Lepidopteran cells
An alternative for the production of recombinant antibodies?

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Abbreviations: AcMNPV, Autographa californica multiple nuclear polyhedrosis virus; BmMNPV, Bombyx mori multiple nuclear polyhedrosis virus; Ig, immunoglobulin; H, heavy chain; L, light chain; scFv, single chain fragment variable; Fab, fragment antibody; PDI, protein disulfide isomerase; HSP70, heat shock protein 70; BiP, binding immunoglobulin protein; GNT-I, N acetylgalcosaminyltransferase I; ER, endoplasmic reticulum; Pfu, plaque forming unit

Monoclonal antibodies are used with great success in many different therapeutic domains. In order to satisfy the growing demand and to lower the production cost of these molecules, many alternative systems have been explored. Among them, the baculovirus/insect cells system is a good candidate. This system is very safe, given that the baculoviruses have a highly restricted host range and they are not pathogenic to vertebrates or plants. But the major asset is the speed with which it is possible to obtain very stable recombinant viruses capable of producing fully active proteins whose glycosylation pattern can be modulated to make it similar to the human one. These features could ultimately make the difference by enabling the production of antibodies with very low costs. However, efforts are still needed, in particular to increase production rates and thus make this system commercially viable for the production of these therapeutic agents.

Production of Recombinant Antibodies using the Baculovirus/Insect Cell System

Baculoviruses are arthropod-specific viruses. Their genome consists of a large circular double stranded DNA molecule that encodes about 150 proteins. During the very late stage of its replication, two non-essential proteins, polyhedrin (PH) and protein 10 (P10) are expressed at a very high level (Fig. 1). Although the polyhedrin protein is not required in vitro, it forms occlusion-bodies in which viral particles are embedded to form polyhedra that are essential in protecting the virus from UV exposure and dehydration in the environment. Baculoviruses isolated from Autographa californica (AcMNPV) or from the silkworm Bombyx mori (BmMNPV) are commonly used for the production of recombinant proteins. The ability of baculovirus to produce two non-essential proteins at very high levels, led to their development as expression vectors. The general principle is to replace PH or P10 genes with a DNA sequence encoding a foreign protein of interest. This replacement is easily promoted by homologous recombination between DNA purified from the wild type virus and a plasmid called “transfer vector.” This vector is a bacterial plasmid containing a fragment of the viral genome encompassing the non-essential gene (i.e., Fragment EcoRI I for the PH gene) flanked with large viral DNA sequences. These flanking sequences allow for a specific homologous recombination and integration of the foreign gene into the genome of the baculovirus. The foreign

Introduction

Antibody-based drugs are now estimated to comprise ~30% of the global biologic drug market because of their success in different therapeutic applications such as cancer, infectious diseases, inflammation and allergy. Mammalian cells, such as Chinese hamster ovary (CHO) cells are commonly used to produce therapeutic antibody molecules at the commercial level. The establishment of stable mammalian immunoglobulin expression cell lines is time-consuming and the production-purification process expensive. To satisfy the growing demand and to lower the cost of production of these molecules, many different systems are being explored. Among these, the baculovirus/insect cell system may offer a good alternative to mammalian expression systems.

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gene is cloned in place of the non-essential gene, conserving all viral 3' and 5' non-coding sequences involved in transcription and translation control. Lepidopteran cells are co-transfected with a mixture of genomic viral DNA and recombinant transfer vector. Homologous recombination takes place between the target gene flanking sequences in the transfer vector and the viral genome. The first attempts performed inserted foreign sequences at the site of the polyhedrin gene. The recombinant viruses were then selected according to their phenotype, i.e., the inability to produce polyhedra, using plaque assays. More recently, new approaches using defective non-infectious viral DNA i.e., linearized genomes or BaciMid-derived systems have been used.6,7 The latter involves the deletion of an essential gene and the insertion of an origin of replication from bacteria (mini-F) into the viral genome allowing the DNA (Bacmid) to replicate in E. coli. Furthermore, the identification of many other non-essential genes has allowed the insertion and simultaneous expression of several genes into a single virus.8 Finally, by using different viral or cellular promoters that present both specific transcriptional patterns and expression rates, it is possible to modulate the production of each gene.9 This highly efficient and very flexible system has therefore been employed to produce full-length immunoglobulins and antibody-derived molecules. The two types of hosts that are used routinely are either established lepidopteran cells (i.e., Sf9, Sf21, Trichoplusia ni) or animals, mostly the silkworm B. mori.

In order to simplify the construction of a recombinant viral genome capable of expressing an antibody, “universal” baculovirus expression cassettes have been designed. An example of these cassettes is presented in Figure 2. A generic cassette consists of the following: (1) a viral promoter (P10 or PH promoter) (2) a sequence encoding an Ig signal peptide (3) two unique restriction sites allowing the insertion of the heavy or light chain variable regions in frame with the upstream signal peptide sequence (4) and a downstream sequence encoding a murine or human heavy or light chain constant region.10 These cassettes are flanked with viral sequences, which direct the integration of the foreign genes into a specific locus. In that case, the two transfer vectors are designed to recombine in two different loci (PH and P10). Some Ig expression cassettes based on backbone vectors containing two back-to-back promoters allow the cloning of the H and L genes within a single transfer vector.11 Although a single vector facilitates the process, the use of separate H and L transfer vectors is more convenient for example when one wishes to test the effect of combining many different H and L chain sequences or to analyze the impact of several mutations on one of the two chains. Indeed in these cases the different plasmid pairs carrying either a light or heavy chain sequence can be rapidly combined with each other in all combinations to be tested. Some vectors have also been constructed for the direct cloning of cDNA encoding scFv or Fab isolated from phage-display libraries.11

The most critical step for the secretion of soluble and functional recombinant antibodies is to simultaneously produce equal amounts of heavy and light chains. Different approaches have been tested to identify the best strategy for this purpose: (1) double infection with two separate heavy and light chain-expressing viruses, each gene being expressed under the control of the same viral promoter (PH or P10 promoter) and (2) the construction of a double-recombinant virus with both chains expressed independently under the same promoter.2,3 Both approaches can present problems. Indeed one of the fundamental characteristics of these viruses, which has led to their use as expression vectors, is that they can induce a very efficient homologous recombination during their replication, thus facilitating the generation of recombinant viruses. This activity depends on the expression of several viral genes.12-14 However, this characteristic can be detrimental when repeated sequences are introduced into the viral genome. While the coexpression of several foreign genes in a single baculovirus ensures that every cell expresses the genes at the same ratio and allows consistent yields of recombinant protein complexes, the duplication of a promoter may induce instability and lead to the rearrangement of the viral genome and loss of genetic information.6 A number of studies have reported that expressing both H and L chains by different promoters with the same transcriptional pattern is a way of overcoming this problem. However, data indicate (Cérutti M, unpublished data) that the preparation of purified recombinant antibodies produced with H chain under the control of the P10 promoter, and L chain under the PH promoter, was highly prone to aggregation. This is because, as shown by Roelvink et al. and Chaabibi et al.26 and contrary to

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**Figure 1.** Baculovirus replication. During the very late stage of replication, two non-essential genes the polyhedrin (PH) and P10 are transcribed at a very high level. Polyhedrin is responsible for the formation of the polyhedra structure in which the virions are embedded. The involvement of P10 remains unclear, but it is generally assumed that it plays a role in cell lysis. These characteristics are the basis for the development of baculovirus as an expression vector.
general assumption, the transcriptional patterns of the P10 and PH genes are similar but not identical.\textsuperscript{16} P10 gene is expressed a few hours before polyhedrin, thus leading to the overexpression of the H chains before the light chains and triggering of secretion of H chains dimers (H\textsubscript{2}).\textsuperscript{15} Since the correct folding of the heavy chain requires the presence of light chains, the unfolded H\textsubscript{2} molecules may be at the origin of the formation of aggregates observed under these conditions.\textsuperscript{17} Aggregation may in turn also explain the low expression rate observed in these conditions.\textsuperscript{18}

In conclusion, to obtain adequate and correctly folded Igs in insect cells, it is important to use promoters that direct the synthesis of very similar amounts of heavy and light chains. When 2 different promoters are used, the time course expression of each promoter has to be considered in order to prevent the premature overproduction of heavy chain when the production of the light chain is still too low.

**Production in cell culture.** Sf\textsubscript{9} and High Five\textsuperscript{TM} (Invitrogen) are commonly used as host cell lines. Although there are exceptions, High Five cells (derived from the lepidopteran *T ni*) are generally more efficient at expressing and secreting recombinant proteins than Sf\textsubscript{9}.\textsuperscript{18,19} However, it should be noted that the N-glycosylation performed by High Five cells is not identical to
and J chain. After binding to the poly-immunoglobulin receptor (plgR) dimeric IgA is transported across the epithelial barrier to be secreted on the luminal side of the mucosa. Upon transport, the plgR is cleaved and the extracellular portion of the molecule, the secretory component (SC), remains bound to the dimeric IgA and released. Binding of the SC to the dimeric IgA forms a secretory IgA (sIgA) and confers to this complex resistance to proteolytic enzymes. Secreted glycosylated human SC has been produced in insect cells, and the SC was able to bind very specifically to dimeric IgA.46

Beside these full-length immunoglobulins, many different antibody-derived molecules have been produced e.g., monoclonal single-chain fragments, scFv,47-50 bi-specific scFv,51 Fab.18,52-54 The expression of scFv is more reliable compared with that of the E. coli system and does not require any re-folding step. Fusion proteins such as chimeric-hormone-antibody molecules that achieved in human cells and may even be immunogenic (see below, “Glycosylation in insect cells”).20 Furthermore, the infectious titers of cell culture supernatant are very low, even at a late time after infection.19 Therefore Hi-Five cells require infection using a higher titer viral supernatant generated by infecting Sf9 cells.

Several immunoglobulin isotypes from many different species, including murine IgG,3,21-25 chimeric IgG,26-30 human IgG,31-39 IgA,40-42 IgM41 and IgE,43-45 as well as pig IgG40,45 have been successfully expressed in insect cells. These molecules are all correctly processed (signal peptide cleaved), glycosylated and assembled (H2L2) with a production rate varying from 0.75 to 100 mg/l, depending on the antibody (Table 1). Carayannopoulos et al. have reported the expression of functional monomeric human IgA as well as dimeric forms after co-infection of insect cells with three different recombinant baculoviruses expressing H, L and J chain. After binding to the poly-immunoglobulin receptor (plgR) dimeric IgA is transported across the epithelial barrier to be secreted on the luminal side of the mucosa. Upon transport, the plgR is cleaved and the extracellular portion of the molecule, the secretory component (SC), remains bound to the dimeric IgA and released. Binding of the SC to the dimeric IgA forms a secretory IgA (sIgA) and confers to this complex resistance to proteolytic enzymes. Secreted glycosylated human SC has been produced in insect cells, and the SC was able to bind very specifically to dimeric IgA.46

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### Table 1. Some examples of antibodies or antibody-derived molecules produced in lepidopteran cells

| Source | Antibody format | Specificity | Host | Expression system | Expression rate | Application | References |
|--------|----------------|-------------|------|-------------------|----------------|-------------|------------|
| Human B-cells | Constant domain of IgE | - | Sf9 cells | Stable cell line IE1 transactivator/HR3 enhancer | 0.1–0.6 mg/l | Basic research | 43, 44 |
| Pig B-cells | Constant domain of IgE | - | Hi-Five cells | - | ND | Basic research | 45 |
| Hybridoma | Fab | Catalytic antibody 6D9 | Hi-Five cells | - | 300 mg/l | Technology research | 54 |
| Hybridoma | scFv | Ginsenoside | B. mori larvae | BmNPV | 650 mg/l of hemolymph | Quality control | 61 |
| Hybridoma | Fab | Botulinum toxin | T ni larvae | AcMNPV | 1g/kg of larvae | Technology research | 64 |

*Fab Library, established from convalescent patients or infected individuals. Hu, human; mu, murine; ch, chimeric; Arsonate, para-azophenylarsonate; HCMV, human cytomegalovirus; PRRSV, Porcine Reproductive and Respiratory Syndrome Virus; BIP, Immunoglobulin heavy chain Binding Protein; HSP70, Heat Shock Protein 70; BSA, Bovine Serum Albumin
Table 1. Some examples of antibodies or antibody-derived molecules produced in lepidopteran cells (continued)

| Source | Antibody format | Specificity | Host | Expression system | Expression rate | Application | References |
|--------|-----------------|-------------|------|-------------------|----------------|-------------|------------|
| scFv/Fab libraries | scFv/Fab→IgG1 | – | Hi-Five cells | 6–18 mg/l | Technology research | 11 |
| Fab library | chlgG | Prion protein | Sf9 cells | 7.2 mg/l | Diagnostic, therapeutic | 29 |
| Fab library | hulgG1 | G1, G2 proteins Hantaan viruses | Hi-Five cells | ND | Neutralising antibodies | 34, 35 |
| Fab library | hulgG1 | HCMV | Sf9 cells | 6 mg/l | Neutralising antibodies | 37 |
| Fab library | hulgG1 | Influenza A HSN1 | Sf9 cells | ND | Neutralising antibodies | 38 |
| Hybridoma | mulgG1 | Arsonate CEA | Sf9 cells | 2–20 mg/l | Technology research | 10, 26 |
| Hybridoma | chlgG1 and Humanized | Human CD29 | Sf9 cells | ND | Anti-cancer | 27 |
| Hybridoma | chlgG2 | Anti-HLADR | Sf9 and Hi-Five cells | 5–10 mg/l | Basic research | 28 |
| Hybridoma | mouse/ Pig Ig | PRRSV | | 30–100 mg/l | Basic research | 30 |
| Hybridoma | chlgA mono ± dimeric | Arsonate | | 0.75 mg/l | Basic research | 40 |
| EBV-immortalized human B-cells | hulgG1 | Rhesus D | Sf9 cells | 10 mg/l | hemolytic disease of newborn | 31 |
| Human B-cells | hulgG1 | cardiolipin | | ND | Basic research | 32, 33, 36 |
| Hybridoma | mulgG1, mulg2a | Arsonate and P. aeruginosa lipoprotein I | | | | |
| Hybridoma | mulg2a | Arsonate | Hi-Five cells | | | |

*Fab Library, established from convalescent patients or infected individuals. Hu, human; mu, murine; ch, chimeric; Arsonate, para-azophenylarsonate; HCMV, human cytomegalovirus; PRRSV, Porcine Reproductive and Respiratory Syndrome Virus; BiP, Immunoglobulin heavy chain Binding Protein; HSP70, Heat Shock Protein 70; BSA, Bovine Serum Albumin*

(Choriogonadotropin fused with mouse IgG Fc domain), scFv anti-TAG72 fused with IL-2, anti-HLADR heavy chain fused with IL-2 and human Fas receptor extracellular domain fused with human IgG1 Fc domain have also been produced.55-58

Production in larvae. Protein production in whole animals has mostly been developed using the silkworm and the BmMNPV baculovirus due to the large size of the B. mori larvae compared with host animals susceptible to AcMNPV. Specific dual expression vectors have been constructed for the production of recombinant proteins in this host since the BmMNPV is highly restricted to B. mori.59,60 Fifth instar silkworms are injected with a recombinant virus and hemolymph is collected 4 to 7 d after infection. The expression rate is very high compared with the secretion observed in a cell culture.61-63 Indeed Reis et al. were the first to report a production of
800 mg/l protein in hemolymph compared with 10 mg/l in the larval body. More recently, this technology has been applied to AcMNPV-derived recombinant viruses by using T. ni larvae. O’Connell et al. have designed the “automated insect rearing system” PERLXpress, an original “scalable technology” for whole insect baculovirus expression. In this case, the larvae are infected orally with highly infectious preoccluded virus. Just 4 d after infection, the expression rate is usually in the range of g of purified Fab/kg of larvae.

**Enhancing the Production and Secretion of Recombinant Antibodies**

Many attempts have been made to optimize the production and secretion of glycoproteins in insect cells. These have included: (1) using alternatives promoters, such as earlier viral or cellular promoters; (2) modifying or exchanging the signal peptide sequence; (3) co-expressing key proteins implicated in the secretion machinery (e.g., chaperone proteins) and (4) generating stably-transformed insect cells. Comparable experiments were conducted in order to increase the secretion of recombinant antibodies. Usually, the authentic signal peptide sequences of secreted proteins are correctly cleaved, generating sequences identical to the N-terminal end of the parental protein. When H and L chains are expressed with a specific signal peptide, both present the expected N-terminal end. Although the exchange of the signal peptide sequence can significantly increase the production of some proteins, no significant enhancement was observed in immunoglobulin production after exchanging the signal peptide of some proteins, no significant enhancement was observed. Although the exchange of the signal peptide sequence can significantly increase the production of some proteins, no significant enhancement was observed.

Assuming that aggregation of overexpressed proteins results from the absence of sufficient levels of ER and/or cytosolic molecular chaperones in infected cells, the co-expression of recombinant chaperones such as BiP, HSP70, PDI, calnexin, calreticulin and ERp57 has been evaluated in both cell culture and infected larvae.

**Molecular chaperone-assisted production.** As early as 1994, the protein biosynthesis pathway of the insect cells has been engineered to improve solubility, assembly and secretion of recombinant Ig. In B-cells, the chaperone BiP, a member of Hsp70 family, was first identified in association with the H chain polypeptide in the ER. This protein may have an essential role at preventing the aggregation of the H chain and by maintaining a specific conformation, until final folding in presence of light chain takes place. When SP9 cells were co-infected with 3 viruses each expressing H, L and murine BiP, Hsu et al. showed that the presence of the chaperone improved the level of soluble and functional antibodies within the cell lysates. The secretion, however, was not significantly enhanced. While the solubility of H and L chains was slightly enhanced when Hsp70 protein, a cytosolic chaperone, was co-expressed with H and L genes, the secretion increased by about 50%. The same approach was taken to produce Ig in silkworm. A 5-fold increase in production of Ig was observed when human chaperones, calnexin, calreticulin and BiP were coexpressed with H and L genes of a human Ig.

Despite chaperone assistance, late during viral infection when the synthesis of the recombinant protein is increasing, the insect physiology is profoundly affected compromising cellular functions such as polypeptide processing, glycosylation and secretion. Therefore as an alternative, stably transformed insect cells have been used to produce recombinant proteins.

**Stably transformed insect cells.** In 1997, McCaroll and King were the first to describe the transfection of Sf9 cells with two plasmids, one containing a selection cassette expressing an antibiotic resistance gene (i.e., neomycin resistance gene) and the other one designed for the expression of the foreign gene. Cellular promoters, such as cytoplasmic actin promoter and/
or promoters from viral immediate-early genes (e.g., ie1 and ie2 from AcMNPV) that are transcribed by the cellular RNA polymerase II were used.\textsuperscript{5,6} Although these promoters drive very high levels of transcription in their natural environment, they allow only very low expression rates of most foreign genes (Table 1). However this system presents a significant advantage, that secreted proteins are processed and secreted more efficiently.\textsuperscript{7,1}

In 2000, Guttieri et al.\textsuperscript{7,2,7,3} have used this new technology to produce antibodies. SF\textsubscript{9} cells were transfected with 3 independent constructs, each containing one expression cassette for H, L and the neomycin resistance gene. Despite a high copy number of H and L genes integrated into the cellular genome (approx 250 copies for both chains) the production rate of IgG was very low at about 0.06 mg/l of cell culture supernatant, which is around 500-fold lower than the production obtained in the baculovirus-insect cell system expressing the same antibody.\textsuperscript{7,4}

More recently, new vectors have been developed for the continuous high-level expression of secreted proteins.\textsuperscript{7,6} These vectors use the property of \textit{B. mori} actin promoter to be stimulated with \textit{BmMNPV} IE1 transactivator via the \textit{BmMNPV} HR3 enhancer. This combination resulted in a 1,000-fold stimulation of foreign gene expression which, at maximum cell density in a shake-culture, can reach a yield of up to 300 mg/l in High Five cells.\textsuperscript{7,4} Thus methods to obtain relatively high yields of Igs in stable cultures are now available.

**Glycosylation in Insect Cells**

Alterating the glycosylation pattern of proteins, including antibodies, may affect their production, secretion and function. While glycans linked to the Fab domains may be directly involved in the interaction with antigenic epitopes, the role of the glycosylation of Asn297 in the CH2 constant domain is essential for many Fc-dependent effector activities. Recently, the fucosylation levels of the N-glycans in human IgG1 was reported to significantly modulate antibody dependent cellular cytotoxicity (ADCC) in vitro and in vivo.\textsuperscript{7,5} Lepidopteran cells have been shown to perform most of the post-translational modifications including N- and O-glycosylations. The N-glycan processing is similar to that of mammals during the early steps of the glycosylation pathway in synthesizing GlcNacMan\textsubscript{3}(Fuc)GlcNac\textsubscript{2}.\textsuperscript{7,6} However, very low levels of GNT-I activity,\textsuperscript{7,6} lack of GNT-II,\textsuperscript{7,7} B1,4 galactosyltransferase,\textsuperscript{7,8} sialyltransferases,\textsuperscript{7,9} and the presence of a Golgi-associated N-acetylgalcosaminidase\textsuperscript{7,8} lead to the formation of a paucimannose structure, Man\textsubscript{3}(Fuc)GlcNac\textsubscript{2}, which has not been described in mammalian cells. Therefore oligomannose-type and partially fucosylated paucimannosic glycans represent the most frequent structures identified in glycoproteins synthesized in infected or non-infected lepidopteran cells. While only α1,6 fucose was found in glycoproteins expressed in \textit{B. mori} and SF\textsubscript{9} cells,\textsuperscript{7,8,7,0} α1,3 and α1,6 fucose are found in \textit{M. brassicae}\textsuperscript{7,8,7,0} and \textit{T. ni} (High fiveTM) cell lines.\textsuperscript{7,0} The presence of α1,3-linked fucose, a potential allergenic epitope in these cell lines, may constitute a limitation to their use for expressing human glycoproteins.\textsuperscript{8,1}

Only little work has been done to characterize the glycosylation pattern of recombinant antibodies produced in insect cells.\textsuperscript{3} Recently, two studies\textsuperscript{8,2,8,2} have reported the presence of paucimannosidic and oligomannosidic glycans including α1,6 fucose without terminal sialic acids. Interestingly, when antibodies are expressed in insects, the higher Ig production rate observed in infected pupa is associated with a better processing of glycans, with five-fold GlcNacMan, GlcNac, structures found on N-glycans, suggesting that glycosylation may promote the expression of a new epitope involved in the secretion process.\textsuperscript{6,3}

Two strategies have been used to “humanize” glycans structures in insect cells; integrating the missing glycosyltransferases into either the cellular genome\textsuperscript{8,5} or the viral genome.\textsuperscript{8,4} Using the latter approach, we have constructed a new baculovirus expressing GNT-I, GNT-II and β1–4 galactosyltransferase (Cérutti M, et al. unpublished data). In order to obtain a stable genetic construct without any duplicated sequences, we chose to direct the gene expression under the control of RNA polymerase II homologous promoters. Three new specific transfer vectors that allow homologous recombination into three dispensable genes were constructed. Structural analysis of these recombinant antibodies shows that the expression of glycosyltransferase activity allows the synthesis of mono- and di-galactosylated antibodies. The impact of these glycosylations on production/secretion rates and on the Fc dependent functions is currently being examined in our laboratory.

**Biological Activities of Recombinant Antibodies Produced in Insect Cells**

The baculovirus expression system has been used for basic research to analyze the molecular basis of specificity,\textsuperscript{2,1,2,4,1,2} autoimmunity,\textsuperscript{3,2,3,3,3,6,3,9} or the function of intact antibodies or of isolated immunoglobulin domains;\textsuperscript{1,8,4,34} It is very easy and fast to generate recombinant viruses expressing H or L bearing specific mutations and to determine the impact of these mutations on the specificity or activity of a given antibody.\textsuperscript{2,1,2,4,1,4}

This strategy has been successfully used to analyze the catalytic activity of the 6D9 monoclonal antibody and the specificity of the PAC1 antibody that binds to the integrin α\textsubscript{II}β3 on activated platelets.\textsuperscript{1,8,2}

Universal expression cassettes have also been designed either to directly clone the VH and VL domains of antibodies expressed by murine cells or by human B-cells isolated from patients,\textsuperscript{2,6,3,2,3,3,6,3,9} or to convert scFv or Fab fragments selected from phage-display libraries into an intact fully human or chimeric IgG.\textsuperscript{2,5,3,4,3,5,3,7,3,8,4,2}

The aim of many of these experiments was to isolate, characterize and validate antibodies for further therapeutic applications in the treatment of human diseases such as hemolytic disease of newborn,\textsuperscript{3,1} cancer,\textsuperscript{2,1,2,7,3,3,6,3,8} and infectious diseases, with the selection of neutralizing antibodies against hemorrhagic fever viruses (e.g., hantaan, Puumala viruses),\textsuperscript{3,4,3,5,7} human cytomegalovirus,\textsuperscript{3,7} influenza virus\textsuperscript{4,6} or prion protein.\textsuperscript{2,9}

Besides having a high specificity and neutralizing activity, which generally depends upon their epitope specificity, some therapeutic antibodies have to express full effector activities such

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as complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC) and phagocytosis in order to function in vivo.

**Antibody dependent cellular cytotoxicity.** ADCC is thought to contribute significantly to the in vivo activity of unconjugated IgG1 antibodies, as shown by the improved response to rituximab of lymphoma patients bearing the high affinity polymorphic form of FcγRIIIA (FcγRIIIA158F), the major receptor expressed by human NK cells.93 This observation has led to the active search for Fc modifications, which would improve binding of IgG1 to both polymorphic forms of this receptor. Several point mutations within the lower hinge and CH2 domains of hlgG1 have been constructed, which show higher affinity for FcγRIIIA and mediate ADCC at lower doses than the corresponding wild type antibodies.73 However, the most effective and widely applied modification of IgG1 for improved ADCC is defucosylation, i.e., removal of the core α1,6 fucose from the N-linked glycan moiety, which results in an approximately 50-fold increase in affinity of IgG1 for FcγRIII.86 Lack of fucose in IgG1 antibodies leads to more efficient ADCC, which is most evident at lower antibody concentrations, or when antigens are expressed at low levels. Furthermore, the inhibition of ADCC observed with fucosylated IgG1 antibodies in the presence of excess free IgG (as would be present in plasma) or C3b deposition following complement activation, in contrast, is not observed when afucosylated antibodies are used. Therefore, ADCC mediated by defucosylated IgG1 takes place even in the presence of excess IgGs or complement, as found in serum. In contrast, defucosylation does not affect binding to FcRn and pharmacokinetics of antibodies, making this format very appealing. Thus, several afucosylated antibodies directed against a variety of targets, such as CD20 (obinutuzumab, GA101), HER2, CCR4 and CD19, have been shown to have improved therapeutic efficacy in tumor models in vivo.85-90 Indeed several such antibodies are in preclinical or clinical development. With regard to other sugar moieties, the presence or absence of galactose or bisecting GlcNAc in IgG1 glycans has limited effects on ADCC, whereas total removal of N-linked glycans from hlgG1 completely abolishes both FcγRIIIA binding and ADCC.91,92

As mentioned above, antibodies produced in insect cells do not generally have the same glycosylation pattern as those produced in CHO; in particular they mostly lack galactose, bisecting GlcNAc and sialic acid, but bear core fucose residues. Such antibodies have been shown to mediate efficient ADCC in vitro, as expected since Gal, GlcNAc and sialic acid do not play a major role in binding to FcγRIII.23,27,28,31 Future work will no doubt see the engineering of insect cells for the production of afucosylated antibodies. Interestingly, different glycosylation patterns of recombinant antibodies have been reported in different insect tissues, such as lack of fucose reported for antibodies produced by *B. mori* silk gland.95

**Complement dependent cytotoxicity.** Complement has been suggested to contribute significantly to the activity of therapeutic antibodies directed against tumor antigens expressed at the cell surface, although some controversy still remains as to its relative importance for in vivo efficacy. Antibodies bearing human IgG1, IgG3 or IgM Fc domains can theoretically bind to hexameric C1q molecule, via contact by its CH2-CH3 domain, and activate the classical pathway of complement. For therapeutic purposes, the IgG1 format has been chosen, due to its greater stability and longer in vivo half-life. Binding of C1q to antigen-bound IgG1 leads to C2, C4 and C3 cleavage, which together form the C5 convertase (C2a-C4b-C3b complex) that binds covalently to the antibody molecule itself and to cell membrane components. Cleavage of C5 by the C5 convertase leads to deposition onto the cell membrane of C5b-C9 fragments polymers (or membrane attack complex) and lysis of the target cell. Not all hlgG1 antibodies activate complement however and it appears that several factors, including antigen density, closeness of the recognized epitope to the plasma membrane, conformation or positioning of bound antibodies and other undefined factors determine whether efficient C1q binding and complement activation does take place with any specific antibody. Indeed even different antibodies of the same Ig class and directed against the same antigen may show widely different capacities to activate complement, as exemplified by anti-CD20 antibodies.84

The glycosylation pattern of the antibody may also significantly influence C1q binding and complement activation. Indeed fully deglycosylated hlgG1 antibodies are inactive in C1q binding and CDC. High mannose type molecules are also defective in C1q binding. Sialic acid is fully dispensable for classical complement pathway activation.89 Galactose and bisecting GlcNAc residues however have a more controversial role.96-98 This may be due in part to the fact that IgG1 lacking galactose (G0) can bind to mannose binding lectin (MBL) and activate the lectin pathway of complement instead of the classical pathway. Nonetheless most studies suggest that removal of Gal leads to decreased C1q binding but increased MBL binding.99 So overall, CDC by some molecules may have contributions from both the classical and lectin pathways.

The influence of Ig glycosylation pattern on complement activation implies that this aspect needs to be taken into account when producing antibodies in insect cells. It is possible either to engineer insect cells expressing specific enzymes that modify the glycosylation pattern of the antibodies produced, or perform in vitro enzymatic modifications of sugar moieties, in order to enable adequate glycosylation according to the functional activity required for the final product (such as introduction of GlcNAc-Gal for CDC or inhibition of fucosylation for ADCC). It is also necessary to carefully probe the effector functions of the antibodies produced in insect cells, in order to verify CDC activity, as required. There are few studies investigating CDC activity of IgG antibodies produced in insect cells.33,26,27,100 In several cases, no CDC activation could be demonstrated, but this was true also for the parent antibody produced in mammalian cells.33,26 In one study, insect cell produced antibodies could indeed induce C1q binding or CDC with human serum.77 However comparison with the same antibody produced in CHO cells was not made, so that the role of a different glycosylation pattern could not be verified. Furthermore, no systematic study and comparison of the effect on CDC of different glycosylated forms produced in insect cells has yet been performed.
**Phagocytosis.** Phagocytosis of opsonised tumor cells is thought to be one of the major immune mediated mechanisms for control of tumor cell growth by unconjugated hlgG1 antibodies such as rituximab or trastuzumab. Phagocytosis is performed mostly by macrophages that in man express the FcγRIIA, FcγRIIA and FcγRI activating receptors, all of which may mediate phagocytosis, and the inhibitory FcγRIIB molecule. As previously mentioned, removal of core fucose residue from hlgG1 specifically affects FcγRIIIA binding, as well as ADCC by NK cells that express only this receptor. Defucosylation of anti-CD20 rituximab or trastuzumab. Phagocytosis is performed mostly by to be one of the major immune mediated mechanisms for control of the Sp2/0 cell line. However, these glyco- systems because their genome can be manipulated very easily and future work should investigate more systematically the capacity of these new yeast cell lines can produce highly uniform N-linked glycans, allowing the biosynthesis of specific glycoforms. These are summarized in Table 2. Yeasts seem to be one of the most promising systems. *Saccharomyces cerevisiae* and *Pichia pastoris*, for example, have many advantages over other eukaryotic systems because their genome can be manipulated very easily and the cells do not require expensive growth media. In 1985, Wood et al. reported the production and secretion of functional glycosylated antibodies in *S. cerevisiae*. Horwitz et al. on the other hand, found the expression level to be very low with only 0.05 mg/l. While an efficient ADCC activity was observed, recombinant IgG1 failed to induce CDC. A 180-fold improvement in secretion was obtained by using a mutant of α mating factor 1 leader peptide and by co-expressing protein disulfide isomerase (PDI). Evans et al. recently reported the production at high yield (40–90 mg/l) of scFv fused to human serum albumin. With regard to glycosylation patterns in yeasts, modifications have been achieved in particular removal of non-human O-glycosylation. However, these glycans being essential for cell survival, any attempt to completely eliminate O-glycosylation were unsuccessful. More recently, by using specific inhibitors of protein-mannosyltransferase I (Pmt) catalyzing the initial reaction of O-glycosylation in yeasts, it is possible to lower the presence of such structures. However, even in these conditions, some yeast O-glycans are still present on recombinant proteins.

The methylotrophic yeast *P. pastoris* has been used to produce high yields of small recombinant proteins. This host presents many advantages over *S. cerevisiae*, as foreign genes are expressed under a strong tightly regulated promoter induced by methanol, the promoter of alcohol oxidase I gene (AOX1), and can be grown to higher density than *S. cerevisiae*. However, the glycosylation pattern of recombinant proteins such as those produced in *S. cerevisiae* is inappropriate, with extra O-glycosylation and the addition of high-mannose type oligosaccharides. Strains of *P. pastoris* have been re-engineered to humanize the glycosylation pathway. As a result of this re-engineering of the secretion pathway, and in contrast to proteins produced by mammalian cells, these new yeast cell lines can produce highly uniform N-linked glycans, allowing the biosynthesis of specific glycoforms. Recombinant immunoglobulins, such as camelid VH, have also been produced in *P. pastoris* with a yield of 10–15 mg/l of secreted and biologically active protein. The quality and the high uniformity of the glycoforms generated by these new strains were used to analyze the impact of N-glycan structures on...
been produced at high titer in the range of 1 g/l for IgG and Fab.124,125 No difference was observed in antigen binding affinity, pharmacokinetic or ADCC. However immunoglobulins were produced as a mixture of glycosylated and aglycosylated ADCC activity of anti-CD20 recombinant antibody.123 All these improvements make this system potentially very attractive.

Other potential alternatives are filamentous fungi such as Aspergillus and Trichoderma spp Fab fragments and IgG have been produced at high titer in the range of 1 g/l for IgG and Fab.124,125 No difference was observed in antigen binding affinity, pharmacokinetic or ADCC. However immunoglobulins were produced as a mixture of glycosylated and aglycosylated

### Table 2. Comparison of the different expression systems used to produce antibodies

| Expression system | Transient or stable Molecule expressed | Average yield | Secretion | Glycosylation pattern | Immune Effector functions | Scale-up | References |
|-------------------|---------------------------------------|---------------|-----------|-----------------------|---------------------------|----------|------------|
| Prokaryotes       | S                                     | IgG           | 1–2g/l    | Secreted in the periplasmic space | NO                        | No unless Fc engineered to bind FcRl. ADCC via DCs | Easy and cost-effective | 144, 145, 148 |
| *E. coli*         |                                       |               |           |                       |                           |          |            |
| Filamentous fungi | S                                     | IgG           | 200–900 mg/l | H chain secreted as a fusion protein released with A. *niger* KexB protease | High-mannose type Presence of galactofuranose Aglycosyl antibodies | ADCC     | Easy and cost-effective | 124, 125 |
| *T. reesi*        |                                       |               |           |                       |                           |          |            |
| *A. niger*        |                                       |               |           |                       |                           |          |            |
| Yeasts            | S or T                                | IgG           | 0.05–9 mg/l | Good secretion with optimized systems | High mannose-type Some extra O-linked glycosylation on secreted H chain. | ADCC     | Easy and cost-effective | 112, 113 |
| *S. cerevisiae*   |                                       |               |           |                       |                           |          |            |
| P. pastoris       | S                                     | IgG           | >1g/l     | Secretion Clone selection required | Some extra O-linked glycosylation With glyco-engineering strains highly uniform human glycoforms | ADCC     | Easy and cost-effective | 121, 123 |
| Plants            | S or T                                | IgG           | S: 1–40 mg/kg T: 200–500 mg/kg (fresh weight) | Extraction from leaves | Immunogenic glycosylation pattern: β1,2 xylose and α1,3 fucose Requires glycoengineering for appropriate human like glycans | ND       | Easy and cost-effective | 131, 132, 134, 135 |
| *N. benthamiana*  |                                       |               |           |                       |                           |          |            |
| *A. Thaliana*     |                                       |               |           |                       |                           |          |            |
| *M. sativa*       |                                       |               |           |                       |                           |          |            |
| Aquatic plant     | S                                     | IgG           | 5–8 g/kg dry weight | Secretion Extraction from plant tissue | Glycoengineering Highly homogeneous glycans | ADCC     | Easy and cost-effective | 137, 138 |
| *L. minor*        |                                       |               |           |                       |                           |          |            |
| Moss              | S                                     | IgG           | ND        | Secretion | Glycoengineering | ADCC     | Easy and cost-effective | 139 |
| *P. patens*       |                                       |               |           |                       |                           |          |            |
| Micro alga        | S                                     | IgG           | 100 mg/kg dry algal biomass | No secretion Accumulation in the chloroplast | No glycosylation | ND       | Easy and cost-effective | 140 |
| *C. reinhardtii*  |                                       |               |           |                       |                           |          |            |

Abbreviations: T, Transient Gene Expression; S, Stable expression; ADCC, Antibody Dependent Cytotoxicity; CDC, Complement Dependent Cytotoxicity; DCs, Dendritic Cells; ES, Embryonic Stem cells
forms, with 50% of N297 glycosylation site unoccupied on heavy chains. Glycans produced by these filamentous fungi are of high mannose-type with terminal galactose in the furanose form (Galβ), with Galβ2Man7GlcNac2 as the most abundant structure. This isomer of galactose is absent in mammals and may be highly immunogenic in humans.

For a long time, plants were considered low-cost alternative for the production of recombinant proteins. In 1989, Hiatt and colleagues were the first to demonstrate the production of a functional recombinant IgG1 in transgenic tobacco. Since then, many groups have expressed recombinant antibodies in transgenic plants. However, these transgenic expression systems have some disadvantages: (1) yield is low, usually in the range 1–40 mg of recombinant antibody per kg of fresh biomass and (2) the process of generating and selecting the best transgenic strain is time consuming and may require more than 2 years. More recently, very efficient transient expression systems have been developed based on A. tumefaciens-mediated delivery (leaf infiltration) or on plant-virus vectors. In contrast with the stably transformed plants, transient expression systems lead to high-yield of functional recombinant proteins 200–500 mg/kg of fresh tissue in only several days (≤2 weeks). However, the presence of non-human immunogenic glycans, such as β1,2 xylose and core α1,3 fucose constituted the major limitation to their use for the production of therapeutics. Knock-out lines for xylose transferase (Xylt1) and fucose transferases (Fuct1) of Arabidopsis thaliana were also generated, allowing the biosynthesis of recombinant antibodies with homogeneous mammalian-like complex glycans. Moreover, transgenic plants with an engineered glycan synthesis pathway have been constructed e.g., by coexpressing human β1,4 galactosyltransferase or by expressing enzymes of the sialic acid synthesis pathway. Application of these new transient expression technologies on an industrial scale and the improvement in glycosylation make this system a highly appealing alternative over mammalian systems.

The small aquatic higher plant Lemna minor is also considered an interesting alternative. By co-expressing H and L chains genes and RNAi targeting Xylt1 and Fuct1, a single major N-glycan species GlcNac2Man2GlcNac2 was produced. Antibodies produced in these conditions lacking fucose presented a higher affinity for FcyRIIIA and a 20–35-fold increase in the ADCC activity. It should be noted that, in contrast to mammalian cells, the glycan profiles are highly reproducible even under different growth conditions.

The moss Physcomitrella patens can be grown in vitro in simple culture medium of inorganic salts. The high degree of homologous recombination observed in its nuclear DNA enables a precise integration of foreign genes and efficient targeted knockouts, such as Xylt1 and Fuct1. Specific inducible promoters and signal peptide sequences have also been identified. Moreover, human genes can be expressed in moss without previous codon adaptation.

Other candidate hosts to reduce production time include microalgae. For example, the unicellular alga Chlamydomonas reinhardtii is able to produce antibodies after complete transformation of the chloroplasts genome. Chloroplast expression presents unique attributes over the transformation of the nuclear genome: the integration of the transgene by homologous recombination, high expression rates, the ability to process polycistrionic transcripts and no gene silencing. Soluble recombinant proteins are accumulated in the chloroplast but are not secreted. Dimerization by disulfide bond formation and multimeric protein complexes can be achieved, leading to the production of functional immunoglobulins. Even though, the chloroplast does not possess any glycosylation machinery.

As for mammalian cells and all stable expression systems such as yeasts or plants, the expression levels may be highly...
affected by the integration site, gene sequence and the copy number of the foreign gene and it may be a very time consuming and expensive to select the best cellular clone that will give the optimal production rate and glycosylation. However, by using new baculovirus/insect cell technologies involving linearized viral DNA or bacmid-derived systems, highly stable and homogeneous recombinant virus population expressing or co-expressing 1 or several foreign genes can be generated in three days. Consequently, the selection of a viral clone is very fast and efficient, the expression rate and glycosylation patterns depending only on the genetic construct inserted into the viral genome.

Surprisingly, *E. coli*, which is traditionally used for the production of small proteins, could be back in competition with the production of non-glycosylated full-size antibodies. Among the protein expression systems, *E. coli* has always been one of the most attractive hosts. It is a rapid and inexpensive method to produce recombinant proteins and the scale-up is easy and cheap. Antibody-derived fragments such as Fab directed against \( \alpha \) vEGF (ranibizumab) and TNF\( \alpha \) (certolizumab pegol), as well as a protein/peptide fused to the Fc domain of human IgG1 (romiplostim) have been successfully developed and approved as therapeutics in human. Furthermore, some researchers have been working on the expression of full-length immunoglobulins in *E. coli*. First attempts were performed by expressing full-length H and L chains as insoluble aggregates. However refolding steps required to reconstitute an active immunoglobulin were very tedious and challenging, and led to very low production rates. Today, by using separated cistrons, adjusting the translation levels of the two chains, removing the rare codons and RNA secondary structure, it is possible to secrete full-length IgG with titer as high as 1–2 g/l. The main limitation of this system is that it produces aglycosyalted antibodies and these lack immune effector activities. Moreover, aglycosylated IgG were shown to be not well-folded and more sensitive to proteases.

As mentioned above, the presence and the composition of the ubiquitous N297 glycans exert a major effect on binding of the antibody to Fc\( \gamma \)Rs; their complete removal abolishes the binding to Fc\( \gamma \)RIIa and Fc\( \gamma \)RI and reduces the interaction with Fc\( \gamma \)RI. However, recent studies demonstrated that some aglycosylated variants of IgG1 are capable of binding to Fc\( \gamma \)Rs, inducing a high ADCC activity. These observations open new avenues in the design of therapeutic antibodies. However, it is worth noting that N297 glycans affect the overall conformation of the immunoglobulin, altering the “open” conformation observed with the fully galactosylated IgG to a “closed” conformation, potentially leading to the unmasking of epitopes or to the induction of new conformational epitopes, which again raise the question of the potential immunogenicity of such molecules.

**Discussion**

The therapeutic use of monoclonal antibodies in the treatment of a wide range of pathologies such as cancer, infections and inflammatory diseases has exploded over the past 15 y. First treatments using murine monoclonal antibodies rapidly demonstrated the immunogenicity of these molecules, limiting their use in humans. Recombinant chimeric, humanized or human monoclonal antibodies have then been produced using mammalian expression systems. Whereas a significant decrease in the incidence of human anti-mouse antibodies (HAMA) response was evident with these recombinant molecules, some anti-glycan response was observed when the antibodies were produced in murine cell lines expressing non-human glycan epitopes such as NS0 or Sp2/0. In addition to these problems, current systems are not sufficient to meet the demand and call for the developments for new, less expensive, simpler and faster systems.

The ideal expression system for the production of recombinant antibodies for human therapy has to meet the following criteria: (1) the molecules produced have to be biologically functional, correctly glycosylated and non-immunogenic, (2) the production rate has to be high enough to meet increasing demand, (3) the selection and characterization steps for each new molecule have to be fast, reliable and cheap and (4) the cost of the whole process, from the establishment of the expression system to the purification steps has to be as contained as possible. The baculovirus/insect cell system may have all the qualities required to produce fully active glycosylated antibodies or antibody-derived molecules. The construction of the recombinant virus is very fast, with only a few days needed to generate stable recombinant viruses. Both generation of new molecules and their production are reliable and very cheap compared with the mammalian system, including the possible use of very inexpensive serum free culture medium. Indeed the main characteristic of the BEVS is the speed with which it is possible to obtain very stable recombinant viruses that are capable of producing fully functional glycoproteins with a human glycan pattern. These specificities may ultimately make a difference, by enabling the development of therapeutic antibodies at a much lower cost. Finally, baculoviruses have a highly restricted host range limited to some invertebrates which makes this system particularly safe. Two vaccines produced in insect cells, Cervarix (GlaxoSmithKline, Cervical cancer) and Provenge (Dendreon, prostate cancer) have received EMA and FDA approval and are commercially available. Many others e.g., FluBlok (Protein Sciences Corporation, Seasonal influenza vaccine) and Diamyd (Diamyd inc., Type 1 diabetes) are in late stages of clinical trials, holding promise for this type of system also for therapeutic antibody production. Given the importance of the therapeutic antibody market, what needs to be established is whether this system is capable of producing very high amounts of recombinant proteins. Efforts are still needed to increase production rates and thus make the system commercially viable for the production of these drugs.

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