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Therapeutic Antibodies and Antibody Fusion Proteins

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Introduction

For the past few decades, antibodies have been anticipated as ideal molecules for the therapy of cancer and infectious disease. Their emergence as an attractive therapy is the result of the evolution of monoclonal antibody technology from fully mouse to fully human antibodies. Today, a large number of antibody-based clinical trials are now well under way, ranging from cancer, autoimmune disease, viral infection, allograft rejection, asthma, stroke, and glaucoma surgery, to name just a few. These trials constitute approximately 30% of all clinical trials registered by the Food and Drug Administration (FDA) in the USA (not including vaccine and gene therapy). Thus, antibodies account for one of the largest groups of biotechnology-derived molecules in clinical trials, and have an estimated potential market of several billion US dollars. This is a big difference to the status of therapeutic monoclonal antibodies seen in the early 1990s, when failing clinical trials, toxicity, and expensive production did not favour the production and marketing of antibodies. When the first monoclonal mouse antibodies entered clinical studies in the early 1980s, they were considered as the stepping-stones for the treatment of cancer. The development of hybridoma technology and the steady advances in monoclonal antibody (MAb) production revitalized the initial concept of Ehrlich concerning the existence of specific cancer

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ADEPT, antibody-directed enzyme prodrug therapy; APC, antigen-presenting cells; CDC, complement-dependent cytotoxicity; CDR, complementarity determining region; CEA, carcinoembryonic antigen; FDA, Food and Drug Administration; HAMA, human anti-mouse antibody; Ig, immunoglobulin; MAb, monoclonal antibody; NK cells, natural killer cells; RIT, radio-immunotherapy; scFv, single-chain Fv fragment; TAA, tumour-associated antigen; TAP, tumour-activated prodrug; VH, variable heavy chain; VL, variable light chain.

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cell-targeted 'magic bullets'. However, it soon became clear that these initial antibodies had limited therapeutical value. The human immune system recognized the monoclonal murine antibodies as foreign material, and produced 'human anti-mouse antibodies' (HAMA) to clear these from the body. Thus, repeated treatment with these antibodies had little therapeutic effect.

With the age of genetic manipulation, recombinant manipulation and production methods were created to tackle all of these problems. First, chimeric antibodies were produced, followed by humanized and, finally, fully human antibodies capable of binding to almost any given antigen. In particular, antibody fragments, capable of keeping their binding capacity, have become increasingly popular. These recombinant antibody fragments are being fused to a range of molecules, such as radioisotopes, drugs, toxins, enzymes, and biosensor surfaces, to name just a few. Indeed, the vast opportunities of fusing functional domains go beyond the capabilities of the naïve antibodies which nature has evolved them for.

However, one must keep in mind that the recombinant antibodies now being approved or tested in clinical trials are considered simple designs, conceived over 10 years ago. The process of 'proof of principle' of a novel therapeutic fusion protein and development into a commercial product requires at least a decade of work. Thus, the majority of antibodies tested today have been derived mainly from well-characterized hybridoma antibodies, and sharing the naïve IgG structure of their parent molecule. We can be sure that the following decades will bring about an increase in fully human recombinant antibodies, introducing fewer side effects, onto the market, and keeping the idea of the 'magic bullet' alive. Heterologous and homologous fusions will also increase their presence, providing novel approaches in therapy.

This review will initially mention the various antibodies already approved by the FDA, and then focus on the various possible antibodies and antibody fragments useful for clinical trials. The number of antibody fusion proteins which have been designed and produced is almost unlimited. At this point, we want to emphasize that a very important class of antibody fusion proteins use the Fc portion of an Ig fused to heterologous targeting or effector domains, such as cell surface receptors, cytokines, or other ligands. This review will not discuss this already extended and rapidly growing family of antibody fusion proteins, but rather focus on the subset which utilizes the Ig antigen-binding domain for targeting. We will focus on fusion proteins of antigen-binding sites used for therapy, including immunotoxins, cytokines, and even possible bispecific fusion antibodies. Subsequent parts of this review will focus on antibody fusion protein expression systems available today, and what the future could possibly have in store for us.

Therapeutic antibodies

ANTIBODIES AND ANTIBODY FRAGMENTS

The IgG molecule is composed of two identical light and heavy chains that are held together by disulphide bonds, having an approximate molecular weight of 150 kDa. Each heavy chain consists of four globular-shaped domains, the variable heavy domain (VH) and the three constant heavy domains (CH1, CH2 and CH3). The light chain consists of two globular-shaped domains, the variable light domain (VL) and

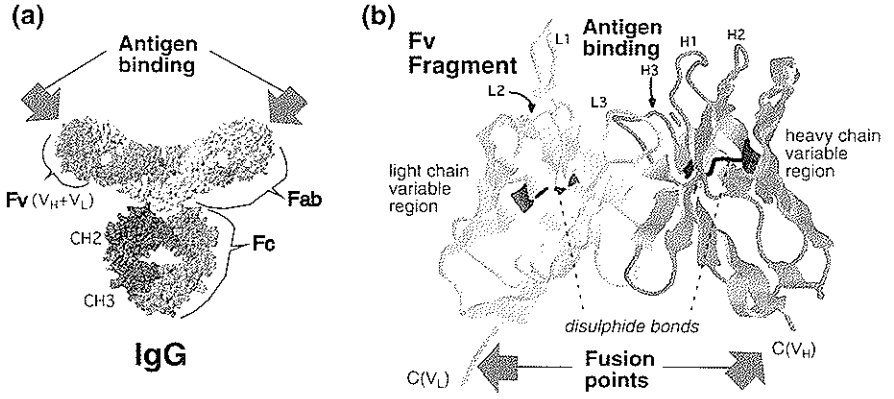

Figure 7.1. (a) Three-dimensional structure of an IgG; (b) alpha C atom backbone of the antigen-binding fragment (Fv fragment) showing the six hypervariable loops (H1–3, L1–3), which constitute the contact area to the antigen. The generation of fusion proteins using an antibody fragment for targeting is facilitated by the opposite locations of the antigen-binding surface and the carboxy-terminus of the variable domains.

the constant light domain (CL). Each VH and VL contains three complementarity-determining regions (CDRs) with hypervariable sequences that can bind to almost every biological structure. These CDRs are brought into close proximity by the folding of the V regions, forming a binding surface at the tip of the molecule that contacts the antigen. The CH2 and CH3 domains are responsible for various non-antigen-binding functions, such as binding to complement and binding to Fc receptors (*Figure 7.1*).

With the advent of hybridoma technology in 1975, it was – for the first time – possible to produce a single, homogeneous antibody of a defined antigen specificity (Köhler and Milstein, 1975). These hybridomas produce murine monoclonal antibodies, limiting their usefulness in therapeutic applications as repeated administration of murine antibodies causes a 'human anti-mouse antibody' response (HAMA) (Courtenay-Luck *et al.*, 1986; Lamers *et al.*, 1995) that can neutralize the therapeutic effect, or possibly even harm the patient.

To reduce antigenicity of murine antibodies, chimeric antibodies have been developed, consisting of mouse variable regions and human constant regions (Morrison *et al.*, 1984). A further step forward was the construction of humanized antibodies, in which only the six CDRs within the VH and VL were murine, whereas all other portions of the molecule were replaced by human sequence (Jones *et al.*, 1986; Studnicka *et al.*, 1994). These humanized antibodies are better tolerated in humans.

One disadvantage of whole IgG molecules is that they do not penetrate solid tumours very well. This problem was tackled by generating smaller antibody fragments that retain the targeting function, i.e. the binding to their specific antigen. These fragments can be made by proteolytic treatment of purified whole antibodies. The digestion of an IgG with the protease pepsin results in the production of a $F(ab')_2$ molecule that contains two antigen-binding sites, which can bind bivalently to the antigen. The treatment of an IgG with the enzyme papain generates monovalent Fab

fragments. Both methods, however, sometimes suffer from unpredictable yields, and are impractical, particularly if an additional proteolytic cleavage site is present in one of the hypervariable loops of a particular antibody. Therefore, a consequent approach is to generate antibody fragments by recombinant means. A major breakthrough in the field of antibody engineering has been the generation of antibody fragments as recombinant proteins in the periplasm of *E. coli* (Better *et al.*, 1988; Huston *et al.*, 1988; Skerra and Plückthun, 1988), where the oxidizing milieu and chaperones for correct folding are provided.

The most prominent recombinant antibody fragment is the single chain Fv fragment (scFv), where the two V region polypeptides (VH and VL) are covalently connected via a peptide linker. The peptide linker connects the C-terminus of VH or VL with the N-terminus of the other domain (Huston *et al.*, 1988; Plückthun, 1991). ScFv fragments, however, despite their simplicity in lab use, are generally not the preferred targeting fragments for therapeutical applications due to their often-limited stability. A structurally stabilized variant is the disulphide stabilized Fv fragment (dsFv), where the VH and VL heterodimer is stabilized by engineering a disulphide bond at the interface of the two domains (Glockshuber *et al.*, 1990; Brinkmann *et al.*, 1993; Reiter *et al.*, 1996). ScFv and dsFv antibodies represent the smallest complete antigen-binding fragments, and are most commonly produced by direct expression of the recombinant fragments in *E. coli*. Single domain antibody fragments have been described as well, but still have to prove their relevance for therapy.

Fab fragments can also be produced as recombinant proteins in *E. coli* (Better *et al.*, 1988). They are composed of two polypeptides (VH-CH1 and VL-CL), connected to each other via the interchain disulphide bond between CH1 and CL. These smaller versions of antibodies retain specificity to the antigen, and improve the potential to penetrate a solid tumour. However, as seen in scFvs and dsFvs, they lack the avidity of a naïve IgG due to their monovalent binding (for a review of various recombinant antibody formats, see Hudson, 1998).

The small Fab (50 kDa) and scFv (29 kDa) fragments are especially useful as tumour-imaging reagents, since they can rapidly penetrate solid tumours, and are quickly cleared from the blood via the kidneys. *In vivo* studies show that multimers in the 60–120 kDa size range, possessing two or more target-binding sites, have higher apparent affinity to the better avidity, and are ideal for tumour-targeting (Hu *et al.*, 1996; Adams and Schier, 1999; Hudson and Kortt, 1999; Viti *et al.*, 1999).

To overcome the limitations of hybridoma technology, recombinant human antibody libraries were developed for the selection of fully human monoclonal antibodies. These libraries contain millions of combinations of human variable region (VH and VL) genes derived from human donors. The antibody fragment gene repertoires are cloned into phagemid vectors, providing the functional antigen-binding moiety to be expressed on the surface of bacteriophage (Barbas *et al.*, 1991; Breitling *et al.*, 1991; Hoogenboom *et al.*, 1991; Marks *et al.*, 1991), where they are selected by affinity enrichment ('panning') on antigen. Alternatively, antibodies are provided in mice by insertion of more or less complete human immunoglobulin gene loci after knocking out the homologous mouse genes (Jakobovits, 1995, 1998; Fishwild *et al.*, 1996). In these systems, human antibody-producing mouse cell lines are generated by conventional immunization and hybridoma production.

ANTIBODY THERAPY

Classical therapeutic treatment, such as surgery, radiation, and chemotherapy, not only fail to cure the great majority of malignant tumours, but their employment could also lead to numerous side effects. With the appearance of MAbs on the market in the 1980s, a new approach was initiated, and the vision of the 'magic bullet' was brought alive. The hype did not last long as failing clinical trials limited the possibilities for treatment. Nevertheless, several optimists kept on, and the antibody-engineering era was born. The types of antibodies approved for clinical trials and in the clinic have been ongoing. Initially, chimerized and humanized full-length monoclonal antibodies (MAbs) were used. Subsequently, these antibodies were reduced to their antigen-binding fragments, and coupled in a variety of conjugates and fusion proteins, ranging anywhere from radioisotopes, drugs, toxins, enzymes, to constructs of diabodies or bispecific antibodies. From a statistical point of view, murine MAbs have been the least successful of the MAb products in trials, with a success rate of only 3%. Chimeric and humanized antibodies have higher success rates of 24% and 25%, respectively (Reichert, 2001). Further, humanized antibodies have shown the highest probability of completing phase 2 and entering phase 3 trials. The future of fully human antibodies remains to be seen. Thus, engineered antibodies have greatly refined and expanded the therapeutic potential of treatment.

FDA APPROVED ANTIBODIES

Sales of antibodies for cancer therapy alone have been estimated from virtually nil in 1996 to now well over US$1 billion. Nevertheless, many of the new antibodies reaching FDA approval are considered conventional designs, conceived over 10 years ago. Human antibodies isolated using phage display or from human Ig transgenic mice are now emerging onto the scene. However, disputes over patent rights are slowing down the application of these promising technologies. Since a decade of work is required for antibodies to reach clinical trials, fully human recombinant antibodies are only now entering the clinic. A tremendous amount of work already published on generation, engineering, and fusion variants builds a strong basis for an explosive growth in the future.

Initially, the types of antibodies entering clinical trials between 1980 and 1992 were predominantly murine MAbs. In 1986, the US Food and Drug Administration approved the first murine antibody onto the market. It was shown that muromonab-CD3 (OKT3®) was able to reverse the effects of acute rejection of kidney transplants (Shield *et al.*, 1996). However, this success was partly dependent on the fact that patients undergoing kidney transplantation were treated with immunosuppressants, and were therefore not as prone to the HAMA response as patients not treated. Between 1988 and 1994, chimeric and humanized antibodies began to emerge. Since 1997, they have become the main source of antibody-based products entering clinical trial studies. Fully human MAbs from transgenic mice are also emerging; however, there are differences between murine and human glycosylation patterns (especially for Galα1-3) that have yet to be resolved before applying *in vivo* therapeutics.

Therefore, therapeutic monoclonal antibodies approved for clinical use today are mainly derived from murine ancestors (*Table 7.1*). Two MAbs are entirely murine (OKT3®, Zevalin™), four have been chimerized, where the human constant domains

Table 7.1. FDA approved therapeutic antibodies

Product name/Company	Product/Indication	Approval	Reference
Orthoclone; OKT® 3 (muromonab)/ Ortho Biotech	Murine MAb; kidney transplant rejection; murine MAb; heart and liver transplant rejection	1986; 1993	(Shield *et al.*, 1996)
ReoPro® (Abciximab)/ Centocor	Chimeric MAb; prevention of blood clotting; refractory unstable angina	1994; 1997	(Glaser, 1996)
Rituxan® (Rituximab)/ Genentec	Chimeric anti-CD20 MAb; non-Hodgkin's lymphoma	1997	(Leget and Czuczman, 1998; Grillo-Lopez *et al.*, 1999)
Zenapax® (Daclizumab)/ Hoffmann-LaRoche	Humanized MAb; prophylaxis of acute renal allograft rejection	1997	(Ekberg *et al.*, 1999)
Simulect® (Basiliximab)/ Novartis	Chimeric MAb; reversal of acute kidney transplant rejection	1998	(Mulloy *et al.*, 1999)
Synagis® (Palivizumab)/ MedImmune	Humanized MAb; respiratory syncytial virus	1998	(Saez-Llorens *et al.*, 1998)
Remicade® (Infliximab)/ Centocor	Chimeric MAb; Crohn's disease; rheumatoid arthritis	1998; 1999	(Maini *et al.*, 1999; Present *et al.*, 1999)
Herceptin® (Trastuzumab)/Genentech	Humanized MAb; metastatic breast cancer	1998	(Goldenberg, 1999; Weiner, 1999)
Thymoglobulin®/Pasteur Meriex Serums et Vaccins, S.A.	Polyclonal antibodies from immunized rabbits; acute renal transplant rejection	1998	(Gaber *et al.*, 1998; Brennan *et al.*, 1999)
Nabi-HB™/Nabi	Polyclonal antibodies from patients with high titers of anti-HBs, exposure to HbsAg	1999	www.fda.gov
Mylotarg™/ Wyeth-Ayerst	Humanized MAb linked to calicheamicin; leukaemia; acute myeloid leukaemia	2000	(Williams and Handler, 2000; Larson *et al.*, 2002)
CroFab®/Protherics	Ovine Fab antibody fragment; minimal and moderate North American Crotalidae envenomation	2000	(Ruha *et al.*, 2002)
Campath® (Alemtuzumab)/ Millenium and ILEX Partners, LP	Humanized MAb; B cell chronic lymphocytic leukaemia	2001	(Thompson, 2001; Osterborg *et al.*, 2002)
DigiFab™/Protherics	Ovine Fab antibody; digoxin toxicity or overdose	2001	(McMillin *et al.*, 2002)
Zevalin™ (Ibritumomab Tiuxetan)/IDEC Pharmaceuticals	Murine MAb linked to chelator tiuxetan; radio-immunotherapy for non-Hodgkin's lymphoma	2002	(Alcindor and Witzig, 2002)

replace murine (ReoPro®, Rituxan®, Simulect®, Remicade®), and five have been humanized, where human residues are being substituted for at least some mouse-specific framework residues in VH and VL (Zenapax®, Herceptin®, Synagis®, Mylotarg™, Campath®). Rituxan® is the first FDA-approved MAb to treat cancer; Zevalin™ is the first FDA-approved radioimmunotherapy to treat non-Hodgkin's

lymphoma; Zenapax® is the first MAb approved to help prevent acute kidney transplant rejection, and Herceptin® is the first antibody-based drug for solid tumour therapy. The first fully humanized anti-CD52 (CAMPATH-1H, Campath®) was approved by the FDA in 2001 for the treatment of B cell chronic lymphocytic leukaemia, and produced by using the anti-CD52 CAMPATH-1 rat antibody as a target and the CDR-grafting technique (Verhoeyen *et al.*, 1988).

In addition, two Fab antibodies of ovine origin have been approved: CroFab®, a snake venom antidote, and DigiFab™ for digoxin toxicity (McMillin *et al.*, 2002). Two polyclonal antibody treatments are also being applied: Thymoglobulin® for acute kidney transplant rejection, and Nabi-HB™ for hepatitis B. From this list, it is evident that only Mylotarg™ can be classified as a 'non-classical' therapeutic antibody variant (not including the radio-labelled antibodies).

Engineering antibody fusion proteins

ANTIBODY FUSION PROTEINS

The effectiveness in the treatment of various diseases by unconjugated/unfused antibodies has been well established, but limited to certain cases. Most obvious, antibodies can act by neutralizing the binding site of a pathogen, such as bacteria or viruses. This requires a significant amount of antibody, since the therapeutic agent is in competition with the pathogen. Other antibodies can activate various effector systems of the body's own immune response against its target. Interestingly, in many cases, the exact mode of action is unknown, despite the clearly demonstrated clinical efficacy.

In order to overcome the principal limitations of 'naïve' Igs or Fabs, researchers have tried to generate new forms of antibodies. One approach is to generate novel antibody formats with improved potential to penetrate a solid tumour. Other proteins, such as cytokines, enzymes, and toxins, can be fused to an antibody or an antibody fragment to evoke a physiological reaction against the target. Subsets of these fusions are homologous fusions, where antibodies of two specificities are combined (bispecific antibodies). A manifold of strategies has been described for the generation of these fragments and fusion proteins. For a review, see Breitling and Dübel (1999), and Dübel and Kontermann (2001).

ANTIBODY CYTOKINE FUSION PROTEINS

Cytokines, such as interleukin-2 (IL-2), interleukin-12 (IL-12), and granulocyte/macrophage colony-stimulating factor (GM-CSF), are locally secreted proteins that enhance the host immune response (T cell, B cell, or natural killer (NK) cells) against a tumour. Systemically administered cytokines can activate a protective immune response against tumours (Tsung *et al.*, 1997; Rosenberg *et al.*, 1998). However, systemic treatment with cytokines can cause severe toxic side effects, so that it is impossible to achieve an effective dose at the tumour site (Siegel and Puri, 1991; Maas *et al.*, 1993). Antibodies specific for different tumour-associated antigens (TAA) genetically fused to cytokines can increase the effective concentration of cytokines at the tumour microenvironment.

IL-2, produced by T helper cells, is a 15 kDa lymphokine that stimulates T cells to proliferate and become cytotoxic for tumours. NK cells can also be stimulated by IL-2, and become cytotoxic for tumour cells (Lotze *et al.*, 1981; Grimm *et al.*, 1982; Hank *et al.*, 1990). When fused to a TAA-specific antibody, IL-2 can be concentrated at the tumour, where the lymphokine elicits a significant antitumour activity. An example for such an antibody fusion protein is the anti-DNS IgG3-IL-2, which is specific for the hapten DNS. Human IL-2 was fused after the CH3 domain of human IgG3 (Harvill and Morrison, 1995).

GM-CSF, a cytokine associated with growth and differentiation of haematopoietic cells, is a potent immunostimulator. It enhances antigen presentation in different cell types, increases proliferation of T cells, and enhances expression of MHC class II on monocytes (Morrissey *et al.*, 1987; Fischer *et al.*, 1988; Santoli *et al.*, 1988). Fusion of GM-CSF to an antibody specific for a TAA will enhance tumour-specific immune response. An example is a chimeric IgG1 fused to human GM-CSF (chCLL-1/GM-CSF), which is specific for human MHC class II of human B cell non-Hodgkin's lymphoma, chronic lymphocytic leukaemia, and multiple myeloma cell lines. Studies suggest that this antibody fusion protein may be useful for the therapy of B cell malignancies (Hornick *et al.*, 1997).

IMMUNOENZYMES

Antibodies or antibody fragments can be chemically or genetically fused to enzymes. These antibody–enzyme fusion proteins – or immunoenzymes – can be used for a variety of therapeutic concepts, most prominently for the treatment of cancer. There are two different basic strategies for delivering the drug to the target.

In the first approach, the enzyme itself is the drug, and is transported to the target by the antibody (direct targeting). Enzymes used for this approach include RNases and DNases. They are chemically or genetically fused to a whole IgG, or to an antibody fragment, such as a Fab or scFv. The antibody specifically binds to an antigen expressed on the tumour cell. After internalization of the fusion protein, the enzyme develops its toxic effect and kills the cancer cell. The most commonly used enzymes are the members of the RNase A superfamily. The bovine pancreatic RNase A and Onconase, a frog ribonuclease isolated from the oocytes of *Rana pipiens*, were the first ribonucleases used as single therapeutic agents in human clinical trials for the treatment of cancer (Aleksandrowicz, 1958; Darzynkiewicz *et al.*, 1988; Mikulski *et al.*, 1993). Other members of the RNase A superfamily are of human origin, e.g. hpRNase (human pancreatic RNase) (Zewe *et al.*, 1997), ANG (human angiogenin) (Strydom *et al.*, 1985), EDN (human eosinophil-derived neurotoxin) (Rosenberg *et al.*, 1989b), and ECP (human eosinophil cationic protein) (Rosenberg *et al.*, 1989a). All these RNases are small, basic proteins (12 kDa) that cleave RNA endonucleolytically after internalization into cells, where they cause protein synthesis inhibition and cell death. The non-human RNases, such as Onconase, are quite homologous to the human RNase A enzymes (60–70% homology and 30% identity in their primary structure), and elicit a surprisingly low anti-enzyme immune response in humans (Fett *et al.*, 1985; Gleich *et al.*, 1986).

Antibody RNase fusion proteins can be generated in several different ways. One

possibility is to fuse the gene encoding an RNase to a whole immunoglobulin molecule. An example of such a fusion protein is the CH2ANG, where the 5' region of the ANG gene has been fused to the 3' region of the CH2 domain of a chimeric anti-human transferrin receptor antibody (Hoogenboom *et al.*, 1990; Rybak *et al.*, 1992). Other RNase fusion proteins consist of an scFv genetically fused to an RNase. These scFv antibody fusions can be constructed with different architectures. One possible configuration of the scFv fusion is VH–VL, with the 3' region of the VL fused to the 5' region of an RNase gene. ScFv–RNase fusions have also been constructed with an opposite orientation of VH and VL in the scFv. An example is the fusion of the genes for three different human RNases (EDN, ANG, or hpRNase) to the 5' region of the VL of an scFv directed against the human transferrin receptor (Zewe *et al.*, 1997).

Another approach for delivering the drug to its target is indirect targeting, which consists of several stages. First, an antibody or antibody fragment fused to an enzyme is administered and allowed to bind to the target antigen at the cell surface. Time is given for unbound fusion protein to clear the circulation. In the next step, an inactive prodrug is administered that is converted to an active drug by the enzyme portion of the fusion protein. The active drug accumulates at the target cells, and causes their death. This technique is called ADEPT (antibody-directed enzyme prodrug therapy) (Bagshawe *et al.*, 1988). The antibodies or antibody fragments used in ADEPT experiments are commonly directed against tumour antigens, such as CEA, growth factor receptors (e.g. EGF, erbB2), and various other antigens on carcinoma cells. These antibodies or antibody fragments are conjugated by chemical or genetic means to different enzymes, such as β-lactamase, glucuronidase, alkaline phosphatase, and carboxypeptidase, which convert an inactive prodrug to an active drug. However, the use of chemical conjugates is limited, since chemical cross-linking agents cause a heterogeneous mixture of conjugate proteins (antibody- and enzyme-linked at different sites; different molar ratios of antibody to enzyme). Therefore, recent research has been directed toward the use of recombinant DNA technology to generate fusion proteins with more uniform properties for ADEPT. One example is the construction of a recombinant fusion protein consisting of an scFv fused to β-lactamase (L6-sFv-BC βL). The gene for β-lactamase was fused to the 3' region of the VH of an scFv directed against the ganglioside antigen present on breast, colon, lung, and ovarian carcinoma cells. In this case, the enzyme portion of the fusion protein catalyses the release of the drug phenylenediamine mustard from the prodrug cephalosporin mustard (Goshorn *et al.*, 1993).

Another example is the fusion protein dsFv3-β-lactamase that is directed against tumour cells overexpressing erbB2 (p185HER2). The β-lactamase gene was fused to the 3' region of the VH, and the VH and VL domains were connected by a disulphide bond. This fusion protein enhanced the killing of the target cells by converting the prodrug cephalosporin doxorubicin into the drug doxorubicin (Rodrigues *et al.*, 1995a,b). A third example is a fusion protein consisting of an scFv directed against p97/melanotransferrin, an antigen expressed on melanomas and carcinomas, linked to the *Enterobacter cloacae* β-lactamase. The β-lactamase gene was fused to the 3' region of the VL domain. The resulting scFv fusion protein, termed L49-scFv–bL, was designed to activate the prodrug cephalosporin nitrogen mustard in the environment of antigen-positive tumour cells (Siemers *et al.*, 1997).

IMMUNOTOXINS

Immunotoxins are fusion proteins consisting of an antibody or antibody fragment chemically or genetically linked to a toxin molecule. These fusion proteins may be potential therapeutics for the treatment of cancer. The antibody or antibody fragment is the targeting moiety that directs the molecule to the tumour cell; the fused toxin then internalizes and kills the target cell (for review, see Kreitman, 1999).

The toxins that are most commonly used to generate immunotoxins are diphtheria toxin (DT) and *Pseudomonas* exotoxin (PE) from bacteria, and ricin isolated from plants. These toxins are multi-domain proteins, composed of functionally similar subunits. To develop cytotoxicity, the toxins require three domains: for binding to the cell surface, for translocation to the cytosol, and for enzymatic inactivation of protein synthesis (Allured *et al.*, 1986; Katzin *et al.*, 1991; Rutenber and Robertus, 1991; Choe *et al.*, 1992). After entering the cell, the toxins induce apoptosis by inactivating protein synthesis. The genes of all three toxins have been cloned and expressed in *E. coli*.

The use of immunotoxins for therapy made by chemical linkage of a toxin to a whole antibody is limited by several problems, such as difficulties in production, and poor tumour penetration, due to the size of the whole complex.

By making immunotoxins with recombinant DNA technology, these limitations are overcome. Recombinant immunotoxins have been made by genetically fusing the bacterial toxins PE and DT to antibody fragments (scFv or dsFv). Recombinant PE immunotoxins consist of recombinant antibody fragments fused to the N-terminus of truncated PE derivatives (PE38 or PE40). In these derivatives, the cell-binding domain of PE had been deleted (Pastan and FitzGerald, 1991; Pastan *et al.*, 1992). In recombinant DT immunotoxins, the antibody fragments have been fused to the C-terminus of DT (Chaudhary *et al.*, 1990, 1991).

An example of a recombinant PE-derived immunotoxin is the anti-Tac(Fv)–PE38, also termed LMB-2, that consists of an scFv directed against CD25 linked to PE38. This immunotoxin may be useful for the treatment of B and T lymphoma and leukaemias (Chaudhary *et al.*, 1989; Kreitman and Pastan, 1995). In a second recombinant immunotoxin, RFB4(dsFv)–PE38 (BL22), PE38 was fused to a dsFv specific for CD22. BL22 may be used for therapy of B leukaemias (Mansfield *et al.*, 1997).

Although some recombinant immunotoxins, including LMB-2 and BL22, are in clinical trials, there are still limitations for their use as therapeutics. Systemically administered immunotoxins cause damage to normal tissue, with attendant side effects. Another unsolved problem is the immunogenicity of the foreign sequences in the toxin portion of the molecule (Kreitman, 1999).

BISPECIFIC ANTIBODIES

In contrast to the antibody fusion proteins that consist of an antibody or antibody fragment fused to a heterologous protein (e.g. enzyme, toxin, cytokine), it is also possible to generate homologous fusions, where antibodies of two specificities are combined. These homologous fusions are termed bispecific antibodies. A major use of these bispecific molecules in cancer therapy is to target a cytotoxic or phagocytic effector cell to a tumour cell. Thus, a bispecific antibody should be able to recognize

an antigen expressed on the surface of the tumour cell, as well as an antigen present on the surface of the effector cell.

Potent effector cells of the immune system are T cells, or NK cells, and myeloid cells (monocytes, macrophages, eosinophils, and neutrophils). In order to stimulate an effector cell, a trigger molecule on its surface must be engaged. Trigger molecules on T cells can be the TCR/CDR3 complex, CD2, and CD28 (Perez *et al.*, 1985; Staerz *et al.*, 1985; Scott *et al.*, 1988; Demanet *et al.*, 1996); trigger molecules on NK cells are CD16 and CD44 (Fanger *et al.*, 1989; Sconocchia *et al.*, 1994). Bispecific antibodies that link the CD3 complex on T cells to tumour-associated antigens expressed on cancer cells can stimulate the T cell, which becomes cytotoxic and kills the tumour cell. Human myeloid cells, such as monocytes, macrophages, and eosinophils, also express cytotoxic and phagocytic trigger molecules on their surface, including CD16, CD32, and CD64 (Fanger *et al.*, 1989, 1994). CD64, for instance, can mediate both antibody-dependent cellular cytotoxicity, and phagocytosis.

Bispecific antibodies can be made by chemical methods, or by using recombinant DNA technology. Due to the problems mentioned above with regard to chemical conjugation, most bispecific antibodies nowadays are generated by recombinant means, which offer all the advantages of recombinant antibody technology (e.g. making fully human antibodies, generating antibody fragments). The most common format for the generation of bispecific antibodies is based on the scFv format. Two scFv antibodies of different specificities are combined and stabilized by engineering either linker peptides or disulphide bonds. Another possibility for combining two scFv fragments is the use of heterologous dimerization domains, such as leucine zippers, or helix turn helix motifs (Kostelny *et al.*, 1992; Pack and Plückthun, 1992). A single chain, bispecific antibody has been directed against BCL1 lymphoma, and CD3 targets T cells to BCL1 lymphoma cells in a mouse model, so that the T cells killed the lymphoma cells. This bispecific antibody had been generated by fusing the DNA encoding an scFv directed against an antigen expressed on murine BCL1 lymphoma to the DNA encoding an anti-mouse CD3 scFv. The two scFv fragments were fused and stabilized by a linker peptide (de Jonge *et al.*, 1995, 1997).

The first recombinant bispecific antibody to enter clinical trials has been directed against CD3 and CD19. This bispecific scFv fragment is highly effective in redirecting primary T cells against CD19-positive lymphoma cells (Löffler *et al.*, 2000).

PEPBODIES AND TROYBODIES

Pepbodies are fusion proteins that consist of recombinant antibody fragments (scFv or Fab) genetically fused to different antibody effector molecules binding peptides. Therefore, pepbodies are able to cross-link an antigen and an antibody effector molecule. These fusion proteins may also be able to initiate antibody effector functions (Lunde *et al.*, 2002a).

Troybodies are antibodies specific for antigen-presenting cells that have a genetically inserted T cell epitope in their constant domain. These antibodies target the integrated T cell epitope to their specific antigen-presenting cell. This is meant to increase the number of T cell epitopes that reach the antigen-presenting cell, and the number of peptide–MHC complexes presented on the antigen-presenting cell is also

increased. The T cell epitopes are then presented to specific T cells. Thus, troybodies are able to enhance T cell activation (Lunde *et al.*, 2002a,b).

Emerging therapies

The generation of fully human antibodies and antibody fragments are becoming commercially available, and many have entered, or are soon to enter, clinical trials. Recent years have seen the production of recombinant antibodies reduced in size, dissected into minimal binding fragments, and rebuilt into multivalent, high avidity, multifunctional reagents. Emphasis has been placed on improving antibody efficiency (affinity and specificity), and on demonstrating their safety and reducing their immunogenicity.

Recombinant designs for antibody fusion are only really limited by one's own imagination, and an expression system capable of producing correctly folded protein products. Antibody fusion can be used, for example, to target transferrin receptors, and thereby cross the blood–brain barrier, carrying EGF-radionuclides and hammerhead ribozymes. Recombinant antibodies have been fused to viral capsid proteins to redirect viruses as gene therapy delivery capsules, and fused to liposome surfaces as drug delivery capsules. ScFv-fusions have been displayed on cell surfaces, and can be used to redirect cytotoxic T cells. ScFv-fusions of IL-2, GM-CSF and IL-12 have been used for cytokine stimulation and T cell proliferation at the target tumour site. Within the group of antibody-mediated drug delivery, the coupling of radionuclides to antibodies under the concept of a radio-immunotherapy (RIT) is, so far, the most commonly used approach. The radioisotopes most often used are iodine-131 and yttrium-90.

Only very few recombinant antibody fusion proteins have reached the clinical stages (*Table 7.2*), and almost all deal with humanized antibodies fused to various target molecules, usually an enzyme or a radionucleotide. It is therefore not surprising to still see so few of these 'high tech' proteins in the clinic today.

CANCER

The most common forms of treatment of cancers known today include surgery, radiation therapy, and chemotherapy. Often, these therapies prove to be ineffective or incomplete, and are often toxic to the patient. If the tumour is inaccessible, surgery is inadequate. Radiation and chemotherapy kill all dividing cells, whether cancerous or not, and many patients suffer from severe side effects. Immunoglobulins have evolved to optimally protect an organism from foreign invaders, rather than to act as an efficient carrier molecule for therapeutic reagents. Despite these potential limitations, our growing understanding of the biological and physiological principles that underlie targeted therapy has led to the development of a generation of novel reagents, and the first encouraging clinical trials.

Most targets for therapeutic antibodies today deal with extracellular molecules (cell-surface antigens) that allow the specific targeting of antibody to tumour tissue. A variety of different agents (e.g. toxins, radionuclides, chemotherapeutic drugs) have been conjugated to mouse and human MAbs for selective delivery to cancer cells (*Table 7.2*). Bispecific antibodies continue to be a major focus in cancer therapy,

Table 7.2. Examples for therapeutic fusion antibodies and conjugates in clinical trails

Fusion antibody	Clinical trial	Type of fusion antibody	Reference
MDX-220	Phase I	Bispecific antibody	*
MDX-H210	Phase II	Bispecific antibody	(Valone *et al.*, 1995; Curnow, 1997)
MDX-447	Phase II	Bispecific antibody	(Baselga, 2002)
MDX-RA	Phase III	Immunotoxin	*
RFB4(dsFv)–PE38 (BL22)	Phase I	Immunotoxin	(Kreitman *et al.*, 2000)
anti-Tac(Fv)–PE38 (LMB-2)	Phase I	Immunotoxin	(Keppler-Hafkemeyer *et al.*, 2000)
Mylotarg	FDA approved 2000	MAb conjugated with the antibiotic calcheamicin	(Williams and Handler, 2000; Larson *et al.*, 2002)
DsFv3–β-lactamase	Phase I	Ab-dependent enzyme mediated prodrug therapy	(Rodrigues *et al.*, 1995a,b; Hakimelahi *et al.*, 2001)
HuC242–DM1	Phase I	Ab-directed prodrug therapy	§
HuN901–DM1	Phase I/II	Ab-directed prodrug therapy	§

* www.newmedicines.org/meds/development/pipeline.phtml
§ ImmunoGen, Inc., www.immunogen.com

particularly for recruitment of cytotoxic T cells, or macrophages, or viruses (adenovirus or adeno-associated virus) for gene therapy. Consequently, approximately half of the estimated 80 antibody derivatives currently in development or under review by the FDA are being studied as treatments for cancer.

Antibody fusion proteins on trial

It is clear that genetically engineered antibody constructs provide an exciting approach to address, and subsequently overcome, some of the problems identified for unmodified IgG. These new constructs should be able to increase the dose fraction localized in tumours versus normal tissue, and thereby improve the delivery capacity. One of the possibilities to overcome the obstacles encountered by conventional radio-immunotherapy (RIT) is to inject antibody and radionuclide separately. The tumour-targeting specificity of the antibody is coupled to streptavidin, and the radionuclide to biotin. Examples include the humanized anti-p185HER2 antibody (humAB4D5-8) that has been used in a phase I clinical trial as a building block to engineer a disulphide-linked Fv (dsFv) β-lactamase fusion protein (dsFv3-β-lactamase) for use in antibody-dependent, enzyme-mediated prodrug therapy using cephalosporin-based prodrugs (Rodrigues *et al.*, 1995b; Hakimelahi *et al.*, 2001). NeoRX Corporation has completed patient enrolment in a phase I clinical trial of Pretarget® Lymphoma, where an anti-CD20-streptavidin fusion protein (B9E9) is used with the radio-therapeutic 90-Y-biotin conjugate to combat adult B cell non-Hodgkin's lymphoma. Still further, conjugates of the L49 monoclonal antibody (binds to the p97 antigen on melanomas and carcinomas) have been formed by attaching *Enterobacter cloacae* β-lactamase (bL) to the L49–Fab' fragment, or by linking the enzyme to L49–sFv using DNA recom-

binant technology. The conjugates have been designed to activate cephalosporin-containing anticancer prodrugs at the surfaces of antigen-positive tumour cells (Kerr *et al.*, 1999).

Tumour-activated prodrug (TAP) technology, developed by ImmunoGen, combines extremely potent, small-molecule drugs with MAbs that recognize and bind directly to tumour cells. Since TAPs are inactive until the drug is released from the antibody inside the target cell, each TAP acts as a prodrug. These drugs are able to kill cancer cells with minimal harm to healthy tissue. In antibody-directed enzyme prodrug therapy, an enzyme conjugated to an antitumour antibody is given i.v., and localizes in the tumour. A prodrug is then given, which is converted to a cytotoxic drug selectively in the tumour. Three phase I trails are under way to test the efficiency of HuC242–DM1 (cantuzumab mertansine) in combating colorectal, pancreatic, and certain non-small-cell lung cancers. The effector molecule, DM1, is a maytansinoid, and a potential inhibitor of cell division that can kill cancer cells at low concentrations. A second product of ImmunoGen is also in a phase I/II trial, huN901–DM1, for treatment of small-cell lung cancer. Another method, where a recombinant fusion protein composed of MFE-23 – an anticarcinoembryonic antigen (CEA) scFv antibody – has been fused to the bacterial enzyme carboxypeptidase G2 (CPG2), was shown to have potential to improve clinical efficiency for ADEPT (Bhatia *et al.*, 2000). For the cytokines, tumour necrosis factor alpha is a pleiotrophic cytokine produced by a variety of human cells, particularly activated macrophages and monocytes. For the ADEPT system, MFE-23::TNFα aims to reduce sequestration and increase tumour concentrations of systemically administered TNFα (Chester *et al.*, 2000; Cooke *et al.*, 2002).

Numerous immunotoxins have been applied, and few have reached phase I trials. To develop a T cell-based therapy for carcinomas, a monoclonal antibody–staphylococcal enterotoxin A recombinant fusion (Fab–SEA) protein was constructed for advanced pancreatic and colorectal cancer. The Fab fragment of the C242 MAb recognizing human colorectal (CRC) and pancreatic carcinomas (PC) has been used, and is now in phase I trials (Giantonio *et al.*, 1997; Nielsen *et al.*, 2000). A future candidate may be LL2, a murine anti-CD22 MAb against B cell lymphoma, covalently linked to the amphibian ribonuclease, Onconase, a member of the pancreatic RNase A superfamily. Since both Onconase and LL2 (humanized LL2: Epratuzumab) are in clinical trials as cancer therapeutics, the covalently-linked agents should be considered for treatment of non-Hodgkin's lymphoma.

Further, therapy with the anti-Tac(Fv)–PE38 immunotoxin (also called LMB-2, an anti-Tac MAb and *Pseudomonas* exotoxin) has been reported to be useful for adult T cell leukaemia, even though a considerable amount of the immunotoxin is needed for the therapy (Kreitman, 2001; Ohno *et al.*, 2002). A second fusion protein, RFB4(dsFv)–PE38, (also called BL22) targets CD22.

Finally, targeted RNases may overcome problems of toxicity and immunogenicity associated with plant or bacterial toxin-containing immunoconjugates. Thus, targeting CD22 on human B cells with a monoclonal antibody conjugate to a cytotoxic RNase causes potent and specific killing of the lymphoma cells *in vitro*. RNA damage caused by RNases could be an important alternative to standard DNA-damaging chemotherapeutics. (Hursey *et al.*, 2002).

Production of antibody fragments and antibody fusion proteins

Various expression systems have been used for the production of recombinant antibodies and their fusion proteins, including bacteria, yeast, baculovirus-infected insect cells, mammalian cells, and transgenic plants and animals. Each system has its advantages and limitations (reviewed in Wright *et al.*, 1992).

PROCARYOTIC EXPRESSION (*E. COLI*)

Well-established procedures for genetic manipulation of DNA, combined with the rapid growth of bacteria generating high yields of recombinant material, have fuelled studies of antibodies and antibody fusion proteins. Most variants have initially been constructed and expressed in *E. coli*. The use of bacteria, however, is limited, since yields of functional antibody fragments produced in the reducing environment of the bacterial cytoplasm are generally low. The vast numbers of refolding strategies have not solved this problem so far. Nevertheless, *in vitro* refolding may prove to be a viable alternative in cases where the correctly folded fusion protein is toxic to the producer cell, as e.g. with RNase fusions.

Therefore, the recombinant antibody is usually fused to a bacterial leader sequence, directing it to the *E. coli* periplasmic space, where proteins can be harvested by osmotic shock (Breitling *et al.*, 1991). The oxidizing environment of this cell compartment favours the formation of disulphide bonds, a prerequisite for correct protein folding. Furthermore, recombinant proteins retain their native amino terminus once the leader sequence is cleaved off. The bacterial system is the preferred system of use for the expression of immuntoxins, since the products can be toxic to mammalian cells. The production levels in shaker flasks usually range from about 0.1 to 10 mg/L, (Carter *et al.*, 1995), but titers of 1–2 grams of soluble and functional antibody per litre have also been reported (Carter *et al.*, 1992). Fermentation of certain optimized molecules can yield larger quantities, but all of these systems are restricted to smaller antibody variants, since complete Ig molecules cannot be produced in this manner.

A significant disadvantage of expression in *E. coli* is the absence of post-translational modification. Although deglycosylation of antibodies has been shown to have no effect on antigen binding, natural effector functions can be hampered (Plückthun, 1991).

Proteus mirabilis

An expression system with future potential is the secretion from the L-form of *Proteus mirabilis*, a bacterium lacking the periplasmic compartment. ScFv yields of up to 200 mg/L have been achieved in lab scale (Rippmann *et al.*, 1998), and production of an scFv fusion to the extracellular domain of tissue factor has been demonstrated (Rippmann *et al.*, 2000).

YEAST

The methylotrophic yeast *Pichia pastoris* is another important tool used in molecular

biology for the generation of recombinant proteins. *P. pastoris* has demonstrated its most powerful success especially in large-scale (fermentation) production (Cregg *et al.*, 2000). To date, well over 200 heterologous proteins have been expressed in *P. pastoris*. One of the advantages is its similarity to *Saccharomyces cerevisiae*, one of the best-characterized experimental systems in modern biology, making genetic manipulation easy. As in *S. cerevisiae*, linear vector DNAs containing the gene of interest can generate stable transformants of *P. pastoris* via homologous recombination. However, unlike fermentative yeasts like *S. cerevisiae*, *P. pastoris* has a strong preference for respiratory growth, a key physiological trait that greatly facilitates culturing at high cell densities (>100 g/L dry cell weight, or 500 OD_{600} units/ml).

For proteins that are secreted or require post-translational modifications and, therefore, cannot be produced in *E. coli*, *P. pastoris* is a possible alternative. As a yeast, *P. pastoris* is a eukaryote capable of many of the post-translational modifications typically performed by higher eukaryotic cells (see mammalian expression). Thus, many proteins that end up as inactive inclusion bodies in bacterial systems are produced as biologically active molecules in *P. pastoris*. However, O- and N-glycosylation in *P. pastoris* and other fungi differs from that of higher eukaryotes, carrying the risk of interference with functionality of the protein product. With *P. pastoris*, heterologous proteins can either be expressed intracellularly, or secreted into the medium. Since *P. pastoris* secretes only low levels of endogenous proteins, a secreted heterologous protein comprises the vast majority of the total protein in the medium. This way, secretion can serve as a first major purification step.

Based on currently available data, there is an approximately 50–75% probability of expressing any protein of interest in *P. pastoris* at a reasonable level. Although there are relatively few examples of expression at 10 g/L, there are many examples of expression in the 1 g/L range, ranking the *P. pastoris* expression system as one of the most productive eukaryotic expression systems available (Cregg *et al.*, 2000).

BACULOVIRUS

Recombinant baculoviruses can also be used to express antibodies and scFv fusion proteins (Holvoet *et al.*, 1991, 1992) in cultured insect cells. Eukaryotic baculovirus-infected cells offer many of the advantages of mammalian cell expression (correct assembly, post-translational modification, secretion) for yielding correctly processed and folded proteins. For this reason, this system is often used when proteins cannot be expressed in bacteria, and when mammalian cell expression is inefficient. Despite this potential and the availability of various baculovirus vectors designed for antibody expression (e.g. Brocks *et al.*, 1997; Liang *et al.*, 2001), the baculovirus system is still limited, compared to other expression systems, because of the narrow range of available promotors (polyhedrin, p10), selectable markers, viral vectors, as well as problems with the ease of manipulation and scale-up (Rybak and Newton, 1999). As with the other non-mammalian cells, glycosylation differs from the human patterns. Expression levels range from 0.1% to 50% of the total insect cell protein (up to 1 mg of protein per $1–2 \times 10^6$ infected cells). Baculovirus systems have not been used to produce therapeutic antibody fusion proteins, but may prove to be a valuable alternative in the future.

MAMMALIAN CELL CULTURE

Intact and fully functional antibodies have been most successfully expressed in mammalian cells. It is possible to first evaluate new genetic constructs in transient expression systems, such as the COS (green monkey kidney) cell system. However, in order to produce material in sufficient quantity, a stable cell line has to be provided. Commonly used cells for this purpose are mouse myeloma (NS0). These cells possess the mechanisms required for correct assembly and post-translational modifications, such as proper folding, proteolytic processing, disulphide bond formation, and glycosylation. These, in turn, can influence biological properties and effector functions, some rather important considerations, especially when the antibody construct is to be used in diagnosis and therapy (Penichet *et al.*, 1999). Secretion through the appropriate intracellular compartments in mammalian cells protects against proteolysis, and results in a protein with the correct amino terminus (Gething and Sambrook, 1992). Moreover, since these proteins are secreted into the culture medium, purification is simplified, as the protein is not co-localized with intracellular proteins.

One problem with expressing proteins in mammalian cells can be the low yield. However, cost-efficient production of antibody–enzyme fusion proteins, for instance, has become available with the development of hollow-fibre bioreactors. In general, antibody yields are very good in these systems, producing about 2–40 mg/L in tissue culture flasks, and 50–1000 mg/L in bioreactors (Stump *et al.*, 1986). One possible drawback in the expression of proteins for clinical use in mammalian cells is the removal of contaminants, such as viral DNA, or products associated with the serum-containing medium. However, it is now possible to grow mammalian cells in serum-free production-media, further simplifying the recovery process. Furthermore, antibodies can be expressed in non-lymphoid cells, such as Chinese hamster ovary (CHO), which can be adapted to grow under serum-free conditions (Cattaneo and Neuberger, 1987) in large fermenters.

TRANSGENIC PLANTS AND ANIMALS

Plants

The inexpensive production of large quantities of protein has led to a new industry: the production of recombinant proteins in transgenic plants and animals. The potential of 'molecular pharming', using transgenic plants and animals as 'bioreactors' to produce proteins for therapy, has been apparent for over a decade, and several proteins produced in these systems are now in clinical trials (e.g. CaroX, Larrick *et al.*, 1998). Plants provide an attractive expression vehicle for numerous proteins, including therapeutic antibodies (Larrick and Thomas, 2001). Plant 'bioreactors' are expected to yield over 10 kg of therapeutic protein per acre in tobacco, maize, soya bean, or alfalfa. Average expression levels of recombinant antibodies in stabile transformed plants are on the level of 1–2% of total soluble protein (TSP) (Fischer *et al.*, 2000). However, some recombinant proteins have reached expression levels of up to 26% TSP (Ziegler *et al.*, 2000).

Genetically engineered transgenic plants have many advantages as sources of

proteins when compared with human or animal fluids/tissues, recombinant microbes, transfected animal cell lines, or transgenic animals. Besides the potential to rapidly upscale production, the cost of producing raw material on an agricultural scale is very low, and there is the possibility, in certain cases, of using the edible plant material directly. Additionally, unlike bacteria, plants are able to produce correctly assembled multimeric proteins and antibody fusions. Finally, plant proteins could be considered safer, as plants do not serve as hosts for human pathogens, such as HIV, prions, or hepatitis viruses.

However, glycosylation – regarding the type of sugars and the number of glycoforms attached – is different in plants and mammals, and gives the major cause for concern regarding the immunogenic potential for therapeutic protein production. To overcome this problem, recent efforts have concentrated on the humanization of plant glycans (Bakker *et al.*, 2001).

Post-translational gene silencing (PTGS)

Silencing of introduced transgenes has frequently been observed in plants, constituting a major commercial problem (Vaucheret and Fagard, 2001). It is often associated with methylation of the transcribed region, and ultimately leads to a sequence-specific RNA degradation. Until now, the most efficient strategy to avoid PTGS was to carefully design transgene constructs, and to thoroughly analyse the transformants at the molecular level (de Wilde *et al.*, 2000). One possibility to minimize gene silencing is the positioning of matrix attachment regions (MARs) on either side of the transgene, usually resulting in higher and more stable expression. Introduction of the helper-component proteinase (HC-Pro) of plant polyviruses also suppresses PTGS, resulting in the loss of small RNAs, and partial loss of methylation (Larrick and Thomas, 2001).

Animals

For the reason mentioned above, transgenic mammals may be an appropriate alternative for the expression of antibodies and antibody fusion proteins. One of the promising approaches for large-scale production has been the secretion of proteins into the milk of transgenic mammals. This has been successfully demonstrated for over two dozen different proteins in either cows, goats, sheep, pigs, rabbits, or mice, and appears particularly promising for the production of large amounts of monoclonal antibodies (Pollock *et al.*, 1999). The genomes have been genetically modified to contain foreign DNA-encoding proteins of interest, and the integrated DNA can be passed on to successive generations. In addition to milk, urine has been suggested as another feasible route for the production of recombinant proteins (Polejaeva *et al.*, 2000). Another approach may be to produce recombinant proteins in transgenic chicken eggs; high-yield protein can be expressed in eggs, harvesting is straightforward, and production is easy to scale-up.

To date, most recombinant proteins produced in transgenic animals have involved the microinjection of a genetic construct, the expression of which is driven by a mammary gland-specific promoter (e.g. for the production of caseins, whey acidic

protein, lactalbumins). Also, the therapeutic proteins produced in transgenic animals undergo post-translational processing characteristic of that animal, although the protein itself is of human sequence. Since carbohydrates in recombinant glycoproteins are somewhat different from those of human origin, there is still the question whether or not this will have adverse effects, such as eliciting an immune response, or conferring altered biodistribution or retention. Although only a few animal-produced protein drugs have been administered to humans, the circulating half-life, or production of antibodies, does not appear to be an issue.

Conclusion: what we can expect

The idea of using antibodies as 'magic bullets' to target toxins or radioisotopes to defined cell types is now over 100 years old. Numerous therapeutic antibodies have been registered as products, and many more are in the pipeline of clinical development. The number of novel antibody formats with potentially favourable characteristics being published today is exceeding by far the realistic number of future clinical trials. With the improved technology for the generation of fully human antibodies, and an increased understanding of antibody function in the therapeutic situation, the concept of the 'magic bullet' can be seen as reliving a renaissance.

With the available data concerning the entire human genome, the generation of a 'complete set' of recombinant antibodies for all possible surface proteins (targets) is made possible. Consequently, the technological developments of antibody fusions will shift from engineering and improving the antigen-binding site to engineering the effector functions. As novel targets are depleting, researchers will increasingly focus their attention on the details of the physiological processes providing therapeutic activity and, consequently, on novel functions added to the targeting component. Microarray technologies will predict activity and toxicity earlier in the developmental process of the agent, allowing the testing of more candidates, fusion partners, or numerous designs than presently possible.

A second, and equally important, area of research will deal with the pharmacokinetic properties of the fusion proteins. With increasing awareness emphasizing the importance of this question, variants of the naïve Ig molecule – such as the various minibody formats, or molecules with mutated Ig regions (to manipulate FcR specificities) – will be systematically produced and compared to existing variants. It can be expected that significant expansions of the therapeutic windows can be achieved, once this broad area of optimization will receive the attention it deserves.

The ideal future therapeutic antibody fusion protein will add novel capabilities to the targeting antibody domain; it will be optimized for best binding to the target tissue, and sport a specifically designed serum half-life. It will consist of domains of reduced immunogenicity, or be of completely human origin. Examples include antibody RNase fusions (which may supersede the classical immunotoxin by avoiding its immunogenicity and unspecific liver toxicity), and completely human-derived bispecific or multispecific antibodies. Lastly, antibody fusion proteins will be produced in recombinant expression systems, allowing mass production at a fraction of today's cost. None the less, it can be assumed that at least another decade must pass before therapeutic antibody fusion proteins will be commonly found in our pharmaceutical repertoire.

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