Transgenic approaches to the increase of disease resistance in farm animals

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Summary
Molecular genetics and reproductive biology techniques enable the transfer of foreign DNA into mammals. Novel approaches to modify disease resistance or susceptibility in livestock are justified by economic and animal welfare concerns. Current research on the improvement of disease resistance by gene transfer focuses on three main strategies, as follows:

a) somatic gene transfer, i.e., nucleic acid vaccines
b) deleterious germ-line gene transfer, i.e., gene knockout
c) additive germ-line gene transfer.

These strategies aim at either the transient or stable expression of components known to influence non-specific or specific host defence mechanisms, or the disruption of genes known to cause susceptibility to disease. Referring to the source of the effective agent and the site and mode of action, the strategies are termed ‘genetic’, ‘congenital’, ‘intracellular’ and ‘extracellular’ immunisation.

Each gene transfer experiment has to be evaluated carefully with respect to the potential to create novel cases of pathogen resistance or to lose species barriers or cell-type restrictions.

Keywords

Introduction

The pioneer experiments which demonstrated that stable gene transfers into mice, gene expression and a phenotypic effect of the transgenes were possible date back more than fifteen years (36, 37, 70). The production of transgenic farm animals – rabbits, pigs and sheep – was reported in 1985 (14, 41). In the following years, this novel technique was also successfully applied to goats and cattle (16).

Gene transfer offers the prospect of developing completely new breeding strategies and novel applications. The use of transgene technology in agriculture for animal production remains very limited compared to the various transgene approaches used in plant production. This is due to the technical problems which persist in the generation of transgenic farm animals at reasonable costs. One general observation is that the ‘bigger’ the mammal, the ‘harder’ it is to successfully transfer gene constructs. The real success of transgenic mammals is undoubtedly in the field of basic research. Relevant biological homologues of human diseases and therapeutics are also created by gene transfer. In mice, a plethora of models generated by gene transfer experiments resulting in either gain or loss of function are available (7, 8, 69). Transgenic farm animals are ready to be used as the source of recombinant proteins of high value for pharmaceutical or nutritional purposes (‘gene farming’, ‘nutraceuticals’) (15, 24, 30). Recently, very encouraging experiments demonstrated the feasibility of xenotransplantation. The hyperacute rejection reaction caused by the primate complement was greatly delayed by expressing complement inhibitors in grafted transgenic pig hearts. Therefore, transgenic pigs expressing the human genes downregulating the complement systems and other genes counteracting the graft rejection reaction might serve as organ donors for human recipients (27, 72). The induction of new genetic traits in domestic animals by transgenesis, however, still awaits success. This is partly due to the technical difficulties but is mainly caused by the lack of valid identified genes. In this paper, the authors focus on the methods of...
transferring genes into farm animals and the possibility of introducing disease resistance traits by this technique.

Gene transfer methods

Gene transfer performed in early embryonic stages aims at the integration of the gene construct into all tissues including the germ line, and is therefore termed 'germ-line gene transfer'. The technique requires profound skills in reproductive technologies, i.e., embryo collection or production, embryo in vitro culture, embryo manipulation and embryo transfer. The reproductive techniques are reviewed extensively elsewhere (9, 16). Somatic gene transfer is applied to neonates at the earliest and results in transient gene expression which lasts from a few days to the entire lifespan of the recipient.

Germ-line gene transfer

Three main routes to germ-line transgenesis in mammals have been described and used routinely, as follows:

a) integration of (retro)viral vectors into an early embryo
b) microinjection of DNA into the pronucleus of a fertilised oocyte
c) incorporation of genetically manipulated embryonic stem (ES) cells into an early embryo or the use of transgenic embryo-derived cells in cloning by nucleus transfer.

Gene transfer by microinjection of DNA constructs into early stage embryos still remains the preferred method for generating transgenic large animals (16). The DNA is microinjected in buffered solution directly into the pronucleus of a fertilised oocyte. The pronuclei of zygotes of some species, e.g., pigs, have to be visualised by centrifugation since the cytoplasm is filled with granula. The equipment required for routine gene transfer programmes by microinjection is listed elsewhere (13, 16). Depending on the species used for gene transfer into farm animals, the efficiency (transgenic neonates/microinjected zygotes) of the technique routinely reaches 0.5%-3%. Data collected during a gene transfer programme indicating the success rates in the differing species are given in Table 1.

Due to safety considerations and the lack of gross advantages in gene transfer efficiency, retroviral vectors are not commonly used. Nevertheless, efforts are being undertaken to develop vector and delivery systems which fulfil the safety and efficiency criteria (77). Other methods, such as using liposomes as a DNA vehicle or techniques originally developed for the transfection of eukaryotic cells, have not yet been established for practical use (13). In 1989, Lavitrano et al. attracted widespread attention by reporting the production of transgenic mice by mixing DNA with spermatozoon prior to in vitro fertilisation (52). Attempts to establish sperm-mediated gene transfer as a routine method in other laboratories have failed to date (80), despite the existence of other reports in addition to the original publication. The reason for this remains unclear. The type and secondary structure of the DNA, the incubation buffer and intermediate factors (embryonic retroponson activity) are discussed as crucial factors. However, the fact that sperm cells are able to take up exogenous DNA spontaneously and transfer it to the oocytes during the process of fertilisation, thereby causing transgenesis in a proportion of the resulting embryos, is well established. This technique was applied to ejaculated semen and resulted in transgenic adult pigs and transformed bovine blastocysts (84). The underlying processes at the molecular level are currently under investigation (59, 107).

A variety of ES cell-like cell lines have been reported in species other than mouse. However, successful germ-line transmission of these cells has not been reported for any of these species (47, 69). The recent progress in cloning animals by nuclear transfer might make the establishment of ES cells in farm animals unnecessary (20, 102). The generation of viable lambs was achieved from the transfer nuclei of embryo-derived epithelial cell lines to an enucleated metaphase-II-arrested oocyte. Further experiments demonstrated the feasibility of genetically modifying the embryo-derived cell lines and the subsequent generation of a transgenic lamb by nucleus transfer cloning (81). Recently a group of scientists in the United States of America (USA) reported the use of this technique in cattle (75).

Table 1

<table>
<thead>
<tr>
<th>Gene transfer statistics</th>
<th>Mouse</th>
<th>Rabbit</th>
<th>Swine</th>
<th>Sheep/goat</th>
<th>Cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of zygotes per donor</td>
<td>15</td>
<td>20</td>
<td>15</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Pregnancy rate after embryo transfer</td>
<td>70%</td>
<td>60%</td>
<td>40-60%</td>
<td>40-60%</td>
<td>50-80%</td>
</tr>
<tr>
<td>Neonates/embryos transferred</td>
<td>10-20%</td>
<td>10%</td>
<td>5%</td>
<td>15%</td>
<td>10%</td>
</tr>
<tr>
<td>Transgene integration frequency (a)</td>
<td>15%</td>
<td>10%</td>
<td>10-15%</td>
<td>5-10%</td>
<td>5-10%</td>
</tr>
<tr>
<td>Gene transfer efficiency (b)</td>
<td>2-8%</td>
<td>1-5%</td>
<td>0.5-1%</td>
<td>1-2%</td>
<td>0.5%</td>
</tr>
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</table>

(a) Transgenic animals/neonates
(b) Transgenic animals/injected embryos transferred
Somatic gene transfer

Somatic gene transfer does not aim at the germ-line integration of the transgenes. Its main application is the field of gene therapy in humans. The methods available for delivering DNA into differentiated mammalian cells can be divided into viral and non-viral methods (62). Viral vector systems have the advantage of being able to enter cells actively; however, they face problems concerning safety and efficiency. The latter problem is mainly due to transcriptional shutdown and low-infectivity of quiescent cells (95). The non-viral methods use direct injection of purified ('naked') or bound DNA into the bloodstream, muscles or other organs and tissues. Particle bombardment with DNA bound to microparticles (commonly gold or wolfram, referred to as the 'gene gun') represents an analogous method for injecting genes into the nucleus of target cells. Jet injection refers to the acceleration of DNA solutions for the delivery to the epidermis (34). Certain methods for controlled delivery of small-molecule drugs or proteins may be useful for delivering genes to cells: these include the binding of DNA to cationic lipids, liposomes, endosomal release peptides, targeting ligands and polymeric carriers (20).

Enhancement of disease resistance by transgenic means

Increased disease resistance can be achieved by introducing resistance-conferring gene constructs into animals or by depleting a susceptibility gene or locus from the animal. Hence gain of function (additive) as well as loss of function (deleterious, knockout) gene transfer experiments can be used. The mechanisms of disease resistance or susceptibility are frequently controlled by complex genetics and are strongly influenced by environmental effects. Gene transfer experiments are often hampered by the lack of identified major genes or loci responsible for resistance traits. Resistance or susceptibility mechanisms may occur at all levels of the pathogen-host interaction, i.e., pathogen entry of the host, distribution in the host, attachment to host organs, tissues and cells, possible penetration into cells, multiplication of the pathogen, maintenance of the pathogen in the host organism and release of the progeny of infectious agents. Candidates for gene transfer experiments include all genes known to modulate non-specific and specific host defence mechanisms, i.e., genes encoding cytokines (58), major histocompatibility complex (MHC) proteins (3, 39, 50, 94), T-cell receptors (TCRs) (76) and proteins conferring specific disease resistance (1, 40, 86). Transgenes may comprise naturally occurring resistance genes or genes which enhance the immune response, or they may consist of in vitro designed gene products (e.g., antisense RNA, DNA vaccines). The different strategies of transgene interference with the pathogen-host interaction are listed in Table II. Referring to the site and mode of action and the source of the effective agent, some of the strategies are termed 'intracellular', 'extracellular', 'genetic' and 'congenital' immunisation (64). The authors discuss the differing transgenic strategies according to the gene transfer method and the immunisation approach.

<table>
<thead>
<tr>
<th>Transgene target</th>
<th>Transgenic strategy</th>
<th>Examples of transgenes encoding</th>
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</thead>
<tbody>
<tr>
<td>Pathogen attachment and penetration into host cells, tissues or organs</td>
<td>Extracellular immunisation</td>
<td>Soluble pathogen receptors</td>
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<td></td>
<td>Intracellular immunisation</td>
<td>Mutated cellular pathogen receptors</td>
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<td></td>
<td>Congenital immunisation</td>
<td>Substances interfering with pathogen entry</td>
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<td></td>
<td>Genetic immunisation</td>
<td>Antibodies</td>
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<td></td>
<td></td>
<td>Antigens</td>
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<tr>
<td>Pathogen replication and progeny release</td>
<td>Intracellular immunisation</td>
<td>Anti-idiotype RNA</td>
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<td></td>
<td></td>
<td>Ribozymes</td>
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<td></td>
<td></td>
<td>Inhibitors of replication</td>
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<tr>
<td>Latency or spread of the pathogen through the host organism</td>
<td>Extracellular immunisation</td>
<td>Products inducing or maintaining latency</td>
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<td></td>
<td>Congenital immunisation</td>
<td>Antibodies</td>
</tr>
<tr>
<td></td>
<td>Genetic immunisation</td>
<td>Antigens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antimicrobial agents</td>
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<tr>
<td>Host immune system</td>
<td>Intracellular immunisation</td>
<td>Cytokines</td>
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<tr>
<td></td>
<td>Extracellular immunisation</td>
<td>Cytokine receptors</td>
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<tr>
<td>Identified host susceptibility genes</td>
<td>Gene targeting (knockout/ gene replacement)</td>
<td>Intracellular effectors of cytokine signalling</td>
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<td></td>
<td></td>
<td>Gene targeting constructs</td>
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authors choose the term 'genetic immunisation' to describe the somatic DNA delivery and utilisation of antigen-encoding gene constructs rather than antigens per se for vaccination. DNA constructs have been delivered to individuals by several routes, including intramuscular, intravenous, intradermal and needleless (jet) injections and micro-bombardment. Depending on the method of administration, the foreign DNA was expressed locally or systemically to levels high enough to induce specific antibody production (23, 28, 29, 92, 93). In addition to the use of naked DNA or bound DNA, nucleic acid vaccines may be targeted to specific tissues, such as mucosal sites, for the induction of local immune responses. With respect to this technique, Sizemore et al. have generated a bacterial vector capable of DNA delivery (82). Mice immunised with recombinant Shigella containing plasmid expression constructs produced cellular immune responses and high titres of specific antibodies. This strategy opens up the possibility for the oral delivery of functional DNA with the potential to induce a local or systemic immune response. Tsukamoto et al. have demonstrated that a single intravenous injection of DNA/lipopolyamine complexes into pregnant mice resulted in successful gene expression in the embryos (91). This opens new aspects for basic research and practical use in neonate vaccination. DNA vaccines have the great advantage of relatively short developmental times, ease of large-scale production and cost-effectiveness in development, manufacture, storage and distribution. DNA vaccine technology also simplifies the process of vaccine antigen discovery. Mixtures of expression plasmids containing fragments of the genome of a given pathogen can be tested for antigen discovery. Using this approach of 'expression library immunisation' (ELI) (6), it should be possible to successively fractionate and test the DNA mixtures and identify the plasmid(s) encoding the protective antigens.

In veterinary medicine, routine vaccination is mostly applied to large populations. Methods for reducing costs and increasing the efficiency of DNA vaccination include jet injection, gene gun or aerosol application of naked or complexed DNA (95). In vivo gene transfer to the airway epithelium was successfully carried out after intratracheal administration in mice (91). Prior to use in farm animals, such delivery methods have to be tested in these species and the necessary safety aspects (biodistribution, long-term expression, DNA persistence, germ-line integration) have to be considered. Currently, non-viral DNA delivery methods are preferred to the viral-based DNA vector systems for safety reasons. The use of viral vectors for immunisation in veterinary medicine is discussed elsewhere (105).

Immunomodulatory molecules, such as cytokines or costimulatory molecules, may be effective at enhancing immune responses against antigens expressed by genetic immunisation. These molecules could be delivered as recombinant polypeptides or could be expressed in situ using plasmid DNA (106). The somatic gene transfer of cytokine transgenes alone might also be used for the enhancement of disease resistance, as discussed below under 'extracellular immunisation'.

**Germ-line gene transfer**

Examples of attempts to introduce disease resistance traits into mammals by germ-line gene transfer are summarised in Table III and described briefly below.

<p>| Table III |</p>
<table>
<thead>
<tr>
<th>Transgenic animals carrying disease resistance enhancing gene constructs</th>
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<tbody>
<tr>
<td><strong>Transgenic strategy</strong></td>
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<tr>
<td><strong>Congenital immunisation</strong></td>
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<tr>
<td><strong>Intracellular immunisation</strong></td>
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<td><strong>Extracellular immunisation</strong></td>
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CRP : C-reactive protein  
TAP : tracheal antimicrobial peptide  
Nramp : natural resistance-associated macrophage protein
Deletive gene transfer

Knockout or replacement of susceptibility or disease genes

The technique of altering the genome by homologous recombination (69) is made possible by the availability of totipotential cells in farm animals or by using the nuclear transfer cloning technique. This allows the targeted disruption of disease causing genes or the replacement of mutant alleles by alleles causing enhanced resistance. A widely discussed example in this context is the generation of prion-resistant flocks and herds. Transmissible spongiform encephalopathies (TSEs) are thought to be caused by a malformed version of the normal cellular prion protein (2, 73, 100). The favoured ‘protein-only’ hypothesis maintains that the prion is devoid of nucleic acids, although the participation of additional infectious agents (51) or the ‘unconventional virus’ hypothesis cannot be ruled out completely. The prion diseases occur in humans (e.g., Creutzfeldt-Jakob disease [CJD]) and animals, including sheep (scrapie) and cattle (bovine spongiform encephalopathy [BSE]). There is now considerable concern that BSE may be transmissible to humans, resulting in a new variant of CJD (19, 44). The observation that mice lacking the endogenous prion protein are resistant to prion disease (100) suggested the generation of prion gene-free sheep and cattle by gene targeting. The availability of the appropriate techniques in sheep and cattle will allow the establishment of proof of principle. However, the production of prion-free farm animals is less practical, since it would be an enormous task to replace the many differing breeds and to preserve the genetic variety. In addition, such animals might suffer some deleterious effect from the depletion of the prion gene, as has been observed in mice (2, 73, 99). However, prion-disease-resistant farm animals would undoubtedly be of benefit for gene farming purposes. For domestic animals, a more efficient approach might be to introduce prion transgenes known to act as dominant-negatives or to replace the existing prion gene alleles by prion gene variants resistant to the development of TSEs.

Additive gene transfer

Congenital immunisation

‘Congenital immunisation’ is defined as transgenic expression and germ-line transmission of a gene encoding an immunoglobulin specific for a pathogen. The animal therefore shows congenital immunity without prior exposure to that pathogen (65). As shown by many investigations, cloned genes coding for monoclonal antibodies can be expressed in large amounts in transgenic mice. The approach was tested in farm animals by expressing the gene constructs encoding mouse monoclonal antibodies in transgenic rabbits, pigs and sheep (55, 98). Both experiments resulted in transgene expression but also revealed some unexpected findings, e.g., aberrant sizes of the transgenic antibody or little antigen-binding capacity. These results were explained by heterologous immunoglobulin chain associations and/or deviant post-translational modifications. Nevertheless, both experiments illustrate the potential of introducing beneficial traits such as germ-line-encoded immunity into farm animals. Whether or not the efforts required for optimising the concept of ‘congenital immunisation’ are justified by its benefits in terms of increased disease resistance in a certain species remains to be investigated. Following this route, one has also to keep in mind that the high mutation rates of most infectious pathogens will readily create novel subtypes able to escape the immunity of the transgenic animal by changing their antigenic determinants. In the transgenic animals generated by Weidle et al. (98), antibodies were not only detected in the blood stream but also in the Colostrum milk (65). In this experiment, the heavy and light chain monoclonal antibody encoding regions were driven by the endogenous mouse immunoglobulin promoters. The expression of antibodies can be more efficiently directed to the mammary gland using mammary gland-specific promoter regions. Milk containing recombinant antibodies would be of high value for the prevention of neonatal diseases in suckling offspring. In addition, this technique is used for the production of monoclonal antibodies for therapeutic or diagnostic use. It is also worth mentioning that the antibody expression technology is applicable to antigens that are non-proteinous.

Intracellular immunisation

The term ‘intracellular immunisation’ was originally proposed for the overexpression in the host of an aberrant form (dominant-negative mutant) of a viral protein that is able to interfere strongly with cellular entry or the replication of the wild-type virus (5). This definition was then extended to include all approaches based on intracellular expression of transgene products which inhibit the multiplication of pathogens in host organisms. Thus different strategies using intracellular antibodies (intrabodies), antisense RNAs and ribozymes, dominant-negative pathogen proteins and host-derived antipathogenic proteins are summarised by this term.

Intrabodies

Recent advances in recombinant antibody technology have allowed the genes encoding antibodies to be modified so that the antigen-binding domain is expressed intracellularly (51). This is achieved by mutation or deletion of the hydrophobic leader sequence for secretion of the recombinant antibodies. Intracellularly, the antibodies can be directed to the desired subcellular compartment by adding the appropriate localisation signal (11). In the whole animal, the expression of an intrabody can be restricted temporally or spatially by using a promoter that is either tissue- (cell-) specific, active at only a certain time in development or upon stimulation, or both. Intrabodies represent a versatile tool to modulate the function of selected intracellular gene products (phenotypic knockout) but also promise to be powerful in the defence against infectious pathogens (10, 60). The recombinant antibodies most often utilised for this approach have been single-chain variable region fragments (scFv), but other formats (whole antibodies, antigen-binding fragments) have...
been used as well. Single-chain Fv proteins are recombinant antibodies derived from monoclonals and are able to bind to their antigens with affinities similar to those of the parental antibodies (37). They consist of only the variable L chain and the variable H chain domains covalently linked by an engineered polypeptide linker. Due to their small size, lack of assembling requirements and easy derivation, these proteins are well suited for the design of new therapeutics and transgenic approaches. At present, intrabodies are mainly investigated for their potential in human gene therapy. In agriculture, the approach has been successfully tested in plants (65), but awaits establishment in transgenic farm animals.

Antisense ribonucleic acid and ribozymes

Antisense nucleic acids comprise short synthetic oligonucleotides (often oligodesoxyribonucleotides) and substantially longer sequences formed by RNA transcribed either in vitro or in vivo. Antisense molecules can inhibit the complementary messenger RNA (mRNA) at multiple levels in the expression and replication of a gene: for example, by blocking the mRNA translation; by increasing the sensitivity of mRNA to cellular double-stranded RNA ribonucleases; by blocking the RNA export from the nucleus; and by base modifications of the RNA. Ribozymes, like antisense RNA, are RNA molecules that bind to their complementary sequences. However, they function additionally to cleave the target RNA (22, 56). Antisense and ribozyme constructs have been tested successfully in transgenic animals (83). Antisense technology was applied in mice and rabbits to prevent infectious diseases. Transgenic mice expressing antisense RNA targeted to the retroviral packaging sequences of Moloney murine leukemia virus did not develop leukemia following challenge with infectious viruses (42). In another report, the nucleocapsid protein gene of the mouse hepatitis virus was the target of inhibition by antisense RNA. The nucleocapsid protein is crucial for the packaging process of the viral genome. Mice expressing the antisense gene construct were shown to be more resistant to infection leading to death than non-transgenic littermates (79). Ernst et al. described the generation of transgenic rabbits expressing an antisense construct complementary to adenovirus h5 RNA (32). Primary cells from transgenic animals were found to be 90% to 98% more resistant to adenovirus infection than cells from control animals. The effect of an antisense RNA transgene targeted against the 5’-untranslated long-terminal repeat of the bovine leukemia virus (BLV) on the virus replication and spread in a rabbit model system was studied (48). BLV, a retrovirus, causes persistent lymphocytosis and B-lymphocyte lymphoma in cattle and sheep, but is also virulent in laboratory animals. Transgenic rabbits showed enhanced resistance to BLV infection compared to wild-type animals. Although the transgenics also produced antibodies to viral proteins, the haematological changes observed in wild-type rabbits were considerably less pronounced in these animals.

The use of antisense RNAs as anti-pathogenic agents has several limitations which are mainly imposed by the stoichiometric nature of the inhibition. Therefore, ribozymes offer the possibility to develop antisense constructs that not only result in RNA-RNA hybrids, but in addition catalytically cleave a phosphodiester bond in the target RNA strand. The catalytic RNA (i.e., the ribozyme) would not be consumed during the cleavage reaction, thus a large number of substrate molecules could be processed (78). So far, there are no ribozyme transgenic animal models reported to show resistance to infectious diseases. A hammerhead ribozyme flanked by antisense sequences directed against regulatory proteins of BLV was shown to inhibit BLV expression in persistently infected cells (21). Taken together with the in vivo data obtained from transgenic rabbits (48), this suggests the possibility of generating transgenic ruminants that will be resistant to BLV-induced diseases.

The creation of animals containing intrabodies, or antisense or ribozyme constructs, against common pathogens is used in cases where the disease-causing organisms are well known. Intrabodies or anti-RNA agents could be raised against conserved epitopes of an essential protein or early RNAs of the pathogen. A more effective strategy would be to provide low levels of transgene products against multiple target proteins or RNAs in the disease-causing organism, thereby reducing the chance that the pathogen would escape by mutagenesis.

Dominant-negative mutant pathogen proteins

The original concept of intracellular immunisation aims at the inhibition of viral multiplication by interference of mutant and wild-type virus proteins. The extended definition includes all approaches using (intra)cellular antiviral agents (35). Clements et al. generated transgenic sheep expressing the maedi-visna virus envelope (E) gene (25). Maedi-visna virus belongs to a subfamily of ovine retroviruses that cause encephalitis, pneumonia and arthritis in sheep. Its E glycoprotein is responsible for binding the virus to host cells. The target cell for maedi-visna virus replication in infected sheep is the monocyte/macrophage. Maedi-visna-infected cells express E protein on their surface which causes immune responses to the virus. Expression of a gene construct consisting of the maedi-visna 3’-untranslated enhancer region fused to the E gene in transgenic sheep had no obvious deleterious effect. Transgene expression was detectable predominantly in macrophages and to a smaller extend in fibroblasts and lymphocytes. Thus, the generated transgenic sheep lines provide a model for studying the potential of a retroviral E glycoprotein to prevent infection and/or to modulate disease in its natural host after virus challenge (26).

Specific disease resistance genes

This term refers to single genetic loci responsible for defined disease resistance traits. Unfortunately there are only rare cases of one gene causing a certain resistance described. Examples are provided by the Mx system (40), the natural
resistance-associated macrophage protein (Nrampl) gene (1) or the retroviral (Friend virus) susceptibility/resistance genes in mice (86).

The Mx system was originally characterised in mice and is known to be able to block the multiplication of certain negative-stranded RNA viruses. The generation of Mx transgenic mice is reviewed elsewhere (4, 40). The attempt to introduce resistance to influenza A viruses into pigs by transferring mouse Mx1 gene constructs into pigs failed. Extensive protein analysis did not detect mouse Mx1 protein in transgenic pigs (62). The underlying reasons may be transgene rearrangement or, more likely, the possibility that the mouse Mx1 promoter does not function optimally in pig cells.

The mouse Nrampl gene (formerly the Bcg/flt/Lsh locus) determines the susceptibility of inbred strains to infection with unrelated intracellular parasites. In livestock species, the locus was also found to be associated with intracellular parasite resistance (33, 46). In mouse inbred strains, the susceptibility to infection is associated with a single amino acid substitution of the protein. Gene transfer of the non-mutated Nrampl gene into susceptible mice conferred resistance to infection with Mycobacterium bovis and Salmonella Typhimurium (38).

Genetic resistance to certain retroviruses has been observed as a polymorphic trait in several experimental species. One of the identified loci in mice, Fv-4, resembles the 3' half of a murine leukaemia virus genome. Expression of the mouse Fv-4 encoding only the viral envelope protein in transgenic mice conferred resistance to infection with ecotropic retroviruses (53, 54). The mechanism of Fv-4 resistance is thought to be related to the phenomenon of viral interference, i.e., competition of the synthesised envelope protein with exogenous virus for the virus receptor. Although the resistance is mediated by an endogenous mouse gene, the strategy resembles the concept of overexpressing dominant-negative pathogen proteins.

Extracellular immunisation

A variety of strategies for the enhancement of disease resistance by transgenesis use gene products which exhibit their antipathogenic activity extracellularly. According to the immunisation strategies described above, the authors refer to methods described next as 'extracellular' immunisation.

Cytokines are potent regulators of the innate and acquired immune response (38). As cytokines form a complex interacting network which regulates cellular growth and differentiation in physiological and pathophysiological states, experiments involving the transfer of cytokine genes have to be carefully designed. Aberrant expression of any cytokine may result not only in the desired positive immune-regulatory effects but also in no effects or in deleterious 'side-effects', as has been experienced in therapeutic uses of cytokines and after deviant expression of cytokines in a variety of transgenic mouse lines (63). Recent progress in understanding the signal transduction pathways and transcription factors activated by cytokines (18, 68) promises to open up new therapeutic approaches as well as novel strategies of gene transfer experiments which will aim at the improvement of the immune response (i.e., the genetic modification of distinct 'cytokine-specific' signalling components) rather than the transfer of cytokine encoding genes per se. In contrast to the germ-line integration of cytokine gene constructs, the transient expression of cytokines by somatic gene transfer has been reported to show the required effects (43, 87).

Peptide-based antimicrobial defence is an evolutionary ancient mechanism of host response found in a wide range of animals (from insects to mammals) and plants (12, 101). These peptides have been classified in four classes based on structural features: the magainins, defensins, melittins and cecropins. These small lytic peptides interact with lipid bilayer membranes to cause osmotic disruption and cell death. Bacterial, protozoal, fungal and damaged eukaryotic cells are most susceptible to disruption. These peptide antibodies are currently thought to solve some of the problems encountered with multidrug-resistant microbes. Recently two transgenic approaches used constructs encoding antimicrobial peptides. Tracheal antimicrobial peptide (TAP) is a member of the β-defensin family which is isolated from the tracheal mucosa of the cow, and exhibits broad-spectrum activity when assayed in vitro against several microbes, including respiratory pathogens. The bovine TAP complementary DNA (cDNA) controlled by the mammary gland-specific WAP (whey acidic protein) promoter was expressed in mice without malign phenotype. The uptake of bovine TAP with the milk also caused no deleterious side-effects in suckling pups. The recombinant bovine TAP purified from mouse milk showed correct folding and processing of the peptide antibiotic and hence antimicrobial activity (104). A similar gene construct might be used in transgenic farm animals for gene farming purposes, i.e., the large-scale production of recombinant TAP. In addition, the mammary gland-specific expression of antimicrobial peptides could be used to prevent enteric infections of neonates, e.g., the enteric Escherichia coli infection in piglets. In the second experiment, a synthetic cecropin-class antimicrobial peptide-encoding sequence was fused to the interleukin (IL) 2 promoter, including the first 20 amino acids of IL-2 serving as a signal peptide for secretion. The expression cassette was chosen to produce the antimicrobial agent in the blood and the lymph. Lymphoid-specific transgene RNA expression could be detected in transgenic mice upon treatment with IL-2 inducers. The antimicrobial activity was tested in a Brucella abortus challenge of the mice. After four weeks, there were significantly lower numbers of bacteria in the spleens of transgenic mice compared to non-transgenic littermates (74). Although some animals showed low-level constitutive transgene expression, the transgenic animals attained normal size, life spans and reproductive performance. The transgenic
mice serve as a model for future use of such gene constructs in gene therapy or the generation of livestock species which are generally more disease-resistant.

Following an acute phase stimulus, such as infection or physical injury, many liver-derived plasma proteins are increased in concentration. These provide enhanced protection against invading micro-organisms, limit tissue damage and promote a rapid return to homeostasis. C-reactive protein (CRP) is one of these acute-phase proteins (85, 88). Mice expressing high levels of rabbit or human CRP under the control of constitutively active promoters were partially protected against lethal challenges by mediators of septic shock, including bacterial endotoxin, and showed a significantly increased resistance to infection with *Streptococcus pneumoniae* (89, 103).

Lactoferrin is an iron-binding glycoprotein found predominantly in milk and granulocytes, and to a lesser extent in exocrine fluids, such as bile and tears. Despite the fact that its specific function has yet to be fully elucidated, lactoferrin has been proposed to play a role in iron uptake by the intestinal mucosa and to act as a bacteriostatic agent by withholding iron from iron-dependent bacteria. In addition, it may be involved in phagocytic killing and immune responses (57). Transgenic mice were produced carrying either human lactoferrin cDNA or genomic sequences controlled by the regulatory regions of the bovine αS1 casein gene. The recombinant human lactoferrin produced in the milk of the transgenic mice did not differ from the human milk-derived protein with respect to biochemical properties and the immunoreactivity (67, 71). The generation of a transgenic bull carrying a mammary gland-specific human lactoferrin construct was also reported (49). However, there is no report on expression studies of transgenic offspring. The experiments described clearly aim at the production of protein with respect to biochemical properties and the specificity and in variations of transgene expression levels in different transgenic lines carrying identical DNA constructs. One approach to achieve a strict spatio-temporal pattern of expression from genes of interest is the use of large gene constructs which provide extensive sequences flanking the coding unit of the gene to avoid unwanted side effects of transgene expression. These constructs could be provided by the currently expanding artificial chromosome vector technology (96). Recently, a report of the first group of yeast artificial chromosome DNA transgenic farm animals (rabbits) has been published (17).

In general, gene transfer experiments in farm animals face several obstacles, as follows:

i) the efficiency of transferring genes by microinjection into pronuclei of zygotes is still too low for routine use in agriculture

ii) DNA microinjection results in random integration of the gene constructs in the host genome. Thus, the transgene expression often underlies 'chromosomal position effects', which results in uncertainty about the expected tissue specificity and in variations of transgene expression levels in different transgenic lines carrying identical DNA constructs.

One approach to achieve a strict spatio-temporal pattern of expression from genes of interest is the use of large gene constructs which provide extensive sequences flanking the coding unit of the gene to avoid unwanted side effects of transgene expression. These constructs could be provided by the currently expanding artificial chromosome vector technology (96). Recently, a report of the first group of yeast artificial chromosome DNA transgenic farm animals (rabbits) has been published (17).

iii) although promising alternative gene transfer methods have been reported, the sperm-mediated transgenesis and gene transfer into foetal cell lines with subsequent nucleus transfer have yet to be evaluated for reliability and efficiency

iv) despite the enormous progress made in the identification of regulatory elements of gene regulation, there is an ongoing demand for basic and applied research on the control of gene expression using laboratory and farm animals

v) the biosafety issues arising with the generation of transgenic farm animals and the food safety evaluation of genetically engineered nutrients remain to be resolved, mainly on the level of politics and legislation (45, 61). The ultimate success of transgenic animals will depend upon the public acceptance of the product.

In addition, gene transfer programmes for the generation of animals carrying disease resistance constructs must consider carefully the possibility of creating or accumulating pathogenic agents able to escape the introduced host defence gene. For example, the strategy of introducing mutated pathogen genes into animals might result in recombination events with wild-type pathogens, thus creating resistant strains or even altered species specificity. A similar scenario has been observed under experimental conditions in intracellularly immunised transgenic plants. Neither transgenesis nor conventional prophylactic or therapeutic measures will be able to overcome the need for optimal...
animal husbandry condition to avoid disease-related problems.

The ongoing genome projects in mammals and the electronically accessible formats of comparative maps will lead to the identification of novel candidate disease-resistance genes and of new regulatory elements for gene transfer experiments (31, 66, 97). While the creation of transgenic farm animals offers tremendous promise for enhancing the quality of production and levels of disease resistance in farm animals, time, money and public acceptance will determine the feasibility of such an undertaking.

Les méthodes transgéniques appliquées à l’amélioration de la résistance aux maladies chez les animaux d’élevage

M. Müller & G. Brem

Résumé
Grâce aux techniques de la génétique moléculaire et de la biologie de la reproduction, on peut transférer de l’acide désoxyribonucléique étranger à des mammifères. Le recours à de nouvelles méthodes visant à modifier la résistance ou la sensibilité aux maladies chez les bovins a pour but d’améliorer l’économie de l’élevage et le bien-être des animaux. Les recherches actuelles sur l’amélioration de la résistance aux maladies par transfert de gènes s’articulent autour des trois principales stratégies suivantes :

a) transfert de gènes somatiques, c’est-à-dire vaccins à acide nucléique ;
b) transfert de gènes invalidés de la lignée germinale (gene knockout) ;
c) transfert de gènes de la lignée germinale avec effet d’addition.

Ces stratégies visent soit à l’expression, provisoire ou permanente, de composants connus pour leur action sur les mécanismes immunologiques, spécifiques et non spécifiques, de l’hôte, soit à l’inactivation de gènes dont on sait qu’ils sont à l’origine de la sensibilité à une maladie. Selon l’origine du gène actif, du site ou du mode d’action, on parle de stratégies d’immunisation « génétique », « congénitale », « intracellulaire » ou « extracellulaire ». Les conséquences de chaque expérience de transfert de gènes doivent être soigneusement évaluées, notamment en ce qui concerne l’apparition possible de nouvelles résistances à des agents pathogènes, la disparition des barrières d’espèces ou encore la perte des restrictions des types cellulaires.

Mots-clés
Constructions de gènes – Gain de fonction – Immunisation – Perte de fonction – Résistance aux maladies – Transgène – Vaccin à ADN.
Métodos transgénicos para incrementar la resistencia a la enfermedad en animales de granja

M. Müller & G. Brem

Resumen
Existen técnicas de genética molecular y de biología de la reproducción que permiten transferir ADN ajeno a un mamífero. Consideraciones de índole tanto económica como de bienestar animal justifican la adopción de sistemas novedosos para modificar la resistencia o la susceptibilidad del ganado a las enfermedades. Tres son las estrategias básicas que sigue actualmente la investigación encaminada a mejorar la resistencia a la enfermedad por transferencia génica. Se trata de las siguientes:

a) transferencia de genes a células somáticas, esto es, vacunas con ácidos nucleicos;

b) transferencia supresora de genes a células de la línea germinal, es decir, supresión específica de genes (gene knockout);

c) transferencia aditiva de genes a células de la línea germinal.

Estas estrategias pueden estar orientadas: bien a la expresión transitoria o estable de moléculas con una influencia conocida sobre los mecanismos de defensa –específicos o inespecíficos– del huésped; o bien a la disrupción de genes con una función conocida causante de susceptibilidad a la enfermedad. En función del origen del agente efector y de su sitio y modo de acción, esas estrategias reciben el nombre de inmunización «genética», «congénita», «intracelular» o «extracelular». Para evitar que un experimento de transferencia génica dé origen a la aparición de nuevas resistencias en un agente patógeno, a la abolición de las barreras entre especies o a la supresión de las restricciones inherentes a cada tipo celular, es preciso evaluar con sumo cuidado los posibles efectos de cada experimento.

Palabras clave
Adquisición de función – Construcciones génicas – Inmunización – Pérdida de función – Resistencia a la enfermedad – Transgen – Vacuna de ADN.

References


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