Immunology and Genetics: Phenotypic, Genetic and Epigenetic Variation of Bovine Immune Responses and Disease Resistance

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Abstract

The immune system integrates innate and adaptive host defense mechanisms and is largely responsible for control of infectious disease. This system is under the tight genetic regulation of hundreds of genes. Some disorders are controlled by one or a few genes, while other more complex infectious diseases are controlled by many genes. Infectious diseases of livestock, including cattle, have become one of the most hazardous and expensive problems facing the agri-food industry. Many of these emerging and re-emerging diseases are zoonotic, causing concern to both animal and human health. Certain infectious diseases can be controlled by traditional methods; however, increasing restrictions on antibiotic use and the sizable costs associated with new drug development are making it more challenging to manage animal health. Therefore, alternative strategies are required for disease prevention that address these concerns for improved food safety and animal well-being. Given the role of the immune system in control of infectious disease, its genetic regulation, and that it is often possible to identify naturally immune individuals within a population, implementing thoughtful genetic strategies to enhance the immune system should help to improve inherent disease resistance. The emerging solutions integrate a variety of molecular and quantitative genetic approaches with both immediate and long-term improvements to animal health. This manuscript describes some of the strategies involving genetic regulation of the immune system.

Résumé

Le système immunitaire intègre des mécanismes innés et évolutifs de défense de l'hôte et est en grande partie responsable du contrôle des maladies infectieuses. Ce système est sous l'étroite régulation génétique de centaines de gènes. Certains troubles sont contrôlés par un ou par quelques gènes, alors que d'autres maladies infectieuses plus complexes sont contrôlées par un grand nombre de gènes. Les maladies infectieuses du bétail, notamment celles des bovins, sont devenues l'un des problèmes les plus dangereux et coûteux auxquels fait face l'industrie agroalimentaire. Plusieurs de ces maladies émergentes et réémergentes sont des zoonoses, ce qui entraîne des préoccupations tant pour la santé humaine que pour la santé animale. Certaines maladies infectieuses peuvent être contrôlées au moyen de méthodes traditionnelles. Toutefois, l'accroissement des restrictions sur l'utilisation des antibiotiques et les coûts appréciables associés à la mise au point de nouveaux médicaments font de la gestion de la santé animale un défi encore plus grand. Pour prévenir les maladies, il est donc nécessaire de disposer d'autres stratégies qui ciblent ces problèmes et assurent une amélioration de la sécurité alimentaire et du bien-être des animaux. Compte tenu du rôle du système immunitaire dans le contrôle des maladies infectieuses, de sa régulation génétique, et du fait qu'il est souvent possible d'identifier des individus naturellement immuns au sein d'une population, la mise en place de stratégies génétiques réfléchies pour renforcer le système immunitaire devrait aider à améliorer la résistance inhérente aux maladies. Les solutions émergentes intègrent une foule de méthodes de génétique moléculaire et quantitative comportant des améliorations tant immédiates qu'à long terme de la santé animale. Le présent document décrit certaines des stratégies mettant en cause la régulation génétique du système immunitaire.

Introduction

There are many emerging and re-emerging diseases, many of which are zoonotic. The increasing restriction on antibiotic use in livestock and sizeable costs associated with new drug development are making it more difficult to manage animal health. Additionally, consumer concern for improved food safety is demanding alternative approaches to disease prevention which do not rely on extensive use of traditional antimicrobials. It is now well accepted that in order to maintain animal health and excellent food quality, genetic selection for production must be accompanied by improvement in health traits.[31,37] In many countries, including Canada, this conclusion has resulted in the inclusion of

health related traits, such as milk somatic cell score (SCS), into selection indices.[35] Nevertheless, there is further need to identify superior markers of animal health to alleviate rising disease concerns. Knowledge of the genetic ability to respond immunologically should facilitate approaches to identify and select animals with enhanced inherent disease resistance while reducing the risks associated with conventional antibiotic and drug therapies. Ideally, these methods will support and enhance traditional approaches to disease prevention.

The immune system is composed of integrated, genetically and environmentally regulated sets of cells and molecules that control the response to external and internal stimuli, including pathogenic microorganisms. Genetic regulation of immune responses and selection for disease resistance in livestock is recognized as a potentially economical and prophylactic approach to improve animal health.[35] There is evidence in a variety of species that selective breeding for high (H) or low (L) immune response influences resistance to infectious disease.[24,55] In most species, including cattle, heritability estimates for antibody and cell-mediated immune responses are stable and moderate-to- high, indicating that genetic selection is feasible.[20,42,46,61] In fact, identification of high immune responders produced benefits in pig and dairy cattle health and production (reviewed Wilkie and Mallard, 2000). Immunologically defined populations of livestock can also be utilized as a tool to identify the proteins and genes that govern these useful phenotypes. Recent studies by our group have allowed refinement of the methods used to identify H and L responder cows and to confirm heritability estimates for primary (0.37) and secondary (0.42) antibody, as well as cell-mediated (0.20-0-49) immune responses.[20,46] Additionally, as a means to discover bovine genes involved in immune response and disease resistance, a number of gene arrays have been developed,[10,14] including a cDNA immune-endocrine thematic microarray.[56] Early evidence indicates sets of genes associated with chronic *Staphylococcus aureus* mastitis,[56,58] as well as other diseases of cattle.[11] Ongoing experiments are beginning to uncover genes associated with innate host defense,[7] high and low SCS[53] and immune responsiveness in Holsteins.[22]

Sources of Phenotypic Variation

Observable traits for an individual, including immune responses, are referred to as the phenotype. For measured traits of relevance to improved performance two factors, genotype and environment, determine phenotypic value of an individual. Genotypic value includes the combined effects of all genes and their interactions, whereas the environment includes the combined effects of all non-genetic factors. The genotypic value (G) is more or less determined at conception, and the environment (E) is the combined effect of all factors that have influenced the phenotypic value (P) of a given trait up to the time when the phenotype is measured.[39] This relationship is commonly expressed in the following equation: P = G + E. The genetic component can be divided into the additive (A), dominance (D) and epistatic (I) fractions. The linear additive fraction is also called the individual's breeding value, and D and I account for the manner in which the genes dominate, combine and interact between loci. A more comprehensive mathematical formula can therefore be written as P = A + D + I + E, in which the proportion of the additive genetic variance in relation to the phenotypic variance (V_A/V_P) is known as heritability (h^2). This information is used to determine an animal's breeding value (EBV, the sum of the effects of all favorable genes), and to select better-quality animal's for breeding.[39] These basic principles of quantitative genetics and animal breeding have been used to improve performance traits of livestock for decades, and have been more recently applied to evaluate the variance associated with health related traits, including immune response.[63,64]

High and Low Immune Response Phenotypes

Quantitative geneticists and animal breeders have discussed the concept of breeding for disease resistance for many decades.[39] High and low immune response phenotypes have been more recently described.[38,42,61,63] The success of breeding livestock, including cattle, for greater disease resistance requires identification of beneficial phenotypes or genes that contribute to resistance and pathogenesis of disease. Also of critical importance is the heritability of selected traits, which for general immunity, as well as adaptive immune responses in dairy cattle has been estimated around 20-40%.[3,20] Ideally, selection using phenotypic information should be made on the basis of estimated breeding values (EBVs). Using the guiding principle that optimal disease resistance should be the function of optimal innate and acquired resistance-mediating defense mechanisms, pigs and dairy cows with high and low immune responder phenotypes have been identified.[64] Pigs selected for nine generations for high immune response showed enhanced response to vaccination, increased rate of gain and improved resistance to natural and artificial challenge with few exceptions.[63,64] Similarly, Holstein dairy cows could be classified into three groups based on immune response during the peripartum period. High responders had no decline in peripartum antibody response, whereas other cows had a decline in response either at week 3 prior to calving or during the week of calving.[61] High immune response phenotypes had improved response to J5 *Escherichia coli* vaccination, more antibody

in milk and colostrum, and less disease occurrence, although this was herd and parity dependent.[61]

Heritability and Genetic Correlations of Immune Response Traits

As with production traits, phenotypic or genetic markers can be used to improve or maintain important heritable health traits. In dairy cattle some traits suggested for selection for resistance to mastitis include clinical occurrence, udder and teat conformation, and SCS.[45] Although mastitis is an important disease of dairy cattle, longterm selection based on SCS may not benefit general improvements in immunity or disease resistance. Focusing on selection for resistance to one pathogen or disease does not necessarily give resistance to others. For example, selection of cows to resist *Brucella* has been partially successful[2] in that macrophages from cattle selected for resistance better inhibited intracellular multiplication of *Brucella abortus, Mycoplasma bovis, Salmonella dublin*, but not *Salmonella typhimurium*.[43] Other examples suggesting a cautionary approach include national sheep flocks genetically selected for resistance to typical strains of scrapie that are now susceptible to emerging strains,[6] as well as current reports suggesting a reciprocal relationship between resistance and susceptibility to West Nile and human immunodeficiency virus (HIV) infection based on the CCR5 delta32 mutation.[18] Consequently, disease specific approaches are likely only appropriate under certain conditions. Since the immune system is central to the prevention and control of infectious disease, other traits that are candidates to more generally improve disease resistance include innate and adaptive resistance mechanisms.[3,63] A heritability of "generalized immunity" for dairy cattle has recently been estimated at 0.20.[3] Additionally, antibody (AMIR)- and/or cell (CMIR)-mediated immune responses, relevant to control of extra- and intracellular pathogens, respectively, have been tested as candidate traits for improving health. AMIR has been used to classify dairy cattle as high, average or low responders.[21,61] Heritability of serum antibody to OVA and an *E. coli* J5 vaccine varied between 0.32-0.88 depending on week relative to calving.[61] Interactions between immune response classification, parity and production were also reported.[62] A study of immune responses in non-peripartum lactating cows estimated variance components for four multiple trait animal models, using the VCE4.2 proceedure. The model for each trait was as follows:

$$y_{injghk} = \mu_{injghk} + c_i + l_n + b_j + s_g + x_h + z_k + e_{injghk} ;$$

where μ_{injghk} = overall mean; c_i = random effect of cow i; l_n = fixed effect of lactation n of cow i; b_j = fixed effect of year of birth j of cow i; s_g = fixed effect of season of birth g of cow i; x_h = fixed effect of year of lactation h of cow i; z_k = fixed effect of season of lactation k of cow i; and, e_{injghk} = random residual effect. Heritabilities for AMIR and CMIR were in the range of 20-40%, depending on antigen and day of test.[20] In a large commercial herd, h^2 for peripartum AMIR and CMIR were lower, ranging from 12-23%.[20] In all cases, heritabilities are sufficient to allow genetic selection to increase both AMIR and CMIR. Although some of the specific genes underlying complex traits, such as immunity, have been identified, practical utilization is currently still challenging because of gene x gene and gene x environmental interactions, as well as problems associated with low penetrance, genetic heterogeneity and intricate bioinformatic analyses.[19] Currently, the best approach may be a genetic selection program for general disease resistance that includes EBVs for conformational health, immune response and production traits.

Canadian Bovine Mastitis Research Network

Recently, the Natural Sciences and Engineering Research Council of Canada (NSERC) has funded a Canadian Bovine Mastitis Research Network (CBMRN, *www.mastitisnetwork.org*) composed of more than 30 researchers across the country studying various aspects of mastitis prevention and control. One of the research themes being investigated is integrative genomic and proteomic strategies to identify immunological profiles associated with enhanced host defense against mastitis pathogens. As one component, *Staphylococcus aureus* as a major pathogen associated with subclinical and persistent mastitis is being examined. In the case of *S. aureus* mastitis, antibiotic treatment is often ineffective and efficacious vaccines are currently unavailable. It has recently been determined that persistent strains tend to survive within host cells and have the characteristics of small-colony variants (SCVs).[36] *S. aureus* SCVs are naturally occurring forms that were first identified over 80 years ago in association with various human diseases that involve persistent and recurrent infections. In human medicine, researchers and clinicians are well aware of *S. aureus* SCVs, but these strains have been overlooked until recently in veterinary medicine when a bovine SCV strain (SCV Heba3231) was isolated from a persistently infected Guelph herd.[5] This isolate had typical gentamicin resistance, slower growth rate and survived longer within cultured bovine aortic endothelial cells without extreme cell damage, compared to its isogenic parent strain or to a prototypic Newbould strain 305.[5] Host response phenotype and immune response gene expression are currently being compared following intramammary infection with the SCV, the parent strain and Newbould 305.

Major Histocompatibility and Other Candidate Genes Associated with High and Low Immune Responsiveness and Mastitis Resistance

Genes of the Major Histocompatibility Complex (MHC), as well as a variety of other candidate genes, have been examined for their association with immune response and disease resistance in cattle. Polymorphisms within the bovine Toll-like receptor (TLR) gene family have also been recently reported.[59,60] For example, several single nucleotide polymorphisms (SNPs) associated with high and low somatic cell counts (SCCs) of Canadian Holsteins were detected within the promoter region of the bovine TLR-4 gene.[53] TLR-4 is a cell surface receptor with the ability to bind lipopolysaccharides of gram-negative bacteria and is critical for initiation of downstream immune responses. Similarly, SNPs detected within the bovine NOD2 gene, which encodes an intracellular pathogen binding and signaling molecule, were associated with somatic cell score, as well as milk production traits.[41]

Molecules encoded by the MHC are well known to be involved in regulation of immune response and disease resistance of mammals, including cattle.[44,55] Numerous studies have reported associations between bovine MHC (BoLA) class I and II molecules and resistance or susceptibility to mastitis,[1,12,23,30,32,52,54,65] bovine leukemia virus,[66] foot and mouth disease,[16] as well as a variety of other pathogens.[55] However, only occasionally is this information useful in commercial settings because of the inverse relationships with more than one disease or immune response phenotype. Nevertheless, in the case where one particular disease is highly prevalent in a given environment and causing major health concerns, it is possible to utilize MHC associations to reduce disease occurrence. The best example in cattle is use of a MHC class II DR/DQ haplotype significantly correlated with resistance to bovine dermatophilosis.[28,29] Additional information is clearly still required to understand the biological and genetic mechanisms associated with these diseases before they can be effectively used to improve livestock health and well-being. It is also worth remembering that MHC genes are only one of the many genes known to be involved in immune response and disease resistance.

Recent experiments by our group[48] examined associations between expression of BoLA DRB3.2 alleleic varients, immune response, SCSs and clinical mastitis. Cows were evaluated *in vivo* for both AMIR and CMIR as the two main components of the immune system that generally predominate in response to extracellular and intracellular pathogens, respectively. In cattle, as well as a number of other mammalian species, antibody responses that are dominated by the IgG_2 isotype, interferon-γ and predominant CMIR are known as type 1 immune responses; whereas, type 2 responses are dominated by AMIR with overriding IgG_1 or IgA isotype production, and interleukin-4. Interestingly, and consistent with the understanding of type 1 and type 2 immune responses,[12] associations between BoLA DRB3.2 alleles and immune responses tended to be opposing for antibody and CMIR.[48] The alleles DRB3.2 *3 and *24 were associated with higher antibody production but lower CMIR, while BoLA allele *22 was associated with lower antibody but higher CMIR. These results clearly support the notion that AMIR and CMIR are genetically independent traits that represent opposing type 1 and type 2 immune responses. This also confirms the value of identifying individuals with the ability to produce both type 1 and 2 responses to improve general disease resistance to both intra- and extra-cellular pathogens.

Gene Discovery using Microarrays

It is not uncommon to study one or a few genes as candidate markers associated with immune response or disease resistance, but it is now also possible to begin to simultaneously evaluate genetic interactions among these genes using genomic tools. Although these genetic interactions are multifaceted, can vary greatly over time and depend on the tissue examined, microarrays offer a novel opportunity to explore sets of genes and their expression patterns. Microarrays commonly include hundreds or thousands of genetic elements placed on glass slides and used to evaluate genetic profiles of livestock, including cattle.[7,17] In some cases, specialized arrays containing immune response elements have been developed.[4,22,50]

To facilitate genetic profiling of cattle, a bovine immune-endocrine array was designed to examine the transcriptional mRNA expression of about 200 genes known to be involved in various aspects of host immunity.[56] In one study, genes ssociated with persistent bovine *S. aureus* mastitis were examined in a case-control design.[57,58] Blood mononuclear cells (BMCs) and milk somatic cells were obtained from Holsteins persistently shedding *S. aureus* and their herd/age/parity/stage of lactation-matched healthy controls. Twenty-two genes from BMCs and 16 genes from milk cells were differentially expressed in case versus control samples. Microarray results were confirmed by real-time PCR and the data interpreted using gene pathway and ontology information. Some genes, such as interleukin-8, have formerly been shown to be involved in other chronic diseases while other genes, including transporters associated with antigen processing and growth hormone, may represent more novel gene combinations associated with persistent bovine mastitis.[58]

As described previously, an immune response index was developed to classify cows as high, low or aver-

age for antibody and cell-mediated immune responsiveness.[21] Cows high for both traits are thought to have the potential to produce more balanced type 1 or type 2 immune responses required to control intra- or extracellular pathogens, increasing their likelihood for improved broad-based disease resistance. By comparing the gene expression patterns of high and low responders, it was possible to begin to identify some of the genes associated with the high and low immune response phenotypes. Preliminary findings indicate differential expression of a variety of molecules involved in cellular communication; for instance, cytokines, chemokines, MHC and T-cell receptor subunits, among cows of high, low or average responder phenotypes.[40]

Single Nucleotide Polymorphism Genotyping

The bovine genome has now been fully sequenced and thousands of SNPs have been identified.[15] Simultaneously, genotyping technology has been created to test individuals for thousands of markers. For example, the *Affymetrix Bovine Genome Array GeneChip*® has been developed in collaboration with researchers, taking into account all the publicly available expressed sequence information. At the moment, this SNP array can be used to study the expression of over 23,000 bovine gene transcripts and evaluate associations with health and production traits of cattle. As new SNPs are found, they are being added to the arrays. SNPs located more or less evenly across the entire genome (at approximately 1 centi-Morgan intervals) can also be used in a novel form of marker- assisted selection (MAS). Identification of SNPs in linkage disequilibrium with quantitative trait loci will facilitate genome-wide MAS[17]. Alternative breeding schemes are being evaluated to integrate optimal genomic selection into current breeding programs. Meuwissen *et al*[33] suggested estimating the effects of each interval for any given trait and found the reliability of these genome-based EBVs (GEBVs) to be ~80%. The cost and labor advantage is that GEBVs with 80% reliability can be obtained at birth by genotyping the calf using available SNPs without massive progeny testing. Additionally, it has been suggested that greater genetic change per year could be realized using GEBVs than with the traditional progeny testing procedures. The increased improvement comes from increased accuracy of GEBVs at an earlier age, allowing the generation interval to be reduced. This procedure should be useful for improvement of all traits, including health, where progress has been difficult.

Epigenetic Information

Epigenetic variation includes alterations in the DNA structure that do not pertain to the actual nucleotide sequence (*www.epigenome.org*). DNA methylation, acetylation, phosphorylation and modification of histones are important sources of epigenetic regulation. These alterations to the DNA structure can decrease (e.g. methylation) or increase (e.g. de-methylation) gene activation or expression due to the changes they induce in chromatin structure. A well-known example is IGF-2, an imprinted gene which is turned off on the maternal chromosome as a result of DNA methylation. Epigenetic patterns are very sensitive to environment influences, and therefore can dramatically and quickly alter an individual's phenotype.

During peripartum, dairy cows experience increased disease susceptibility associated with calving stress and suboptimal immune responses.[61] Cytokine secreting CD4+ T helper 1 (Th1) cells responding to intracellular pathogens initiate a characteristic IFN-γ, IgG2-associated type 1 immune response. Conversely, Th2 cells tend to secrete IL-4, IL-5, IL-10 and IL-13 and respond to extracellular pathogens, supporting a type 2, predominately antibody-mediated immune response in which IgG1, IgE and IgA isotypes prevail. Experimental evidence in dairy cows indicates a shift from Th1 to Th2 immediately following calving.[51] This shift may increase disease risk, particularly when IgG2 is required. In mice and humans, cytokine expression is influenced by epigenetic mechanisms such as DNA methylation.[9] Therefore, in a recent study,[49] IL-4 and IFN-γ cytokine promoter methylation patterns were examined during the peripartum period in order to examine potential epigenetic influences on bovine cytokine gene expression of dairy cows. Blood was collected four weeks before and four days after calving, and CD4+ T-cells isolated. Genomic DNA extracted from T-cell mitogen (ConA)- stimulated and unstimulated CD4+ cultured cells were exposed to sodium bisulphite. Bisulphite- converted and unconverted DNA was PCR-amplified from IFN-γ and IL-4 promoter regions. PCR products were cloned, sequenced and CpG dinucleotides identified. Preliminary results indicate that prepartum, ConA stimulation was associated with decreased methylation at four of the five CpG dinucleotides examined, suggesting increased transcriptional accessibility. Conversely, postpartum ConA stimulation increased methylation.[49] Changes in cytokine expression patterns during the peripartum period of dairy cows may relate to epigenetic changes in promoter methylation patterns, and needs to be examined further to determine how these alterations may influence peripartum health.

Proteomics

Proteomics—high through-put protein expression profiling—has the potential to extend the understanding of biological processes beyond the transcriptome, since gene transcription does not always directly correlate with

protein expression. For example, genes can be alternatively spliced, yielding a variety of protein variants, or transcripts may be stored for later release or not released as protein. Traditionally, to learn more about protein expression, two-dimensional gel electrophoresis (2D-GE) combined with mass spectroscopy has been utilized, but it is restricted to proteins ranging from 10-200 kDa with isoelectric points between 4-10, and is not well suited for separating acidic, basic, or hydrophobic membrane protein. It is also labour intensive. In contrast, the newer protein chip chromatographic retention technologies based on surface-enhanced laser desorption/ionisation, time-of-flight mass spectrometry (SELDI-TOF-MS) are more user friendly and are less restricted by size or nature of the protein. This technology has been effectively used to evaluate protein profiles between phenotypically diverse groups and to characterize protein interactions in a number of species, including sheep,[27] pigs and cattle.[26] In the context of cattle identified with increased risk of mastitis, or expressing high and low immune response phenotypes, studies are now under way using proteomic methods, such as SELDI-TOF-MS, to identify proteins associated with these potentially useful phenotypes.

Conclusions

Dairy cows can be classified using a mathematical index into three groups according to antibody and cell-mediated immune responses (Group 1>Group 2>Group 3). Milk whey and colostral antibody to test antigens and J5 vaccine antigens also reflected this ranking. With few exceptions, cows with the highest rank (high immune response) have the lowest occurrence of disease, including mastitis.

Antibody and cell-mediated immune responses of livestock, including cattle, are highly heritable and amenable to genetic selection. Substantial genetic variation exists among individuals and between breeds, making genetic selection for high immune response possible, with beneficial effects noted in both health and production traits. Many genes regulate host defense, and immune response gene expression profiles vary among individuals selected for various traits, including immune responsiveness. Epigenetic modification can also influence immune response gene expression, and needs to be investigated further.

Emerging and re-emerging diseases are a concern to both human and veterinary medicine. Certain infectious diseases are well controlled by traditional methods, however the increasing restrictions on antibiotic use and costs associated with new drug development are making it more challenging to manage arising health issues. Various approaches are being investigated to adequately address these concerns, particularly as they relate to animal health. Combining quantitative and molecular genetic techniques in animal breeding with improved knowledge of the genes and proteins that underlie resistance to complex infectious disease can help to improve food quality, as well as animal health and well-being.

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