Disease resistance in farm animals

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Abstract. Genetic variations in disease resistance of farm animals can be observed at all levels of defence against infectious agents. In most cases susceptibility to infections has polygenic origins. In domestic animals only a few instances of a single genetic locus responsible for disease resistance are known. A well-examined example is the Mx1 gene product of certain mice strains conferring selective resistance to influenza virus infections. Attempts to improve disease resistance by gene transfer of different gene constructs into farm animals include the use of monoclonal antibody gene constructs, transgenes consisting of antisense RNA genes directed against viruses and Mx1 cDNA containing transgenes.

Key words. Disease resistance; genetic variations; Mx system; breeding techniques; transgenes.

Introduction

The control or elimination of infectious agents in farm animals has always depended on the use of vaccines and drugs, quarantine safeguards and eradication. However these measures have failed to eradicate some of the major infectious diseases of livestock. Basic research into the mechanisms of disease defences will provide new approaches for eliminating these diseases.

In domestic animals disease resistance can be achieved by vaccination and/or genetic improvement. It has been shown that vaccination programmes can be very successful, but in a number of instances they have been impossible due e.g. to the non-availability of appropriate vaccines, or logistic or management problems in extensive animal agriculture. Some programmes have also been unfavourable because of immunosuppressive effects of vaccines, or outbreaks of disease despite vaccination. In some cases progress has not been made because vaccination programmes have been declared to be illegal, as was the case, for example, with vaccination against foot-and-mouth disease in the US.

Differential genetic disease resistance in farm animals has been known for a long time[46]. The impetus to study the genetic control of immune responsiveness in farm animals was provided over 25 years ago by the discovery of 'immune response'(*Ir*) genes[71].

Continuation and enhancement of research towards improving disease resistance of livestock is justified not only on the ground of animal welfare and economical reasons but also by recent advancements in molecular biology promising higher success rates. The costs of disease have been estimated to account for 10–20% of total production values[111]. Genetic improvement leading to disease resistance may therefore contribute considerably to reduce these costs. Recently developed techniques in molecular biology allow mechanisms of disease resistance and linkage of known genetic loci with disease susceptibilities to be studied in detail. Such techniques can be used also to clone specific disease resistance genes, which will be a prerequisite to the improvement of genetic disease resistance by employing new breeding strategies such as marker assisted selection or gene transfer strategies.

This paper discusses conventional breeding strategies for disease traits and outlines the principles underlying the genetics of disease resistance in farm animals with special regard to principle mechanisms of disease resistance and to infectious agents (non-infectious diseases and genetic disorders are not included). A brief survey of methods allowing identification of disease resistance genes will be presented and an example of a distinct resistance phenotype caused by a single genetic locus, i.e., the Mx1 gene product of certain mice strains conferring selective resistance to influenza viruses, is discussed. This review concludes with an overview of attempts to improve disease resistance of livestock by the application of modern gene transfer techniques.

Problems encountered with conventional disease resistance breeding programmes

For practical purposes investigators will normally focus their attention on two phenotypic disease susceptibility classes (afflicted/not afflicted animals) in which continuous variation is disregarded. However, due to the existence of unknown continuity-causing factors which become noticeable above a certain threshold level disease susceptibility may vary discontinuously, thus necessitating genetic analyses to be carried out quantitatively rather than qualitatively. The continuous variable underlying these phenomena has been termed *liability*. Falconer[30] has calculated the correlation of liability between relatives of any specified sort and the heritability. If a trait like disease resistance must be included in a breeding programme animal breeders will have to consider the four criteria which they already use when dealing with productive or reproductive traits:

- Genetic variability and heritability
- Economic value
- Possibilities and costs for recording data
- Usage of marker traits and genes

Shook[111] has discussed these four criteria with special attention given to disease resistance traits. Although dis-

ease trait heritabilities are normally low, the genetic variation of disease incidences is economically important and justifies the inclusion of disease traits in breeding programmes. Unfortunately standards for recording and accumulating field data for disease have not yet been set up[111]. However, several systems for collecting disease data in breeding populations have been developed and tested recently[12, 25, 26].

Improvement of disease resistance in livestock by genetic means is a difficult and time-consuming task requiring long-term strategies. Progress in resistance breeding is limited and delayed by at least two factors:

- Inadvertent enhancement of susceptibility to a disease by selection for specific resistance to another disease;
- lack of strategies allowing selection for overall resistance.

Selection for disease resistance by employing conventional breeding strategies has at least two advantages:

1) All genetic host factors influencing resistance or susceptibility are automatically included, and 2) selection for the resistant trait is independent of shifts in environmental factors and disease profiles over time.

The principal disadvantages of direct breeding methods are introduced by the following factors:

- low heritability of disease traits (especially when recorded as all-or-nothing responses), thus necessitating expensive progeny testing with prolonged generation intervals;
- age and sex restriction of disease traits, which may also affect the generation interval;
- heterogeneity of disease traits which may also be moderately defined; - genotypes which result in high productive yield but which may increase susceptibility to severe diseases (e.g. slight correlation between milk yield and mastitis).

Genetics of disease resistance in farm animals

The first studies indicating the existence of genetic variations in resistance or susceptibility to infections can be dated back to the beginning of this century[46, 47]. Almost fifty years ago evidence for specific disease resistance was deduced from findings that mice being highly resistant to one infectious pathogen were highly susceptible to other infections.

It has been observed that generalized disease resistance is relatively independent of the nature of the infectious agent. It is caused by the cooperative effects of many genes and is strongly influenced by environmental factors. Antigenic drift of the pathogens usually only has minor or no effects on this polygenic type of defence mechanisms which include a variety of different physiological and anatomical characteristics acting together to invoke resistance. An example of such polygenic traits are trypano-tolerant cattle breeds (e.g., N'Dama and West African Shorthorn) which, in addition to their disease resistance also possess the capacity to tolerate heat, the ability to conserve water, and low maintenance requirements[84].

Disease resistance or susceptibility to a certain disease or pathogen is usually controlled by a major single locus. The defence mechanism may be modulated however by unidentified loci, including genetic regulatory elements, and by environmental factors. The expression of the resistance locus may be a specific predisposing or conditioning factor among a series of other factors. The mechanism underlying resistance can frequently be explained by the presence or absence of certain molecules in the host which are critical for infection, recognition, or elimination of the pathogen. Thus, in contrast to general disease resistance, the trait depends more on genetic and antigenic drift of the pathogen. Typical examples are the major histocompatibility complex and disease associations[23], the resistance to neonatal diarrhoea due to *E. coli* K88 in pigs[108], and the susceptibility of mice to influenza viruses[65].

It is obvious that there are types of resistance or susceptibility which may not conform to the two types defined above. Examples are non-specific defence mechanisms influenced by the expression of major genes such as the activity of lysozymes, interferons, phagocytes etc. and also monogenic deficiencies leading to general susceptibility.

Defence mechanisms against infectious diseases and their involvement in differential disease resistance of farm animals

The defence against pathogens starts at the sites of entry. The surface protection mechanisms can be grouped into two caregories: physical-chemical (desiccation, desquamation, pH extremes, secretion of mucus or other fluids containing, for example acids and enzymes, and body temperature) and biological (competition of pathogens with resident microflora and antibiosis). An example for these types of resistance mechanisms is the defence of dairy cattle against microorganisms causing mastitis[78] and the variation in tick resistance in cattle breeds[107].

The second level of defence mechanisms is characterized by the presence or absence of pathogen receptors. Receptor proteins are mostly membrane-bound, but also exist as soluble proteins with high specific affinities for hormones, antibodies, enzymes, and other biologically active compounds. Pathogens take advantage of existing receptor proteins to facilitate opportunistic penetration in hosts. Only a few instances exist where genetic variation in receptors for pathogens is known to occur in farm animals. Receptors providing access to the host (or host cells) were found in chicken for avian leukosis viruses (ALV) and Rous sarcoma viruses (RSV)[92], and in pigs for enterotoxic *Escherichia coli*[108].

Defences preventing the establishment and reproduction of pathogens are caused mainly by general immune re-

sponse mechanisms and the expression of the major histocompatibility complex (MHC). Resistance mechanisms at this level are very complex and have been reviewed extensively elsewhere [52, 100].

Macrophages frequently serve as accessory or effector cells and are thus involved in numerous immune reactions. The activation and action of macrophages is complex and depends on the stage of maturation, the presence of exogenous and endogenous stimuli, and the susceptibility of pathogens to the toxic reaction of macrophages [9, 38]. The existence of genetic variations in the phagocytic activities of macrophages were shown by Meyer et al. [76] who selected mice for high and low clearance rates of injected high-molecular-weight carbohydrates.

After infection by a pathogen macrophages activate diverse T- and B-lymphocytes which eventually initiate a variety of humoral and cellular responses.

A plethora of molecules that modulate immune reactions have been identified. They include interferons [22], neuropeptides [50], hormones [50], and interleukins [24, 34, 70, 112].

The principle function of the B-cell population is the production of immunoglobulin antibodies directed to protein or carbohydrate moieties of antigens. T-cells either function as effector cells that are involved in cytotoxic mechanisms on target cells and in delayed hypersensitivity reactions, or regulatory cells that stimulate or downregulate both B- and T-cells. The effector molecules involved in pathogen destruction are, for example, oxidative components, enzymes (e.g. arginase) and tumour necrosis factors (TNFs) [85, 93, 100].

Humoral responses to antigens of restricted specificity appear to be controlled by specific single immune response genes (*Ir* genes), whereas responses to heterologous antigens are mediated in each instance by about ten independent genetic loci [10]. Extensive selection studies in mice have indicated that antibody production is negatively correlated with the microbicidal activities of macrophages, whereas cell-mediated immunity is not affected. These findings can be extended to other species including chicken [113], guinea pig, rat, swine, and Japanese quail [144].

Following the discovery that major histocompatibility complex (MHC) gene products play an important role in directing and controlling the immune response, and that they influence resistance and susceptibility to disease [21], several associations of MHC haplotypes with diseases have also been found in farm animals [56, 60, 89, 140]. In vertebrates the MHC consists of several closely linked genetic loci which encode cell-surface glycoproteins and serum proteins known as histocompatibility antigens. In mammals the MHC locus consists of genes divided into class I, II, and III and in chicken they are known as class I, II, and IV.

Class I MHC antigens play a key role in cell-mediated immunity (i.e., antiviral and antitumorigenic receptor responses). Class I molecules serve as 'restriction elements' for cytotoxic T-lymphocytes ($CD8^+$ CTLs); i.e., the receptor on the effector T-cell recognizes an antigen only in association with a MHC class I gene product [52]. Class I molecules are found on almost all surfaces of nucleated cells; however, expression at the highest concentration takes place on the membranes of T- and B-cells and macrophages [52].

Class II molecules are important for humoral immunity, determining the concentration of serum antibodies, and are also involved in helper/inducer T-cell functions ($CD4^+$ cells) in the presentation of foreign antigens. Recognition of the antigen-MHC class II complex by helper T-cells results in the synthesis and secretion of a variety of glycoproteins, known as lymphokines and cytokines. These soluble mediators enhance and modify the immune response by regulating proliferation and differentiation of a series of lymphoid and hematopoietic cells [81]. Expression of class II molecules is restricted to specific cell types such as macrophages, B-cells, dendritic cells, certain activated T-cells, and specialized ephithelial cells [52].

Class III MHC genes code for serum proteins of the complement system (C2, C4, factor B, and steroid 21-hydroxylases) [138].

Other mechanisms provoking resistance against the establishment and reproduction of pathogens include natural killer cells that are present in the host regardless of its prior exposure to antigens against which they are active. Natural killer cells seem to play a role in resistance to Marek's disease in poultry [110]. In cattle a major gene has been shown to regulate the lysozyme system, a general bacteriolytic mechanism [63].

Examples for disease resistance in farm animals

The genetic complexity of the specific and unspecific, humoral and cellular defence mechanisms against pathogens explains the variation in resistance to infectious agents between breeds and within breeds. Indeed, there are a lot of well-documented examples of genetic variation in hosts for susceptibility to pathogens. In farm animals genetic variation in resistance to nearly all kinds of infectious agents has been described for ecto- and endoparasites, bacteria, viruses, and the unusual pathogen(s) causing scrapie in sheep.

Wakelin [142] has reviewed the genetic control of susceptibility and resistance to parasitic infections.

Examples of differential resistance against bacterial infections are resistance to mastitis in dairy cattle [69], resistance to neonatal scours in swine [37, 105, 108] and resistance to pullorum disease in fowl [47], or resistance to fowl cholera [55]. The genetic control of resistance to *Brucellae* spp. infections has been studied in swine [16] and cattle [135].

Genetic variations in the protection against viruses have been observed for Marek's disease [4], avian leukosis virus (ALV), and Rous sarcoma virus (RSV) in chicken [19, 96, 117], equine sarkoidosis [59], caprine arthritis encephalitis (CAE) [104], African swine fever [64], bovine

leukosis virus (BLV)[11,61,62], and myxomatosis in rabbits[115].

The resistance of certain mice strains and other mammals to orthomyxo- and rhabdoviruses[119,124] will be discussed below in greater detail.

Scrapie susceptibility in sheep also shows genetic variations[42,79].

Identification of disease resistance genes

Gavora[35] has specified the essential steps in the study of genetic resistance to disease (fig. 1). The first step is to define the phenotype of disease resistance. The choice and definition of disease resistance parameters should be as simple as possible because large numbers of individuals will have to be screened. Important elements in the determination of resistance characteristics are the symptoms and the time course of the disease, the impairment of productive capacity, titration of pathogen doses in challenge experiments, and the measurement of the basic components of the immune response. In the absence of more detailed knowledge, challenge experiments with a pathogen may be the best starting point for research and selection for disease resistance improvement, because it provides a complete spectrum of the disease susceptibility in the examined species or breed.

Festing and Blackwell[31] have reviewed the methods available for the establishment of the mode of inheritance of resistance to pathogens.

The identification of marker traits – DNA and protein polymorphisms in particular – associated with genetic resistance is an important step in research on disease resistance. The use of genetic markers allows genetic linkage studies, and such studies will simplify the selection of disease-resistant animals by circumventing most of the problems of conventional breeding strategies. Marker-assisted selection (MAS)[64] is carried out by using single markers for well-defined defence mechanisms under the influence of a major locus. The study of polygenically controlled disease resistance (probably accounting for the majority of cases) requires the use of a series of markers for so-called quantitative trait loci (QTL)[36]. Molecular markers can be identified by DNA analysis employing restriction fragment length polymorphisms (RFLPs)[7,145], DNA-fingerprinting with highly informative locus-specific polymorphic probes, e.g., variable number of tandem repeats (VNTRs), or minisatellite DNAs[51], polymerase chain reaction (PCR)[48] and other techniques of modern molecular biology[57]. The strategy of dissecting quantitative traits into several discrete Mendelian factors facilitates mapping of economically important traits in farm animals, including disease resistance loci[6,8]. The progress of this approach will depend on the availability of physical genomic maps[33,87].

A classical example of linkage between disease susceptibility and a genetic marker in livestock is the resistance of the B[21] haplotype of the chicken MHC to the neural lymphoma, Marek's disease (MD)[132]. MHC associations with farm animal disease candidates have been found in many species (table 1). Reviews of the MHC and its disease resistance associations in livestock have been published elsewhere[55,60,89,140]. Marker associations are also useful in mapping and cloning of single genes or gene clusters involved in defence or pathogenesis[114]. An impressive example is the identification of the cystic fibrosis gene in humans[101].

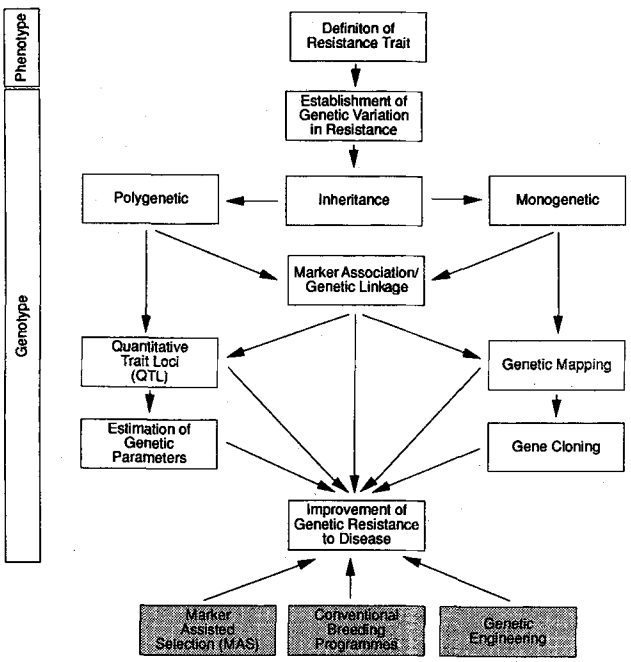

Figure 1. Essential steps in the study of genetic resistance to disease and strategies for improvement of disease resistance (after Gavora[35]).

Table 1. MHC associations of mammalian livestock with disease resistance

Various species	MHC association with disease defence traits	References
Cattle (BoLA)	Enzootic bovine leukosis	11, 61, 62
	Lymphosarkoma and persistent lymphocystosis	41, 60, 61
	Mastitis	78, 88, 116, 118
	Bovine virus diarrhoea	20
	Respiratory diseases	89
	Ticks (*Boophilus microplus*)	131
	Worms (*Cooperia* spp., *Haemonchus placel*)	130
	Ocular carcinoma	129
Sheep (OLA)	Scrapie	79
	Differential immune response to vaccinations	90
Goat (CLA)	Caprine arthritis encephalitis	104
Horse (ELA)	Sarcoids	28, 59
	Allergic diseases	58
Pig (SLA)	Piglet mortality	98
	Differential immune response to vaccinations	103, 139
	Sinclair swine cutaneous malignant melanoma	136
	Trichinella spiralis	68

The involvement of a single genetic locus responsible for disease resistance or pathogenesis has been demonstrated for a few infectious diseases. Well-known examples in livestock are the dominant alleles responsible for receptors providing access to host cells for certain retroviruses in chicken[19], and for enterotoxic *E. coli* in swine[37].

Mx system

The Mx (*myx*ovirus-resistant[66]) system of mice is one of the rare examples of a distinct resistance phenotype linked to a single genetic locus[65-67]. Mx proteins belong to a variety of interferon-stimulated proteins.

A large number of RNA and DNA viruses induce the synthesis of interferons (IFNs) after infection of vertebrate cells[94]. IFNs mediate a transient antiviral state by increasing the expression of a number of IFN-dependent proteins[99,119]. Little is known about the actions of IFN-induced cellular genes that cause the antiviral state. The double-stranded RNA-dependent enzymes (2′-5′)-oligoadenylate [2,5-A_n] synthetase and the P1/elF-2α protein kinase may play important roles in the regulation of viral macromolecular synthesis (inhibition of initiation of mRNA translation) and degradation (mRNA, rRNA)[94,119]. The extent to which these enzymes are involved in the antiviral actions of IFN in vivo has yet to be resolved. At present the Mx protein is the best example of an IFN-induced protein which can be attributed to a specific IFN function.

Mx system of mice

Genetic analysis has established that in mice the antiviral state directed against influenza A and B viruses is controlled by the autosomal dominant $Mx1^+$ allele. The $Mx1^+$ mRNA is mainly induced by interferons α and β (type I IFNs)[120,125] and encodes a 72 kD nuclear protein[27]. The $Mx1^+$ gene comprises at least 55 kb of chromosomal DNA and consists of 14 exons[45].

Mice strains with the $Mx1^-$ alleles are sensitive to influenza virus infections. The $Mx1^-$ genotype is characterized either by large deletions or a nonsense mutation within the Mx1 gene that abolish synthesis of functional Mx1 protein[122].

It is well established that Mx1 protein is both necessary and sufficient to promote resistance to influenza virus infections. Constitutive expression of Mx1 protein in influenza-susceptible cells lacking the protein is sufficient to protect against influenza viruses[86,125]. Moreover, micro-injection of anti-Mx1 antibodies into Mx^+ cells blocks the antiviral state of IFN-treated cells[2]. $Mx1^+$ inhibits influenza virus replication at an early step; however, the precise mechanisms by which Mx1 protein is acting are not known. It has been reported that $Mx1^+$ inhibits primary transcription of the virus[54] or alternatively prevents translation of viral proteins[77].

The Mx1 gene is located on the distal portion of mouse chromosome 16[97,126]. It is closely linked to a related gene called Mx2[127]. IFN-treated cells from influenza resistant mouse strains fail to express Mx2 transcripts. The Mx2 protein has not yet been characterized.

Haller et al.[39] have tested the susceptibility of wild mice to influenza virus. The authors have shown that approximately 75% of tested mice were resistant to influenza virus infections and were able to synthesize Mx1 protein, as expected for mice carrying the dominant $Mx1^+$ allele in either homozygous or heterozygous form.

Mx homologues in other organisms

Southern analysis has indicated that Mx homologues exist in all examined eukaryotes (table 2)[82,119]. Mx-related structures have been described in greater detail in humans[1,44], cattle[43], pigs[17,83], rats[72], fish[128] and even

Table 2. Mx systems in eukaryotes

Species	Mx gene(s)	Protein localization	Function	References
Human	MxA cDNA	Cytoplasm	Inhibition of orthomyxo- and rhabdovirus replication	1, 44
	MxB cDNA	Cytoplasm	Unknown	1, 44
Mouse	$Mx1^+$ cDNA/genomic	Nucleus	Inhibition of orthomyxovirus replication	45, 125
	$Mx1^-$			122
	Mx2 cDNA	Cytoplasm (?)	Unknown	127
Rat	3 cDNAs	Mx1: nucleus	Inhibition of orthomyxo- and rhabdovirus replication	72
		Mx2: cytoplasm	Inhibition of orthomyxovirus replication	
		Mx3: cytoplasm	Unknown	
Hamster		Cytoplasm	Unknown	82
Cattle		2 Mx-related cytoplasmic proteins	Correlation with action of IFN on orthomyxovirus replication	43
Goat		Cytoplasm	Unknown	82
Pig	Mx1 and 2 (partial genomic clones)	Cytoplasm	Unknown	17, 82, 83
Horse		Cytoplasm	Unknown	119
Fish	Mx gene	Unknown	Unknown (inducibility by dsRNA)	128
Yeast	Mx cDNA	Cytoplasm	Essential function in protein sorting	102

in yeast[102]. All well examined mammals express at least two Mx-related genes. Unlike the murine Mx1 protein, most of the other known Mx proteins are located in the cytoplasm. In humans two cytoplasmic Mx proteins have been identified[1,44,123]. Human MxA protein protects cultured cells both against orthomyxoviral and rhabdoviral infections, whereas murine Mx1 protein fails to inhibit rhabdovirus replication[91].

Human MxA, like murine Mx1, appears to act at an early stage of the viral infection cycle. The human MxB protein does not appear to be involved in the inhibition of viral infections[91].

Little is known about the function of Mx proteins in farm animals. The antiviral action of bovine IFN-α on influenza virus has been found to be correlated with the induction of two Mx-related proteins in bovine cells. Both proteins are located in the cytoplasm, as are the homologous human proteins[43].

DNA, RNA and protein studies in IFN-treated porcine peripheral blood lymphocytes have indicated the existence of two Mx genes in pigs. Polyclonal anti-mouse Mx1 antibodies[121] immunoprecipitate in IFN-stimulated porcine cells a protein of approximately 75 kD[83]. DNA sequence analysis of some exons of the porcine Mx genes has revealed an overall homology of about 80% to known Mx sequences of other mammals[17] (Müller et al., unpublished data). The involvement of the porcine Mx system in specific defence of viral infections has not yet been studied.

The ubiquity of Mx proteins and the lack of involvement in viral defence of some Mx proteins suggest that Mx family members may carry out functions that are fundamental to cellular physiology in addition to providing resistance to viruses[102].

Gene transfer strategy

Five classes of mammalian genes are currently seen as possible candidates for gene transfer experiments because they have been implicated in regulating disease resistance:

- MHC genes
- T-cell receptor genes
- immunoglobulin genes
- genes encoding lymphokines[18]
- specific disease resistance genes

The detailed aspects of the transfer of immune system genes into the germ line of laboratory animals are outlined in the reviews by Bluethmann and Iglesias (this issue). Thus some attempts at improving disease resistance via gene transfer in livestock will be discussed in the following.

Intracellular immunization of mammals against virus infections

An interesting approach to the establishment of resistance to virus infections in mammals is known as 'intracellular immunization'[5]. Studies involving the use of cultured cells infected with herpes virus[32] and HIV-1[137] have shown that endogenously produced viral proteins and also their mutated forms that dominantly interfere with the corresponding wild-type virus proteins protect against infections by the cognate virus. By using transgenic chicken that express the avian leukosis virus envelope glycoprotein Salter and Crittenden[106] have demonstrated the defence against ALV. Baltimore[5] has suggested to apply the strategy of intracellular immunization for gene therapy in order to overcome viral diseases against which conventional immunization has proven to be difficult.

Monoclonal antibodies expressed in transgenic animals

A further possibility for protection of animals against infectious diseases could be a strategy called 'in vivo immunization'. It is based on the expression of definitive antibody genes in transgenic livestock. As shown by many investigations, cloned genes of monoclonal antibodies can be expressed in large amounts in transgenic mice after transfer of suitable gene constructs into the germ line. These mice produce antibodies against specific antigens without any prior immunization or contact[133]. Most experiments in mice cited above have focussed attention only on genes for heavy or light chains of antibodies and the primary events induced at the genomic level rather than a full study of the exact composition of secreted antibodies. In an attempt to extend these investigations to other animals genes for the light and the heavy chain of a mouse monoclonal antibody directed against 4-hydroxy-3-nitro-phenylacetate were introduced not only in mice but also into the germ line of rabbits and pigs. Serum antibody titers have been achieved in transgenic rabbits and pigs, respectively of 100 μg/ml and 1000 μg/ml[146]. Unfortunately, only a few bands observed in isoelectric focussing gels were identical to those of the purified mouse antibody. One possible explanation may be that the levels of κ chain expression were insufficient for complete allelic exclusion. The incorporation of additional sequences in the corresponding gene constructs, e.g., downstream light chain enhancer elements[75], might be helpful in overcoming this low expression level. In principle it should be possible in the future to develop gene constructs allowing expression in transgenics sufficient to protect animals against the severe attack by viruses or bacteria in a manner equivalent to immunization. This strategy would be particularly useful for protection against diseases where vaccination is not allowed, difficult or impossible.

Transgenic animals with antisense RNA genes directed against viruses

The use of antisense polynucleotides provides another powerful possibility for the protection against viral infections[73,143].

In prokaryotes a large number of natural antisense RNAs (asRNA; also complementary RNA, cRNA) have

been found to regulate among other things the replication of plasmids, or the translation of mRNA of bacteria, bacteriophages, and transposons[49,95]. In eukaryotic cells asRNA molecules have been discovered in both nuclear and cytoplasmic extracts[49]. The functional importance of these RNAs is still unknown and genes encoding asRNA have not yet been identified. Natural or artificial antisense polynucleotides are capable of specifically suppressing the expression of corresponding genes. The modulation of gene activity in eukaryotic cells by antisense transcription is associated both with the inhibition of mRNA translation and with the disruption of splicing and transport of mRNA into the cytoplasm. In prokaryotes, of course, only the first of these mechanisms is relevant[49,73].

The ability of asRNAs and complementary oligodeoxynucleotides to inhibit replication of viral genomes and expression of their genes has been demonstrated for a number of animal, plant, and bacterial viruses in cell culture experiments[80]. The attempts to create an antiviral state in animals based on asRNA transgenes seems to be a logical consequence of the cell culture experiments[29,80,134]. Ernst et al.[29] have described the generation of transgenic rabbits expressing an asRNA gene directed against adenovirus h5 (Ad5). The asRNA gene was controlled by the murine metallothionein-1 (MT-1) promoter. Despite rearrangements, deletions, amplifications, and a reduction of copy numbers observed during the generation of transgenic founder animals and transgenic offspring, some rabbits had integrated intact asRNA gene copies and stably transmitted the transgenes to their offspring. The resistance to Ad5 infections was tested in primary kidney cell cultures of transgenic rabbits and non-transgenic control animals. Cell lines correctly processing the asRNA transgene have been estimated to be 90–98% more resistant to Ad5 than normal kidney cell lines.

Ernst et al.[29] have demonstrated the feasibility of using asRNA genes to protect against viral replication. However the gene constructs used so far have to be improved to guarantee their stability in the host genome. In addition the use of regulatory elements which are naturally active during defence against infections (e.g., promoters of IFN genes or IFN-dependent genes) may be preferable to constitutive or house keeping gene promoters such as the metallothionein promoter.

Transgenic animals with the murine influenza resistance gene Mx1

Orthomyxovirus infections are common and occasionally cause epidemic diseases, not only in man, but also in a number of domestic animals, such as chicken (fowl plaque), horse (epizootic cough, equine influenza), and swine (hog flu, swine influenza)[109]. In the mid eighties the cloning and functional characterization of the 'anti-influenza' gene Mx1 in mice[125] and the progress made in the transfer of genes into farm animals[13,40] has presented the promising possibility of attempting to improve disease resistance characteristics in farm animals by gene transfer. In the first series of gene transfer experiments in pigs, the gene constructs consisted of the murine Mx1 cDNA placed behind the human metallothionein II_A promoter or the SV40 early enhancer/promoter (Weissmann and Noteborn, 1986, pers. communication). The result of these experiments indicated that high expression of the Mx1 protein during embryogenesis may be deleterious: the efficiency of gene transfer with the constitutively or highly expressing Mx1 gene constructs decreased dramatically in comparison to results obtained with other gene constructs[14]. All transgenic pigs harbouring the Mx constructs either had extensive rearrangements that abolished Mx1 protein expression or did not express Mx1 protein despite the fact that the insertion was intact and stable (Müller et al., unpublished data). Arnheiter et al.[3] have made similar observations in their attempts to generate transgenic mice constitutively expressing Mx1 protein.

The use of a virus-responsive regulatory element – the murine Mx1 promoter itself[45] – linked to the Mx1 cDNA (fig. 2) for micro-injection into pronuclei of fertilized porcine oocytes has yielded eight transgenic pigs. The efficiency of the gene transfer (transgenic piglets/embryos transferred) was 0.5%[14].

The correct integration of the gene construct and the stable transmission to progeny has been examined by differential restriction analysis, Southern blotting (fig. 2), and PCR. The transgenic founder animals contained 10–30 intact integrated copies and transmitted the transgenes stably to their offspring.

The inducibility of the transgenes has been determined in vitro by culturing peripheral blood lymphocytes (PBLs) which had been treated with native porcine IFNs or double-stranded RNA (pol[I]-poly[C]), and in vivo by intravenous application of porcine IFNs ($2-5 \times 10^5$ IFN/m^2 body surface) (Müller et al., unpublished data). After IFN induction, total RNA of PBLs was prepared and subjected to Northern analysis using probes of the gene construct (fig. 3). Two founder animals and their offspring showed an IFN-inducible increase of transgene mRNA levels.

Protein analysis of Mx1 gene constructs have been carried out by methionine labelling of cultured blood cells, followed by immunoprecipitation of proteins with anti-mouse Mx antibodies and by indirect immunofluorescence of tissue sections derived from piglets treated in vivo by IFN (Müller et al., in cooperation with O. Haller, unpublished data). To date, an increase of murine Mx1 protein in the examined transgenic cells and tissues has not been detected. The most probable reason is that the response of the transgenes to IFN is too low to result in detectable amounts of protein. In murine $Mx1^+$ cells Mx mRNA induction leads to levels corresponding to approximately 0.1% of the polyadenylated mRNA in the cells, or about 1000 Mx mRNA molecules per cell[119].

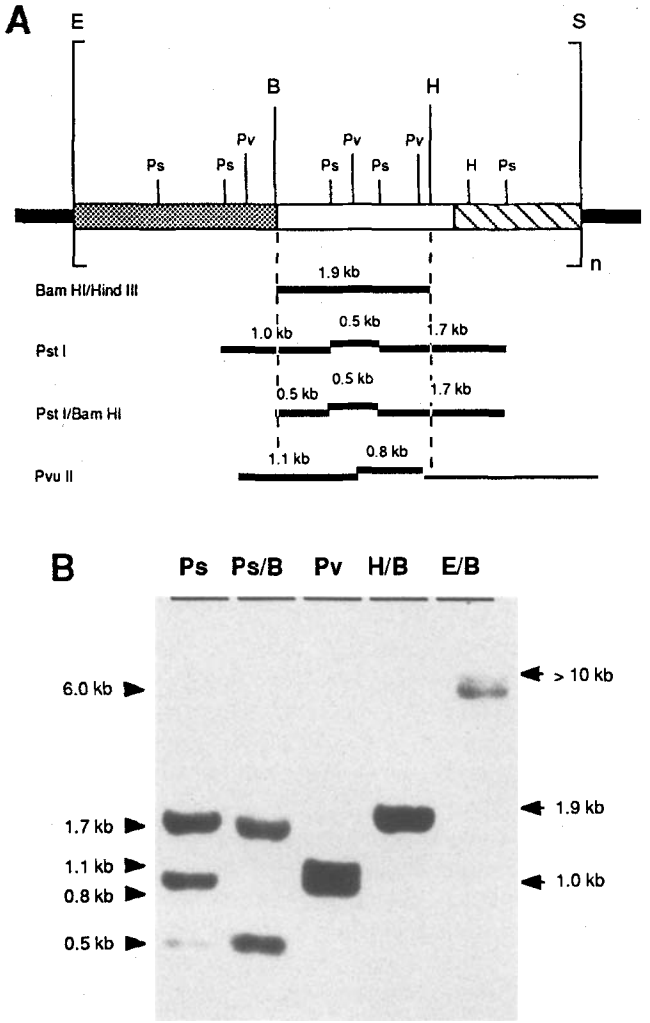

Figure 2. Structure of the injected Mx1 gene construct and Southern analysis of a transgenic pig. *A* Structure and restriction pattern of the Mx1 gene construct. The gene construct contains 2.4 kb of the mouse Mx1 promoter[45] (grey box) linked to the mouse Mx1 cDNA[125] (open box), and the intron-containing mouse β-globin sequences providing the polyadenylation signal[53] (hatched box). The fat lines show the expected bands after cleavage with the indicated endonucleases and hybridization with the mouse Mx1 cDNA (*Bam*HI/*Hin*dIII fragment). *B* Southern analysis of 30 µg Mx1 transgenic porcine DNA cleaved with the appropriate endonucleases. The hybridization probe is described above. The autoradiogramme was developed after 18 h of exposure.
Abbreviations: B = *Bam*HI; E = *Eco*RI; H = *Hin*dIII; Ps = *Pst*I; Pv = *Pvu*II; S = *Sal*I.

The estimated mRNA levels in transgenic cells are considerably lower.

Arnheiter et al.[3] have compared the accumulation of transgenic Mx1 RNA and protein with the corresponding levels in naturally $Mx1^+$ cells. The authors have found in addition to non-expressing transgenics, high- and low-responder animals. The high-responder animals had RNA and protein levels corresponding to 60–70% of $Mx1^+$ mice, whereas the low-responders had levels about 10–20% or below. These data indicate that transgene expression and inducibility depends on the position of the transgene in the genome. What is important for the resistance to influenza virus in transgenics are the levels to which Mx1 protein can be induced. High-responders are protected against virus infection, while low-responders are not protected, or are protected only when infected with high virus doses. This indicates that only the efficient transgene induction is capable of producing the desired effect.

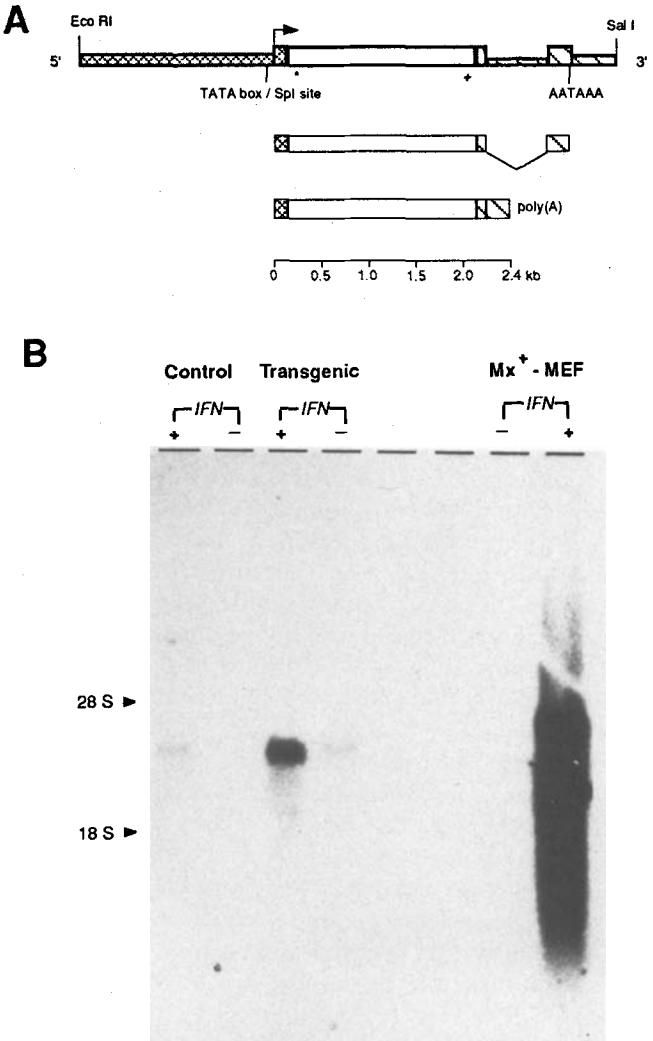

Figure 3. Expected transgene RNAs and RNA analysis of transgene expression. *A* The double-hatched boxes represent the promoter region (lower box) and the 5′ untranslated region of the Mx1 cDNA (upper box), the upper box the encoding Mx1 region, the hatched upper box translated mouse β-globin regions, and the hatched lower boxes the mouse β-globin intron or the 3′ untranslated sequences. Top panel: injected gene construct; middle panel: pre-mRNA; bottom panel: mRNA. *B* Northern analysis of total RNA (25 µg per lane) from peripheral blood lymphocytes of control and transgenic pigs either left untreated (IFN–) or induced with interferon (IFN+). Total RNA (25 µg per lane) from mouse Mx^+ embryo fibroblasts (Mx^+-MEF) was used as positive control. The blot was probed with the Mx1 cDNA probe (*Bam*HI/*Hin*dIII fragment). The autoradiogramme was developed after 18 h of exposure.

Conclusions

Our knowledge of the biological and molecular basis of infectious agents and of the defences against infectious diseases has increased considerably during the past decade and now provides means to modify disease resistance by employing genetic techniques in a more direct approach than has previously been possible. While conventional breeding programmes have mainly concentrated on 'classical' productive and reproductive traits, the new strategies for improving disease resistance will allow *direct selection* for disease resistance traits by using molecular markers (e.g. RFLPs, VNTRs, minisatellite DNAs) and transgenic animals.

The transfer of 'resistance' genes into farm animals is a good example for the potential of these new approaches. The studies reported here demonstrate the general feasibility of gene transfer. Further gene mapping and cloning and especially studies on the mechanisms of regulation of gene expression, defence molecules, interplaying mechanisms, network of pathogenesis etc. will be required. A landmark in livestock gene transfer would be the generation of transgenic chimaeras by injection of genetically manipulated embryonic stem (ES) cells into embryos. In mice this technique has already been established [141]. In farm animals it is probably only a question of time until the first ES-cells will be available. Phenomenons such as extensive rearrangements, non- or aberrant expression of the integrated genes, or deleterious effects of transgene expression could be avoided by the use of precisely defined regulatory elements and extensively tested gene constructs.

Despite the great advances in molecular animal breeding [15] much molecular biological research will be needed before such systems can be implemented routinely. Social and ethical issues associated with the use of these new techniques will also have to be discussed.

Acknowledgments. We thank O. Haller and P. Staeheli for polyclonal anti-mouse Mx antibodies, cooperation and helpful discussions, and A. Arthur and H. Ibelgaufts for critical reading of the manuscript.

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0014-4754/91/090923-12$1.50 + 0.20/0

Transgenic vertebrates. Conclusions and outlook

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Transgenic vertebrates in basic and applied research

Generation of transgenic animals is a fascinating way to examine the effect of genes and their products on developmental, morphogenetic and physiological processes in the live animal. This kind of research is in part dependent on the feasibility to control the level of expression of the transgene. As summarized by Rusconi in this review, much has been learned in the past few years about regulation of transgenes. New control regions have been found and several (to some extent) inducible transcription systems have been developed. Nevertheless, gene expression of most gene constructs used at present in transgenics cannot be controlled at will. This circumstance hampers research at the basic level and more seriously at the applied level. For example, in studying B cell development in transgenic mice it would be helpful if the transcriptional onset of the transgenes (such as H chain genes) could be predicted or, still better, be induced at a specific developmental stage. On the other hand, studies in which the effects of (over)expression of transgenes (such as interleukin genes) are examined in vivo may still yield useful information in spite of varying expression levels of the transgenes (see Iglesias, this issue). The same is true of studies on allelic exclusion which denotes the phenomenon of only the maternal or paternal allele of a gene being expressed in any given cell. This is the case for genes that encode T cell receptor (TCR) proteins. Transgenic mice having integrated a productively rearranged TCRβ transgene show different levels of expression of the transgene. This variation helped to interpret the process of allelic exclusion. When transgene expression was high, the endogenous TCRβ genes were suppressed. When it was low, both transgenic and endogenous TCRβ chains could be detected on the surface of the same cell, indicating that allelic exclusion is a regulated event and not a matter of probability (see Bluethmann, this issue).

An important new method in the field of gene transfer is the possibility of gene targeting by homologous recombination. This demanding technique (emphasized in the chapters by Rusconi, Bluethmann and Wilmut et al.) may solve many of the problems of varying expression of transgenes and also become the method of choice for gene disruption, alteration or replacement. It is hoped that in the future the efficiency of this method can be improved and that it can be applied to animal species other than mice as well.

A considerable number of investigations on transgenesis in vertebrates deals with fish (cf. Houdebine and Chourrout, this issue). Generation of transgenic fish seems not to be a major problem in spite of the invisibility of the pronuclei in most (yolk-rich) fish eggs. However, expression of the transgenes is usually poor, probably due to the fact that most of the transgenes used so far were constructed for work with mammals and contain mam-