TRANSGENIC DRUGS- AN OVERVIEW

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ABSTRACT

In the present review transgenic drugs, i.e plant origin and animal origin, transgenic drugs are extensively used to study in vivo gene function as well as to model human diseases. The technology for producing transgenic drugs exists for a variety of animal and plant species. Embryonic stem cell technology has been most often used to produce null mutants (gene knockouts) but may also be used to introduce subtle genetic modifications down to the level of making single nucleotide changes in endogenous genes. Methods are also available for inducing conditional gene knockouts as well as inducible control of transgene expression. Here, we review the main strategies for introducing genetic modifications into the animals and plants species. We also review a number of recent methodologies for the production of transgenic drugs including retrovirusmediated gene transfer, RNAi-mediated gene knockdown and somatic cell mutagenesis combined with nuclear transfer, methods that may be more broadly applicable to species where both pronuclear injection and ES cell technology have proven less practical. Genetic engineering of plants represents the next stage of evolution in our continuing efforts to improve plants used for the production of food and animal feed. Agricultural biotechnology is a powerful technique offering great potential for agricultural sustainability and safe production of foods with increased nutritive value, improved flavour, prolonged freshness, and even disease-fighting properties, but it is not without controversy. The potential for the transgenic crops to alleviate human hunger, and the controversies which are invariably based on visions of the new technology from widely different ethical perspective which have divided both the public and the scientific communities are discussed. But, critical to its adoption and acceptance is by providing choice and accurate information to consumers from scientists, policy makers, industry and the press. The present review addresses the prospects of the technology, and the polemics concerning its adoption.

Keywords: Conditional gene inactivation, Gene targeting, Inducible transgene expression, Pronuclear injection, Transgenic animal, transgene, pharmaceutical crops, risks, prospects and controversies.

INTRODUCTION

Transgenic drugs are from animal as well as from plant origin. Some definition are given below to understand the process easily;

Biotechnology

Biotechnology is the use of living systems and organisms to develop or make products, or "any technological application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use". Depending on the tools and applications, it often overlaps with the (related) fields of bioengineering, biomedical engineering, biomanufacturing, etc. The wide concept of "biotech" or "biotechnology" encompasses a wide range of procedures for modifying living organisms according to human purposes, going back todomestication of animals, cultivation of the plants, and

"improvements" to these through breeding programs that employ artificial selection and hybridization. Modern usage also includes genetic engineering as well as cell and tissue culture technologies. The American Chemical Society defines biotechnology as the application of biological organisms, systems, or processes by various industries to learning about the science of life and the improvement of the value of materials and organisms such as pharmaceuticals, crops, and livestock.[3] As per European Federation of Biotechnology, Biotechnology is the integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services.[4] Biotechnology also writes on the pure biological sciences (animal cell culture, biochemistry, cell biology, embryology, genetics, microbiology, andmolecular biology). In many instances, it is also dependent on knowledge and methods from outside the sphere of biology including:

- bioinformatics, a new brand of computer science
- bioprocess engineering
- biorobotics
- chemical engineering

Transgenesis

Transgenesis is the process of introducing an exogenous gene, called a transgene, into a living organism so that the organism will exhibit a new property and transmit that property to its offspring. Transgenesis can be facilitated by liposomes, enzymes, plasmid vectors, viral vectors, pronuclear injection, protoplast fusion, and ballistic DNA injection. Transgenesis can occur in nature. Transgenic organisms are able to express foreign genes because the genetic code is similar for all organisms. This means that a specific DNA sequence will code for the same protein in all organisms. Due to this similarity in protein sequence, scientists can cut DNA at these common protein points and add other genes. An example of this is the "super mice" of the 1980s. These mice were able to produce the human protein tPA to treat blood clots.

Transgenesis can be done in animals as well as in plants so to understand we have to go through both origins.

TRANSGENIC ANIMALS AND THEIR APPLICATION IN MEDICINE

With the advent of transgenic technology and its application in many laboratories around the world, there is an increase in the generation and use of genetically modified animals in biomedical, pharmaceutical research and safety testing. This development has been additionally accelerated by the decoding the genome of man, mouse and rat.[1] Pharmaceutical companies are faced with the challenge that about 10% of compounds tested in clinical trials make to the market and, out of these, a minority will generate significant profit. The costof identifying new drug is immense, about United States (US) $ 800 million and 80% of this cost is spent in clinical trials and development. Transgenic technology has potential to influence the attrition rate in pharmaceutical research by increasing the quality of both targets and compounds. [2] To date most of the medicines are synthetically produced and will continue in future. However, the challenge for the pharmaceutical industry is the development of 'Biotech medicines' which include therapeutic proteins such as enzymes and antibodies. [3] The global market for recombinant proteins from domestic animals is expected to exceed US$1 billion in 2008 and reach US$18. 6 billion in 2013.[4] The two major animal systems of production are pharmaceutical proteins in milk and egg white from transgenic animals. [1] Since the first transgenic mice were generated in 1982, transgenic animal models have been

used extensively to investigate biomedical important mechanisms underlying a variety of diseases, to develop and evaluate new therapies. [5] Thus transgenic animals have the ability to fulfill the needs of the pharmaceutical industry and in coming years they are looked as potential contributors to the drugs and research in medicine.

Making of Transgenic Animals

There are three types of laboratory animal models mentioned in the literature. They are spontaneous, induced and transgenic. Spontaneous models shape up as a result of naturally occurring mutations. Induced models are produced by a laboratory procedure like administration of a drug or chemicals, feeding of special diets or surgical procedure. The third category includes transgenic models.[6] Transgenic refers to insertion of cDNA (complimentary deoxyribonucleic acid) made from specific mRNA (messenger ribonucleic acid) into cells.[7] A transgenic animal is defined as an animal which is altered by the introduction of recombinant DNA through human intervention. [8] Following sequence is generally adopted for the development of transgenic animals irrespective of species: [1, 9]

- Identification and construction of the foreign gene and any promoter sequences
- Introduction of DNA directly into the pronucleus of a single fertilized egg by various methods
- Implantation of these engineered cells into surrogate mothers
- Bringing the developing embryo to term, proving that the foreign DNA has been stably and heritably incorporated into the DNA of at least some of the newborn offspring.
- Demonstrating that the gene is regulated well enough to function in its new environment.

The foreign DNA can be inserted into the pronucleus or cytoplasm of the embryo using microinjections or transposon. Other methods of DNA transfer are by lentivirus, sperms, pluripotent cells and cloning. The last three methods allow random gene addition and targeted gene integration via homologous recombination or gene replacement thus causing mutation. [1,6] Targeted mutation refers to a process whereby a specific gene (removal of a gene or part of a gene) is made nonfunctional (knocked-out) or less frequently made functional (knocked-in). [4, 7] A transgenic organism carrying more than one transgenes is known as multiple transgenic. 4These methods do not create new species, but only offer tools for producing new strains of animals that carry novel genetic information. [10]

Transgenic Animal models of various diseases

An animal model is a living, non-human animal used for research and investigation of human disease, for the purpose of better understanding the disease without the added risk of causing harm to a human being during the entire drug discovery and development process. Transgenic animal models are created by the insertion of a particular human DNA into fertilized oocytes which are then allowed to develop to term by implantation into the oviducts of pseudo pregnant females.[6] There are different models of transgenic animals for various diseases.

A. Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS): Tg26 HIVAN Mouse Model was the first transgenic model developed in 1991 for HIV. Since than many models have developed, Rosenstiel et al gives summary of 32 transgenic murine HIVAN models developed. [11] These transgenic animals can express HIV-1 proteins; develop symptoms and immune deficiencies similar to the manifestations of AIDS in humans. [12]

Other models are AIDS Mouse and Smart Mouse. [6]

B. Alzheimer's disease: No animal models existed for the disease before transgenic technology was employed. Immunization of Amyloid precursor protein A42 in transgenic mice showed that vaccination against Alzheimer's disease could have potential as a therapeutic approach.[2, 6] Different animal models like Alzheimer's mouse, amyloid pathology mouse models like PDAPP mice, Tg2576 mice, [13, 14] TAU transgenic mouse models like ALZ7 mice, 7TauTg mice and presenilin transgenic models like ApoE knockout are developed to study Alzheimer's disease. [13, 15]

C. Cardiovascular disease: Various transgenic animal models for gain and or loss of function of angiotensin, endothelin, natriuretic peptides, catechoalmines, Calcium binding-signaling, sodium channel transporters, and nitric oxide synthesis involved in cardiovascular regulation are used to study cardiovascular diseases.[16] Transgenic models of heart failure and hypertrophy like Gene overexpression of Calmodulin, Gene mutation of alpha cardiac myosin heavy chain and Knockout gene model of transforming growth factor are developed.[17] Mutation of the ApoE gene that is critical for uptake of chylomicrons and very lowdensity lipoprotein particles, results in a model that develops atherosclerotic lesions histologically similar to those found in humans.[2]

D. Diabetes Mellitus: Transgenic models are developed for studying the genes, and their role in peripheral insulin action. Models of insulin secretion such as glucokinase, islet amyloid polypeptide, and hepatic glucose production in type 2 diabetes are developed. [18]A transgenic mouse model that expressed Insulin Dependent Diabetes Mellitus by inserting a viral gene in the animal egg stage is also developed.[19] There areother models like beta receptor knockout mouse, uncoupling protein (UCP1) knockout mouse,acute and chronic models for the evaluation of antidiabetic agents. [18, 20, 21, 22]

E. Angiogenesis: Mouse models of angiogenesis, arterial stenosis, atherosclerosis, thrombosis, thrombolysis and bleeding addresses techniques to evaluate vascular development.[23] Inhibition of angiogenesis is currently one of the biggest opportunities for new cancer therapies. With the help of angiogenesis transgenic animal models inhibitors are identified which act on specific mechanisms of angiogenesis. [2]

F. Cancer diseases: Oncomouse was first transgenic animal to be patented. Its germ cells and somatic cells contain an activated human oncogene sequence introduced into the animal at an early embryonic stage to ensure that the oncogene is present in all the animal cells.[6] Mechanisms for tumor progression and metastasis via E-cadherin, and other adhesion molecules is possible by various transgenic knockout models. [7] Transgenic animal models are used in the assessment of mutagenicity, carcinogenicity and tools for understanding metabolic enzymes and receptors. [24] There are transgenic animal modelsfor mutagenicity assays approved by the World Health organization like LACItransgenic model (Big Blue® construct) and LACZ transgenic model (Muta™Mouse construct). [25]Variety of transgenic animals have been generated by different strategies in experimental immunotherapy of cancer, each aims to activate different immune system components. Some of them are transgenic rodent models expressing tumor associated antigens like MUC1 transgenic mice, Oncogene transgenic mice to study immunotherapeutic strategies, transgenic mice expressing immune effector cell molecules like Fc-receptor transgenic mice [5] The preclinical transgenic model of Matrix Metalloproteinase (MMP) inhibitors studies the bioactive products of MMP and their possible effects on cell physiology. [26] Animal models for Huntington's disease, skeletal muscle disease and other diseases are also developed.[27, 28] Disease models are needed in medicine so that one can discover the targets for drug development. Adding and deleting genes in these animalsprovide them new properties that make them useful for better understanding of disease or manufacturing a cure. It is not ethical or safe to perform the

initial tests in humans, so transgenic animals are used. As the testing of new vaccines and drugs must first be performed on animals, these disease models are indispensable. [6]

Products from Transgenic Animals

Most transgenic species are studied for research applications as well as potential commercial pharmaceutical productively. Here are some of the transgenic animals and their products in development.

Goats: Monoclonal Antibodies (MAbs) , Ig fusion proteins, tPA (tissue Plasminogen Activator), ATryn (recombinant human antithrombin III) is the first transgenic recombinant protein from transgenic animal approved by the United States Food and Drug Administration (USFDA) in January 2009. [9,29]

Chickens and Eggs: vaccines; interferons, cytokines; Human Serum Albumin (HSA), insulin, MAbs. [9]

Pigs: organs for xenotransplantation, human hemoglobin, human protein C. [9,30,31]

Cows: Factors VIII and IX, protein C, recombinant antithrombin III (rATIII), recombinant HSA, and human milk protein. [9]

Mice: expression of malaria protein for vaccine development; MAbs, ATIII, beta interferon; cystic fibrosis transmembrane regulator; Factor X, HSA, tPA, myelin basic protein; prolactin; fibrinogen and antineoplastic urinary protein. [9]

Rabbits: recombinant human C1 inhibitor, human erythropoietin, human alpha antitrypsin, human interleukin 2, tPA, alpha glucosidase, and human growth hormone. [9]

Sheep: sheep milk includes fibrinogen (major constituent, with thrombin and Factor XIII) human Factor VII, Factor IX, activated protein C and alpha-1-antitrypsin. [9,31]

Other Species: Frogs, nematodes, and marine invertebrates have been used to study various promoter elements and gene transfer technology. [9] Currently in most pharmaceutical companies, relatively large numbers of targets are validated to varying extents and progressed to the high throughput screening stage. The drugs acting on these targets are then used in clinical trials where the attrition rate is generally high, this makes it extremely costly. It is believed that the greater use of transgenic models could reduce the required throughput for achieving success and thereby significant impact on costs.[10]

Drugs from Transgenic Animals and Other Applications:

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 is now used by most of the diabetic patients. This success was limited as bacteria cannot synthesize complex proteins such as monoclonal antibodies or coagulation blood factors that must be matured by post-translational modifications to be active or stable in vivo. These can be fully achieved only in mammalian cells which can be cultured in fermenters or used in living animals. Several transgenic animals can produce recombinant proteins but presently two systems started being implemented. The first is milk of transgenic mammals and the second system are chicken egg white. A variety of recombinant proteins which includes antibodies, vaccines, blood factors, hormones, growth factors, cytokines, enzymes, milk proteins, collagen, fibrinogen and others are being developed in transgenic animals. [1,3,31]

The mammary gland is the preferred production site, because of the quantities of protein that can be produced in this organ and established methods for extraction and purification of these proteins. Products derived from the mammary gland of transgenic goats and sheep are ATIII, α-antitrypsin and tPA. ATIII is employed for the treatment of heparin-resistant patients

undergoing cardiopulmonary bypass. The enzyme α-glucosidase from the milk of transgenic rabbits has been successfully used for Pompe's disease. [4, 31] Blood, seminal plasma, urine, silk gland and insect larvae haemolymph are other theoretically possible systems. Blood most of the time cannot store high levels of recombinant proteins which are naturally unstable also biologically active proteins in blood may alter the health of the animals. Milk avoids essentially these problems and is presently the most mature systems to produce recombinant proteins from transgenic organisms. Now experiments validate egg white as a source of foreign proteins including recombinant vaccines. [1,31]

Blood replacement

The current production system for blood products is donated human blood, and this is limiting because of disease concerns, lack of qualified donors, and regulatory issues. Genetically engineered animals, such as cattle carrying human antibody genes which are able to produce human polyclonal antibodies, have the potential to provide a steady supply of polyclonal antibodies for treatment of various infectious and medical conditions like organ transplant rejection, cancer, and autoimmune diseases and other diseases. [32] There are currently at least 33 different drugs in clinical testing including several in pivotal trials that contain variable regions from transgenic mice encoded by human sequences. Also there are 17 therapeutic MAbs approved by the USFDA which are in different phases of drug development. [33] Functional human haemoglobin has been produced in transgenic swine. The transgenic protein purified from the porcine blood showed oxygenbinding characteristics similar to natural human haemoglobin but only a small proportion of porcine red blood cells contained human form of haemoglobin. [4]

Xenotransplantation of porcine organs to human patients

Today more than 250,000 people are alive only because of the successful transplantation of an appropriate human organ (allotransplantation). However, progress in organ transplantation technology has led to an acute shortage of appropriate organs, and cadaveric or live organ donation does not meet the demand. To close the growing gap between demand and availability of appropriate human organs, porcine xenografts from domesticated pigs are considered to be the best alternative.[4] Essential prerequisites for a successful xenotransplantation are: [4, 34]

1. Prevention of transmission of zoonoses
2. Compatibility of the donor organs in anatomy and physiology
3. Overcoming the immunological rejection of the transplanted organ.

The immunological hurdles are:

(a) Hyperacute rejection response (HAR) occurs within seconds or minutes.
(b) Acute vascular rejection occurs within days.
(c) Cellular rejection occurs within weeks after transplantation.
(d) Chronic rejection is a complex immunological process resulting in the rejection of the transplanted organ after several years. Due to demand and unavailability of appropriate organs, xenotransplantation is considered as the solution of choice. The pig seems to be the optimal donor animal because their organs have a similar size as human organs, porcine anatomy and physiology is same and maintenance is possible at high hygienic standards at relatively low costs. [34, 35] Two main strategies have been successfully explored for long-term suppression of HAR, the knockout of α-gal epitopes which are the antigenic structures on the surface of the porcine cells that cause HAR and synthesis of human complement regulatory proteins in transgenic pigs. [31, 34, 36]

Problems with drugs from transgenic animals:

Erythropoietin could not be expressed in the mammary gland of transgenic cattle. The recovery rates of Factor VIII protein were low. [34] Another concern is leakage of a target protein into the circulation by way of the mammary epithelial cells and as measured by increased plasma levels of the protein designed to be expressed only in the animal's milk. [9] There is also a risk of transmission of infection from animal to man. [31] There are some unique concerns such as premature lactational shut down observed in some animals expressing recombinant proteins in their mammary gland. [37] While there are problems associated with transgenic animals, the benefit derived from them is far superior and with the increase in technology this could be solved.

Drugs from Transgenic Animals in Clinical Trials

The approval of ATryn (rATIII) by USFDA has opened gates for other drugs from transgenic animals.[29] Recombinant C1 inhibitor produced in the milk of transgenic rabbits has completed phase III trials and is expected to receive registration. [4, 31] A topical antibiotic against Streptococcus mutans, for prevention and treatment of dental caries, has completed phase III trials. [34] Vaccine used in Alzheimers disease has restored neurological performance in the mice, and is currently in phase II human clinical trials. 6α-Glucosidase from the rabbit is in clinical trial phase II/III for Pompe's disease. [31, 34] Products such as α-anti-trypsinused for cystic fibrosis, α-AT deficiency and tPA used for coronary clots are currently in phase II/III clinical trials and are expected to be on the market within the next few years. [34]

Ethics in Transgenic Animals

Genetic modification of micro-organisms and plants has least concern with regards to ethics but when it comes to genetic modification of animals and particularly humans, more objections are registered. There remains concern that with advances in transgenic animal technologies the number of animals used for research may actually increase rather than reduced because of a wider range of diseases and conditions. [38] Ethical concern with oncomouse is that it usually suffers in order to collect relevant information, which contradict the principles of animal rights.[6] Adenopolyposis coli knockout mutant mice are clinically normal until the intestinal polyps develop, after which mice become anemic and lose weight. Each newly created transgenic strain has the potential to cause poor health and suffering in the animals hence measures need to be undertaken to minimize animal suffering. [39]

Other ethical concerns are breaching species barriers and animal life should not be regarded as a chemical product subject to genetic alteration and patentable for economic benefit. Also genetic engineering of animals interferes with the integrity or telos of the animal. Telos is defined as the set of needs and interests which are genetically based, environmentally expressed, and which collectively constitute or define the form of life or way of living exhibited by that animal, and whose fulfillment or thwarting matter to that animal. [37] In India attitude towards animals is tinged with religious and ethical colour which makes religious sentiments and public awareness necessary to be taken in consideration.[40] The 3R (Reduction, Refinement, Replacement) aim to minimize pain experienced by the animals used in experiments.29Inspite of the problems listed above, transgenic animals may represent a "refinement" in comparison to some other traditional experimental models of disease where animals bear a heavy load of suffering. A genotype is an excellent model of disease for selected body functions at the molecular or cellular level while the corresponding phenotype

is completely healthy. Thus it becomes necessary to consider the moral implications of producing such a species as well as measures of reducing animal suffering. [39]

Patents on Transgenic animals

Patenting of animal models is the need of hour, because it is an indispensable tool for screening of novel molecule to various diseases. A human pathological condition in animals is most important to determine the therapeutic efficacy of novel molecule. They allow facilitation of the screening process to eliminate inactive moieties and assess the pharmacologist to identify the therapeutic potential and characterize the toxicological profile of novel chemical or biological entities. [6] The two major aspects in granting patents to animal model are morality and reproducibility. [6] Other concerns like restrictive licensing of the patents can hinder transfer of knowledge. [41] Preclinical animal models are important in drug discovery, because they lay the fundamental for human trials. Patents remain one of the important ways of recovery of the investments made by the Pharmaceutical companies in research. The Indian Patent Law section 3i and 3 j states that all the surgical processes and animals are not patentable, hence animal models are not patentable in India. If the suitable amendments are made then animal models can be patentable in India and it would open novel vistas in the research arena in India. [6]

Regulation in Transgenic Animals

Pharmaceutical research on animals in India depends on Department of Biotechnology, National Institute of Immunology and Environment Protection Act. An Animal Welfare Board is constituted, with a Committee for the Control and Supervision of Experiments Animals (CPCSEA) which is in charge of legal and ethical aspects or animal research. General Guidelines on caring for animals in research are in accordance with the International Committee for Laboratory Animal Science (ICLAS) Guidelines. However there is no specific law or guidelines for cloned and transgenic animals. In 2000, Indian Council of Medical Research Report promotes transgenic animal research as long as it would pursue a higher scientific goal. [40, 42] With changes in the overall process of drug discovery, US patents of animal models encourage scientists in America and Europe to produce animal models which are very close to human disease and hence contribute significantly to the process of drug discovery. [6] The Indian regulatory authorities need to be prepared for such challenges of ethics, regulation and patents in transgenic animals.

Future prospects

Xenogenic cells, in particular from the pig, hold great promise with regard to successful cell therapy for human patients. Porcine islet cells transplanted to diabetic patients has shown to be partially functional over a period of time. Porcine fetal neural cells have been transplanted into the brain of patients suffering from Parkinson's disease, Huntington's disease, stroke and focal epilepsy. The advantages of porcine neural cells over their human counterparts are the abundant availability. [34]The pig could be a useful model for studying defects of growth-hormone releasing hormone, which are implicated in conditions such as Turner syndrome, hypochrondroplasia, and intrauterine growth retardation.[4] Eggs provide other non-invasive harvesting medium. Significant quantities of two human proteins, interferon beta-1a and a humanized monoclonal antibody (miR24) were expressed in
the whites of eggs laid by transgenic hens. miR24 is being developed for malignant melanoma. [3] There are transgenic animals for the development of unique biological materials

like polymers based on spider silks that may be useful as suture or plastic materials in facial and orthopedic restorative surgery. [43,37] It is impractical to obtain sufficient quantities of plasma butylcholinesterase (BChE) to treat humans exposed to organophosphorus agents in agriculture and chemical warfare, the production of recombinant BChE in milk of transgenic animals is under investigation. [44] It is 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. [1] Thus transgenic animals have the capacity of mass production and an effective alternative to various human byproducts. Improvements in transgenic technology include inducible gene expression, artificial chromosomes and advancement in nuclear transfer. [34, 45, 36]

Emerging transgenic technologies: [4, 31, 45]

- Lentiviral transfection of oocytes and zygotes,
- Chimera generation via injection of pluripotent cells into blastocysts
- Culture of spermatogonia and transplantation into recipient males
- Ribonucleic acid interference

Researchers are using transgenic animals to develop therapies for a wide range of diseases discussed above and other diseases like Anaemia, Emphysema, Haemophilia, Malaria and Rheumatoid arthritis. [3]

CONCLUSION

Major prerequisites for success and safety of transgenic animals will be a continuous refinement of reproductive biotechnologies. In coming years genetically modified animals will play a significant role in the field of biomedicine especially in drug development, animal disease models, xenotransplantation, antibody production, gene pharming and blood replacement. [4] The regulatory aspects and ethics should be given due consideration while using transgenic animals. From research, pigs and transgenic animals derived products like milk, eggs seems to be promising in developments of therapeutic strategies. Drugs from transgenic animals can minimize the attrition rate in clinical trials by increasing the quality of the target and compound combinations making the transition from discovery into development. Transgenic technology can impact at many points in the discovery process, including target identification and target validation. It also provides models designed to alert researchers early to the potential problems with drug metabolism and toxicity which will help in providing better models for human diseases. [2] Transgenic in general is a rapidly advancing field, and within 20 years of its inception it has produced the first USFDA approved drug for transgenic animals. Thus the use of transgenic animals has the capacity to overcome the current and future needs in medicine and is now a necessity rather than a matter of choice.

TRANSGENIC PLANTS AND THEIR APPLICATION IN MEDICINE

Subunit vaccines are commercially produced in genetically engineered bacteria, yeast or mammalian cells. With the advent of genetic engineering of higher plants, attempts have been made to add transgenic plants to the list. The goal is to produce plant organs (leaves, fruit), crude extracts (dry protein powder) or purified proteins that upon oral or parenteral administration deliver one (or more) immunogenic protein(s) in a manner that triggers an immune response. The applications of plants as protein production systems are wide and varied. The first demonstration of expression of a vaccine antigen within plants occurred in 1990 when Curtiss and Cardineau expressed the *Streptococcus mutans* surface protein

antigen A (SpaA) in tobacco [67]. This demonstration was closely followed by plant expression of the hepatitis B surface antigen (HbsAg) [46, 47], the *E. coli* heat–labile enterotoxin responsible for diarrhoea [46], the Norwalk virus capsid protein [46] and the rabies virus glycoprotein [48], Proteins produced in these plants induced synthesis of antigen specific mucosal IgA and serum IgG when delivered orally to mice and humans. References [49, 50], list proteins that have been expressed in genetically-modiied plants (GM-plants) and are now being tested for their potential use as human or animal vaccines. The production of autoantigens in plants for oral tolerance therapy of autoimmune diseases has also been shown to be feasible [50, 51], In addition, attention is being directed to the production of epitopes in plants that target cytotoxic activity against tumours. Plants can also serve as bioreactors for the production and scale-up of functional antibodies used in immunotherapy [52], however the focus within this paper will be restricted to plant-derived therapeutics for active immunisation. Approaches to meet the present public concern on the use of GM-plants and the spread of GM-pollen have been proposed [49, 50 , 53, 54] .

Is there a need for plant-derived vaccines?

Definitely, the answer is yes. As outlined in Table 1, the production of recombinant vaccines in plants may overcome some of the major difficulties encountered when using traditional or subunit vaccines in developing and developed countries. In developing countries difficulties include vaccine affordability, the need for "cold chains" from the producer to the site of use of the vaccine and the dependence on injection. Plant-derived vaccines do not face these issues. In developed countries plant-derived vaccines offer increased safety, envisaged low cost of program for mass vaccination, and the wider use of vaccination for veterinary use [55].

Table 1: Technical and social benefits envisaged in plant-derived edible vaccines

No.	Benefit	Characteristics
1	Oral delivery	The plant cell wall, consisting essentially of cellulose and sugars, provides protection in the stomach and gradual release of the antigen in the gut
2	Use as raw food or dry powder	The vaccinogenic plant tissue may be used as raw food, dried or, alternatively, proteins may be partially or fully purified and administered in capsules as dry powder
3	No need for "cold chain"	The vaccinogenic plant parts or plant extracts can be stored and shipped at room temperature
4	Mucosal and serum immune response	Plant-derived vaccines are primarily designed to trigger the mucosal immune system (IgA), thus preventing pathogen entry at mucosal surfaces; they also elicit serum and, possibly, cytotoxic responses
5	Cost efficiency	Production cost will be reduced 100–1000 times as compared with that of traditional vaccines
6	Optimised expression system	Plants may be engineered to accumulate the antigen in convenient intracellular compartments (endoplasmic reticulum, chloroplast)
7	Ease of genetic manipulation	Procedures essentially rely on established molecular and genetic manipulation protocols; these are already available in developing countries
8	Ease of production and	GM-plants can be stored as seeds. Unlimited vaccine

	scale-up	quantity can be produced from these in limited time; production and management is suitable for developing countries
9	Safer than conventional vaccines	Lack of contamination with mammalian pathogens
10	Ideal to face bio-weapons	Safety and cost efficiency propose plants plant-derived vaccines as an ideal tool to face bio-terrorism
11	Ideal for veterinary use	Cost affordable Ready for use as food additive

Plant species

To date many plant species have been used for vaccine production. Early studies used tobacco and potato but now tomato, banana, corn, lupine, lettuce and others are being used for this purpose [46, 49, 50]. The choice of the plant species (and tissue in which the protein accumulates) is important and is usually determined through how the vaccine is to be applied in the future. For example an edible, palatable plant is necessary if the vaccine is planned for raw consumption. This limitation is overcome in non-edible plants by vaccine antigen extraction and purification. Antigen extraction is often performed when using tobacco, a plant that offers considerable experimental advantages such as ease of transformation and extensive genomic sequence knowledge. Heat treatment is feasible only if there is no deleterious effect on antigen stability. Recently, a "cooked" GM corn snack that accumulates the *E. coli* heat–labile enterotoxin has been proposed. In the case of vaccines for animal use, the plant should preferentially be selected among those consumed as normal component of the animals' diet.

What are the targets for plant-derived vaccines?

Vaccines against infectious diseases
There is a large and fast growing list of protective antigens from microbial and viral pathogens that have been expressed by plants. The initial focus was upon human pathogens. However, today attention has also spread to animal pathogens (e.g. Newcastle and foot and mouth disease). There is no limit to the number and range of antigens that can be produced in plants if the DNA sequences coding for the appropriate genes are available.

Vaccines against autoimmune diseases
Transgenic plants expressing autoantigens are being produced in attempt to cure diseases in which the immune system recognises the body's own proteins as foreign. The diseases include arthritis, multiple sclerosis, myasthenia gravis, and type I diabetes. The rational is that an appropriate oral dose of a plant-derived autoantigen will inhibit the development of the autoimmune disease. Pioneering and recent work is described in [50, 51].

Vaccines against human tumours
Particular proteins have been shown to over-express on the cell surface of many tumours, including melanoma and breast cancer. Naturally acquired, actively induced or passively administered antibodies against these antigens have been able, in some cases, to eliminate circulating tumour cells and micrometastasis. However, cancer vaccine development is complicated due to the tumour antigens also being auto-antigens [56, 57].

In the last decade, immunologists have identified and characterised epitopes specific for different human tumours.

For instance, an epitope specific, cytotoxic T lymphocyte response in mice was stimulated after injecting naked recombinant plasmid DNA carrying a poly-epitope isolated from a human melanoma tumour [58]. This DNA is now being integrated into the nuclear and chloroplast DNA of tobacco in attempt to develop a plant-derived melanoma vaccine (collaboration: Pasteur Institute, Paris, University of Milano, Italy, and University of Central Florida, USA).

The biotechnological approach: construction of appropriate gene expression cassettes, plant transformation, and efficiency of antigen expression

The production of a vaccine in plants depends upon the availability of a DNA sequence coding for a protective antigen and on the construction of an expression "cassette" suitable for plant transformation. Stable plant transformation currently offers two options: insertion of the foreign gene into the nuclear genome or into the chloroplast genome. Transient plant transformation has also been used for plant expression of vaccine antigens through integration of the gene of interest into a plant virus and subsequent infection of susceptible plants. Plants producing two or more antigens may also be obtained through transformation with multiple gene constructs or through sexual crossing. The strategies for plant expression cassette construction and plant transformation depend on the desired goal. Points worth noting are summarised in Table 2.

Stable integration of genes into the plant genome
The quantity of plant tissue that may constitute a vaccine dose must be of practical size both for field production and for consumption. Since the demonstration that low levels of a recombinant hepatitis B surface antigen (HbsAg) could be produced in GM potato and that the antigen assembled into spherical particles similar to those seen in .level [46, 52, 63].
A number of factors may modulate gene expression in plants. They include: codon usage; promoter, leader and polyadenylation signals; DNA sequences that target antigen accumulation to a specific tissue or cell compartment and others found listed in Table 2. The use of carrier proteins may also be required, especially for small, non-particulate subunit vaccine antigens. The observation that the LT-B, CT-B and HBsAg antigens are highly immunogenic when assembled into multi-subunit structures led to the finding that these structures may act as carriers for different candidate epitopes [46, 47].

The site of gene integration into the genome also influences epitope and transgene accumulation in plants. *Agrobacterium tumefaciens* infection is most frequently used to achieve permanent integration into the nuclear DNA, where integration occurs at random chromosomal sites. A second promising approach is based on the integration of the gene or epitope into the circular chloroplast DNA (cpDNA) that is present in multiple copies within defined plant cells. In this case transformation is usually achieved through the use of the "particle gun" and results in site-specific integration [49, 60]. Both nuclear and chloroplast genomes accept large and multiple gene inserts [46, 62].Advantages envisaged for cpDNA transformation are manifold: the cpDNA molecule (a circular DNA molecule of about 150 Kb) is fully sequenced in a number of important plants and is present to up to 10.000 copies per cell. Furthermore, it has been shown that chloroplasts can properly process eukaryotic proteins, including correct folding and disulfide bridges [62]. Integration into cpDNA has two important advantages, the first being the foreign sequence

is targeted, by homologous recombination through the use of appropriate flanking sequences, to a precise cpDNA site. This eliminates variability in gene expression and gene silencing, which may occur in the case of gene integration in the nuclear DNA. The second advantage lies in the increased accumulation of the recombinant protein (up to 46% of total soluble protein, as compared with 0.01–0.4% with nuclear inserted genes). Apparently, accumulation of the foreign protein in the chloroplast does not significantly impair photosynthetic efficiency.

The current limitation to frequent use of cpDNA transformation is that although cpDNA transformation is routine in tobacco, it is more difficult and still requiring optimization in other edible plant species [59, 60, 62].

Table 2: Gene constructs, expression signals and peptide design for optimal vaccine-production in GM-plants

No.	Purpose	Approach and notes	References[a]
1	Optimise codon usage	Adapt codon usage to that preferred by plant genes	46
2	Optimise epitope sequence	Adapt A + T composition to that found in plant genes Eliminate sequences that destabilise or splice mRNA Minimise secondary structure hairpins	46
3	Select promoter	This may be: plant constitutive, tissue specific, inducible by environmental factors	46
4	Use leader and 3_-polyadenilation signals	Alternative signals affect protein accumulation Use TEV (the 5_untranslated region of the tobacco etch virus)	52, 59
5	Target protein to the chloroplast	Integrate the DNA sequence in the nuclear DNA and use an N-terminal chloroplast transit peptide: the protein is accumulated in the chloroplast	
6	Target protein to the endoplasmic reticulum	Use an endoplasmic reticulum retention signal, such as SEKDEL	46
7	Integrate the epitope DNA in the chloroplast DNA	Integrate the DNA sequence in the chloroplast DNA under appropriate expression signals: the protein will be synthesised and accumulated in the chloroplast	59, 60
8	Integrate the epitope DNA into a plant virus vector	Use a viral promoter when the epitope is integrated into a plant virus Use a defective virus for improving yield and for environmental safety	54, 61
9	Express polycystronic mRNA	Integrate into the plant DNA a poly-epitope under a single expression signals	46, 62

10	Choose selectable marker genes	Use an appropriately selected gene Remove the gene after selection	49, 63

[a] The cited references give further recommended readings on construction of plant expression cassettes, expression signals and peptide design listed in the table.

The use of plant viruses as transient expression vectors

Plus-sense, single-stranded plant RNA viruses have been proposed as an effective alternative to produce vaccine antigens in plants. In this technique the epitope of interest is engineered into a plant virus, usually within the coat protein gene. Infection of a susceptible non-GM-plant results in intracellular production and accumulation of the epitope. The epitope sequence, as well as the viral genome, never become integrated into the plant genome and hence are only expressed by the generation of infected cells [46, 54, 61].

A recombinant cowpea mosaic virus was shown to elicit protective immunity in mink when engineered to express the antigenic epitope against mink enteritis virus [54]. Other successes are listed in [46, 54]. A limitation of the recombinant cowpea mosaic virus approach is the failure of the virus to assemble when peptides of more than 25 amino acids are incorporated into their coat protein. More flexibility was obtained when epitope sequences were inserted at the N-terminal end of the coat protein of the alfalfa mosaic virus (A1MV). Recombinant AlMV has enabled expression of significant quantities of rabies virus and HIV epitopes upon integration of their respective coding sequence into the A1MV coat protein, and infection of tobacco plants. The extra sequences were found to protrude from virion surface without interfering with virus assembly [54]. Results of these studies demonstrated that in order to retain antigenic capacity, the virus particle must retain its potential to self assemble while displaying the antigenic epitope on its surface. Recombinant A1MV coat protein molecules have also demonstrated the ability to assemble into particles containing three different epitopes from HIV and rabies [54, 61]. This demonstrates the ability of plant viruses to produce multicomponent vaccines. Claimed advantages of transient viral expression of transgenes over transgenic plants are: shorter time for cloning of the foreign gene in the viral genome as compared with time required to transform plants, the ease at which antigen production can be scaled up and the wide host range of plant viruses that allow the use of multiple plant species as biofactories [54].

Oral delivery, mucosal and systemic antibody responses

Most infectious agents enter the body through mucosal membranes. Induction of mucosal immunity is best achieved by direct vaccine delivery to mucosal surfaces. This stimulates production of sIgA, the predominant antibody isotope in mucosal secretion. Whilst effective inducers of systemic immunity, vaccines delivered by injection are not efficient at inducing mucosal responses [46, 50, 52, 54, 62].

Plant-derived vaccines have demonstrated the ability to induce both systemic and mucosal immune responses [46, 64]. The major obstacle to oral vaccination is the digestion of the antigenic protein in the stomach. Vaccines derived and delivered by plant cells have been shown to overcome this problem through the protective effect of the plant cell wall. Like liposomes and microcapsules, the plant cell wall allows gradual release of the antigen onto the vast surface area of the lower digestive tract. Further problems may be associated with poor immunogenicity or the induction of tolerance. Binding to a targeting molecule or carrier peptide, such as HbsAg, has been shown to overcome poor immunogenicity of orally

delivered subunit vaccines [46, 50]. In specific circumstances, for example cancer therapy, injection of the drugs, after purification from the producing plant, may be preferred.

Safety and public acceptance

Plant-derived vaccines are certified free from animal pathogen contaminants. Furthermore plant DNA is not known to interact with the animal DNA and plant viral recombinants do not invade mammalian cells. Further safety of plant-derived vaccines is obtained through following the same regulations established for traditional vaccines. Nevertheless, the present concern over the use of GM-plants is now affecting research in this important field, especially in Europe.

One of the fears is that GM-pollen may outcross with sexually compatible plants (related crops or weeds) and affect biodiversity. In order to address this alarm, several pollen containment approaches have been developed. These are essentially based on the exploitation of different forms of male sterility (suicide genes, infertility barriers, apomixis). An alternative way of solving the problem is engineering vaccines into the cpDNA, which is not transmitted to the sexual progeny through the pollen grains [59, 60]. An additional safety feature would be the recognition of GM-plants that produce vaccines by the addition of genes encoding coloured plant pigments [50].

It is important to recognise that plants that produce vaccines are medicinal plants and should be grown, processed and regulated as pharmaceutical products. It is thought that pharmaceutical crops will be able to be grown on relatively small extensions of land, preferably contained within greenhouses using controlled environmental conditions. In the majority of earlier papers, level of antigen accumulation in the plant organ was in the order of 0.1–0.4% of total soluble protein [46], while the more recent developments on cpDNA integration promises to increase this value to 30% or more [65]. At the latter value, land requirements for industrial plant-derived vaccine-production will be in the order of a few thousand square meters. This will definitely enable vaccine-producing plants to be set apart from field grown crop plants and offer added safety when engineered plant viruses are used for transient antigen expression. A further point of public concern in GM-plants is the presence of antibiotic resistance genes (used as selective marker in most transgenic plants). Approaches have now been developed to generate GM-plants (with both nuclear or cpDNA integration) that do not carry these genes [49, 63, 65].

Future perspectives

Although still at an early stage of development, the experimental know-how and results strongly suggest that plant-derived edible vaccines are likely to become a reality in the next few years. Future research will demonstrate if these vaccines meet the standards of quality (purity, potency, safety and efficacy) defined for vaccines by the World Health Organization [66].

When is this expected to happen? A realistic appraisal of the state of the art should consider that after the ongoing event of *discovery* (i.e. the demonstration that plants can be engineered as to produce edible vaccines that trigger an immune response in mice and humans), we are now confronted with the successive problems of *clinical trials*, *process development*, *registration* and *marketing*. *Clinical trials* with populations at risk are already under way in some laboratories. The definition of the overall immune response to plant-derived edible

vaccines is of the utmost importance. With the growing availability of plant-derived vaccines, this will soon be verified. *Process development* primarily concerns achieving sufficiently high levels of expression of the recombinant antigen, and defining the optimal way of antigen administration. Solutions to the first point are well under way, as described above, while approaches to the second will be manifold. While the initial concept was to induce an immune response by directly feeding a crude edible plant portion (fruit, leaf, tuber), it is now felt that this may not be the ideal solution as it would be difficult to standardise antigen concentration in different harvests of the same crop. Furthermore, fresh products may have short shelf life. Dried products, for instance banana slices, may offer a partial solution, but the best solution (as for shelf-life, stability and title standardisation) would be delivery in the form of a dry powder. This can be achieved by using low cost food processing technology. A dried tomato powder has been stored for one year in C. Arntzen's laboratory without loss of antigen activity. In cases in which effectiveness is much more relevant than cost, for example with cancer antigens, administration may be through injection of appropriately purified antigens.

Field and clinical trials are required to define the risk/benefit ratio of a GM-plant before *registration* is granted. In most countries of the world, plants engineered to produce vaccines fall under the very restrictive rules set up to control GM-crop plants. The present concern, especially in Europe, over the use of biotechnology for the genetic improvement of crop plants also negatively affects the acceptance of GM-plants for medicinal use. As a consequence, while the demonstration that plant-derived vaccines are effective on populations at risk is expected to arrive within 1–2 years, a further quarantine of 2–3 years will be required in order to fulfil requirements for *registration* and *marketing*. It is hoped that simpler rules will be set up for GM-plants producing vaccines and that they are seen as clearly and legally distinct from GM-plants grown for nutrition purposes. Important social questions still exist. Who will benefit from this development? Who will be able to perform research, produce and control plant-derived edible vaccines? Will the resultant vaccines be affordable to developing countries? Definitely, the answer is that there is no danger of monopoly in the hands of powerful economic groups. Many countries in the world are already greatly involved in research on plant vaccines; these include the USA, the European Community, China, Japan, India, Korea and others. The reason for this is that the applications are based on established gene cloning and plant transformation technology and that development requires relatively limited investment.

A unique opportunity against the threat of bio-weapons

A number of infectious diseases, including smallpox, anthrax and plague have recently raised concern for their possible use in actions of bio-terrorism. Nations at risk are now faced with the need to be ready to vaccinate part or all of their population within limited periods of time. This means that millions of vaccine doses have to be prepared, stored and renewed at intervals of time. The economic and technical benefits offered by plant-derived vaccines (Table 1) propose these vaccines as ideal substitutes for traditional vaccines. Research on plants that produce antigens against major pathogens feared in case of bio-terrorism is already under way.

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