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Production systems for recombinant antibodies

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1. ABSTRACT

Recombinant antibodies are the fastest growing class of therapeutic proteins. Furthermore, antibodies are key detection reagents in research and diagnostics. The increasing demand for antibodies with regards to amount and quality resulted in the development of a variety of recombinant production systems employing Gram-negative and Gram-positive bacteria, yeast and filamentous fungi, insect cell lines as well as mammalian cell lines. More recently, antibodies were also successfully produced in transgenic plants and animals. Currently, the production of recombinant antibodies for therapy is performed in mammalian cell lines to reduce the risk of immunogenicity caused by non-human post-translational modifications, in particular glycosylation. However, novel strategies already allow human-like glycosylation patterns in yeast, insect cell lines and transgenic plants. Furthermore, therapeutic strategies not requiring glycosylation of the Fc portion have been conceived, most prominently using bispecific antibodies or scFv fusion proteins, which can be produced in bacteria. Here, we review all current antibody production systems considering their advantages and limitations with respect to intended applications.

2. INTRODUCTION

Recombinant antibodies and antibody fragments are important tools for research, diagnostic and therapy. Currently, scientists and medics employ antibodies in their routine laboratory work for a large number of standard assays like immunoblot analysis, flow cytometry or immunohistochemistry. After completion and refinement of the human genome (1, 2), the demand for specific binders dramatically increased for systematic analysis of the huge number of proteins encoded by individual genes and open reading frames (ORFs) with respect to their function, cellular localization and regulation. Moreover, recombinant antibodies can be used for the diagnosis of diseases by detection of pathogens or toxins (e. g. (3)). For the last few decades, antibodies have been anticipated as molecules well suited for various therapeutic approaches (4, 5). The major therapeutic applications of antibodies are inflammatory (6) and tumour diseases (7). Over 30% of biopharmaceuticals in clinical trials are recombinant antibodies (8). The market for therapeutic antibodies is estimated at 30 billion US dollars in 2010 (9). Recombinant antibodies even have the potential to be used in consumer products, like in shampoos to prevent dandruff (10) or in

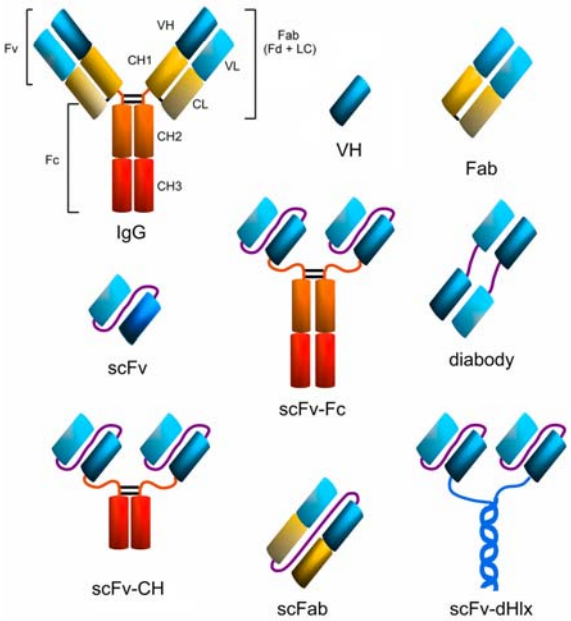

Figure 1. Nomenclature for a selection of recombinant antibody formats engineered for different applications, in comparison to native IgG (upper left corner). Variable regions are given in turquoise, constant regions in yellow, orange and red, artificial peptide linkers in purple. dHLX represents amphipathic helices used for dimerization of scFv fragments.

toothpaste to protect against tooth decay caused by caries (11,12), which will require very large quantities and appropriate antibody production systems.

Polyclonal antibodies are widely used as detection reagents in research and diagnostics but they are a batch-dependent limited resource and also contain antibodies with unknown specificities. In addition, polyclonal non-human antibodies are very immunogenic restricting their therapeutic application to acute intoxications, for example, after snake bites (13). A big milestone in antibody generation was the generation of monoclonal antibodies (mAbs) by hybridoma technology which is based on the fusion of antibody producing spleen cells from immunized mice or rats with immortal myeloma cell lines (14). However, the hybridoma technology has some limitations concerning the inefficient immune response to highly toxic or conserved antigens. In addition, today all novel therapeutic antibodies are of human origin or at least partially humanized to reduce the risk of immunogenicity. The generation of hybridomas from human B cells remains difficult but B cells of transgenic mice with the human IgG gene repertoire can be used for the generation of murine hybridomas producing human immunoglobulins (15-20). However, this method still requires immunization and a successful *in vivo* antibody response. *In vitro* antibody selection technologies such as antibody phage display and ribosomal display circumvent the *in vivo* immune response and allow the generation of human antibodies (21-25). Furthermore, antibody display technologies allow improvement of antibodies in terms of affinity, stability, solubility or even its "humanness".

New recombinant antibody generation technologies increased the number of antibodies for different applications but also raised demands for appropriate production systems which are the focus of this review. The production of antibodies in high amounts is a challenge due to the high complexity of immunoglobulins. Immunoglobulin G (IgG) is a heterotetrameric molecule formed by the assembly of two different polypeptide chains connected by inter-chain disulfide bonds which form many domains with numerous non-covalent interactions and disulfide linkages. Antibody light chain (LC) and heavy chain (HC) consist of several "immunoglobulin fold" domains, each requiring an intra-domain disulfide bond for stabilization (26). Efficient and correct formation of the large number of disulfide bonds together with the folding and assembly of four chains to one IgG molecule requires an oxidizing environment and the assistance by a complex folding apparatus.

Many hosts for *in vitro* selection or production of antibodies do not have optimal conditions for the expression of full size IgGs. This problem was partially addressed by the use of smaller antibody fragments (Figure 1) which are still able to mediate antigen binding properties. These antibody fragments can be used for many research and diagnostic applications, and therapeutic treatments where binding to an epitope is sufficient for the therapeutic effect, for example, to achieve virus or toxin neutralization or receptor blocking. Furthermore, the smaller fragments are key elements for the success of most of the *in vitro* antibody selection systems, like phage display, cell surface display or ribosomal display (21-25, 25a).

The Fv fragment is the smallest antigen binding fragment of immunoglobulins retaining its complete antigen binding site, and constists only of the variable (V) regions. To overcome the low stability of Fv fragments, a soluble and flexible 15 - 25 amino acid peptide linker is used to connect the V regions to form a scFv fragment (27), or the constant domains CL and CH_1 are added to the V regions to obtain a Fab fragment (see Fig 1). Currently, scFv and Fab are the most widely used antibody fragments, in particular of those produced in prokaryotes. Other antibody formats have been produced in prokaryotic and eukaryotic cells, for example, disulfide-bond stabilized scFvs (ds-scFv) (28), single chain Fab fragments (scFab) combining scFv and Fab properties (29), multimeric antibody formats such as dia- tria- or tetrabodies generated by reducing the linker length in scFvs (30-33), minibodies (miniAbs) comprising different formats consisting of scFvs linked to oligomerization domains such as immunoglobulin CH_3 domain (30), leucin zipper, helix turn helix motif or streptavidin, or scFv-scFv tandems to enhance the apparent affinity by avidity effect (34, 34b, 35). Moreover, there are bispecific antibody formats

combining two different antigen binding domains in one molecule which can be used to cross-link two different targets (36, 37). The smallest antibody fragments are V_{HH} of camelide heavy chain antibodies (38) and similar single domain antibodies (dAb) (39).

Production levels of up to gram per liter amounts were achieved for smaller antibody fragments even in bacteria (40, 41). However, for many therapeutic applications, the immunoglobulin Fc moiety is essential, particularly, if antibody mediated effector functions or enhanced serum half life are required. For example, antibody dependent cellular cytotoxicity (ADCC) requires specific N-linked glycosylation in the Fc domain which can only be processed in eukaryotic hosts. The fusion of antibody fragments to the Fc domain (e. g. V_{HH}-Fc, scFv-Fc) is used to gain effector functions and avidity almost indistinguishable from IgGs (38, 42-44) whilst requiring the production of only a single polypeptide.

3. ANTIBODY PRODUCTION IN PROKARYOTIC HOSTS

3.1. Gram-negative bacteria

The Gram-negative bacterium *Escherichia coli* is the most widely used production system for recombinant proteins, reaching volumetric yields of more than 10 g/L for cytoplasmic expression. Key to the success for the first expression of a functional antibody fragment in *E. coli* described by Skerra and Plückthun in 1988 (45) was the translocation of both V chains into the periplasmic space of *E. coli* where the oxidizing environment allowed the correct formation of disulfide bonds and the assembly to a functional Fv fragment. This strategy also allowed the first expression of functional Fab fragments in *E. coli* (46).

The expression of recombinant antibodies in the reducing cytoplasmic compartment mostly results in non-functional aggregates (47). Recovery of functional antibody fragments from cytoplasmic inclusion bodies by complete denaturation and refolding (48) is not very efficient. Only heavily engineered cystein free variants of a few scFvs were successfully produced in the cytoplasm of *E. coli*, not requiring refolding (49, 50). *E. coli* strains with mutations in the glutathione and thioredoxin reductase in combination with coexpression of cytoplasmic chaperones GroEL/ES, trigger factor, DnaK/J as well as signal sequence-less variants of periplasmic chaperones DsbC and Skp increased yield of correctly folded Fab with Skp showing the most significant effect. A maximum yield of 0.8 mg functional Fab per liter per $O.D._{600}$ was achieved (51).

Despite these efforts, most antibody fragments are produced in the periplasm of *E. coli* using N terminal leader sequences targeting the periplasmic *Sec* pathway, for example, signal peptides derived from outer membrane protein A (*OmpA),* alkaline phosphatase A (*PhoA)* or pectate lyase B (*PelB)* (52-55). After expression, recombinant antibodies are usually isolated from the periplasmic fraction (56, 57), and in some cases from culture supernatant (58, 59). Periplasmic expression and yield of functional scFv fragments could be improved by co- or overexpression of GroES/L or of the peptidylprolyl-cis,trans-isomerase FkPa (60-62).

The Fab format requires the expression, periplasmic transport, correct folding and assembly of two different polypeptide chains. Among the different vector formats and arrangements, bicistronic vectors with the first cistron encoding the light chain and the second cistron encoding the Fd fragment (consisting of Vh and CH1) were optimal (56). Recently, even aglycosylated full size IgG were successfully produced in *E. coli* (63). Interestingly, the production of full size IgG in *E. coli* was only possible after fine adjustment of the translation strength of both IgG chains by introduction of silent mutations into the translation initiation region of the leader sequence to achieve optimal periplasmic transport (63, 64).

Since antibody expression in *E. coli* is related to host toxicity resulting in very low yields, vector mutation and plasmid loss, it must be well repressed before production phase and requires an adjustable induction during the production. These critical parameters can be addressed by the promoter system, plasmid copy number and other parameters (65, 65a, 65b). Periplasmic antibody production is usually performed at 20 to 30°C rather than at 37°C to prevent overloading of *E. coli*'s secretory pathway and intracellular aggregation. However, very high yields of antibody fragments produced in *E. coli* are mainly provided by high cell density fermentation in bioreactors. The high yield of 1.2 g/L for a hapten-specific scFv produced in a bioreactor (65) compared to 16.5 mg/L obtained by optimized shake flask production (66) can be mostly addressed to the over 100-fold higher cell density in the bioreactor.

Cell-wall-less L-forms of the Gram-negative bacterium *Proteus mirabilis* were used for the production of miniAbs and scFvs (35, 67), with total and functional scFv yields of 83-127 mg/L and 9 -12 mg/L, respectively (67).

3.2. Gram-positive bacteria

Gram-positive bacteria directly transfer secretory proteins into the medium due to the lack of an outer membrane, which may facilitate the production and purification of antibody fragments. The Gram-positive bacteria *Bacillus brevis* (68, 69) and *Bacillus subtilis* (70, 71) have been successfully used for the production of different antibody fragments. Recently, *Bacillus megaterium*, which does not produce alkaline proteases and provides high stability of plasmid vectors during growth (72) and which allows stable transgene expression during long term cultivation in bioreactors, was employed for the production of antibody fragments. Functional scFv fragments could be directly isolated from the supernatant (73).

Lactobacilli are "generally regarded as safe" (GRAS) microorganisms and have also been tested for antibody production. To date, two lactobacillus strains were used for the production of scFvs, *Lactobacillus zeae / casei* (74, 75) and *Lactobacillus paracasei* (38, 76). The GRAS status of lactobacilli allows their direct use for oral application, for example, for the production of anti-*Streptococcus mutans* antibody fragments to prevent tooth decay (74).

4. ANTIBODY PRODUCTION IN EUKARYOTIC HOSTS

Eukaryotic hosts provide a more sophisticated protein folding and secretion apparatus than prokaryots. Correct post-translational processing of IgG, particularly glycosylation, is only provided by eukaryotic cells. However, their capacity to generate human glycosylation patterns shows great variations (5). The immunoglobulin Fc part contains site specific N-linked glycosylations which are important for the interaction with other components of the immune system, like the recognition by Fc receptor bearing immune cells. The IgG glycosylation pattern can vary which influences effector properties of IgGs (77). Elimination of core fucosylation from IgG improved binding to Fc-gamma receptors and ADCC activity (78). Therefore, the capacity of the host system to achieve the required glycosylation is an important parameter for a number of therapeutic antibodies and their application.

4.1. Yeast

The heterologous production of recombinant proteins in yeasts is attractive because it combines the advantages of short generation time, ease of genetic manipulation, robustness and simple medium requirements of unicellular microbial hosts with enhanced post-translational folding, processing and secretion capacity of eukaryots. Many yeast strains have GRAS status and do not produce endotoxins which could facilitate the admittance for the production of heterologous proteins for *in vivo* diagnostics and therapy. Another advantage of yeasts are the low number of secreted proteins, facilitating the downstream processing of secretory produced heterologous proteins.

Beside the well-characterised baker's yeast *Saccharomyces cerevisiae*, non−*Saccharomyces* yeast strains are available for recombinant polypeptide production including *Pichia pastoris, Hansenula polymorpha, Schizosaccharomyces pombe* (78)*, Schwanniomyces occidentalis, Kluyveromyces lactis* and *Yarrowia lipolytica (*79). However, mostly *S. cerevisiae* and *P. pastoris* have been studied for recombinant antibody production. *P. pastoris* shows a better overall capacity of producing and secreting heterologous proteins than *S. cerevisiae*. It also prefers respiratory growth leading to higher cell densities of more than 100 g/L dry weight (80). One of the most prominent features of *P. pastoris* is the metabolization of methanol as sole carbon source. The alcohol oxidase 1 (AOX1) promoter is tightly controllable by methanol and commonly used for recombinant protein expression in *P. pastoris*.

The first expression of a scFv fragment in *P. pastoris* was shown by Ridder *et al.* in 1995 (81). In *P. pastoris*, different scFvs were produced with a yield of 70 mg/L (82) to 250 mg/L (83). Under optimized fermentation conditions even up to 1.2 g/L functional scFv were obtained (84). In *S. cerevisiae* over 100 mg/L of a llama V_{HH} were produced in shake flask culture (85). DNA shuffling of three homologous V_{HH} clones did not only improve affinity and thermal stability but also expression yield (86).

The secretory production of recombinant antibodies in yeast requires a N terminal signal sequence targeting yeast's secretory pathway. Initially, the secretory signal sequence derived from the *S. cerevisiae* mating factor alpha (alpha-factor) prepro-peptide has been used. Proteolytic cleavage sites (KEX2 often in combination with STE13 sites) introduced after the secretory signal sequence led to incomplete or incorrect proteolytic processing (87), but simple signal peptides could be used instead.

In the case of Fab fragments or IgGs, both antibody chains must be fused to the alpha-factor prepropeptide leader sequence and co-transformed into yeast. The yield of Fabs produced in yeast ranged from 1 to 50 mg/L in shake flasks to 0.5 g/L in bioreactors. There are only rare data about IgG expression in yeast. In an early study, a mouse-human chimeric antibody and its Fab fragment were produced in *S. cerevisiae* with yields of 50 – 80 µg/L and 0.2 mg/L, respectively. The chimeric IgG mediated tumor specific binding and ADCC but no CDC (88). In contrast, scFv-Fc fusion proteins were produced with more than 10-30 mg/L in *P. pastoris* (89).

Overexpression of the chaperone immunoglobulin binding protein BiP or protein disulfide isomerase PDI in *S. cerevisiae* increased scFv secretion titers 2 to 8 fold for five scFvs with an average yield of 20 mg/L in shake flask culture (90).

Reported disadvantages of yeasts include lower transformation frequencies than *E. coli*, failure to express AT-rich genes due to premature transcriptional termination, inefficient secretion of some larger proteins (>30 kDa), proteolysis of secreted proteins during high-cell density fermentation, and inappropriate glycosylation of human glycoproteins. Yeasts also tend to hyperglycosylate heterologous proteins even at positions not glycosylated in the native host, and immunogenicity and different pharmacokinetics can be caused by carbohydrate structures not not observed in higher eucaryotes (216). However, *P. pastoris* mediates much lower hyperglycosylation than *S. cerevisiae* and often exhibits N-linked carbohydrate structures similar the mammalian high-mannose core unit $Man_{5-6}GlcNAc_2$ (91). Also, there are strong efforts to accomplish humanized glycosylation pattern in *P. pastoris* (92-95). Recently, the production of a human IgG showing humanized N-linked glycosylation in *P. pastoris* has been reported (96). Unlike full size IgGs produced in

wildtype yeast, those produced in glycoengineered yeast had functional antibody-mediated effector functions.

4.2. Filamentous fungi

Filamentous fungi of the genera *Trichoderma* and *Aspergillus* have a high capacity to secrete large amounts of protein and metabolites into the medium (97). They have been widely used in food industry, for example, *A. niger* for the citric acid production. Furthermore, *A. niger (*subgenus *A. awamori*) and *A. oryzae* are classified as GRAS for some of their products and, thus, are suitable hosts for the production of recombinant proteins. In *A. niger* yields of up to 1.2 g/L IgG were obtained. Two promoters are used for the expression of antibodies, the glucoamylase promoter glaA (98) and the endoxylanase A promoter exlA (99). After fusion of the antibody chains to the C terminus of glucoamylase, which is highly secreted by *Aspergillus*, the yield was increased five fold to 50 mg/L (100). To release the antibody, protease cleavage sites like KexB are introduced between antibody chain and glucoamylase (98). In *Trichoderma reesei*, 150 mg/L of a Fab fused to cellobiohydrolase I was produced, whereas without a fusion partner the yield was only 1 mg/L (101). *A. awamori* was used for the production of scFvs, llama V_{HH} and antibody enzyme fusion proteins (97, 99, 102). Some proteolytic degradation was observed. *Chrysosporium lucknowense C1* is a triple protease deletion mutant (Delta-alp1, Delta-pep4, Delta-alp2) which is has been used for small scale production, for screening, as well as for large scale protein production in bioreactors (103).

4.3. Insect cells

Insect cells are a versatile eukaryotic system for heterologous protein expression. Insect-specific viruses from the family of *Baculoviridiae*, particularly the *Autographa californica* nuclear polyhedrosis virus (AcNPV), are origin for most used insect expression vectors. Baculoviruses are highly species-specific and are considered as safe for humans, mammals and plants. Therefore, no special safety precautions must be considered. A Baculovirus based Hepatitis virus C vaccine is promising as the first human therapeutic produced in insect cells (104). Non-essential Baculovirus genes in the tissue culture life cycle, like *Polyhedrin*, *P10* or *Basic* can be replaced even by large heterologous genes. Heterologous proteins can be produced at levels ranging between 0.1% and 50% of the total insect cell protein. Recombinant Baculoviruses are usually used for the infection of insect cell lines like Sf-9 and Sf-21 of *Spodoptera frugiperda* or High Five™ (BTI-TN-5B1-4) of *Trichoplusia ni*. Important parameters for optimising the production are the multiplicity of infection (m.o.i.) and the protein production time which seems to be limited to 96 h after infection due to the start of cell lysis. Protease inhibitors are recommended because cell lysis is associated with the release of viral proteases. Yields of up to 32 mg/L of secreted monomeric anti-phOx scFv were obtained in a 6 L bioreactor with 10^9 cells per liter after 72 h with m.o.i. of 1 (105). The production of various IgGs reached yields of 6 – 18 mg/L (106). IgGs produced in High Five cells showed terminal galactosyl residues beta (1,4)-linked to the biantennary GlcNAc residues but the absence of sialylation, formation of paucimannosidic structures and the presence of potentially allergenic alpha1,3-fucose linkages were the differences observed relative to mammalian glycosylation pattern (107). However, IgG produced in insect cells were able to mediate effector functions like complement binding (108, 109). Ten µg/mL of an anti-Rhesus D antibody produced in Sf9 cells mediated lysis of Rh^+ red blood cells by ADCC (110). Insect cell protein expression systems were improved using protease deficient Baculovirus strains or insect cell lines with additional glycosyltransferase gene modifications to obtain glycosylation pattern comparable to mammalian cell lines (111).

After expression of IgG in insect cells driven by the strong *Polyhedrin* promoter an extensive aggregation was observed probably due to the overloading of the cellular folding and post-translational processing apparatus (112). Recombinant overexpression of the ER resident chaperone BiP significantly enhanced levels of soluble and secreted IgG in *T. ni* cells (113). Enhanced secretion of IgG as well as IgG recovery from insoluble aggregates was also achieved by co-expression of PDI. Overexpression of the human cytosolic chaperone hsp70 increased solubility and secretion of murine IgG in *T. ni* cells by enhancing the solubility of the light chain precursor (114).

As an alternative to the Baculovirus system which often suffers from a strong intracellular protein aggregation and from strong use of cellular metabolism resulting in a high diversity of post-translational modified products, insect cell expression plasmids can be transiently or stably transfected into Schneider 2 (S2) cells of *Drosophila melanogaster*. Secretory production requires a signal sequence like the honeybee melittin leader. Stable transfection of *Drosophila* cell lines with monomeric and dimeric antibody fragments resulted in yields of up to 25 µg/mL (115).

4.4.Mammalian cells

Despite high production costs due to expensive medium and cultivation requirements as well as difficult handling, today 60-70 % of recombinant protein pharmaceutics and all currently approved therapeutic antibodies are produced in mammalian cell lines. The success of mammalian production systems is due to their advanced folding, secretion and post-translational apparatus which leads to producing antibodies which are indistinguishable from those produced in the human body. Moreover, the still continuing progress of the mammalian cell culture technology has already reached antibody production levels of about 5 g/L, 10-fold higher than what they were some years ago (116). Major parameters responsible for this development are improved generation of high producer cell lines and prolonged

cultivation at very high cell densities during production. The productivity of mammalian cells increased from 10 pg antibody per cell per day in 1986 to about 90 pg/cell/d in 2004 which is still in the scale of earlier reports for a myeloma cell line producing 20 to 80 pg/cell/d (117). However, improved production processes increased cell densities from 2 x 10^6 cells/mL in 1986 to 10^7 cells/mL in 2004. In addition, improved cell viability allows production times at high cell densities for almost 3 weeks. To reduce costs as well as risks of contamination by pathogens or bovine spongiform encephalopathy (TSE/BSE) agents, media without supplementation of animal sera or serum components had been developed (118). Moreover, research is still progressing in process optimization and genetic engineering of production cell lines regarding glycosylation homogeneity as well as improved metabolism, reduced apoptosis and inducible cell cycle arrest for prolonged protein production (119,120).

However, mammalian cell lines are still under critical observation regarding to unknown viral contamination (121,122) and release of factors with oncogenic or pathogenic potential. Therefore, most mammalian cell lines used for recombinant protein production are derived from normal diploid mammalian cells by a known transformation mechanism but not from tumor tissues. For recombinant protein production, Chinese hamster ovary cells (CHO) cells, mouse myeloma NS0 cells, baby hamster kidney (BHK) cells and two cell lines generated from human embryonal tissue by transformation with Adenovirus 5 (Ad5) DNA, the human embryonic kidney cell line HEK-293 and the human retinal cell line Per.C6 (Crucell, NL) gained regulatory approval for recombinant protein production (123). More recently, the HKB11 cell clone, derived from a hybrid cell fusion of HEK 293S with the Burkitt's lymphoma line 2B8, showed 10-fold higher product yield than CHO cells in serum free media (124, 125). Although glycosylation pattern of mammalian glycoproteins is very similar to that in humans (126), even small differences can influence pharmacokinetics and effector functions of antibodies.

Large scale antibody production usually needs long term production stability which requires chromosomal integration of antibody genes into the host cell genome. That demands a long term screening of large numbers of clones to identify stable high producer clones. There are many strategies to overcome negative effects of the integration site by introduction of protective cis-regulatory elements including insulators, boundary elements, scaffold / matrix attachment regions (S/MARs) (127), chromatin opening elements (128) and antirepressor elements (129) into the vector which reduce influence of heterochromatin and stabilize transgene expression (130, 131). Silencing can also be blocked by inhibition of histon deacetylation using butyrate (132) which could enhance the protein expression levels of the cells (133) but can also induce apoptosis. Recombination enzymes, like bacteriophage P1 Cre recombinase, lambda phage integrase, or yeast Flp recombinase can efficiently catalyse the site specific integration in chromosomal recombination site (134). However, cell lines with chromosomal recombination cassettes optimized for high level protein expression have not been available, so far.

In contrast to the chromosomal integration site, genetic elements driving the transcription seem to play a minor role for a stable high transgene expression. In most cases, strong promoters like the immediate early cytomegalovirus (CMV) or the cellular elongation factor (EF) 1-alpha promoter are implemented. Polyadenylation sites usually derived from the simian virus (SV) 40 or the bovine growth hormone (BGH) improve mRNA stability and enhance translation efficiency. Furthermore, splicing of mRNA is known to promote mRNA packaging and transfer into cytosol to stabilize and enhance gene expression as well as to reduce silencing of heterologous transgenes (135, 136). For IgG expression, two different genes must be stably transfected into one cell clone, either by co-transfection or by using bicistronic expression vectors. Bicistronic vectors employing internal ribosomal entry sites (IRES) allow the translation of two or more cistrons from the same transcript (137). The encephalomyelitis virus (ECMV) IRES has shown the highest efficiency in various mammalian cell lines. Mutated IRES derivatives even allow the control of translation efficiency in relation to the cap dependent cistron. The ratio between light and heavy chain has great impact on the secretion level of functional IgG (137b). The long term stability of ECMV IRES containing bicistronic constructs have been demonstrated even in the absence of selection pressure over months (137).

Production cell lines are usually the result of a year-long, tedious process of repeated cycles of gene amplification. Here, two systems are used with most success, the dihydrofolate reductase (DHFR) and the glutamyl synthetase (GS) selection systems (138). Yield and functionality of an IgG1 produced in *dhfr*- CHO and GS-NSO were equivalent (139). Prolonged fed-batch production of three to four weeks using GS-NS0 cells yields up to 1.8 g/L antibody (140) or even up to 2.7 g/L final antibody concentration after improved feeding with NS0 cells containing 3 vector copies per cell (141). Anti-$p185^{HER-2}$ scFv-CH3 minibodies (10H8) were expressed with up to 60 mg/L in NS0 cells (142).

Particularly in research and development, transient or semi-stable production systems are often used to circumvent the time consuming selection of single stable expression cell clones requiring several months. Transient productions in scales of hundreds of milligrams are possible in mammalian cells (143). Derivatives of the human embryonic kidney (HEK) cell line 293 transformed either with the simian virus 40 (SV40) large T antigen (HEK293T) or the Epstein Barr virus (EBV) nuclear antigen EBNA-1 (HEK293-EBNA) combine high transfection efficiency with the semi-stable episomal propagation of expression plasmids containing the origin of replication of SV40 or EBV,

respectively, allowing transient production for more than two weeks. Transient transfection of HEK293T cells can be efficiently performed using cationic liposomes (Lipofectamine, HEKfectin, etc.) or polyethylenimines (PEI) (144). Calcium phosphate (CaPi) transfection is also efficient for HEK293 cells and inexpensive (145). Vectors with compatible restriction cassettes allow one step cloning of scFv fragments from phage display libraries into the scFv-Fc format. Small scale production in HEK293T cells was also used for the screening of functional IgG clones after two-step subcloning of Fab gene fragments immediately after phage display selection (146). Transient productions in tissue culture plates using adherent HEK293T cells yielded 1 and 20 mg/L of IgG and scFv-Fc, respectively (137 and our own unpublished results). Transient productions in liter scales are mostly performed in stirred-tank bioreactors (STR) but with an increasing degree in disposable plastic bioreactors like WAVE^{TM} (147).

4.5. Transgenic plants

Particularly, if very large amounts of biopharmaceuticals are required and production costs are to be considered, plants are an attractive alternative even for recombinant antibody production. All other production systems, notably mammalian cell systems, can not be up-scaled without limitation. Moreover, up-scaling of biofermentation processes often does not proportionally reduce production costs. Against that, the growing of plants can be easily expanded simply by using larger fields without much higher costs. Theoretically, the costs of an IgA produced in transgenic plants are only 1 to 10 % in comparison to hybridoma production, even acknowledging the more expensive downstream process in plants (148).

Dicotyledonous plants, like tobacco, are mainly transformed using the soil bacterium *Agrobacterium tumefaciens*. T-DNA vectors derived from the tumor inducing (Ti) plasmid of *A. tumefaciens* are used for the gene transfer. Under control of a viral plant promoter, mostly the constitutive cauliflower mosaic virus (CaMV) 35S promoter, the antibody gene is cloned into a region termed as T-DNA which is flanked by two 25 base pair long imperfect repeats. The T-DNA is transferred to the nucleus of the plant cell by the combined action of virulence (*vir*) genes provided by the T-DNA vector or a separate plasmid and subsequently integrated into the plant genome by non-homologous recombination. Transformed cells are selected by resistance markers and complete transgenic plants are regenerated from transformed calli (149). Rapid transient antibody productions in plants has also been performed using modified plant viral vectors (150, 151) with production levels of up to 0.7 % of total protein. Monocotyledonous plants are mostly transfected by particle gun (152).

Recombinant production of antibodies in plants has been mostly performed in tobacco, in *Nicotiana tabacum* (153-160) and *Nicotiana benthamiana* (161). The first IgG was produced in tobacco by Hiatt *et al.* in 1989 (153). The production of an anti-cutinase scFv in tobacco could be increased from 0.01 % to 1 % of total soluble protein by fusion with the endoplasmic reticulum (ER) retention signal KDEL (156, 157). IgGs were also produced in suspension cultures of tobacco (162). Antibody production in plant seeds immediately offers excellent storage conditions until downstream processing. A scFv produced in the seed of tobacco reached protein levels from 0.43 % to 0.67 % of total soluble protein (163). Alternatively, antibody encoding genes were also introduced into the chloroplast genome allowing high copy numbers, increased expression levels and prevention of ex-crossing by pollen count (164). In potato plants (*Solanum tuberosum*) antibody genes were transferred into chloroplasts as well as amyloplasts (165) and in *Arabidopsis thaliana* Fab fragments were produced in apoplasts (166). Other plants successfully used for antibody production include soybean (*Glycine max*) (167), rice (*Oryza sativa*), wheat (*Triticum aestivum*) (168), lettuce (*Lactuca sativa*) (169), petunia (*Petunia hybrida*) (170) and alfalfa (*Medicago sativa*) (171) (Tab. 1). Furthermore, there are antibody production systems using aquatic plants like the algea *Chlamydomonas reinhardtii* (172) and the common duckweed (*Lemna minor*). In addition, *Lemna minor* has been stably modified to process human N-linked core glycosylation by RNA interference targeting expression of the endogenous alpha-1,3-fucosyltransferase and beta-1,2-xylosyltransferase transcripts (173). Large Scale Biology (U.S.A.) produces anti-idiotypic antibodies in tobacco which are already in clinical phase II for the treatment of Non-Hodgkin lymphoma (174, 175). CaroRX developed by Planet Biotechnology (Hayward, CA, U.S.A.) is produced in transgenic tobacco. It is a complex secretory IgA–like molecule consisting of a hybrid antibody comprising C-gamma1 and -2 domains of the heavy chain of an IgG fused to C-alpha2 and -3 of IgA, plus a J chain and a secretory component analog. It binds to the streptococcal antigen I/II of *Streptococcus mutants*, the major causative agent of bacterial tooth decay. CaroRX already demonstrated prevention of *S. mutans* adherence to tooth enamel in clinical phase II studies. Additionally, topical oral treatment in human subjects was safe and effective (11, 12).

4.6. Transgenic animals

The generation of transgenic animals is very difficult but allows the production of recombinant antibodies in milk or eggs. The production is highly scalable and downstream processing is less expensive than from plants. The oral administration of milk from transgenic mice secreting IgG1 or IgA derivatives of the corona virus neutralizing mAb 6A.C3 provided protection against coronavirus infections. Both antibody chains were under control of the whey acidic protein and beta-lactoglobulin promoter. Neutralizing activity corresponded to immunoglobulin concentrations of up to 5 to 6 mg per mL (176). Moreover, 0.8 g of immunoenzyme fusion (177) and 0.5 mg of a chimeric anti-CD19 IgG per mL milk were produced in

transgenic mice (178). However, there are concerns relating to potential product immunogenicity caused by different glycosylation patterns characteristic for proteins produced in transgenic milk in comparison to native human serum proteins. The presence of N-glycolyl neuraminic acid could potentially trigger immunological complication or other side effects in man (179).

Another technology for recombinant protein production in transgenic animals is the targeted expression in the naturally sterile egg white of transgenic chicken. Each egg contains about 3 to 4 g protein with half of it expressed by both *Ovalbumin* alleles. The production is scalable and regulatory paths to current GMP have been paved by companies producing vaccines in eggs. Recently, transgenic chicken were generated expressing immunoglobulin chain genes under control of the *Ovalbumin* gene promoter including all known regulatory elements responsible for its oviduct specificity and steroid dependent induction in laying hens (180). Up to 3 mg per egg of fully assembled IgGs were obtained. Antigen binding was not altered and ADCC was even enhanced whereas half-life in mouse serum was half of that of natural antibodies. Further technical advances and detailed product characterization, particularly antigenicity, remain to be assessed (181).

Another unique possibility is the immunization of transgenic large animals containing human chromosomal immunoglobulin loci as a novel source for human polyclonal antibodies (hPABs) for therapy. Transgenic calves containing a human artificial chromosomes with the entire unrearranged sequences of the human immunoglobulin HC and lambda LC locus were already generated producing functional human immunoglobulins (182). Furthermore, there are first issues to knock out bovine immunoglobulin μ heavy chain locus and bovine prion gene to address the potential BSE risk in cattle (183).

An overview of recombinant antibodies produced in different hosts is shown in Table 1. It must be discriminated between the yield of functional antibodies after purification and the total yield. Furthermore, intrinsic properties of the indiviual antibody molecule can dramatically influence its expression.

5. CONCLUDING REMARKS

Although at present, mammalian cell lines are the only system used for the production of therapeutic antibodies, alternative expression hosts like yeasts, insect cells as well as transgenic plants are under development which can produce antibodies with human-like glycosylations suitable for therapeutic application at much lower production costs. For some therapeutic applications, mostly in *in vitro* diagnostics and strategies that rely on antigen neutralization, “correct” post-translational modifications are irrelevant and production systems employing hosts like bacteria, yeasts, filamentous fungi and insect cells can be used. They have many advantages over mammalian cells, such as easier handling, higher robustness and reduced production costs. Transgenic plants and animals have the potential for the production of antibodies in theoretically unlimited amounts. A comparison of key features of the different production systems is given in Table 2.

The Gram-negative bacterium *E. coli* is the best genetically examined organism and provides a large set of molecular-biological tools for genetic engineering. Antibody fragments like scFvs or Fabs are usually produced in *E. coli* by periplasmic expression. Particularly, in combination with antibody phage display *E. coli* allows fast, robust and reliable small scale antibody production of the selected antibodies. High yields of 1 - 2 g/L of functional scFv or Fab produced in *E. coli* are strongly dependent on the individual antibody fragment and require optimized high cell density fermentation.

Gram-positive bacteria are well suited for biotechnological processes because of their powerful secretion apparatus and lack of outer membrane. However, antibody production systems employing Gram-positive bacteria are still in the developmental stage.

Yeasts combine short generation time, ruggedness and low nutrient requirements of microbial prokaryots with the eukaryotic folding and secretion capacity. The simple generation of stable transgenic yeast clones has capacity for through-put processes, but far beyond *E. coli*. Despite the availability of modified yeast strains which make human-like glycosylations possible, production of complex full size IgGs still remains a challenge.

Filamentous fungi require higher efforts to generate transformed clones in comparison to yeast. However, they are promising candidates for biotechnological production because of their long biotechnological tradition and their high secretory capacity. *A. niger* has already been used to produce large amounts of functional IgG with great potential for improvement.

Insect cells have a better suited protein folding and secretion apparatus than prokaryots and lower eukaryots, as well as higher robustness combined with less sophisticated requirements for fermentation than mammalian cells. However, development of stable insect cell lines and process technology is not as far developed as for mammalian cells.

Mammalian cell lines are still the system of choice for the production of therapeutic antibodies, because they have the best equipped folding and secretion apparatus for antibodies and are capable to produce human glycosylations, provide easy

Table 1. Production of recombinant antibodies in different hosts

Host	Antigen	Antibody format (clone)	Production system	Yield	Ref
Gram-negative bacteria					
Escherichia coli	digoxin	Fab (26-10)	shake flask	0.8 mg/L/O.D.$_{600}$	51
Escherichia coli	CD18	F (ab')$_2$	fermentor	2.5 g/L	41
Escherichia coli	lysozyme	scFv (D1.3)	400 mL shake flask	0.3 – 1.0 mg/L	73, 184
Escherichia coli	MUC1	V_{HH}	100 L shake flask	10 mg/L	185
Escherichia coli	p815^{HER2}	Fab	10 L fermentor	1-2 g/L	40
Escherichia coli	PPL	VL dAb	1.5 L fermentor	35-65 mg/L	186
Escherichia coli	phOx	scFv	50 mL shake flask	16.2 mg/L	66
Escherichia coli	phOx	scFv	3 L fermentor	1.2 g/L	65
Escherichia coli	scorpion toxin Cn2	scFv; Fab (BCF2)	n.d.	0.3 mg/mL; 1.0 mg/L	187
Escherichia coli	tissue factor	IgG	10 L fermentor	130-150 mg/L	63
Escherichia coli	TAG-72	Fv (B72.3)	shake flask; fermentor	40 mg/L; 450 mg/L	188
Escherichia coli	HIV capsid	Fab, engineered	shake flask	12 mg/L	189
Proteus mirabilis	FAP	scFv (OS4)	50 mL shake flask	~12 mg/L	67
Proteus mirabilis	phosphorylcholine	scFv-dHLX	n.d.	10-18 mg/L	35
Gram-positive bacteria					
Bacillus brevis	uPA	Fab	2 L shake flask	100 mg/L	68
Bacillus megaterium	lysozyme	scFv (D1.3)	400 mL shake flask	0.41 mg/L	73
Bacillus subtilis	digoxin	scFv	n.d.	12 mg/L	70
Lactobacillus paracasei	rotavirus	V_{HH}	n.d.	~ 1 mg/L	38
Streptomyces lividans	lysozyme	Fv	n.d.	~ 1 mg/L	190
Eukaryots					
Yeast					
Pichia pastoris, Kluyveromyces lactis	catalytic antibody	scFv (4B2)	n.d.	1.3-4 mg/L	191
Yarrowia lipolytica, Kluyveromyces lactis	human Ras	scFv	shake flasks	10-20 mg/L	192
Pichia pastoris	MUC1	V_{HH}	baffle flask	10-15 mg/L	193
Pichia pastoris	p185^{HER-2}	scFvs (C6.5; C6ML3-9)	2 L baffle flask	70 mg/L; 9 mg/L	82
Pichia pastoris	Desipramine; CD7	scFvs (G5;3A1f)	baffle flask	60-250 mg/L	83
Pichia pastoris	human CD33	scFv (p67)	shake flask	11-48 mg/L	87
Pichia pastoris	serpins	scFv	shake flask	25 mg/L	194
Pichia pastoris	PSA	scFv	n.d.	20 mg/L	195
Pichia pastoris	Fc epsilon RI	Fab	100 mL shake flask	10-40 mg/L	196
Pichia pastoris	anti-idiotypic	Fab (3H6)	fermentor	260 mg/L	197
Pichia pastoris	atrazine	Fab (K411B)	5 L fermentor	40 mg/L,	198
Pichia pastoris	HBsAg	Fab	shake flask; 5 L fermentor	50 mg/L; 420-458 mg/L	199, 200
Pichia pastoris	GST	scFv-Fc	5 L fermentor	10-30 mg/L	89
Pichia pastoris	ED-B (B-fibronectin)	scFv- $(G_2S_2)_2$- GS-C_{1-3}	shake flask; 4 L fermentor	5-20 mg/L; 60 mg/L	201
Pichia pastoris	TAG-72	tetravalent scFvs	20–30% tetramers	15-20 mg/L	202
Saccharomyces cerevisiae	HCG; RR6	V_{HH} (H-14; R2)	100 mL shake flask	up to 100 mg/L	85
Saccharomyces cerevisiae	L6 tumor antigen	chimeric IgG/ Fab (L6)	ADCC but not CDC	50-80 mug/L/ 100-200 mug/L	203
Filamentous Fungi					
Aspergillus niger var. awamori	ErbB2	hIgG1; Fab (trastuzumab)	50 mL shake flask	0.9 g/L; 1.2 g/L	98
Aspergillus niger var. awamori	lysozyme	scFv (D1.3)/D1.3-glucoamylase	n.d.	10/50 mg/L	100
Aspergillus niger var. awamori	RR6 azo-dye hapten	V_{HH} (R2)	shake flask	7.5 mg/L	102
Trichoderma reesei	phOx	Fab	15 L fermentor	1 mg/L	101
Insect cells					
Drosophila melanogaster, DS2	ACMV	scFv, scFv-C_{kappa} (S20)	25-cm^2-flasks	25 mg/L	115
D. melanogaster, SC-2	E-selectin	scFv	n.d.	0.1-0.4 mg/L	204
Spodoptera frugiperda, Sf9	phOx	scFv	6 L bioreactor	32 mg/L	105
S. frugiperda, Sf9	hanta-, hepatitis-, rabbies virus	Several IgGs	T75 flasks	6 – 18 mg/L	106
S. frugiperda, Sf9	arsonate	IgG1	n.d.	5 mug/L	112
S. frugiperda, Sf9	rhesus D	IgG1	n.d.	10 mg/L	110
Trichoplusia ni	lipoprotein I	Murine IgG2a	150 mL shake flasks	n.d.	113
Mammalian cells					
Transient					
HEK293T	various	IgG and scFv-Fc	LF, HEKfectin, tissue culture plates	1 – 20 mg/L	137, unpubl.
HEK293E	n. d.	hIgG	PEI, 10 L shake bottle	80 mg/L	143
HEK293E	n. d.	hIgG	CaPi, 110 L STR	7.7 mg/L	205
CHO-K1	several antigens	IgG	LF, 1 L spinner flask	20 mg/L	206
CHO-K1-S	TAG72	Chimeric IgG4 (B72.3)	PEI, 10 L, shake flask	39 mg/L	207
Stable					
CHO	TAG-72	Fv (B72.3)	roller bottle	4 mg/L	188
GS-NS0	TAG-72	cIgG4 (cB72.3)	fermentor, fed-batch, air-lift	560 mg/L	208
NS0	p185^{HER-2}	scFv-CH3 (10H8)	n.d.	60 mg/L	142

SP2/0	CEA	hIgG (hMN14)	serum free	300 mg/L	209
GS-NS0	different	IgG	7 L STR, fed-batch	600 mg/L	210
GS-NS0	n. d.	IgG	fermentor, fed-batch	1 g/L	211
GS-NS0	n. d.	IgG	fermentor, fed-batch	1.8 g/L (3-4 wks)	140
GS-NS0	n. d.	IgG	fermentor, fed-batch	2.7 g/L	141
CHO-S	n. d.	hIgG	fermentor	4.7 g/L	116
Transgenic plants					
Arabidopsis thaliana	CK	Fab (MAK33)	leaves	1.3, 3-6 % total soluble protein	166, 212
Medicago sativa	human globulin	IgG (C5-1)	leaves	0.1-1 % total soluble protein	171
Nicotiana tabacum	MUC1	VHH	leaves	28-136 mg/kg	213
Nicotiana tabacum	cutinase	scFv (21C5)	leaves	1 % soluble protein	157
Nicotiana tabacum	CEA	scFv; IgG (T84.66)	leaves	5 mg/kg; 1 mg/kg	159
Nicotiana tabacum	CK	Fab (MAK33)	leaves	0.044 % total soluble protein	212
Nicotiana tabacum	phosphonate ester	catalytic IgG	leaves	1.3 % total protein	153
Nicotiana tabacum	TM	IgG (mAb24)	leaves	0.04-0.16 % total protein, 1.56 mg/kg	214
Nicotiana tabacum	TMV	IgG (rAb24)	suspension culture	37.6 mg/kg	162
Oryza sativa	CEA	scFv (T84.66)	leaves	1.5-29 mg/kg	168
Petunia hybrida	dihydroflavonol 4-reductase	scFv	leaves	1 % total soluble protein	170
Triticium aestivum	CEA	scFv (T84.66)	leaves	50-900 mug/kg	168
Transgenic animals					
Mus sp.	TGEV	IgA (6A.C3)	milk	5 – 6 g/L	176
Mus sp.	TFR	IgG-RNase (E6)	milk	0.8 g/L	177
Mus sp.	CD19	chimeric IgG	milk	0.5 g/L	178
Mus sp.	CD6	chimeric IgG	milk	0.4 mg/L	215
Gallus sp.	Dansyl; PSMA	chimeric IgG; hIgG1 (F1)	egg white	0.5 – 3.4 mg/egg	180

Abbreviations: ACMV, African cassava mosaic virus; CaPi, Calcium phosphat transfection; CEA, carcinoembryonic antigen; FAP, fibroblast activation protein alpha; GS, glutamyl synthetase amplification system; HCG, human pregnancy hormone; RR6, azo-dye hapten; HCG, human chorionic gonadotropin hCK, human kreatin kinase; HIV, human immunodeficiency virus; LF, lipofectamine; lipoprotein I, lipoprotein I of *P. aeruginosa;* PEI, polyethylenimine transfection; phOx, 2-phenyloxazoline-5-on; PPL, peptostreptococcal protein L; PSA, prostate-specific antigen; PSMA, prostate specific membrane antigen; serpins, serpins of *Mamestra configurata*; SP2/0, murine myeloma cell line; STR, stirred-tank bioreactor; TFR, transferin receptor; TMV, tobacco mosaic virus; uPA, urokinase type plasminogen activator.

Table 2. Recombinant production systems

Organism	Growth	Generation of cells	Yield	Glycosylation	Whole ig (1)
In vitro					
reticulocyte lysate	not necessary	not necessary	very low	-	-
Prokaryotic organisms					
E.coli					
Cytoplasm	very fast	simple	high/ req. S--S refolding	-	-
Periplasm, soluble	very fast	simple	low/medium	-	-
Periplasm, inclusion bodies	very fast	simple	high/ req. refolding	-	-
Other Gram-negative	very fast	simple	low/ medium		
Gram-positive	fast	simple	low/medium	-	-
Eukaryotic organisms					
Yeasts	medium	1-n months	medium	different /engineered	
Filamentous fungi	medium	> 1 month	low/medium	different	+
Baculovirus / insect cells	medium	> 1 month	low	different	+
Mammalian cells					
transient	medium	simple	medium	+[2]	+
stable		medium	ca. 1 year	high	+[2]
Transgenic plants	slow	6 mo – > 1year	high	different	+
Transgenic animals	slow	> 1 year	high	+	+

[1] Procaryotic organisms are typically used to produce scFv , Fab frgaments or diabodies. A few examples of complete IgG production in procariotic organisms have been indeed described, but so far not matured into broadly applicable procedures, [2] Glycosylation of the Fc part of therapeutically approved IgG produced in CHO cells is different to human glycosylation but the differences are not believed to affect product safety but can lower efficiency, e.g. ADCC. Consequently, human cell lines like HEK293 or PER.C6™ are increasingly used, or engineering of the glycosylation machinery is evaluated.

downstream processing, highest product quality as well as lowest immunogenicity. However, large scale protein production in mammalian cells requires very high technological efforts and is very expensive and time consuming. To date, up to 5 g per liter functional IgG production have been reported which has not been achieved by any other biofermentation system and, notwithstanding this, there are still efforts to increase even further the yield of mammalian antibody production. However, up-scaling of mammalian production does not dramatically reduce the production costs.

Transgenic plants represent the most scalable production system with least production costs. Growing of plants can be easily expanded by simply using larger fields without significant higher costs, only downstream processing is connected with higher effort. Still, the

growing of transgenic plants is politically not accepted in several countries.

Currently, the generation of transgenic animals is very complex and difficult. However, antibody production in milk or eggs is also highly scalable. Down-stream processing of antibodies is much easier than that of transgenic plants and might not be required for topical and oral applications.

There is no 'universal' production system, that can guarantee high yields of recombinant antibody, particularly since every antibody-based molecule itself will cause its own issues in terms of expression as a result of quite different folding efficiencies of individual antibodies. In the very near future, recombinant antibodies from other production systems than mammalian cell culture will surely find their way into the therapeutic application. Agrarian mass production systems like transgenic plants or animals may even allow reductions in production cost to a level allowing the use of recombinant antibodies for consumer applications.

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Abbreviations: IgG: immunoglobulin G; mAb: monoclonal antibody; Fv: fragment variable; scFv: single chain Fv; Fab: fragment antigen binding; Fc: fragment crystallizable

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