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Production of pharmaceutical proteins by transgenic animals

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Abstract

Proteins started being used as pharmaceuticals in the 1920s with insulin extracted from pig pancreas. In the early 1980s, human insulin was prepared in recombinant bacteria and it is now used by all patients suffering from diabetes. Several other proteins and particularly human growth hormone are also prepared from bacteria. This success was limited by the fact that bacteria cannot synthesize complex proteins such as monoclonal antibodies or coagulation blood factors which must be matured by post-translational modifications to be active or stable in vivo. These modifications include mainly folding, cleavage, subunit association, γ-carboxylation and glycosylation. They can be fully achieved only in mammalian cells which can be cultured in fermentors at an industrial scale or used in living animals. Several transgenic animal species can produce recombinant proteins but presently two systems started being implemented. The first is milk from farm transgenic mammals which has been studied for 20 years and which allowed a protein, human antithrombin III, to receive the agreement from EMEA (European Agency for the Evaluation of Medicinal Products) to be put on the market in 2006. The second system is chicken egg white which recently became more attractive after essential improvement of the methods used to generate transgenic birds. Two monoclonal antibodies and human interferon-β1a could be recovered from chicken egg white. A broad variety of recombinant proteins were produced experimentally by these systems and a few others. This includes monoclonal antibodies, vaccines, blood factors, hormones, growth factors, cytokines, enzymes, milk proteins, collagen, fibrinogen and others. Although these tools have not yet been optimized and are still being improved, a new era in the production of recombinant pharmaceutical proteins was initiated in 1987 and became a reality in 2006. In the present review, the efficiency of the different animal

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systems to produce pharmaceutical proteins are described and compared to others including plants and micro-organisms.
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Keywords: Proteins; Recombinant; Pharmaceutical; Transgenic; Animals; Milk; Egg white; Monoclonal antibodies; Vaccines

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Résumé

Les protéines d'intérêt pharmaceutique ont commencé à être utilisées au cours des années 1920 avec l'insuline extraite des pancréas de porcs. Au début des années 1980, l'insuline humaine a commencé à être préparée à partir de bactéries recombinantes et désormais, tous les diabétiques utilisent cette hormone. Plusieurs autres protéines et notamment l'hormone de croissance humaine, ont été préparées à partir de bactéries recombinantes. Ces premiers succès ont rapidement montré la limite des bactéries qui sont incapables de synthétiser des protéines ayant une structure complexe comme les anticorps ou les facteurs de coagulation sanguine. En effet, pour être stables et actives in vivo, ces protéines doivent subir de multiples modifications post-traductionnelles. Les principales modifications sont le repliement, le clivage, l'association des sous-unités, la γ-carboxylation et la glycosylation. Elles ne se produisent complètement que dans des cellules de mammifères cultivées dans des fermenteurs à l'échelle industrielle ou appartenant à des animaux transgéniques. Plusieurs espèces d'animaux transgéniques peuvent produire des protéines recombinantes mais actuellement deux systèmes ont commencé à être exploités. Le premier est le lait des animaux de ferme transgéniques qui sont étudiés depuis 20 ans. Ce système a permis à une protéine, l'antithrombine III humaine, de recevoir l'autorisation de mise sur le marché par l'EMEA (European Agency for the Evaluation of Medicinal Products) en 2006. Le second système est le blanc d'œuf de poulets transgéniques qui est devenu récemment plus attractif après que les méthodes de préparation d'oiseaux transgéniques aient été améliorées. Deux anticorps monoclonaux et de l'interféron-β1a humain ont été obtenus dans le blanc d'œuf de poulets. Une grande variété de protéines recombinantes a été préparée à titre expérimental avec ces deux systèmes et quelques autres. Ces protéines comprennent des anticorps monoclonaux, des vaccins, des facteurs sanguins, des hormones, des facteurs de croissance, des cytokines, des enzymes, des protéines du lait, du collagène, du fribrinogène et d'autres encore. Bien que ces outils n'aient pas été optimisés et soient encore en cours d'amélioration, une nouvelle ère dans la production de protéines recombinantes pharmaceutiques a commencé en 1987 et est devenue une réalité en 2006. Dans cette revue, l'efficacité des différents systèmes animaux capables de produire des protéines pharmaceutiques sont décrits et comparés aux autres incluant les plantes et les microorganismes.
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Mots clés : Protéines ; Recombinantes ; Pharmaceutiques ; Transgéniques ; Animaux ; Lait ; Blanc d'oeuf ; Anticorps monoclonaux ; Vaccins

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1. Introduction

Human communities have extensively used for centuries plant extracts as pharmaceuticals. These pharmaceuticals did not include proteins which were unknown, denatured during extraction and not active by the oral route. The first protein used as a therapeutical molecule was insulin extracted from pig pancreas. Although these

preparations did not raised major problems for decades, human insulin was obtained from recombinant bacteria in the early 1980s. This protein which proved of better quality than the conventional insulin, has been adopted by all patients suffering from diabetes. Human growth hormone was prepared by the same way soon after. Both insulin and human growth hormone obtained from tissue extracts were not limiting but their quality was greatly improved by using recombinant bacteria. The major improvement for human growth hormone was that it was no more contaminated by human prions. The case of bovine growth hormone is somewhat different. This protein extracted from recombinant bacteria could not be contaminated by prions but this system was the only capable of providing farmers with enough quantity of the hormone to enhance milk production in ruminant herds.

Bacteria soon appeared severely limited by their poor capacity to synthesize some proteins and particularly those which have a complex structure. Some proteins are so abundantly produced in bacteria that they form aggregates from which it is difficult to isolate the proteins of interest without denaturating them. Bacteria cannot always fold proteins in an appropriate manner and assemble subunits to form biologically active molecules. Moreover, bacteria can eliminate signal peptides but cannot cleave preproteins like native coagulation growth factors. Bacteria are also unable to perform the post-translation modifications of proteins, namely of glycosylation, γ-carboxylation, phosphorylation, sulphatation and others. Other systems have thus been implemented and proved more or less capable of producing at a low cost large amount of active proteins having an appropriate biochemical structure.

2. The different production systems

The different systems to produce recombinant proteins which are being studied or used are summarized in Table 1. The advantages and limits of bacteria have been discussed above.

Yeast was expected to have some advantages over bacteria. Indeed, these cells are eukaryotes and they may potentially synthesize and secrete large amount of matured recombinant proteins. Yeast proved unable to fold and secrete monoclonal antibodies in an appropriate manner. Moreover, some yeast did not glycosylate proteins or they added sugars which are not found in human proteins. Interestingly, genetically modified yeast expressing foreign genes coding for enzymes responsible for glycosylation proved able to secrete substantial amounts of recombinant proteins having carbohydrates almost similar to those found in human proteins [1]. This suggests that yeast could become an essential system to produce pharmaceutical proteins in the coming years. However, recombinant hepatitis B vaccine prepared from yeast does not contain the disulphide bridges which have to be created in vitro. This modification is likely not possible with all the proteins.

The baculovirus-Sf9 insect cells system is very popular and used extensively in laboratories to prepare limited amounts of proteins. This tool still suffers from several limitations. The cells which harbour the baculovirus do not survive to the infection which must be repeated with new cells and virus preparation if large amount of proteins are

Table 1
Comparison of the different systems to produce recombinant pharmaceutical proteins

Points to consider	Production systems					
	Bacteria	Yeast	Insect cells + baculovirus	Animal cells (CHO cells	Transgenic plants	Transgenic animals
Theoretical production level	+++++	+++++	+++	+	+++++	+++++
Practical production level	++ (+)	++ (+)	+	+	++	++++
Investment cost	+++++	+++++	++	+	++++	+++
Production cost	+++++	+++++	++	++	+++++	++++
Flexibility	+++++	+++++	++	+	+++++	++++
Line conservation	+++++	+++++	+++	+++	+++++	+++++
Line stability	+++++	+++++	++++	+++	+++++	+++++
Delay for the first production	+++++	+++++	+++	+++++	++++	+++ (+)
Scaling up	+++++	+++++	++	+	+++++	++++
Collection	+++++	+++++	+++++	+++++	+++++	++++
Effect on organism	+++ (+)	+++ (+)	+++ (+)	+++ (+)	+++ (+)	+++
Post-translational modifications	+	++	+++	++++	+++	++++
Glycosylation	+	++	+++	++++	++	++++
Stability of product	+++++	+++++	+++	+++	++++	++++
Purification	+++	+++	+++	++++	+++	+++
Contaminant pathogens	+++++	+++++	+++++	++++	+++++	++++
Intellectual property	++++	+++	+++	++	+++	+++
Products on the market	++++	+++	+++	+++++	+	+++

needed. On the other hand, insect cells do not add to proteins all the sugars which are present in human proteins. For these reasons, the baculovirus system offers little advantage if any over CHO cells and it is only marginally used to prepare pharmaceutical proteins.

Animal or human cells and particularly CHO cells (Chinese Hamster Ovary) have been used for two decades successfully. The major advantage of these cells is that the proteins secreted in the culture medium are post-translationally modified essentially as their native counterparts. It remains that the sugars appear not completely added to the recombinant proteins. The major drawback of the mammalian cell culture systems is their relatively low production levels. Consequently, the use of these systems is limited by their high cost and their incapacity to produce more than a few kilograms of recombinant proteins per year. In addition, although the conditions of animal cell culture were improved, the production of recombinant proteins and their glycosylation may be not stable and reliable.

Transgenic plants offer interesting possibility to produce recombinant proteins as transgenesis is relatively easy in plants. A number of enzymes used for research or for diagnosis are currently being produced by transgenic plants at an industrial scale. Producing pharmaceuticals in plants is a more ambitious project. This system offers several advantages but also serious limits [2]. Various transgenic plant species can be obtained. Foreign proteins may be stored in leaves, in seeds or both, according to the promoter used. Leaves are very abundant but it may be difficult to purify the protein of interest from them due to the presence of proteases or substances like polyphenols which are not well-tolerated by patients. The amount of recombinant proteins which can be prepared in plants is virtually unlimited and the production cost is low. Moreover, agriculture techniques offer a great flexibility for scaling up. Leaves and mainly seeds containing the proteins of interest

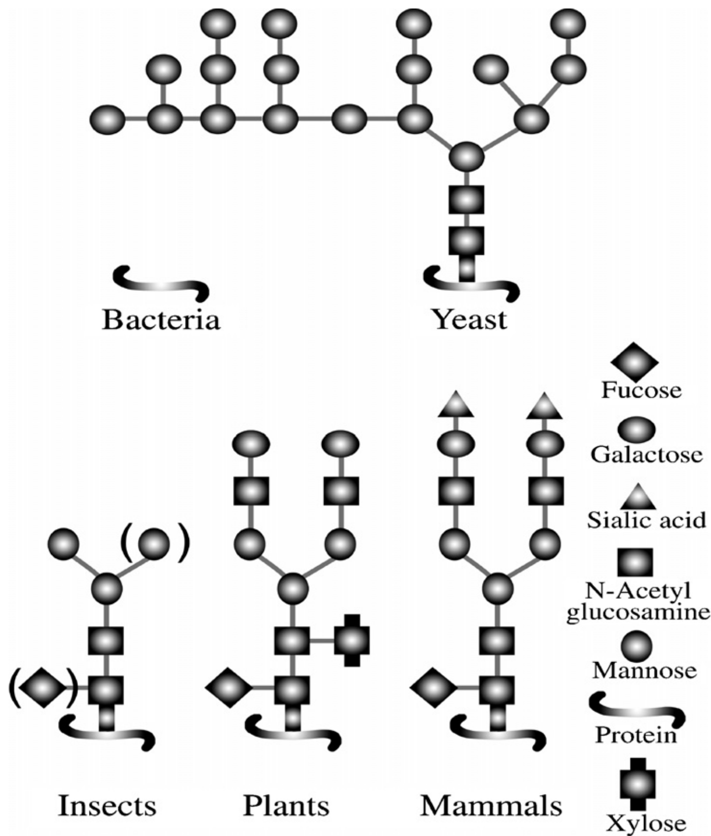

Fig. 1. Comparison of the glycosylation of recombinant proteins produced by different systems.

can be stored easily. It is also simple to rescue the plant lines and establish master banks allowing a reproducible production of proteins. Plant cells are able to fold proteins and associate subunits as efficiently as animal cells. On the contrary, plants cells add carbohydrates to protein chains but not as animal cells do. Proteins synthesized in plant cells have no terminal sialic acid and they contain xylose which may induce deleterious immune response in patients (Fig. 1). Experiments are in progress to modify protein glycosylation by transferring various genes responsible for the addition of sugars to proteins in a way similar to what happens in mammalian cells [3]. Proteins prepared from plants have very little chance to contain pathogens for humans or animals. Using transgenic plants to prepare recombinant proteins raise little ethical problems. One major concern is the uncontrolled dissemination of the proteins thus of the antigens when plants are cultured in open fields [4]. Low amount of antigens might induce a tolerance in humans or a basal unknown vaccination. This problem cannot be solved easily. Plants may be sterile to prevent any dissemination of the transgene. Another proposition which has been retained by several companies restricts the production of recombinant proteins by plants not used for human feeding, such as tobacco or alfalfa [5]. One possibility to suppress the problem

consists of keeping the plants in greenhouses. This is technically possible but would enhance markedly the production cost, reducing the attractiveness of transgenic plants for this purpose. A satisfactory approach could be to use plants which can be cultured easily, in large quantity and at a low cost in confined areas. Encouraging experiments have shown that duckweed and microalgae could provide humans with large amount of proteins produced in perfectly well-controlled conditions [6]. Another possibility would be to use cultured plant cells. Recent studies suggest that this perspective offers attractive alternative in some cases [7]. Recently, a system called magnifection was shown to allow the rapid production (within 2 weeks) of grams of functional antibodies in plants [8]. This system involves the transient high-level co-expression of the transgenes (for example immunoglobulin heavy- and light-chains) through the use of plant viral vectors delivered by *Agrobacterium* to the plant body. Although encouraging, these results cannot predict when or if recombinant proteins prepared from transgenic plants will be able to reach the market.

Transgenic animals offer particularly attractive possibilities to prepare recombinant pharmaceutical proteins. The advantages are the high and low cost production as well as the high quality of the proteins. A drawback may be the difficulty to separate the human proteins from their animal counterpart. Special care must also be taken to check that animal pathogens active in humans are not present in the protein preparations. Moreover, some of the recombinant proteins may be active and deleterious for transgenic animals. The comparison of the different possible animal systems is depicted in the following section.

3. The different transgenic animal systems

Milk is presently the most mature system to produce recombinant proteins from transgenic organisms [9–11]. Blood, milk [9–11], egg white [12–14], seminal plasma [15], urine and silk gland [16], insect larvae haemolymph [17] are other theoretically possible systems (Table 2). Silk gland is a promising system in particular cases. Preliminary results indicate that active human factor VII can be found in different tissues of a transgenic fish (tilapia). It is not known if this system may be improved and scaled up (McLean unpublished data). Blood cannot most of the time store high levels of recombinant proteins which are naturally too unstable. Moreover biologically active proteins in blood may alter the health of the animals. Milk avoids essentially these problems. Several mammalian species (rabbits, pigs, sheep, goats and cows) are currently being studied or used to produce recombinant proteins in their milk. Rabbits offer a number of advantages: easy generation of transgenic founders and offspring, high fertility, relatively high milk production, insensitivity to prion diseases, no transmission of severe diseases to humans. Pigs are more costly but produce higher amounts of milk than rabbits. Ruminants are potentially the most appropriate species to produce large amount of proteins but they need cloning or lentiviral vectors to integrate foreign genes, their reproduction is relatively slow, they do not glycosylate proteins as well as rabbits and pigs and they are sensitive to prion diseases (Tables 3 and 4).

Until recently, egg white was considered as a promising system strongly limited by the great difficulty of generating transgenic birds. This difficulty appears now surmounted. Lentiviral vectors proved efficient in chicken as in mammals ([14,18] and this issue). More

Table 2
Comparison of the different transgenic animal species to produce recombinant pharmaceutical proteins

Points to consider	Production systems						
	Blood	Milk	Egg white	Seminal Plasma	Urine	Silk gland	Drosophila larvae
Theoretical production level	+++++	+++++	+++++	+++	++	++	++
Practical production level	++	++++	+++ (+)	+	+	++	+
Investment cost	+++	+++	+++	+	+	+++	+++
Production cost	++++	++++	++++	++	+	+++++	++++
Flexibility	+++++	+++++	+++++	++	+	+++++	++++
Line conservation	+++++	+++++	+++++	+++++	+++++	+++++	+++++
Line stability	+++++	+++++	+++++	+++++	+++++	+++++	+++++
Delay for the first production	+++	+++	+++	++	+	++++	++++
Scaling up	++++	++++	++++	++	+	++++	+++
Collection	+++++	++++	+++++	+++	+++	+++++	+++++
Effect on organism	++	+++	+++ (+)	+++ (+)	+++ (+)	++++	++++
Post-translatlonal modifications	+++++	++++	+++ (+)	+++ (+)	+++ (+)	++ (+)	++ (+)
Glycosylation	++++ (+)	++++	+++	+++ (+)	+++ (+)	++	++
Stability of product	+++	++++	++++	+++ (+)	+++ (+)	+++ (+)	+++ (+)
Purification	++	+++	+++	++ (+)	++ (+)	+++	++ (+)
Contaminant pathogens	++	+++	+++	+++	++	++++	++++
Intellectual property	++++	+++	+++	+++	+++	+++	+++
Products on the market	+	++++	++	+	+	++	+

Table 3
Comparison of the time required to obtain recombinant proteins in different transgenic animal species

	Rabbit	Pig	Sheep	Goat	Cow
Gestation time (months)	1	4	5	5	9
Age at sexual maturity (months)	5	6	8	8	15
Time between gene transfer and first lactation (months)	7	16	18	18	33
Number of offspring	8	10	1–2	1–2	1
Annual milk yield (liters)	15	300	500	800	8000
Recombinant protein per female per year (kg)	0.02	1.5	2.5	4	40

impressively, pluripotent cell lines from primordial germ cells (PG cells) have been established in chicken. These cells harbouring foreign genes can be reintroduced in early embryos and participate to the development of chimeric transgenic chickens [13]. In a previous experiment, the same group showed that chimeric transgenic chicken generated by using non-pluripotent cells was able to secrete a monoclonal antibody in egg white [12]. These experiments validate egg white as a source of foreign proteins including recombinant vaccines.

4. The methods to generate transgenic animals

The generation of transgenic farm animals may be achieved according to species by DNA microinjection into embryo pronuclei, by using lentiviral vectors or transposons, by

Table 4
Possible level of recombinant protein production in milk of different transgenic animal species

Protein	Estimated need (kg year^{-1})	Species	Herd size
Human serum albumin	100,000	Cow	5,400
α-1-Antitrypsin	5,000	Sheep	4,300
Monoclonal antibody	100	Goat	58
Anti-thrombin-III	75	Goat	43
Factor IX	2	Pig	4
Protein Cl inhibitor	1	Rabbit	50

incubating sperm with DNA followed in vitro fertilization using ICSI (Intracytoplasmic Sperm Injection), by transferring the foreign gene into pluripotent cells (embryonic stem cells or primordial germ cells) followed by the generation of chimeric animals harbouring normal and transformed cells, by transferring the foreign gene into somatic cells used for the generation of cloned animals using nuclear transfer. These methods have been described in recent reviews [19–21]. They are summarized in Fig. 2.

Microinjection into pronuclei is very poorly efficient in ruminants and some other species. It is still being used successfully in mice, rats, rabbits, pigs and fish. In order to increase the integration frequency, foreign genes can be introduced in vectors containing natural elements favouring DNA integration such as transposons and lentiviral vectors ([14,18] and this issue). The latter proved highly efficient in ruminants and pigs. This technique is being adopted by experimenters even if these vectors have limited capacity to harbour foreign DNA and if the number of integration sites in the same animal is presently difficult to control.

DNA transfer via sperm has been developed mainly in pigs and mice. It may simplify transgenesis in some cases. An improve protocol consist of initially degrading sperm membrane, to incubate sperm in the presence of DNA and to fertilize oocytes using ICSI ([22] and this issue). ICSI gave a good yield of transgenic pigs [23].

The utilisation of cells as carrier for the foreign genes has been used in mice for almost 20 years. In this case, pluripotent cells capable of participating to the development of chimeric transgenic animals are being used. This method is laborious and used only for gene targeting and in practise essentially to inactivate genes (gene knock out).

For unknown reasons, it has not been possible to obtain and use pluripotent cells from embryos (ES cells: embryonic stem cells) in species other than mice. A recent study has shown that, in chicken, it was possible to establish pluripotent cell lines (EG cells) from the pluripotent cells which are present in foetal gonads (PGC: primordial germ cells). This made it possible the generation of transgenic birds which are candidates to produce recombinant proteins in egg white ([13] and this issue).

The cloning technique used to generate Dolly the sheep is being used to generate transgenic ruminants and pigs. This technique allows gene addition but also gene targeting by homologous recombination. This makes it possible gene knockout. Gene targeting is also a way to integrate foreign genes in genomic sites known to favour their expression.

The generation of transgenic animals remains relatively laborious and costly but it is no more a hurdle to the production of recombinant proteins.

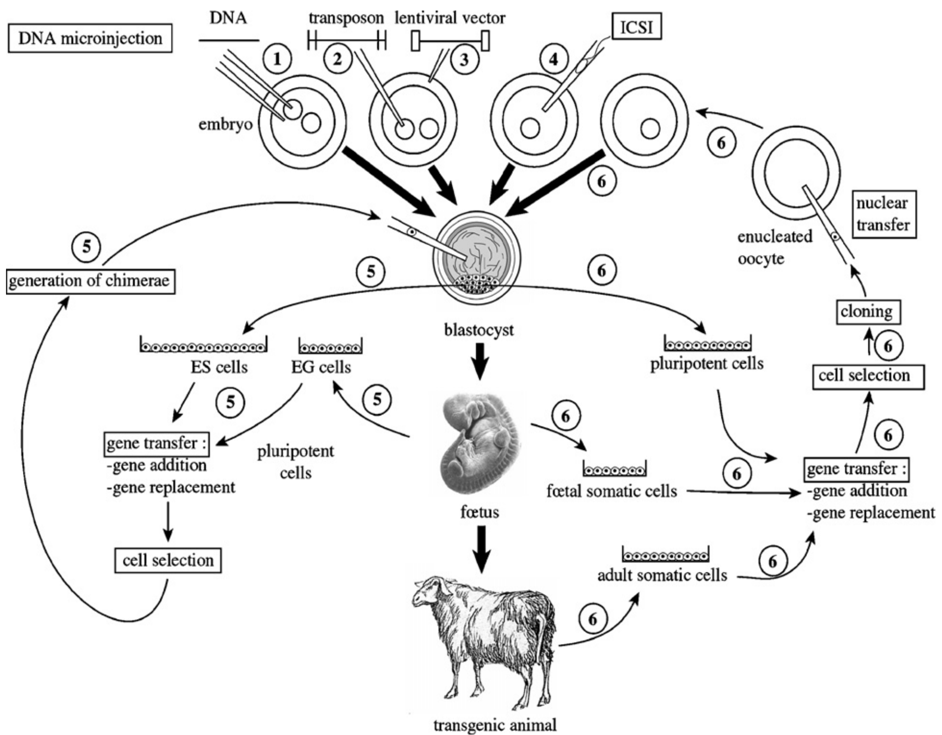

Fig. 2. Different methods to generate transgenic animals. (1) DNA transfer via direct microinjection into a pronucleus or cytoplasm of embryo; (2) DNA transfer via a transposon: the gene of interest is introduced in the transposon which is injected into a pronucleus; (3) DNA transfer via a lentiviral vector: the gene of interest is inserted into a lentiviral vector which is injected between zona pellucida and membrane of oocyte or embryo; (4) DNA transfer via sperm: sperm is incubated with the foreign gene and injected into oocyte cytoplasm for fertilization by ICSI (Intracytoplasmic Sperm Injection); (5) DNA transfer via pluripotent cells: DNA is introduced into pluripotent cell lines (ES: embryonic stem cells: lines established from early embryo, EG: embryonic germ cells: lines established from the primordial germ cells of foetal gonads). The pluripotent cells containing DNA are injected into early embryos to generate chimeric animals harbouring the foreign gene; (6) DNA transfer via cloning: the foreign gene is introduced into somatic cells, the nucleus of which are introduced into the cytoplasm of enucleated oocytes to generate transgenic clones. Methods 4, 5 and 6 allow random gene addition and targeted gene integration via homologous recombination for gene addition or gene replacement including gene knock out and knock in.

5. Optimization of transgene expression

To be expressed in a reliable manner, a transgene must ideally contain a promoter, enhancers, insulators, introns and a transcription terminator [20]. Expression in milk is achieved successfully with promoters from milk protein genes. Expression in egg white is possible using the potent promoter of ovalbumin gene. Using long genomic DNA fragments containing the promoter of interest generally enhances greatly the expression of foreign cDNA. This proved to be the case for the promoter of one milk protein gene, WAP gene (Whey Acidic Protein) [24]. This suggests that elements from long DNA fragments

will be identified and used in future to construct compact vectors expressing transgenes in a reliable manner ([25] and this issue).

Constructing an efficient expression vector to produce a therapeutic protein is not a standard operation. Two examples may illustrate this point. Recombinant vaccines against malaria are presently under study [26]. One of the proteins was initially obtained in mouse milk [27] it is now being produced in goat milk. Unexpectedly, the antigen produced in mouse milk lost its vaccinating properties when glycosylated. The second example is the production of VP2 and VP6 proteins from rotavirus in transgenic rabbit [28]. Rotavirus has a genome formed of several independent RNA fragments. This virus is replicated in cytoplasm and its proteins are not individually secreted. The following modifications of the VP2 and VP6 nucleotide sequence were performed: elimination of the splicing sites and of several *N*-glycosylation sites, addition of a peptide signal and adaptation of codons to optimize the expression of the two cDNAs in the mammary gland of the animals. The modified cDNAs were introduced into a vector designed according to the criteria defined above [20]. These gene constructs made it possible the co-secretion in milk of the two viral proteins at concentration up to 500 μg/ml. These proteins were able to protect mice against the virus completely or partially according to the mode of administration [42].

6. Possible improvement of the methods

Data shown in Table 5 report some of the major projects in development based on production of recombinant proteins in milk. The projects implying production in egg white and depicted above could be added on this list.

Despite important progress in the design of vectors for the expression of transgenes in milk or egg white must still be improved. Indeed, the level of foreign proteins found in milk or egg white is sometimes low for unknown reasons and it is not always strictly mammary specific. The position site effect must have a strong impact on transgene expression. The use of long genomic DNA fragments depicted above is one way to obtain a more reliable expression of the transgenes. The use of episomal vectors independent of integration sites and foreign gene targeting to known active genome sites are other possibilities under study [20].

A level of 1 mg per ml of milk or even lower appears acceptable economically. At higher concentration, the recombinant proteins may not be fully matured and particularly glycosylated. The mammary cellular machinery is likely saturated and cannot fully glycosylate the extra proteins. The recombinant protein ATryn (human antithrombinIII) produced in goat milk contains less sialic acid than its native counterpart is a case in point [29]. Similarly, the human inhibitor C1 produced in rabbit milk is not fully sialylated [30]. Monoclonal antibodies secreted in chicken egg white do not contain sialic acid [12]. This diminishes markedly the half-life of the proteins in patients and may complicate their use by clinicians. The transfer of genes coding for glycosylation enzymes improved carbohydrate transfer to proteins synthesized in yeast [1] but also in CHO cells [31]. The optimization of glycosylation in plants is in course using the transfer of genes coding for the glycosylation enzymes [3]. The same could be achieved in mammals and chicken to improve glycosylation in milk and egg white, respectively.

Table 5
Some of the pharmaceutical proteins under study produced in milk

Proteins	Company	Animal	g/l	Glycosylation	Development
ATryn	GTC	Goat	3	<NANA, >NGNA	EMEA (2006)
InhibitorC1	Pharming	Rabbit	8	<NANA	Phase III
Fibrinogen	Pharming	Rabbit	?		Phase III
Malaria antigen	GTC	Goat	?	No	Clinical
Anti-CD137	GTC	Goat	?	?	Clinical
Albumin	GTC	Goat	?	No	Clinical
α-AT	GTC	Goat	?	?	Clinical
BChE	PhAth	Goat	?	?	Preclinical
RotavirusVP2/VP6	BPT	Rabbit	0.5	No	Preclinical
Blood factor	BPT	Rabbit	3	<NANA	Preclinical
TNAP	AM Pharma	Rabbit	<0.1	?	Preclinical

A more subtle glycosylation effect on antibody action has been recently observed. It has been shown that the addition of *N*-acetylglucosamine through gene transfer in CHO cells allowing the formation of a third carbohydrate antenna in antibodies, enhances their capacity to induce the ADCC (Antibody-Dependent Cellular Cytotoxicity) immune cellular reaction [32]. In fact, the absence of fucose in antibodies has a stronger effect on their capacity to stimulate ADCC reaction [33]. It was also demonstrated that sialylated antibodies have a lower affinity for Fc-FcγR receptors and a higher capacity to induce anti-inflammatory reaction. The presence of some pathogens in animals induces a desialylation of antibodies to enhance their cytotoxic action. Hence, recombinant monoclonal antibodies may be engineered to induce an anti-inflammatory reaction by adding sialic acid with or without fucose. Similarly, monoclonal antibobodies may contain no fucose and no sialic acid to favour their cytotoxic effect and reduce proinflammatory reactions [33]. The last form of antibodies appears the most appropriate to destroy tumour cells. Interestingly, the recombinant monoclonal antibodies found in egg white are devoid of sialic acid and fucose suggesting that they are potent tools to destroy tumour cells [12].

Other post-translational modifications of proteins are crucial. This is namely the case for some specific cleavage and for γ-carboxylation [34]. The complete maturation of human protein C found in the milk of transgenic mice was achieved by crossing these animals with others expressing the furin transgene in their mammary gland [34,35]. This pioneer work indicates that living fermentors such as mammary gland can be engineering to perform the post-translational modifications of recombinant proteins.

7. Other uses of recombinant proteins in milk

The modification of milk composition may have applications other than preparing pharmaceutical proteins. Some of the studies in course aim at increasing the overall nutritional values of pig milk to provide piglets with enough feed. Other modifications of ruminant milk composition may improve quality of the curd. Several experiments led to the secretion in ruminant milk of antibacterial proteins such as human lactoferrin and lysozyme or lysostaphin having antibacterial activities. These molecules protect to some

degree milk, mammary gland and potentially consumers from bacterial infection. A monoclonal antibody against coronavirus secreted in mouse milk protects pups against infection by the virus. Cow milk devoid of the allergenic protein β-lactoglobulin is under study. Milk having low lactose concentration might be helpful for consumers who are intolerant to lactose. Milk enriched in ς-3 fatty acids have been obtained in transgenic mice ([19] and this issue). This shows that the techniques originally designed to produce pharmaceutical proteins in milk can be extended to improve milk as a food or to use it as a vehicle to provide consumers with molecules beneficial in some way for their health.

8. Conclusions and perspectives

The two major animal systems to produce pharmaceutical proteins in milk and egg white have recently been technically improved and their use as an essential source of new medicaments has become very likely. The different mammals which participate to this industrial activity are rabbits, pigs, sheep, goats and cow (Tables 3 and 4). Each of these species offers advantages and drawbacks. Rabbits are sufficient to produce several kilos of proteins per year. This species is particularly flexible allowing a rapid generation of founders and scaling up. For very high protein production, larger animals are needed. It is now proven that the amount of human antithrombin III obtained per year from transgenic goats is equivalent to this resulting from 90,000 human blood samplings. A possible alternative could be to use recombinant adenoviral vectors capable of infecting ruminant mammary gland [36]. This approach is laborious but could be helpful when a protein has a deleterious effect on animals and can be only transiently produced.

The proteins which have been prepared in milk are mainly naturally secreted. In the case of lysostaphin, rotavirus vaccine and a few others, the proteins became secreted efficiently after adding a signal peptide and they were found in the aqueous phase of the milk. In two cases, CFTR [37] and alkaline phosphatase [38], the proteins were hydrophobic and concentrated in the membrane of milk fat globules. This may facilitate or complicate the purification of the recombinant proteins. As a general rule, it must be considered that the purification protocol of recombinant proteins from milk is not standard and must be designed on a case by case basis. Recombinant proteins are stable in milk but they may be more or less trapped by caseins which are extremely abundant.

Rabbits and pigs are not sensitive to prions and the PrP gene required for the development of prion diseases has been knocked out in cows [39]. Guidelines to breed animals producing pharmaceutical proteins and to control the absence of pathogens in the purified protein fractions have been defined. It may thus be considered that the preparation of pharmaceutical proteins from milk is a safe process. The same is potentially true for the proteins which are being prepared from egg white [40].

The study of the biochemical and biological properties of the recombinant proteins is of paramount importance before they can be put in the market. This problem is particularly complex as natural and recombinant proteins often exist under different forms. This is particularly the case as far as glycosylation is concerned. The case of ATryn is a good example [29]. Indeed, ATryn contains two forms of sialic acid: *N*-acetylneuraminic acid (NANA) and *N*-glycosylneuraminic acid (NGNA). Only the NANA form is present in

humans. The second form NGNA may induce an immune response in patients with not predictable side-effects. Interestingly, recombinant proteins prepared in rabbit and pig milk contain only the NANA form in human proteins. Despite these imperfections, ATryn was accepted by EMEA which argued that the protein has little chance to induce deleterious effects and could be helpful for patients. This decision suggests that EMEA does not consider that a recombinant pharmaceutical protein must absolutely be structurally similar to its native counterpart but it must meet satisfactory clinical and biosafety criteria on a case by case basis. The fact that ATryn (human antithrombin III) was accepted by EMEA is an important message to the involved biotechnology companies.

Using transgenic animals as a source of pharmaceutical proteins raise minor general biosafety and ethical problems. Indeed, the escape of the animals in environment is extremely unlikely and in the majority of the cases, animals do not suffer from expressing a foreign protein in their milk or eggs. Several proteins raised specific problems. Human erythropoietin produced in rabbit milk altered their health [41]. Less intense side effects were observed when human growth hormone was produced in rabbit milk and the high producers of human EC superoxide dismutase failed to lactate in a normal way [unpublished data].

The number of companies involved in the production of recombinant pharmaceutical proteins is expected to increase. This will result from the improvement of the different systems and from the fact that the oldest patents are becoming obsolete in the coming years. Competition may thus become very intense. Some experts consider that the demand of pharmaceutical protein production might increase faster than the capacity of the companies to produce proteins [10]. The different systems would be then all helpful for one or two decades.

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