MOLECULAR APPROACHES TO DISEASE RESISTANCE

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INTRODUCTION

Genetic resistance to infectious diseases has been a subject of many controversies. The finding in the last 30 years of a number of polymorphisms in genes strongly influencing the outcome of the infectious process in various species has given a definitive response to the question of the existence of genetic resistance to infection. Looking for such genes has now been largely recognized has a powerful tool for the analysis of host/pathogens interaction mechanisms. In domestic species, direct selection of resistant populations is considered as a promising strategy against a number of infectious diseases. In these species, the use of genetic resistance should have advantages in a number of cases. The first idea coming to mind concerns its use against diseases for which we do not have any other possibility, neither vaccine nor therapeutics having been found. Transmissible Spongiform Encephalopathies (TSE) may represent the archetype of such diseases. The second area of major interest for the use of genetic resistance in farm animals are diseases due to a variety of pathogens using similar mechanisms to invade the host and/or to determine lesions as in the case of gastrointestinal nematodes. A third major goal of this approach is the possibility to decrease the carrier state in contaminated animals. Carriage of infectious pathogens by animals without clinical sign is largely responsible for the transmission to human of a number of infectious diseases. In the present communication devoted to the interest of using molecular tools for the discovery and the use of resistance genes in domestic animals, we will take a number of examples from studies on these pathogens of concern both for farm animals and human.

OBSERVATION OF THE VARIABILITY IN MOUSE LINES AND NATURAL POPULATIONS

Among the first reports establishing the existence of a genetic component in resistance to infectious diseases are the pioneer papers of Webster (1933) and Gowen (1948) who compared the relative susceptibility to salmonellosis or viral infections of a number of mouse lines. Although they crossed together some of these lines and suggested through F2 analysis the possibility of discrete genes controlling susceptibility to diseases, they lack markers to perform co-segregation experiments and thus failed to formerly demonstrate their existence. Protein polymorphisms evidenced by electrophoresis provided such markers at the beginning of the sixties. Combined with segregation analysis of well defined phenotypes related to disease

susceptibility, they allowed the location of some genes of resistance to disease in inbred lines of mice, e.g. the *Ity*, *Bcg* and *Lsh* genes (which were considered as identical or closely linked on mouse ch 1) respectively controlling mouse susceptibility to *Salmonella typhimurium*, *Mycobacterium bovis* and *Leishmania donovani* (Rosenstreich *et al.*, 1982), the *Lps* gene (mouse ch 4) regulating the host response to the lipopolysacharide component of bacterial membranes (O'Brien, 1981), the Xid gene (mouse ch X) involved in the regulation of antibody synthesis (O'Brien, 1981). These two genes are also involved in susceptibility to Salmonellosis and several other bacterial diseases. The Mx gene (mouse ch 16) that controls mouse susceptibility to several viral infections was also identified at the beginning of the eighties (review by Staeheli, 1990). In the same time, comparison of selected lines or breeds, or analysis of the variability observed in natural populations suggested the existence of discrete genes controlling the susceptibility to some infectious or parasitic diseases of farm animals (reviewed in Lantier and Vu Tien Khang, 1988). However, very few genes could be identified, essentially because of the very low number of genes or markers available on the farm animals genetic maps. One example of such a gene primarily detected in farm animals by the Neuropathogenesis Unit (Edinburgh) is the *SIP* gene controlling the duration of Scrapie incubation period in cheviot sheep (Foster *et al.*, 1988).

DIRECT CLONING FROM PROTEIN DATA : THE EXAMPLE OF THE PRP GENE

Scrapie is a fatal, progressive sheep and goat neuropathology. It belongs to the group of Transmissible Spongiform Encephalopathy (TSE) of animals and human. These diseases are characterized by the accumulation in or close to vacuolar lesions of the PrP, the protease resistant protein (Goldmann *et al.*, 1991) or prion protein (Carlson *et al.*, 1991). By cloning the PrP gene from a cDNA library using hybridisation with oligonucleotides designed from the reverse translation of the PrP protein sequence, Oesh *et al.* (1985) evidenced that the PrP protein corresponds to the pathological isoform of a post-translationaly modified host encoded protein. The genes controlling the duration of the scrapie incubation period in mice (sinc) and sheep (sip) are thought to be identical to the PrP gene (Moore *et al.*, 1998). A number of observations has shown the cosegregation of polymorphisms of the PrP gene and of the scrapie incubation period in mice and sheep (Carlson *et al.*, 1991 ; Goldmann *et al.,* 1991 ; Clouscard *et al.*, 1995). In human, the polymorphism of the PrP gene also influences individual susceptibility and clinical features of the disease. The PrP encoding gene is highly conserved in mammals and is also found in birds (Lee *et al.*, 1998). The profound effect of mutations of the PrP gene on sheep susceptibility to scrapie (Elsen *et al.*, 1999 ; Andréoletti *et al*., 2000) and the importance of BSE for human health has lead several groups in GB, Netherland and France to propose the selection of resistant sheep as a new mean to control sheep TSE, *i.e*. both scrapie and an eventual BSE strain that could have accidentally been transmitted to a sheep flock. Such a prophylactic action is presently under application in these three countries.

However, the PrP gene polymorphism does not explain the total variability observed in natural or experimental TSE. Several groups are consequently now looking for other genes influencing the TSE pathogenesis in the vicinity of the PrP locus (Moore *et al.*, 1999) or on the whole genome (see the QTL approach below).

THE REVERSE GENETIC APPROACH

The search for genetic markers of resistance to disease and comparative mapping. The tools that now permit the reverse genetic approach (or positional cloning approach) to clone and sequence the located and phenotypically identified genes became available during the eighties. Polymorphism of nucleotide sequences such as RFLP (restriction fragment length polymorphisms) could be detected using specific digestion of the DNA through restriction enzymes and then hybridisation techniques (Southern blot) using molecular probes. Such polymorphisms are much more frequent than protein polymorphisms, allowing a considerable enhancement of the number of available genetic markers in chromosome region of interest. This approach was applied to the precise location of the *Ity/Lsh/Bcg* genes on mouse chromosome 1 (Schurr E, 1990). The enrichment in genetic markers of region of interest also led to the concept of the conservation through the evolution of a number of chromosomal segments. This concept of comparative mapping has become of major importance for the study of genetic resistance to infectious disease allowing the transposition to large animals and human of information from location on the mouse genome of susceptibility genes. For example the mouse chromosome 1 fragment carrying the *Ity/Lsh/Bcg* genes (cloned as *Nramp1*, see bellow) has been shown to be partially conserved in human (Schurr *et al.*, 1990) and domestic animals including birds (Pitel *et al.* 1994 ; Cellier *et al*., 1996 ; Hu *et al.*, 1995 ; Girard-Santosuosso *et al.*, 1997). Because the genetic maps of human and model genomes are the object of constant developments, this concept of comparative mapping has now become a powerful mean to get genomic information and speed up gene identification in less studied species.

Positional cloning and conservation of resistance genes. The discovery of the PCR technology gave to the molecular biologist a number of new genetic markers such as the microsatellites and SNPs (single nucleotide polymorphisms). These new tools facilitate the definition of small chromosomal regions surrounding genes of interest. The larger the analysed segregating population is, the smaller is the interesting chromosomal fragment, thus facilitating the application of molecular strategies of physical mapping**.** A positional cloning approach using a large mouse population segregating for genetic markers of the *Ity/Bcg/Lsh* genes allowed the group of Ph Gros and E Skamene to define a small region surrounding this locus. The use as one of the parental line of a feral mouse line increased the probability to observe polymorphisms at each of the available genetic marker. Cloning of large genomic fragment combine with an exon trapping strategy allowed this group to identify the mouse *Nramp1* gene (Vidal *et al.*, 1993) as a candidate gene. In inbred mouse lines, a polymorphism of this gene was found to be responsible for the resistance (*Nramp* $^{Gly\ 169}$) and the susceptibility (*Nramp* $^{Asp\ 169}$) to intracellular pathogens (Vidal *et al*., 1993, 1995 ; Malo *et al.*,1994). However the definitive demonstration of the *Nramp1* gene being responsible for the resistance/susceptibility to *Salmonella*, *Mycobacteria* and *Leishmania* only came from gene inactivation and reconstitution in knock out mice (Vidal *et al.*, 1995). Because of their potential economic and health interest, the identification in laboratory rodents or human of genes influencing the outcome of infectious diseases prompted research groups working on farm animals to look for the existence of such genes in these species (Staeheli, 1990 ; Lantier *et al.*, 1990 ; Malo *et al.*, 1995 ; Feng *et al*., 1996 ; Qureshi *et al*., 1996 ; Barthel *et al*., 2001). The availability of genomic sequences greatly facilitated this work. Hypothesising sequence conservation between

species, a number of research groups have for example cloned the NRAMP1 gene in bovine (Feng *et al*., 1996), sheep (Bussmann *et al*., 1998), and chicken (Hu *et al*., 1995). This fruitful approach has been extended to a variety of disease susceptibility genes and species. Thus the TolR4 (formerly the mouse Lps gene) has been shown to influence the outcome of salmonella infection in mice and chicken (Hu *et al*., 1997 ; Qureshi *et al*., 1999), probably through the activation of an adaptative immune response by recognizing a conserved microbial structure, and to participate to the mouse pulmonary resistance to *Pasteurella pneumotropica* in conjunction with *Nramp1* and the MHC class II genes (Chapes *et al*., 2001).

THE QUANTITATIVE TRAIT LOCI (QTL) APPROACH

The development of genetic map in domestic animals (Barendse *et al.*, 1997) was a prerequisite for the considerable development in such species of the QTL approach, or "Genome scan". First applied to mouse studies (Lander and Schork, 1994), this approach corresponds to generalization of the molecular tools and statistical methodologies used in studies looking for the cosegregation of genetic marker and a candidate gene. Based on the analysis of a progeny from a parent heterozygous both for the marker and the candidate gene, it evaluates for each locus the statistical difference at the phenotypic level between individuals receiving one or the other of the two alleles of the genetic marker. In mammals, the simplest system may result from the progeny analysis of a back cross between a F1 from two inbred line with different susceptibility to a given disease and the disease susceptible parent line. In the case of a single gene with a dominant resistance allele there will be a statistically significant relationship of the resistance phenotype with the linked genetic marker allele from the resistant parental line. Similar analysis can be extended to the analysis of F2 population or of progenies from crosses between outbred populations (Haley *et al*., 1993, LeRoy and Elsen, 1993). In the case of resistance to disease in domestic animals a QTL approach has been able to define a new salmonella resistance gene in chicken (SAL1, Mariani *et al*., 2001). In the case of larger animals with long generation intervals such as ruminants, the cost and the duration of such experiments that requires large sized populations has led to protocols devoted to the identification of QTL for a variety of unrelated characteristics: wool traits, carcass quality and salmonella resistance in sheep (Moreno *et al*., 2001; Ponz *et al*., 2001) or milk quality, fertility and parameters of mastitis susceptibility in bovines (Zhang *et al*., 1998). Another possibility might be to first define chromosomal region of interest in mice in order to limit the expensive work of genotyping. As an example of such strategy, QTL analysis have been performed in mice with the aim to identify regions of the genome outside the PRNP gene that support the variability observed in natural populations infected by the agents of the bovine or ovine TSE. Three complementary studies have been published (Manolakou *et al*., 1998, Stephenson *et al*., 2000, Llyod *et al*., 2001) and we performed an additional one (see the communication of Moreno et al in this congress). Although they used similar approaches, these four studies were based on complementary experimental protocols (Scrapie or BSE agent, laboratory or feral mouse lines). A dozen of new QTL were detected, some of them by several studies. Although this result confirms that other genes than the PRNP one are influencing the duration of the incubation period, their identification through a positional cloning approach remains a long process. The relative importance of each of these QTL in ruminant populations cannot be inferred from mouse studies. One can imagine that two possibilities should be explored. The first one is to confirm the existence of QTL in ruminant chromosomal regions homologous to

the one defined in mice through familial analysis. Such a protocol is presently in progress in a sheep flock naturally infected by the scrapie agent (Elsen *et al*., 1999). The second complementary strategy consists in researching candidate genes in these chromosome regions.

THE CANDIDATE GENE APPROACH AND THE ANALYSIS OF MECHANISMS OF RESISTANCE TO INFECTION

The major difficulty encountered by such approach is linked to the size of the putative QTL region. As for positional cloning, the first step consist in accumulating new markers in the target portion of the genome in order to better define the QTL location. Simultaneously, one can have a look on genes potentially concerned with the disease resistance/susceptibility phenotype. Of special interest in this case are the genes involved in immune mechanisms related to the infectious process. Beside the genes already identified as "disease susceptibility" genes such as the already mentioned NRAMP1 or PrP genes, polymorphisms of effector or regulatory molecules may affect the efficiency of the host immune response against viral, bacterial or parasitic diseases. Pathogenesis studies with knock out mice in which one or several of the interferon (IFN) genes themselves or of the genes coding for the receptors to this cytokines family have been eliminated illustrate the central role of interferon in host mechanisms of resistance to a number of parasitic, bacterial or viral diseases (Samuel, 2001 ; Dessein *et al*., 2001). The role of Interferon gamma in human susceptibility to Mycobacterial diseases is the object of a continuous interest, genetic deficiency in this cytokine or in its receptor inducing mortality in children infected or even vaccinated with *Mycobacteria* (Abel et Casanova, 2002 ; Dupuis *et al*., 2000). However, the elucidation of the mode of action of this cytokine family is difficult to determine because of the number of regulatory and effector molecules activated by interferons. The Mx proteins are among the few effector of the interferon with known antiviral activities. The Mx gene has been described a long time ago as controlling mouse susceptibility to a number of viral infection in mice. It corresponds to a highly conserved family of interferon (IFN) responsive genes that code for structurally related nuclear and cytoplasmic proteins collectively referred to as Mx proteins. The Mx1 and Mx2 murine genes show a high degree of sequence similarity and are both located on chromosome 16 (Staeli, 1990). Ortholog genes have been cloned in fish, birds, mammals (including farm animals, Ellinwood *et al*., 1998) and human (Hefti *et al*., 1999) and a number of polymorphisms have been identified. Recent results using transgenic mice suggest that Mx proteins have antiviral properties on their own and should represent an interesting molecule in term of improvement of the resistance of farm animals to a number of viral diseases.

THE EXPRESSION PATTERN APPROACH

The emergence of new tools and approaches for study of the host pathogens interactions, including functional genomic, should lead to further insights into the structure-function relationship of a number of genes of susceptibility to diseases and to the identification of key components of the disease resistance mechanisms, which could represent target for an effective selection for multi-resistance to pathogens of importance in animal populations. The major advantage of these new molecular biology techniques is their possibility of simultaneous application to large numbers of samples. According to each technology, one can compare large numbers of individuals, laboratory rodents strains or cell lines submitted to various stress conditions and test for the expression of thousands of target molecules or genes. Approaches

such as the hybridisation of differential mRNA on high-density oligonucleotides arrays have been applied to the "profiling" of the host response to variations of physiological and pathologic conditions. However one of the difficulties of these approaches resides in the definition of criteria used to classify genes as "regulated", *i.e.* to distinguish real activation from back-ground and minimize the number of false positive. This is a complex problem when comparing outbred animals submitted to a variety of natural uncontrolled stimuli. In order to simplify experimental model, one may use animal lines in controlled environment or *in vitro* culture systems. This approach has been developed by the group of C Nathan to investigate the macrophage response to Interferon gamma (IFNg) and/or *Mycobacterium tuberculosis* (Mtb) infection (Ehrt *et al*., 2001). Macrophages are both a target cell for intracellular pathogens and the "chef d'orchestre" of the induction of the immune response in the early phase of the infectious process. Their activation increases phagocytosis, synthesis of inflammatory and regulatory mediators, and production of the bactericidal derivatives of Nitric Oxyde (NO) and Reactive Oxygen Intermediate (ROI). These bactericidal compounds, respectively encoded by genes NO-synthase (iNOS) and phagocyte oxydase (Phox), play an essential role in host defense against a variety of pathogens. Microarray experiments reported in the paper from Ehrt et al (2001) showed that macrophage activation induced the suppression or the induction of a total of about 2000 genes. Mtb mimicked or synergized with IFNg rather than antagonized its action, confirming the central role of this cytokine in the resistance to intracellular pathogens. However the same strategy applied to macrophages deficient in iNOs and phox reveales that these two enzymes or more probably the cascade of their products help orchestrate the profound remodelling of the transcriptome that underline macrophage activation, suggesting a modified view of signal transduction by protein-protein interaction relays.

CONCLUSION

Understanding of infectious disease pathogenesis requires identification and characterization of host/pathogen interactions. Through evolution, a series of innate immune defense mechanisms have evolved to protect the host against the constant threat of microbial injury and direct the development of specific adaptive immune responses (Qureshi *et al*., 1999). Genetic analyses of host resistance in animal models or natural populations submitted to high infection pressure have provided new insights in the mechanisms of host immune response and demonstrated the feasability of selection for disease resistance in domestic animals. Rapid advances are now being made in the integration of dense genetic maps and complete sequence of model and human genomes. Comparative genomics and sequence analysis will play an increasingly important role in facilitating the transfer of new knowledge from the best known models to farm species of economic importance. However, farm animals may have also a pivotal role to play in this knowledge acquisition through their particular capacity to be both a target species of veterinary importance and animal models for other organisms including human. As such, they should beneficiate of the application of the new technologies of functional genomic.

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