implies that hybridoma clones at rather low frequency spontaneously perform class switching to the subsequent isotype encoded by the heavy chain immunoglobulin gene locus. These rare variants can be traced, enriched, and established by limiting dilution in combination with isotype-specific ELISA (Spira et al. 1984). Many variations of standard indirect ELISA (with immobilized antigen), such as sandwich and competitive ELISA, were suggested, allowing an increase in sensitivity that under normal conditions is not necessary. In addition, for the detection of antibodies directed against cell membrane-exposed antigens cellular ELISA protocols have been developed (Feit et al. 1983). These are especially suitable if a homogeneous cell population, as for instance a gene transfected cell line, is available for screening.

2.4.5.2 Flow Cytometry

The invention of the fluorescence-activated cell sorter (FACS) was a hallmark in the analysis of cell membrane antigens detected by polyclonal antibodies in the beginning and subsequently by mAbs (Bonner et al. 1972, Parks and Herzenberg 1984). First a cell suspension (for instance white blood cells) is incubated with hybridoma supernatant. Depending on the nature of the antigen recognized, a certain proportion of cells will bind the respective antibody to which a second layer, a fluorescently labeled anti-immunoglobulin (e.g. goat anti-mouse IgG coupled to fluorescein or phycoerythrin), is added. Single cells are then passed through a laser beam leading to excitation of the fluorochrome. Taking advantage of highly sophisticated optical and electronic devices, emitted fluorescence from stained cells is measured by a photomultiplier. Usually forward and side scatter signals providing information on the size and granularity of cellular subpopulations are also monitored. Dead cells can be discriminated in parallel by propidium iodide, a fluorescent dye that stains DNA and that is only taken up by injured cells.

In essence, fluorescence-activated cell analyzers and cell sorters provide extremely valuable tools for the rapid, reliable and quantitative screening of antibodies interacting with cell surface receptors. In addition, flow cytometry offers a broad spectrum of applications in immunology, cell biology, and other disciplines. Modern instruments equipped with argon and krypton lasers make the simultaneous use of multiple antibody–fluorochrome conjugates possible, thus allowing multi-parameter analysis of cellular subsets.

2.4.5.3 Immunohistology and Immunocytology

If morphological aspects play a major role in antibody screening immunohistology and immunocytology may provide appropriate methods. Several techniques have been advised to fix tissue or cell suspensions. In traditional immunohistology formalin-fixed and paraffin-embedded sections are mostly used. Unfortunately, many mAbs raised against protein antigens will not work with such material due to the destruction of native conformation by the fixation process. On the other hand, antibodies raised against protein-derived peptides are likely to react with paraffin sections because they often recognize linear epitopes of the antigen. Frozen sections fixed with acetone are well suited for initial antibody screening. Likewise, cells to be used for immunocytology should be fixed with acetone and subsequently air-dried. For this, adherent cells can be grown on coverslips, whereas cells growing in suspension are immobilized on a glass slide using a cytocentrifuge. The staining procedure is reminiscent of that described for ELISA. Briefly, it consists of a first incubation with the hybridoma-derived antibody followed by a secondary enzyme- or less frequently fluorochrome-labeled reagent. If the hybridoma secretes a mouse mAb, a secondary reagent like goat anti-mouse IgG conjugated to alkaline phosphatase or horseradish peroxidase may be applied. The reaction's sensitivity can be enhanced by employing pre-formed complexes consisting of enzyme and anti-enzyme antibodies (APAAP,

alkaline phosphatase anti-alkaline phosphatase and PAP, peroxidase anti-peroxidase). Another option for signal augmentation is the use of biotinylated secondary reagents that exhibit high-affinity binding to streptavidin linked to the enzyme. In contrast to ELISA, for immunohistology and immunocytology an insoluble substrate is needed that forms an insoluble colored precipitate at the site where the antibody has bound.

2.4.5.4 Cytotoxicity Assays

In case antibodies need to be selected that do not only bind to a particular cell type but in addition should fix complement, a screening assay for cytotoxicity may be performed. This type of test, aimed at tracing CDC, is based on the measurement of cell membrane leakiness following complement attack. Target cells are plated in microtiter wells and mixed with hybridoma supernatants together with a source of complement proteins like rabbit or guinea-pig serum. If the antibody under investigation is able to activate the complement cascade, cell lysis will occur within minutes to hours. Killed target cells can be microscopically visualized by addition of dyes such as trypan blue or acridine orange. For quantitative evaluation either the chromium release test (that requires initial labeling of target cells with radioactive chromium-51) or flow cytometric staining with propidium iodide may be applied.

Similar assays have been established to detect antibodies capable of inducing ADCC. Here, target cells are lysed by Fc receptor-bearing effector cells (mainly natural killer (NK) cells and monocytes) that are attracted by and interact with the Fc portion of the antibody bound to the target cell (Sondel and Hanks 2001).

In very rare instances, antibody binding per se can cause target cell destruction. One prominent example is the crosslinking of certain death receptors such as CD95 exposed on the surface of lymphoid cells leading to apoptosis (Schulze-Bergkamen and Krammer 2004).

2.4.5.5 Screening for Function

A great variety of different assays have been set up to identify hybridoma antibodies with special features. To mention only few examples related to oncology here, those screening procedures relying on biological interference with tumor cell growth are of particular interest. A simple method to monitor cell proliferation is incorporation of ^3H -thymidine or bromodesoxyuridine into cellular DNA that can be quantified by beta-counting or ELISA, respectively. Antibodies directed against the growth factor interleukin 6 (IL-6) or its cellular receptor, IL-6R, can be recognized by growth inhibition of a sensitive multiple myeloma target cell. Similarly, epidermal growth factor receptor (EGFR) constitutes a growth-promoting receptor on colon cancer cells that can be used for screening. Recently, antibodies interfering with angiogenesis that bind to vascular endothelial growth factor (VEGF) or its receptors on endothelial cells have been successfully identified and brought to the clinics.

2.4.6

Cloning

Rapid cloning of hybridoma cultures is mandatory to select for stable antibody-secreting cell lines. There are mainly two reasons for single-cell cloning: first, as mentioned before, early hybridomas are sequestering chromosomes to stabilize their genetic inventory. Second, the culture of interest may contain two or even more individual antibody-producing hybrids, making the maintenance of the desired clone not an easy task. The method of choice for single-cell cloning is limiting dilution. In principle, hybridoma cells are distributed in 96-well plates so that one well will contain theoretically 0.5 or 1 cell. Based on the individual cloning efficiency of a particular hybridoma culture that actually is not known at time of cloning, few to many of the seeded cells will give rise to cell clones. Because single hybridoma cells are dependent on several poorly characterized growth factors and in addition require “cellular togetherness,” feeder cells have to be added. Feeder cell cultures are usually prepared from BALB/c spleen cells, thymocytes, or peritoneal macrophages. After 10–14 days clones are reanalyzed for specificity. Ideally, every growing clone should secrete the mAb of interest. To be on the safe side, hybridoma cultures should be cloned at least twice. Interspecies hybrids like mouse/rat or mouse/human hybrids are often unstable and need repeated recloning to preserve antibody production. It is advisable to reclone any hybridoma before starting mass production.

2.4.7

Expansion and Freezing of Hybridoma Clones

After cloning and reanalysis cultures of interest are slowly expanded. Vigorous dilution can cause sudden death of the culture. At this time point hypoxanthine thymidine (HT) medium is gradually replaced by normal medium. To be on the safe side one should freeze a small cell aliquot as backup as soon as possible. If afterwards hybridoma cells divide rapidly, a couple of samples can be frozen and stored in liquid nitrogen for decades. Freezing medium usually consists of 20–90% fetal calf serum and 10% dimethyl sulfoxide.

One of the most adverse events during hybridoma culture is contamination with mycoplasma (Hay et al. 1989; Rottem and Barile 1993). Since mycoplasma infection can interfere with numerous cellular, biochemical, and molecular biological assays, early detection is essential. It is advisable to check all permanently growing cell lines in a laboratory on a regular basis by a sensitive method such as polymerase chain reaction (PCR) or ELISA (Uphoff et al. 1992). Some antibiotics can eliminate mycoplasma from cell culture but this needs a long lasting treatment procedure without guarantee of success and carries the risk of inducing resistant variants. Prevention of contamination by regular testing and clean cell culture working is of course the best way to solve the problem.

2.5

Purification and Modification of Monoclonal Antibodies

2.5.1

Mass Culture and Purification of Monoclonal Antibody

Once the hybridoma is established, large quantities of antibody can be produced employing modern cell culture devices for long-term propagation. At least two systems are on the market meeting the demands of laboratory-scale production because they can simply be installed in a normal CO₂ incubator and do not require complicated pumping and other sophisticated equipment. Both, the miniPERM modular minifermenter (Falkenberg et al. 1995) and the two-chamber cell culture device CELLline 1000 (Trebak et al. 1999) are easy to handle and allow culturing of hybridoma cells at high density (above 10⁷ cells per mL). Harvest of the antibody-enriched product can be performed several times until productivity ceases. The antibody yield throughout is comparable to the formerly favored ascites production in mice that now is prohibited in most Western countries by animal protection laws.

Purification of mouse monoclonal IgG antibodies by affinity chromatography over protein A–Sepharose represents the method of choice (Seppälä et al. 1981). There are suitable protocols available for isolation of all different IgG isotypes. Mouse IgG antibodies are very robust molecules, easy to handle and of high efficiency in many biological assays. By contrast, only the rat IgG2c subtype will bind with sufficient strength to protein A, for the remaining IgG isotypes of rat protein G–Sepharose is the best-suited affinity matrix (Björck and Kronvall 1984). Monoclonals of IgM isotype, however, cause problems in purification, storage and handling. Therefore, one should carefully decide whether an IgM reagent is really useful for a certain application. If the epitope of interest is located on the carbohydrate or glycolipid portion of an antigen one has certainly to deal with IgM antibodies. IgM antibodies can be purified by a combination of gel filtration and ion exchange chromatography or alternatively by affinity chromatography on immobilized mannan-binding protein (Neuens et al. 1992).

2.5.2

Fragmentation of Monoclonal IgG Antibodies

There are certain applications where smaller versions of an antibody may perform better (e.g. in immunoscintigraphy). Furthermore, in some settings it is necessary to exploit monovalent binding of the antibody, thereby preventing crosslinking of the antigen. Another reason to use immunoglobulin fragments is to get rid of the antibody Fc portion that may bind in a nonspecific fashion to Fc receptors exposed on the surface of myelomonocytic cells.

Digestion of IgG (MW approx. 150 kDa) with the thiol protease papain results in two monovalent Fab fragments (MW approx. 50 kDa). The nonspecific protease pepsin cuts below the first disulfide bond in the hinge region, giving rise to a

$F(ab')_2$ fragment (MW approx. 100kDa). Fragmentation of mouse mAbs is not an easy task and the protocol has to be adapted for each individual antibody. There is, however, a clear hierarchy with regard to the immunoglobulin subclasses (Parham 1983; Lamoyi and Nisonoff 1983). More recently a method has been reported that cleaves mouse IgG2b antibodies with lysyl endopeptidase to obtain $F(ab')_2$ fragments (Yamaguchi et al. 1995).

2.5.3

Labeling of Monoclonal Antibodies

For many applications, such as multicolor staining of cells, enzyme immunoassay, or affinity determination of antibody by Scatchard plot, directly labeled monoclonals are needed. Conjugation of purified antibody with either fluorescein isothiocyanate (FITC) and biotin or iodine-125 can be easily performed. FITC binds by a hydrolysis reaction to the free amino group of lysines in the immunoglobulin. Biotin connected by a spacer of variable length to succinimide ester is also covalently bound to the antibody via lysine. The biotin–streptavidin system is especially attractive because of its flexibility. In essence, streptavidin binds with such high affinity to biotin that this bond is rapidly formed and irreversible. In addition, one biotin can accommodate four streptavidins, leading to an amplification effect. There are many streptavidin conjugates commercially available containing fluorescent dyes, enzymes, or even particles for electron microscopy. If the antibody of choice contains many lysines in its combining site, conjugation may abolish binding activity. By contrast, sodium ^{125}I -iodide is coupled to immunoglobulin via tyrosine residues, usually by an oxidation reaction (for instance the chloramine-T method, the Iodogen method, or application of Iodobeads). Alternatively, radiolabeling of antibodies can be achieved via lysine by the Bolton–Hunter procedure (Bolton and Hunter 1973). Enzyme conjugation of antibodies is technically more demanding because the labeled products have to be separated from the unlabeled by biochemical means. Many conjugates of high quality are now commercially available and thus individual enzyme labeling of monoclonals is performed only in special cases.

2.6

Monoclonal Antibodies for Tumor Therapy

2.6.1

Leukocyte Differentiation Antigens

Shortly after the invention of mAb technology in laboratories all around the world a huge variety of reagents were raised against white blood cells and normal as well as malignant cells from numerous tissues. This led to a Babylonian confusion with regard to antibody names and designation of detected antigens. The problem was approached by the organization of the well-known Workshops and

Conferences on Human Leukocyte Differentiation Antigens (HLDA), the first of which took place 1982 in Paris, France, and the most recent 8th meeting was held 2004 in Adelaide, Australia (Bernard et al. 1984; Zola et al. 2005). Antibody samples submitted to the Workshop were grouped into panels and simultaneously analyzed by a couple of reference laboratories with expertise for particular methods such as flow cytometry, immunohistology, biochemistry, or molecular genetics. The results were subsequently compared and statistically evaluated. This allowed the identification of distinct “clusters of differentiation” that became the basis of CD antigen nomenclature. To date, 339 CD antigens have been defined and characterized in depth by approaches taking advantage of immunology, cell biology, biochemistry, and molecular biology.

Monoclonal antibodies have proven to be unique reagents for the analysis of surface antigens on lymphocytes that are expressed on certain stages of lymphocyte differentiation and maturation. Using a whole panel of such antibodies has facilitated the phenotyping of functional subpopulations of normal lymphocytes. Likewise, the malignant counterparts derived from the respective stages of differentiation can be classified. Figure 2.7 illustrates how individual B cell antigens show up and vanish during B cell development. The identification and diagnosis of distinct entities among malignant lymphomas is essentially based on immunohistological staining with a set of antibodies recognizing lymphocyte differentiation antigens (Harris et al. 2001). More recently, classical immunophenotyping

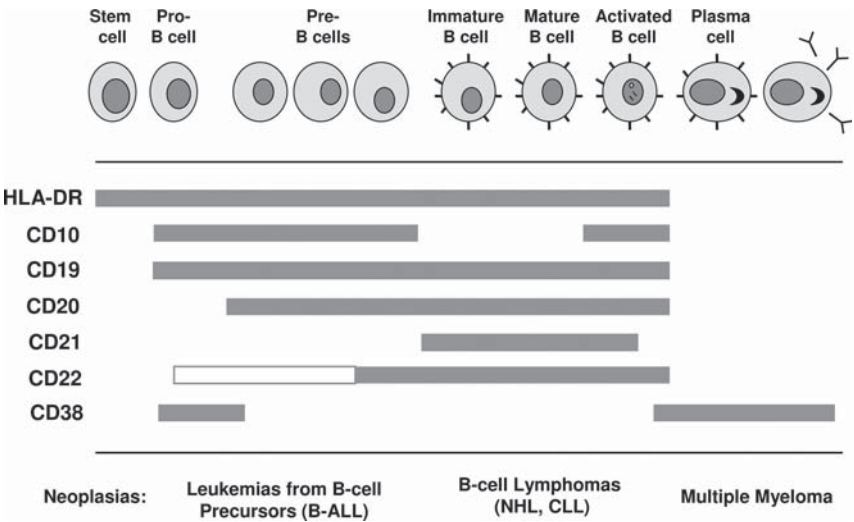


Fig. 2.7 Differentiation antigens of human B lymphocyte. Cluster of differentiation antigens (CD antigens) are expressed on certain maturation stages during B-cell ontogeny (upper panel). Monoclonal antibodies to CD antigens are also reactive with tumor-derived samples of corresponding counterparts (bottom panel), allowing for lymphoma phenotyping.

Table 2.1 Target antigens on lymphocytes for monoclonal antibody therapy.

Antigen	Expression	Structure	Function	Antibody	Indication
CD3	Mature T cells	Complex of five polypeptides	Signal transduction, TCR coreceptor	Muromonab	Transplant rejection
CD20	B cells	Pp35/37 kDa	Ion channel for Ca ²⁺	Rituximab	B-NHL
CD22	Mature B cells	Gp130/140 kDa heterodimer	Modulation of B cell activation, cell–cell adhesion	Epratuzumab	B-NHL
CD33	Monocytes, myeloid cells	Gp67	Cell–cell adhesion?	Gemtuzumab	AML
CD25	Activated B and T cells	Gp55 kDa	α-chain of interleukin 2 receptor	Daclizumab	Transplant rejection
CD52	Lymphocytes, monocytes	Gp21–28 kDa	Unknown	Alemtuzumab	B-CLL, T-lymphoma

Gp, glycoprotein; Pp, phosphoprotein (number given is the molecular weight under reducing conditions); TCR, T-cell receptor; NHL, Non-Hodgkin's lymphoma; AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia.

of lymphomas was complemented by gene expression profiling technology (Staudt and Dave 2005).

Only a small proportion of the 339 CD antigens have yet evolved as valuable targets for antibody therapy not only of malignant lymphomas but also of certain autoimmune diseases and for the prevention of allograft rejection. In Table 2.1 the characteristics of six antigens that serve as targets for therapeutic antibodies already on the market are listed. These antigens differ with respect to their tissue distribution and show different traits concerning stability of surface expression and internalization. The CD22 antigen, for example, has a high internalization rate, making it an exquisite candidate for manufacturing an immunotoxin that has to reach the cytosol of a target cell to become effective (Messmann et al. 2000; Kreitman et al. 2001).

2.6.2

Epithelial Differentiation Antigens

The most frequent tumor type in humans, carcinoma, is derived from epithelial cells. Therefore, tremendous efforts have been made to identify tumor-associated or even tumor-specific membrane antigens on epithelial tumors by means of mAbs. With time it has emerged that all antigens initially regarded as tumor-specific were actually differentiation antigens and are also expressed on certain normal cells. Today it is clear that tumor-specific antigens recognized by antibodies most likely do not exist. The same experience emerged from studies focusing on other tumor types such as melanoma and brain tumors. We have

learned, however, that differentiation antigens, although not tumor-specific, are valuable targets for antibody-based tumor therapy.

Members of the epidermal growth factor receptor (EGFR) family, in particular, such as HER2/neu and EGFR, hold great promise as therapeutic targets since they are overexpressed in a variety of solid tumors (Hynes and Lane 2005). The induction of antitumor responses using the antibodies trastuzumab and cetuximab are discussed in Volume III in Chapters 14 and 4. Further good candidates for antibody therapy of solid tumors are the epithelial cell adhesion molecule, Ep-CAM, and the carcinoembryonic antigen, CEA. Ep-CAM represents a very stable marker even in highly de-differentiated adenocarcinomas and its overexpression is associated with poor prognosis in breast cancer (Gastl et al. 2000) whereas CEA appears to be a suitable target for radioimmunotherapy of colorectal and medullary thyroid cancer (Mayer et al. 2000; Sharkey et al. 2005).

2.6.3

Mechanisms of Action of Monoclonal Antibodies

The antitumor effects of antibodies can be induced by direct and indirect mechanisms (Table 2.2). In some instances antibody binding per se will lead to cell death. For instance, if a surface receptor is crosslinked that transmits an apoptosis signal, programmed cell suicide is started. Likewise binding to growth receptors or their ligands might abrogate vital signals required for cell proliferation. Antibodies against EGFR family members are prominent examples of this mode of action, as already mentioned. Recently, reagents interfering with angiogenesis have become increasingly attractive (Ferrara et al. 2003). Antibodies specific for VEGF or its receptors can prevent tumor vessel formation and thus deprive the tumor of nutrients. A rather special case is represented by anti-idiotypic antibodies in B cell lymphoma. By mechanisms that are poorly understood, these antibodies are able to facilitate long lasting growth control of tumor cells (Davis et al. 1998).

The classical effector functions of antibody are CDC and ADCC. Depending on the isotype of the therapeutic antibody, complement component C1q is activated and triggers a cascade of enzymatic reactions resulting in recruitment of phagocytes and formation of a membrane-attack complex that finally leads to the

Table 2.2 Therapeutic effects of monoclonal antibodies.

Direct effects	Induction of apoptosis Inhibition of proliferation Blockade of growth factors or growth factor receptors Interference with angiogenesis
Indirect effects	Complement-dependent cytotoxicity (CDC) Antibody-dependent cellular cytotoxicity (ADCC) Vehicle for toxins, radionuclides and cytostatic drugs Anti-idiotypic antibody formation Effector cell targeting using bispecific antibodies

lysis of tumor cells (Gelderman et al. 2004). In case of ADCC tumor cell-bound antibodies interact via their Fc portion with Fc receptors expressed at high density on NK cells, neutrophils, and monocytes (Ravetch and Bolland 2001). Upon activation, these effector cells release cytotoxic granules from the cytosol delivering a kiss of death to the tumor target. Unfortunately, many antibodies elicit neither direct nor indirect effects, this holds especially true for murine antibodies. However, these reagents can be successfully used as carriers for toxins, radionuclides, or chemotherapeutic substances. There has been much debate on the issue whether anti-idiotypic networks, forming an internal image of tumor antigens, really contribute to tumor regression. Finally, bispecific antibodies are synthetic molecules that carry two different antigen binding sites. By virtue of their dual specificity they can trigger effector cells via a membrane receptor and at the same time link them to a tumor cell. This interaction leads to the subsequent destruction of the tumor cell.

2.6.4

Human Monoclonal Antibodies

Great efforts have been made to take human myeloma cells in culture suitable for cell fusion in order to raise human mAbs. These attempts were largely hampered by the fact that most of the laboriously established lines later turned out to be Epstein–Barr virus (EBV) transformed lymphoblastoid B cell cultures. Although some human cell lines capable of producing human hybridomas have been described, for instance SK-007 (Olsson and Kaplan 1980), GM1500 (Croce et al. 1980) LICR-LON-Hmy2 (Edwards et al. 1982) and Karpas 707 (Karpas et al. 1982), the overall experience remains disappointing. In addition, for ethical reasons it is not possible to immunize a human volunteer with an experimental antigen. As already mentioned, *in vitro* immunization was not able to solve the problem due to predominant IgM responses.

In an alternative attempt, antigen-specific B lymphocytes were isolated from the peripheral blood of human donors and immortalized by EBV to establish permanent cell lines (Steinitz et al. 1977). Unfortunately, the production rate of the lines was low and decreased with time. It further turned out that the EBV-transformed lines were extremely difficult to clone. To circumvent those problems, the EBV hybridoma technique was developed, which combined EBV-induced immortalization of human antibody-secreting cells with fusion of a variant of the human myeloma line GM1500 to obtain human–human hybrids (Kozbor and Roder 1981). This method, however, is complex and often leads to instable hybridomas that require repeated recloning. Lacking a human non-secretor myeloma cell line with high fusion frequency the production of human mAbs by the hybridoma technique was no longer pursued for many years. Recently, the EBV method has been improved to immortalize memory B cells from a patient with severe acute respiratory syndrome (SARS) coronavirus infection. Neutralizing mAbs of high affinity against the virus, conferring protection in a mouse model, were successfully isolated (Traggiai et al. 2004).

At present, there are at least three alternative core technologies available allowing for the creation of human mAbs. The variable regions or only the CDRs from mouse heavy and light chains can be grafted onto a human IgG scaffold giving rise to chimeric or humanized antibodies, respectively (Carter 2001). Screening of large recombinant antibody libraries is exploited to build human antibodies with high specificity and affinity (Hoogenboom 2005). Transgenic mice carrying human immunoglobulin genes will respond to immunization with the production of entirely human antibodies. After fusion with mouse myeloma cells, these human antibodies are secreted by resulting hybridomas (Lonberg 2005). In addition, recombinant antibodies containing minimal binding fragments can be reconstructed to multivalent high-affinity reagents (Holliger and Hudson 2005).

2.7

Outlook

Monoclonal antibodies secreted by hybridoma cells have led to a revolution in biology, medicine, and many applied sciences due to their excellent specificity. After a first wave of innovation based on mouse monoclonals, molecular biology has provided tools for reshaping the antibody molecule to obtain chimeric, humanized, and fully human antibodies as well as recombinant antibody fragments. Therapeutic antibodies have evolved as effective pharmaceutical compounds not only for the treatment of malignant tumors but also of autoimmune diseases and infections. Currently we are encountering a third wave of scientific advancement by subtle antibody engineering, making it possible to tune the molecule in a way that it can meet special therapeutic demands (Weiner and Carter 2005). In the end there is no doubt that antibody-based therapeutics will play an outstanding role in several fields of modern medicine.

Acknowledgments

I would like to thank Dr Reinhard Schwartz-Albiez for critical reading of the manuscript.

References

- | | |
|--|---|
| <p>Abbas, A.K., Lichtman, A.H. (2003) <i>Cellular and Molecular Immunology</i>, 5th edn. Philadelphia: Saunders.</p> <p>Adams, G.P., Weiner, L.M. (2005) Monoclonal antibody therapy of cancer. <i>Nat Biotechnol</i> 23: 1147–1157.</p> | <p>Barreto, V.M., Ramiro, A.R., Nussenzweig, M.C. (2005) Activation-induced deaminase: controversies and open questions. <i>Trends Immunol</i> 26: 90–96.</p> <p>Barry, M.A., Barry, M.E., Johnston, S.A. (1994) Production of monoclonal</p> |
|--|---|

- antibodies by genetic immunization. *BioTechniques* 16: 616–618.
- Bazin, H., Pear, W.S., Klein, G., SÜmegi, J. (1990) Rat immunocytomas (IR) In: *Rat Hybridomas and Rat Monoclonal Antibodies* (Bazin, H., ed.), pp. 53–68. Boca Raton: CRC Press.
- Bernard, A., Boumsell, L., Dausset, J., Milstein, C., Schlossman S.F. (1984) *Leucocyte Typing*. Berlin, Heidelberg: Springer-Verlag.
- Bolton, A.E., Hunter W.M. (1973) The labeling of proteins to high specific radioactivities by conjugation to a 125I-containing acylating agent. *Biochem J* 133: 529–539.
- Björck, L., Kronvall, G. (1984) Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J Immunol* 133: 969–974.
- Björk, I., Petersson, B.A., Sjöquist, J. (1972) Some physicochemical properties of protein A from *Staphylococcus aureus*. *Eur J Biochem* 29: 579–584.
- Bonner, W.A., Hulett, H.R., Sweet, R.G., Herzenberg, L.A. (1972) Fluorescence activated cell sorting. *Rev Sci Instrum* 43: 404–409.
- Borrebaeck, C.A. (1983) In vitro immunization of mouse spleen cells and the production of monoclonal antibodies. *Acta Chem Scand B* 37: 647–648.
- Brekke, O.H., Sandlie, I. (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Disc* 2: 52–62.
- Carter, P. (2001) Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer* 1: 118–129.
- Cohen, S., Porter, R.R. (1964) Structure and biological activity of immunoglobulins. *Adv Immunol* 3: 287–349.
- Coligan, J.E., Bierer, B.E., Margulies, D.H., Shevach, E.M., Strober, W. (2004) *Current Protocols in Immunology*. New York: John Wiley and Sons.
- Croce, C.M., Linnenbach, A., Hall, W., Stepkowski, Z., Koprowski, H. (1980) Production of human hybridomas secreting antibodies to measles virus. *Nature* 288: 488–489.
- Davis, T.A., Maloney, G.G., Cerwinski, D.K., Liles, T.M., Levy, R. (1998) Anti-idiotypic antibodies can induce long-term complete remissions in non-Hodgkin's lymphoma without eradicating the malignant clone. *Blood* 15: 1184–1190.
- DeNardo, G.L., Bradt, B.M., Mirick, G.R., DeNardo, S.J. (2003) Human antiglobulin responses to foreign antibodies: therapeutic benefit? *Cancer Immunol Immunother* 52: 309–316.
- Edelman, G.M., Cunningham, B.A., Gall, W.E., Gottlieb, P.D., Rutishauser, U., Waxdal, M.J. (1969) The covalent structure of an entire γ G immunoglobulin molecule. *Proc Natl Acad Sci USA* 63: 78–85.
- Edwards, P.A.W., Smith, C.M., Neville, A.M., O'Hare, M. (1982) A human-human hybridoma system based on a fast-growing mutant of the ARH-77 plasma cell leukemia-derived line. *Eur J Immunol* 12: 641–648.
- Ehrlich, P. (1900) On immunity with special reference to cell life. *Proc R Soc Lond* 66: 424–448.
- Eichmann, K. (2005) *Köhler's Invention*. Basel-Boston-Berlin: Birkhäuser-Verlag.
- Engvall, E., Perlman, P. (1971) Enzyme-linked immunosorbent assay (ELISA): Quantitative assay of immunoglobulin G. *Immunochemistry* 8: 871–879.
- Falkenberg, F.W., Weichert, H., Krane, M., Bartels, I., Palme, M., Nagels, H.-O., Fiebig, H. (1995) In vitro production of monoclonal antibodies in high concentration in a new and easy to handle modular minifermenter. *J Immunol Methods* 179: 13–29.
- Fazekas de St. Groth S., Scheidegger, D. (1980) Production of monoclonal antibodies: strategy and tactics. *J Immunol Methods* 47: 129–144.
- Feit, C., Bartal, A.H., Tauber, G., Dymbort, G., Hirshaut, Y. (1983) An enzyme-linked immunosorbent assay (ELISA) for the detection of monoclonal antibodies recognizing antigens expressed on viable cells. *J Immunol Methods* 58: 301–308.
- Ferrara, N., Gerber, H.-P., LeCouter, J. (2003) The biology of VEGF and its receptors. *Nat Med* 9: 669–676.
- Freund, J. (1956) The mode of action of immunologic adjuvants. *Adv Tuberc Res* 7: 130–148.
- Galfrè, G., Milstein, C., Wright, B. (1979) Rat x rat hybrid myelomas and a monoclonal

- anti-Fd portion of mouse IgG. *Nature* 277: 131–133.
- Gastl, G., Spizzo, G., Obrist, P., Dünser, M., Mikuz, G. (2000) Ep-CAM overexpression in breast cancer as a predictor of survival. *Lancet* 356: 1981–1982.
- Gelderman, K.A., Tomlinson, S., Ross, G.D., Gorter, A. (2004) Complement function in mAb-mediated cancer immunotherapy. *Trends Immunol* 25: 158–164.
- Glennie, M.J., van de Winkel, J.G.J. (2003) Renaissance of cancer therapeutic antibodies. *Drug Disc Today* 8: 503–510.
- Goding, J.W. (1996) *Monoclonal Antibodies: Principles and Practice*. 3rd edn. London, San Diego: Academic Press.
- Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T.M., Sutcliffe, J. G., Lerner, R.A. (1982) Immunogenic structure of the influenza virus hemagglutinin. *Cell* 28: 477–487.
- Grohmann, U., Romani, L. Binaglia, L., Fioretti, M.C., Puccetti, P. (1991) Intrasplic immunization for the induction of humoral and cell-mediated immunity to nitrocellulose-bound antigen. *J Immunol Methods* 137: 9–15.
- Hämmerling, G.J., Hämmerling, U., Kearney, J.F. (Eds) (1981) *Monoclonal Antibodies and T-cell Hybridomas*. Amsterdam: Elsevier/North-Holland Biomedical Press.
- Harlow, E., Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Harris, N.L., Stein, H., Coupland, S.E., Hummel, M., Favera, R.D., Pasqualucci, L., Chan, W.C. (2001) New approaches to lymphoma diagnosis. *Hematology (Am Soc Hematol Educ Program)*, 194–220.
- Hay, R.J., Macy, M.L., Chen, T.R. (1989) Mycoplasma infection of cultured cells. *Nature* 339: 487–488.
- Holliger, P., Hudson, P. (2005) Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 23: 1126–1136.
- Hoogenboom, H. (2005) Selecting and screening recombinant antibody libraries. *Nat Biotechnol* 23: 1105–1116.
- Hynes, N.E., Lane, H.A. (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 5: 341–354.
- Janeway, C.A., Travers, P., Walport, M., Shlomchik, M.J. (2005) *Immunobiology. The Immune System in Health and Disease*, 6th edn. London: Garland Science.
- Jerne, N.K. (1955) The natural selection theory of antibody formation. *Proc Natl Acad Sci USA* 41: 849–857.
- Karpas, A., Fischer, P., Swirsky, D. (1982) Human plasmacytoma with an unusual karyotype growing in vitro and producing light-chain immunoglobulin. *Lancet* I: 931–933.
- Kearney, J.F., Radbruch, A., Liesegang, B., Rajewsky, K. (1979) A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J Immunol* 123: 1548–1550.
- Kennett, R.H., McKearn, T.J., Bechtol, K.B. (Eds) (1980) *Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analyses*. New York: Plenum Press.
- Khazaeli, M.B., Conry, R.M., LoBuglio, A.F. (1994) Human immune response to monoclonal antibodies. *J Immunother* 15: 42–52.
- Köhler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.
- Köhler, G., Milstein, C. (1976) Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur J Immunol* 6: 511–519.
- Köhler, G., Howe, S.C., Milstein, C. (1976) Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. *Eur J Immunol* 6: 292–295.
- Kozbor, D., Roder, J.C. (1981) Requirements for the establishment of high-titered human monoclonal antibodies using the Epstein-Barr virus technique. *J Immunol* 127: 1275–1280.
- Kreitman, R.J., Wilson, W.H., Bergeron, K., Raggio, M., Stetler-Stevenson, M., Fitzgerald, D.J., Pastan, I. (2001) Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N Engl J Med* 345: 241–247.
- Lamoyi, E., Nisonoff, A. (1983) Preparation of F(ab')₂ fragments from mouse IgG of

- various subclasses. *J Immunol Methods* 56: 235–243.
- Ledbetter, J.A., Herzenberg, L.A. (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol Rev* 47: 63–90.
- Levy, R., Miller, R.A. (1990) Therapy of lymphoma directed at idiotypes. *J Natl Cancer Inst Monogr* 10: 61–68.
- Littlefield, J.W. (1964) Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* 145: 709–710.
- Lonberg, N. (2005) Human antibodies from transgenic animals. *Nat Biotechnol* 23: 1117–1125.
- Mayer, A., Tsiompamou, E., O'Malley, D., Boxer, G.M., Bhatia, J., Flynn, A.A., Cester, K.A., Davidson, B.R., Lewis, A.A., Winslet, M.C., Dhillon, A.P., Hilson, A.J., Begent, R.H. (2000) Radioimmunoguided surgery in colorectal cancer using a genetically engineered anti-CEA single-chain Fv antibody. *Clin Cancer Res* 6: 1711–1719.
- McLaughlin, P., Grillo-Lopez, A.J., Link, B. K., Levy, R., Czuczman, M.S., Williams, M.E., Heyman, M.R., Bence-Bruckler, I., White, C.A., Cabanillas, F., Jain, V., Ho, A.D., Lister, J., Wey, K., Shen, D., Dallaire, B. (1998) Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 16: 2825–2833.
- Melchers, F., Potter, M., Warner, N.L. (Eds) (1978) Lymphocyte hybridomas. *Current Topics in Microbiology and Immunology*, Vol. 81. Berlin, Heidelberg, New York: Springer-Verlag.
- Messmann, R.A., Vitetta, E.S., Headlee, D., Senderowicz, A.M., Figg, W.D., Schindler, J., Michiel, D.F., Creekmore, S., Steinberg, S.M., Kohler, D., Jaffe, E.S., Stetler-Stevenson, M., Chen, H., Ghetie, V., Sausville E.A. (2000) A phase I study of combination therapy with immunotoxins IgG-HD37-deglycosylated ricin A chain (dgA) and IgG-RFB4-dgA (Combotox) in patients with refractory CD19(+), CD22(+) B cell lymphoma. *Clin. Cancer Res* 6: 1302–1313.
- Milstein, C. (1999) The hybridoma revolution: an offshoot of basic research. *BioAssays* 21: 966–973.
- Nadler, L.M., Stashenko, P., Hardy, R., Kaplan, W.D., Button, L.N., Kufe, D.W., Antman, K.H., Schlossman, S.F. (1980) Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. *Cancer Res* 40: 3147–3154.
- Nevens, J.R., Mallia, A.K., Wendt, M.W., Smith, P.K. (1992) Affinity chromatographic purification of immunoglobulin M antibodies utilizing immobilized mannan binding protein. *J Chromatogr* 597: 247–256.
- Ohnishi, K., Chiba, J., Goto, Y., Tokunaga, T. (1987) Improvement in the basic technology of electrofusion for generation of antibody-producing hybridomas. *J Immunol Methods* 26: 181–189.
- Olsson, L., Kaplan, H.S. (1980) Human-human hybridomas producing monoclonal antibodies of predefined antigenic specificity. *Proc Natl Acad Sci USA* 77: 5429–5434.
- Parham, P. (1983) On the fragmentation of monoclonal IgG1: IgG2a and IgG2b from BALB/c mice. *J Immunol* 131: 2895–2902.
- Parks, D.R., Herzenberg, L.A. (1984) Fluorescence-activated cell sorting: theory, experimental optimization, and application in lymphoid cell biology. *Methods Enzymol* 108: 197–241.
- Pasqualini, R., Arap, W. (2004) Hybridoma-free generation of monoclonal antibodies. *Proc Natl Acad Sci USA* 101: 257–259.
- Pontecorvo, G. (1975) Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. *Somatic Cell Genet* 1: 397–400.
- Ravetch, J.V., Bolland, S. (2001) IgG Fc receptors. *Annu Rev Immunol* 19: 275–290.
- Raybould, T.J.G., Takahashi, M. (1988) Production of stable rabbit-mouse hybridomas that secrete rabbit MAb of defined specificity. *Science* 240: 1788–1790.
- Roitt, I., Brostoff, J., Male, D. (2001) *Immunology*, 6th edn. St Louis: Mosby.
- Ross, J.S., Schenkein, D.P., Pietrusko, R., Rolfe, M., Linette, G.P., Stec, J., Stagliano, N.E., Ginsburg, G.S., Symmans, W.F., Pusztai, L., Hortobagyi, G.N. (2004) Targeted therapies for cancer 2004. *Am J Clin Pathol* 122: 598–609.

- Rottem, S., Barile, M.F. (1993) Beware of mycoplasmas. *Trends Biotechnol* 11: 143–151.
- Sanchez-Madrid, F., Szklut, P., Springer T.A. (1983) Stable hamster-mouse hybridomas producing IgG and IgM hamster monoclonal antibodies of defined specificity. *J Immunol* 130: 309–317.
- Schmitt, J.J., Zimmermann, U. (1989) Enhanced hybridoma production by electrofusion in strongly hypo-osmolar solutions. *Biochim Biophys Acta* 983: 42–50.
- Schulze-Bergkamen, H., Krammer, P.H. (2004) Apoptosis in cancer – implications for therapy. *Semin Oncol* 31: 90–119.
- Seppälä, I., Sarvas, H., Péterfly, F., Mäkelä, O. (1981) The four sub-classes of IgG can be isolated from mouse serum by using protein A-Sepharose. *Scand J Immunol* 14: 335–342.
- Sharkey, R.M., Hajjar, G., Yeldell, D., Brenner, A., Burton, J., Rubin, A., Goldenberg, D.M. (2005) A phase I trial combining high-dose 90Y-labeled humanized anti-CEA monoclonal antibody with doxorubicin and peripheral blood stem cell rescue in advanced medullary thyroid cancer. *J Nucl Med* 46: 620–633.
- Sondel, P.M., Hank, J.A. (2001) Antibody-directed, effector cell-mediated tumor destruction. *Hematol Oncol Clin North Am* 15: 703–721.
- Spira, G., Bargellesi, A., Teillaud, J.L., Sharff, M.D. (1984) *J Immunol Methods* 74: 307–315.
- Spitz, M., Spitz, L., Thorpe, R., Eugui, E. (1984) Intrasplenic primary immunization for the production of monoclonal antibodies. *J Immunol Methods* 11: 39–43.
- Staudt, L.M., Dave, S. (2005) The biology of human lymphoid malignancies revealed by gene expression profiling. *Adv Immunol* 87: 163–208.
- Steinitz, M., Klein, G., Koskimies, S., Mäkelä, O. (1977) EB virus-induced B lymphocyte cell lines producing specific antibody. *Nature* 269: 420–422.
- Stern, M., Herrmann, R. (2005) overview of monoclonal antibodies in cancer therapy: present and promise. *Crit Rev Oncol/Hematol* 54: 11–29.
- Traggiai, E., Becker, S., Subbarao, K., Kolesnikova, L., Uematsu, Y., Gismondo, M.R., Murphy, B.R., Pappuoli, R., Lanzavecchia, A. (2004) An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med* 10: 871–875.
- Trebak, M., Chong, J.M., Herly, D., Speicher, D.W. (1999) Efficient laboratory-scale production of monoclonal antibodies using membrane-based high-density cell culture technology. *J Immunol Methods* 230: 59–70.
- Uphoff, C.C., Brauer, S., Grunicke, D., Gignac, S.M., MacLeod, R.A.F., Quentmeier, H., Steube, K., Tummier, M., Voges, M., Wagner, B., Drexler, H.G. (1992) Sensitivity and specificity of five different mycoplasma detection assays. *Leukemia* 6: 335–241.
- von Behring, E., Kitasato, S. (1890) Über das Zustandekommen der Diphtherie-Immunität und der Tatanus-Immunität bei Thieren. *Dtsch Med Wochenschr* 16: 1113–1114.
- Weiner, L.M., Carter, P. (2005) Tunable antibodies. *Nat Biotechnol* 23: 556–557.
- Yamaguchi, Y., Kim, H.H., Kato, K., Masuda, K., Shimida, I., Arata, Y. (1995) Proteolytic fragmentation with high specificity of mouse immunoglobulin G. *J Immunol Methods* 181: 259–267.
- Zola, H., Swart, B., Nicholson, I., Aasted, B., Bensussan, A., Boumsell, L., Buckley, C., Clark, G., Drbal, K., Engel, P., Hart, D., Horejsi, V., Isacke, C., Macardle, P., Malavasi, F., Mason, D., Olive, D., Saalmueller, A., Schlossman, S.F., Schwartz-Albiez, R., Simmons, P., Tedder, T.M., Uguccioni, M., Warren, H. (2005) CD molecules 2005: human cell differentiation molecules. *Blood* 106: 3123–3126.