



Harnessing Evolution to Make Medicines

Nobel Lecture, December 8, 2018 by

Gregory Winter

MRC Laboratory of Molecular Biology, Cambridge and Trinity College,
Cambridge, UK.

INTRODUCTION

Antibodies are part of our natural defence against infectious agents such as viruses and bacteria and are raised by the immune system in response to infection or vaccination. Indeed, the immune system is a simple system for the fast evolution of antibodies against infectious agents; it generates a diverse range of antibodies and selects those that bind to the infectious agent.

Although Nature has developed antibodies to protect against infectious disease, Man has further developed and evolved antibodies for treatment of *non*-infectious disease, such as inflammatory disorders and cancer (Winter & Milstein, 1991). Man-made antibodies have been used to block the biology of protein receptors and ligands involved in inflammatory disorders, cell growth and T-cell activation; or to kill target cells by recruiting immune effector functions. Indeed, the development of antibodies for treatment of non-infectious disease has revolutionised the pharmaceutical industry, an industry previously dominated by chemical drugs, particularly for treatment of auto-immune inflammatory diseases and cancer. For example, in recent years the antibody Humira (or adalimumab), used for treatment of rheumatoid arthritis, has been the world's best-selling pharmaceutical drug. Of the top ten best selling drugs in 2016, six were antibodies (Table 1).

<u>TRADE NAME</u>	<u>DISEASE</u>	<u>COMPANY</u>	<u>SALES (\$bn)</u>
1. Humira*	rheumatoid arthritis	AbbVie	16.1
2. Harvoni	hepatitis C	Gilead	9.1
3. Enbrel*	rheumatoid arthritis	Amgen/Pfizer	8.9
4. Rituxan*	NHL	Roche/Biogen	8.6
5. Remicade*	rheumatoid arthritis	J&J/Merck	7.8
6. Revlimid	multiple myeloma	Celgene	7.0
7. Avastin*	cancers	Roche	6.7
8. Herceptin*	breast cancer	Roche	6.7
9. Lantus	diabetes (insulin)	Sanofi	6.0
10. Prevnar	pneumonia (vaccine)	Pfizer	5.7

Table 1. Sales of top ten pharmaceutical drugs, 2016. Antibodies are marked*. Source: Genetic Engineering and Biotechnology News.

The development of such antibody pharmaceuticals has required multiple technological inventions, and a molecular understanding of the disease and of antibody structure, function and genetics.

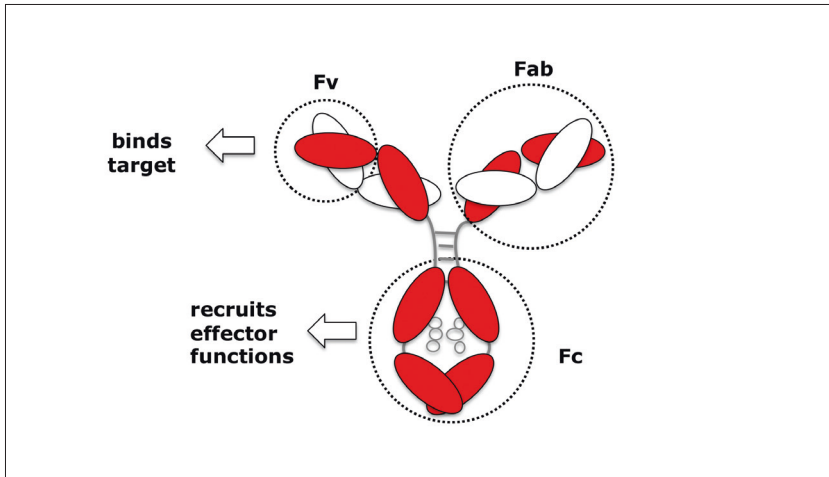


Figure 1. Structure of IgG. Heavy chains (red), light chains (white) with classical antibody fragments (Fv, Fab and Fc) marked.

ANTIBODY STRUCTURE, FUNCTION AND GENETICS

An IgG antibody (Figure 1) is a large (150,000 Da) Y-shaped molecule, two arms and a stem, comprising four chains, two heavy and two light of linked protein domains. The heavy and light chain variable domains (abbreviated VH and VL) at the end of the arms come together to form a

protein scaffold of beta-sheet, surmounted by six loops of variable sequence. Antibodies protect against infectious agents by binding to the target through the variable loops and blocking the process of infection. In addition, the antibody stem can recruit immune effector functions such as complement activation, phagocytosis and antibody-dependent cellular cytotoxicity (ADCC) to kill the infectious agent.

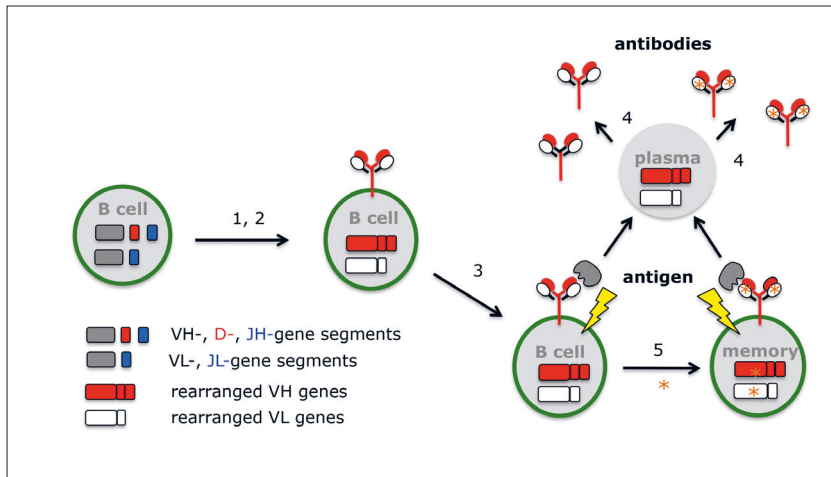


Figure 2. Strategy of the immune system for making antibodies. (1) rearrangement of V-gene segments (Hozumi 1976); (2) surface display of antibody on B-cell; (3) antigen-driven selection; (4) secretion of soluble antibody from plasma cell; (5) affinity maturation.

The diversity of the antigen-binding loops is created through rearrangement of different germ-line segments of DNA during human B-cell development (Figure 2). In heavy chains it is created through combinations of V_H , D and J_H gene segments, each of which has multiple members, to give the rearranged V_H genes. In light chains it is created through combinations of two sets of gene segments (V_K and J_K ; V_λ and J_λ) to give the rearranged V_L genes. Editing at the segment junctions generates further genetic diversity, as does the random combination of heavy and light chain genes in each B-cell. After DNA rearrangement, the encoded antibody is expressed on the surface of the B-cell. As the process takes place independently in many B-cells, it generates a library of cells in which each member expresses a unique antibody on its surface.

When the displayed antibody binds to cognate antigen, the B-cell is stimulated to differentiate. It gives rise to plasma cells, which are factories for production of antibodies against the antigen, and to memory B-cells. In memory B-cells, the antibody genes are targeted for random mutation, and the mutant antibodies displayed on the cell surface. On further

encounter with antigen, the mutant antibodies compete for limiting antigen; those cells displaying antibodies with the highest binding affinity are favoured for further rounds of differentiation. This leads to an antibody response that improves with repeated immunisation, a process known as affinity maturation. For general review, see Schroeder & Cavacini, 2010.

ANTIBODY ENGINEERING

The first step in the creation of antibody pharmaceuticals was the invention of hybridoma technology by Koehler and Milstein (1975) at the MRC Laboratory of Molecular Biology. Mice were immunised with the target antigens, the spleens harvested, and the responding B-cells immortalised by cell fusion. The hybrid cells (or hybridomas) were then screened to identify those making monoclonal antibodies against the target cells. Although this technology generated many useful research reagents, including against human proteins and cells, the mouse monoclonal antibodies were seen as foreign when injected into patients, compromising their use in the clinic. Attempts to make human monoclonal antibodies against human cancer cells proved impossible, not least because the human immune system has tolerance mechanisms that prevent it making antibodies against self-antigens.

By the mid 1980s, solutions began to emerge through the application of protein engineering, in which the antibody genes were altered and the altered antibodies expressed in a host cell. Attempts were made to express antibodies in bacteria, but this gave very poor yields and the antibodies

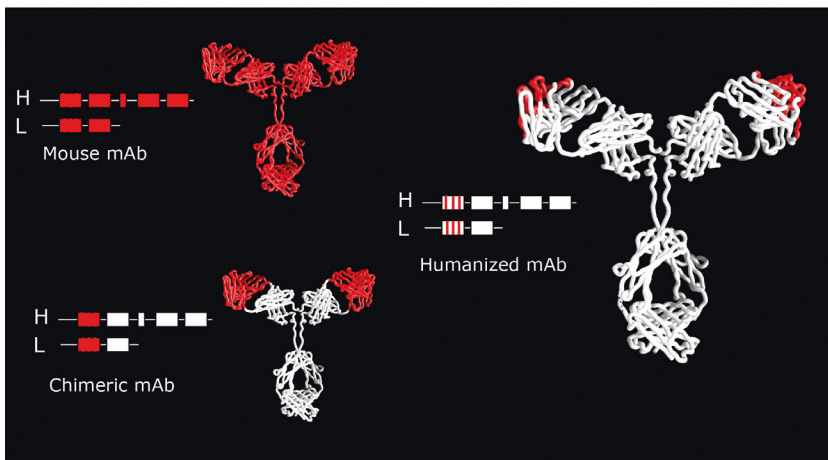


Figure 3. Humanising rodent monoclonal antibodies. IgG polypeptide backbone and gene structure, mouse origin (red) and human origin (white).

had to be refolded from intracellular inclusion bodies (Boss *et al.*, 1984; Cabilly *et al.*, 1984). Lymphoid cells proved to be more suitable hosts for the expression, secreting folded and functional antibodies into the medium (Rice & Baltimore, 1982; Neuberger, 1983; Ochi *et al.*, 1983; Oi *et al.*, 1983).

The first focus of the protein engineers was to turn mouse monoclonal antibodies into their human counterparts (Winter, 1989; Winter & Milstein, 1991) (Figure 3). First came the simple chimeric antibodies in which entire antigen-binding domains were transplanted from mouse to human antibodies. Such antibodies were one-third mouse and two-thirds human in origin, binding to the same antigen as the mouse hybridoma and triggering human effector functions (Boulianne *et al.*, 1984, Morrison *et al.*, 1984, Neuberger *et al.*, 1985).

Then came “humanised” antibodies, in which only the antigen-binding loops were grafted from the mouse antibodies into human antibodies (Jones *et al.*, 1986; Riechmann *et al.*, 1988; Verhoeyen *et al.*, 1988).

As the inventors of humanised antibodies (which are up to 95% human in origin), we argued that as the antigen-binding loops differ between human antibodies, such humanised antibodies might be regarded as synthetic human antibodies. One of these humanised antibodies (Riechmann *et al.*, 1988), directed against the lymphocyte antigen CD52, was used to treat patients with non-Hodgkins lymphoma. It was tolerated over the 30-day course of treatment and destroyed a large mass of spleen tumour (Hale *et al.*, 1988). This appears to have been the first clinical use of an engineered antibody. Later, clinicians at the Cambridge Department of

Cinquaero (<i>reslizumab</i>) (IL5/asthma)	Lucentis (<i>ranibizumab</i>) (VEGF-A/AMD)
Nucala (<i>mepolizumab</i>) (IL5/asthma)	Avastin (<i>bevacizumab</i>) (VEGF-A/several C)
Xolair (<i>omalizumab</i>) (IgE/asthma)	Herceptin (<i>trastuzumab</i>)(HER2/HER2+ BC)
Lemtrada (<i>alemtuzumab</i>) (CD52/MS)	Perjeta (<i>pertuzumab</i>) (HER2/HER2+ BC)
Tysabri (<i>natalizumab</i>) (VLA-4/MS,CD)	Gazyvaro (<i>obinutuzumab</i>) (CD20/CLL)
Ocrevus (<i>ocrelizumab</i>) (CD20/MS)	Empliciti (<i>elotuzumab</i>) (SLAMF7/MM)
RoActemra (<i>tocilizumab</i>) (IL-6R/RA)	Tecentriq (<i>atezolimumab</i>) (PD-L1/NSCLC)
Cimzia (<i>certolizumab pegol</i>)(TNF/RA, CD)	Keytruda (<i>pembrolizumab</i>) (PD-1/melanoma)
Entyvio (<i>vedolizumab</i>) (I- α 4 β 7/CD)	Synagis (<i>palivizumab</i>) (RSV/RSV infection)
Soliris (<i>eculizumab</i>) (C5/PNH)	

Table 2. Humanised antibodies approved by the US Food and Drug Administration. Each antibody listed in order of trade name, non-proprietary name, pharmaceutical target and disease area. MS = Multiple Sclerosis, CD = Crohn’s Disease, RA = Rheumatoid Arthritis, PNH = Paroxysmal Nocturnal Haemoglobinuria, AMD = Acute Macular Degeneration, BC = Breast Cancer, CLL = Chronic Lymphocytic Leukaemia, MM = multiple myeloma, NSCLC = Non Small Cell Lung Cancer, RSV = Respiratory Syncytial Virus.

Clinical Neurosciences developed this antibody (alemtuzumab, marketed as Lemtrada) for treatment of relapsing forms of multiple sclerosis. Many other antibodies have been humanised and approved as pharmaceutical drugs (Table 2). But in the late 1980s we didn't know that humanised (or even chimeric) antibodies would be so well tolerated in patients. We therefore began to think about ways of making fully human antibodies. A possible solution emerged from a methodological improvement in making engineered mouse antibodies.

ANTIBODY LIBRARIES

One of the rate-limiting steps in making engineered antibodies was the isolation of the rearranged VH- and VL-genes from the mouse hybridoma. For this purpose, we decided to explore the use of the polymerase chain reaction (PCR), in which target regions of DNA are amplified by repeated cycles of polymerase extension of two flanking primers (Saiki *et al.*, 1988). By comparing the nucleotide sequences of many different antibodies, we identified regions at the ends of both heavy and light chain genes that seemed sufficiently conserved to allow the design of a simple set of PCR primers. There was no way of avoiding some primer/template mismatches; indeed, we took advantage of the mismatched regions to incorporate restriction sites for cloning the amplified DNA into expression vectors. After exploring a range of experimental conditions, we identified a set of primer sequences that allowed us to amplify the rearranged VH- and VL-genes of several mouse hybridomas (Orlandi *et al.*, 1989).

This method made it so much easier to clone and express antibody genes that we speculated about making recombinant antibodies directly from libraries of V-genes from the spleens of immunised mice, thereby by-passing hybridoma technology (Orlandi *et al.*, 1989). We also realised that a similar approach might lend itself to making human antibodies, including those against human self antigens. However, we first needed to find an expression host suitable for the mass screening of libraries of recombinant antibodies. We alighted on bacteria, as it had just been shown that antibody fragments could be secreted in a folded and functional form into the bacterial periplasm by attaching a signal sequence (Better *et al.*, 1988; Skerra & Pluckthun, 1988).

We tailored a plasmid vector for the bacterial expression of antibody fragments from the VH- and VL-genes as amplified by our PCR primers. After cloning the antibody genes from the hybridoma (D1.3) (Amit *et al.*, 1986), we established that the D1.3 antibody Fv fragment (associated VH and VL domains) was secreted from the transfected bacteria into the culture supernatant, and bound to hen egg lysozyme, as in the original hybri-

doma. We also discovered that the secreted VH domain bound to hen egg lysozyme in the absence of the VL domain, with a loss of only about ten-fold in binding affinity.

We then immunised mice with protein antigens and made a VH gene expression library from the immunised spleens. Screening of the library revealed VH domain fragments with binding activities to the immunising antigen at a frequency of about 1/100. This seemed promising, and we wondered whether such single domain antibodies (or dAbs) might provide a platform of small high affinity protein domains for a range of applications. However, the isolated VH domains were “sticky”, presumably due to the exposed hydrophobic surface normally capped by the VL domains. Although we believed that the poor biophysical properties of mouse (and human) VH domains could be overcome (Ward *et al.*, 1989), we did not pursue the further development of a dAb platform for some years (Jespersen *et al.*, 2004a; Jespersen *et al.*, 2004b; Ueda *et al.* 1984). Indeed, camels were found to have naturally occurring antibodies devoid of light chains, and the camel VH domains to have good biophysical properties (Hammers-Casterman *et al.* 1993).

In the meantime, the group of Richard Lerner (Scripps Research Institute) and the biotechnology company Stratagene had developed an interest in antibody libraries. They took the next step and created libraries of heavy and light chain (Fab) pairs from the V-genes from spleens of lymphocytes immunised with hapten. The pairings were generated randomly, and the libraries referred to as random combinatorial libraries. After mass screening of the Fab fragments, they identified several hapten binders at a frequency of 1/10,000 (Huse *et al.*, 1989). Again, this seemed promising, but we thought it unlikely that the screening method would be sufficiently powerful to find human antibodies against human self-antigens.

We therefore looked at the more powerful strategy used by the immune system itself. Could we develop a B-cell mimic, essentially a “genetic display package”, with antibody displayed on the outside of the package to encounter antigen, and the antibody genes packaged within? We considered several possibilities, including the display of antibodies on mammalian cells, bacteria and bacterial viruses. Of these the filamentous bacteriophage seemed the most attractive.

PHAGE DISPLAY

Four years earlier George Smith had shown that peptides could be displayed on filamentous bacteriophage by genetic fusion to a coat protein (p3) that mediates the bacterial infectivity of the phage. He had shown that phage displaying peptide epitopes could be selected and enriched by binding to cognate antibodies (Smith, 1985). Instead of peptides, we won-

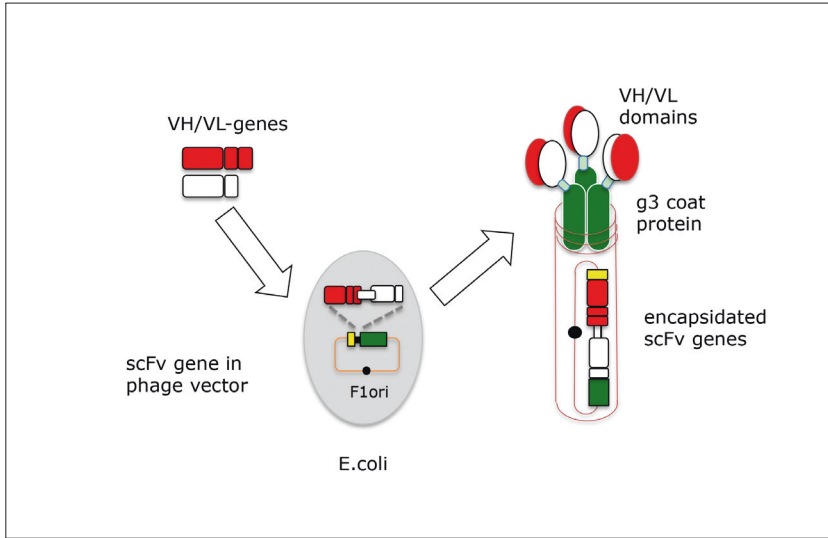


Figure 4. Cloning scFv fragment for phage display. VH genes and VH domain (red), VL genes and VL domain (white), phage 3 protein (green), leader sequence (yellow). Phage truncated and only 3 copies of p3 shown for simplicity of representation.

dered whether we could display antibody fragments on phage, and select the “phage antibodies” by binding to antigen?

We did not know whether the displayed antibodies would fold or be efficiently incorporated into the phage coat. Nor did we know whether the antibody-p3 fusions would survive proteolysis in the bacterial periplasm or mediate phage infectivity. We therefore checked with our model D1.3 antibody fragment. To avoid dissociation of the domains, we decided to link the VH and VL domains together with a short flexible peptide, so creating a single chain Fv fragment (scFv) (Bird *et al.*, 1988; Huston *et al.*, 1988). We then fused the antibody gene to the phage p3 gene in a phage vector (Figure 4). After transfection of the recombinant vector DNA into bacteria, we tested the recombinant phage for binding to hen egg lysozyme using an ELISA assay. As we had hoped, the recombinant phage bound specifically to hen egg lysozyme (McCafferty *et al.*, 1990). We now mixed the phage antibody with a large excess of wild-type phage and showed that we could enrich the phage antibody by rounds of selection on antigen-affinity columns. Indeed, the phage antibodies were enriched one thousand-fold in a single round of selection, and one million-fold over two rounds of selection.

We then used the phage to display and enrich libraries of antibody fragments from immunised mice. We decided to follow the mouse antibody response to the hapten phenyloxazolone (phOx), as this response had been characterised in molecular detail using hybridoma technology

(Berek *et al.*, 1985). We started with a random combinatorial library of about 2×10^5 phage antibody clones derived from spleen IgG mRNA of a mouse immunised with the hapten (Clackson *et al.*, 1991). After affinity selection, we identified multiple binders. Although we had expected to find binders, we had not anticipated how readily we would find them. On the one hand, we had expected the Ig mRNA to be enriched towards antibodies directed to the immunising hapten, particularly due to the elevated Ig mRNA in plasma cells (Schibler *et al.*, 1978; Hawkins & Winter, 1992). On the other hand, we had expected the frequency of original VH and VL pairings (and comprising those elicited by immunisation) to be low given the random combinatorial process and the number of B-cells used to make the library.

Sequence analysis of the binders revealed a further factor – “promiscuity” – in which the same VH-gene was paired with different VL-genes, and vice-versa. Indeed, after shuffling one such pairing with the original VH- gene or VL-gene libraries respectively, we found binders in which each domain had made new pairings. After recloning the antibody genes for bacterial expression as soluble antibody fragments, we identified pairings with binding affinities comparable to those of hybridomas ($K_d = 10$ nM) made from the same spleens (Gherardi & Milstein 1992). Together with the results from the Scripps (Huse *et al.*, 1989), this confirmed that the library technology had the potential to bypass hybridoma technology, at least for making mouse monoclonal antibodies against haptens.

HUMAN ANTIBODIES FROM PHAGE LIBRARIES

We then faced up to the next challenge – to make human antibodies and to do so without immunisation. We first made some technical improvements to make the libraries more diverse and larger. To make the libraries more diverse, we took the peripheral blood lymphocytes from unimmunised human donors and amplified the rearranged human VH- and VL-genes from naïve and primary response B-cells (IgM) cDNA (Marks *et al.*, 1991a) using a set of “family-based” PCR primers (Marks *et al.*, 1991b). To make the libraries larger, we turned to phagemid vectors in which the antibody fusion is encoded by a phagemid, and a helper phage provides other g3 subunits (Bass *et al.*, 1990). The phagemid vectors have higher bacterial transfection efficiencies than phage vectors, allowing the creation of larger libraries. The phagemid vectors also simplified the expression of soluble fragments. By interposing an amber stop codon between the antibody gene and the p3 protein, we could switch between display on phage in amber suppressor strains of bacteria (and helper phage), or secretion of soluble fragments in non-suppressor strains (Hoogenboom *et al.*, 1991).

From a library of 10^7 phagemid clones, we isolated human antibody fragments binding to a foreign antigen (turkey egg lysozyme), a hapten (phOx) (Marks *et al.*, 1991a), and to several human self-antigens (Griffiths *et al.*, 1993). Although the binding of the soluble fragments was specific for each target, the binding affinities of the antibody monomers were in the micromolar range. We set about improving these affinities by subjecting the selected clones to further rounds of diversification and selection, as in the immune system.

For example, starting with the human phOx antibody (binding affinity $K_d = 320$ nM), we reshuffled the VH gene with the entire VL gene library, selecting for hapten-binding under stringent conditions (Marks *et al.*, 1992). This led to new VL partners, and an antibody fragment with a 20-fold improved binding affinity, which we improved a further 15-fold by shuffling with a library of the VH gene segments. Overall, we had achieved 300-fold improvement in binding affinity ($K_d = 1$ nM).

In another example, we grew the phage displaying the human phOx antibody in a bacterial mutator strain. After multiple rounds of growth and stringent selection with the hapten, we achieved a 100-fold improvement in binding affinity ($K_d = 3.2$ nM). This was comparable to the affinities of mouse monoclonal antibodies made by repeated immunisation to the same hapten. We could even construct a genealogical tree from the sequences of the mutants at different rounds and identify four sequentially acquired mutations which were together responsible for the improved affinity (Low *et al.*, 1996).

In the selection process, we typically used longer washes, more disruptive washing conditions or lower concentrations of antigen to distinguish between phage antibodies with different affinities, (Hawkins *et al.*, 1992). In this context the phagemid vectors had a further advantage over phage vectors – “monovalent” display (Lowman & Wells, 1991; Lowman *et al.*, 1991). With phage vectors, we would expect five antibody heads on each phage and potentially highly avid binding to solid phase antigen. In practice few phage have five antibody heads – they suffer proteolysis, and the phages bind to solid phase antigen with variable avidities, making it difficult to distinguish between those binders with high affinity and those with high avidity. The use of phagemid vectors, in which incorporation of the helper phage p3 reduces the phage valency, leads to so-called “monovalent” phage, and helps to eliminate the avidity component from affinity selections.

SYNTHETIC HUMAN ANTOBODIES

As well as antibody libraries from the rearranged VH- and VL-genes from human B-cells, we developed synthetic human antibody libraries. We cre-

ated the synthetic libraries from the human gene segment building blocks (V_H , D and J_H ; V_K and J_K ; V_L and J_L) – such libraries have the advantage that the composition and diversity of the library can be predetermined. In 1989, the sequences of most of the human VH- and VL-genes was unknown, so we first had to clone and sequence all the human V-gene segments, starting with the V_H segments (Tomlinson *et al.*, 1992). To the ends of each of 49 human V_H gene segments (Hoogenboom & Winter, 1992; Nissim *et al.*, 1994) we introduced an artificial D-segment comprising random nucleotide sequence of variable lengths, and a J-segment. These rearranged VH gene segments were combined with a single fixed rearranged VL gene and cloned for display on phage. From a library of 10^8 phagemid clones we obtained micromolar binders to haptens and proteins, comparable in affinity to libraries of similar size made from naturally rearranged V-genes.

After cloning the V_L - gene (Williams & Winter, 1993) and V_K - gene (Cox *et al.*, 1994) segments, and building the corresponding synthetic rearranged VL gene repertoires, we set about creating an even larger combinatorial library. However, we were limited by the efficiencies of transformation of *E. coli* by phagemid DNA. We therefore developed a new strategy (Waterhouse *et al.*, 1993) in which we infected bacteria harbouring a heavy chain library (on a plasmid) with a light chain library (on a phage). The two chains were combined on the same (phage) replicon within the bacterium by Cre catalysed recombination at *loxP* sites, creating combinatorial libraries, potentially as large as the number of bacteria infected. In this way we created a library of $> 10^{10}$ clones (Griffiths *et al.*, 1994) including almost all the human V-gene segments. Selection of this library yielded antibodies to a wide range of antigens, against both foreign and self-antigens. The binding affinities were in the nanomolar range, and comparable to the affinities of hybridomas after affinity maturation. Indeed, using such large libraries reduced the need for affinity maturation.

THERAPEUTIC ANTIBODIES FROM PHAGE

In parallel with our work in the MRC, the start-up company Cambridge Antibody Technology (CAT) was working with commercial partners to develop human therapeutic antibodies. Their most spectacular success was the development of the phage antibody adalimumab, which is targeted against TNF α and used for treatment of autoimmune inflammatory diseases such as rheumatoid arthritis, psoriasis and Crohn's disease. When CAT started the work on adalimumab in the early 1990s, the random combinatorial libraries of human antibodies were not large or diverse enough. We therefore tried a different strategy, starting with a

mouse monoclonal antibody against $\text{TNF}\alpha$ (Jespers et al., 1994), as illustrated in Figure 5. For example, the mouse heavy chain can be paired with a library of human light chains and selected for binding to $\text{TNF}\alpha$. The selected human light chain can then be paired with a library of human heavy chains, and selected for a fully human antibody that binds to the same antigen. This chain shuffling strategy, essentially a molecular version of Theseus's paradox (or grandfather's axe paradox), synthesises a human antibody using a mouse antibody as template. It was this strategy that gave rise to adalimumab.

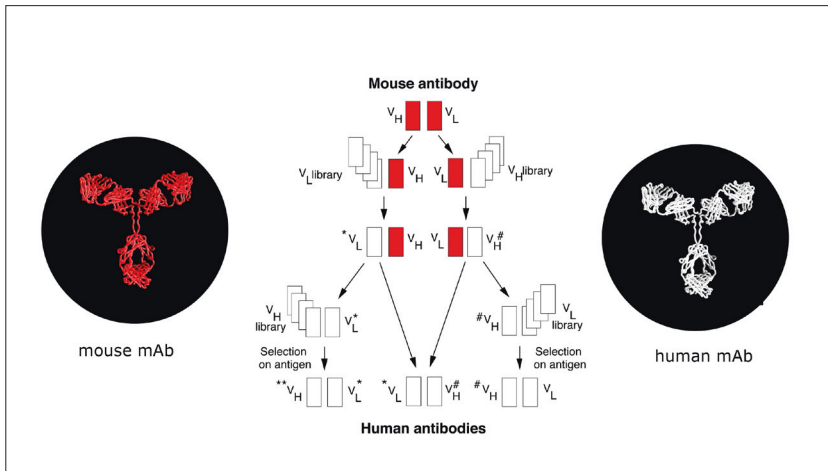


Figure 5. Humanising antibodies by chain shuffling. IgG polypeptide backbone and V_H and V_L gene structure, mouse origin (red) and human origin (white).

Subsequently Cambridge Antibody Technology (Medimmune/Astra-Zeneca) has focused on creating very large libraries ($>10^{11}$ clones). Table 3 shows the pharmaceutical target classes to which human phage antibodies have been made using the same library (J. Osbourn, Medimmune, personal communication), and Table 4 those human therapeutic antibodies already approved by the US Food and Drug Administration.

Growth factor: PIGF, VEGF-2, GDF-8
 Chemokine: CXCL13
 Ion Channel: P2X4
 Receptor: IL-21R, PSGL-1, TRAIL-R1, GM-CSFa2
 GPCR: GLP1R, GIPR
 Cytokine: IL-6, Blys, APRIL
 Protease inhibitor: PAI-1
 Peptide: Ghrelin, NKB, gp41

Table 3. Pharmaceutical target classes against which phage antibodies have been raised using the same large library. In order target class and molecular target.

Humira (*adalimumab*) (TNF/RA,CD)
 Benlysta (*belimumab*) (BAFF/SLE)
 Cablivi (*caplacizumab*) (vWF/TTP)
 Tremfya (*guselkumab*) (IL23/Psoriasis)
 Takhzyro (*Janadelumab*) (PK/HAE)
 Portrazza (*necitumumab*) (EGFR/NSCLC)
 Cyramza (*ramucirumab*) (VEGFR2/Cancer)
 Lumoxiti (*moxetumumab pasudotox*)(CD22/HCL)
 Bavencio (*avelumab*) (PD-L1/MCC)
 ABthrax (*raxibacumab*) (Anthrax/Inhalational anthrax)

Table 4. Human antibodies made by phage display and approved by the US Food and Drug Administration. Each antibody listed in order of trade name, non-proprietary name, pharmaceutical target and disease area. SLE = Systemic Lupus Erythematosus, TTP= Thrombotic Thrombocytopenic Purpura, HAE = Hereditary Angioedema, HCL = Hairy Cell Leukaemia, MCC = Merkel Cell Carcinoma. Other abbreviations as in Table 2.

In conclusion, antibody libraries and phage display have provided the key elements for the creation of a fast evolutionary system for the generation of fully human antibody medicines.

REFERENCES

- Amit A.G., Mariuzza R.A., Phillips S.E.V. & Poljak R.J. (1986) "Three-dimensional structure of an Antigen-Antibody Complex at 2.8Å Resolution." *Science* **233**, 747–753
- Bass S., Greene R. & Wells J.A. (1990) "Hormone phage: An enrichment method for variant proteins with altered binding properties." *Proteins* **8**, 309–314.
- Berek C., Griffiths G.M. & Milstein C. (1985) "Molecular events during maturation of the immune response to oxazolone". *Nature* **316**, 412–418.
- Better M., Chang C.P., Robinson R.R. & Horwitz A.H. (1988) "Escherichia coli secretion of an active chimeric antibody fragment." *Science* **240** (4855), 1041–1043.
- Bird R.E., Hardman K.D., Jacobson J.W., Johnson S., Kaufman B.M., Lee S., Lee T., Pope S.H., Riordan G.S. & Whitlow M. (1988) "Single chain antigen-binding proteins." *Science* **242**, 423–426.
- Boss M.A., Kenten J.H., Wood C.R. & Emtage J.S. (1984) "Assembly of functional antibodies from immunoglobulin heavy and light chains synthesized in *E. coli*." *Nucl. Acids. Res.* **12**(9), 3791–3806
- Boulianne G.L., Hozumi, N. & Shulman M.J. (1984) "Production of functional chimeric mouse/human antibody." *Nature* **312**, 643–646.
- Cabilly S., Riggs, A.D., Pande H., Shively J.E., Holmes W.E., Rey M., Perry J., Wetzel R. & Heynecker H.L. (1984) "Generation of antibody activity from immunoglobulin polypeptide chains produced in *Escherichia coli*." *Proc. Natl. Acad. Sci. USA* **81**, 3273–3277
- Clackson T., Hoogenboom H.R., Griffiths A.D. & Winter G. (1991). "Making antibody fragments using phage display libraries." *Nature* **352**(6336), 624–628.
- Cox J.P., Tomlinson I.M. & Winter G. (1994). "A directory of human germ-line V kappa segments reveals a strong bias in their usage." *Eur J Immunol* **24**(4), 827–836.
- Gherardi E. & Milstein C. (1992) "Original and artificial antibodies" *Nature* **357**, 201–202.
- Griffiths A.D., Malmqvist M., Marks J.D., Bye J.M., Embleton M.J., McCafferty J., Baier M., Holliger P., Gorick B.D., Hughes-Jones N.C., Hoogenboom H.R. & Winter G. (1993). "Human anti-self antibodies with high specificity from phage display libraries." *EMBO J* **12**, 725–734.
- Griffiths A.D., Williams S.C., Hartley O., Tomlinson I.M., Waterhouse P., Crosby W.L., Kontermann R.E., Jones P.T., Low N.M., Allison T.J., Prospero T.D., Hoogenboom H.R., Nissim A., Cox J.P., Harrison J.L., Zaccolo M., Gherardi E. & Winter G. (1994). "Isolation of high affinity antibodies directly from large synthetic repertoires." *EMBO J* **13**(14), 3245–3260.
- Hale G., Dyer M.J., Clark M.R., Phillips J.M., Marcus R., Riechmann L., Winter G. & Waldmann H. (1988). "Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H." *Lancet* **2**(8625), 1394–1399.
- Hamers-Casterman C., Atarhouch T., Muyldermans S., Robinson G., Hamers C., Bajzana Songa E., Bendahman N. & Hamers R (1993). "Naturally occurring antibodies devoid of light chains." *Nature* **363**, 446–448.
- Hawkins R.E. & Winter G. (1992). "Cell selection strategies for making antibodies from variable gene libraries: trapping the memory pool." *Eur J Immunol* **22**(3), 867–870.
- Hawkins R.E., Russell S.J. & Winter G. (1992). Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J Mol Biol* **226**(3), 889–896.

- Hoogenboom H.R., Griffiths A.D., Johnson K.S., Chiswell D.J., Hudson P. & Winter G. (1991). "Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains." *Nucleic Acids Res* **19**(15), 4133–4137.
- Hoogenboom H.R. & Winter G. (1992). "By-passing immunisation. Human antibodies from synthetic repertoires of germline V_H gene segments rearranged *in vitro*." *J Mol Biol* **227**(2), 381–388.
- Huse W.D., Sastry L., Iverson S.A., Kang A.S., Alting-Mees M., Burton D.R. Benkovic S.J. & Lerner R.A. (1989) "Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda" *Science* **246**, 1275–1281
- Huston J.S., Levinson D., Mudgett-Hunter M., Tai M., Novotny J., Margolies M.N., Ridge R., Brucoleri R.E., Haber E., Crea R & Oppermann H. (1988) "Protein engineering of antibody binding sites: recovery of specific activity in an anti-dioxin single-chain Fv analogue produced in *Escherichia coli*." *Proc.Natl.Acad. Sci. USA* **85**, 5879–5883
- Jespers L., Schon, O, James, L.C., Vepintsev D. & Winter G (2004). "Crystal structure of HEL4, a soluble, refoldable human VH single domain with germ-line scaffold." *J Mol Biol* **337**, 893–903.
- Jespers L., Famm K., Schon, O. & Winter G. (2004). "Aggregation resistant proteins selected by thermal cycling." *Nature Biotech.*, **22**, 1161–1165.
- Jespers L.S., Roberts A., Mahler S.M., Winter G. & Hoogenboom H.R. (1994). "Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen." *Biotechnology (NY)* **12**(9), 899–903.
- Jones P.T., Dear P.H., Foote J., Neuberger M.S. & Winter G. (1986). "Replacing the complementarity-determining regions in a human antibody with those from a mouse." *Nature* **321**(6069), 522–525.
- Kohler G. & Milstein C. (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity." *Nature* **256**, 495–497.
- Low N.M., Holliger P. & Winter G. (1996). "Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain." *J Mol Biol* **260**(3), 359–368.
- Lowman H.B & Wells J.A. (1991). "Monovalent phage display: a method for selecting variant proteins from random libraries." *Methods* **3**, 205–216
- Lowman H.B., Bass S.H., Simpson N. & Wells J.A. (1991). "Selecting high affinity binding proteins by monovalent phage display." *Biochemistry* **30**, 10832–10838
- Marks J.D., Hoogenboom H.R., Bonnert T.P., McCafferty J., Griffiths A.D. & Winter G. (1991a). "By-passing immunization. Human antibodies from V-gene libraries displayed on phage." *J Mol Biol* **222**(3), 581–597.
- Marks J.D., Tristem M., Karpas A. & Winter G. (1991b). "Oligonucleotide primers for polymerase chain reaction amplification of human immunoglobulin variable genes and design of family-specific oligonucleotide probes." *Eur J Immunol* **21**(4), 985–991.
- Marks J.D., Griffiths A.D., Malmqvist M., Clackson T.P., Bye J.M. & Winter G. (1992). "By-passing immunization: building high affinity human antibodies by chain shuffling." *Biotechnology (NY)* **10**(7), 779–783.
- McCafferty J., Griffiths A.D., Winter G. & Chiswell D.J. (1990). "Phage antibodies: filamentous phage displaying antibody variable domains." *Nature* **348**(6301), 552–554.
- Morrison, S.L., Johnson M.J., Herzenberg L.A. & Oi V.T. (1984). "Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains." *Proc. Natl. Acad. Sci. USA* **81**(21), 6851–6855

- Neuberger M.S. (1983). "Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells." *The EMBO Journal* **2**(8), 1373–1378
- Neuberger M.S., Williams, S.G.T, Mitchell E.B, Jouhal S.S., Flanagan J.G. & Rabbitts T.H. (1985). "A hapten-specific IgE antibody with human physiological effector function." *Nature* **314** (6008) 268–270.
- Nissim A., Hoogenboom H.R., Tomlinson I.M., Flynn G., Midgley C., Lane D. & Winter G. (1994). "Antibody fragments from a 'single pot' phage display library as immunochemical reagents." *EMBO J* **13**(3) 692–698.
- Ochi O., Hawley R.G., Hawley T., Schulman M.J., Traunecker A, Kohler G. & Hozumi N. (1983). "Functional immunoglobulin M production after transfection of cloned immunoglobulin heavy and light chain genes into lymphoid cells." *Proc. Natl. Acad. Sci. USA* **80**, 6351–6355.
- Oi V.T., Morrison S.L., Herzenberg L.A. & Berg P. (1983). "Immunoglobulin gene expression in transformed lymphoid cells." *Proc. Natl. Acad. Sci. USA* **80**, 825–829.
- Orlandi R., Gussow D.H., Jones P.T. & Winter G. (1989). "Cloning immunoglobulin variable domains for expression by the polymerase chain reaction." *Proc Natl Acad Sci USA* **86**(10) 3833–3837.
- Riechmann L., Clark M., Waldmann H. & Winter G. (1988). "Reshaping human antibodies for therapy." *Nature* **332**(6162) 323–327.
- Rice D. & Baltimore D. (1982). "Regulated expression of an immunoglobulin k gene introduced into a mouse lymphoid line." *Proc Natl Acad Sci USA* **79**, 7862–7865
- Saiki R.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B. & Ehrlich H.A. (1988). "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase." *Science* **239**(4839), 487–91
- Schibler U., Marcu K.B. & Perry R.P. (1978). "The synthesis and processing of the messenger RNAs specifying heavy and light chain immunoglobulins in MPC-11 cells." *Cell* **15**, 1495–1509
- Schroeder H.W. & Cavacini L. (2010). "Structure and Function of Immunoglobulins" *J Allergy Clin Immunol.* **125**(202):S41–S52.
- Skerra A. & Pluckthun A. (1988). "Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*." *Science* **240**(4855), 1038–1041
- Smith G.P. (1985). "Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface." *Science* **228**(4705), 1315–7.
- Hozumi N. & Tonegawa S. (1976). "Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions." *Proc Natl Acad Sci USA* **73**, 3628–32
- Tomlinson I.M., Walter G., Marks J.D., Llewelyn M.B. & Winter G. (1992). "The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops." *J Mol Biol* **227**, (3) 776–798.
- Ueda H., Kristensen P. & Winter G. (2004). "Stabilization of antibody VH-domains by proteolytic selection." *J Mol Catalysis B: Enzymatic*, **28**, 173–179.
- Verhoeyen M., Milstein C. & Winter G. (1988). "Reshaping human antibodies: grafting an anti-lysozyme activity." *Science* **239**(4847), 1534–1536.
- Ward E.S., Gussow D., Griffiths A.D., Jones P.T. & Winter G. (1989). "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*." *Nature* **341**(6242), 544–546.
- Waterhouse P., Griffiths A.D., Johnson K.S. & Winter G. (1993). "Combinatorial infection and in vivo recombination: A strategy for making large phage antibody repertoires." *Nucleic Acids Res* **21**(9), 2265–2266.

- Williams S.C. & Winter G. (1993). "Cloning and sequencing of human immunoglobulin V lambda gene segments." *Eur J Immunol* **23**(7), 1456–1461.
- Winter G. (1989). "Antibody engineering." In: *Phil Trans R Soc Lond B Biol Sci* **324**(1224), 537–547.
- Winter G. & Milstein C. (1991). "Man-made antibodies." *Nature* **349**(6307), 293–299.