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TITLE:
Expression of Recombinant Antibodies

ABSTRACT:
Recombinant antibodies are highly specific detection probes in research, diagnostics, and have emerged over the last two decades as the fastest growing class of therapeutic proteins. Antibody generation has been dramatically accelerated by in vitro selection systems, particularly phage display. An increasing variety of recombinant production systems have been developed, ranging from Gram-negative and positive bacteria, yeasts and filamentous fungi, insect cell lines, mammalian cells to transgenic plants and animals. Currently, almost all therapeutic antibodies are still produced in mammalian cell lines in order to reduce the risk of immunogenicity due to altered, non-human glycosylation patterns. However, recent developments of glycosylation-engineered yeast, insect cell lines, and transgenic plants are promising to obtain antibodies with “human-like” post-translational modifications. Furthermore, smaller antibody fragments including bispecific antibodies without any glycosylation are successfully produced in bacteria and have advanced to clinical testing. The first therapeutic antibody products from a non-mammalian source can be expected in coming next years. In this review, we focus on current antibody production systems including their usability for different applications.

Introduction:
Today, antibodies are used for several applications in research, diagnostics, and therapy. They are used in many standard assays such as immunoblot, flow cytometry, or immunohistochemistry. In addition this, the emerging field of proteome research has a huge need of binders against different protein antigens and splice variants (1, 2). Moreover, recombinant antibodies are used for the diagnosis of different pathogens (3–5) or toxins (6, 7). In the past decade, several antibodies for therapeutic applications have been developed (8, 9), primarily targeting inflammatory or tumor diseases (10). In 2010, sales of approved therapeutic monoclonal antibodies in the USA and EU reached 50 billion US dollars (11).
For the detection of different antigens, polyclonal antibodies are widely used in research and diagnostics. These sera contain a large and diverse amount of different antibodies with unknown specificities. However, polyclonal non-human antibodies may exhibit an immune response in human beings that hampers the therapeutic use for example after snake bites (12). Therefore, the production of monoclonal antibodies (mAbs) by hybridoma technology was a significant milestone (13) for the generation of antibodies for therapeutic use. As this technology is based on the fusion of antibody producing spleen cells from immunized mice or rats with immortal myeloma cell lines, its main obstacle is the inefficient immune response to highly toxic or conserved antigens. In addition, nearly all antibodies which are currently in clinical development are of human-origin or at least humanized in some aspect (9, 14, 15) to prevent immunogenicity. Consequently, transgenic animals, especially mice, have been developed which contain a human immunoglobulin gene repertoire (16, 17) solving the problem of immunogenicity but not the need of an efficient immune response after immunization. Finally, in vitro selection technologies such as antibody phage display or ribosomal display provide a solution for the generation of human antibodies (18–22). These new antibody generation technologies have increased the amount of antibodies for different applications and, therefore, also the need of efficient production systems.
Immunoglobulin G (IgG) is a heterotetrameric molecule consisting of two heavy and two light chains, respectively, which are connected via disulfide bonds. Heavy and light chains (HC and LC) also contain intramolecular disulfide bonds for stabilization (23). These structural properties require a sophisticated folding apparatus as well as an oxidizing environment for the generation of disulfide bonds. Consequently, many traditionally expression hosts do not provide these mechanisms for efficient production of IgGs. Therefore, smaller antibody fragments have been developed which combine easier production with full antigen binding capacity of an IgG (Figure 1). In addition, the development of smaller fragments was the basis for most of the in vitro antibody generation systems (18–22). These antibody fragments can be used for applications, where epitope binding is sufficient for the desired effect including therapeutic applications such as virus neutralization or receptor blocking.

The smallest antigen binding fragment of immunoglobulins maintaining its complete antigen binding site is the Fv fragment, which consists only of variable (V) regions. A soluble and flexible amino acid peptide linker is used to connect the V regions to a scFv (single chain fragment variable) fragment for stabilization of the molecule (24), or the constant (C) domains are added to the V regions to obtain a Fab fragment (Figure 1). Today, scFv and Fab are the most widely used antibody fragments which are produced in prokaryotes. Other antibody formats have been produced in prokaryotic and eukaryotic cells, for example, disulfide-bond stabilized scFv (ds-scFv) (25), single chain Fab fragments (scFab) combining scFv and Fab properties (26) as well as di- and multimeric antibody formats like dia-, tria-, or tetra-bodies (27, 28) or minibodies (miniAbs) comprising different formats consisting of scFvs linked to oligomerization domains like immunoglobulin CH3 domain (28), leucin zipper, helix turn helix motif streptavidin, or scFv-scFv tandems (29–31). Bispecific antibody formats combine two different antigen binding domains in one molecule (32–34). The smallest antibody fragments are VHHs of cameloide heavy chain antibodies (35) and single domain antibodies (dAb) (36, 37).
For most therapeutic applications, the Fc moiety of an immunoglobulin is essential for the method of action as it mediates the effector functions such as cellular dependent cytotoxicity or the activation of the complement system. Therefore, antibody fragments have been fused to the Fc domain to regain effector functions and avidity (38, 39). Figure 1 depicts some of these antibody formats that have been developed for different applications.

Gram-negative bacteria ::: Antibody Production in Prokaryotic Hosts:
Escherichia coli is the most important production system for recombinant proteins reaching volumetric yields in the gram per liter scale for extracellular production (40–42). For production of functional antibody fragments, the key to success was the secretion of both V chains into the periplasmic space of E. coli where the oxidizing environment allows the correct formation of disulfide bonds and the assembly to a functional Fv fragment (43). This strategy also allowed the first expression of functional Fab fragments in E. coli described in 1988 (44).
The production of recombinant antibodies in the reducing cytoplasmic compartment results mostly in non-functional aggregates (45). Recovery of functional antibody fragments from cytoplasmic inclusion bodies by complete denaturation and refolding (46) is often not efficient. Stable cysteine free mutants of some scFvs were successfully produced in the cytoplasm of E. coli (47, 48). 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 the yield of functional Fab (49).
For the production of camelid single domain antibodies (VHH), coexpression of Erv1p sulfhydryl oxidase increased the yield in the cytoplasm (50).
Despite these efforts, most antibody fragments are produced in the periplasm of E. coli using N-terminal leader sequences targeting the periplasmic Sec pathway (51), for example signal peptides derived from outer membrane protein A (OmpA), alkaline phosphatase A (PhoA), or pectate lyase B (PelB) (52–54). Also the SRP pathway can be used for antibody fragment production (55). After expression, recombinant antibodies are usually isolated from the periplasmic fraction (56, 57) but also from the culture supernatant (58–61).
The yield of functional scFv fragments has been improved by co- or overexpression of GroES/L, peptidyl prolyl-cis,trans-isomerase FkPa, or other folding helper proteins (62–66). Functional expression can also be increased by optimization of cultivation parameters, such as temperature, media, or additives. Here, the optimal parameters are dependent on the individual antibody fragment (58, 67). The production system itself influences the production rate. Very high yields of antibody fragments produced in E. coli are mainly provided by high-cell density fermentation in bioreactors: the expression of a hapten-specific scFv produced in a bioreactor (68) lead to yields up to 1.2 g/L compared to 16.5 mg/L yield of the same antibody obtained by optimized shake flask production (69), which can be mostly addressed to the over 100-fold higher cell density in the bioreactor. A recent production system is the LEX bubble column bioreactor. Yields in the LEX system of an anti-MUC1 scFv was ~30–40× higher and yields of an anti-lysozyme antibody was about 2× higher compared to shake flask incubation (67, 70). E. coli strain optimization, e.g., plasmid stability, can additionally improve production yield (71).
The Fab format requires 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 are optimal (56). Even aglycosylated full-size IgGs were successfully produced in E. coli (72, 73). In our view, the raison d'être of complete IgG production in E. coli is doubtful.
Cell wall-less L-forms of the Gram-negative bacterium Proteus mirabilis were used for the production of miniAbs and scFv (30, 74), but yield of total scFv and of functional scFv were different and ranged from 83 to 127 mg/L of total scFv to just 9–12 mg/L of functional scFv (74). However, quite recently scFv were produced successfully in Pseudomonas putidas with a yield of 0.5–3.6 mg/L. Interestingly, production yields were decreased by using scFv genes codon optimized for P. putidas (75).

Gram-positive bacteria ::: Antibody Production in Prokaryotic Hosts:
Gram-positive bacteria directly secrete proteins into the medium due to the lack of an outer membrane which could facilitate production of antibody fragments. The Gram-positive bacteria Bacillus brevis (76, 77), Bacillus subtilis (78, 79), and Bacillus megaterium (80–85) have already been successfully used for the production of different antibody fragments. In addition, B. megaterium does not produce alkaline proteases and provides high stability of plasmid vectors during growth allowing stable transgene expression during long term cultivation in bioreactors (86).
Lactobacilli are also tested for antibody production and are "generally regarded as safe" (GRAS) microorganisms. To date, two lactobacillus strains were used for the production of scFvs, Lactobacillus zeae/casei (87, 88), and Lactobacillus paracasei (35, 89). The GRAS status of lactobacilli allows their direct use for oral application for example for production of anti-Streptococcus mutans antibody fragments to prevent tooth decay (88).

Yeasts ::: Eukaryotic Hosts Used for Antibody Production:
Eukaryotic cells have developed an advanced folding, post-translational, and secretion apparatus which enhances the secretory production of antibodies, including full immunoglobulins compared to bacteria. Yeasts combine the properties of eukaryotic cells short generation time and ease of genetic manipulation with the robustness and simple medium requirements of unicellular microbial hosts. Moreover, yeasts have been used for fermentation in food production for several millennia in human history; they do not produce bacterial endotoxins and have gained the GRAS status paving the way toward production of therapeutic proteins (90, 91). Pichia pastoris represents the major yeast strain used for recombinant antibody production (92). Other yeasts like Saccharomyces cerevisiae, Hansenula polymorpha, Schizosaccharomyces pombe (93, 94), Schwanniomyces occidentalis, Kluyveromyces lactis, and Yarrowia lipolytica (95) have also been described for protein production but have played only a minor role. P. pastoris shows overall optimal capacity for the production and secretion of heterologous proteins than S. cerevisiae and does not secrete large amounts of its own protein which simplifies the downstream processing. Moreover, P. pastoris prefers respiratory growth resulting in high-cell densities of more than 100 g/L dry weight (96). Probably the most prominent feature of P. pastoris is the metabolization of methanol as sole carbon source. The alcohol oxidase 1 (AOX1) promoter is strictly controllable by methanol and commonly used for recombinant protein expression. The secretory production of heterologous proteins including antibodies requires an aminoterminal signal sequence targeting the yeast's secretory pathway. S. cerevisiae mating factor alpha (alpha-factor) pre-pro peptide is the most commonly used secretory signal sequence and is followed by appropriate proteolytic cleavage sites sensitive for the Golgi resident endoprotease KEX2 for efficient release of antibodies during secretion, which is often used in combination with ST13 exoprotease sites (97). Expression of scFv antibody fragments in P. pastoris was first shown by Ridder et al. in 1995 (98). Yields for different scFvs ranged from 70 mg/L (99) to 250 mg/L (100). Up to 8 g/L functional scFv were obtained under optimized conditions in bioreactors with coexpression of BiP (101). Llama VHHs achieved over 100 mg/L yield in S. cerevisiae even in shake flask cultivation (102). Production of more complex, yet still single-gene-encoded formats such as dimeric scFv-Fc antibodies in P. pastoris achieved production levels of 10–30 mg/L (103). Antibody formats encoded by two genes such as Fab and IgG required the fusion of the two different antibody chains to the aminoterminal secretory signal sequence and their cotransformation. The yield of Fabs produced in yeast ranged from 1 to 50 mg/L by shake flask cultivation and up to 0.5 g/L in bioreactors (96).
Limited data concerning full-sized IgG expression in yeast is available. In an early study, a mouse-human chimeric antibody and its Fab fragment were produced in S. cerevisiae with a yield of 50–80 μg/L IgG and 200 μg/L Fab, respectively. The chimeric IgG mediated tumor specific binding and ADCC (antibody dependent cellular cytotoxicity) but no CDC (complement dependent

cytotoxicity) (104). Using P. pastoris up to 1.4 g/L of a human IgG1 could be expressed in a 40-L bioreactor (105).
Lower transformation rates compared to E. coli must be considered for antibody library generation rather than for antibody production. Moreover, the frequency of homologous transformation in yeast is higher compared to higher eukaryotes facilitating the process of making stable expression clones. Specific issues of heterologous protein expression in yeast can be circumvented by optimizing gene sequences, for example by avoiding AT-rich stretches which can cause premature transcriptional termination. The productivity of antibody fragments in yeasts was increased by DNA shuffling (106).
Inefficient secretion of larger heterologous proteins (>30 kDa), proteolysis of secreted proteins during high-cell density fermentation, and inappropriate glycosylation of human glycoproteins are serious issues which required engineering of yeast strains. Overexpression of the chaperone immunoglobulin binding protein (BiP) or protein disulfide isomerase (PDI) in S. cerevisiae increased scFv secretion titers twofold to eightfold, with an average yield of 20 mg/L in shake flask culture (107). Yeasts tend to hyperglycosylate heterologous proteins even at positions not glycosylated in the native mammalian host, which can influence activity of antibodies and is a potential source of immunogenicity or adverse reactions in human patients. P. pastoris exhibits much lower hyperglycosylation than S. cerevisiae, and its N-linked carbohydrate structures are already similar to the mammalian high-mannose core unit Man5–6GlcNAc2 (108). Moreover, genetically modified glyco-engineered P. pastoris strains have been generated which produce humanized glycosylation patterns (109–113). The therapeutic IgG antibodies produced in glyco-engineered yeast achieved results that were comparable to its counterpart Trastuzumab that has been produced in mammalian cells (114). Unlike IgGs produced in wildtype yeast, those produced in glyco-engineered yeasts were able to mediate antibody-mediated effector functions.
Production processes employing glyco-engineered yeasts are currently optimized for commercial antibody production (115) as well as for high throughput screening (116).

Filamentous fungi ::: Eukaryotic Hosts Used for Antibody Production:
Filamentous fungi of the genera Trichoderma and Aspergillus have the capacity to secrete large amounts of proteins and metabolites into the medium (117). They are widely used in the food and biotechnological industry, for example A. niger for citric acid production. Moreover, A. niger (subgenus A. awamori) and Aspergillus oryzae gained obtained GRAS status. Two promoters are typically used for the expression of antibodies in fungi: the glucoamylase promoter (glaA) (118) and the endoxylanase A promoter (exlA) (119). Antibody chains are usually fused to the aminoterminus of glucoamylase in Aspergillus and cellobiohydrolase I in Trichoderma spec., respectively, in order to obtain optimal secretion (120). Moreover, protease cleavage sites like KexB are introduced to release the antibody from glucoamylase before secretion (118). Yields of up to 1.2 g/L IgG were achieved in A. niger when both antibody chains were fused to glycoamylase. In Trichoderma reesei, 150 mg/L of a Fab fragment was obtained when both chains were fused with cellobiohydrolase I increasing yields 100-fold higher than with its natural signal peptide (121). A. awamori was used for the production of several scFvs, llama VHHs and antibody enzyme fusion proteins (117, 119, 122). A yield of 73.8 mg/L of an anti-EGFR-VHH was achieved in A. oryzae by using a Taka-amylase A signal sequence and 28 amino acids from the aminoterminal region of Rhizopus oryzae lipase (123).
Fungal proteases can result in protein degradation which was addressed by deletion mutants. Chrysosporium lucknowense C1 contains a triple protease deletion (Delta-alp1, Delta-pep4, Delta-alp2) and was successfully used in small-scale productions for screening as well as in high scale bioreactor productions (124).

Protozoa ::: Eukaryotic Hosts Used for Antibody Production:
Recently, the eukaryotic parasite Leishmania tarentolae has been explored as an expression system for different recombinant proteins (125, 126). One major advantage of this expression system is the mammalian-like glycosylation pattern: this protozoa is able to perform O-glycosylation as well as N-glycosylation, which is highly conserved in mammalians (127). Consequently, L. tarentolae has been begun to be used for the production of recombinant antibodies: analysis of different signal peptides lead to a protein yield of 2–6 mg/L purified scFv (128).

Insect cells ::: Eukaryotic Hosts Used for Antibody Production:

Insect cells represent a very versatile eukaryotic expression system. They can be efficiently transfected with insect-specific viruses from the family of Baculoviridae, particularly the Autographa californica nuclear polyhedrosis virus (AcNPV). Baculoviruses are highly species-specific and are considered as safe for humans, mammalians and plants. Infection of human hepatocytes and mammalian cell lines including stable transduction has been demonstrated in cell culture without evidence of viral replication or gene expression under the control of baculoviral promoters (129, 130). Non-essential baculovirus genes involved in the viral life cycle, like Polyhedrin, P10, or Basic can be replaced by heterologous genes. The flexible viral envelop allows packaging of large heterologous gene sequences of more than 20 kb. Heterologous genes under the control of the strong polyhedron promoter are expressed at levels ranging from 0.1 to 50% of the total insect cell protein. Baculoviral protein expression is normally performed in insect cell lines like Sf-9 and Sf-21 of Spodoptera frugiperda, DS2 cells of Drosophila melanogaster, or High Five cells (BTI-TN-5B1-4) of Trichopulsia ni. High Five cells have certain advantages over Sf-9 cells for recombinant protein expression because they secrete up to 25-fold higher protein levels (131), have a more rapid doubling time, allow quick adaptation to serum-free medium and grow in suspension culture. In contrast, Sf-9 and Sf-21 cells are recommended for producing high-titer viral stocks due to higher transfection efficiency. Recombinant protein production can be performed in small-scale using plates or shake flasks as well as in large scale using Spinner flasks or bioreactors. Important parameters for optimizing baculoviral protein production are multiplicity of infection (m.o.i.), production length (usually up to 96 h), addition of protease inhibitors due to the release of viral proteases, temperature (usually 25–30°C), and media pH (pH 6.0–6.4).
Secreted monomeric anti-phOx scFv were obtained at levels of up to 32 mg/L in a 6-L bioreactor with 109 cells per liter after 72 h with an m.o.i. of 1 (132). Production yields of 6–18 mg/L have been achieved for various IgGs (133). Immunoglobulins produced in High Five cells showed mammalian-like terminal galactosyl residues β(1,4)-linked to the biantennary GlcNAc residues. In contrast, the absence of sialylation, the formation of paucimannosidic structures and the presence of potentially allergenic α(1,3)-fucose linkages are different to mammalian glycosylation (134). Nevertheless, IgGs produced in insect cells were able to mediate effector functions like complement binding (135, 136) and ADCC (137). Insect cell protein expression was improved using protease deficient baculovirus strains or cell lines with additional glycosyltransferase gene modifications to obtain glycosylation patterns comparable to mammalian cell lines (138–141). Expression of IgGs in insect cells under control of the strong Polyhedron promoter resulted in an extensive aggregation, probably caused by overloading the cellular folding and post-translational processing apparatus (142).
Overexpression of the ER resident chaperone binding protein (BiP) significantly enhanced levels of soluble and secreted IgGs in T. ni cells (143). Enhanced secretion of IgGs was also achieved by coexpression of protein disulfide isomerase (PDI) or the human cytosolic chaperone hsp70 in T. ni cells (138).
Due to strong usage of the cellular metabolism during baculoviral protein expression a high diversity in the post-translational modification was observed. Alternatively to baculoviral expression, insect cells can also be transfected with expression plasmids in a transient or stable manner. Here, usually Schneider 2 (S2) cells of D. melanogaster are used. 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 (144). Immunoglobulin G production using the baculovirus expression system demonstrated IgG effector function such as complement binding (135, 136). 10 µg/mL of anti-Rhesus D antibody produced in Sf-9 cells mediated lysis of Rh+ red blood cells by ADCC (137).

Stable production of antibodies in mammalian cells ::: Mammalian cells ::: Eukaryotic Hosts Used for Antibody Production:
The generation of stable master cell lines is a prerequisite for GMP compliant IgG production in the therapeutic sector in order to guarantee long term production stability. Here, the antibody gene expression cassettes have to be stably integrated into the host cell genome.
Strong promoters like the immediate early cytomegalovirus (CMV) or the cellular elongation factor (EF) 1-alpha promoter and polyadenylation sites from the simian virus (SV) 40 or the bovine growth hormone (BGH) for improved mRNA stability and translation efficiency are usually implemented into the expression vector. Furthermore, splicing of mRNA is known to promote mRNA packaging and transfer into the cytosol in order to stabilize and enhance gene expression as well as to reduce silencing of heterologous transgenes (153, 154). For IgG expression, two

different genes must be stably transfected into one cell clone, either by cotransfection 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 (155). The encephalomyelitis virus (ECMV) IRES has shown the highest efficiency in various mammalian cell lines. Mutated IRES derivatives 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 IgGs (156). The long term stability of ECMV IRES containing bicistronic constructs has been demonstrated even in the absence of selection pressure over months (157).
There are different methods to enhance antibody expression by increasing the number of antibody gene copies in the genome through gene amplification. The two major systems on the market are based on dihydrofolate reductase (DHFR) or glutamyl synthetase (GS) selection. Yield and functionality of an IgG1 produced in dhfr– CHO and GS-NSO are equivalent (158) and reached 1.8 g/L in GS-NS0 cells (159). However, gene amplification also causes genetic instability, and after removing the selection pressure the yield of antibodies can be reduced again. Moreover, high producer cell lines often contain only a few copies of the antibody genes. For example, up to 2.7 g/L final antibody concentration were obtained from NS0 cells containing three vector copies per cell (160). Other factors than the number of gene copies play an important role to achieve high production levels of antibodies. Therefore, industrial antibody expression platforms employ efficient screening systems in order to isolate the best of the high producers. However, there are also strategies to facilitate the isolation of high producer clones (161). To overcome negative effects of the integration site, protective cis-regulatory elements include insulators, boundary elements, scaffold/matrix attachment regions (S/MARs) (162), chromatin opening elements (163), and antirepressor elements (164) were introduced into the vector which reduced the influence of heterochromatin and stabilize transgene expression (165, 166). Silencing can be blocked by inhibition of histone deacetylation using butyrate (167) which could enhance the protein expression levels of the cells (168) but can also induce apoptosis.
Recombination enzymes, like bacteriophage P1 Cre recombinase, lambda phage integrase, or yeast Flp recombinase can efficiently catalyze the site specific integration into defined chromosomal recombination exchange cassettes which have been introduced into producer cell lines (169, 170). The 2A/furin technology allows expression of both IgG chains as a single gene due to post-translational auto-cleavage of the viral protease 2A encoded by the linker and subsequent processing by the Golgi protease furin (171, 172).

Transient production of antibodies in mammalian cells ::: Mammalian cells ::: Eukaryotic Hosts Used for Antibody Production:
The generation of high producer cell lines has been dramatically improved and accelerated (161, 173), however it is still too expensive, time-consuming and laborious for research applications, or if large numbers of individual antibodies have to be produced. Here, transient and semi-stable mammalian antibody expression is much more suitable because it allows fast and parallelized production without any need to generate producer cell lines (174). Moreover, transient mammalian antibody production can be scaled up by employing batch or fed-batch bioreactor processes to more than 150 L production volumes (175). Therefore, transient antibody production is suitable for small-scale production in antibody screening (176), but also capable to generate grams of antibodies (177–179).
The human embryonic kidney (HEK) 293 cell lines have been widely used for transient protein expression because they can be very efficiently transfected with plasmid DNA. Some derivatives were further transformed either with the simian virus 40 (SV40) large T antigen, termed HEK293T, or with the Epstein Barr virus (EBV) nuclear antigen 1 (EBNA1), termed HEK293E, in order to mediate semi-stable episomal propagation of vectors containing an origin of replication (ori) of SV40 or EBV, respectively. Transient transfection of plasmid DNA in HEK293 cells can also be performed in large scale by calcium phosphate transfection (180), cationic liposomes, and polymers like polyethyleneimine (PEI) (181, 182).
Recently, transient production of IgG-like scFv-Fc antibodies in the HEK293-6E cell line, a genetically modified variant with a truncated version of EBNA1 growing in suspension and chemically defined serum-free medium (183, 184), achieved volumetric yields of up to 0.6 g/L by simple shake flask cultivation. Improved production media, fed-batch supplementation, and well-controlled bioreactor processes allow higher cell densities and prolonged production time, both enhancing the yield. Backliwal and colleagues (177) combined optimized PEI-based transfection at high-cell densities with the coexpression of cell cycle regulators p18 and p21, acidic fibroblast growth factor, valproic acid supplementation, consequent maintenance at high-cell densities of

cells/milliliter and up-scaling to 2 L and achieved production levels of more than 1 g IgG within 2 weeks after transient transfection.

Transgenic plants ::: Transgenic Organisms:
The development of transgenic plants for the expression of recombinant antibodies is becoming interesting, especially when high amounts are required. Up-scaling of this production system can be achieved more easily compared to other systems such as mammalian cell culture, where up-scaling of the fermentation process leads to increasing production costs. In theory, the costs of an IgA expressed in plants are only 1–10% compared to the expression in hybridoma cells (185).
The generation of genetically modified dicotyledonous plants is mainly done by the transfer of the expression cassette of the transgene with the help of Agrobacterium tumefaciens. In principle, the gene of interest is cloned into the T-DNA of a binary plasmid (186, 187) which is flanked by two 25 bp imperfect repeats. In most cases, the expression of the transgene is under the control of one or two (188) copies of the constitutive cauliflower mosaic virus (CaMV). In addition, a selection marker is located on the T-DNA and transferred into the host genome for effective screening of successfully transformed plants. After integration of the T-DNA into the host genome by non-homologous recombination complete plants can be regenerated from transformed pieces of the plant [RB (189)]. As this procedure requires several months of transformation and special regeneration protocols, transient expression systems have been developed which allow time saving production of recombinant proteins: McCormick and colleagues designed a tobacco mosaic virus (TMV) based vector for the secretory expression of different scFvs for the treatment of non-Hodgkin's lymphoma (190). Expression yields in Nicotiana benthamiana were up to 100–800 μg/mL in the crude secretory extract. Same technique has been applied for the expression of idiotype-scFvs for personalized vaccination of follicular B-cell lymphoma patients in a phase I clinical study (191). In this study, nearly half of the treated patients developed an antigen specific immune response despite differences in glycosylation pattern.
Differences in the glycosylation pattern between mammalia and plants are one of the main obstacles researchers have to overcome when developing therapeutic antibodies expressed in plants. Although plants are able to perform complex glycosylation, differences in glycosylation patterns, in particular β1,2-xylose and α1,3-fucose, can lead to immunogenicity of the therapeutic proteins (192–194). Therefore, different strategies have been developed to express recombinant proteins with a more mammalian-like glycosylation pattern. The first one is the retention of the protein in the endoplasmic reticulum (eR) as eR-associated N-glycosylation leads to the generation of oligomannose-type N-glycans which are identical in plants and mammalians (192, 195). One side effect of this localization is the accumulation to higher levels in the eR (196, 197). A second approach for the expression of proteins with mammalian-like glycosylation patterns is the usage of glyco-engineered plants. In most cases, RNA interference (RNAi) is used for the down-regulation of endogenous beta1,2-xylosyltransferase and alpha1,3-fucosyltransferase leading to a reduction of the xylosylated and core-fucosylated N-glycans (198–200). A second type of glyco-engineering in plants is the coexpression of genes which facilitates the expression of human-like N-glycans (201) or even the in planta protein sialylation by the coexpression of six mammalian genes (202). An increasing effort has been put into the adaptation of N-glycosylation, but there are also some efforts in the engineering of sialylated mucin-type O-glycans to achieve the most human-like glycosylation patterns (203, 204). Alternatively, non-glycosylated antibodies which mediate protection against an inhalation anthrax spore challenge in non-human primates showed an improvement of the half-life in serum (205). Rodriguez and colleagues showed that the aglycosylated form of Nimotuzumab (currently in a phase II clinical study in the USA and Canada) produced in tobacco shares the in vitro and in vivo properties as well as the antitumor effect in nude mice with the glycosylated form (206).
Transient expression of an antibody in plants can be achieved using viral vectors. The main problem with this approach is the low infectivity with these vectors. Therefore, the more efficient transfer of A. tumefaciens was combined with the speed and high expression rate of plant RNA viruses (207). This system has been used for the expression of monoclonal antibodies in Nicotiana benthamiana with yields up to 0.5 g/kg fresh weight (208).
In principle, most plantibodies are expressed in tobacco (N. tabacum or N. benthamiana), but there are also production systems in Lemna minor (duckweed) (209–211), rice cell culture (212), Arabidopsis thaliana seeds (213, 214), Medicago sativa (alfalfa) (215), lettuce (216), and maize (217). HIV-1 neutralizing antibody 2G12 was expressed in the endosperm of maize and showed similar or even better neutralizing properties as its CHO-derived counterpart (218).

Besides the transfection or transformation of whole plants or at least organs, monoclonal BY-2 tobacco cell lines that grow in suspension have been developed (219). Flow cytometric analysis has been used to enrich cells expressing a fluorescent marker which was located on the same T-DNA with the antibody gene. Using this method for the enrichment of high expressing cells, production could be increased up to 13-fold and was shown to be stable for 10–12 months. Much effort has been set into the establishment and development of plants producing antibodies for therapy, but so far none of these products has appeared on the market, despite of the estimated dramatic reduction of production costs (220). Nevertheless, at least two plant derived antibodies have been used in clinical trials: CaroRX was developed by Planet Biotechnology (Hayward, CA, USA) and is expressed in transgenic tobacco (221). This antibody binds to the streptococcal antigen I/II of S. mutans, the major causative agent of bacterial tooth decay and prevents the attachment of S. mutans to tooth enamel. CaroRX has entered clinical phase II (222, 223). A second plant-made anti-idiotype antibody against non-Hodgkin-lymphoma (NHL) which was successfully tested in clinical phase I study has been mentioned above (191, 224).

Transgenic animals ::: Transgenic Organisms:
In recent years the idea of expressing human antibodies in transgenic animals has increased. On the one hand the humanization of antibodies for therapeutics derived from hybridoma technology is still a laborious and time-consuming procedure which often requires the generation and characterization of a set of different humanized versions of the antibody. On the other hand the mouse or rat derived antibodies may elicit an immune response in patients (225, 226). One method for the generation of fully human binders is the antibody phage display technology (21). Beside this, several researchers developed transgenic animals for the production and expression of human monoclonal and polyclonal antibodies: therefore, human antibodies have mostly been expressed in the milk of transgenic mice (227–230), goats (231), or even in eggs of transgenic chickens (232).
The first step toward the generation of human antibodies in animals by immunization was the transfer of a human minilocus containing unrearranged immunoglobulin variable, diversity, and joining elements linked to a human μ-chain into mice (233). In this study, approximately 4% of the extracted B-lymphocytes expressed human antibodies. The immunization of larger animals containing human chromosomal immunoglobulin loci would enable the production of even larger amounts of antibodies. Therefore, transgenic cattle were developed by the transfer of a human artificial chromosome vector containing the entire unrearranged sequences of the human immunoglobulin heavy and lambda light chain loci (234). For the improvement of the human antibody proportion and for safety reasons regarding the potential risk of BSE, the bovine immunoglobulin μ heavy chain locus and the bovine prion protein have been knocked out (235, 236). Finally, transgenic cattle carrying human immunoglobulin heavy and kappa-light chain loci have been used for immunization with anthrax protective antigen. The resulting polyclonal antibody mixture consisted of entirely human and chimeric immunoglobulins that showed high activity and were protective in an in vivo mouse challenge models (237). Rabbits and cattle were used for expression of a bispecific scFv targeting the melanoma-associated proteoglycan and the human CD28 molecule on T cells (238). The usage of different animals as a source for the generation of human polyclonal sera has already been initiated: the immunoglobulin gene loci have been knocked out in livestock such as pigs or rabbits (239–241). For a review of approaches for the generation of transgenic animals expressing polyclonal human antibodies see Houdebine (242).
Much of the energy in transgenic production has been set on the development of humanized mice or rats. The well established property of generating hybridoma cells from these species facilitates a streamlined approach for the generation of a cell line which stably expresses monoclonal antibodies (17, 243, 244). Using humanized mice, an anti-HIV-1 gp140 antibody was identified, but in contrast the low number of antigen specific hybridomas occurring during the generation of the clones has been observed (245). Therefore, the usage of humanized rats has been suggested to circumvent these problems and first antibodies have been developed with sub-nanomolar affinities using the so called OmniRat (246).

Concluding Remarks:
Today, mammalian cell lines represent the most widely used expression system for the production of recombinant antibodies. Several other hosts are being developed which are even able to produce antibodies with human-like glycosylation patterns. In addition to this, there are several applications where the glycosylation pattern does not play a critical role, such as for in vitro

diagnostics or in research. Therefore, bacteria, yeasts, filamentous fungi, and insect cells can be employed in order to lower the production costs of these products. In principle, transgenic plants and animals have the highest potential for up-scaling processes to theoretically unlimited production amounts. An overview of recombinant antibodies produced in different hosts is shown in Table 1. There, however, it must be discriminated between the yield of functional antibodies after purification and the total yield.

Antibody phage display is now a widespread method for the development of antibody fragments such as scFv or Fab. The expression host used in this technology is E. coli which is known to be the best genetically examined organism providing a large set of molecular biological tools for genetical engineering. Consequently, both antibody generation and production can be performed without changing the production system. Using high-cell density fermentation, the yield can be up to 1–2 g/L depending on the individual antibody fragment. Antibody fragments expressed in E. coli are mainly secreted into the periplasm and have to be extracted from there. Gram-positive bacteria lack the outer membrane and are well suited for biotechnological processes due to their powerful secretion apparatus which allow easy purification directly from the cultivation supernatant. However, antibody production systems employing Gram-positive bacteria are still in the developmental stage. However, larger antibody formats are very difficult to express in bacteria, if they can be expressed at all. Furthermore, the lack of a glycosylation apparatus limits their use, if effector functions are needed.

Yeasts, as an eukaryotic organism, has the capacity to perform post-translational modifications. In addition, they can be used even in high throughput processes and glyco-engineering enables the expression of recombinant proteins with human-like glycosylation. Nevertheless, the production of full-size immunoglobulins remains a challenge. Compared to yeasts, filamentous fungi are more difficult for the generation of transformed clones, but they have a long tradition for the usage in biotechnology and they have partially been used for the expression of IgGs. In contrast, the development of protozoa as an expression system for recombinant proteins and antibodies has just been started and is still in a developmental stage. However, the mammalian-like glycosylation pattern presents them a promising candidate for further exploitation.

Insect cells contain a better suited protein folding and secretion apparatus than prokaryotes. Their high robustness combined with less sophisticated requirements for fermentation provide some advantages compared to mammalian cells. However, the development of stable insect cell lines and process technology is not developed as far. Consequently, mammalian cell lines are most widely used for the production of therapeutic antibodies as they provide a sophisticated folding and secretion apparatus as well as human-like glycosylation. For the production of high levels of recombinant antibodies high technical efforts are needed leading to relatively high costs. The maximum reported yield of functional IgG was 5 g/L which cannot be achieved using other expression systems so far, but up-scaling of the production does not lead to a high reduction of the production costs.

For an efficient reduction of production costs, transgenic plants can be used as they represent a highly scalable expression system; cultivation can be easily expanded without a gross increase in costs. In contrast, the generation of transgenic plants remains very complex and difficult. The most important obstacle of transgenic plants is the downstream processing as tons of plant material may have to be processed. However, antibody production in milk or eggs of animals would also be highly scalable and permits easy downstream processing. Several livestock animal species have been developed for the expression of recombinant proteins, but generation of transgenic animals also is very laborious. An interesting approach is the combination of human transgenic animals with hybridoma technology for the development of human antibodies.

In principle, there is no “universal” production system which can guarantee high yields of recombinant antibody, particularly as every antibody-based molecule itself will cause its own issues in terms of expression.

Conflict of Interest Statement:
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.