# Current methods for the generation of human antibodies for the treatment of autoimmune diseases

Jane Osbourn, Lutz Jermutus and Alex Duncan

**Autoimmune diseases are a significant area of unmet medical need in the Western World, but human antibodies are an emerging drug class that could address this demand. Some autoimmune diseases, such as rheumatoid arthritis, are currently benefiting from antibody treatment and new and existing technologies for antibody generation could facilitate the production of effective human antibodies as future drug candidates for other autoimmune diseases. Several methods of generating human antibodies for use as therapeutics have been established, the most commonly used being phage display and transgenic mouse technologies and more recently, cell-free display technologies have also emerged. In this review, we explain the principles behind the various methods of antibody generation and highlight some potential benefits of certain approaches in the context of treatment of autoimmune disease.**

**Jane Osbourn***
**Lutz Jermutus**
**Alex Duncan**
Cambridge Antibody Technology, Milstein Building
Granta Park, Cambridge
UK CB1 6GH
*e-mail: jane.osbourn@cambridgeantibody.com

The American Autoimmune Related Diseases Association (www.aarda.org) estimates that there are >80 autoimmune diseases, which, collectively, after heart disease and cancer, cause one third of all premature deaths and disability in the Western World. Among the most prevalent are Graves' Disease, rheumatoid arthritis (RA), Hashimoto's thyroiditis, multiple sclerosis (MS), Raynaud's syndrome, systemic lupus erythematosus (SLE), Sjogren's syndrome, scleroderma and Crohn's disease [1].

## Disease mechanisms

The normal function of the immune system is to respond to invading microorganisms by producing antibodies or sensitized lymphocytes to recognize and destroy them. Autoimmune diseases occur when the normal function of the immune system is disrupted and antibodies to the patient's own cells and tissues are produced. These antibodies can interfere with cellular function and initiate tissue destruction, producing inflammation in the surrounding tissue or organ. The disease-producing processes in autoimmunity are referred to as hypersensitive reactions, which are similar to those observed in allergy, except that in autoimmune disorders, the hypersensitivity response is to the body itself, rather than to an outside substance.

Autoimmune disorders are classified into organ-specific and non-organ-specific types. In organ-specific types, the reaction is directed against one particular organ – examples include Hashimoto's thyroiditis (thyroid) and Addison's disease (adrenal glands). In non-organ-specific disorders, the effects are widespread throughout the body, for example SLE and RA. Most autoimmune diseases are chronic, requiring lifelong care and monitoring, and no cure for any of these diseases has yet been developed.

## Current treatments

In autoimmune disorders, such as RA, established treatments relieve pain and inflammation, or suppress the immune response. Pain-relief drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids reduce inflammation but are also associated with a wide range of side-effects. Commonly used NSAIDs, such as the cyclooxygenase 2 (COX-2) inhibitors, can cause a variety of problems, including gastrointestinal toxicity, which results in abdominal pain, nausea, indigestion and ulcers [2,3]. Corticosteroids, such as prednisone, are highly effective at relieving pain but can cause weight gain [4] and, in some patients, can contribute to the development of osteoporosis [5].

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Alternative RA treatments, aimed at slowing joint destruction, include a range of widely used disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate and chlorquine. Although effective, these drugs must be taken for several months before they show any positive effects. They are also non-specific to the site of inflammation and therefore, cause side effects ranging from abdominal discomfort, rashes, gastrointestinal toxicity and nausea to decreases in platelets, or white and red blood cell counts [6].

The variety of severe side effects associated with RA treatments has prompted a move towards the development of drugs that directly target the causes of the disease rather than attempting to alleviate the symptoms. For RA treatment, these 'biological response modifiers' (BRMs) directly target the cytokines, such as tumour necrosis factor (TNF-α), that are involved in the development and progression of the disease. Treatments include Enbrel®, a TNF-α receptor protein [7], and the antibody drugs Remicade® [8] and HUMIRA™ [9], which bind specifically to TNF-α. In clinical trials, these BRMs have demonstrated efficacy in reducing the symptoms of RA and in inhibiting joint erosion. Antibodies are particularly suited to the role of a BRM, because they can bind specifically and with high affinity to a wide range of biological molecules and, because they have a longer half-life than small-molecule treatments.

## Monoclonal antibodies

The production of hybridoma cell lines is perhaps the most established technique for generating monoclonal antibodies [10]. Hybridomas are generated by immunizing a mouse with a target antigen, then fusing the antibody-producing B cells of that mouse with mouse tumour cells. The hybridoma possesses the immortal growth properties of the tumour cell and thus, can be cultured indefinitely, and can secrete antibody with the specificity of the original B cell. The homogenicity and specificity of monoclonal antibodies that are produced from hybridomas makes them particularly suitable for *in vivo* administration for therapeutic purposes. However, clinical studies using mouse monoclonal antibodies have been disappointing, because the human immune system recognizes the mouse antibodies as 'foreign'. This results in rapid clearance of the mouse protein by the immune system of the patient and, in some cases, can lead to the induction of a severe allergic reaction to the mouse antibodies – an effect known as the human anti-mouse antibody (HAMA) response.

## Chimaeric monoclonal antibodies

To reduce the potential for HAMA responses, variable regions from mouse antibody genes can be recombined with constant regions from human antibody genes. The recombinant gene encodes a chimaeric mouse–human antibody that has antigenic specificity derived from the mouse, but a human isotype. Hence, the resultant protein has human effector functions and fewer mouse antigenic determinants, and is therefore less likely to be immunogenic in humans. Mouse variable regions can also cause an immune response in humans, and chimaeric antibodies containing only mouse complementarity determining regions (CDRs) have subsequently been developed. CDRs are the regions of the antibody molecule that are largely responsible for antibody–antigen binding and including only these elements rather than the full variable region of the antibody, further reduces the risk of immunogenicity. Replacing some mouse CDR sequences with human sequences can further humanize antibodies; mouse residues are minimized but retained at the key binding regions, to maintain affinity.

By the start of 2003, nine monoclonal antibodies had been granted a biologics licence approval (BLA) by the US Food and Drug Administration (FDA) (www.fda.gov) and all of these were of the chimaeric mouse–human or humanized type. These antibodies are being successfully used to treat a variety of conditions, including leukaemia, organ rejection, non-Hodgkin's lymphoma and breast cancer. In addition, one of these chimaeric antibodies, Remicade®, is approved for the treatment of the autoimmune disorders, RA and Crohn's disease, and there are also several other chimaeric or humanized antibodies currently in clinical trials for the treatment of a range of autoimmune diseases (Table 1).

Many chimaeric antibodies still cause adverse events, such as headache, fever, chills [11] and respiratory infection [12] and, in some patients, these events can be associated with allergic reactions to the drug. One solution to some of the immunogenic responses induced by chimaeric antibody drugs is to develop human antibody therapeutics, which contain no mouse-derived sequences. Transgenic mice and phage display techniques are the key technologies that have been widely used to isolate such human antibodies.

## Transgenic mice

Transgenic mouse technology for antibody drug discovery involves the introduction of human antibody genes into the mouse genome [13,14]. The mice are engineered to suppress their own antibody production and large sections of the human antibody heavy and light chain loci are then transferred into these animals; the human antibody genes are functional in the context of the mouse machinery for antibody recombination and expression. The mouse antibody-

**Table 1. Chimaeric and humanized antibodies developed for the treatment of autoimmune diseases**

| Product name | Generic name | Antibody type | Indication | Clinical development phase | Company |
|---|---|---|---|---|---|
| Remicade® | Infliximab | Anti-TNF-α | Crohn's Disease, RA, psoriasis | FDA approved | Centocor |
| Campath® | Alemtuzumab | Anti-CD52 | MS | FDA approved | BTG, ILEX, Millennium, Schering AG |
| Raptiva™ | Efalizumab | Anti-CD11 | Psoriasis | Phase III<br>BLA filed | Xoma, Serono, Genentech |
| Antegren® | Natalizumab | Anti-$\alpha_4$ integrin | MS | Phase III | Elan, Biogen, Genzyme |
| Humicade™ | CDP 571 | Anti-TNF-α | Crohn's Disease | Phase III | Celltech, Biogen, Genzyme |
| CDP 870 | CDP 870 | Anti-TNF-α | RA and Crohn's Disease | Phase III | Celltech, Pharmacia |
| Antegren® | Natalizumab | Anti-$\alpha_4$ integrin | MS, Crohn's Disease | Phase III | Elan, Biogen, Genzyme |
| MLN02 | MLN02 | Anti-$\alpha_4\beta_7$ integrin | Crohn's Disease | Phase II | Millennium, Genentech |
| HuZAF | Fontolizumab | Anti-interferon-γ | Crohn's Disease, psoriasis | Phase II | PDL |
| IDEC-151 | Clenoliximab | Anti-CD4 | RA | Phase II | IDEC |
| Zenapax® | Daclizumab | Anti-IL-2 | Uveitis | Phase II | PDL |
| 5G1.1 | Eculizumab | Anti-C5 | RA | Phase II | Alexion |
| MEDI-507 | Siplizumab | CD2 agonist | Psoriasis | Phase II | Biotransplant, MedImmune |

Abbreviations: IL, interleukin; MS, multiple sclerosis; RA, rheumatoid arthritis; TNF, tumour necrosis factor.

producing B cells can then be used to generate hybridoma cell lines for human monoclonal antibody production. In the usual time span required for hybridoma generation and characterization, a transgenic mouse can produce a range of specific antibodies from which potential drug candidates can be identified. If further optimization of the antibody is required, the genomic information that encodes the antibody of interest must be cloned and alternative antibody engineering approaches employed.

Companies using this approach include Abgenix (http://www.abgenix.com) and Medarex (http://www. medarex.com). Medarex has licensed its technology to Genmab A/S (http://www.genmab.com) and also collaborates with Kirin Brewery (http://www.kirin.co.jp/). These companies have taken antibody drugs into various stages of clinical trials, with Medarex having two antibodies to treat different autoimmune diseases in mid-stage clinical trials (Table 2). No significant allergic reactions have been noted so far and thus, the safety profiles seem better than with chimaeric antibodies.

## Phage display

The other established method of developing human monoclonal antibodies is phage display technology. This

**Table 2. Human antibodies in development to treat autoimmune diseases**

| Product name | Generic name | Antibody type | Indication | Method of generation | Clinical development phase | Company |
|---|---|---|---|---|---|---|
| HUMIRA™ | Adalimumab | Anti-TNF-α | RA | Phage display | FDA approved | CAT, Abbott |
| HuMax-CD4 | | Anti-CD4 | Psoriasis | Transgenic mice | Phase II | Genmab, EISAI |
| HuMax-IL15 | | Anti-IL-15 | RA, psoriasis | Transgenic mice | Phase II | Genmab, Medarex |
| HUMIRA™ | Adalimumab | Anti-TNF-α | Juvenile RA, Crohn's Disease | Phage display | Phase II | CAT, Abbott |
| CAT-192 | Metelimumab | Anti-TGF-β | Scleroderma | Phage display | Phase II | CAT, Genzyme |
| ABT-874 | | Anti-IL-12 | Crohn's disease, RA | Phage display | Phase II | CAT, Abbott, Wyeth Research |

Abbreviations: IL, interleukin; MS, multiple sclerosis; RA, rheumatoid arthritis; TGF, tissue growth factor.

**Figure 1.** A filamentous phage displaying an antibody protein at its tip. An antibody variable region sequence is inserted into the phage genome linked to the gene 3 coat-protein sequence. The antibody sequence is then 'displayed' as a gene 3 fusion protein on the outside of the phage. The key feature of phage display is that the phenotype and genotype are linked in a single molecule.

technique, as first described for antibodies in 1990 [15], exploits the ability of filamentous bacteriophage, such as M13, to express foreign proteins on the phage surface (Figure 1), enabling the gene and gene product to be physically linked.

Phage antibody libraries are produced by cloning variable regions of human antibody genes, derived from naïve, immunized or synthetic antibody repertoires, into the phage coat-protein genes of phagemid vectors. These libraries are introduced into *Escherichia coli* cells, which are then superinfected with helper phage, to facilitate the replication and assembly of viable phage particles. Phage libraries contain a huge variety of antibody variable region sequences [16] and some libraries now contain $>10^{11}$ unique phage antibodies. Each phage particle in the library encapsidates the DNA that encodes the antibody expressed on its surface and antibodies specific to a defined target can be selected from these large phage libraries by allowing the library to bind to immobilized antigen – phage antibodies that bind to the antigen are thereby captured and non-binding phage washed away. Antibody variable regions selected using this technique can be reformatted into IgG molecules by combination with human antibody constant regions and subsequently expressed in mammalian cell lines.

The great advantage of phage display is that once a library has been created, it can be used to select for antibodies that bind to any target antigen of interest, with the selection and primary screening process being completed in only 2–3 weeks.

Selection from the library can also be tailored to produce a range of output populations, depending on the desired characteristics of the antibody drug. For example, it might be necessary to generate an antibody with the ability to bind to a specific epitope on the target molecule, the ability to cross-react with a rodent antigen, or the ability to recognize a given cell type to name but a few. When an antibody with the desired characteristics has been isolated, the phage system also provides the format for the engineering of antibody affinity or potency. The diversity of antibody populations generated using phage display means that antibodies that bind to different parts of the same target antigen molecule can be isolated. This also makes the technique highly applicable to functional genomics, because it can rapidly generate lead candidate antibodies to large numbers of proteins in high throughput.

Several biotechnology companies use phage display technology to produce human antibody therapeutics, including Cambridge Antibody Technology (CAT, http://www.cambridgeantibody.com), Crucell (http://www.crucell.com), Dyax (http://www.dyax.com) and MorphoSys (http://www.morphosys.de/). The world's first human antibody to treat RA, HUMIRA™, is now on the market. This antibody was isolated and optimized by CAT in collaboration with Abbott Laboratories (http://abbott.com). All the human antibodies that are currently in clinical trials (from Phase II) for autoimmune diseases are listed in Table 2.

HUMIRA™ demonstrates the effectiveness of human monoclonal antibodies derived from phage display. Published data from a study of 271 patients showed that HUMIRA™ improved the symptoms of RA in up to 50% of patients. In addition, only minor side effects, such as injection-site reactions, rashes and headaches were reported, suggesting that the drug is safe and well tolerated at the doses used [9].

Abbott submitted information from this trial and several others to the FDA and European Agency for the Evaluation of Medicinal Products (EMEA, http://www.emea.eu.int/), obtaining marketing approval from the FDA on 31 December 2002. This made HUMIRA™ the first human monoclonal antibody therapy on the market for the treatment of an autoimmune disease.

## Emerging *in vitro* display technologies for creating human antibodies

Transgenic mice and phage display are both cell-based development technologies, and hence, they are limited by the physical constraints of getting DNA into a cellular host. To overcome this restriction, two new cell-free translation technologies have been developed, known as 'covalent display' and 'ribosome display'. These techniques promise the rapid construction of display libraries, containing up to $10^{14}$ molecules – significantly larger than phage display libraries – and thus, provide an increased probability that the desired specificity and potency required in the therapeutic candidate can be directly selected.

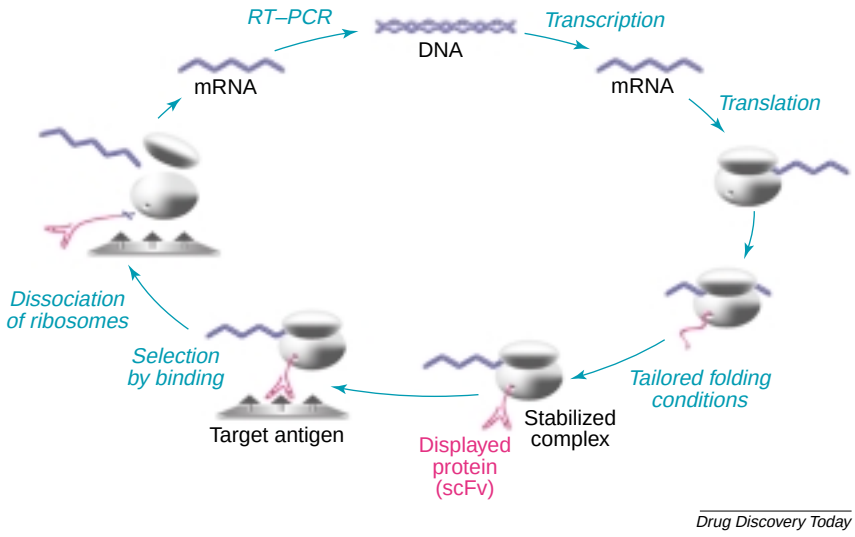


**Figure 2.** The ribosome display process. This cell-free process, analogous to phage display, involves the following steps: i) *in vitro* transcription of an antibody DNA library; ii) *in vitro* translation of mRNA molecules that contain a short spacer sequence and lack a stop codon; iii) folding of antibody molecule while tethered to the ribosome and encoding mRNA; iv) selection of antibody–ribosome–mRNA complexes by binding to immobilized target antigen (any non-binding antibodies are washed away); v) release of selected mRNA molecules by alteration of wash conditions and; vi) generation of DNA by RT–PCR for use in further rounds of selection.

### *Ribosome display*

Ribosome display works by exploiting the cellular role of ribosomes to synthesize proteins. Ribosomes do this by binding to mRNA and travelling along the molecule, reading the nucleotide sequence and assembling a chain of amino acids as they move. In the cell, the fully formed protein is then released and the mRNA dissociates from the ribosome. The ribosome display system uses mRNA encoding a repertoire of antibody genes in a cell-free expression system (either *E. coli* or eukaryotic cell lysate), so enabling assembly of antibody molecules *in vitro*.

A short mRNA spacer is included at the beginning of the antibody sequence to ensure that the antibody molecule is fully extruded from the ribosomal tunnel. Cellular mRNA molecules have a start and stop codon on either side of the protein coding sequence; in the ribosome display system, the stop codon at the end of the antibody-coding region is removed, preventing the release of the newly translated antibody protein. The antibody remains tethered to the ribosome, which, in turn, remains associated with the corresponding antibody gene in the form of mRNA (Figure 2). Conditions in the cell-free lysate environment can be tailored to ensure the correct folding of the antibody molecule.

The ribosome display selection process is analogous to that of phage display, with antibody–ribosome– mRNA complexes being selected by binding to immobilized target antigen and any non-binding antibodies being washed away. The selected mRNA can be released from the ribosomes by alteration of the salt concentration of the wash buffer. RT–PCR can then be performed to generate a population of selected DNA that can be used as the starting point for further rounds of selection

Using ribosome display, single-chain antibodies specific for a yeast transcription factor GCN4 have been selected [17]. In addition, ribosome display has been successfully used to generate anti-progesterone antibody fragments [18]. There is also evidence that ribosome display can produce variable domain (CTLA-4) libraries and could be used to provide specific targeting to either novel or refractory cancer markers [19]. Ribosome display technology (acquired and developed for commercial purposes by CAT) has the potential to become a versatile tool for the development of human monoclonal antibodies [20] with picomolar affinities [21].

The main advantage of this technique is that it has the potential to generate larger libraries of antibodies than phage display, hence producing still more diverse selectable populations, while offering many of the same benefits of a phage display library. These include the ability to produce and screen the library for binding activities in a matter of weeks and the provision of a format for the directed evolution of antibodies to improve desirable characteristics, such as affinity, stability and potency [22].

### *Covalent display*

Other emerging display technologies, known as covalent display, have been developed by Isogenica [23] (http:// www.isogenica.com) and Phylos [24] (http://www.phylos.com).

The Phylos PROfusion™ mRNA display system relies upon puromycin-mediated covalent coupling of protein to the RNA that encodes it [25], and is conducted entirely as isolated biochemical reactions, outside of the host cells. The process begins with oligonucleotide synthesis to construct a population of DNA molecules, each containing defined regions with signals for RNA transcription, protein translation, purification tags and PCR amplification. The molecules also have regions of diversity, which encode for a protein scaffold and a unique protein-binding site [26].

The library DNA is transcribed to generate RNA and a peptide acceptor is covalently attached to the 3′ end of mRNA, using a synthetic linker. During *in vitro* translation in a cellular lysate, the ribosome moves along the mRNA to generate a protein.

When the ribosome has reached the end of the encoding mRNA, it pauses, allowing time for the peptide acceptor to covalently link to the C-terminus of the protein. The result is a collection of up to $10^{13}$ possible binding proteins, each chemically linked to the RNA that encodes it.

The molecules can then be purified from the reaction mixture using target proteins that are captured on magnetic beads. The beads are washed to remove nonspecific or weakly binding molecules and the nucleic acid of the PROfusion™ molecules that bind to the target molecule are recovered by PCR, creating a new pool of nucleic acids enriched with high-affinity binders for the target of interest.

The Isogenica system uses a replication initiator protein (P2A) from the *E. coli* phage, P2, to initiate rolling circle replication by binding to the viral origin and introducing a nick in the DNA. This exposes the 3′-OH group, which then initiates DNA synthesis [27–29]. Nicking also exposes a 5′ phosphate, which attaches to a tyrosine residue in the active site of P2A, thus binding the P2A to the DNA molecule from which it has been expressed. This enables pools of polypeptides that are fused to P2A to be synthesized *in vitro*, so that they become attached to their own coding sequence and can be selected on an immobilized antigen.

Recently, the Phylos PROfusion™ mRNA display system has been used to produce protein microarrays [30]. However, neither of the technologies described has, to date, generated a therapeutic antibody, although development is ongoing.

## Summary and prospects

One chimaeric monoclonal antibody (Remicade®) has been FDA-approved as a treatment for RA and Crohn's disease and 11 are in mid–late-stage clinical trials; to date, chimaeric or humanized antibodies are the most commercially advanced types of antibody therapeutics for the treatment of autoimmune diseases. This is due partly to the fact that methods for generating chimaeric antibodies have been available for longer than the alternative technologies and have, therefore, produced more antibodies for clinical development. Many of these antibodies have significant side effects relating to their immunogenicity in human patients.

With the approval of HUMIRA™ (the first human antibody to treat RA) and an additional four human antibodies in Phase II or III trials for the treatment of autoimmune diseases, transgenic mice and phage display systems are now proving effective in the field of drug development. The benefit of these technologies is the ability to generate wholly human antibodies, which do not produce the immunogenic side effects that are seen with infusions of chimaeric antibodies. Short lead-generation times for antibodies made by phage display or transgenic mouse technology and, in the case of phage display, the ability to further optimize these lead candidates, makes the possibility of high potency therapeutics from these methods a reality.

Owing to their relatively recent emergence, neither covalent nor ribosome display has so far produced any human antibodies for use as clinical candidates in autoimmune disease. However, both technologies can produce libraries that are potentially larger than either phage display or chemical libraries, and so they could, in the next five years, become more widely adopted. Successful technical developments already demonstrated within the ribosome display system could mean that these technologies become the systems of choice for the generation and optimization of human antibodies against new drug targets for autoimmune diseases in the near future.

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