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Preparation of recombinant vaccines

Eric Soler[1,*] and Louis-Marie Houdebine[2]
[1]*Cell Biology Department, Erasmus MC, dr. Molewaterplein 50, 3015 GE, Rotterdam, The Netherlands*
[2]*Biologie du Développement et Reproduction; Institut National de la Recherche, Agronomique, 78350 Jouy en Josas, France*

Abstract. Vaccination is one of the most efficient ways to eradicate some infectious diseases in humans and animals. The material traditionally used as vaccines is attenuated or inactivated pathogens. This approach is sometimes limited by the fact that the material for vaccination is not efficient, not available, or generating deleterious side effects. A possible theoretical alternative is the use of recombinant proteins from the pathogens. This implies that the proteins having the capacity to vaccinate have been identified and that they can be produced in sufficient quantity at a low cost. Genetically modified organisms harboring pathogen genes can fulfil these conditions. Microorganisms, animal cells as well as transgenic plants and animals can be the source of recombinant vaccines. Each of these systems that are all getting improved has advantages and limits. Adjuvants must generally be added to the recombinant proteins to enhance their vaccinating capacity. This implies that the proteins used to vaccinate have been purified to avoid any immunization against the contaminants. The efficiency of a recombinant vaccine is poorly predictable. Multiple proteins and various modes of administration must therefore be empirically evaluated on a case-by-case basis. The structure of the recombinant proteins, the composition of the adjuvants and the mode of administration of the vaccines have a strong and not fully predictable impact on the immune response as well as the protection level against pathogens. Recombinant proteins can theoretically also be used as carriers for epitopes from other pathogens. The increasing knowledge of pathogen genomes and the availability of efficient systems to prepare large amounts of recombinant proteins greatly facilitate the potential use of recombinant proteins as vaccines. The present review is a critical analysis of the state of the art in this field.

Keywords: vaccine, recombinant proteins, adjuvants, epitope carrier, VLP, transgenic animals.

Introduction

The pioneering work of E. Jenner, L. Pasteur and others made it possible the eradication of smallpox from the earth by vaccinating a large number of people. Other diseases like hepatitis B and gastroenteritis induced by rotavirus might also be markedly reduced using vaccination.

The method commonly used to prepare vaccines consists of obtaining sufficient amount of attenuated or inactivated pathogens and administering this material to humans or animals. Attenuated forms of the pathogen are generally obtained by natural mutation followed by a selection. The number of random mutants may be increased by using mutagenic chemicals or

*Corresponding author. Tel: 31-10-408-7352.
E-mail: e.soler@erasmusmc.nl (E. Soler).

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irradiation. Alternatively, known virulent genes may be removed from the pathogen genome using genetic engineering. The native pathogen may also be inactivated by physicochemical treatment. This approach suffers from several limitations. Attenuated exploitable forms of the pathogen may not be obtained in all cases. The tools commonly used for that purpose are animal cell lines and chicken eggs. The live vaccines obtained in this way are generally potent but their composition is complex and they may induce severe deleterious effects precluding their use. This was the case for a live-attenuated vaccine against rotavirus, which induced severe intestine inflammation (intussusceptions) [1]. New vaccines still containing attenuated retrovirus are under development with expected reduced side effects [2,3]. Even in case of success, possible unknown side effects may persist with live vaccines. One is that the vaccinated persons are effectively protected but still shedding wild active viruses contributing to support the epidemic. Another problem generated by the use of attenuated or inactivated pathogen is that it is difficult to make a distinction between animals or humans who are vaccinated and those who are infected. Indeed, the same antibodies against the pathogens are present in the blood of both categories of animals or people. The absence of one gene of the pathogen may make the distinction possible between vaccinated and infected individuals. Alternatively, serum antibody markers resulting from the immunization by a foreign antigen added to the vaccine may also distinguish vaccinated and infected individuals.

Viral vectors can be used to express genes coding for vaccinating proteins from another pathogen. This system cumulates the advantage of using the efficiency of the viral vector to transfer and express the foreign gene. Several strains of vaccinia virus and adenoviruses from different origins are being used successfully to vaccinate animals. Naked DNA under the form of plasmids and harboring genes coding for vaccinating proteins is also a simple, versatile and safe tool to vaccinate animals. This method still needs to be improved before being approved for animals and humans.

A possible alternative consists of using subunits of the pathogens containing one or a few proteins organized as in the pathogens and forming virus-like particle (VLP) in the case of viruses [4]. This approach is expected to be safe as the material does not contain nucleic acids from the pathogen or from the vectors used to carry the gene coding for vaccinating proteins, which may be plasmids or viral vectors. This approach also makes it possible the distinction between vaccinated and infected individuals as antibodies are raised against most of the pathogen proteins after infection and only against a few of them after vaccination. The preparation of recombinant vaccines may be efficient but not easy to implement. Indeed, a long study may be necessary to define which proteins have a sufficient vaccinating capacity and ideally are efficient against most if not all the forms of the pathogens. The proteins cannot generally be obtained from the pathogen in sufficient quantity. Systems capable of providing large amounts of recombinant proteins at a low

cost must then be implemented. The isolated proteins are also generally less potent to induce an immune response than the whole pathogens, mainly if they are living. This implies the use of potent adjuvants, which must be devoid of deleterious side effects. The vaccinating proteins must therefore be purified to prevent any immune response against their contaminants. The vaccinating potency of recombinant proteins is largely unpredictable and this obliges experimenters to evaluate the efficiency of various modes of administration with different adjuvants.

The increasing knowledge of pathogen genomes offers multiple possibilities to identify proteins and even epitopes capable of inducing a protection against the pathogens. It has become possible to test one by one the different antigens of a pathogen. This approach implies the systematic cloning of the genes coding for putative vaccinating proteins, the preparation of the corresponding proteins and the evaluation of their capacity to be used as vaccines. This brute-force method already resulted in an unprecedented burst of new antigen discovery. A less laborious approach recently met a great success to identify new antigens from group A *Streptococcus*. This method consists of releasing fragments of the surface antigens by treating the bacteria with proteases. The peptides were identified by mass spectrometry and the corresponding genes were cloned to prepare and evaluate the corresponding antigens. This method allowed the fast identification of antigens for vaccination [5]. This method should be applied for a number of pathogens [6].

Several systems are becoming efficient to produce large amount of recombinant proteins including vaccines. Among these systems are transgenic animals and plants.

A pathogen protein having potent vaccination properties may theoretically be used as carriers for epitopes from other pathogens. In practice, the generation of fusion proteins harboring the epitopes and capable of inducing a protection against the pathogen is not an easy task.

The present review examines the different steps in the preparation and the evaluation of recombinant proteins to be used as vaccines.

The different systems to produce recombinant vaccines

Different systems are being implemented to produce recombinant proteins for experimental use or for biotechnological applications. They include peptide chemical synthesis, microorganisms, animal cells, plant cells, transgenic plants and transgenic animals (Table 1).

Peptide chemical synthesis

A number of peptides covering a pathogen protein known to induce vaccination may be chemically synthesized and tested for their capacity to induce a protection against a pathogen. This was achieved with the fragments of

Table 1. Comparison of the different systems for the production of recombinant proteins.

Production systems (Points to consider)	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	+ + + + +	+ + + + +	+ + + + +	+ + + +	+ + + + +	+ + + +
Dissemination in environment	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ +	+ + + + +
Intellectual property	+ + + +	+ + +	+ + +	+ +	+ + +	+ + +
Products on the market	+ + + +	+ + +	+ + +	+ + + + +	+	+ +

Note: The best parameters have the largest cross number.

VP6 proteins from rotavirus [7,8]. Essential epitopes of a protein for vaccination may thus be determined. This also makes it possible the identification of the mechanisms of the immune response induced by each epitope. The peptides containing relevant epitopes may then be chemically synthesized and chemically linked to carrier proteins. The resulting material may be used as vaccine. Alternatively, fusion recombinant proteins containing the epitopes of interest and a carrier protein known to induce vaccination may be prepared (see below).

Microorganisms

Microorganisms were the first to be used to produce recombinant proteins. Human insulin has been prepared for the last twenty years by bacteria. Although highly efficient for some proteins, bacteria show limited possibilities due to the fact that they cannot fold properly a number of proteins and proceed to posttranslational modifications [9]. Bacteria may produce so high amount of recombinant proteins that they form inclusion bodies precluding an easy purification. Some proteins are toxic for bacteria and cannot be prepared in this way. Interestingly, VP6 protein prepared from bacteria proved to have vaccinating capacity almost similar to the protein prepared from insect Sf9 cells infected by recombinant baculovirus harboring the corresponding viral gene [7,8–10,11].

Yeast may be easily transformed but they often produce limited amount of recombinant proteins, which are not glycosylated or unduly glycosylated. Interestingly, several genes coding for glycosylating enzymes have been transferred into yeast, which has become capable of adding several of the carbohydrates present in human proteins [12]. It is interesting to mention that a part of the hepatitis B vaccine is prepared from recombinant yeast. It is also important to note that the viral protein prepared from yeast does not form correctly polymers by disulfide bridges. The protein must therefore be chemically reduced to allow an appropriate formation of disulfide cross-links and VLPs having full vaccinating potency.

Animal cells

Various animal cells are currently being used to prepare recombinant proteins for experimental studies or for biotechnical applications [13]. It is interesting to note that most of the recombinant proteins used as pharmaceuticals are being prepared from animal cells.

One of the cell systems frequently used in laboratories to prepare viral proteins is the baculovirus-Sf9 cell system. This system is relatively simple to use and it proved efficient to prepare well-assembled viral proteins forming VLPs [4]. To reach this goal, the viral genes are introduced into baculovirus by homologous recombination in insect Sf9 cells. The resulting viral particles

are used to infect a large number of Sf9 cells that produce high amounts of viral proteins, which are not secreted but stored in cytoplasm to form spontaneously well-shaped VLPs. The VLPs can be isolated from cell lysate and purified using different protocols. One of them consists of fractionating VLPs in cesium chloride gradients. Several VLPs prepared in this way show structure similar to native corresponding viral complex as judged by electron microscopy and biochemical analysis. The baculovirus system makes it possible the preparation of VLPs from a broad variety of viruses having or not a simple or a double capsid and an envelope [4]. This tool allows the preparation of VLPs in sufficient quantity to determine their structure and to evaluate their vaccinating properties. The baculovirus-Sf9 system cannot be easily scaled up to prepare vaccines at an industrial scale.

Mammalian cells can be used to prepare recombinant proteins. CHO (Chinese Hamster Ovary) cells are most frequently used to prepare pharmaceutical proteins. One of the advantages of these cells is that they proceed to most of the posttranslational modifications of proteins. However, glycosylation of recombinant proteins secreted by CHO may be incomplete due to a saturation of the glycosylating enzymes. The extremity of the carbohydrate moiety of the secreted proteins does not contain quantitatively the terminal sialic acid. The addition of genes coding for glycosylating enzymes improves the quality of the secreted proteins [14,15]. Moreover, human cells synthesize sialic acid under the NANA (*N*-acetylneuraminic acid) form as do rabbit and chicken cells [16,17] whereas ruminant cells synthesize also the *N*-glycosylneuraminic acid [18].

The glycosylation of proteins is essential for the activity of some proteins. Non-glycosylated proteins have a short half-life *in vivo*. Unexpectedly, a peptide which is a candidate to become a vaccine against malaria looses its capacity to vaccinate mice under a glycosylated form [19]. This exemplifies the necessity to control glycosylation of recombinant vaccines in some cases.

In one case, for the preparation of a vaccine against hepatitis B, animal cells are used and provide a vaccine essentially similar to this obtained with yeast.

Although efficient, CHO cells remain a costly and poorly flexible system to prepare recombinant proteins. Indeed, a 100,000 l fermentor costs 400 million dollars and five years are needed to build such a tool.

Transgenic plants

The first transgenic plants were obtained in 1983. Apart from their use for basic studies, transgenic plants are increasingly used to improve food production. The idea of using transgenic plants as the source of recombinant proteins has become a reality. A number of enzymes used for research or for diagnosis are currently being produced at an industrial scale.

Producing pharmaceuticals in plants is a more ambitious project. This system offers several advantages but also serious limits [20]. Various plant species can be obtained as transgenics. Two essential methods are implemented to transfer genes to plants. One of these tools is the *Agrobacterium tumefaciens* system which contains a natural vector able to transfer a foreign gene into the plant genome. The other known as biolistics consists of projecting minute bullets covered by DNA into plant cells. In both cases, viable plants are developed from transformed somatic cells. A large number of transgenic plants can be obtained making it possible the selection of those in which the transgene is intact and functional.

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 polyphenol, 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 or seeds containing the proteins of interest 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 those forming antibodies essentially 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. 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 mammalian cells [21].

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 [22]. 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 companies involved in the production of recombinant proteins by plants is to limit the gene transfer to plants not used for human feeding such as tobacco or alfalfa [23]. This does not stop completely the uncontrolled diffusion of the antigen. 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 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 [24].

Another possibility would be to use cultured plant cells. Recent studies suggest that this perspective offers attractive alternative in some cases [25].

Several antigens potentially to be used for vaccination have been produced in transgenic plants. The capsid protein VP6 of rotavirus has been found in tubers and leaves of transgenic potatoes [26,27]. The amount of VP6 was 0.01% of soluble tuber proteins in the first case and 0.02% and 0.06% in tubers and leaves in the second case. Oral immunization with tuber tissues generated measurable titers of both anti-VP6 IgG in serum and IgA in intestine. This proof of concept is insufficient to conclude that this method may contribute to a vaccine.

A fragment of S protein from SARS virus (severe acute respiratory syndrome) was found in transgenic tomato and nicotine-free tobacco. Oral administration of transgenic tomatoes to mice induced synthesis of IgA antibodies suggesting that mucosal immune response was triggered after oral administration. Parenteral administration of transgenic tobacco to mice was followed by the presence of IgG antibodies in serum [28].

The protein G of the rabies virus was obtained in tobacco at the concentration of 0.38% of soluble proteins. Intraperitoneal injection of tobacco extract in mice in the presence of complete Freund adjuvant induced a total protection against the virus [29].

To produce anti-hepatitis B vaccine at a lower price, the antigen was produced in transgenic potatoes. The viral protein was directed to the endoplasmic reticulum by adding to the cDNA a signal peptide and the KDEL signal. Retention of the antigen in the reticulum was observed. Oral immunization of mice in the presence of cholera toxin induced the secretion of a high-antibody titer, which was still increased by boosting with parenteral administration of the potato extract [30,31]. The retention of the antigen in the reticulum may have played the role of a bioencapsulation and favored the immune response.

The synthesis in transgenic rice of epitopes known to induce a tolerance toward Japanese cedar antigen was achieved. The rice extract given orally to mice inhibited Th2-mediated IgE responses to the antigen [32,33].

Recently, a system called magnifection was shown to allow the rapid production (within two weeks) of gram of functional antibodies in plants [34]. This system involves the transient high-level co-expression of the transgenes (for example immunoglobulin heavy- and light-chains) through the use of plant viruses vectors delivered by *Agrobacterium* to the plant body.

Although encouraging, these results cannot predict when or if recombinant vaccines prepared from transgenic plants will be able to reach the market.

Transgenic animals

The first transgenic animals were generated in 1980 and the idea of using these animals as the source of recombinant proteins was proposed two years later when the giant mice having high concentration of growth hormone were obtained. In 1985, it was shown that the DNA microinjection used to generate transgenic mice could be extrapolated to rabbits, sheep and pigs. The use of farm transgenic animals to produce recombinant proteins appeared realistic and the choice of milk as the vehicle was made in 1986. One year later, two proteins were produced in the milk of transgenic mice. This proof of concept was followed by the industrial development of the method. Only in 2006 one protein, human antithrombin III, has been approved by the European agency EMEA to be on the market. In the mean time a large part of the technical obstacle has been crossed. These problems are (i) the establishment of transgenic lines (ii) the secretion of the foreign proteins at a high level (iii) the purification of the recombinant proteins and (iv) the validation of the proteins as therapeutics on a case by case basis.

Generation of 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 incubating sperm with DNA followed by *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 harboring normal and transformed cells, by transferring the foreign gene into somatic cells and by the generation of cloned animals using nuclear transfer. These methods have been described in recent reviews [35–37]. They are summarized in Fig. 1.

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. To increase the integration frequency, foreign genes can be introduced in integrating vectors such as transposons and lentiviral vectors. The latter proved highly efficient in ruminants and pigs. This technique is being adopted by experimenters even if these vectors have limited capacity to harbor foreign DNA and if the integration number is presently difficult to control.

DNA transfer via sperm has been developed mainly in pigs and mice. It may simplify transgenesis in some cases.

The utilization of cells as carrier for the foreign genes has been used in mice for almost twenty 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 practice essentially to inactivate genes (gene knockout).

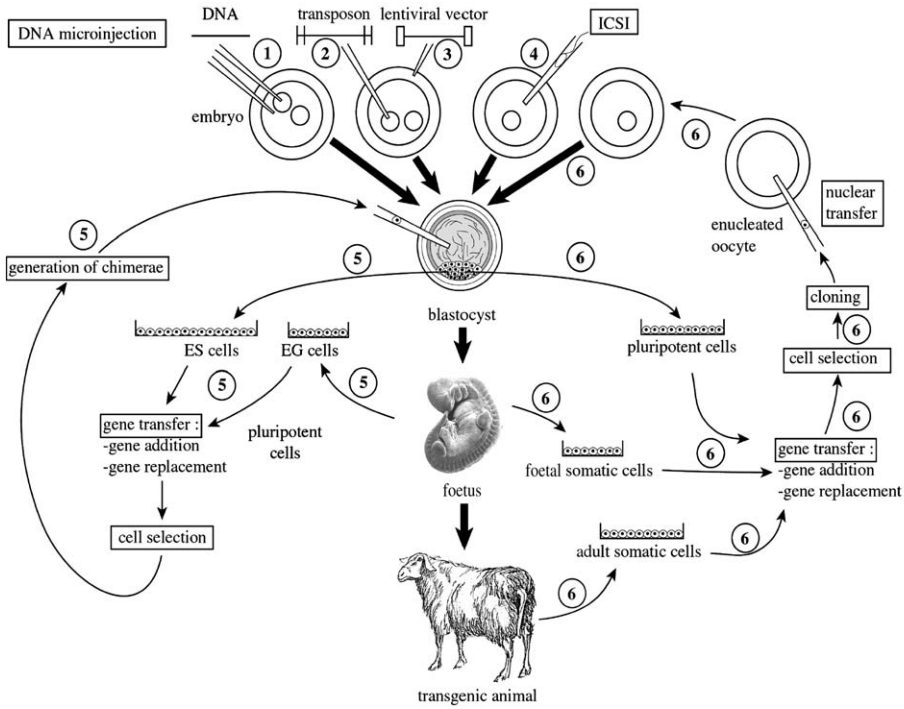

Fig. 1. Different methods to generate transgenic animals: (1) DNA transfer via direct microinjection into pronucleus or cytoplasm of embryo; (2) DNA transfer via a transposon: the gene of interest is introduced in the transposon, which is injected into a pronucleus; (3) DNA transfer via a lentiviral vector: the gene of interest is 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 cloning: the foreign gene is introduced into a somatic cell, the nucleus of which is introduced into the cytoplasm of an enucleated oocyte to generate a transgenic clone; (6) 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 fetal gonads). The pluripotent cells containing DNA are injected into an early embryo to generate chimeric animals harboring the foreign gene. Methods 4, 5 and 6 allow random gene addition and targeted gene integration via homologous recombination for gene addition or gene replacement including gene knockout and knockin.

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 and quails it was possible to establish pluripotent cell lines (EG cells) from the pluripotent cells which are present in fetal gonads (PGC: primordial germ cells). This made it

possible the generation of transgenic birds, which are candidates to produce recombinant proteins in egg white.

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.

The different sources of recombinant proteins

Milk is presently the most mature system to produce recombinant proteins from transgenic organisms [38]. Blood, milk [38], egg white [39,40], seminal plasma [41], urine and silk gland [42] and insect larvae hemolymph [43] are other theoretical 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 store high levels of recombinant proteins most of the time. Moreover, 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 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, and 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. 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. More impressively, pluripotent cell lines have been established in chicken and quail. These cells harboring foreign genes can be reintroduced in early embryos and participate to the development of chimeric transgenic animals [40]. 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. This antibody was functional but a reduced half-life due to the lack of sialic acid in the terminal end of the carbohydrate chain [39]. These experiments validate egg white as a source of foreign proteins including recombinant vaccines.

Table 2. Comparison of the different sources of recombinant proteins from transgenic animals.

Production systems (Points to consider)	Blood	Milk	Egg white	Seminal plasma	Urine	Silk gland	Drosophila larva
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 animal	+ +	+ + +	+ + + (+)	+ + + (+)	+ + + (+)	+ + (+)	+ + + +
Post translational modifications	+ + + + +	+ + + +	+ + + (+)	+ + + (+)	+ + + (+)	+ (+)	+ + (+)
Glycosylation	+ + + + (+)	+ + + +	+ + +	+ + + (+)	+ + + (+)	+ + (+)	+ +
Stability of product	+ + +	+ + + +	+ + + +	+ + + (+)	+ + + (+)	+ + + (+)	+ + + (+)
Purification	+ +	+ + +	+ + +	+ + (+)	+ + (+)	+ + +	+ + (+)
Contaminant pathogens	+ +	+ + +	+ + +	+ + +	+ +	+ + +	+ + + +
Dissemination in environment	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + +	+ + + + +
Intellectual property	+ + + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Products on the market	+	+ + + +	+ +	+	+	+ +	+

Note: The best parameters have the largest cross number.

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 [36,44].

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) [45]. This suggests that elements from long-DNA fragments will be used in future to construct compact vectors expressing transgene in a reliable manner.

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 [46]. One of the proteins was initially obtained in mouse milk [19]; 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 milk [47]. 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 slicing 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 [44]. 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 (see below).

A number of experiments have shown that the posttranslational modifications of recombinant proteins secreted in milk may be incomplete. This indicates that the cellular machinery of mammary gland is not sufficient to mature completely proteins when they are secreted at a high level. Experiments carried out several years ago showed that human protein C found in mouse milk was only partly cleaved. This maturation process was complete in transgenic mice expressing *furin gene* coding for a cleavage enzyme [48]. This pioneer work indicates that living fermentors such as mammary gland can be engineered to perform the posttranslational modifications of recombinant proteins.

Vaccine adjuvants, formulation and delivery

Identifying and producing vaccinating proteins can be a long and difficult task. But once it is done, other important challenges need to be achieved.

Choosing a suitable adjuvant to enhance the immune response against vaccine antigens together with choosing the right way to deliver the vaccine in recipients are critical for its efficiency. Most of the recombinant proteins or subunit vaccines are poorly immunogenic by themselves compared to whole killed or live-attenuated pathogens. They lack important features commonly present in pathogens like lipopolysaccharides (LPS) or unmethylated CpG-containing-DNA that are able to activate the innate immune system and shape the adaptive immune response. For toxicity reasons, whole killed or attenuated pathogens cannot be used in many cases. Adjuvants are then needed to increase the immunogenicity of the subunit vaccines. The common role of adjuvants is to enhance the immune response to weak antigens, and they also are implicated in the orientation of the response to a defined type: cellular or humoral, Th1- or Th2-biased response. The field of adjuvant research is very active and several new candidates are being developed and tested in animals and humans. In addition, the choice of formulation and vaccine delivery is crucial to induce an appropriate protective immune response (local or systemic). It should also be easy to handle and in the best case be needle-free and non-invasive to avoid pain and requirement for sterile material and trained medical workers (this is especially important for vaccines targeting developing countries). The following section summarizes the recent advances in these fields.

Vaccine adjuvants

Aluminum

Despite extensive evaluation of several candidates over the past few years, the aluminum-based mineral salts (also called alum) are the only adjuvants approved by the US Food and Drug Administration (FDA) for human use. Alum is well tolerated and presents a good safety record. However, it is a relatively weak adjuvant for antibody induction against recombinant vaccines. It induces mainly a Th2 immune response and is not efficient for activation of cellular immunity (Th1) [49]. Thus alum adjuvant is suitable when antibody-based protective immunity is required (for example induction of neutralizing antibodies), but alum lacks the ability to induce mucosal IgA. This can impede efficiency of several vaccines where a strong mucosal immunity is needed to prevent pathogen entry and replication into host. This is for example the case for rotaviruses that replicate in the intestine causing severe gastroenteritis, and for which intestinal IgA were shown to protect against disease [50]. Other limitations of alum adjuvants are increased IgE production, allergenicity and neurotoxicity [49,51,52]. Alum also cannot be effective in some vaccine formulations [49]. Despite its extensive use for many years, alum mechanism of action is not completely understood. Adsorption of antigens onto alum results in the formation of a depot at the site of injection. The particulate structure of the alum/antigen complex may

facilitate uptake by antigen presenting cells and alum could activate complement and macrophages [49]. The saponin Quil A, derived from the bark of a Chilean tree, *Quallaja saponaria*, or purified extracts none as QS-21 have been evaluated as alternatives to alum for cell-mediated responses activation. The observed toxicity (local reactions, hemolysis) associated with these adjuvants renders their use in humans limited to life threatening diseases like cancer or HIV infection [53].

ISCOMS

Immunostimulatory complexes (ISCOMS) are adjuvants composed of hydrophobically associated cholesterol, phospholipids and quillaja saponins that form a stable cage-like structure in which the antigens can be enclosed [54]. ISCOM-based vaccines are able to induce strong antibody and cellular immune response. It has been shown with a number of different antigens in several animal models including non-human primates (reviewed in [55]). In mouse, ISCOM-based vaccines were shown to be potent inducers of Th1 immune responses, contrarily to aluminum-based vaccines. In non-human primates, strong long-lasting CD4+ and CD8+ responses were observed following immunization with the core protein of the hepatitis C virus (HCV) complexed to the ISCOMATRIX (a preformed ISCOM preparation) in addition to humoral responses [56]. The mechanism of action of ISCOM is not fully understood. It is believed that because of their particulate structure their uptake by antigen-presenting cells is more efficient. The saponin component also has potent adjuvanticity (see above), and it has been shown that ISCOM activate the innate immune system through an IL-12-dependent mechanism [57].

In humans, a number of clinical studies were conducted with different vaccine-based ISCOM (reviewed in [55]). Antibody and/or cellular responses were induced in most of the recipients, and faster antibody responses of higher intensity were observed in people vaccinated with an influenza/ISCOM-based vaccine [58]. ISCOM-based vaccines have been administered to several recipients and showed to be safe with low reactogenicity. Common adverse events were reaction at the site of injection and myalgia of mild intensity and of short duration. ISCOMS appear to be interesting candidates for human use. In particular, the ISCOMATRIX adjuvant has been well characterized and appears to be stable and easy to handle [55]. Finally, ISCOMS benefit from robust and reproducible manufacturing procedures that can be scaled up for industrial production.

CpG Oligodeoxynucleotides

CpG dinucleotides-containing oligodeoxynucleotides (CpG ODN) possess adjuvant activity and were shown to be efficient in different vaccine formulations in animals and humans. CpG ODN are currently evaluated in clinical

trials in humans in the field of infectious diseases, cancer treatment and asthma/allergy.

CpG ODN are very potent at orienting the immune system toward a Th1-biased response and can therefore be of primary interest for vaccines where a Th1-biased reaction is needed to achieve protective immunity. Furthermore, CpG ODN are able to stimulate mucosal immunity. CpG ODN even showed greater efficiency when administered with other adjuvants like alum or in formulation like lipid emulsions or nanoparticules, which can be necessary to induce a protective response when the antigen is weak. Studies in mice showed that CpG ODN can boost both humoral and cell-mediated immune responses against a broad range of proteins or vaccines. For example inclusion of CpG ODN in a SARS coronavirus subunit vaccine composed of a fragment of the spike protein in alum, increased IgG2a titers (representative of a Th1-like response) and interferon-γ (INF-γ) secreting cells [59]. The same observations were reported with several other subunit vaccines against different pathogens (hepatitis A and B virus [60–63], herpes virus [64] and rotavirus [65]).

The exact mechanism of action of CpG ODN is not precisely elucidated, but it is known that CpG ODN act mainly through activation in the innate immunity. The innate compartment of the immune system evolved to recognize general structures commonly found on a broad range of pathogens. These include the structure of the bacterial and of many viruses DNA, which unlike vertebrate genomic DNA, contain a high proportion of unmethylated CpG dinucleotides. Bacterial and other pathogens DNA can be recognized directly by the innate immune system through the interaction with the Toll-like receptor 9 (TLR9) which, in humans, is present in B-cells and plasmacytoid dendritic cells (pDC). In mice, TLR9 is also expressed in monocytes and in myeloid dendritic cells. The effect of TLR9 activation is the induction of a proinflammatory (IL-1, IL-6, IL-18, TNF-α) and a Th1-biased cellular and humoral immune response (reviewed in [66]). CpG ODN mimic the presence of bacterial DNA and primarily trigger activation of the innate immune system. CpG ODN are rapidly internalized by immune cells where they are bound by TLR9. The TLR9 activation caused by CpG ODN administration can enhance antigen-specific humoral or cellular immune response against co-administered antigens. Contrarily to humans, TLR9 in mice is not only expressed in B-cells and pDC but also in monocytes and myeloid dendritic cells. This observation renders difficult the extrapolation of the encouraging results obtained in mice to humans because these cells may play important roles in vaccination efficiency. However, data obtained from clinical trials in humans showed efficacy of CpG ODN adjuvants. Co-administration of CpG ODN with hepatitis B vaccine (Engerix-B) to healthy adult volunteers, either alone or in combination with alum, resulted in increased IgG titers compared to the control group receiving Engerix-B alone [67]. Furthermore, hepatitis B-specific surface antigen antibodies appeared

earlier when immunizations were carried out with CpG [67,68]. Inclusion of CpG adjuvant also increased the proportion of antigen-specific high-avidity antibodies [69]. An accelerated antibody response combined with increased magnitude and avidity was also observed when healthy volunteers were immunized with the anthrax vaccine adsorbed (AVA) when CpG ODN were included [70]. So far, treatments with CpG ODN were well tolerated, and the adverse effects observed among recipients only included pain and erythema at the site of injection, and mild to moderate flu-like symptoms that did not last and did not impede daily life activities [66]. Taken together, these results underline the potential of CpG ODN adjuvants both in animals and humans. Although more studies are needed and important points remain to be addressed (like the possibility to induce autoimmune diseases in recipients [66]), CpG ODN appear to be promising tools. Interestingly, CpG ODN could benefit from the large-scale – good manufacturing practices – industrial production technologies developed during the past few years for the antisens drug development (which have been approved by the US FDA) [71].

Bacterial toxins

Two bacterial toxins were identified as powerful mucosal adjuvants: the cholera toxin (CT) and the related heat labile enterotoxin (LT) produced by *Escherichia coli*. Both toxins consist of a catalytic subunit A (CTA or LTA) associated with a pentameric cell-binding B subunit. CTA and LTA subunits possess an ADP-ribosyl transferase enzymatic activity resulting in permanent adenylate cyclase activation in targeted cells, increased cAMP production and hypersecretion of salt and water into the bowel [72,73]. The CTB and LTB parts allow the binding to cell surface through their association with GM1 gangliosides, which result in the internalization of the toxic A subunit. These toxins are internalized by polarized epithelial cells and it is thought that co-administered antigens may follow the same route. These toxins induce strong systemic and mucosal immune responses and increased responses against co-administered antigens. Vaccinations with CT and LT as adjuvants produced Th1 and Th2 responses. They showed excellent efficacy in inducing protective immunity when administered via the nasal and rectal route and to a lesser extent via the oral route [65,74,75]. However, the strong toxicity of these molecules precludes their use in humans (ingestion of 5 µg of CT in human would result in the induction of a 5-l watery diarrhea). Several less toxic derivatives that retain adjuvanticity were generated by site-directed mutagenesis. These mutants comprise the LT K63, LT R72 and LT R192G forms of LT. The LT K63 and LT R72 bear single amino acid substitutions in the catalytic A subunit. Both mutants differ in the residual enzymatic activity, which positively correlates with their adjuvanticity. LT R192G contains a single amino acid substitution in a protease sensitive portion of the catalytic A subunit [76]. This mutant with reduced enterotoxicity shows great adjuvanticity when delivered mucosally either by the nasal, oral or rectal

route [10,74,75,77]. Interestingly, intranasal delivery of antigens in combination with CT, LT or its derivatives induces mucosal responses even at distant sites. When rotavirus virus-like particles (VLP) were administered intranasally, strong mucosal and systemic responses were induced together with intestinal IgA production [74,78].

Encouraging results were obtained using a strategy consisting of fusing the enzymatically active A subunit of CT to a B-cell-targeting moiety (D) of *Staphylococcus aureus* protein A. This adjuvant, called CTA1-DD, is far less toxic than the intact CT and contrarily to CT produces a balanced Th1/Th2 response [79]. It was also shown to give comparable protection against rotavirus infection when compared with LT R192G or CpG [80]. Furthermore, in mice receiving a nasal administration of the universal influenza vaccine M2e-HBc combined with CTA1-DD, a complete protection against a lethal infection was observed, together with a reduction of morbidity, in the context of a Th1-type immunity [81].

The B subunits of CT (CTB) and LT (LTB) could also serve as mucosal adjuvants. CTB and LTB contain adjuvant activity when administered by the nasal route. Mice vaccinated with an influenza virus vaccine with LTB showed higher systemic and mucosal antibody responses than mice receiving the vaccine alone [82]. Interestingly, recent study showed that the fusion of CTB to CpG ODN (CpG-CTB) resulted in an enhancement of the immunostimulatory effect of CpG ODN, with a more potent stimulatory effect of pro-inflammatory cytokine and chemokine responses in human and mouse splenocytes [83].

It is worth being mentioned that in addition to CT and LT (and their derivatives), a third toxin called Zonula occludens toxin (Zot) showing adjuvant activity has been identified [84]. Zot is a single polypeptide chain encoded by the filamentous bacteriophage CTXΦ and expressed by *Vibrio cholerae*. Zot binds a receptor on intestinal epithelial cells and increases mucosal permeability by acting on the structure of epithelial tight junctions. This phenomenon is believed to allow penetration of antigens into the tissue where they can interact with immune cells. It is also possible that Zot does not only act as a co-delivery system for antigens but may also have immunomodulatory properties by activating antigen-presenting cells. Interestingly, the effect that Zot exerts on tight junctions is reversible and does not cause tissue damage.

Several other bacterial toxins having adjuvant activity have also been identified and studied by different groups (reviewed by [85]) but their mechanisms of action still need to be clarified.

Monophosphoryl lipid A

LPS is a major constituent of the Gram-negative bacteria. LPS are considered to be endotoxins and induce strong pro-inflammatory reaction. LPS have strong adjuvant properties but excess production of pro-inflammatory

cytokines linked to repeated administration of LPS leads to endotoxin shock characterized by inflammation, profound hypotension and organ failure [86]. Because of this elevated toxicity, LPS cannot be used in humans. An LPS-mimetic compound called monophosphoryl lipid A (MPL), exhibiting adjuvanticity and low toxicity has been generated. MPL, like LPS act by interacting with Toll-like receptor 4 (TLR4) on antigen presenting cells resulting in the release of pro-inflammatory cytokines like TNF-α, IL-6, IL-10 and INF-γ, which will ultimately enhance the adaptive immune response (humoral and cellular). In preclinical studies, MPL has been shown to generate Th1-type immune response to antigens [87]. The molecular mechanisms resulting in the lower toxicity of MPL versus LPS are not clear; but recently, Okemoto and collegues [88] showed that contrarily to LPS, MPL activation of macrophages does not result in the release of IL-1β (a pleiotropic pro-inflammatory cytokine involved in the endotoxin shock [89]), nor the activation of caspase-1 (also involved in the induction of endotoxin shock).

MPL adjuvant, or synthetic analogue components (RC-529) formulations have often been used in clinical trials in combination with alum and QS21 [90,91]. The adjuvant designated AS04 composed of an association of alum salts with MPL has been shown to increase antibody responses against a papillomavirus subunit vaccine in humans [92]. This formulation also led to a long-lasting immunity to the vaccine (at least 3.5 years), and an increase of memory B-cells when compared to alum salt only formulations [91–93].

More than 12,000 subjects received MPL-formulated vaccines for herpes virus [94], hepatitis B virus (HBV) [95], papillomavirus [91–93]) and extensive clinical data are available for this adjuvant. In addition, MPL is presently approved in Europe for use in combination with allergy vaccines [96].

Formulation and delivery

At present different strategies are developed to optimize antigen stability and bioavailability in the host. Most of them rely on the entrapment of the antigens into polymer-based particles in the case of microspheres, or into lipid-based membranous vesicles in the case of liposomes. Microspheres are composed of biodegradable polymers, mainly polylactide (PLA) or poly(DL-lactide-co-glycolide) (PLGA). The polymers degrade *in vivo* to form non-toxic lactic and glycolic acids. Administered microspheres allow controlled antigen release: it may form a depot at the site of injection, allowing the slow release of the antigen for extended periods. It can thus minimize the number of doses required for immunization. Liposomes are bilayered vesicles composed of phospholipids and other sterols surrounding an aqueous center where the antigens can be entrapped. Liposomes allow for prolonged release times of antigens.

Microspheres and liposomes present several advantages like increased resistance to degradation of the antigens in the gastro-intestinal tract, controlled

antigen release minimizing the number of doses, particle uptake by immune cells, and ability to induce cytotoxic T-lymphocytes responses.

Adjuvants can also be entrapped in the particles to enhance immune responses against delivered antigens and one may include this type of formulation to increase vaccine efficiency.

The choice of site of vaccine delivery is particularly important. Usually, vaccines are delivered by the parenteral route (either by subcutaneous or intra-muscular injection). This immunization regimen often leads to induction of systemic immune responses and circulating antibodies but a poor mucosal immunity. This type of immunization is suitable when serum neutralizing antibody induction is needed to prevent pathogens to replicate or to reach their target cells in the host. This is for example the case for HBV vaccine delivered parenterally by injection, where neutralizing antibodies mediate protection. However, it is generally considered that in order to produce protective immunity it is best to vaccinate via the natural route of infection of pathogens. Most pathogens infect hosts via the mucosal epithelium that represents 90% of the body surface: respiratory tract (respiratory syncitial virus), gastrointestinal (enterotoxigenic *E. coli*, rotavirus), vaginal (papillomavirus, HIV) or rectal mucosa (HIV). At present, a great challenge for vaccination is to stimulate a strong mucosal immunity to prevent pathogen entry into host.

The easiest way to administer a vaccine is through oral delivery. However some limitations do exist. These include degradation of the antigens in the harsh gastrointestinal environment (acidity, bile salts and pancreatic secretions), and induction of oral tolerance to the antigens. One major feature of the mucosa-associated lymphoid tissue is the homing of circulating activated B-cells at distant effector sites from the site of induction [97]. This feature allows, for example, for the production of intestinal or vaginal IgA after intranasal immunization [74,78,98]. Intranasal immunization has been widely used in mouse and is recognized as a very potent induction site for protective immunity in a number of cases. However, this immunization strategy may not be well adapted for humans. Indeed, the nasal epithelium is in close contact with the olfactory bulb and the central nervous system (CNS). The close vicinity of these structures renders the intranasal delivery of bacterial toxin-based adjuvants a dangerous approach for mass vaccination since toxins and co-administered antigens could penetrate the CNS [99,100], (and see also NIAID July 9, 2001 meeting summary at http://www3.niaid.nih.gov/research/topics/enteric/meetings.htm).

Alternative immunization sites could be used to overcome this problem, for example the vaginal or the intrarectal delivery of antigens. The latter has recently been shown to be efficient for vaccination against the enteric pathogen rotavirus [65,75,119]. Recently the transcutaneous route has been shown to stimulate mucosal responses [101,102].

Mucosal immunization offers a number of important advantages including non-invasive (needle-free) easy administration (intranasal, oral or intra-rectal/vaginal) of vaccines. It can also be conceived that mucosal vaccines could be self-delivered without the use of sterile equipment (syringes) and trained medical workers, which may be a real advantage for vaccination in developing countries.

The use of virus-like particles as foreign antigen carrier systems

Virus-like particles (VLPs) are non-infectious, non-replicating analogues of pathogenic viruses. VLPs are formed *in vitro* by the self-assembly of viral capsid proteins. A number of VLPs from different viruses have been described to date like papillomaviruses, rotaviruses, Norwalk viruses, hepatitis B and E viruses, and parvoviruses to name a few. Some of them are used as vaccines (papillomavirus and hepatitis B virus). The repetitive structure of the arranged capside proteins in VLPs (as in native virus particles) favors activation of B-cells and antibody production [103–105]. Some VLPs can also efficiently activate cytotoxic T-cell responses in the absence of infection and intracellular replication [106–109]. VLPs are attractive tools to present foreign epitopes to the immune system. Some chimeric VLPs have been described for hepatitis B virus [110–112], hepatitis E virus [113], rotavirus [75,114], and parvovirus [115] among others.

HBV VLPs consisting of the fusion of HCV epitopes to the HBV core protein have been used in mice immunization. Both anti HBV and anti-HCV epitope responses were observed [116]. HBV VLPs were also used to carry large polypeptides like GFP (Kratz) or the ectodomain of the outer surface protein A (OspA) from *Borrelia burgdorferi*, the causative agent of Lyme disease. HBV/OspA hybrid VLPs immunization could protect mice against challenge with *Borrelia burgdorferi* [111]. In another study, inclusion of a B-cell epitope tag into hepatitis E virus (HEV) VLPs induced specific antibody responses against both the VLP and the B-cell epitope. Sedlik *et al.* showed that porcine parvovirus VP2 capsid protein carrying a CD8+ T-cell epitope from the lymphocytic choriomeningitis virus nucleoprotein retain its capacity to assemble into VLPs. Immunization of mice with these hybrid VLPs resulted in strong cytotoxic T-lymphocytes responses against the CD8+ epitope and protected mice against a lethal challenge with the lymphocytic choriomeningitis virus [117]. It is important to mention that in some of these experiments, vaccinations were successfully conducted without the use of adjuvant, underlining the immunostimulatory effect of VLPs on the foreign epitopes.

Thus, combining the presentation of antigens in an immunogenic repetitive structure (like VLPs) with the use of powerful adjuvants should result in

increased efficiency of immune system activation against otherwise poorly immunogenic soluble antigens. This approach could be a nice strategy for the elaboration of combined multivalent vaccines, presenting the advantage of vaccinating against both the carrier (VLP) and the introduced epitopes.

Conclusion

Recombinant vaccines have well-identified theoretical advantages over conventional live vaccines. Yet, recombinant vaccines remain scarce. Vaccine against hepatitis B is one of them. A vaccine against RHDV (rabbit hemorrhagic disease virus) is also used to vaccinate rabbits. An efficient vaccine against poultry Newcastle disease has been prepared in transgenic plant and approved but not put on the market so far [118]. No more than four plant-derived recombinant vaccines have reached clinical development [20]. The vaccine against malaria produced in goat milk is under clinical study whereas the vaccine anti-rotavirus produced in rabbit milk is under preclinical study.

Identifying a relevant antigen capable of becoming efficient is the result of a relatively long-term study. Yet, such antigens have been characterized and could be prepared. Validating a mode of administration and determining the valuable adjuvant require specific studies on animal models. Such models are not always relevant. Mice are most frequently used species for this purpose. These animals often give only limited information. Infection by rotavirus is not followed by diarrheas. Other species not so easily used such as pigs or monkeys are then required.

The different systems for the production of recombinant vaccines have been markedly improved during the last decades. Additional progress is expected but the state of the art in this field is no more a hurdle. About 475,000 l of animal cell fermentors are available and could contribute more extensively to the production of recombinant vaccines. Production in the yeast *Pichia pastoris* is getting more and more efficient and reliable.

Transgenic plants are still facing important problems. The production level remains often low. The glycosylation problem is not expected to be solved in a near future and the uncontrolled dissemination of antigens may not find solution other than the implementation of confined areas. The only marketed proteins produced in plants are enzymes for industrial applications. Two proteins only are under clinical study, dog lipase for patients suffering from cystic fibrosis and a monoclonal antibody directed against *Streptococcus mutans* and preventing tooth decay [118] and none of them has been approved yet.

The production in milk is the most mature system and is available to produce reliable recombinant vaccines at a low cost. The spectacular advance for generating transgenic chicken and for expressing monoclonal antibodies in egg white (3 mg per egg) suggest that this system will soon contribute to boost the production of recombinant proteins in transgenic animals. The fact

that Atryn (human antithrombin III) produced in goat milk has been approved by EMEA contributes companies and investors to be more confident in transgenesis to produce biopharmaceuticals.

Technical gaps cannot therefore account for the slow development of recombinant vaccines. Economical reasons are the major limitation in this field. It is important to note that the vaccines all included are at the eighth position in the classification of the biopharmaceuticals [118]. The vaccine business is in the hand of five major companies, which focus their effort on influenza and childhood diseases. The demand of vaccines including recombinant vaccines remains relatively modest as these biopharmaceuticals require relatively a high investment in research. The amount of product to be prepared is relatively low. Vaccination is a preventive operation. This implies a very low level of risk. The price of vaccines is expected to be low, especially when they are to be used in developing countries. The recent rotavirus vaccines are being used in several countries despite the risk of intussusceptions as the risk due to the vaccination is significantly lower than the risk of infection.

Recombinant vaccines appear to be a better tool than conventional vaccines in a number of cases. Their development might become more rapid during the coming decade as, in an increasing number of countries, governments recommend or require systematic vaccination for entry of children into schools. Recent world epidemics such as SARS or influenza incline government to support the development of new vaccines. The threat of bioterrorism is going in the same direction.

The development of recombinant vaccines thus depends on political decision but technical improvements are still needed to improve the efficiency of recombinant vaccines and to lower their production cost.

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