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Review

The production of pharmaceutical proteins from the milk of transgenic animals *

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Summary — The preparation of recombinant proteins of pharmaceutical interest from the milk of transgenic animals is becoming a reality. No protein has reached the market yet but several have been prepared in large quantities not only from laboratory animals but also from ruminants (goat and sheep) and pigs. Rabbit appears more and more to be an intermediate animal well adapted for the preparation of limited amounts of proteins. Several problems remain to be solved to optimize the method. The expression level of genes of interest associated with milk protein gene control regions is usually unpredictable. The recombinant proteins secreted in milk are not always in a satisfactory biochemical form. Cleavage and glycosylation are not always carried out correctly. The problem of the possible presence of agents pathogenic for humans in proteins extracted from milk is not completely solved. Prions have not been found in mammary glands and other milk pathogens may be controlled using good practice in breeding.

recombinant protein / transgenic animal / milk

Résumé — Production de protéines d'intérêt pharmaceutique à partir du lait d'animaux transgéniques. La préparation des protéines recombinantes d'intérêt pharmaceutique à partir du lait d'animaux transgéniques est en train de devenir une réalité industrielle. Aucune protéine n'est encore sur le marché mais plusieurs sont produites en abondance non seulement par des animaux de laboratoire mais aussi par des ruminants (chèvre et mouton) ainsi que par des porcs. Le lapin apparaît de plus en plus comme un animal intermédiaire bien adapté pour la préparation de quantités limitées de protéines. Des problèmes restent encore à résoudre pour optimiser la méthode. Le taux d'expression des gènes d'intérêt associés aux éléments régulateurs des gènes des protéines du lait reste le plus souvent imprévisible. Les protéines produites dans le lait ne sont pas toujours sous une forme biochimique satisfaisante. Certains clivages ainsi que certaines glycosylations ne sont parfois réalisés que partiellement. Le problème de la présence éventuelle d'agents pathogènes pour l'homme dans les préparations de protéines recombinantes issues du lait n'est pas complètement résolu. Cet obstacle ne paraît pas infranchissable. Les prions ne semblent en effet pas présents dans la glande mammaire et le lait et la présence d'autres agents pathogènes peut probablement être contrôlée par de bonnes pratiques d'élevage.

protéine recombinante / lait / animal transgénique

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The idea that the milk from transgenic animals could be the source of recombinant pharmaceutical proteins was suggested several years ago. The first experimental demonstration that this goal can be reached was given by Simons *et al* (1987) who succeeded in producing sheep β-lactoglobulin in the milk of transgenic mice.

To obtain a protein not naturally secreted in milk, gene constructs containing the regulatory region of a milk protein gene fused to the coding region of the gene of interest must be prepared. The promoter regions from most of the milk protein genes from different species are used for this purpose. The promoter from αS_1-β-caseins, β-lactoglobulin, whey acidic protein (WAP), α-lactalbumin and even the long terminal repeat (LTR) from mouse mammary tumor virus (MMTV) have thus been fused to a variety of genes and used to generate transgenic mice, rats, rabbits, goats, sheep, pigs and cows. Probably more than 50 foreign proteins have been produced in this way in the milk of transgenic mammals. The published data was reported in a recent review (Houdebine, 1994). Since this review was published, additional proteins have been obtained in milk. Among these proteins are human lysozyme (Maga *et al*, 1994), human insulin-like growth factor-1 (IGF-1) (Maga *et al*, 1994), bovine chymosin (Brem *et al*, 1995), human plasminogen activator (Riego *et al*, 1993; Ebert *et al*, 1994), human growth hormone (Tojo *et al*, 1993; Ninomiya *et al*, 1994), human γ-interferon (Dobrovolsky *et al*, 1993), human lactoferrin (Platenburg *et al*, 1994), human erythropoietin (Hyttinen *et al*, 1994; Castro *et al*, 1995), human protein C (Drohan *et al*, 1994; Paleyanda *et al*, 1994; Wei *et al*, 1995), and human albumin (Barash *et al*, 1993; Hurwitz *et al*, 1994).

Although little doubt remains on the use of transgenic animals as living fermentor, a certain number of problems are still to be solved before this method can become a common industrial process to produce recombinant proteins.

THE GENERATION OF TRANSGENIC MAMMALS

The first transgenic mouse was obtained 13 years ago. The method originally defined for mouse and relying on the direct microinjection of gene into the pronucleus of one-cell embryos has been extended to other mammals. Minor adaptations are sufficient to obtain many transgenic rats and rabbits. For farm animals, the yield of transgenics is dramatically low in most cases. Injecting a polycation-DNA complex instead of DNA into cytoplasm, although of moderate efficiency, leads to the generation of transgenic animals. This approach may improve the situation when pronuclei are not visible (Velander *et al*, unpublished data). The availability of embryos is a limiting factor for ruminants. It is now possible to generate a number of one-cell-stage embryos from cow ovary at a low cost by performing *in vitro* oocyte maturation and fertilization and to use them for transgenesis (Krimpenfort *et al*, 1991; Hyttinen *et al*, 1994). The *in vitro* generation of embryo from ovary has been extended successfully to sheep and goat (N Crozet *et al*, personal communication). The same is not true for porcine embryos at present.

To tentatively reduce the number of recipient females, embryos can be cultured until the blastocyst stage. Only those that were not damaged by the micromanipulation remain at the end of the culture and are candidates to be transferred to females.

The identification of transgenic embryos at the blastocyst stage is theoretically possible by using PCR, starting from a few blastomers. In practice, this method is poorly reliable, due to traces of remaining unintegrated DNA which gives numerous false positives (King and Wall, 1988; Ninomiya *et*

al, 1989). Another method based on the discrimination of DNA methylation in the original insert and in the integrated gene may be used (Cousens *et al,* 1994). Fluorescence *in situ* hybridization, which proved to be a simple and reliable method to detect transgenes in newborn animals (Swiger *et al,* 1995), might be helpful in identifying transgenic embryos using isolated blastomers. The coinjection of reporter genes, such as that coding for vargula luciferase but not involving an invasive test on blastocysts, may be an attractive alternative (Thompson *et al,* 1995). This reporter gene has been associated with the heat stock protein 70 gene promoter and the matrix-attached regions (MAR) from human interferon-β gene. With such a gene construct, the vargula luciferase can be synthesized, secreted under heat stock and measured in the blastocyst culture medium. The presence of the MARs in the gene construct favours the expression of the integrated DNA and is expected to identify the blastocysts in which the microinjected DNA is integrated and to eliminate those in which unintegrated DNA is still present. A reliable and easy method to identify genuine transgenic blastocysts therefore remains unavailable for the time being.

The transfer of foreign genes through embryonic stem (ES) cells or primordial germ cells (McLaren, 1992) remains impossible so long as these cells cannot be obtained in a reliable manner in species other than mouse. The recent observation that spermatogonia can colonize a foreign testis after a direct microinjection in this tissue and give birth to normal mice in good yield opens new avenues (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Gene transfer through spermatogonia awaits additional experiments showing that these cells can be cultured, transfected and retain their capacity to differentiate to functional spermatozoa after reimplantation into recipient testis.

THE CONSTRUCTION OF EFFICIENT VECTORS

Many experiments have led to the conclusion that native genes are often efficiently expressed as transgenes whereas their cDNA counterparts are not. The presence of intron, which is generally not required in cultured cells, is thus of paramount importance for transgenes (Brinster *et al,* 1988; Palmiter *et al,* 1991). Several studies did not reveal which combinations of promoters, introns and terminators are the best to obtain high and predictable expression of cDNA in transgenic animals (Palmiter *et al,* 1991). Selecting the best introns, 5'- and 3'-untranslated regions and terminators to be associated with the promoter of a given milk protein gene is a possible approach. *In vitro* and *in vivo* studies have thus shown that the intron from SV40 early genes is much less efficient than the intron from SV40 late genes and the SIS generic intron (Petitclerc *et al,* 1995). Some gene constructs containing a given cDNA associated with efficient introns and transcription terminators may however be much less efficient *in vivo* than the corresponding genomic fragment, although they were significantly more potent in cultured cells (Petitclerc *et al,* 1995). Introducing the foreign cDNA in the middle of milk protein genes, keeping some of their introns and exons in the cDNA situated before and after proved to be efficient in several cases (Brem *et al,* 1994; Drohan *et al,* 1994; Hyttinen *et al,* 1994; Maga *et al,* 1994; Brem *et al,* 1995). Using P1 phage or YAC vectors may be useful if long fragments of DNA surrounding milk protein genes or if long foreign genes are to be used. The coinjection of 2 overlapping complementary regions which recombine *in vivo* may also be performed. This simple procedure proved to be efficient in several species, including rabbit to regenerate functional genes from 2 pieces. At least 60% of the transgenic rabbits obtained after coinjections of overlapping DNA fragments containing the rabbit WAP

promoters and human factor VIII cDNA harboured a reconstituted complete gene (Attal *et al,* unpublished results).

Promoters from milk protein genes used so far show quite different potency. The most efficient promoters seem to be those from ruminant αS_1-casein (Brem *et al,* 1994; Platenburg *et al,* 1994), β-casein (Ebert *et al,* 1994; Maga *et al,* 1994), sheep β-lactoglobulin (Barash *et al,* 1993; Brash *et al,* 1994; Hurwitz *et al,* 1994; Platenburg *et al,* 1994) and rabbit WAP (Bischoff *et al,* 1992; Devinoy *et al,* 1994). A distribution of the upstream regulatory regions specific of each species may explain some of these differences.

The expression of transgenes is generally highly influenced by their chromatin environment. A few genes are expressed independently of the integrating site. This is the case for sheep β-lactoglobulin (Whitelaw *et al,* 1992) and rat WAP gene (Krnacik *et al,* 1995). This is not true when the β-lactoglobulin gene promoter is associated with a foreign gene (Barash *et al,* 1994). In most cases, the DNA sequences responsible for transgene insulation have not been defined. AT-rich MAR have been shown to amplify and insulate integrated genes. This was observed to some extent when chicken lysozyme MAR insulating region was associated with mouse WAP promoter (McKnight *et al,* 1992). No effect was seen when the MAR located in the 3'-OH of human apolipoprotein B 100 gene and in the SV 40 genome was added of both side of constructs containing the rabbit WAP gene promoter (Attal *et al,* unpublished data). Insulating transgenes therefore remains possible so far only in a limited number of cases.

Introducing a foreign cDNA within a milk protein gene by using ES cells and homologous recombination should provide efficient promoter, introns, terminators and insulators. This technical approach was successfully used to replace mouse α-lactalbumin and β-casein genes by homologous inactive mutated genes (Kumar *et al,* 1994; Stinnakre *et al,* 1994). These preliminary successes suggest that foreign proteins will be produced in milk of farm animals when functional ES cells are available.

THE PREDICTION OF GENE CONSTRUCT EFFICIENCY

Evaluating the potency of gene constructs *in vitro* before using them to generate transgenics would be very helfpul. Indeed, a relatively long time elapses between the construction of the genes and the measurement of the foreign proteins in milk (especially of course when males are founders), even when transgenic mice are used.

The mouse mammary cell line HC11 (Ball *et al,* 1988) can be utilized to evaluate the efficiency of gene constructs. All researchers admit however that the correlation between the potency of a construct in HC11 cells and transgenics is poor (Petitclerc *et al,* 1995).

Transgenic mice are usually predictive of what will be observed in other transgenic mammals. In one case at least, however, a gene construct containing the mouse WAP promoter and the human protein C gene was poorly efficient in mice and of quite acceptable potency in the pig (Velander *et al,* 1992). Unexpectedly also, the same gene construct was ectopically expressed in sheep (Wall *et al,* 1995) but not in pig.

Introducing a gene directly into the mammary gland is possible. This was achieved using cationic lipids (MA Sirard, personal communication), biolistics (Furth *et al,* 1992), and retroviral vectors (Archer *et al,* 1994). The expression of the gene was then low. It could be higher if gene transfer was carried out with the available potent adenoviral vectors. Whatever happens, this procedure is not expected to be able to replace transgenesis to express large amounts of foreign proteins in many animals and it has little

chance of allowing a good prediction of the efficiency of gene constructs used as transgenes. Indeed, these methods are *in vivo* transfection and the foreign genes are not then submitted to the numerous modifications of chromatin which occur during embryo development and organogenesis. The direct gene transfer into functional mammary gland may however provide interesting information on the capacity of the mammary cell to correctly modify a given foreign protein post-translationally.

THE CHOICE OF THE ANIMAL SPECIES

For the production of very large amounts of foreign protein (more than 100 tons per year), transgenic cows seem the most appropriate. For quantities higher than 1 kg per year, sheep, goats or even pigs are valuably used. When no more than 1 kg of recombinant protein per year is needed, rabbits which can be milked with an adapted machine (Lebas, 1970; Duby *et al,* 1993), may be attractive, due to the relatively low cost of transgenic generation; rabbit also appears to be appropriate as a transition animal to obtain easily sufficient amounts of recombinant proteins for the study of their biochemical and biological properties before using larger animals to produce these proteins on an industrial scale.

Mice can provide only very limited amounts of milk. Milking these animals is possible. It is also easy to collect the whole milk from mammary glands by keeping them on ice for a few hours (Stinnakre *et al,* 1992).

THE BIOLOGICAL EFFECTS OF THE RECOMBINANT PROTEINS ON HEALTH OF TRANSGENIC ANIMALS

Most of the recombinant proteins secreted in milk of transgenic animals are to be used potentially as pharmaceuticals for humans. Most of these proteins are biologically active in various mammals. The ectopic and non-temporally regulated expression on the transgenes proved to have detrimental effects on animals. Mouse WAP led to milchlos phenotype (Burdon *et al,* 1991). Human growth hormone (hGH) induced subfertility and gigantism in mice (Devinoy *et al,* 1994). Human erythropoietin cDNA fused to rabbit WAP promoter led to a marked accumulation of red blood cells, a delay in growth, a severe subfertility and a premature death of transgenic rabbits (Attal *et al,* unpublished data).

Ectopic or non-temporally regulated expression of transgenes might be significantly attenuated by adding insulators to the constructs (see above) or by controlling the expression of the transgenes by an external inducer such as tetracyclin (Furth *et al,* 1994).

An induced homologous recombination using the cre-lox system (Barinaga, 1994) might also contribute to reduced expression of the transgenes out of the lactation period.

An improvement of the specificity of transgene expression might be obtained by replacing a milk protein gene by the gene of interest. This was achieved recently in mice expressing human α-lactalbumin gene instead of their own (Stacey *et al,* 1994, 1995). It should be noted that this approach remains limited to mice so long as ES cells are not available in other species. Moreover, the *in situ* association of a foreign gene of interest with the regulatory sequence of a milk protein gene using homologous recombination may be not efficient in all cases.

On the other hand, whey proteins such as α-lactalbumin or WAP are spontaneously transferred from milk to blood during lactation (Grabowsky *et al,* 1991). This is also the case for recombinant proteins such as human growth hormone (GH) (Devinoy *et*

al, 1994). In some cases, even when the expression of the transgene is prefectly controlled, the health of the animals may be severely altered by the recombinant proteins transferred from milk to blood.

PURIFICATION OF THE RECOMBINANT PROTEINS FROM MILK

Milk is not an exceedingly complex biological fluid. It contains a large quantity of a few proteins and only limited proteolytic activity. Conventional methods to purify proteins seem appropriate to isolate recombinant proteins from whey (Ebert *et al,* 1991; Wilkins and Kuys, 1992). In particular cases, when recombinant proteins are hydrophobic, they are secreted with lipid globules. This was the case for human cystic fibrosis transmembrane conductance regulator (CFTR) (Di Tullio *et al,* 1992).

The major concern with recombinant proteins from animal origin is not the classical biochemical purity. The presence of pathogens in the purified proteins may be detrimental for humans as soon as the proteins are injected, and in some cases, such as albumin or haemoglobin, in very large amounts. The presence of prions is a potential problem. The capacity of these molecules to be pathogens for other species is not well documented. In case this problem cannot be solved the inactivation of the PrP gene by homologous recombination can be envisaged although presently only in mouse. In the mouse, the inactivation of this gene did not alter greatly the health of the animals. However, the problem may be not so crucial since no prion has been found so far in mammary gland extract and milk from cows suffering from bovine spongiform encephalopathy. Transgenic mice harbouring sheep PrP gene instead of their own might be used to detect the presence of prion in fractions isolated from sheep milk.

THE BIOCHEMICAL STRUCTURE OF THE RECOMBINANT PROTEINS FROM MILK

Animals cells rather than bacteria or yeast are used to produce a certain number of recombinant proteins to tentatively obtain polypeptides with all the post-translational modifications. It is by no means certain that the mammary gland can perform all these modifications in a convenient manner. This seems unfortunately to be the case. Human-α_1-antitrypsin from sheep milk (A Colman, unpublished data), human anti-thrombin III from goat milk (ES Cole, unpublished data) are not fully glycosylated. The *in vitro* enzymatic addition of the missing terminal sialic acid increases significantly the half-life of the protein *in vivo* without apparently altering its biological activity.

Human protein C precursor from pig milk is not completely processed and not fully γ-carboxylated (Drohan *et al,* 1994).

Most likely, the mammary cells of different species do not have exactly the same enzymatic equipment for protein maturation. It may be therefore hazardous in some cases to extrapolate from model animals such as the mouse and the rabbit to predict the structure of a given recombinant protein in pig or ruminants.

Modifying the enzymatic equipment of the mammary cell through transgenesis to improve maturation of recombinant proteins is conceivable. It may however lead to disfunction of the mammary gland in some cases and a long investigation is needed before such an approach can be envisaged.

The fact that recombinant proteins from milk do not always have the same structure as their native counterpart may be of limited importance. The possible side-effects of the recombinant proteins extracted from milk must thus be evaluated case by case.

ALTERNATIVE PRODUCTION SYSTEMS

Milk is presently considered as the best biological fluid to produce recombinant proteins from animals. Blood may be appropriate in some cases, when the recombinant proteins are not too unstable or too toxic for the animals. This was the case for human α_1-antitrypsin which has been produced from liver at the concentration of 1 mg/ml with apparently a glycosylation similar to the native protein (Massoud *et al,* 1991). Human haemoglobin (Swanson *et al,* 1992) and peptides have also been produced in reticulocytes from transgenic animals. The purification of human proteins from the blood of transgenic animals may not be easy in some cases. Blood may thus be of limited interest in practice.

Egg white might become, in the future, a good source of recombinant proteins by transgenic birds.

Transgenic plants can also be the source of recombinant proteins. Hepatitis B surface antigen has been prepared in this manner (Mason *et al,* 1992). Plants may be appropriate when very large amounts of simple polypeptides must be prepared without any pathogens for humans. Human albumin and haemoglobin are good candidates to be extracted from transgenic plants.

OTHER USES OF TRANSGENESIS TO EXPRESS FOREIGN GENES IN THE MAMMARY GLAND

Gene transfer is used to study growth, differentiation (Jhappan *et al,* 1993; Pierce *et al,* 1993) and oncogenesis (Cardiff and Muller, 1993) of the mammary gland or other aspects of mammary gland function. Milk represents about 30% of the proteins of human food in rich countries. Its composition may be changed to improve yield or quality of the product for dairy industry. This may be achieved by modifying the concentration or amino acid composition of milk proteins.

Milk is not only a food. It contains various growth factors, anti-pathogen proteins such as lysozyme, lactoferrin, antibodies. Milk may become a vehicle to provide humans with number of factors and it may thus become a novel food (Clark, 1992; Yom *et al,* 1993). Conventional gene transfer, targeted gene knock-out and mutation in farm animals may greatly contribute to reaching this goal.

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